

ALAN EMANUEL SILVA CERQUEIRA

**MICROBIOTA ASSOCIADA A ABELHAS SEM FERRÃO: DIVERSIDADE,
COMPOSIÇÃO E RESPOSTA A IMPACTOS AMBIENTAIS**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientadora: Cynthia Canêdo da Silva

Coorientador: Weyder Cristiano Santana

**VIÇOSA - MINAS GERAIS
2021**

**Ficha catalográfica elaborada pela Biblioteca Central da Universidade
Federal de Viçosa - Campus Viçosa**

T

C416m
2021

Cerqueira, Alan Emanuel Silva, 1989-

Microbiota associada a abelhas sem ferrão [recurso eletrônico]: diversidade, composição e resposta a impactos ambientais / Alan Emanuel Silva Cerqueira. – Viçosa, MG, 2021.

1 tese eletrônica (98 f.): il. (algumas color.).

Inclui apêndices.

Orientador: Cynthia Canedo da Silva.

Tese (doutorado) - Universidade Federal de Viçosa.

Inclui bibliografia.

DOI: <https://doi.org/10.47328/ufvbbt.2021.029>

Modo de acesso: World Wide Web.

1. *Melipona*. 2. *Tetragonisca angustula*. 3. Bactérias.
4. Fungos. 5. Intestinos. 6. Mel. 7. Simbiose. 8. Barragem de
rejeitos. I. Universidade Federal de Viçosa. Departamento de
Microbiologia. Programa de Pós-Graduação em Microbiologia
Agrícola. II. Título.

CDD 22. ed. 595.799

ALAN EMANUEL SILVA CERQUEIRA

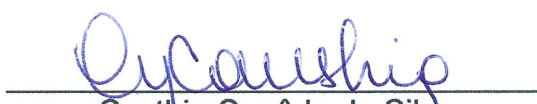
**MICROBIOTA ASSOCIADA A ABELHAS SEM FERRÃO: DIVERSIDADE,
COMPOSIÇÃO E RESPOSTA A IMPACTOS AMBIENTAIS**

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

APROVADA: 22 de julho de 2021.

Assentimento:


Alan Emanuel Silva Cerqueira
Autor


Cynthia Canêdo da Silva
Orientadora

A Deus,

*Aos meus pais Manoel Cerqueira e Maria Auxiliadora,
Aos meus irmãos, sobrinhos, tios, primos, amigos, esposa e toda minha família,
A meu irmão Ismário, pelo seu legado de luta pela família, trabalho e arte,
Aos meus amigos e colegas,*

Dedico.

AGRADECIMENTOS

A Deus por me abençoar, iluminar, proteger e guiar em todos os momentos.

Aos meus pais e anjos da guarda Manoel e Dora, por todo amor, carinho, apoio, orações e incentivo, com os quais compartilho o mérito de todas as conquistas.

Aos meus irmãos Yves e Júlia, minha sobrinha/irmã Juli, meus sobrinhos Clarinha, Yane, Yres, Paulo Arthur, Luquinhas, Pedrinho e Yan, meus tios, primos e toda minha família por todo amor, incentivo e acolhimento. Amo muito todos vocês.

Ao meu irmão Ismário (in memoriam), pelo amor, apoio, incentivo e pelo legado de cuidado com a família, poesias, músicas e, sobretudo, de nunca desistir.

À minha esposa, amiga, companheira e parceira de todas as horas, Wiane Meloni Silva e sua família, por todo amor, apoio e incentivo durante a minha jornada.

Aos meus amigos pelo apoio, pelos momentos compartilhados e por contribuírem para a construção do meu caráter como pessoa e como profissional.

À Professora Cynthia Canêdo da Silva pela orientação, ensinamentos, oportunidade, paciência, confiança e apoio durante os 7 anos de mestrado/doutorado.

Ao Professor Weyder C. Santana pela coorientação, ensinamentos e incentivo. À Professora Nancy Moran e Dr. Tobin Hammer, pela colaboração e coorientação no doutorado sanduíche, na The University of Texas at Austin, USA. E, à Professora Maria Catarina Megumi Kasuya, por todo apoio, ensinamentos, incentivo e por ter me recebido de braços abertos no seu laboratório.

Aos amigos do LAMAP e LAMIC pelos ensinamentos, convivência, parceria, trocas e bons momentos compartilhados.

Aos meliponicultores pelo provimento de abelhas e apoio ao trabalho.

À Universidade Federal de Viçosa, pela oportunidade de realizar a pós-graduação.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código de Financiamento 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

Ao CNPq, pela concessão da bolsa de estudos e a CAPES, pela concessão da bolsa de estudos de doutorado sanduíche no exterior (CAPES-Print).

BIOGRAFIA

Alan Emanuel Silva Cerqueira, filho de Manoel dos Santos Cerqueira Filho e Maria Auxiliadora Silva Cerqueira, é natural de Salvador/BA onde se graduou em 2013 como Bacharel em Ciências Biológicas pela Universidade Federal da Bahia. No ano de 2014, ingressou no mestrado em Microbiologia Agrícola na Universidade Federal de Viçosa - MG se tornando mestre no ano de 2016. Em seguida, ingressou no doutorado em agosto deste mesmo ano, submetendo-se à defesa de tese em julho de 2021 para obtenção do título de Doutor em Microbiologia Agrícola pela Universidade Federal de Viçosa - MG. Durante este período, realizou Doutorado Sanduíche - CAPES/PRINT no laboratório da professora Nancy A. Moran - Department of Integrative Biology - University of Texas at Austin – USA.

RESUMO

CERQUEIRA, Alan Emanuel Silva, D.Sc, Universidade Federal de Viçosa, julho de 2021. **Microbiota associada a abelhas sem ferrão: diversidade, composição e resposta a impactos ambientais.** Orientadora: Cynthia Canêdo da Silva. Coorientador: Weyder Cristiano Santana.

As abelhas eusociais corbiculadas (tribos Apini, Bombini e Meliponini) possuem uma microbiota distintiva, especializada e dominante no intestino que, conforme descrito em *Apis* (Apini) e *Bombus* (Bombini), contribuem para a saúde e nutrição destes animais. Estudos sugerem uma composição microbiana intestinal mais variável nas abelhas sem ferrão (Meliponini), mas ainda há uma carência de pesquisas com essas abelhas. Abelhas sem ferrão são o maior grupo de abelhas eussociais corbiculadas, consistindo em importantes polinizadores da vegetação nativa e de culturas agrícolas de ecossistemas tropicais. Além disso, são importantes em uma perspectiva socioeconômica como fonte de renda para os meliponicultores pela comercialização de pólen, cera e mel. Entretanto, as abelhas sem ferrão têm sofrido os efeitos das atividades antrópicas tais quais o desmatamento e acúmulo de químicos agrícolas e metais no ambiente. Agentes estressores interferem na composição microbiana e saúde das abelhas, mas tais efeitos ainda são pouco conhecidos nas abelhas sem ferrão. Esta tese visou preencher lacunas associadas ao estudo dos microrganismos associados às abelhas sem ferrão, do seu alimento e das consequências de impactos ambientais na composição e diversidade microbiana. Assim, este trabalho foi dividido em três capítulos, construídos com uma abordagem metataxonômica, com o objetivo de obter (1) uma visão geral sobre diversidade, composição e origem de microrganismos associados a *Melipona* e seu alimento; (2) investigar com maior esforço amostral a perda de simbiontes intestinais e o possível surgimento de novos simbiontes em *Melipona*; e (3) avaliar as diferenças na microbiota intestinal de *Tetragonisca angustula* vivendo em locais não impactados e impactados pelos rejeitos de mineração oriundos do rompimento da barragem de Fundão em Mariana, Minas Gerais, Brasil. Dentre os resultados do capítulo 1, detectamos uma maior diversidade microbiana e maior especificidade de microrganismos à espécie em estudo nas abelhas em comparação ao mel – que tende a ser mais influenciado por microrganismos ambientais. Fungos filamentosos predominam nas abelhas e são

próximos a isolados ambientais, de plantas ou associados às colmeias, possivelmente obtidos durante o forrageio. As leveduras são mais prevalentes no mel e próximas a isolados de colmeias e isolados associados ao seu metabolismo. Bactérias filogeneticamente próximas a isolados de plantas e ambientais fructofílicas são abundantes no mel, ao passo que nas abelhas, predominam bactérias simbiontes intestinais e outras próximas a isolados de plantas/ambiente. Curiosamente, *Gilliamella* e *Snodgrassella*, duas importantes bactérias membros da microbiota core das abelhas eussociais corbiculadas, não foram encontradas em *Melipona*, observação que nos levou a concluir, no capítulo 2, com maior esforço amostral, a perda da associação entre esses simbiontes intestinais e este clado de abelhas sem ferrão. Por fim, no capítulo 3, observou-se alteração na abundância relativa de bactérias "core" (presentes em todas as amostras - possivelmente simbiontes) associadas ao intestino de *T. angustula* além de uma redução da riqueza de bactérias "estritamente ambientais" (menos comuns, possivelmente transitórios) em abelhas vivendo em áreas impactadas pelos rejeitos de mineração, possivelmente como consequência da exposição a esses ambientes. Os resultados aqui obtidos contribuíram para um melhor entendimento sobre os microrganismos associados às abelhas sem ferrão e abrem perspectivas para o desenvolvimento desta linha de pesquisa.

Palavras-chave: *Melipona*. *Tetragonisca angustula*. Microbioma. Bactérias. Fungos. Intestino. Mel. Simbiose. Barragem de Fundão. Rejeitos de mineração.

ABSTRACT

CERQUEIRA, Alan Emanuel Silva, D.Sc., Universidade Federal de Viçosa, July, 2021. **Stingless bees associated microbiota: diversity, composition, and response to environmental impacts.** Adviser: Cynthia Canêdo da Silva. Co-adviser: Weyder Cristiano Santana.

The microorganisms associated with eusocial corbiculate bees (Apini, Bombini and Meliponini) contribute to their health and nutrition. This microbiota is distinctive, specialized, and dominant in the bee gut. However, most studies were carried out with *Apis* (honeybees) and *Bombus* (bumblebees). Studies suggest a more variable gut microbial composition in stingless bees (Meliponini), but there is still a lack of research. Stingless bees are the largest group of eusocial corbiculate bees, consisting of important pollinators of native vegetation and agricultural crops in tropical ecosystems. In addition, they are important from a socioeconomic perspective as a source of income for stingless beekeepers through the sale of pollen, wax, and honey. However, stingless bees have been impacted by the effects of human activities such as deforestation and accumulation of agricultural chemicals and metals in the environment. Environmental stressors interfere in the microbial composition and health of bees, but such effects are still understudied in stingless bees. This thesis aimed to fill gaps associated with the study of microorganisms associated with stingless bees, their food, and the consequences of environmental impacts on microbial composition and diversity. Then, this work was divided in three chapters, built with a metataxonomic approach, with the objective of obtaining (1) an overview of the composition, diversity and origin of microorganisms associated with *Melipona* bees and its food; (2) investigate with greater sampling effort the loss of gut symbionts and the possible emergence of new symbionts in *Melipona*; and (3) evaluate the differences in the gut microbiota of *Tetragonisca angustula* living in areas impacted and not impacted by the mining tailings of the Fundão dam collapse, located in Mariana, Minas Gerais - Brazil. Among the results of Chapter 1, we detected greater microbial diversity and greater specificity of microorganisms to the species under study in bees compared to honey – which tends to be more influenced by environmental microorganisms. Filamentous fungi predominate in bees and are close to environmental, plant or hive-associated isolates, possibly obtained during foraging. Yeasts are more prevalent in honey and

close to hive isolates and isolates associated with honey metabolism. Bacteria phylogenetically close to plant isolates and environmental fructophilic bacteria are abundant in honey, while in bees, gut symbiotic bacteria and others close to plant/environmental isolates predominate. Interestingly, *Gilliamella* and *Snodgrassella*, two important bacteria that compose the eusocial corbiculate bees core microbiota, were not found in *Melipona*, an observation that led us to conclude, in chapter 2, with greater sampling effort, the loss of the association between these gut symbionts and this clade of stingless bees. Finally, in chapter 3, we observed an alteration in the relative abundance of core bacteria (those present in all samples - possibly symbionts) associated with the gut of the stingless bee *T. angustula*; and a reduction in the richness of the "strictly environmental" bacteria (less common, possibly transient) in bees living in areas impacted by the mining tailings, which is possibly a consequence of the bee exposure to these environments. The results obtained here contributed to a better understanding on the stingless bee associated microorganisms and open perspectives for the development of this research field.

Keywords: *Melipona*. *Tetragonisca angustula*. Microbiome. Bacteria. Fungi. Gut. Honey. Symbiosis. Fundão dam collapse. Mining tailings.

SUMÁRIO

INTRODUÇÃO GERAL	12
Chapter 1: <i>Melipona</i> stingless bees and honey microbiota reveals the diversity, composition, and possible origin of associated microorganisms.....	14
ABSTRACT	14
Introduction.....	15
Material and methods	17
a. Sampling and DNA extraction.....	17
b. DNA sequencing.....	18
c. Amplicon sequencing analysis.....	19
d. Alpha and Beta Diversity and Community composition analysis	19
e. Identification of the most abundant OTUs and their origin by heatmap and phylogenetic analysis	20
Results and Discussion	21
1. Sequencing and dataset summary	21
2. Bees and honey alpha diversity.....	21
3. Replicates from the same species tend to be less dissimilar in bees than honey samples.	23
4. Bee and honey samples from the same species harbor dissimilar microbial composition, which tend to be closer between samples from same type.	24
a. Filamentous fungi are more abundant in bee samples, possibly acquired from environment/plants by foraging activity.	24
b. Yeasts are widespread, comprises higher proportion of honey microbiota, and are mostly obtained from plants/environment or persisting insect and hive associated symbionts.	29
c. Bee and honey microbiome are associated with either symbiotic transmitted or plant/environmentally acquired bacteria.	31
5. Absence of core symbionts points toward a microbiome disruption in <i>Melipona</i> bees.	36
6. Conclusions	37
Acknowledgments:	38
References:.....	39
Supplementary material	44
Chapter 2: Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees.	54
ABSTRACT	54
Introduction.....	55
Methods and Results.....	56

Discussion	59
Acknowledgments	60
Conflict of interest.....	60
References	61
Supplementary Methods	63
Supplementary References	66
Supplementary Figures	67
Supplementary Tables	70
Chapter 3: Stingless bees gut microbiota is altered in areas impacted by mining tailings from the Fundão dam collapse in Mariana, Brazil.....	73
ABSTRACT	73
Introduction.....	74
Methods	76
Results	81
Discussion	90
Acknowledgments	93
References	94
Conclusões gerais	97

INTRODUÇÃO GERAL

O microbioma associado a abelhas eusociais corbiculadas, tais como as do gênero *Apis* (“honeybees” – tribo Apini), *Bombus* (“bumblebees” – tribo Bombini) e algumas abelhas sem ferrão (“stingless bees” – tribo Meliponini) normalmente possui baixa diversidade microbiana em comparação com outros animais e dominância de determinadas espécies bacterianas, que evoluem junto com o hospedeiro em função de serem transmitidas pelo contato social entre as abelhas. Algumas outras bactérias são obtidas do ambiente e compõem a microbiota dominante. O mel, alimento consumido pelas abelhas, também possui baixa diversidade microbiana, dentre outros, devido ao alto teor de compostos antimicrobianos resultantes da ação fermentativa de microrganismos adaptados.

A simbiose entre abelhas e microrganismos favorece a saúde dos hospedeiros em diversos aspectos. Os microrganismos atuam na proteção contra patógenos e parasitas, podem ser fonte de nutrientes, auxiliar na digestão do pólen e mel ingerido, no efluxo de substâncias tóxicas etc. No entanto, a maioria dos estudos sobre microbiomas de abelhas é feita com *Apis* e *Bombus*, e há uma escassez de estudos sobre o microbioma de abelhas sem ferrão.

Abelhas sem ferrão (Meliponini) são importantes polinizadores dos ecossistemas neotropicais, auxiliando na agricultura e preservação de espécies nativas. Além disso, são importantes em uma perspectiva socioeconômica como fonte de renda para os meliponicultores pela comercialização de pólen, cera e mel. Como habitam florestas, essas abelhas sofrem os impactos de ações antrópicas, como o desmatamento que, além de destruir suas colônias, reduzem suas fontes de alimento. Além disso, por acumularem poluentes durante as atividades de forrageio, essas abelhas são afetadas pela exposição a agroquímicos e metais. Maior susceptibilidade a patógenos, associados a um déficit nutricional, e alteração no microbioma de *honeybees* e *bumblebees* em decorrência da exposição a poluentes, já foram observados. Entretanto, há carência de estudos sobre os impactos de estressores ambientais na microbiota intestinal de abelhas sem ferrão.

Com o objetivo de ampliar os conhecimentos acerca dos microrganismos associados a abelhas sem ferrão, no primeiro capítulo, amostras de mel e abelhas do gênero *Melipona* foram coletadas de três espécies localizadas em áreas de Mata

Atlântica pertencentes a UFV. Essas amostras foram analisadas quanto a composição, diversidade, e possível origem de seus fungos e bactérias associadas, obtendo-se uma visão geral sobre a microbiota de abelhas e seu alimento.

A partir dos resultados do primeiro capítulo, onde, dentre outros, se percebeu a possível ausência em *Melipona* de *Snodgrassella* e *Gilliamella*, importantes simbiontes bacterianos ancestralmente associadas ao clado de abelhas corbiculadas eussociais, um outro estudo foi direcionado para a investigação dessa possível ruptura de simbiose. Nesse sentido, no segundo capítulo, expandiu-se a amostragem, abrangendo 23 populações coletadas em quatro diferentes biomas compreendendo pelo menos 8 espécies diferentes. Além da confirmação da ausência de *Snodgrassella* e *Gilliamella* em *Melipona*, indicativos de um possível estabelecimento de novas simbioses foram observados, abrindo perspectivas para futuros estudos. Os resultados do segundo capítulo, aqui apresentados, são reproduzidos do original, publicado no formato de *Brief Communication* dia 18 de maio de 2021 na revista *The ISME Journal* (disponível em <https://doi.org/10.1038/s41396-021-01000-1>).

Por fim, no terceiro capítulo, visto que abelhas sem ferrão são capazes de acumular metais, buscou-se entender o impacto causado pelo rejeito de mineração oriundo do rompimento da barragem de Fundão em Mariana, Minas Gerais, Brasil, na microbiota do intestino de *Tetragonisca angustula*. Este desastre foi considerado o maior no contexto socioambiental do Brasil, poluindo rios, destruindo florestas e lançando lama com elevado teor de metais no ambiente. Áreas impactadas e não impactadas foram comparadas quanto a diferenças na comunidade microbiana geral, nas bactérias “core” (presentes em todas as amostras) e nas bactérias “estritamente” ambientais – aqui definidas como bactérias “não core” distintas das comumente encontradas na literatura, e nos dois capítulos anteriores, como associadas às abelhas eussociais corbiculadas. Em geral áreas impactadas e não impactadas diferem quanto a composição microbiana, quanto a proporção das linhagens de bactérias “core” e quanto a diversidade das “estritamente” ambientais. Portanto, ressalta-se a importância de estudos sobre a microbiota intestinal de abelhas sem ferrão na avaliação dos impactos causados pelo desastre de Mariana. Além disso, reforça-se a necessidade de mais estudos sobre a importância da microbiota intestinal para a saúde das abelhas sem ferrão, e acerca do possível isolamento e aplicação de linhagens potencialmente tolerantes (com proporção aumentada nas áreas impactadas) como potenciais probióticos em abelhas sob condição de estresse.

Chapter 1: *Melipona* stingless bees and honey microbiota reveals the diversity, composition, and possible origin of associated microorganisms.

ABSTRACT

Eusocial corbiculate bees, which comprise the honeybees, bumblebees and stingless bees' tribes, have low microbial diversity and a dominant gut microbiome. These microbes are socially transmitted but also obtained from the environment. Honey environment usually consist of a challenging environment, also with low diversity, due to the elevated antimicrobial properties derived from the activity of inoculated nectar fermenting microbes, such as lactic acid bacteria (LAB). The stingless bees genus *Melipona* (Meliponini) occurs in Neotropical ecosystems, acting as pollinators of crops and native plants, and also as socioeconomic activity for stingless beekeepers in Brazil. However, despite the nutritional and health importance of bee associated microorganisms, already described for honeybees and bumblebees, the microbial composition, diversity, and its contribution to the health of stingless bees still require to be more studied. Here, we studied the microbes associated with bee individuals and their honey of three *Melipona* species, to provide an overview about fungi and bacteria associated with this understudied clade and investigate possible origin of symbionts. We observed that bees harbor higher microbial alpha diversity than honey possibly due to honey high antimicrobial properties. Also, bee microbiota tends to be more species-specific than honey, that might be more influenced by environmental microbes. Filamentous fungi predominate in bees, being acquired from the environment/plants during foraging or hive associated fungi. Yeasts are widespread but are more prevalent in honey samples and close to isolates previously found in hive environment and associated with honey metabolism. Concerning bacteria, honey has high abundance of species more transiently associated with bees, plant derived and environmental fructophilic species. Bees are more associated with gut symbiotic and environmental/plant derived bacteria. Intriguingly, *Melipona* bee's microbiota is different than other eusocial corbiculate bees', virtually lacking important symbionts, which deserve further investigation by increasing studied species, biomes, and sites.

Keywords: *Melipona*, fungi, bacteria, microbiome, hive.

Introduction

Eusocial corbiculate bees, which comprise the honeybees, bumblebees and stingless bees tribes, usually have low microbial diversity, with few bacterial species as a core microbiome, including *Snodgrassella alvi*, *Gilliamella apicola*, *Lactobacillus* spp. Firm-4 and Firm-5 (phylum Firmicutes) and *Bifidobacterium* spp. (phylum Actinobacteria) (KOCH; SCHMID-HEMPEL, 2011a; KWONG et al., 2017; KWONG; MORAN, 2016). Less prevalent, but also corbiculate associates include the Acetobacteraceae Alpha 2.1 - *Commensalibacter* sp. and Alpha 2.2 - *Parasaccharibacter apium*, *Bombella favorum*, *Bombella mellum*, *Bombella apis* (phylum Proteobacteria) (KWONG et al., 2017; KWONG; MORAN, 2016; NOWAK et al., 2021). In addition, other Proteobacteria such as *Frischella perrara* (Orbaceae family) and *Bartonella apis* (Rhizobiaceae family) are also gut associates for honeybees (KWONG et al., 2017; KWONG; MORAN, 2016; NOWAK et al., 2021).. Gut bacteria contribute to bee health by promotion of immune response (KATSNELSON, 2015), host expression of antimicrobials peptides (KWONG; MANCENIDO; MORAN, 2017), by formation of biofilms in bees gut ileum (ENGEL; MARTINSON; MORAN, 2012a; POWELL et al., 2016), efflux of toxic substances (ENGEL; MARTINSON; MORAN, 2012), and defence against parasite infections (KATSNELSON, 2015; KOCH; SCHMID-HEMPEL, 2011b).

Microorganisms are also associated with nutrition and metabolism of pollen and honey. Gut microbiota harbor a variety of genes related to carbohydrates and peptides transport (ENGEL; MARTINSON; MORAN, 2012), acts in pollen fermentation and spoilage avoidance (VÁSQUEZ; OLOFSSON, 2009), conversion of nectar to honey (OLOFSSON; VÁSQUEZ, 2008a) and degradation of pollen pectin (ENGEL; MARTINSON; MORAN, 2012). Microbes can be also ingested as a source of nutrients for larvae to reach pupal status (MENEZES et al., 2015; PALUDO et al., 2018) and by adults as source of proteins (MODRO et al., 2009).

Usually, the core microbiota is generalist - suggesting their presence in basal corbiculate strains, but the presence of host-specific and tribe-specific strains indicate that there is a vertical social transmission that contributed to microorganism-host coevolution (KOCH; SCHMID-HEMPEL, 2011a; KWONG et al., 2017). In social bees, the microbial acquisition occurs mainly via the oral trophallaxis from nurse workers to

newly emerged workers, via contact with hive material such as honey and bee bread, and specially through fecal-oral transmission between different aged bees - from older to younger (KWONG; MORAN, 2016; POWELL et al., 2014). On the other side, there is also evidence of environmental acquisition of microbes such as lactic acid bacteria including *Lactobacillus* present in flowers (FREDERICK et al., 2012), and some yeasts that occur in flowers and can also be found in pollen and honey provisions in the beehives and/or associated with bees (BRYSCH-HERZBERG, 2004; LACHANCE et al., 2010).

Honey environment usually has elevated acidity and antimicrobial activity (VILLACRÉS-GRANDA et al., 2021) derived from the activity of inoculated nectar fermenting microbes, such as lactic acid bacteria (LAB) (DE VUYST; LEROY, 2007; OLOFSSON et al., 2016; SYED YAACOB et al., 2018). LAB include *Lactobacillus*, *Bifidobacterium* (OLOFSSON et al., 2016), *Fructilactobacillus* (ZHENG et al., 2020), *Fructobacillus* (SYED YAACOB et al., 2018), *Bacillus* (NGALIMAT et al., 2019) found in stingless bees honey. Due to challenging environment, honey is expected to have low microbial diversity.

In face of anthropic impacts on bees, such as deforestation that affects nutrition increasing susceptibility to pathogens and parasites (AIDAR, 1996; BROWN; ALBRECHT, 2001; DOLEZAL; TOTH, 2018) and chemical pollution that compromise foraging, brain development and decrease beneficial microorganisms (MORAIS et al., 2018; MOTTA; RAYMANN; MORAN, 2018; SEIDE et al., 2018; TOMÉ et al., 2012) studies on the microbiome are important for the bee's preservation. However, the majority of bee microbiome studies are made with honeybees and bumblebees (MENEGATTI et al., 2018) and the microbial composition, diversity and its contribution to the health of stingless bees still require to be more studied.

Stingless bees from the genus *Melipona* (Meliponini) occurs in Neotropical ecosystems, acting as pollinators of native plants and of around 15-26 crops in Brazil (GIANNINI et al., 2015) increasing quantity and quality of fruits and seeds (SANTOS et al., 2009; SILVA-NETO et al., 2019). In addition, bee products such as pollen and honey can be commercialized by stingless beekeepers (SEBRAE, 2015), thus *Melipona* has a wide environmental and socioeconomic importance in Brazil. Despite the importance of microorganisms for social bee's health and nutrition, there is a lack of studies on stingless bee microbiota. Here, we studied the microbes associated with

bee individuals and their honey, using three *Melipona* species living in the same area: *Melipona bicolor bicolor* (Mbb), *Melipona mondury* (Mm) and *Melipona quadrifasciata anthidioides* (Mqa). In this sense, we compared the alpha and beta diversity, the microbial community composition – including indicator species, and the possible origin of the most abundant microorganisms between bee individuals and their honey provisions. Our results will contribute to the field of *Melipona* bees and honey microbiome, by understanding their associated microbes and possible origin, providing insights, and directing future research.

Material and methods

a. Sampling and DNA extraction

The samples were collected from beekeeping boxes of *Melipona quadrifasciata anthidioides*, *Melipona mondury* and *Melipona bicolor bicolor* living under natural feeding, avoiding the influence of external food sources on the microbiota, at the research station “Mata do Paraíso” (Atlantic Forest Biome) of Federal University of Viçosa, located at the State of Minas Gerais – Brazil. We sampled a total of 9 beekeeping boxes, three for each bee species (triplicate), at which forager bees were taken from the entrance and stored in 96% ethanol, and honey were collected from honey pots using sterile syringes. A total of 18 samples, comprising triplicates of 6 treatments are under study (Table 1).

Table 1 - Treatments under study

Treatment	Bee specie	Sample Type	Amount	Nº of samples
1	<i>Melipona bicolor bicolor</i>	Bee	5 individuals	3
2	<i>Melipona mondury</i>	Bee	5 individuals	3
3	<i>Melipona quadrifasciata anthidioides</i>	Bee	5 individuals	3
4	<i>Melipona bicolor bicolor</i>	Honey	20mL	3
5	<i>Melipona mondury</i>	Honey	20mL	3
6	<i>Melipona quadrifasciata anthidioides</i>	Honey	20mL	3

For each sample, we disinfested 5 bees with 1% sodium hypochlorite, washed in distilled sterile water, pooled together, and macerated with liquid nitrogen for the cell lysis. Concerning the honey, 20 mL from each beekeeping box was diluted with 180 mL sterile distilled water (1:10) and filtered to minimize the amount of carbohydrates

and retain microbial cells. Then, the filter was cut and pieces vortexed in Phosphate Buffered Saline (PBS) to resuspend bound microbial cells. Total DNA was extracted from bee and honey samples using a chloroform/isoamylalcohol (24:1) solution, as follows: bee lysate and the honey filtrate were then transferred to sterile 50 mL conical centrifuge tubes at which PBS buffer were added and a centrifugation at 12000 rpm for 5 minutes was done. The supernatant was discarded, and the pellet was resuspended in PBS, vortexed and mixed with 100 μ L lysozyme (100 mg mL⁻¹). The mixture was incubated under water bath for 30 minutes at 37°C and agitated in every 10 minutes. Afterwards, 50 μ L of proteinase K (10 mg mL⁻¹) and 200 μ L sodium dodecyl sulphate (SDS) 10% (final concentration 2%) were added and incubated at 60°C for 30 minutes (agitated every 10 minutes). Then, 3 cycles of freeze-thaw were performed to increase cell lysis: 2 minutes in liquid nitrogen and 2 minutes at 65°C. Saturated phenol at pH 8 was added and kept under vigorous agitation for 30 minutes. The sample was, then, centrifuged for 5 minutes at 12,000 rpm, the supernatant was collected and chloroform:isoamylalcohol (24:1) was added. After 5 minutes at the same speed centrifugation the supernatant was mixed with NaCl 5 M. Then, cold ethanol was added to precipitate the DNA. A final centrifugation for 20 minutes was made, and the ethanol discarded. The pellet was washed with 70% ethanol. After drought of ethanol the DNA was resuspended in 25–50 μ L of sterile ultrapure water.

b. DNA sequencing

The ITS1F/ITS2 primers set targeting a shorter region of the ITS (Internal Transcribed Spacer) (SMITH; PEAY, 2014) and 515F/806R primers set targeting 16S rRNA gene V4 variable region with barcode on the forward primer were used for fungal and bacterial amplicon generation, respectively. PCR was performed using HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 minutes, followed by 30-35 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 5 minutes was performed. After amplification, purified DNA of multiple samples with equal proportions based on their molecular weight and DNA concentration were pooled together and Illumina DNA library prepared. Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) on a MiSeq Illumina System following the manufacturer's guidelines.

c. Amplicon sequencing analysis

The MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA) were used to process sequencing data. First, the paired-ends sequences were joined, the barcodes depleted and sequences shorter than 150 bp and with ambiguous base calls removed. Then, sequences denoising, OTUs clustering at 3% divergence (i.e., 97% similarity) and chimera removal were performed, and the remaining OTUs were taxonomically classified using BLASTn tool against a curated database derived from Ribosome Database Project (RDPII) and NCBI (<http://rdp.cme.msu.edu>, www.ncbi.nlm.nih.gov).

d. Alpha and Beta Diversity and Community composition analysis

Initially, non-targeted/contaminant reads such as Mitochondria/Chloroplast, were removed from the dataset and the fungal (ITS) and bacterial (16S) Operational Taxonomic Units (OTU) tables (i.e., tables containing information about the number of reads per sample per OTU) merged into a single table, representing the whole microbiota. Then, low abundant sequences (<100 reads in total) were removed from the dataset and samples rarefied to same number of reads using the R package *mctoolsr* (available at: <https://github.com/leffj/mctoolsr>). Rarefaction curves were made with the R package “Vegan” (OKSANEN et al., [s.d.]) and Good’s coverage estimator, which is the number of OTUs sampled at least twice in each sample (i.e. non singleton OTUs) divided by the total number of sequences (GOOD; TOULMIN, 1956), were calculated using the package “QsRutils” version 0.1.3. Then, alpha diversity metrics such as Richness – number of unique OTUs per sample, Simpson’s $(1 - D)$ – that quantifies evenness, and Shannon – which combines richness and evenness, were calculated using *mctoolsr* R package at OTU level. The dissimilarity in the community composition between samples (i.e., beta diversity) was accessed based on a Bray-Curtis dissimilarities matrix after performing square-root transformations on the OTUs and plotted in a Non-Metric Multidimensional Scaling (NMDS) using *mctoolsr* R package. Aimed at describing the differences in microbial community composition between bee and honey samples, the most abundant fungal and bacterial family were plotted in barplots using “*mctoolsr*” R package. We additionally visualized the occurrence of habitat specialist OTUs, i.e., OTUs with statistically significant association with honey or bee samples, by using the R package “*indicspecies*” (DE

CÁCERES; LEGENDRE, 2009). Each habitat specialist OTU was considered as an indicator OTUs.

e. Identification of the most abundant OTUs and their origin by heatmap and phylogenetic analysis

Finally, we inferred the source of microorganisms associated to bee individuals and honey, by evaluating the possible origin of the most abundant OTUs. In this sense, using phyloseq (available at: <https://joey711.github.io/phyloseq/>) (MCMURDIE; HOLMES, 2013) implemented in ggplot2 (WICKHAM, 2016), we, first, plotted the most abundant fungal and bacterial OTUs in a heatmap for observation of the differences in their incidence between bee and honey samples. Then, we constructed phylogenies as follows: selected OTUs were submitted to BLASTN similarity searches against GenBank Nucleotide Collection and Reference Sequence (sequences from type material) databases, and the most similar sequences aligned with the query OTUs were downloaded. Afterwards, OTUs and downloaded sequences were aligned by ClustalW, had the nucleotide substitution models determined by calculation of likelihood scores and selection according to Akaike information criterion (AIC), and Maximum Likelihood phylogenetic trees were made with bootstrap of 1000 replications using MEGA software (KUMAR; STECHER; TAMURA, 2016). The information about the isolation substrate/host of the most phylogenetically closely related downloaded sequence to each OTU (Table S2 and Figures S3-S7) together with the heatmap allowed us to infer the most abundant OTUs in bees and honey samples, their taxonomic classification and where they are possibly obtained from.

The significances of the differences in family/OTUs relative abundances and in diversity indexes between bee and honey samples of all analysis were accessed by Mann-Whitney (p-value <0.05) test calculated with “mctoolsr” R package or PAST software.

Results and Discussion

1. Sequencing and dataset summary

The sequencing of the 16SrRNA region resulted in 1,023,420 and 808,132 reads classified in OTUs for bee and honey samples, respectively. On the other side, sequencing of ITS1 rDNA region resulted in 1,531,254 and 978,968 reads classified in OTUs for bee and honey samples, respectively. We merged and rarefied fungal and bacterial data, and each sample totalized 103,215 reads that were used as input dataset for the study of overall bee and honey microbiota. The number of fungal and bacterial OTUs is represented in Table S1. Rarefaction curves harbored a discrete trend of plateau formation, but Good's coverage index indicate that the sampling effort was satisfactory (Fig. S1). The proportion of bacteria and fungi in the dataset is represented in Fig. S2.

2. Bees and honey alpha diversity.

Our study contributed to the understanding of the underexplored stingless bee's microbiome, focusing not only in bacterial but also in the usually neglected fungal microbes, additionally expanding to honey associated microbes. Low diversity associated to bee species and honey species were expected, since most studies to date well documented the low diversity and occurrence of core bacterial species associated to honeybees (*Apis*) and bumblebees (*Bombus*) gut (KOCH; SCHMID-HEMPEL, 2011a; KWONG et al., 2017; MARTINSON et al., 2011; MORAN et al., 2012; MOTTA; RAYMANN; MORAN, 2018; RAYMANN; MORAN, 2018; RIBIÈRE et al., 2019; SABREE; HANSEN; MORAN, 2012), and due to the highly unfavorable environment for microbial survival after honey ripening (ANDERSON et al., 2011; OLOFSSON; VÁSQUEZ, 2008b; WEN et al., 2017).

We observed that regardless of the bee species, mean microbial alpha diversity (Shannon), OTUs richness and equitability in OTUs abundance (Simpson) tend to be higher in bees than honey (Fig.1), but without significant difference (Mann-Whitney pairwise test $p\text{-value} > 0.05$) due to great variability among samples.

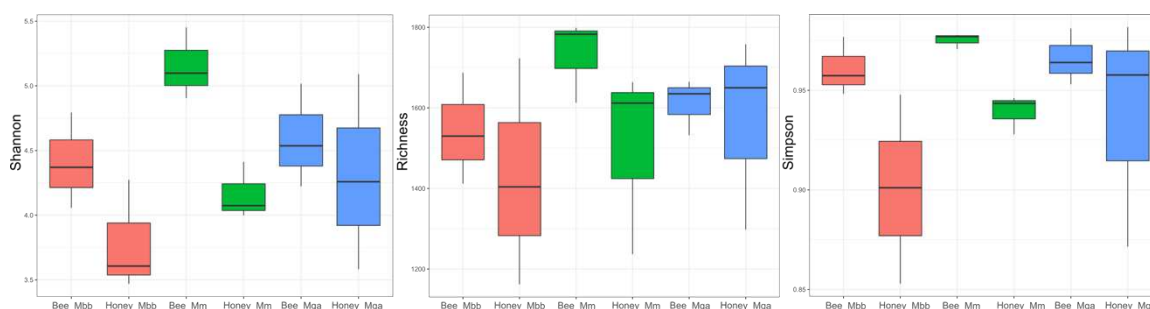


Figure 1 – Box plots representing the Shannon, Richness and Simpson diversity indexes. Y-axis represents the indexes values and x-axis represents bee or honey samples from Mbb – *Melipona bicolor bicolor*, Mm – *Melipona mondury*, Mqa – *Melipona quadrfasciata anthidioides*. The x-axis represent the index values and y-axis the categories. The interquartile ranges (IQR) between the first and third quartiles are represented by top and bottom boxes boundaries, respectively. The horizontal lines inside boxes represent the median diversity values. The lowest and highest diversity values are show in the ends of the vertical lines.

Nevertheless, this tendency of lower diversity in honey, may be justifiable, since stingless bees honey, although with higher water content and less sugar concentration than honeybees' – important features that prevent microbial growth, has elevated acidity and antimicrobial activity (VILLACRÉS-GRANDA et al., 2021), as a result of the metabolism microbes that act in honey production by nectar fermentation. These microorganisms include lactic acid bacteria (LAB), common symbionts of the honey stomach of social bees, that are inoculated in nectar and produce bioactive compounds – such as organic acids, enzymes, hydrogen peroxide, antimicrobial peptides, bacteriocins, among others (DE VUYST; LEROY, 2007; OLOFSSON et al., 2016; SYED YAACOB et al., 2018). Described LAB include *Lactobacillus*, *Bifidobacterium* (OLOFSSON et al., 2016) and also *Fructobacillus* species – abundant in stingless bees honey (SYED YAACOB et al., 2018). Other LAB, such as *Bacillus* species from stingless bees honey has been also described to produce different antimicrobial compounds against a broad spectrum of threatening microbes (NGALIMAT et al., 2019). In fact, this genus has been already found as major constituents of honey of honeybees (WEN et al., 2017). Active microorganisms are usually found in fresh honey but metabolites persist in ripened stored honey, thus providing unfavorable survival condition to microorganisms (ANDERSON et al., 2011; OLOFSSON; VÁSQUEZ, 2008b; SYED YAACOB et al., 2018). In this sense, altered fungal and bacterial composition has been observed during ripening process including significant decrease in fungal diversity (WEN et al., 2017). These features might explain why honey have slightly lower microbial diversity, richness, and evenness of OTUs.

3. Replicates from the same species tend to be less dissimilar in bees than honey samples.

The microbial composition in the bee individuals, have a clearer grouping by the species, i.e., bees from the same species have a less dissimilar microbial composition between each sampled colony (replicates). This is not the case for honey samples, at which samples from colonies of different species overlap in community composition (Figure 2 – colored shadows).

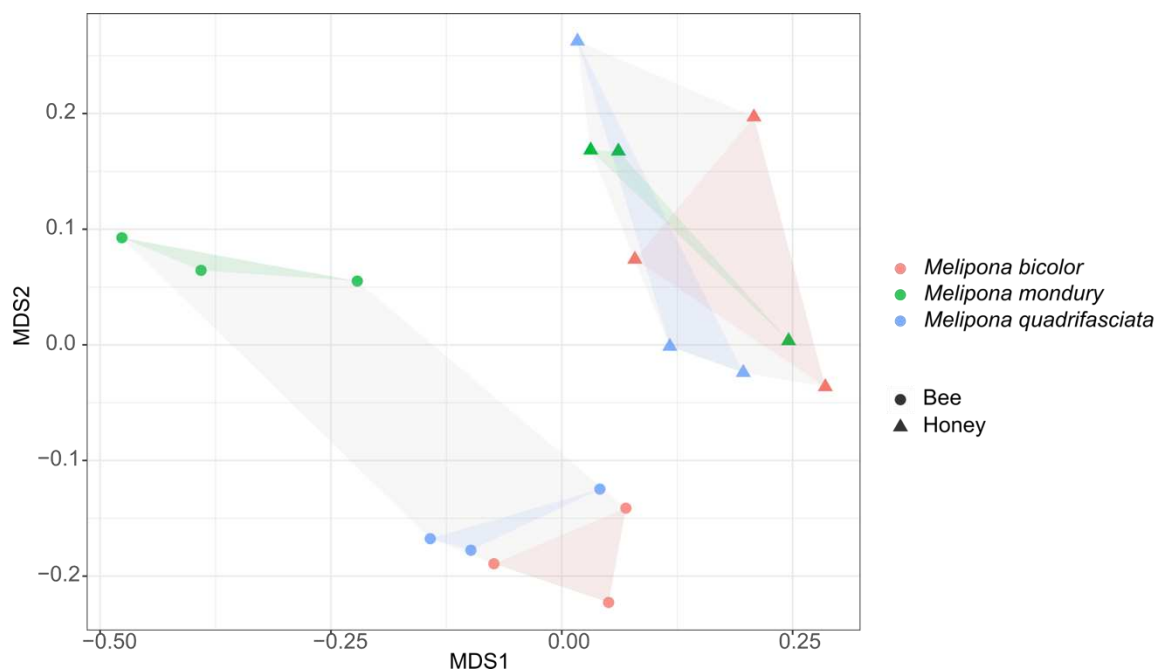


Figure 2 – Non-metric multidimensional analysis based on Bray-Curtis dissimilarity matrix calculated after performing square-root transformations on a rarefied OTU table. Samples closer to each other have less community dissimilarity, harboring a more similar microbial composition. Colored shadows connect samples from the same bee species. Gray shadows connect samples from the same type (i.e. honey or bee).

These observations indicate that bee microbiome tend to be more specific of a host species, which is in accordance with previous observations that bee sociality contributes to microbial vertical transmission between colony members, leading to a co-diversification and co-adaptation between host and symbiont during evolution (revised by KWONG; MORAN, 2016). Thereby, different host species tend to have different bacterial strains of a same species (KOCH et al., 2013), and the microbial community of distantly located populations of a same host species, do not significantly diverge (KWONG et al., 2017). Our honey samples, otherwise, had unclear separation by bee species, thereby indicating less specific microbiome. As mentioned above,

ripened honey environment is not favorable to microbial proliferation and when fresh are predominantly composed by lactic acid bacteria, osmophilic yeasts, and fructophilic microbes (OLOFSSON et al., 2016; SYED YAACOB et al., 2018; DE PAULA et al., 2021). Although some of these microbes inhabit the honey stomach of bees, some are obtained from flowers during nectar foraging activity. Since our sampled species were living in the same area, they share same floral resources at some degree, thus eventually bring colony the same strains from the environment, what can justify overlap in honey composition among different species.

4. Bee and honey samples from the same species harbor dissimilar microbial composition, which tend to be closer between samples from same type.

Community composition of bee and honey samples formed two distinct groups (Fig.2 – gray shadows), thus there is a tendency of grouping by sample type, not by bee species (except for *Melipona mondury* bees samples that grouped separately). Aimed to verify which microbes drive these differences between bees and honey samples, we compared the most abundant families (Fig.3), the habitat specific - indicator OTUs (Fig.4) and the most abundant fungal and bacterial OTUs in the dataset. Also, we evaluated the phylogenetic affiliation of the most abundant OTUs to infer the possible source/origin of microbes present in bees and honey samples (Fig.5).

a. Filamentous fungi are more abundant in bee samples, possibly acquired from environment/plants by foraging activity.

Taken together, our results indicated that the filamentous fungi Cladosporiaceae, Nectriaceae, and Aspergillaceae are significantly higher (Mann-Whitey p-value <0.05) in bees and low abundant in honey samples (Fig.3).

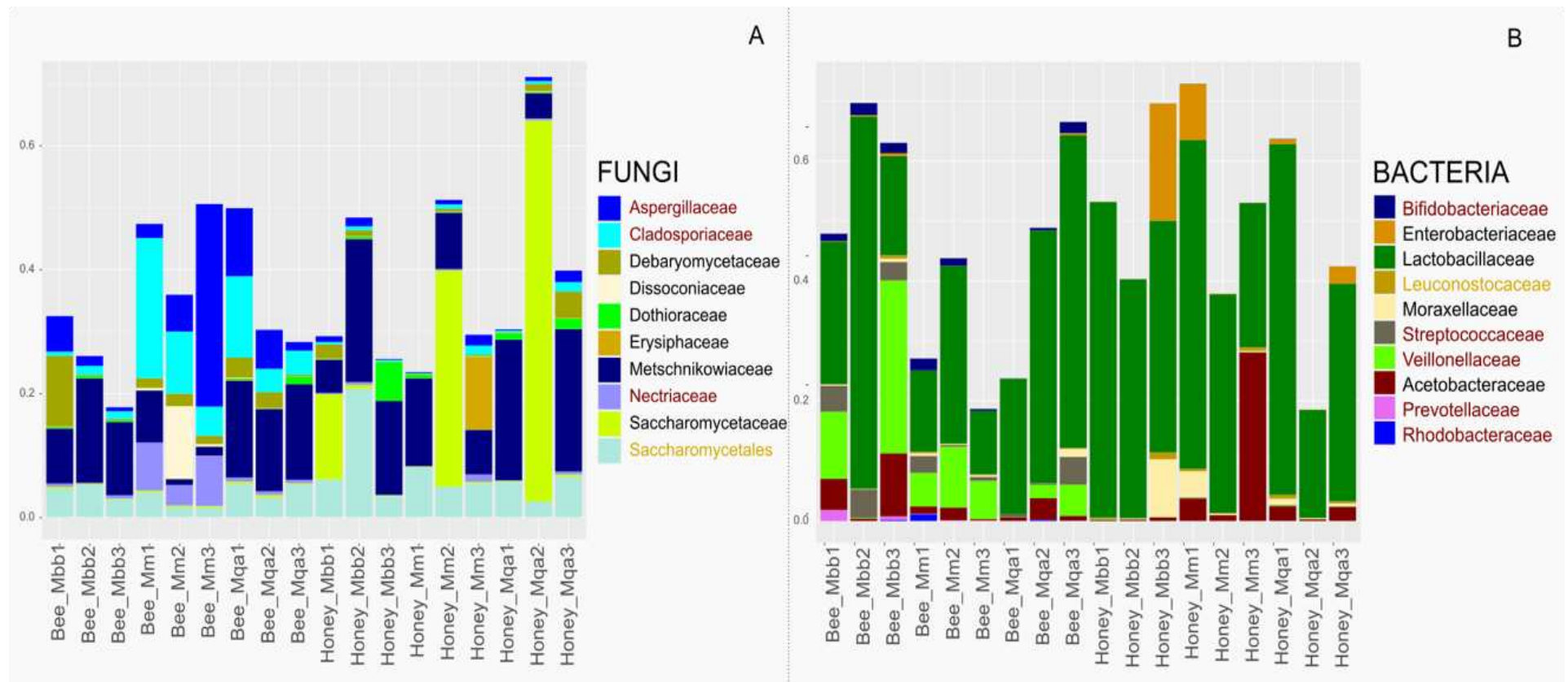


Figure 3 – Relative abundance of the most abundant families of fungi and bacteria in the dataset. Y-axis represents relative abundance in relation to the total merged dataset, and x-axis represents bee or honey samples from Mbb – *Melipona bicolor bicolor*, Mm – *Melipona mondury*, Mqa – *Melipona quadrifasciata anthidioides*. Microbial families written in red color have higher abundance in bees and in yellow color have higher abundance in honey samples (Mann-Whitney p-value <0.05).

Some OTUs classified in genera belonging to these families, such as *Penicillium* and *Monascus* (Aspergillaceae); *Cladosporium* (Cladosporiaceae); *Fusarium* and *Cylindrocladiella* (Nectriaceae), were considered indicator OTUs for bees (Fig.4).

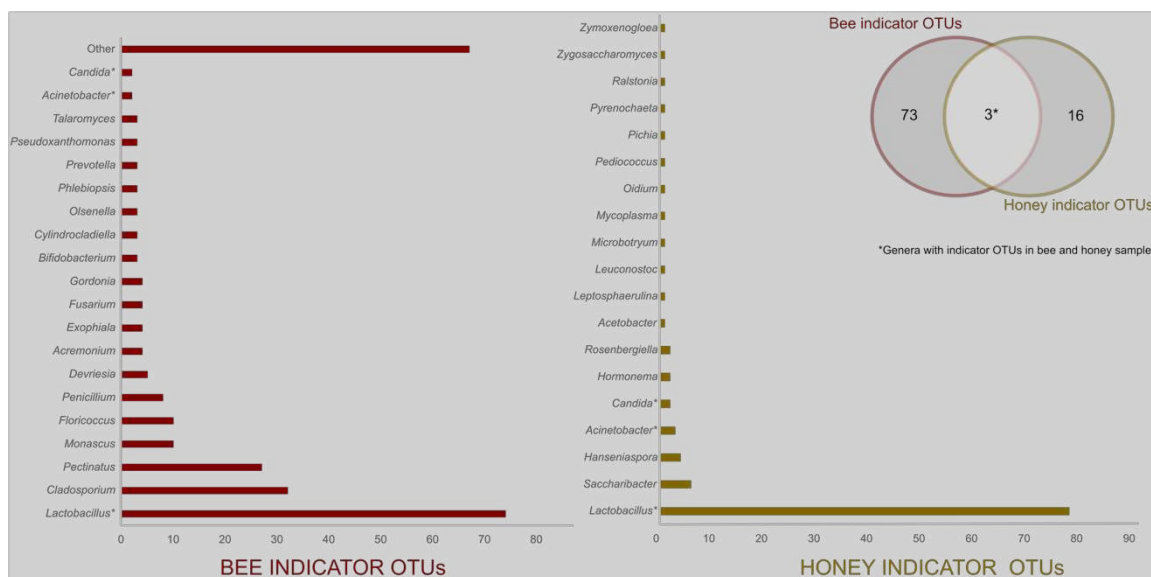


Figure 4 – Number of habitat specific (indicator) OTUs. Barplots represent the number of indicator OTUs and its identification in genus level, for bees (red colored) and honey (yellow colored). Circles represent Venn diagram, showing the amount of genera containing indicator OTUs only for bee (red circles), only for honey (yellow circle) or for both samples (intersection, indicated by the asterisks).

In addition, some of the *Cladosporium* OTUs belonged to the most abundant in the dataset and had significant higher abundance in bee samples (Fig.5 - red colored OTUs).

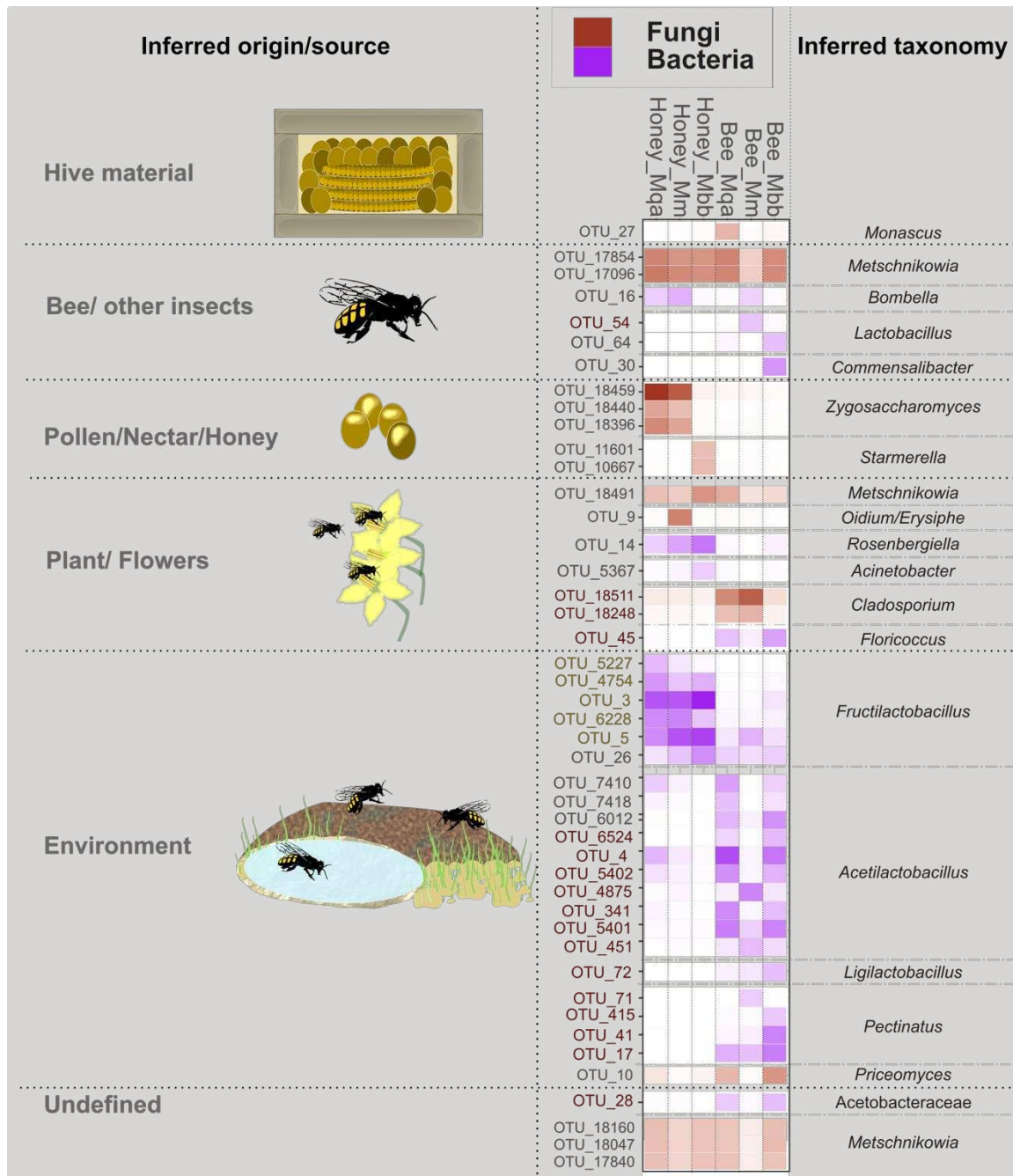


Figure 5– Most abundant OTUs and their possible origin. Heatmap: indicates log-transformed mean fungal (red-colored squares) and bacterial (purple-colored squares) relative abundances for each treatment. Rarefied dataset was used and some of the most abundant OTUs (OTUs with mean relative abundance over 1% in at least one treatment) of the most abundant fungal and bacterial genera are represented. Darker squares correspond to higher log-transformed mean relative abundances for a given OTU in treatment. Red and yellow colored OTUs are significantly higher abundant in bees and honey, respectively (Mann-Whitey p-value <0.05). Draws represent the possible source of acquisition of each OTU, based on the isolation substrate of the phylogenetically closer sequence (check Figures S3-S7 for phylogenies and Table S2 for isolation substrate of each sequence used in phylogenetic trees). Possible sources include Bee/Other insects (bee and other insects associated microbes, including body and gut); Pollen, Nectar, or honey found in bee nests; Plant/Flowers (plant associated microbes, including those in pollen and nectar from flowers); Hive material (microbes from the bee nest environment); and Environment (microbes from all other substrates not included in other categories such as soil, water, oil, beverages, vinegar etc.). Legend: *Mbb* – *Melipona bicolor bicolor*; *Mqa* – *Melipona quadrifasciata anthidioides*; *Mm* – *Melipona mondury*.

Cladosporium has already been found in honeybees located in very close areas to our study site (MODRO et al., 2009), which collected the fungus in a similar manner as pollen collections, i.e. by the formation of pellets. Due to food scarcity and proximity to *Cladosporium* reservoirs (e.g. coffee plantation), *Cladosporium* pellets were used as food source for bees because of its high nutrient and low mineral content (MODRO et al., 2009). Due to its high availability, filamentous fungi fragments are usually brought to colony by stingless bees together with pollen, and spores are believed to be a complement to stingless bees diet (ELTZ; BRÜHL; GÖRKE, 2002; OLIVEIRA; MORATO, 2000). Among the most abundant *Cladosporium* OTUs found in our dataset, OTUs 18511 and 18248 are significantly higher in bees (Mann-Whitney p-value<0.05) and grouped in a clade composed by plant isolates (Fig.S3 and Table S2). Therefore, these fungi might be transiently found in bees and have been collected from plants during pollen foraging, brought to colony and possibly used as food source. More studies will give insights into this association.

Aspergillaceae most abundant genus is *Monascus*. *Monascus* OTUs were found as indicator for bee species (Fig.4). In fact, *Monascus* has already been seen in stingless bees, associated to hive material, being transmitted as bees recycle the material for construction of new brood cells (MENEZES et al., 2015). In addition *Monascus* has been isolated from honey, pollen and inside nests of *Melipona scutellaris* living in Brazilian Atlantic forest (BARBOSA et al., 2017). Hive associated *Monascus* act in the modulation of steroid biosynthesis by *Zygosaccharomyces* in stingless bees (PALUDO et al., 2019). The production of antibacterial compounds (JUZLOVÁ; MARTÍNKOVA; KREN, 1996) and mycotoxin citrinin, which is toxic to animals, but can inhibit gram-positive bacteria (CHEN; INBARAJ, 2016) have already been described for *Monascus* species. Most abundant *Monascus* OTUs found in our datasets are phylogenetically closer to isolates obtained from hive material (OTU_27) and environment (OTU_3), thus indicating that our sampled *Melipona* species possibly keep hive associated *Monascus*, obtained by contact with hive material, and acquire environmental strains (Fig.5, Fig.S4 and Table S2). We believe that *Monascus* is probably a beneficial fungus associated with *Melipona* hive environment and its presence on bees might be derived to bees handling with hive material.

Other filamentous fungi, such as Erysiphaceae and Dothioraceae, did not significantly differed (Mann-Whitney p-value >0.05) but are slightly more abundant in

honey (Fig.3). A tendency of association with honey is corroborated by occurrence of indicator OTUs from these families, such as *Oidium* (Erysiphaceae) and *Hormonema* (Dothioraceae) (Fig.4). Erysiphaceae most abundant OTUs (OTU_9) is not statistically different but also tend to be higher in honey. OTU_9 had unclear phylogenetic classification as *Oidium/Erysiphe* but formed a clade with plant isolates, thus is probably brought to colony from plants by foraging activity (Fig.5, Fig.S3, Table S2). Whether saprophytic or beneficial to honey depends on more evidence.

b. Yeasts are widespread, comprises higher proportion of honey microbiota, and are mostly obtained from plants/environment or persisting insect and hive associated symbionts.

We observed that honey fungal composition, is mostly dominated by yeasts, although some groups as Metschnikowiaceae are widespread in bees and honey samples. Concerning this family, no indicator OTUs of Metschnikowiaceae were found associated with bee or honey samples (Fig.4), indicating absence of habitat specificity for OTUs of this family in both sample types. In fact, *Metschnikowia* genus (Metschnikowiaceae) most abundant OTUs have high incidence in bees and honey samples of the three *Melipona* species (Fig.5). Some OTUs may correspond to yeasts brought to colony by foraging activity. This is the case for OTU 18160, that formed a clade close to isolates from pollen of flowers, water, and a leaf beetle (considered undefined origin in Fig.S5) and OTU 18491 close to yeasts from rotten tree trunks (Fig.5, FigS5, Table S2). Indeed, *Metschnikowia* yeasts are usually encountered in nectar of flowers (SJAMSURIDZAL et al., 2013), at which are associated to the emission of volatile compounds that are attractive to bee pollinators (RERING et al., 2018), and are major fungal genus found in honey (WEN et al., 2017). Since are able to live in high sugar concentration environments, producing sugars naturally found in honey – they possibly have a role in the final composition of honey (GARCIA-GONZALEZ et al., 2019; GARCIA-GONZALEZ et al., 2020).

We also found *Metschnikowia* OTUs 17854 and 17096, that are closer to isolates from Muroid fly, representing lineages associated to insects (Fig.5, Fig S5, Table S2). *Metschnikowia* yeasts has been found in pollen of the carpenter bee *Ceratina australensis* nests (MCFREDERICK; REHAN, 2019) and at the proboscis and

in the digestive tract of bumblebees (BRYSCH-HERZBERG, 2004). We cannot infer whether they are transiently or symbiotically associated to *Melipona*, but since are close insect associated yeasts, they possibly occur in gut and are inoculated in honey.

Our data suggest significant higher abundance of Saccharomycetales yeasts in honey samples. Despite that, these yeasts were present in all analyzed samples (Fig.3). and the presence of two *Candida* indicator OTUs for bee and honey samples (Fig.4), suggest occurrence of habitat specific species for each sample type. Indeed, *Candida* yeasts are commonly associated to floricolous insects, not only already isolated from honey but also from the proboscis and digestive tract of bumblebees (BRYSCH-HERZBERG, 2004). In addition, stingless bees such as *Melipona quadrifasciata*, *Melipona rufiventris* and *Trigona* sp. are also source of *Candida* isolates (LACHANCE et al., 2010). Some *Candida* species, similarly to *Metschnikowia*, depend on bumblebees as host for hibernation in winter, but use flower nectar to boom its reproduction during warm seasons – at which they can be found in bee surface (BRYSCH-HERZBERG, 2004). *Candida* are commonly found in flowers (LACHANCE et al., 2010), thus the high occurrence of these yeasts in honey samples, and also in bees, can indicate that they are acquired from flower nectar during foraging. We cannot infer, however, if the yeasts found in our bee samples are associated with our *Melipona* species gut or transiently present in the bee surface or mouth, since the guts were not separated from the whole body in our analysis.

Saccharomycetaceae are also more prevalent in honey and virtually absent in bee samples (Fig. 3). The occurrence of an indicator OTU (Fig.4) and three high abundant OTUs - 18459, 18440 and 18396 (Fig.5) of *Zygosaccharomyces* (Saccharomycetaceae) associated to honey samples, highlight the prevalence of this genus in this sample type. These abundant OTUs are phylogenetically closer to *Zygosaccharomyces mellis* previously isolated from honey samples (Fig.5, Fig.S5, Table S2). *Zygosaccharomyces* are high osmotolerant yeasts yet associated with eusocial corbiculate bees colonies. This genus is associated to fermentation of *Apis cerana* honey (CHIKANO; TAKAHASHI, 2020) and can inhabit ripe and unripe honey of stingless bees (revised by DE PAULA et al., 2021) and some species are fructophilic (LEANDRO et al., 2011). In addition, *Zygosaccharomyces* has been observed to grow inside brood cells over the larval food of *Scaptotrigona depilis* stingless bee, being ingested as source of sterols, necessary for larvae to reach pupal status (MENEZES

et al., 2015; PALUDO et al., 2018). Thereby, our *Melipona* samples might maintain positive association with *Zygosaccharomyces* yeasts in their honey, and these yeasts are probably kept and recycled with hive material.

Saccharomycetaceae OTUs 11601 and 10667 were found among the most abundant in our dataset and is also abundant in honey samples (Fig.5). These yeasts formed a clade close to *Starmerella cellae* isolated from Pollen-nectar provisions of the solitary bee *Centris tarsata* living in Brazil (Fig.S5, TableS2). Indeed, *Starmerella* yeasts are commonly found associated to stingless bee honey and adult bees, including *Melipona* species (TEIXEIRA et al., 2003) and have specialized niche and fructophily (preference for fructose over glucose) as main metabolism (GONÇALVES et al., 2020). Thus, our findings might indicate maintenance of association between *Starmerella* yeasts, and our stingless bee honey.

Debaryomycetaceae yeasts could be found in bees and honey samples but tend to be higher in bees (Fig.3). The most abundant OTU of this family, *Priceomyces* OTU_10, is slightly high abundant in bees and form a clade with *Priceomyces fermenticarens* isolated from lichen on tree and *Priceomyces melissophilus* isolated from honeybee gut, both in South Africa (Fig.5, Fig S5, Table S2). The latter species has already been isolated from floral nectar (CANTO; HERRERA; RODRIGUEZ, 2017). Thus, this OTU might represent an environmentally acquired microbe, that possibly associate to gut but whether symbiont, or transiently acquired during foraging on plants depends on more evidence.

c. Bee and honey microbiome are associated with either symbiotic transmitted or plant/environmentally acquired bacteria.

Among bacteria, some genus, such as Acetobacteraceae, Lactobacillaceae, Enterobacteriaceae and Moraxellaceae did not statistically differ (Mann-Whitney p-value >0.05) between bee and honey samples (Fig.3) but comprised higher proportion of honey samples. Acetobacteraceae and Lactobacillaceae are widespread in both sample types. Members of Acetobacteraceae family has already been described as abundant in guts of adult bees, in larvae, and in nectar, hive materials and pollen, composing the dominant members of bee gut microbiota (revised by KWONG; MORAN, 2016). Important Acetobacteraceae species are divided in two clades, the

Alpha 2.1 related to *Gluconobacter*, composed by *Commensalibacter* sp. and found in bees guts and hive environment, capable of using a large range of carbon substrate and more specialized to hindgut; and Alpha 2.2, composed by *Parasaccharibacter apium*, *Bombella favorum*, *Bombella mellum* and *Bombella apis*, more transiently associated to bees than Alpha 2.1; possibly adapted to fast-resource utilization in high acidic and sugary environments and common members of food stores, royal jelly, hypopharyngeal glands, forager crop (honey stomach), larvae and queen gut microbiota of honeybees. *P. apium* is usually negligible in nurse and forager mid and hindgut (revised by CORBY-HARRIS et al., 2014; revised by KWONG; MORAN, 2016; BONILLA-ROSSO et al., 2019; revised by NOWAK et al., 2021). Acetobacter-like OTU has been described as associated with Meliponini bees. High levels of environmental Acetobacteraceae are also associated with bees presenting disrupted microbial community due to parasite infection (CARIVEAU et al., 2014).

We observed habitat specificity (indicator OTUs) of Acetobacteraceae in honey samples including 6 *Saccharibacter* and 1 *Acetobacter* OTUs (Fig.4). Among the most abundant OTUs in the dataset the OTU_16 is closely related to Alpha 2.2. This clade consist of two sub-lineages separated by *Bombella intestine* (isolated from the crop of a bumblebee) and related to flower associated *Saccharibacter floricola* (BONILLA-ROSSO et al., 2019). The same pattern was observed; thus, we infer that OTU_16 is Alpha 2.2. As mentioned above, this clade is found in bee honey stomach and food stores, thus the occurrence of OTU_16 in honey and bee samples is in accordance with the niche occupied by Alpha 2.2 (Fig. 5, Fig. S6, Table S2). Acetobacteraceae OTU_30 has phylogenetic proximity with *Commensalibacter intestini* isolated from *Drosophila* midguts (OTU_30) which is close to the Alpha 2.1 clade, commonly referred as *Commensalibacter* sp. (CORBY-HARRIS et al., 2014; BONILLA-ROSSO et al., 2019; revised by NOWAK et al., 2021). This clade harbors generalist bacteria found in hive environments, but is considered a dominant component of the honeybee gut microbiota (revised by KWONG; MORAN, 2016). This OTU was more abundant in bees, which is in accordance with a stable association between Alpha 2.1 and bees (KEŠNEROVÁ et al., 2020) maybe representing a *Commensalibacter* sp (Alpha2.1) gut bacteria of *Melipona* (Fig 5., Fig. S6, Table S2). Acetobacteraceae OTU_28 is significantly higher (Mann-Whitney p-value<0.05) in bees than honey but has undefined classification and origin (Fig 5., Fig. S6, Table S2).

Lactobacillaceae family includes *Lactobacillus* genus, already described as core gut bacteria in social bees - mentioned as Firm-4 and Firm-5 clades; (KWONG et al., 2017; KWONG; MORAN, 2016; MARTINSON et al., 2011) and common lactic acid bacteria (LAB) capable of forming biofilms in the honey stomach of social bees, that are inoculated in nectar for honey production (OLOFSSON et al., 2016). Our data indicated high number of *Lactobacillus* OTUs associated with *Melipona* bee and honey environments. 74 and 78 OTUs are habitat specific for bees or honey, respectively (Fig.4). We also observed high number of Lactobacillaceae among the most abundant OTUs (Fig.5). Some of these are phylogenetically close to environmental *Fructilactobacillus* and significantly associated (Mann-Whitney p-value <0.05) to honey (Fig. 5 yellow colored OTUs, Fig S7, Table S2). *Fructilactobacillus* is a novel genus that contains bacteria previously classified as *Lactobacillus* that have preference for growth using fructose as electron acceptor. Thus, most species of this genus is fructophilic and has been described associated to flowers, insects, spoiled and fermented foods (ZHENG et al., 2020). As soon as fructose is the main sugar component of stingless bee honey (DE SOUSA et al., 2016; VILLACRÉS-GRANDA et al., 2021), prevalence of such environmental *Fructilactobacillus* mostly in honey samples, indicate that they are brought to colony by nectar foraging and act in nectar fermentation and honey production in *Melipona*.

Other Lactobacillaceae OTUs are highly abundant in bees, either significantly (Fig.5 red colored OTUs) or not (Fig.5 black OTUs). Among them, *Lactobacillus* OTUs 54 and 64 have unclear phylogenetic position but both form clades closely related to *Lactobacillus* isolates obtained from social bees gut, *Lactobacillus apis* – from honeybees and *Lactobacillus bombicola* – from bumblebees (Fig. S7 and Table S2). These species are both classified as *Lactobacillus* Firm-5, most abundant core gut bacteria associated to eusocial corbiculate bees, capable of producing antimicrobial substances and probably defending hosts, to uptake sugars by phosphotransferase systems and to biosynthesize/use disaccharides for energy storage, among others (revised by KWONG; MORAN, 2016). *Lactobacillus* OTUs 54 and 64 are more abundant in bees, the first with statistic difference (Mann-Whitney p-value >0.05), reinforcing their association to Firm-5 and gut origin. Remaining Lactobacillaceae OTUs were taxonomically close to environmental isolates (Fig.5, Fig S7, Table S2) such as *Ligilactobacillus* and *Acetilactobacillus*. The social behavior of social bees contributes to the maintenance of a distinctive host-specific microbiota, transmitted

between members of the hive by oral trophallaxis, contact with hive material and fecal-orally. However, environmental microorganisms that are generalists, capable of inhabiting different hosts and environments, thus not co-diversified with bee hosts, are capable of associating with social bees (crop) and hive environment, as is the case for *Apilactobacillus kunkeei* (revised by KWONG; MORAN, 2016; TAMARIT et al., 2015). In facultatively social *Megalopta* bees, social and non-social colonies had similar set of environmentally and maternally acquired microorganisms, including *A. kunkeei* (previously *Lactobacillus kunkeei* [ZHENG et al., 2020]) that dominated some samples, indicating that environmental transmission is more responsible for microbial associations in these bees than social transmission (MCFREDERICK et al., 2014). The occurrence of many environmental *Lactobacillus* OTUs mostly in our bee samples, possibly indicate that environmental microorganisms are important components of *Melipona* microbiome.

Enterobacteriaceae family tended to be higher in honey (Fig.3), and *Rosenbergiella* OTUs were found as being habitat specific (indicator) for honey (Fig.4). In addition, *Rosenbergiella* OTU_14 is among the most abundant OTUs in the dataset, more abundant in honey samples and is closely related to *Rosenbergiella epipactidis* isolated from floral nectar, other plant isolates and a honeybee mouth isolate (Fig.5, Fig S6, Table S2). Indeed, *Rosenbergiella* is commonly found in floral nectar (HALPERN et al., 2013), bee nests (ROTHMAN et al., 2018) and wild bee pollen (MCFREDERICK; REHAN, 2019), thus our *Melipona* honey samples may have acquired *Rosenbergiella* from nectar, brought to colony by foraging activity. The occurrence of a bee mouth isolate in the clade (Fig. S6, Table S2), might indicate a transient occurrence in the mouth because of floral nectar transportation.

The same tendency was observed for Moraxellaceae, higher abundant in honey (Fig.3), with indicator OTUs associated to honey habitat, as is the case for *Acinetobacter* OTUs (Fig.4), and with an *Acinetobacter* OTU 5367 that is slightly more abundant in honey sample. This OTU formed a clade with bee gut (crop to hindgut) and floral nectar isolates, being phylogenetically closer to *Acinetobacter nectaris* isolated from floral nectar (Fig.5, Fig.S6, Table S2). Some *Acinetobacter* OTUs were also indicator for bee samples (Fig.4), suggesting that some lineages can be specific for bees. In fact, some *Acinetobacter* species are found in the intestinal tract of honeybees (KIM et al., 2014). On the other side, this genus is a common member of floral nectar of insect-pollinated plants (ÁLVAREZ-PÉREZ et al., 2013). Therefore, the

prevalence of Moraxellaceae (including high abundant *Acinetobacter* OTU 5367) in honey might indicate that it is brought to colony from nectar obtained by foraging activity. However, occurrence of indicator OTUs in bees and proximity of OTU 5367 to floral nectar and bees can be an indicative that this floral acquired bacterium can remain associated to *Melipona* gut. More information is needed to understand if this association is transient or symbiotic.

Bifidobacteriaceae, Veillonellaceae, Streptococcaceae, Prevotellaceae, Rhodobacteraceae families are significantly more abundant (Mann-Whitney p-value <0.05) in bees than honey (Fig.3), of which we highlight the first 3 families - because of their high abundance. Bifidobacteriaceae indicator OTUs were found in bees, such as the three *Bifidobacterium* OTUs (Fig.4). *Bifidobacterium* is known as “Bifido”, and composes the gut core microbiome of eusocial corbiculate bees (KWONG et al., 2017; MORAN et al., 2012; RAYMANN; MORAN, 2018; RIBIÈRE et al., 2019; SABREE; HANSEN; MORAN, 2012). Although we did not evaluate the colony health status, such as rapid colony number building up and the availability of pollen and honey (RIBIÈRE et al., 2019), the high abundance of gut core lactic acid bacteria such as *Bifidobacterium* (and also *Lactobacillus*) in the three bee species, can be an indicative of bee health, since it protects against pathogens (FORSGREN et al., 2009), helps in pollen fermentation into bee bread and in its spoilage avoidance – inoculated by regurgitation (VÁSQUEZ; OLOFSSON, 2009), and acts in the conversion of nectar to honey (OLOFSSON; VÁSQUEZ, 2008b). Therefore, association between our bee samples and *Bifidobacterium* indicates maintenance of this ancient symbiosis in *Melipona* bees.

Many indicator OTUs for Veillonellaceae genus were also found in bees, as is the case for the 32 OTUs *Pectinatus* associated with bees. None was found in honey (Fig.4). In addition, four *Pectinatus* OTUs are among the most abundant and are significantly more abundant in bees (Mann-Whitney p-value <0.05) than honey (Fig.5). Taken together, these results present a strong association between this genus and bees, but virtual scarcity in honey samples. *Pectinatus* has already been detected in honeybees (KWONG et al., 2017) and stingless bees (BOTINA et al., 2019; KWONG et al., 2017) and is considered as an environmental bacteria, thus possibly acquired during forage or from hive material (KWONG et al., 2017). Indeed, our most abundant *Pectinatus* OTUs are phylogenetically close to environmental isolates (Fig. S7, Table S2), thus our data also suggest *Pectinatus* as being obtained from environment and

being associated to bees, not honey. Since free-living environmental microorganisms can turn into facultative or obligatory host mutualists (revised by DREW; STEVENS; KING, 2021; HOSOKAWA et al., 2016), the degree of association between *Melipona* bees and *Pectinatus*, i.e., whether transiently or symbiotically, deserve more studies.

Similarly, many Streptococcaceae OTUs were found as indicator for bees, as is the case for the 10 *Floricoccus* OTUs (Fig.4). In addition, *Floricoccus* OTU_45 is among the most abundant, is significantly higher (Mann-Whitney p-value <0.05) in bees' samples (Fig.5) and formed a clade closely related to *Floricoccus penangensis* and *Floricoccus tropicus* both isolated from fresh flowers (Fig S7, Table S2). These results suggest that *Floricoccus* are obtained by bees during foraging activity in plants and kept in bees. Again, we cannot infer if this strong association between bees and *Floricoccus* is symbiotic or transient, but as mentioned above, free-living to host-symbiont switch is common in nature.

5. Absence of core symbionts points toward a microbiome disruption in *Melipona* bees.

Core bacteria such as *Gilliamella* (core group "Gamma1") from the Orbaceae and *Snodgrassella* (core group "Beta") from Neisseriaceae families, that coevolved during bees diversification (MORAN et al., 2012) and are frequently found in honeybees - *Apis* (KWONG et al., 2017; MORAN et al., 2012; RAYMANN; MORAN, 2018; RIBIÈRE et al., 2019; SABREE; HANSEN; MORAN, 2012), bumblebees - *Bombus* (KWONG et al., 2017; ROTHMAN et al., 2019, 2020) and some stingless bees - Meliponini (KWONG et al., 2017), were not found in our dataset. These bacteria are known to form biofilms in the bee gut protecting against pathogens, and in the case of *Gilliamella* to degrade pollen pectin (Engel et al., 2012). *Gilliamella* and *Snodgrassella* absence in *Melipona* has already been reported but only a few species were studied (DÍAZ et al., 2017; KOCH et al., 2013). Thereby, the absence of *Gilliamella* and *Snodgrassella* in our data can suggest that these groups are not core members of *Melipona* microbiota, possibly being lost in these species' evolution.

Other interesting features detected in our samples are, the expected absence of honeybee specific *Bartonella* (Bartonellaceae) and *Frischella* (Orbaceae) (KWONG et al., 2017), and the predominance of environmental members of Lactobacillaceae and Firm-5 *Lactobacillus* but virtual absence of Firm-4 among the most abundant.

Therefore, *Melipona* microbiome tends to differ from other eusocial corbiculate bees, including stingless bees, in such a way that widely distributed *Pectinatus* and *Floricoccus*, environmental Lactobacillaceae and Acetobacteraceae and even fungi, possibly arose as new symbionts in *Melipona*.

However, we understand that our limited sampling of *Melipona* species prevent us to infer about symbiont loss. Additionally, although diversity and microbial composition of microbiota has a lot of similarity between gut and the whole-body (RIBIÈRE et al., 2019), the use here of the whole-body instead of only gut, might have masked occurrence of such gut symbionts. We plan to expand sampling to different areas, biomes, and species, using dissected gut to deeply investigate about the *Melipona* core gut microbial community and about such microbiome disruption.

6. Conclusions

In conclusion, we have shown that bee microbiome tends to have higher alpha diversity, and to be more species-specific than honey, that might be more influenced by environmental microbes, shared between different bee species living in the same area. Concerning microbial composition, filamentous fungi tend to be more proportionally abundant in bees than in honey and are acquired from the environment/plants during foraging or are hive associated fungi. Yeasts, otherwise, can be widespread but comprise higher proportion of honey samples. Some yeasts are virtually absent in bees and abundant in honey, vice-versa. The honey abundant yeasts are close to isolates previously found in hive environment and associated to honey metabolism. High abundant yeasts in bees are environmental. Concerning bacteria, honey has high abundance of OTUs close to Acetobacteraceae Alpha 2.2 (*Bombella*), plant derived *Rosenbergiella* (Enterobacteriaceae) and *Acinetobacter* (Moraxellaceae), and environmental *Fructilactobacillus*. Bees, otherwise, are composed by bacteria close to gut symbionts such as Alpha 2.2/ Alpha 2.1 Acetobacteraceae, *Bifidobacterium* and *Lactobacillus* Firm5. OTUs close to environmental/plant derived Lactobacillaceae, *Pectinatus* (Veillonellaceae) and *Floricoccus* (Streptococcaceae) are also abundant in bees. Intriguingly, *Melipona* bees differ from other eusocial corbiculate bees and important symbionts are absent, such as *Gilliamella* and *Snodgrassella*. *Lactobacillus* Firm4 is also not among the most

abundant. Increasing sampling species, biomes and sites may give clues on a possible microbiome disruption in *Melipona* species.

Acknowledgments:

We acknowledge the economic and structural support of the Federal University of Viçosa. The financial support was provided by the CNPq, CAPES Finance code 001 and FAPEMIG.

References:

- AIDAR, D.S. (1996). A mandaçaia: Biologia de abelhas, manejo e multiplicação artificial de colônias de *Melipona quadrifasciata* Lep. (Hymenoptera, Apidae, Meliponinae). Série Monografias 4, **Braz, Journ. Genetics**, 104p.
- ÁLVAREZ-PÉREZ, S. et al. *Acinetobacter nectaris* sp. nov. and *Acinetobacter boissieri* sp. nov., isolated from floral nectar of wild Mediterranean insect-pollinated plants. **International Journal of Systematic and Evolutionary Microbiology**, v. 63, n. PART4, p. 1532–1539, 2013.
- ANDERSON, K. E. et al. An emerging paradigm of colony health: Microbial balance of the honey bee and hive (*Apis mellifera*). **Insectes Sociaux**, v. 58, n. 4, p. 431–444, 2011.
- BARBOSA, R. N. et al. Phylogenetic analysis of *Monascus* and new species from honey, pollen and nests of stingless bees. **Studies in Mycology**, v. 86, p. 29–51, 2017.
- BONILLA-ROSSO, G. et al. Acetobacteraceae in the honey bee gut comprise two distant clades with diverging metabolism and ecological niches. **bioRxiv**, v. 41, n. 0, p. 1–50, 2019.
- BOTINA, L. L. et al. Behavior and gut bacteria of *Partamona helleri* under sublethal exposure to a bioinsecticide and a leaf fertilizer. **Chemosphere**, v. 234, p. 187–195, 2019.
- BROWN, J. C.; ALBRECHT, C. The effect of tropical deforestation on stingless bees of the genus *Melipona* (Insecta : Hymenoptera : Apidae : Meliponini) in central Rondonia , Brazil. **Science**, v. 28, n. 5, p. 623–634, 2001.
- BRYSCH-HERZBERG, M. Ecology of yeasts in plant – bumblebee mutualism in Central Europe. **FEMS Microbiology Ecology**, v. 50, p. 87–100, 2004.
- CANTO, A.; HERRERA, C. M.; RODRIGUEZ, R. Nectar-living yeasts of a tropical host plant community: Diversity and effects on community-wide floral nectar traits. **PeerJ**, v. 2017, n. 7, p. 1–22, 2017.
- CARIVEAU, D. P. et al. Variation in gut microbial communities and its association with pathogen infection in wild bumble bees (*Bombus*). **The ISME Journal**, p. 2369–2379, 2014.
- CHEN, B. H.; INBARAJ, B. S. **12 - Nanomaterial-based sensors for mycotoxin analysis in food**. [s.l.] Elsevier Inc., 2016.
- CHIKANO, M.; TAKAHASHI, J. ICHI. Complete mitochondrial DNA sequence of the yeast *Zygosaccharomyces siamensis* (Saccharomycetes: Saccharomycetales) from fermented honey of the *Apis cerana japonica* in Japan. **Mitochondrial DNA Part B: Resources**, v. 5, n. 3, p. 2645–2647, 2020.
- CORBY-HARRIS, V. et al. Origin and effect of Alpha 2.2 Acetobacteraceae in honey bee larvae and description of *Parasaccharibacter apium* gen. nov., sp. nov. **Applied and Environmental Microbiology**, v. 80, n. 24, p. 7460–7472, 2014.
- DE PAULA, G. T. et al. Stingless bees and microbial interactions. **Current Opinion in Insect Science**, v. 44, p. 41–47, 2021.
- DE SOUSA, J. M. B. et al. Sugar profile, physicochemical and sensory aspects of monofloral honeys produced by different stingless bee species in Brazilian semi-arid region. **LWT - Food Science and Technology**, v. 65, p. 645–651, 2016.
- DE VUYST, L.; LEROY, F. Bacteriocins from lactic acid bacteria: Production, purification, and food applications. **Journal of Molecular Microbiology and Biotechnology**, v. 13, n. 4, p. 194–199, 2007.

- DÍAZ, S. et al. Report on the microbiota of *Melipona quadrifasciata* affected by a recurrent disease. **Journal of Invertebrate Pathology**, v. 143, p. 35–39, 2017.
- DOLEZAL, A. G.; TOTH, A. L. Feedbacks between nutrition and disease in honey bee health. **Current Opinion in Insect Science**, v. 26, p. 114–119, 2018.
- DREW, G. C.; STEVENS, E. J.; KING, K. C. Microbial evolution and transitions along the parasite–mutualist continuum. **Nature Reviews Microbiology**, v. 0123456789, 2021.
- ELTZ, T.; BRÜHL, C. A.; GÖRKE, C. Collection of mold (*Rhizopus* sp.) spores in lieu of pollen by the stingless bee *Trigona collina*. **Insectes Sociaux**, v. 49, p. 28–30, 2002.
- ENGEL, P.; MARTINSON, V. G.; MORAN, N. A. Functional diversity within the simple gut microbiota of the honey bee. **Proceedings of the National Academy of Sciences**, v. 109, n. 27, p. 11002–11007, 2012.
- FORSGRÉN, E. et al. Original article Novel lactic acid bacteria inhibiting *Paenibacillus* larvae in honey bee larvae. **Apidologie**, v. 41, p. 99–108, 2009.
- FREDERICK, Q. S. M. C. et al. Environment or kin : whence do bees obtain acidophilic bacteria ? p. 1754–1768, 2012.
- GARCIA-GONZALEZ, M. et al. Efficient production of isomelezitose by a glucosyltransferase activity in *Metschnikowia reukaufii* cell extracts. **Microbial Biotechnology**, v. 12, n. 6, p. 1274–1285, 2019.
- GARCIA-GONZALEZ, M. et al. Molecular characterization and heterologous expression of two α -glucosidases from *Metschnikowia* spp, both producers of honey sugars. **Microbial Cell Factories**, v. 19, n. 1, p. 1–14, 2020.
- GIANNINI, T. C. G. et al. Crop pollinators in Brazil : a review of reported interactions. **Apidologie**, v. 46, p. 209–223, 2015.
- GONÇALVES, P. et al. The Wickerhamiella/Starmerella clade—A treasure trove for the study of the evolution of yeast metabolism. **Yeast**, v. 37, n. 4, p. 313–320, 2020.
- HALPERN, M. et al. *Rosenbergiella nectarea* gen. nov., sp. nov., in the family Enterobacteriaceae, isolated from floral nectar. **International Journal of Systematic and Evolutionary Microbiology**, v. 63, n. PART 11, p. 4259–4265, 2013.
- HOSOKAWA, T. et al. Obligate bacterial mutualists evolving from environmental bacteria in natural insect populations. **Nature Microbiology**, v. 1, n. 1, p. 1–7, 2016.
- JUZLOVÁ, P.; MARTÍNKOVÁ, L.; KREN, V. Secondary metabolites of the fungus *Monascus* : a review. **Journal of Industrial Microbiology**, v. 16, p. 163–170, 1996.
- KATSNELSON, A. The puzzle in a bee 's gut. **Nature**, v. 521, n. 2014, p. S56, 2015.
- KEŠNEROVÁ, L. et al. Gut microbiota structure differs between honeybees in winter and summer. **ISME Journal**, v. 14, n. 3, p. 801–814, 2020.
- KIM, P. S. et al. *Acinetobacter apis* sp. nov., isolated from the intestinal tract of a honey bee, *Apis mellifera*. **Journal of Microbiology**, v. 52, n. 8, p. 639–645, 2014.
- KOCH, H. et al. Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. **Molecular Ecology**, v. 22, p. 2028–2044, 2013.
- KOCH, H.; SCHMID-HEMPEL, P. Bacterial Communities in Central European Bumblebees : Low Diversity and High Specificity. **Invertebrate Microbiology**, v. 62, p. 121–133, 2011a.
- KOCH, H.; SCHMID-HEMPEL, P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 48, p. 19288–19292, 2011b.
- KWONG, W. K. et al. Dynamic microbiome evolution in social bees. **Science Advances**, v. 3, n. 3, p. 1–17, 2017.
- KWONG, W. K.; MANCENIDO, A. L.; MORAN, N. A. Immune system stimulation by

- the native gut microbiota of honey bees Subject Category : Subject Areas : **R. Soc. open sci**, v. 4, p. 1–9, 2017.
- KWONG, W. K.; MORAN, N. A. Gut microbial communities of social bees. **Nature Reviews Microbiology**, v. 14, p. 374–384, 2016.
- LACHANCE, M.-A. et al. The use of parsimony network analysis for the formal delineation of phylogenetic species of yeasts : *Candida apicola* , *Candida azyma* , and *Candida parazyza* sp . nov . , cosmopolitan yeasts associated with floricolous insects. **Antonie van Leeuwenhoek**, v. 97, p. 155–170, 2010.
- LEANDRO, M. J. et al. The osmotolerant fructophilic yeast *Zygosaccharomyces rouxii* employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters. **Microbiology**, v. 157, n. 2, p. 601–608, 2011.
- MARTINSON, V. G. et al. A simple and distinctive microbiota associated with honey bees and bumble bees. **Molecular Ecology**, v. 20, n. 3, p. 619–628, 2011.
- MCFREDERICK, Q. S. et al. Host species and developmental stage, but not host social structure, affects bacterial community structure in socially polymorphic bees. **FEMS Microbiology Ecology**, v. 88, n. 2, p. 398–406, 2014.
- MCFREDERICK, Q. S.; REHAN, S. M. Wild Bee Pollen Usage and Microbial Communities Co-vary Across Landscapes. **Microbial Ecology**, v. 77, p. 513–522, 2019.
- MENEGATTI, C. et al. *Paenibacillus polymyxa* Associated with the Stingless Bee *Melipona scutellaris* Produces Antimicrobial Compounds against Entomopathogens. **Journal of Chemical Ecology**, v. 44, p. 1158–1169, 2018.
- MENEZES, C. et al. A Brazilian social bee must cultivate fungus to survive. **Current Biology**, v. 25, n. 21, p. 2851–2855, 2015.
- MODRO, A. F. H. et al. Saprophytic Fungus Collection by Africanized Bees in Brazil. **Neotropical Entomology**, v. 38, n. 3, p. 434–436, 2009.
- MORAIS, C. R. DE et al. Ecotoxicological effects of the insecticide *fi pronil* in Brazilian native stingless bees *Melipona scutellaris* (Apidae : Meliponini). **Chemosphere**, v. 206, p. 632–642, 2018.
- MORAN, N. A. et al. Distinctive Gut Microbiota of Honey Bees Assessed Using Deep Sampling from Individual Worker Bees. **PLoS ONE**, v. 7, n. 4, p. 1–10, 2012.
- MOTTA, E. V. S.; RAYMANN, K.; MORAN, N. A. Glyphosate perturbs the gut microbiota of honey bees. **Proceedings of the National Academy of Sciences**, v. 115, n. 41, p. 10305–10310, 2018.
- NGALIMAT, M. S. et al. Characterisation of bacteria isolated from the stingless bee, *Heterotrigona itama*, honey, bee bread and propolis. **PeerJ**, v. 2019, n. 8, p. 1–20, 2019.
- NOWAK, A. et al. Characterization of *Apis mellifera* Gastrointestinal Microbiota and Lactic Acid Bacteria for Honeybee Protection-A Review. **Cells**, v. 10, n. 3, p. 1–29, 2021.
- OLIVEIRA, M. L.; MORATO, E. F. Stingless bees (Hymenoptera, Meliponini) feeding on stinkhorn spores (Fungi, Phallales): robbery or dispersal? **Revista Brasileira de Zoologia**, v. 17, n. 3, p. 881–884, 2000.
- OLOFSSON, T. C. et al. Lactic acid bacterial symbionts in honeybees - an unknown key to honey's antimicrobial and therapeutic activities. **International wound journal**, v. 13, n. 5, p. 668–679, 2016.
- OLOFSSON, T. C.; VÁSQUEZ, A. Detection and Identification of a Novel Lactic Acid Bacterial Flora Within the Honey Stomach of the Honeybee *Apis mellifera*. **Current Microbiology**, v. 57, n. 4, p. 356–363, 2008a.
- OLOFSSON, T. C.; VÁSQUEZ, A. Detection and identification of a novel lactic acid

- bacterial flora within the honey stomach of the honeybee *Apis mellifera*. **Current Microbiology**, v. 57, n. 4, p. 356–363, 2008b.
- PALUDO, C. R. et al. Stingless bee larvae require fungal steroid to pupate. **Scientific Reports**, v. 8, n. 1, p. 1–10, 2018.
- PALUDO, C. R. et al. Microbial community modulates growth of symbiotic fungus required for stingless bee metamorphosis. **PLoS ONE**, v. 14, n. 7, p. 1–12, 2019.
- POWELL, J. E. et al. Routes of Acquisition of the Gut Microbiota of the Honey Bee *Apis mellifera*. **Appl. Environ. Microbiol.**, v. 80, n. 23, p. 7378–7387, 2014.
- POWELL, J. E. et al. Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. **Proceedings of the National Academy of Sciences**, v. 113, n. 48, p. 13887–13892, 2016.
- RAYMANN, K.; MORAN, N. A. The role of the gut microbiome in health and disease of adult honey bee workers. **Current Opinion in Insect Science**, v. 26, p. 97–104, abr. 2018.
- RERING, C. C. et al. Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator. **New Phytologist**, p. 750–759, 2018.
- RIBIÈRE, C. et al. Gut and Whole-Body Microbiota of the Honey Bee Separate Thriving and Non-thriving Hives. **Microbial Ecology (2019)**, v. 78, p. 195–205, 2019.
- ROTHMAN, J. A. et al. Floral and Foliar Source Affect the Bee Nest Microbial Community. **Microbial Ecology**, v. 78, p. 506–516, 2019a.
- ROTHMAN, J. A. et al. The bumble bee microbiome increases survival of bees exposed to selenate toxicity. **Environmental Microbiology**, v. 21, n. 9, p. 3417–3429, 2019b.
- ROTHMAN, J. A. et al. The direct and indirect effects of environmental toxicants on the health of bumblebees and their microbiomes. **Proc. R. Soc. B.**, v. 287, 2020.
- SABREE, Z. L.; HANSEN, A. K.; MORAN, N. A. Independent Studies Using Deep Sequencing Resolve the Same Set of Core Bacterial Species Dominating Gut Communities of Honey Bees. **PLoS ONE**, v. 7, n. 7, p. e41250, 2012.
- SANTOS, S. A. B. et al. Pollination of tomatoes by the stingless bee *Melipona quadrifasciata* and the honey bee *Apis mellifera* (Hymenoptera, Apidae). **Genetics and Molecular Research**, v. 8, n. 2, p. 751–757, 2009.
- SEIDE, V. E. et al. Glyphosate is lethal and Cry toxins alter the development of the stingless bee *Melipona quadrifasciata*. **Environmental Pollution**, v. 243, p. 1854–1860, 2018.
- SEBRAE (2015, September 15). Potencialidades da meliponicultura: criação de abelhas nativas. Retrieved from <http://www.sebrae.com.br/sites/PortalSebrae/artigos/potencialidades-da-meliponicultura-criacao-de-abelhas-nativas,36267383f9cbe410VgnVCM1000003b74010aRCRD>
- SILVA-NETO, C. D. M. et al. The stingless bee mandaçaia (*Melipona quadrifasciata* Lepelletier) increases the quality of greenhouse tomatoes. **Journal of Apicultural Research**, v. 58, n. 1, p. 9–15, 2019.
- SMITH, D. P.; PEAY, K. G. Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. **PLoS ONE**, v. 9, n. 2, 2014.
- SYED YAACOB, S. N. et al. Identification of *Lactobacillus* spp. and *Fructobacillus* spp. isolated from fresh *Heterotrigona itama* honey and their antagonistic activities against clinical pathogenic bacteria. **Journal of Apicultural Research**, v. 57, n. 3, p. 395–405, 2018.

- TAMARIT, D. et al. Functionally structured genomes in *Lactobacillus kunkeei* colonizing the honey crop and food products of honeybees and stingless bees. **Genome Biology and Evolution**, v. 7, n. 6, p. 1455–1473, 2015.
- TEIXEIRA, A. C. P. et al. *Starmerella meliponinorum* sp. nov., a novel ascomycetous yeast species associated with stingless bees. **International Journal of Systematic and Evolutionary Microbiology**, v. 53, p. 339–343, 2003.
- TOMÉ, H. V. V et al. Imidacloprid-Induced Impairment of Mushroom Bodies and Behavior of the Native Stingless Bee *Melipona quadrifasciata anthidioides*. **PLoS ONE**, v. 7, n. 6, p. 1–10, 2012.
- VÁSQUEZ, A.; OLOFSSON, T. C. The lactic acid bacteria involved in the production of bee pollen and bee bread. **Journal of Apicultural Research and Bee World**, v. 48, n. 3, p. 189–195, 2009.
- VILLACRÉS-GRANDA, I. et al. Honey quality parameters, chemical composition and antimicrobial activity in twelve Ecuadorian stingless bees (Apidae: Apinae: Meliponini) tested against multiresistant human pathogens. **Lwt**, v. 140, n. July 2020, 2021.
- WEN, Y. et al. The microbial community dynamics during the vitex honey ripening process in the honeycomb. **Frontiers in Microbiology**, v. 8, n. AUG, p. 1–12, 2017.
- ZHENG, J. et al. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. **International Journal of Systematic and Evolutionary Microbiology**, v. 70, n. 4, p. 2782–2858, 2020.

Supplementary material

Table S1 - Number of fungal and bacterial OTUs of each sample in the merged/rarefied dataset. Legend: Bee represents a pool of five bee individuals from a single colony and Honey represents 50 mL of honey taken from a single hive. Mbb 1-3 represents the *Melipona bicolor bicolor* samples; Mm 1-3 represents *Melipona mondury* samples; Mqa 1-3 represents the *Melipona quadrifasciata anthidioides* samples.

N° of OTUS	Mbb1	Mbb 2	Mbb3	Mm 1	Mm 2	Mm 3	Mqa 1	Mqa2	Mqa 3
Bee fungal OTUs	1271	995	1112	1436	1205	1427	1405	1283	1128
Honey fungal OTUS	1077	1414	849	854	1353	1270	916	1372	1389
Bee bacterial OTUs	416	417	418	363	408	356	260	352	404
Honey bacterial OTUs	327	309	313	383	311	342	382	278	369

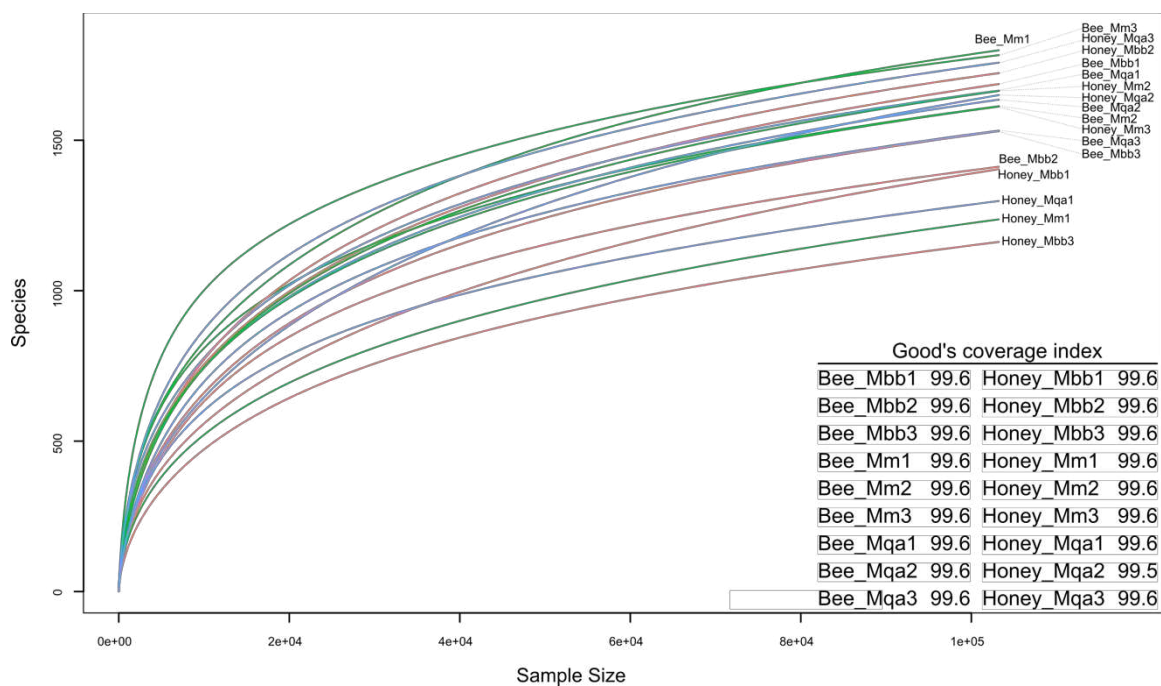


Figure S1 – Rarefaction curves and good's coverage indexes. Legend: Bee represents a pool of five bee individuals from a single colony and Honey represents 50 mL of honey taken from a single hive. Mbb 1-3 represents the *Melipona bicolor bicolor* samples; Mm 1-3 represents *Melipona mondury* samples; Mqa 1-3 represents the *Melipona quadrifasciata anthidioides* samples.

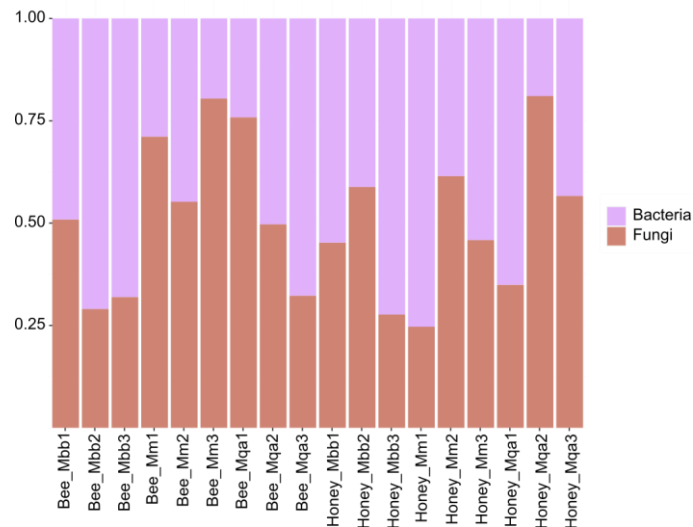


Figure S2 – Proportion of fungal and bacterial in the merged dataset. Legend: Bee represents a pool of five bee individuals from a single colony and Honey represents 50 mL of honey taken from a single hive. Mbb 1-3 represents the *Melipona bicolor bicolor* samples; Mm 1-3 represents *Melipona mondury* samples; Mqa 1-3 represents the *Melipona quadrifasciata anthidioides* samples.

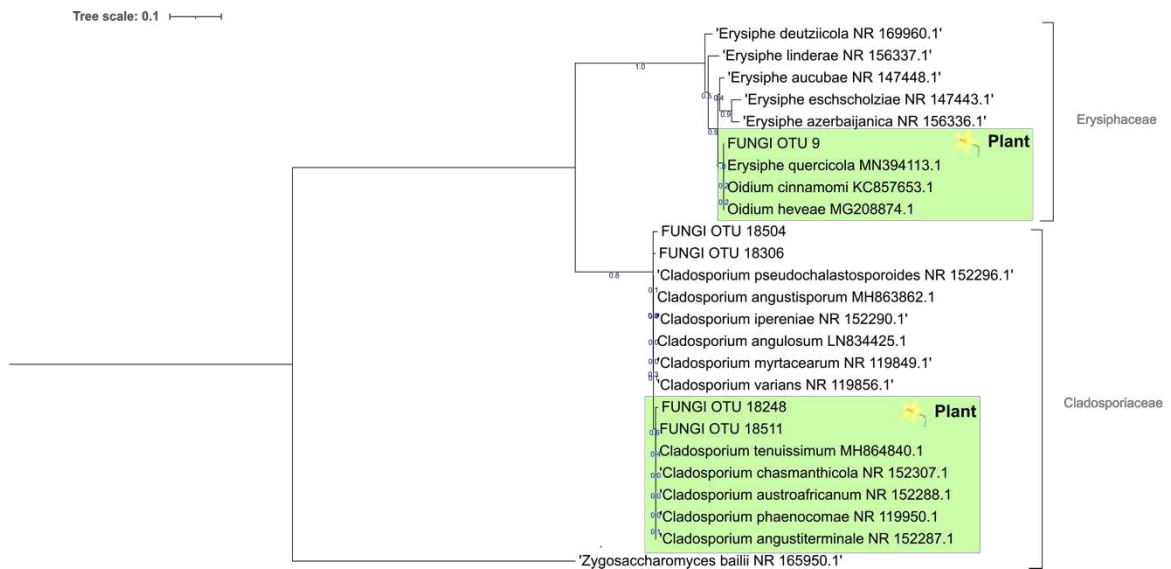


Figure S3 – Phylogenetic tree of the most abundant filamentous fungi OTUs in the dataset. Bootstrap values are shown in blue letters. Sequences used in the tree were obtained in the Genbank database and labeled taxa (green box) are only the most closely related to *Melipona* OTUs. Their source of isolation is presented (information in Table S2). Brackets represent the family which taxa belong. The root was placed on the branch leading to *Zygosaccharomyces bailii*.

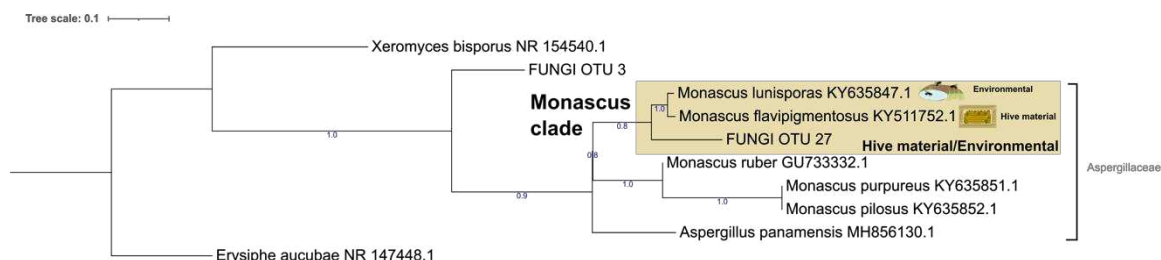


Figure S4 – Phylogenetic tree of the most abundant Aspergillaceae OTUs in the dataset. Bootstrap values are shown in blue letters. Sequences used in the tree were obtained in the Genbank database and labeled taxa (brown box) are only the most closely related to *Melipona* OTUs. Their source of isolation is presented (information in Table S2). Brackets represent the family which taxa belong. The root was placed on the branch leading to *Erysiphe aucubae*.

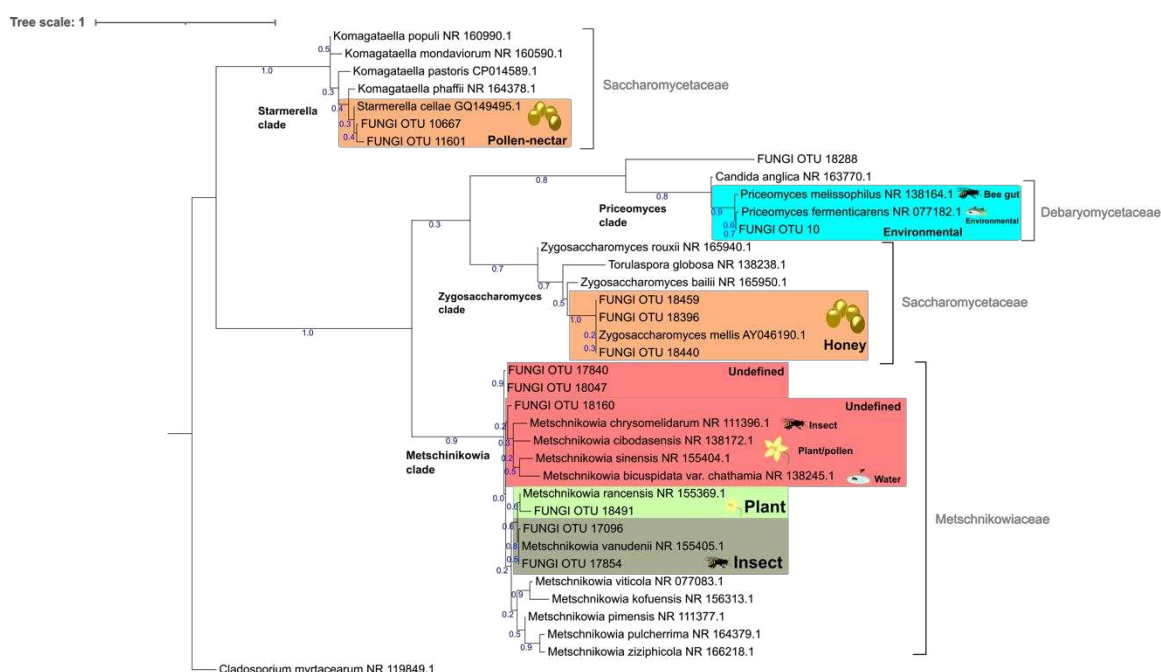


Figure S5 – Phylogenetic tree of the most abundant yeasts OTUs in the dataset. Bootstrap values are shown in blue letters. Sequences used in the tree were obtained in the Genbank database and labeled taxa (colored boxes) are only the most closely related to *Melipona* OTUs. Their source of isolation is presented (information in Table S2). Brackets represent the family which taxa belong. The root was placed on the branch leading to *Cladosporium myrtacearum*.

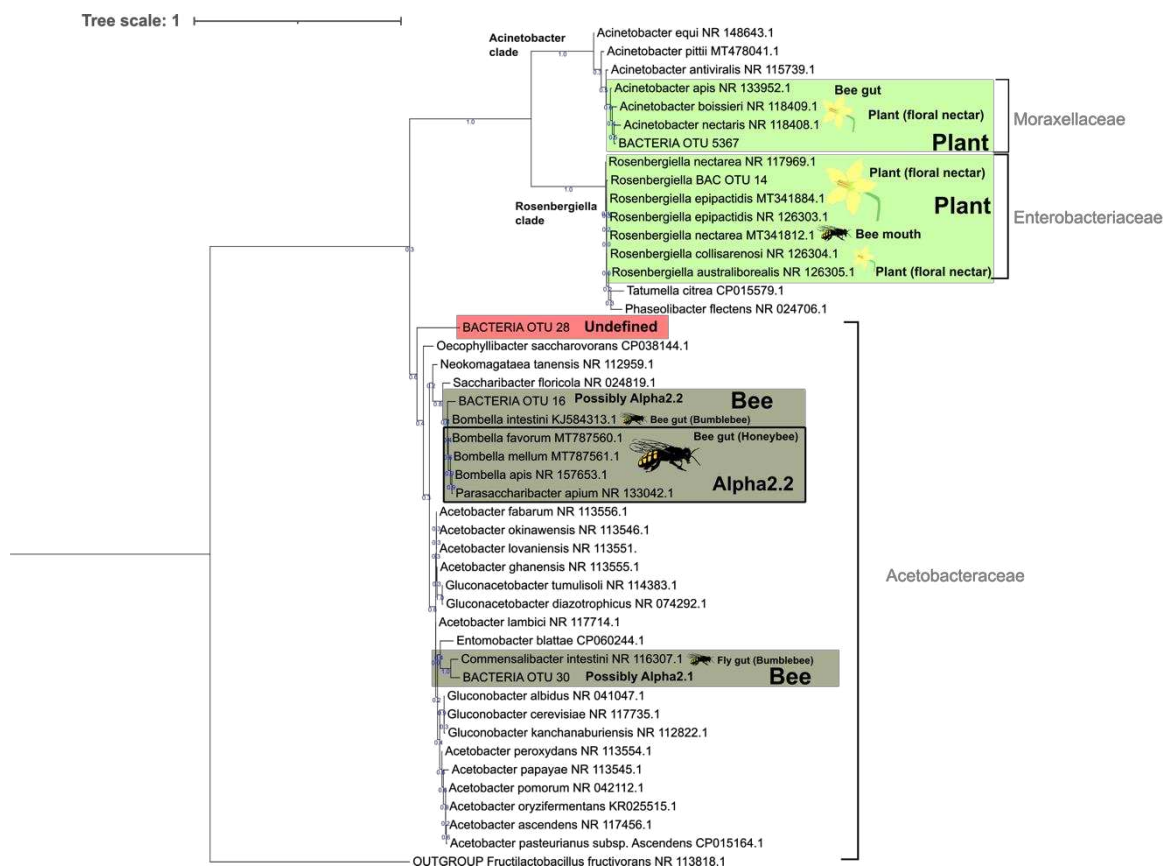


Figure S6 – Phylogenetic tree of the most abundant Proteobacteria OTUs in the dataset. Bootstrap values are shown in blue letters. Sequences used in the tree were obtained in the Genbank database and labeled taxa (colored boxes) are only the most closely related to *Melipona* OTUs. Their source of isolation is presented (information in Table S2). Brackets represent the family which taxa belong. The root was placed on the branch leading to *Fructilactobacillus fructivorans*.

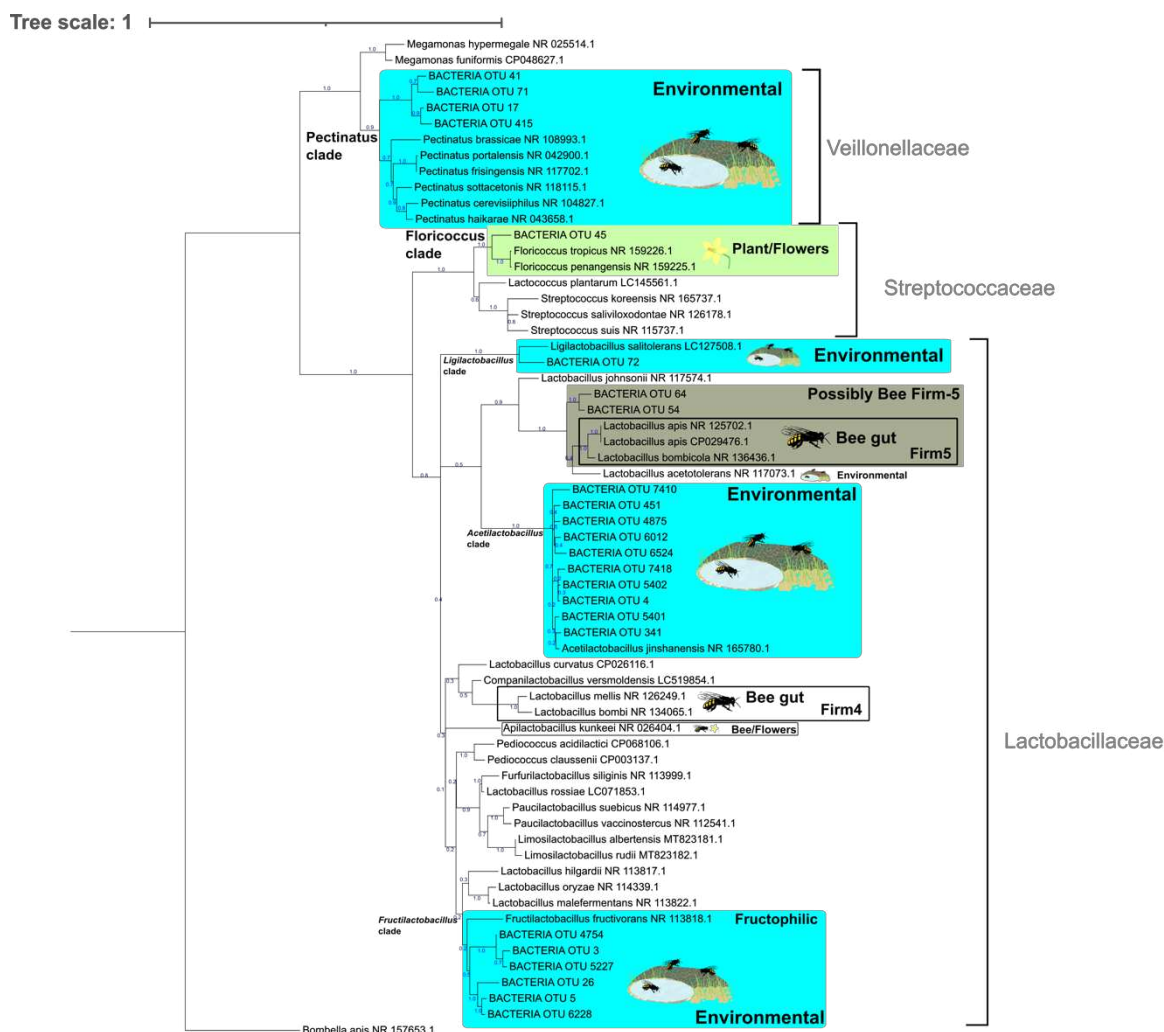


Figure S7 – Phylogenetic tree of the most abundant Firmicutes OTUs in the dataset. Bootstrap values are shown in blue letters. Sequences used in the tree were obtained in the Genbank database and labeled taxa (colored boxes) are only the most closely related to *Melipona* OTUs. Their source of isolation is presented (information in Table S2). Brackets represent the family which taxa belong. The root was placed on the branch leading to *Bombella apis*.

Table S2. GenBank sequences used for analyses.

Species name on the tree	Accession	Isolation substrate	Microbial type
Yeasts tree			
<i>Metschnikowia cibodasensis</i>	NR_138172.1	Plant (pollens) - Isolated from flowers Saurauia pendula - Cibodas Botanic Garden, Indonesia	Yeast
<i>Metschnikowia vanudenii</i>	NR_155405.1	Insect - Walkplate" sampling of mucoid fly collected from A. syriaca	Yeast
<i>Metschnikowia viticola</i>	NR_077083.1	Plant - Berries of grape (Vitis vinifera), red cultivar, Hungary	Yeast
<i>Metschnikowia bicuspidata</i> var. <i>chathamia</i>	NR_138245.1	Water - Lake water, Chatham Island -New Zealand	Yeast
<i>Metschnikowia rancensis</i>	NR_155369.1	Plant - Rotten tree trunk of Laurelia sempervirens, Lake Ranco, Chile	Yeast
<i>Metschnikowia sinensis</i>	NR_155404.1	Plant -From jujube fruit (Ziziphus jujuba Mill.) surface in unmanaged orchard, Shanxi Province, Jiaocheng - China	Yeast
<i>Metschnikowia ziziphicola</i>	NR_166218.1	Plant - from jujube fruit (Ziziphus jujuba Mill.) surface in unmanaged orchard, Shanxi Province, Jiaocheng - China	Yeast
<i>Metschnikowia pulcherrima</i>	NR_164379.1	Plant - berries of Vitis labrusca (Concord grapes) - California - USA	Yeast
<i>Metschnikowia pimensis</i>	NR_111377.1	Insect - gut of Chrysoperla carnea (female), Arizona - USA	Yeast
<i>Metschnikowia chrysomelidarum</i>	NR_111396.1	Insect - unidentified leaf beetle (Coleoptera, Chrysomelidae), Barro Colorado Island - Panamá	Yeast
<i>Metschnikowia kofuensis</i>	NR_156313.1	Plant/fruit - berries of Vitis coignetiae (wild grapes), Yamanashi Prefecture, Kofu - Japan	Yeast
<i>Priceomyces fermenticarens</i>	NR_077182.1	Lichen - lichen, on tree, South Africa	Yeast
<i>Priceomyces melissophilus</i>	NR_138164.1	Gut of Apis mellifica var. adonsonii (honeybee) - South Africa	Yeast
<i>Candida anglica</i>	NR_163770.1	Cider - apple cider - UK	Yeast
<i>Zygosaccharomyces mellis</i>	AY046190.1	Honey - USA	Yeast
<i>Zygosaccharomyces bailii</i>	NR_165950.1	Unkown	Yeast
<i>Torulaspora globosa</i>	NR_138238.1	Soil -- St. Thomas - West Indies (ilhas do caribe)	Yeast
<i>Zygosaccharomyces rouxii</i>	NR_165940.1	Plant/Fruit - Concentrated black-grape must - Italy	Yeast
<i>Komagataella pastoris</i>	CP014589.1	Plant: Exudate from chestnut tree - near Lyon, France	Yeast
<i>Komagataella phaffii</i>	NR_164378.1	Plant: Quercus kelloggii (black oak), California USA	Yeast
<i>Komagataella mondavium</i>	NR_160590.1	Plant: Exudate of cottonwood tree (Populus deltoides), USA: Davis, CA	Yeast
<i>Komagataella populi</i>	NR_160990.1	Plant: Exudate of cottonwood tree (Populus deltoides), USA: Illinois, Peoria	Yeast
<i>Starmerella cellae</i>	GQ149495.1	Nest (pollen): Pollen-nectar provisions of Centris tarsata (solitary bee), Ecological Station of the Universidade Federal de Minas Gerais, Brazil.	Yeast
<i>Cladosporium myrtacearum</i> _OUTGROUP	NR_119849.1	Plant- Corymbia foelscheana - Northern Territory, Emerald Springs, Australia	Filamentous fungi

Filamentous fungi tree			
Oidium heveae (Non-type)	MG208874.1	Plant: Hevea brasiliensis, China	Filamentous fungi
Oidium cinnamomi (Non-type)	KC857653.1	Plant: Cinnamomum camphora, Taiwan	Filamentous fungi
Erysiphe aucubae	NR_147448.1	Plant: Aucuba japonica - Japan: Shiga Prefecture, Maibara-shi, Green Park Santo	Filamentous fungi
Erysiphe azerbaijanica	NR_156336.1	Plant: Castanea sativa - Azerbaijan: Shaki	Filamentous fungi
Erysiphe eschscholziae	NR_147443.1	Plant - Eschscholzia californica - Switzerland	Filamentous fungi
Erysiphe deutzicola	NR_169960.1	Plant - Deutzia parviflora - China	Filamentous fungi
Erysiphe linderae	NR_156337.1	Plant - Lindera praecox - Japan: Mie Prefecture, Suzuka-shi, Kusiro-yama	Filamentous fungi
Erysiphe quercicola (Non Type)	MN394113.1	Plant - Ziziphus jujuba - Pakistan	Filamentous fungi
Cladosporium tenuissimum	MH864840.1	Plant: fruits of Lagerstroemia sp., Louisiana,Baton Rouge - USA	Filamentous fungi
Cladosporium angustisporum	MH863862.1	Plant: Alloxyylon wickhamii - N.Queensland, Daintree N.P., Australia	Filamentous fungi
Cladosporium angulosum	LN834425.1	Animal: Human bronchoalveolar lavage fluid, USA	Filamentous fungi
Cladosporium chasmanthicola	NR_152307.1	Plant - Leaves of Chasmanthe aethiopica - South Africa: Western Cape, Brackenfell	Filamentous fungi
Cladosporium austroafricanum	NR_152288.1	Plant - leaf litter - South Africa - Western Cape Province, Cape Town, next to M3 road	Filamentous fungi
Cladosporium pseudochalastosporoides	NR_152296.1	Plant - Pine needles - Mexico	Filamentous fungi
Cladosporium ipereniae	NR_152290.1	Plant - Puya species - La Serrana, Chile	Filamentous fungi
Cladosporium varians	NR_119856.1	Plant - Leaves of Catalpa bungei - St.Petersburg,botanical garden of the academy, Russia	Filamentous fungi
Cladosporium myrtacearum	NR_119849.1	Plant- Corymbia foelscheana - Northern Territory, Emerald Springs, Australia	Filamentous fungi
Cladosporium angustiterminale	NR_152287.1	Plant - Banksia grandis - Western Australia, Augusta	Filamentous fungi
Cladosporium phaenocomae	NR_119950.1	Plant - leaf bracts of Phaenocoma prolifera, Western Cape Province, Hermanus, Fernkloof	Filamentous fungi
Zygosaccharomyces bailii _ OUTGROUP	NR_165950.1	Nature Reserve - South Africa	Yeast
Unkown			
Monascus tree			
Monascus flavipigmentosus	KY511752.1	Nest (colônia de Melipona): Inside nest of Melipona scutellaris; Recife, Pernambuco,Brazil	Filamentous fungi
Monascus lunisporas	KY635847.1	Ração Mofada: Mouldy feed for race horses - Japan	Filamentous fungi
Monascus pilosus	KY635852.1	Plant/Fermentada: Sorghum vulgare fermented grain, Mansyu I - Japan	Filamentous fungi
Monascus purpureus	KY635851.1	Plant/fermentada: Fermented rice grain, 'ang-quac' (purple coloured rice) - Kagok-Tegal, imported from China, Prov. Quouan-toung, Java	Filamentous fungi
Aspergillus panamensis	MH856130.1	Soil - Panama	Filamentous fungi
Xeromyces bisporus	NR_154540.1	Plant: mouldy stick of liquorice	Filamentous fungi
Monascus ruber	GU733332.1	Soil - Poona, Maharashtra	Filamentous fungi
Erysiphe aucubae_OUTGROUP	NR_147448.1	Plant: Aucuba japonica - Japan: Shiga Prefecture, Maibara-shi, Green Park Santo	Filamentous fungi

Proteobacteria tree			
<i>Oecophyllibacter saccharovorans</i>	CP038144.1	Animal: from weaver ant <i>Oecophylla smaragdina</i> in Kuala Lumpur, Malaysia, Kuala Lumpur	Bacteria
<i>Bombella intestini</i>	KJ584313.1	Animal: isolated from the crop of a bumblebee (<i>Bombus lapidarius</i>) in 2013 in Ghent, Belgium.	Bacteria
<i>Bombella mellum</i>	MT787561.1	Animal: isolate from honeycombs of <i>Apis mellifera</i> , Germany	Bacteria
<i>Bombella apis</i>	NR_157653.1	Animal: The midgut of a honeybee (<i>Apis mellifera</i>), South Korea	Bacteria
<i>Bombella favorum</i>	MT787560.1	Animal: isolate from honeycombs of <i>Apis mellifera</i> , Germany	Bacteria
<i>Saccharibacter floricola</i>	NR_024819.1	Plant: Pollen of Japanese flowers, Kanagawa Prefecture, Japan.	Bacteria
<i>Parasaccharibacter apium</i>	NR_133042.1	Isolated from Honeybee larvae, associated to nurse crops, hypopharyngeal glands (HG), and royal jelly (RJ). Flower of candle bush, <i>Senna alata</i> , Koh-Tan, Hat Khanom-Mu Ko Thale Thai National Park, Nakhon Si Thammarat, Thailand	Bacteria
<i>Neokomagataea tanensis</i>	NR_112959.1		Bacteria
<i>Rosenbergiella epipactidis</i> (Non Type)	MT341884.1	Plant: Floral nectar of <i>Antirrhinum</i> sp., Spain: Barbate, Cadiz	Bacteria
<i>Rosenbergiella nectarea</i> (Non-Type)	MT341812.1	Animal: Mouth of a honeybee (<i>Apis mellifera</i>), USA: Stanford Campus, Stanford, California	Bacteria
<i>Rosenbergiella epipactidis</i>	NR_126303.1	Plant: Floral Nectar of <i>Epipactis palustris</i> , France: Dune du Perroquet, Bray-Dunes	Bacteria
<i>Rosenbergiella collisarenosi</i>	NR_126304.1	Plant: Floral Nectar of <i>Epipactis palustris</i> , Ter Yde, Oostduinkerke Belgium	Bacteria
<i>Rosenbergiella nectarea</i>	NR_117969.1	Plant: flower nectar of <i>Amygdalus communis</i> (almond) in northern Israel	Bacteria
<i>Rosenbergiella australiborealis</i>	NR_126305.1	Plant: Flower Nectar of <i>Protea roupelliae</i> (Proteaceae), mount Gilboa South Africa	Bacteria
<i>Phaseolibacter flectens</i>	NR_024706.1	Plant: (bean pod) Australia Host	Bacteria
<i>Tatumella citrea</i>	CP015579.1	Plant: Mandarin orange, Japan	Bacteria
<i>Acinetobacter apis</i>	NR_133952.1	Animal: intestinal tract (from the crop to the hindgut) of <i>Apis mellifera</i> , Pocheon, Korea	Bacteria
<i>Acinetobacter nectaris</i>	NR_118408.1	Plant: Floral nectar of <i>Muscari comosum</i> (L.) Mill. (Hyacinthaceae), surroundings of Donˆana's Natural Park (Huelva province, SW Spain)	Bacteria
<i>Acinetobacter boissieri</i>	NR_118409.1	Plant: Floral nectar of <i>Fritillaria lusitanica</i> Wikstr. (Liliaceae), surroundings of Donˆana's Natural Park (Huelva province, SW Spain)	Bacteria
<i>Acinetobacter antiviralis</i>	NR_115739.1	Plant: from Tobacco plant roots	Bacteria
<i>Acinetobacter equi</i>	NR_148643.1	Animal: faeces of a horse collected in Minsleben (Germany)	Bacteria
<i>Acinetobacter pittii</i> (NonType)	MT478041.1	Soil: Rhizospheric soil of mangroves, India: NAVI MUMBAI	Bacteria
<i>Acetobacter peroxydans</i>	NR_113554.1	Water: Ditch water, Delft Netherlands	Bacteria
<i>Acetobacter ascendens</i>	NR_117456.1	Quick vinegar	Bacteria
<i>Acetobacter oryzifermentans</i>	KR025515.1	Vinegar, South Korea	Bacteria
<i>Acetobacter papayae</i>	NR_113545.1	Plant/Fruit: isolated from a papaya fruit collected in Okinawa Prefecture in Japan	Bacteria
<i>Acetobacter pomorum</i>	NR_042112.1	Vinegar: Submerged cider vinegar fermentation at a factory, Esslingen Germany	Bacteria
<i>Acetobacter pasteurianus</i> subsp. <i>Ascendens</i>	CP015164.1	-	Bacteria
<i>Commensalibacter intestini</i>	NR_116307.1	Animal (Gut): isolated from <i>Drosophila</i> midguts	Bacteria
<i>Acetobacter fabarum</i>	NR_113556.1	food/Plant: isolated from a Ghanaian cocoa bean heap fermentation	Bacteria
<i>Acetobacter lovaniensis</i>	NR_113551.1	Soil: sewage on soil, Becquevoort, Belgium	Bacteria
<i>Acetobacter okinawensis</i>	NR_113546.1	Plant: isolated from a piece of the stem of sugarcane collected in Okinawa Prefecture in Japan	Bacteria
<i>Acetobacter lambici</i>	NR_117714.1	Beverage: isolated from fermenting lambic beer	Bacteria
<i>Acetobacter ghanensis</i>	NR_113555.1	Fermented cocoa beans, Ghana	Bacteria
<i>Gluconacetobacter diazotrophicus</i>	NR_074292.1	Plant/Roots: Sugarcane roots, Alagoas, Brazil	Bacteria

<i>Gluconacetobacter tumulisoli</i>	NR_114383.1	Soil: Japan	Bacteria
<i>Gluconobacter kanchanaburiensis</i>	NR_112822.1		Bacteria
<i>Gluconobacter cerevisiae</i>	NR_117735.1		Bacteria
<i>Entomobacter blattae</i>	CP060244.1		Bacteria
<i>Gluconobacter albidus</i>	NR_041047.1		Bacteria
<i>Fructilactobacillus fructivorans</i> _ OUTGROUP	NR_113818.1	Beverage: Spoiled sake, Japan	Bacteria

Firmicutes tree

<i>Floricoccus penangensis</i>	NR_159225.1	Plant: isolated from fresh flowers of durian (<i>Durio zibethinus</i>) in an orchard in Teluk Bahang, Penang, Malaysia	Bacteria
<i>Floricoccus tropicus</i>	NR_159226.1	Plant: from fresh flowers of Hibiscus (<i>Hibiscus rosa-sinensis</i> L.) in the campus ground of Universiti Sains Malaysia, in Penang, Malaysia	Bacteria
<i>Lactococcus plantarum</i>	LC145561.1	Plant/food: Frozen peas	Bacteria
<i>Streptococcus suis</i>	NR_115737.1	Animal: Pig	Bacteria
<i>Streptococcus salivloxodontae</i>	NR_126178.1	Animal: isolated from the elephant oral cavity in a zoo (Ichihara Elephant Kingdom, Chiba, Japan)	Bacteria
<i>Streptococcus koreensis</i>	NR_165737.1	Animal/Human: isolated from the human subgingival dental plaque of a periodontitis lesion, in the Republic of Korea	Bacteria
<i>Pectinatus frisingensis</i>	NR_117702.1	Beverage: Turbid beer, Finland	Bacteria
<i>Pectinatus haikarae</i>	NR_043658.1	Air/Beverage: brewery bottling hall in Finland. Strains have also been isolated from spoiled German and Finnish beers	Bacteria
<i>Pectinatus cerevisiophilus</i>	NR_104827.1	Beverage: Packaged beer	Bacteria
<i>Pectinatus sottacetonis</i>	NR_118115.1	Food (tanque de fermentação de pickles): Isolated from a commercial pickle spoilage tank in North Carolina, USA.	Bacteria
<i>Pectinatus brassicae</i>	NR_108993.1	Food: salty pickle wastewater, China	Bacteria
<i>Megamonas funiformis</i>	CP048627.1	Zhejiang Province, Cixi	Bacteria
<i>Pectinatus portalensis</i>	NR_042900.1	Animal: human faeces (healthy adult), Japan	Bacteria
<i>Megamonas hypermegale</i>	NR_025514.1	Wastewater: isolated from a wastewater treatment plant collecting the sewage and wastes of a Southwestern Spanish city, Jerez de la Frontera	Bacteria
<i>Ligilactobacillus salitolerans</i>	LC127508.1	Animal: Chicken caecum	Bacteria
<i>Lactobacillus johnsonii</i>	NR_117574.1	Plant/Fungi Mushroom bed, spent mushroom substrates. Hara-mura, Nagano, J Japan	Bacteria
<i>Lactobacillus apis</i> (Non Type)	CP029476.1	Animal: Human blood, Isolated from human blood and feces of chicken, mice, calf, and pig.	Bacteria
<i>Lactobacillus apis</i>	NR_125702.1	Animal: Gut of Honeybee (<i>Apis mellifera</i>), Switzerland: Lausanne	Bacteria
<i>Lactobacillus bombicola</i>	NR_136436.1	Stomach of Honeybees	Bacteria
<i>Lactobacillus acetotolerans</i>	NR_117073.1	Animal: isolated from the gut of a <i>Bombus lapidarius</i> bumblebee in 2013 in the region of Ghent, Belgium	Bacteria
<i>Acetilactobacillus jinshanensis</i>	NR_165780.1	fermented broth of rice vinegar	Bacteria
<i>Lactobacillus curvatus</i>	CP026116.1	Vinegar: Solid-State Vinegar Culture of Zhenjiang Aromatic Vinegar, Zhenjiang, Jiangsu province, China	Bacteria
<i>Companilactobacillus versmoldensis</i>	LC519854.1	Milk, Germany	Bacteria
<i>Lactobacillus mellis</i>	NR_126249.1	Food: Poultry salami, Germany	Bacteria
<i>Lactobacillus bombi</i>	NR_134065.1	Honey stomach of the honeybee <i>Apis mellifera</i>	Bacteria
		Animal: Digestive tract of a bumblebee queen (<i>Bombus terrestris</i>)	Bacteria

<i>Apilactobacillus kunkeei</i>	NR_026404.1	Partially fermented grape juice.	Bacteria
<i>Pediococcus acidilactici</i>	CP068106.1	Barley (cevada)	Bacteria
<i>Pediococcus clausenii</i>	CP003137.1	Beverage: spoiled beer	Bacteria
<i>Furfurilactobacillus siliginis</i>	NR_113999.1	Wheat sourdough in Daejeon, South Korea	Bacteria
<i>Lactobacillus rossiae</i>	LC071853.1	Fermento: wheat sourdough (fermento de trigo), Italy	Bacteria
<i>Paucilactobacillus suebicus</i>	NR_114977.1	apple mash, Germany	Bacteria
<i>Paucilactobacillus vaccinoferus</i>	NR_112541.1	Animal: Cow Dung (Esterco), Atsugi-shi Kanagawa Japan	Bacteria
<i>Limosilactobacillus albertensis</i>	MT823181.1	Animal: stomach of a hamster in the USA	Bacteria
		Animal: the faecal sample of striped mouse (<i>Rhabdomys pumilio</i>) raised at Henry Doorly Zoo and Aquarium (Omaha, NE, USA)	
<i>Limosilactobacillus rudii</i>	MT823182.1		Bacteria
<i>Lactobacillus hilgardii</i>	NR_113817.1	Beverage: beer	Bacteria
<i>Lactobacillus oryzae</i>	NR_114339.1	Plant/Rice: fermented rice grains in Tochigi, Japan	Bacteria
<i>Lactobacillus malefermentans</i>	NR_113822.1	Beverage: Fermented beverages, beer	Bacteria
<i>Fructilactobacillus fructivorans</i>	NR_113818.1	Beverage: Spoiled sake, Japan	Bacteria
<i>Bombella apis</i> OUTGROUP	NR_157653.1	Animal: The midgut of a honeybee (<i>Apis mellifera</i>), South Korea	Bacteria

Chapter 2: Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees.

This chapter is reproduced from the accepted manuscript and supplementary material of the reference below:

CERQUEIRA, A. E. S.; HAMMER, T.J.; MORAN, N. A.; SANTANA, W. C.; KASUYA, M. C. M.; SILVA, C. C. Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees. **ISME J**, v.15, p. 2813–2816, 2021.
<https://doi.org/10.1038/s41396-021-01000-1>.

The original published paper is available at the following link:

<https://www.nature.com/articles/s41396-021-01000-1>

ABSTRACT

Animal-microbe symbioses are often stable for millions of years. An example is the clade consisting of social corbiculate bees—honeybees, bumblebees, and stingless bees—in which a shared ancestor acquired specialized gut bacteria that subsequently diversified with hosts. This model may be incomplete, however, as few microbiomes have been characterized for stingless bees, which are diverse and ecologically dominant pollinators in the tropics. We surveyed gut microbiomes of Brazilian stingless bees, focusing on the genus *Melipona*, for which we sampled multiple species and biomes. Strikingly, *Melipona* lacks *Snodgrassella* and *Gilliamella*, bacterial symbionts ubiquitous in other social corbiculate bees. Instead, *Melipona* species harbor more environmental bacteria and bee-specific *Starmerella* yeasts. Loss of *Snodgrassella* and *Gilliamella* may stem from ecological shifts in *Melipona* or the acquisition of new symbionts as functional replacements. Our findings demonstrate the value of broadly sampling microbiome biodiversity and show that even ancient symbioses can be lost.

Keywords: *Melipona*, corbiculate bees, microbiome, symbiosis, *Snodgrassella*, *Gilliamella*

Introduction

Microbial symbionts have contributed to the diversification of many animal lineages. Host-microbe associations are often stable (BOURGUIGNON et al., 2018; MORAN; MCCUTCHEON; NAKABACHI, 2008); however, ancient symbionts can be lost, and sometimes replaced by novel symbionts (MATSUURA et al., 2018; CHONG; MORAN, 2018). These transitions may be driven by erosion of symbiont functionality (BENNETT; MORAN, 2015) or shifts in host ecology (SUDAKARAN; KOST; KALTENPOTH, 2017). Although symbiont turnover has been documented for intracellular symbionts, its prevalence among gut symbionts is unclear. Here we report the dissolution of a long-standing symbiosis between social bees and specialized gut bacteria.

The eusocial corbiculate bees (Apidae) contain distinctive gut bacterial symbionts that are restricted to bees and that have co-diversified with their hosts (KWONG et al., 2017; KWONG; MORAN, 2016; MARTINSON et al., 2011). In *Apis mellifera* (honeybees) and *Bombus impatiens* and *B. terrestris* (bumblebees), *Snodgrassella*, *Gilliamella* and *Lactobacillus* have been observed to contribute to host health (KOCH; SCHMID-HEMPEL, 2011; MOCKLER et al., 2018; ROTHMAN et al., 2019; ZHENG et al., 2017). However, gut microbiomes of stingless bees (Meliponini), the most diverse clade of highly social bees and major pollinators in the tropics (GIANNINI et al., 2015), are understudied, and seem to vary more than microbiomes of other social bees (DÍAZ et al., 2017; KOCH et al., 2013; KWONG et al., 2017; LEONHARDT; KALTENPOTH, 2014). Here, we characterize gut bacteria and fungi associated with the genus *Melipona* from multiple Brazilian biomes.

Methods and Results

We sampled 3 colonies from each of 23 *Melipona* populations representing 4 Brazilian biomes (SISBIO/ICMBio authorization #69500-1). Three colonies of *Apis mellifera* and 8 of the stingless bee *Tetragonisca angustula* were also collected from one site. A pool of guts from five individuals/colony had regions of bacterial 16S rRNA and fungal ITS1 genes sequenced to characterize gut microbial community composition (Table S1), and we analyzed these data (SRA accession #PRJNA678404) together with previously published data (KWONG et al., 2017) (see Supplementary Methods). Our results show that *Melipona* lack *Snodgrassella* and *Gilliamella*, core gut symbionts of honeybees, bumblebees, and some other stingless bee clades (Fig. 1A). As expected, honeybee-specific *Bartonella apis* was also absent. These taxa were significantly lower in relative abundance in *Melipona* as compared with *A. mellifera* (Kruskal-Wallis $p < 0.05$) (Fig. 1A).

The most abundant and consistent bacteria in *Melipona* belong to *Lactobacillus*, Bifidobacteriaceae, Acetobacteraceae, and *Floricoccus* (Fig. 1B). For the core *Melipona* bacterial taxa (i.e., taxa containing exact sequence variants [ESVs] in all *Melipona* samples), we constructed phylogenies using the most abundant ESVs and related sequences from GenBank to differentiate widespread environmental taxa from specialized associates of social bees. *Melipona*-associated *Lactobacillus* and Acetobacteraceae include putatively environmental ESVs as well as bee-specific ESVs (Fig. 1B, Fig. S1, Fig. S2, Table S2). Bifidobacteriaceae ESVs in *Melipona* are closely related to bee isolates (Fig. 1b, Fig. S3, Table S2). In contrast, the core *Floricoccus* ESV is closely related to environmental isolates (Fig. 1B, Fig. S4, Table S2).

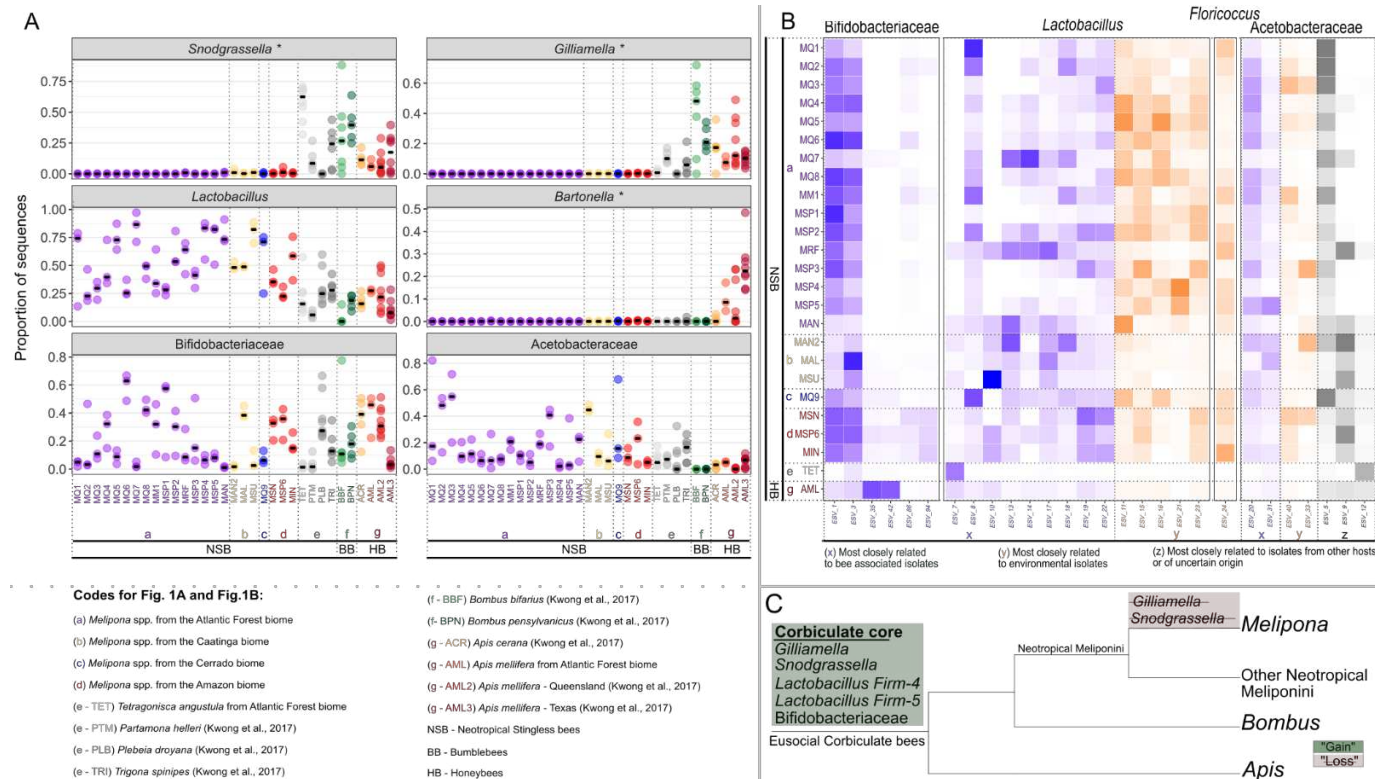


Figure 1 – Gut bacterial community composition and a model of symbiont gain and loss in eusocial corbiculate bees. (A) Distribution of six bee-specific gut bacterial symbionts across Neotropical stingless bees (NSB), bumblebees (BB) and honeybees (HB). Bee populations are on the x-axis (more detail in Table S1). Previously published data (KWONG et al., 2017) are included for comparison. The y-axis shows the relative abundance of each bacterial group as the proportion of sequences. Circles represent replicate bee samples, and black bars represent the median proportion for each bee population. Taxa with significantly higher relative abundances in *Apis mellifera* than *Melipona* are denoted with an asterisk. (B) Heatmap comprising the most abundant ESVs belonging to *Melipona* core bacterial taxa (i.e. taxa present in all samples of all *Melipona* populations). The x-axis represents ESVs labeled by the source from which close relatives were isolated (Figs. S1-S4), and the y-axis represents each bee population sequenced. Darker squares correspond to higher log-transformed mean relative abundances for a given ESV in each bee population. (C) Phylogeny of eusocial corbiculate bees (based on (RAMÍREZ et al., 2010)) showing the gains (KWONG et al., 2017; KWONG; MORAN, 2016; MARTINSON et al., 2011) and our hypothesized losses of core symbionts. A color version of this figure is available in the Supplementary Material.

The fungal gut microbiome of *Melipona* is mainly composed of *Starmerella*, *Lachancea*, *Zygosaccharomyces*, and a few other yeasts (Fig. 2). Of these, *Starmerella* are noteworthy in that they form bee/beehive-specific clades (Fig. S5), were present in all *Melipona* samples and comprised a higher proportion of gut microbiomes in *Melipona* than in other social bee species (Kruskal-Wallis $p < 0.05$) (Fig. 2). Apparent symbiont loss from *Melipona* (Fig. 1C) is not due to incomplete sampling, as rarefaction curves confirm sufficient characterization of bacterial communities (Table S3, Fig. S6).

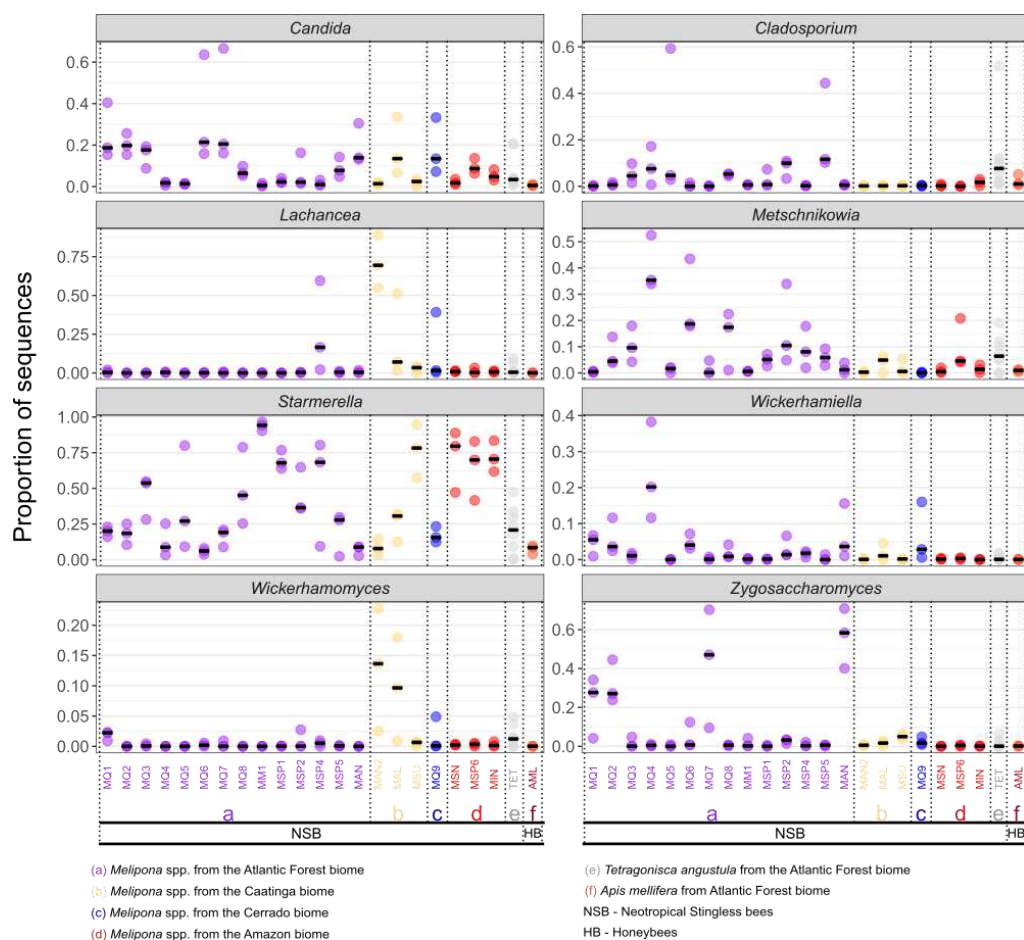


Figure 2 - Distribution of eight dominant fungi across *Melipona*, *Tetragonisca angustula* and *Apis mellifera*. The x-axis represents sequenced bee populations (Table S1). The y-axis shows the relative abundance of each fungal genus as the proportion of sequences for each bee population. Circles represent replicate bee samples, and black bars represent the median proportion for each bee population. A color version of this figure is available in the Supplementary Material.

Discussion

Although present in our *A. mellifera* and *T. angustula* samples, *Snodgrassella* and *Gilliamella* were extremely rare or absent in samples of 23 *Melipona* populations from four biomes in Brazil (Fig. 1A). When present, the low abundance likely reflects artefacts such as cross-contamination. These findings, together with previous work (DÍAZ et al., 2017; KOCH et al., 2013), suggest that *Snodgrassella* and *Gilliamella* have been lost entirely, or almost entirely, from the sampled species of *Melipona*.

This shift is unlikely to have occurred in the common ancestor of stingless bees, because several other Neotropical stingless bees, including *T. angustula* and other species occurring in Brazil, do maintain these associations (KWONG et al., 2017) (Fig. 1A and C). Furthermore, such losses are not geographically restricted as they were observed in *Melipona* from four Brazilian biomes (Fig. 1A) and from Central America (KOCH et al., 2013). While a few other stingless bee genera have been reported to lack *Snodgrassella* and *Gilliamella*, based on limited sampling (KOCH et al., 2013; KWONG et al., 2017; LEONHARDT; KALTENPOTH, 2014), our finding of consistent absence from *Melipona* provides the strongest evidence of symbiont loss in a major social bee clade. Since *Snodgrassella* and *Gilliamella* can contribute to the health of honeybees and bumblebees (KOCH; SCHMID-HEMPEL, 2011; MOCKLER et al., 2018; ROTHMAN et al., 2019; ZHENG et al., 2017), it is possible that *Melipona* has undergone ecological shifts that release them from dependence on symbiont-based nutrition or defense. Alternatively, either persisting members of the ancestral microbiome, or newly acquired symbionts, may compensate for their absence. *Melipona* retains bee-specialized strains of *Lactobacillus* (Fig. S1), Acetobacteraceae (Fig. S2), and Bifidobacteriaceae (Fig. S3), which in theory could have gained new functions, such as metabolic and protective capabilities. *Melipona* has also gained new microbial associations, such as environmental strains of *Lactobacillus*, Acetobacteraceae, and *Floricoccus*. These bacteria are widespread in *Melipona* but only occasionally found in *T. angustula* and *A. mellifera* (Fig. 1B). Although phylogenetic evidence suggests they are acquired from the environment, the prevalence of these taxa points toward a stable, and possibly functional association with *Melipona*.

Furthermore, certain fungi are also associated with *Melipona* (Fig. 2). *Starmerella*, the most abundant and widespread of these, has been found in stingless bee adults, pollen provisions and honey (TEIXEIRA et al., 2003), suggesting specialization for the bee niche (Fig. S5, Table S2). In other insects, fungi have occasionally replaced degenerating bacterial symbionts (MATSUURA et al., 2018), and dependence on a fungal symbiont is documented for another stingless bee, *Scaptotrigona depilis* (PALUDO et al., 2018). Experimental work on *Melipona* and other stingless bees could give insights into the functional basis and dynamic nature of bee microbiomes.

Acknowledgments

We acknowledge the UFV, the financial support from CNPq, CAPES – Finance Code 001 and FAPEMIG, a USDA NIFA postdoctoral fellowship (2018-08156) to TJH, and NIH award R35GM131738 to NAM. We thank Marina Cunha, Gil Viana, José Souza, Eduardo Ferreira, Leandro Campos, Marcelo Silva, Antônio Alves, Sidcley de Lucena, Flávio Yamamoto, Hilton Gomes, Kalhil França, Gilvan Santos and Helder Resende for providing bees, and Fernando da Silveira for Amazonian bee identification.

Conflict of interest

We declare no competing financial interests.

References

- BENNETT, G. M.; MORAN, N. A. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. **Proceedings of the National Academy of Sciences of the United States of America**, v. 112, n. 33, p. 10169–10176, 2015.
- BOURGUIGNON, T. et al. Rampant host switching shaped the termite gut microbiome. **Current Biology**, v. 28, p. 649–654, 2018.
- CHONG, R. A.; MORAN, N. A. Evolutionary loss and replacement of Buchnera, the obligate endosymbiont of aphids. **The ISME Journal**, v. 12, p. 898–908, 2018.
- DÍAZ, S. et al. Report on the microbiota of *Melipona quadrifasciata* affected by a recurrent disease. **Journal of Invertebrate Pathology**, v. 143, p. 35–39, 2017.
- GIANNINI, T. C. G. et al. Crop pollinators in Brazil : a review of reported interactions. **Apidologie**, v. 46, p. 209–223, 2015.
- KOCH, H. et al. Diversity and evolutionary patterns of bacterial gut associates of corbiculate bees. **Molecular Ecology**, v. 22, p. 2028–2044, 2013.
- KOCH, H.; SCHMID-HEMPEL, P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 48, p. 19288–19292, 2011.
- KWONG, W. K. et al. Dynamic microbiome evolution in social bees. **Science Advances**, v. 3, n. 3, p. 1–17, 2017.
- KWONG, W. K.; MORAN, N. A. Gut microbial communities of social bees. **Nature Reviews Microbiology**, v. 14, p. 374–384, 2016.
- LEONHARDT, S. D.; KALTENPOTH, M. Microbial communities of three sympatric Australian stingless bee species. **PLoS ONE**, v. 9, n. 8, p. 1–6, 2014.
- MARTINSON, V. G. et al. A simple and distinctive microbiota associated with honey bees and bumble bees. **Molecular Ecology**, v. 20, n. 3, p. 619–628, 2011.
- MATSUURA, Y. et al. Recurrent symbiont recruitment from fungal parasites in cicadas. **Proceedings of the National Academy of Sciences of the United States of America**, v. 115, n. 26, p. E5970–E5979, 2018.
- MOCKLER, B. K. et al. Microbiome structure influences infection by the parasite *Crithidia bombi* in bumble bees. **Applied and Environmental Microbiology**, v. 84, n. 7, p. 1–11, 2018.
- MORAN, N. A.; MCCUTCHEON, J. P.; NAKABACHI, A. Genomics and evolution of heritable bacterial symbionts. **The Annual Review of Genetics**, v. 42, p. 165–90, 2008.
- PALUDO, C. R. et al. Stingless bee larvae require fungal steroid to pupate. **Scientific Reports**, v. 8, n. 1, p. 1–10, 2018.
- RAMÍREZ, S. R. et al. A molecular phylogeny of the stingless bee genus *Melipona* (Hymenoptera: Apidae). **Molecular Phylogenetics and Evolution**, v. 56, n. 2, p. 519–525, 2010.
- ROTHMAN, J. A. et al. The bumble bee microbiome increases survival of bees exposed to selenate toxicity. **Environmental Microbiology**, v. 21, n. 9, p. 3417–3429, 2019.
- SUDAKARAN, S.; KOST, C.; KALTENPOTH, M. Symbiont acquisition and replacement as a source of ecological innovation. **Trends in Microbiology**, v. 25, n. 5, p. 1–16, 2017.
- TEIXEIRA, A. C. P. et al. *Starmerella meliponinorum* sp. nov., a novel ascomycetous yeast species associated with stingless bees. **International Journal**

of Systematic and Evolutionary Microbiology, v. 53, p. 339–343, 2003.

ZHENG, H. et al. Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. **Proceedings of the National Academy of Sciences of the United States of America**, v. 114, n. 18, p. 4775–4780, 2017.

Supplementary Methods

We sampled bee colonies from 23 *Melipona* populations from four Brazilian biomes: Atlantic Forest, Caatinga (dry Tropical Forest in semiarid region), Cerrado (Brazilian Savanna) and Amazon (SISBIO/ICMBio authorization number 69500-1). For each population (i.e., bees from the same species living in the same site) 3 bee colonies, consisting of bees living in beekeeping boxes, were sampled. Additionally, 3 colonies of honeybees (*Apis mellifera*) and 8 of the stingless bee *Tetragonisca angustula* were sampled and used as comparison groups (Table S1).

Bee specimens with similar morphology were grouped into morphotypes and identified based on taxonomic keys (CAMARGO; PEDRO, 2013) and by comparison with specimens previously identified by specialists. A total of 14 morphotypes, comprising 8 identified species from the four *Melipona* sub-genera (RAMÍREZ et al., 2010) and 6 morphotypes whose identification could not be confirmed (referred to as "*Melipona* cf. = *conferatum*") were sampled (see Table S1 for details).

Some bees were identified by collaborators: bees sampled in Amazonian biome (coded MSN and MIN) were identified by Professor Fernando Amaral da Silveira from the Federal University of Minas Gerais – Brazil; bees from the Santa Tereza city in the Atlantic forest biome (coded MQ7, MRF and MAN) were identified by Professor Eduardo Antonio Ferreira from the Federal Institute of Espírito Santo – Brazil; *Tetragonisca angustula* bees (coded TET) were identified by Professor Helder Canto Resende from the Federal University of Viçosa – Brazil. Voucher specimens were deposited in the scientific collection of the Regional Museum of Entomology of the Federal University of Viçosa (UFVB) - Brazil.

From each of the 80 bee colonies we sampled, 10-20 bees were taken from the box entrance and placed in sterile tubes containing 95% ethanol. Then, 5 bees from each box were dissected using sterile forceps and iris scissors with a stereoscopic microscope. Each set of 5 guts was pooled together as a single sample, an approach used to ensure a sufficient yield of DNA for PCR amplification. Then, we extracted total DNA using the kit NucleoSpin® Soil (Macherey-Nagel) preceded by a proteinase K treatment for 4 hours at 56°C. The DNA was submitted for 250 bp paired-end amplicon sequencing at Novogene Corporation Inc (Sacramento, CA, USA) using an Illumina NovaSeq 6000 Sequencing System. The primer pairs 341F

(CCTAYGGGRBGCASCAG) – 806R (GGACTACNNGGGTATCTAAT) and ITS5-1737F (GGAAGTAAAAGTCGTAACAAGG) – ITS2-2043R (GCTGCGTTCTTCATCGATGC) were used, targeting the 16S rRNA V3-V4 and ITS1 rDNA regions, respectively. Sequencing of the 16S rDNA gene region was successful for all the samples, but two samples (coded MRF and MSP3) were not sequenced for the fungal ITS region due to poor amplification during library preparation (Table S1). The data (SRA accession number PRJNA678404) were processed with the DADA2 (CALLAHAN et al., 2016) package in R following the DADA2 microbiome pipeline for 16S (available at: <https://benjjneb.github.io/dada2/tutorial.html>) and for ITS (available at: https://benjjneb.github.io/dada2/ITS_workflow.html). Briefly, DADA2 was used to filter, trim, de-replicate and denoise sequences. For fungi, trimming was not performed, and adapters were removed using Cutadapt (MARTIN, 2011). After removing chimeras, we then used these merged paired-end reads to construct an exact sequence variant (ESV) table. Finally, the taxonomy was assigned to ESVs by naive Bayesian classifier method implemented in DADA2 using trained SILVA database for bacteria and trained UNITE database for fungi.

For data analysis we used the R packages “mctoolsr” (available at: <https://github.com/leffj/mctoolsr>), “phyloseq” (MCMURDIE; HOLMES, 2013), “ggplot2” (WICKHAM, 2016) and “vegan” (OKSANEN et al., [s.d.]). First, mitochondria, chloroplasts, and low abundance ESVs (those with <100 total reads across all samples) were removed from the dataset (Table S3). We then made rarefaction curves, which demonstrate that our sequencing depth adequately captured the majority of bacterial diversity (Fig. S6). Then, we visualized the differences in bacterial (Fig. 1A) and fungal (Fig. 2) community composition between bee species at the genus level and, for bacteria, we compared our results to previously published data (KWONG et al., 2017). The significance of the differences in the relative abundance of microbial taxa between our *Melipona*, *Apis mellifera* and *Tetragonisca angustula* samples was determined using a Kruskal-Wallis test. For the core *Melipona* bacterial taxa (i.e., taxa containing ESVs present in all samples of all *Melipona* species) we compared the log-transformed mean relative abundances of the most abundant ESVs in each bee population (Fig. 1B). Furthermore, these ESVs were submitted to BLASTN similarity searches against GenBank at NCBI Reference Sequence Database at which we could identify and download sequences from type isolates aligned to them. Downloaded

sequences (Table S2) were aligned using ClustalW and the Maximum Likelihood phylogenetic tree made with bootstrap of 1000 replications using MEGA software (KUMAR; STECHER; TAMURA, 2016). By this approach we could determine the possible origin of dominant ESVs in *Melipona*, specifically whether they are related to widespread environmental taxa or to specialized associates of social bees (Fig. S1-S4). A similar approach was used to determine the possible origin of the most abundant ESVs of *Starmerella*, the only core *Melipona* fungal taxon (Fig. S5). Finally, we made a simplified representation of the phylogenetic relationship between eusocial corbiculate bees (based on (RAMÍREZ et al., 2010)) showing the gains (KWONG et al., 2017; KWONG; MORAN, 2016) and our hypothesized losses of corbiculate core symbionts (Fig. 1C).

Supplementary References

- CALLAHAN, B. J. et al. DADA2 : High-resolution sample inference from Illumina amplicon data. **Nat Methods**, v. 13, n. 7, p. 581–583, 2016.
- CAMARGO, J. M. F.; PEDRO, S. R. M. **Meliponini Lepeletier, 1836**. Disponível em: <<http://www.moure.cria.org.br/catalogue>>. Acesso em: 20 jan. 2021.
- KUMAR, S.; STECHER, G.; TAMURA, K. MEGA7 : Molecular Evolutionary Genetics Analysis Version 7 . 0 for Bigger Datasets. **Molecular biology and evolution**, v. 33, n. 7, p. 1870–1874, 2016.
- KWONG, W. K. et al. Dynamic microbiome evolution in social bees. **Science Advances**, v. 3, n. 3, p. 1–17, 2017.
- KWONG, W. K.; MORAN, N. A. Gut microbial communities of social bees. **Nature Reviews Microbiology**, v. 14, p. 374–384, 2016.
- MARTIN, M. Cutadapt Removes Adapter Sequences From High-Throughput Sequencing Reads. **EMBnet journal**, v. 17, n. 4, p. 5–7, 2011.
- MCMURDIE, P. J.; HOLMES, S. phyloseq : An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. **PLoS ONE**, v. 8, n. 4, p. e61217, 2013.
- OKSANEN, J. et al. **vegan: Community Ecology Package**. [s.l: s.n.].
- RAMÍREZ, S. R. et al. A molecular phylogeny of the stingless bee genus *Melipona* (Hymenoptera: Apidae). **Molecular Phylogenetics and Evolution**, v. 56, n. 2, p. 519–525, 2010.
- WICKHAM, H. **ggplot2: Elegant Graphics for Data Analysis**. [s.l.] Springer-Verlag New York, 2016.

Supplementary Figures

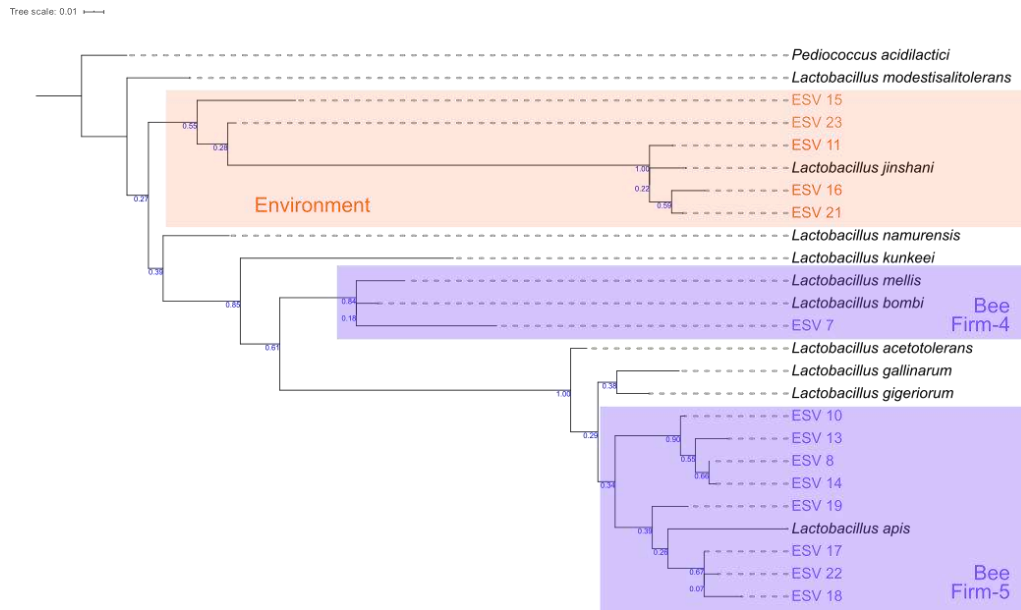


Figure S1 - Phylogenetic tree of the most abundant *Lactobacillus* ESVs found in the samples of the *Melipona* bee populations. Sequences used in the tree were obtained in the GenBank database (accession numbers and other information in Table S2). Bootstrap values are shown in blue letters. ESVs in orange are phylogenetically closer to isolates obtained from environmental samples. ESVs in purple are closer to isolates obtained from honeybees or bumblebees (*Lactobacillus* Firm-4 or Firm-5). Only taxa most closely related to *Melipona* ESVs are labeled. The root was placed on the branch leading to *Pediococcus acidilactici*.

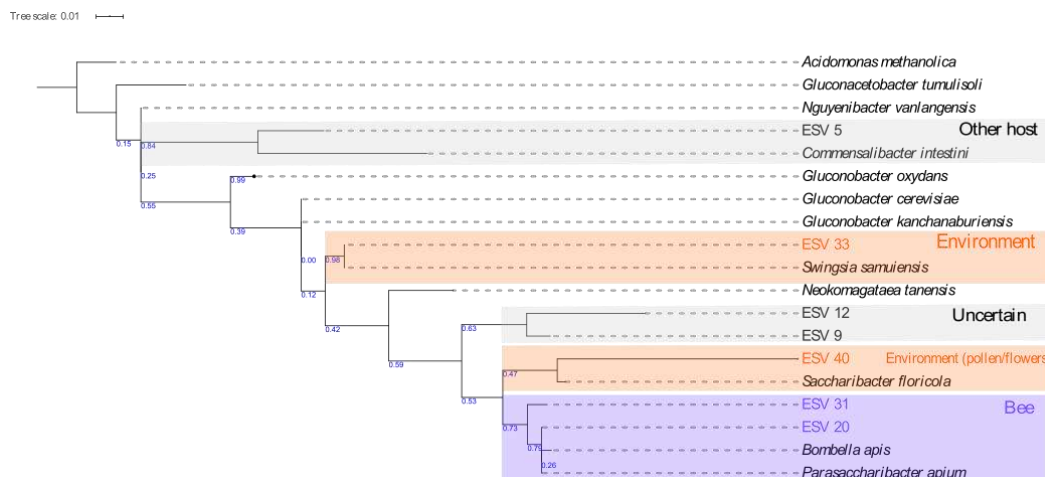


Figure S2 - Phylogenetic tree of the most abundant Acetobacteraceae ESVs found in the samples of the *Melipona* bee populations. Sequences used in the tree were obtained in the Genbank database (accession numbers and other information in Table S2). Bootstrap values are shown in blue letters. ESVs in orange are phylogenetically closer to isolates obtained from environmental samples. ESVs in purple are closer to isolates obtained from bees. ESVs in gray are closer to isolates from alternative hosts (ESV 5) or have uncertain phylogenetic proximity to environmental – pollen/flowers or bee associated isolates (ESV 9 and ESV 12). Only taxa most closely related to *Melipona* ESVs are labeled. The root was placed on the branch leading to *Acidomonas methanolica*.

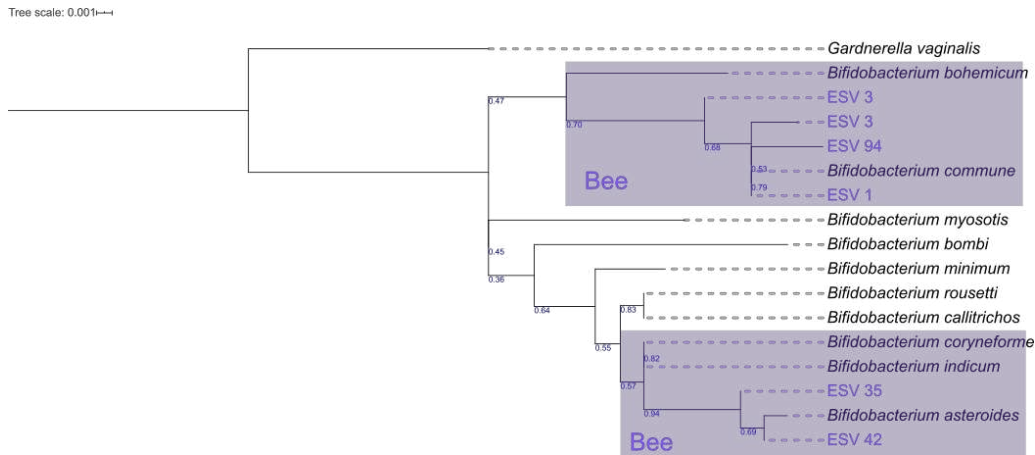


Figure S3 - Phylogenetic tree of the most abundant Bifidobacteriaceae ESVs found in the samples of the *Melipona* bee populations. Sequences used in the tree were obtained in the Genbank database (accession numbers and other information in Table S2). Bootstrap values are shown in blue letters. ESVs in purple are phylogenetically closer to bacteria isolated from bees. Only taxa most closely related to *Melipona* ESVs are labeled. The root was placed on the branch leading to *Gardnerella vaginalis*.

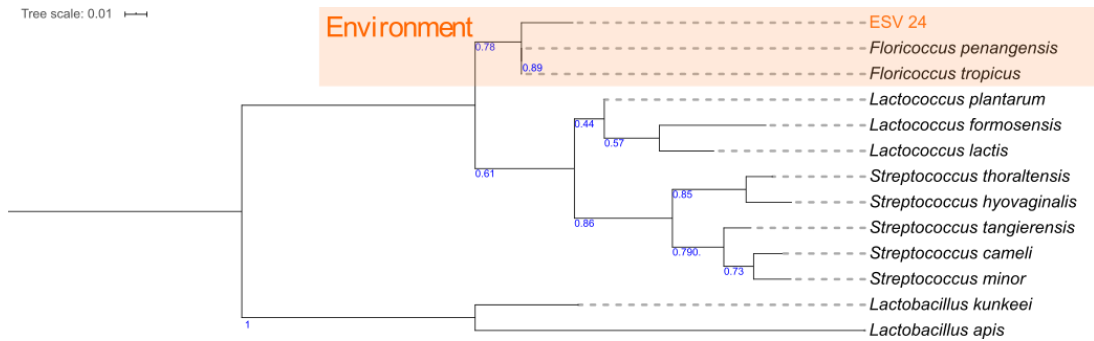


Figure S4 - Phylogenetic tree of the core *Floricoccus* ESV found in the samples of the *Melipona* populations. Sequences used in the tree were obtained in the Genbank database (accession numbers and other information in Table S2). Bootstrap values are shown in blue letters. The ESV in orange is closely related to environmental isolates. Only the taxon most closely related to *Melipona* ESV is labeled. The root was placed on the branch leading to *Lactobacillus kunkeei* and *Lactobacillus apis*.

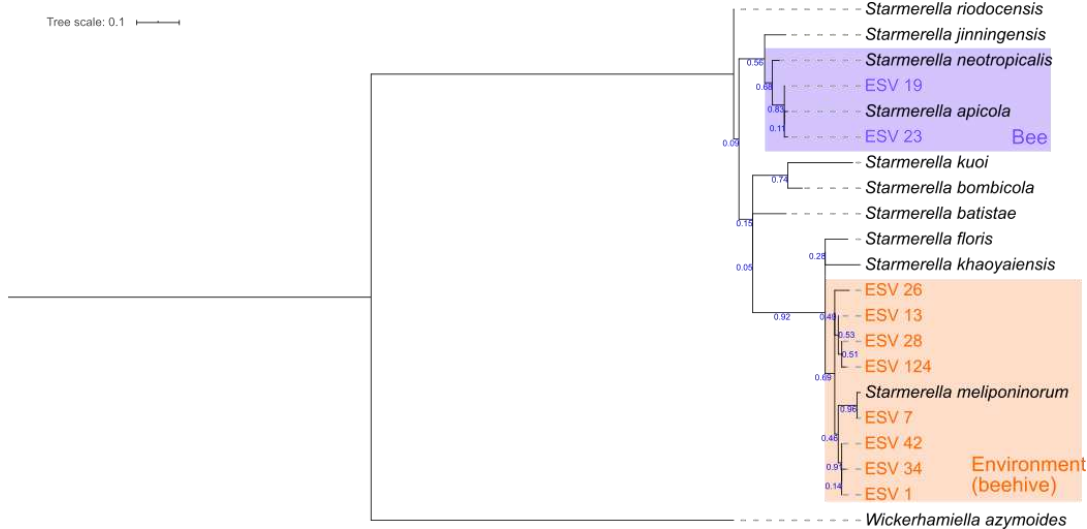


Figure S5 - Phylogenetic tree of the most abundant *Starmerella* ESVs found in the samples of the *Melipona* populations. Sequences used in the tree were obtained in the Genbank database (accession numbers and other information in Table S2). Bootstrap values are shown in blue letters. ESVs in orange are phylogenetically closer to isolates obtained from environmental (beehive) samples. ESVs in purple are closer to isolates obtained from bees. Only taxa most closely related to *Melipona* ESVs are labeled. The root was placed on the branch leading to *Wickerhamiella azymoides*.

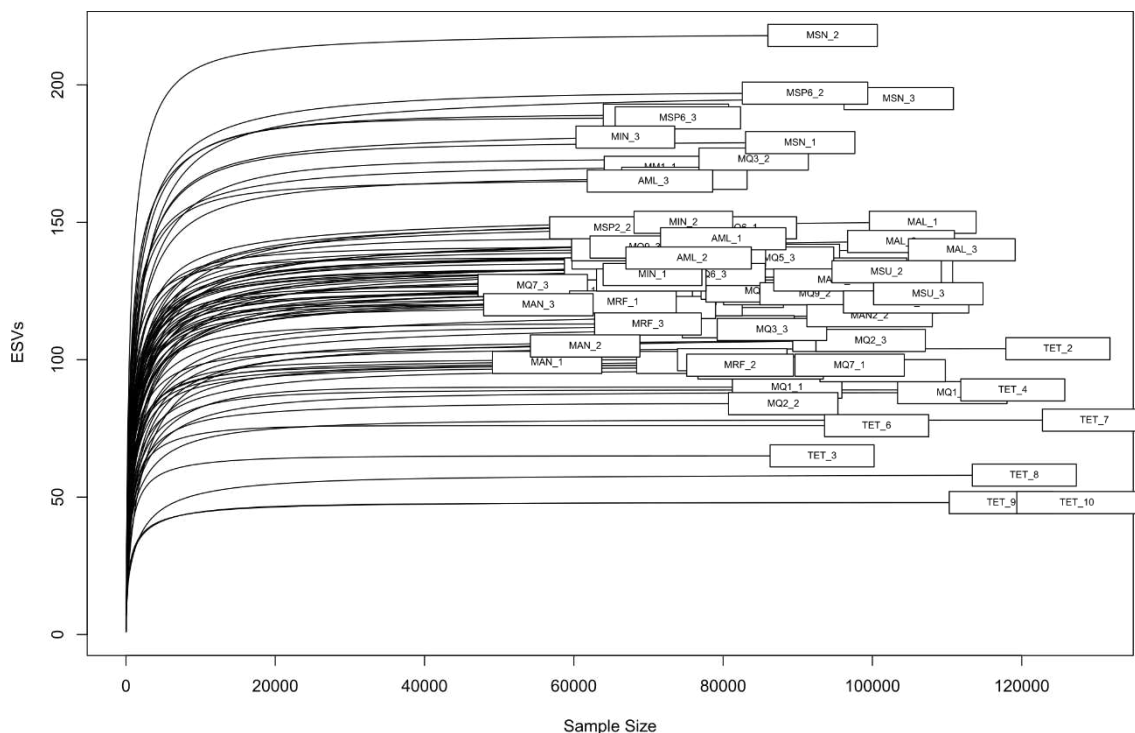


Figure S6 - Rarefaction curves of the 80 samples after removing Mitochondria/Chloroplasts and low abundance ESVs (<100 reads across all samples) from the bacterial dataset. The curves indicate that the samples reached a plateau, thus, overall bacterial diversity is adequately captured by our sequencing.

Supplementary Tables

Table S1 - Bee populations sampled in this study.

Code	Code NCBI*1	Sampling date	Sampling Biome	Sampling city	Sampling state	Population	Morphotype	Bee species	Sub-genus	Sequenced samples	
										16S	ITS
MQ1	MQA1	May-19	Atlantic Forest	Magé	Rio de Janeiro	1	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ2	MQA2	May-19	Atlantic Forest*2	São Sebastião do Paraíso	Minas Gerais	2	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ3	MQA3	May-19	Atlantic Forest*2	Antônio dos Santos/Caeté	Minas Gerais	3	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ4	MQA4	Apr-19	Atlantic Forest	Viçosa	Minas Gerais	4	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ5	MQA5	May-19	Atlantic Forest	Cotia	São Paulo	5	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ6	MQA6	May-19	Atlantic Forest	São Paulo	São Paulo	6	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ7	MQA7	Apr-19	Atlantic Forest	Santa Tereza	Espírito Santo	7	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MQ8	MQQ	May-19	Atlantic Forest	Cotia	São Paulo	8	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MM1	MM1	Aug-18	Atlantic Forest	Viçosa	Minas Gerais	9	2	<i>Melipona mondury</i>	<i>Michmelia</i>	3	3
MSP1	MM2	May-19	Atlantic Forest	Cotia	São Paulo	10	3	<i>Melipona cf. mondury</i>	<i>Michmelia</i>	3	3
MSP2	MRF_SP	May-19	Atlantic Forest	São Paulo	São Paulo	11	4	<i>Melipona cf. rufiventris</i>	<i>Michmelia</i>	3	3
MRF	MRF	Apr-19	Atlantic Forest	Santa Tereza	Espírito Santo	12	5	<i>Melipona rufiventris</i>	<i>Michmelia</i>	3	-
MSP3	MSC	May-19	Atlantic Forest	Cotia	São Paulo	13	6	<i>Melipona cf. scutellaris</i>	<i>Michmelia</i>	3	-
MSP4	MBS	May-19	Atlantic Forest	São Paulo	São Paulo	14	7	<i>Melipona cf. bicolor</i>	<i>Eomelipona</i>	3	3
MSP5	MRG	May-19	Atlantic Forest	Cotia	São Paulo	15	8	<i>Melipona cf. marginata</i>	<i>Eomelipona</i>	3	3
MAN	MAN	Apr-19	Atlantic Forest	Santa Tereza	Espírito Santo	16	9	<i>Melipona mandacaia</i>	<i>Melipona</i>	3	3
MAN2	MAN2	May-19	Caatinga	Petrolina	Pernambuco	17	9	<i>Melipona mandacaia</i>	<i>Melipona</i>	3	3
MAL	MAL	May-19	Caatinga	Petrolina	Pernambuco	18	10	<i>Melipona asilvai</i>	<i>Eomelipona</i>	3	3
MSU	MSU	Aug-19	Caatinga	Mossoró	Rio Grande do Norte	19	11	<i>Melipona subnitida</i>	<i>Melipona</i>	3	3
MQ9	MQA8	Dec-18	Cerrado	Passos	Minas Gerais	20	1	<i>Melipona quadrifasciata</i>	<i>Melipona</i>	3	3
MSN	MSN	Jun-19	Amazon	Iranduba	Amazonas	21	12	<i>Melipona seminigra merrillae</i>	<i>Michmelia</i>	3	3
MSP6	MFL	Jun-19	Amazon	Iranduba	Amazonas	22	13	<i>Melipona cf. rufiventris</i>	<i>Michmelia</i>	3	3
MIN	MIN	Jun-19	Amazon	Iranduba	Amazonas	23	14	<i>Melipona interrupta</i>	<i>Melikerria</i>	3	3
TET	TET	Oct-19	Atlantic Forest	Camargo/Ouro Preto	Minas Gerais	-	-	<i>Tetragonisca angustula</i>	-	8	8
AML	AML	Jul-19	Atlantic Forest	Viçosa	Minas Gerais	-	-	<i>Apis mellifera</i>	-	3	3

*1 Population code deposited in NCBI-SRA (available at <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=PRJNA678404>)

*2 Atlantic forest in transition with Cerrado biome.

Table S2 - GenBank sequences used for analyses.

Species name on the tree	Accession	Isolation substrate
Lactobacillus tree		
<i>Lactobacillus jinshani</i>	NR_165780.1	Solid-State Vinegar Culture of Zhenjiang Aromatic Vinegar
<i>Lactobacillus apis</i>	NR_125702.1	Stomach of Honeybees
<i>Lactobacillus bombi</i>	NR_134065.1	Digestive tract of a bumblebee queen (<i>Bombus terrestris</i>)
<i>Lactobacillus mellis</i>	NR_126249.1	Honey stomach of the honeybee <i>Apis mellifera</i>
<i>Lactobacillus kunkeei</i>	NR_026404.1	Partially fermented grape juice.
<i>Lactobacillus modestisalitolans</i>	NR_136786.1	Traditional Fermented Foods
<i>Lactobacillus namurensis</i>	NR_042514.1	Sourdough
<i>Lactobacillus gallinarum</i>	NR_042111.1	Chicken intestine
<i>Lactobacillus acetotolerans</i>	NR_117073.1	fermented broth of rice vinegar
<i>Lactobacillus gigeriorum</i>	NR_117057.1	Chicken Crop
<i>Pediococcus acidilactici</i>	NR_042057.1	Barley
Acetobacteraceae tree		
<i>Bombella apis</i>	NR_157653.1	The midgut of a honeybee.
<i>Commensalibacter intestini</i>	NR_116307.1	<i>Drosophila melanogaster</i> gut
<i>Gluconacetobacter tumulicola</i>	NR_114383.1	Soil
<i>Gluconobacter cerevisiae</i>	NR_117735.1	The brewery environment
<i>Gluconobacter kanchanaburiensis</i>	NR_112822.1	Fermented fruits (spoiled fruits), <i>Artocarpus heterophyllus</i>
<i>Gluconobacter oxydans</i>	NR_118196.1	Involved in industrial vinegar
<i>Neokomagataea tanensis</i>	NR_112959.1	Flower of candle bush, <i>Senna alata</i>
<i>Nguyenibacter vanlangensis</i>	NR_125459.1	Rice rhizosphere
<i>Parasaccharibacter apium</i>	NR_133042.1	Isolated from Honeybee larvae, associated to nurse crops, hypopharyngeal glands (HGs), and royal jelly (RJ).
<i>Saccharibacter floricola</i>	NR_024819.1	Pollen in flowers
<i>Swingsia samuiensis</i>	NR_132672.1	A flower of golden trumpet (<i>Allamanda cathartica</i>)
<i>Tanticharoenia sakaeratensis</i>	NR_041601.1	Soil
<i>Acidomonas methanolica</i>	NR_112209.1	A septic methanol processing mixture
Bifidobacteriaceae tree		
<i>Bifidobacterium commune</i>	NR_136422.1	The bumblebee gut
<i>Bifidobacterium asteroides</i>	NR_044154.1	The Honey Stomach of the Honeybee <i>Apis Mellifera</i>
<i>Bifidobacterium bohemicum</i>	NR_108439.1	The digestive tract of three bumblebee species
<i>Bifidobacterium coryneforme</i>	NR_044690.2	The Hindgut of honeybee
<i>Bifidobacterium indicum</i>	NR_043439.1	The hindgut of honeybee
<i>Bifidobacterium minimum</i>	NR_044692.2	Sewage
<i>Bifidobacterium roussetti</i>	NR_164634.1	Faeces of the Egyptian fruit bat <i>Rousettus aegyptiacus</i>
<i>Bifidobacterium myosotis</i>	NR_147760.1	The faeces of common marmosets (<i>Callithrix jacchus</i>)
<i>Bifidobacterium bombi</i>	NR_104872.1	The Digestive tract content of the bumblebee <i>Bombus lucorum</i>
<i>Bifidobacterium callitrichos</i>	NR_113172.1	The faeces of common marmoset (<i>Callithrix jacchus</i>)
<i>Gardnerella vaginalis</i>	NR_118377.1	Human vaginal secretions
Floricoccus tree		
<i>Floricoccus penangensis</i>	NR_159225.1	Flowers of Durian tree and <i>Hibiscus</i>
<i>Floricoccus tropicus</i>	NR_159226.1	Flowers of Durian tree and <i>Hibiscus</i>
<i>Lactococcus formosensis</i>	NR_114366.1	Fermented broccoli stems
<i>Lactococcus plantarum</i>	NR_118903.1	Frozen peas
<i>Lactococcus lactis</i>	NR_113960.1	Human
<i>Streptococcus tangierensis</i>	NR_134818.1	Raw camel milk
<i>Streptococcus thoraltensis</i>	NR_026368.1	The intestine of a pig
<i>Streptococcus hyovaginalis</i>	NR_044912.1	Isolated from the vagina of a pig
<i>Streptococcus cameli</i>	NR_134817.1	Raw camel milk
<i>Streptococcus minor</i>	NR_025729.1	The tonsils of a dog
Starmerella tree		
<i>Starmerella apicola</i>	NR_130681.1	Gut of bee
		Adult of the stingless bee <i>Trigona</i> sp. in a <i>M. quinquefolia</i> flower
<i>Starmerella floris</i>	NR_155820.1	
<i>Starmerella khaoyaiensis</i>	NR_155821.1	Flower
<i>Starmerella meliponinorum</i>	NR_137869.1	Pollen provisions of <i>Tetragonisca angustula</i>
<i>Starmerella neotropicalis</i>	NR_160316.1	Adult bee, <i>Melipona quinquefasciata</i>
<i>Starmerella riocensis</i>	NR_137870.1	Pollen-nectar provisions of the solitary bee <i>Megachile</i> sp
<i>Starmerella bombycola</i>	NR_121483.1	Honey of bumblebee (<i>Bombus</i>)
<i>Starmerella kuoi</i>	NR_164377.1	Concentrated grape juice
<i>Starmerella batistae</i>	NR_155813.1	Larval provisions, larvae and pupae of the solitary bees <i>Diadasina distincta</i> and <i>Ptilotrix plumata</i>
<i>Starmerella jinningensis</i>	NR_155850.1	Flowers of <i>Erianthus rufipilus</i>
<i>Wickerhamiella azymoides</i>	NR_164509.1	Ripe fruits of <i>Eugenia uniflora</i> and the larva of the fly <i>Anastrepha mucronota</i> found in <i>Peritassa campestris</i> fruits

Table S3 - Number of ESVs and reads in the original bacterial dataset, after initial filtration of mitochondria/chloroplasts, and after subsequent low abundance ESVs filtering.

	Original dataset	# / % after filtering mitochondria and chloroplasts	# / % after filtering low abundance ESVs
ESVs	2852	2806 / 98.39%	794 / 28.30%
READS	6,911,317	6,894,536 / 99.76%	6,865,138 / 99.57%

Chapter 3: Stingless bees gut microbiota is altered in areas impacted by mining tailings from the Fundão dam collapse in Mariana, Brazil.

ABSTRACT

Stingless bees (Meliponini) are highly diverse and important pollinators in the tropics. These bees inhabit forests in tropical ecosystems and thus are affected by anthropic activities such as deforestation and use of agrochemicals. Furthermore, stingless bees are also capable of accumulating metals, already described as damaging reproduction, survival and foraging in bees. *Tetragonisca angustula* is a stingless bee species that occurs from southern Brazil to Central America and has been already described as a bioindicator of environmental pollution. Eusocial corbiculate bees (honeybees, bumblebees and stingless bees) harbor a beneficial gut-associated microbiota that is either socially transmitted or acquired from the environment. However, these microorganisms are affected by environmental stressors such as metals, and exposed bees can have compromised associations. In this sense, we studied the differences in the microbiota between impacted and not-impacted areas by mining tailings from the mud tsunami generated by the collapse of Fundão dam in Mariana, considered the worst social and environmental impact in Brazil. Changes in the relative abundance of core bacteria (present in all samples – possibly symbionts) and in the composition of “strictly environmental” bacteria (less common – possibly transient) associated with *T. angustula* were observed in impacted areas compared to not-impacted areas. Thereby, *T. angustula* microbiota has potential to be included in the monitoring of environmental impacts of the Fundão dam collapse, since its alteration might be harmful to bee’s health and could lead to reduced pollination services, compromising the recovery of impacted areas.

Keywords: *Tetragonisca angustula*, eusocial corbiculate bees, microbiome, symbiosis, environmental impact, mud tsunami

Introduction

Stingless bees (Meliponini) comprises a monophyletic tribe (RASMUSSEN; CAMERON, 2010; ROMIGUIER et al., 2016), with high diversity and importance as pollinators of crops and native plants in tropical and subtropical ecosystems around the globe (GIANNINI et al., 2015; HEARD, 1999; SLAA et al., 2006). Since inhabit forests, stingless bees suffer directly and indirectly the impacts of deforestation, that destroys habitat and colonies decreasing the population and genetic variability (BROWN; ALBRECHT, 2001). The plant diversity loss also reduces the availability and variety of sources of pollen and nectar, which are the basis of bee nutrition. Nutritional deficiency has been shown to weaken social bees immune response that become more susceptible to fungal, bacterial and viral pathogens in addition to parasitic arthropods (DOLEZAL; TOTH, 2018).

Furthermore, other anthropogenic environmental stressors such as exposure to pesticides can be related to large-scale bee loss, as detected in 2006/2007 with honeybees (*Apis mellifera*) in the United States, a phenomenon called "Colony Collapse Disorder" (CCD) (VANENGELSDORP et al., 2009). Chemicals are also deleterious to stingless bees as occur with Fipronil that affect *Melipona scutellaris* foraging activity (MORAIS et al., 2018) and Imidacloprid (neonicotinoid) that causes mortality and sublethal effects on brain development and mobility of young *Melipona quadrifasciata anthidioides* adults (TOMÉ et al., 2012). Also, larval food contamination by herbicides such as glyphosate and Cry toxins from *Bacillus thuringiensis* transgenic plants is lethal and alters the development of bees, respectively, as is the case for *Melipona quadrifasciata* (SEIDE et al., 2018).

Metals are also important pollutants capable of bioaccumulating in bees (ZARIC et al., 2018). Increasing heavy metal concentrations are capable of affecting wild bees, by decreasing reproduction (reduced number of brood cells) and survival of offspring in contaminated areas (MORÓN et al., 2014). Honeybees treated with metals also had high number of dead pupae (HLADUN et al., 2016).

The effect of environmental stressors is also evident on the gut microbiota of social bees, important contributors to bees health and nutrition (ENGEL; MARTINSON; MORAN, 2012; KATSNELSON, 2015; KOCH; SCHMID-HEMPEL, 2011; KWONG; MANCENIDO; MORAN, 2017; KWONG; MORAN, 2016; OLOFSSON; VÁSQUEZ,

2008; POWELL et al., 2016; VÁSQUEZ; OLOFSSON, 2009). This is the case for the agrochemical glyphosate that affect the gut microbial abundance and composition in honeybees, decreasing beneficial microbes such as *Snodgrassella alvi* and leading to a higher susceptibility and mortality due to opportunistic pathogens, for example *Serratia marcescens* (MOTTA; RAYMANN; MORAN, 2018). Also, chemicals and metals (sequestered by plants growing in contaminated soils) commonly found in pollen and nectar, can alter the bumblebee gut microbiome, causing gut dysbiosis and indirectly affecting bee health (ROTHMAN et al., 2020).

Stingless bees, especially *Tetragonisca angustula*, are also capable of accumulating metals and are directly affected by the level of anthropogenic activities in such a way that the more human-influenced the landscape is, the higher the source of pollution detected in bees. Thus, these bees are important tools for environmental monitoring studies, being used as bioindicators in Atlantic Forest (BARBOSA et al., 2021). *T. angustula*, popularly known as “Jataí”, is an important pollinator occurring from southern Brazil to Mexico (PRONI; MACIEIRA, 2004). This species has a generalist habit capable of pollinating many different plant species (VIEIRA et al., 2020). However, despite being able to accumulate metals, little is known about the effects of environmental stressors in the stingless bee’s gut microbiome, an important tool for studying host-adapted microbial communities, due to the occurrence of a simple and distinctive microbiota, comprised by dominant species clusters mostly transmitted by sociality – favoring co-diversification and co-adaptation with host; and capable of being cultured in laboratory (KWONG; MORAN, 2016; MARTINSON et al., 2011; ZHENG et al., 2019). Besides the occurrence of this so-called host-adapted core gut microbiota (composed mainly by *Snodgrassella*, *Gilliamella*, *Lactobacillus* Firm-4 and Firm-5 and *Bifidobacterium*), environmental strains are also found in association with eusocial corbiculate bees (including honeybees, bumblebees and stingless bees), and are probably acquired by contact with hive material and during foraging activity (CERQUEIRA et al., 2021; KWONG et al., 2017). Here, we aimed to contribute to this research field and evaluated the possible relationship between environmental impacted areas and alteration in gut microbiota of *T. angustula*.

For this purpose, *T. angustula* were collected in areas impacted and non-impacted by mining tailings resultant of Fundão Dam collapse. On November 5th, 2015, the rupture of the Fundão Dam, part of Germano mining complex, located in the district of

Bento Rodrigues in the municipality of Mariana – Minas Gerais, Brazil, released at least 34-50 million cubic meters of mining tailings that travelled more than 600 Km, through three rivers (Gualaxo Norte, Carmo and Rio Doce) until reaching the coast. This mud tsunami is considered the largest environmental and socio-economic disaster in Brazil, causing 19 casualties, destruction of Bento Rodrigues district and 207 properties (GOMES et al., 2017; IBAMA, 2015; OMACHI et al., 2018), covering a riverside area of 1176.6 ha with contaminated mud (OMACHI et al., 2018). The disaster deposited significant amounts of metals on estuaries and river ecosystems (GOMES et al., 2017) alluvial sediments (DUARTE et al., 2021) and soils (FERREIRA et al., 2021) and destroyed 476 ha of Atlantic forest (OMACHI et al., 2018). By evaluation of gut microbiota, we compared the bacterial composition and diversity of bees living in impacted and not impacted areas, aimed at understanding how core and environmental bacteria associated with *T. angustula* differ between impacted and not-impacted areas from Fundão Dam collapse. Since environmental stressors are capable to affect social bees gut associated microorganisms (MOTTA; RAYMANN; MORAN, 2018; ROTHMAN et al., 2020) and *T. angustula* can accumulate pollutants (BARBOSA et al., 2021) we hypothesize that bees living in impacted areas have altered microbial composition/diversity in relation to not impacted areas.

Methods

To understand the possible influence of environmental impact in the microbiome of stingless bees, we sampled 23 colonies of *Tetragonisca angustula*, popularly known as “Jataí”, living in eight different areas in an Atlantic forest biome located at the state of Minas Gerais – Brazil, comprising three different categories (C1, C2 and C3): C1 consist of three areas impacted by the mining tailings as a result of Fundão dam rupture in November 5th, 2015; C2 – two not-impacted areas at Mariana city; C3 – three not-impacted areas at Ouro Preto city and surrounding areas (Figure 1, Table1).

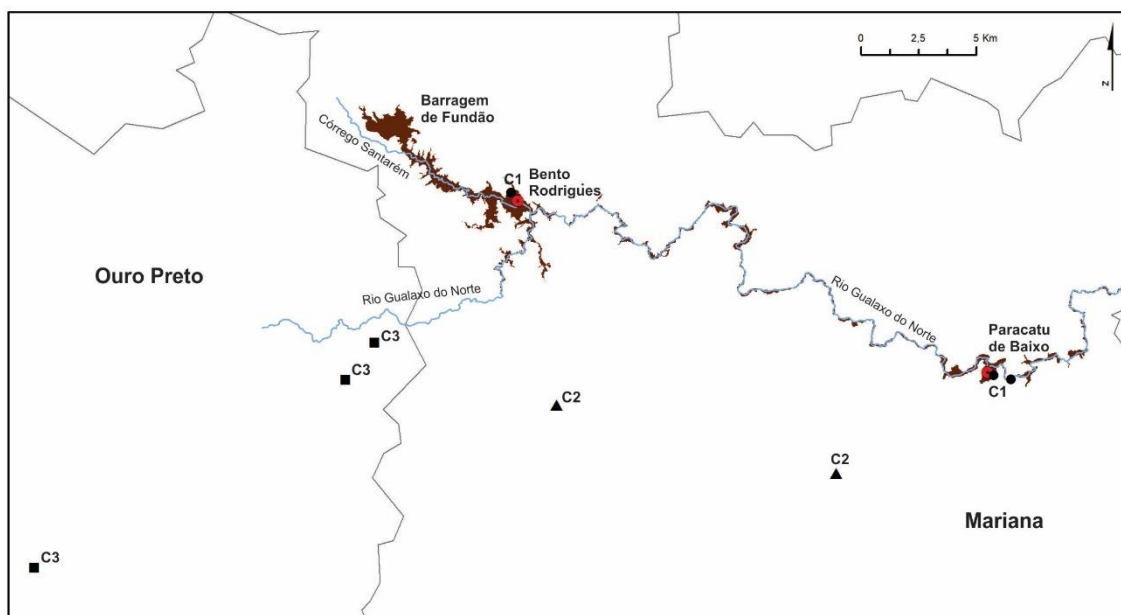


Figure 1 – Sampling locations at Minas Gerais state. Red circles represent Bento Rodrigues and Paracatu de Baixo, districts impacted by the mining tailings. Legend: C1/black circles – areas impacted by the mining tailings; C2/triangles – not-impacted areas surrounding Mariana city; C3/squares – not-impacted areas surrounding Ouro Preto city. Figure provided by Helder Canto Resende.

Table 1 – Information about sampling

CODE	REPETITION	CATEGORY	TYPE	LOCATION	DATE	LATITUDE	LONGITUDE
C1_Impacted_NPB_1	C1_1	C1	Impacted	Near Paracatu de Baixo	16/10/2019	S 20° 18' 23.1"	W 43° 13' 22.6"
C1_Impacted_NPB_2	C1_2	C1	Impacted	Near Paracatu de Baixo	16/10/2019	S 20° 18' 23.1"	W 43° 13' 22.6"
C1_Impacted_BR_1	C1_3	C1	Impacted	Bento Rodrigues	31/07/2018	20°14'00.5"S	43°25'06.7"W
C1_Impacted_BR_2	C1_4	C1	Impacted	Bento Rodrigues	17/09/2018	20°14'00.5"S	43°25'06.7"W
C1_Impacted_PB_1	C1_5	C1	Impacted	Paracatu de Baixo	18/03/2018	20°18'17.7"S	43°13'47.5"W
C1_Impacted_PB_2	C1_6	C1	Impacted	Paracatu de Baixo	15/09/2018	20°18'17.7"S	43°13'47.5"W
C2_Not-Impacted_M_NBR_1	C2_1	C2	Not-impacted	Mariana - near Bento Rodrigues	15/09/2018	S 20° 23' 05.8"	W 43° 32' 27.9"
C2_Not-Impacted_M_NBR_2	C2_2	C2	Not-impacted	Mariana - near Bento Rodrigues	14/10/2019	S 20° 23' 05.8"	W 43° 32' 27.9"
C2_Not-Impacted_M_NBR_3	C2_3	C2	Not-impacted	Mariana - near Bento Rodrigues	14/10/2019	S 20° 23' 05.8"	W 43° 32' 27.9"
C2_Not-Impacted_M_NBR_4	C2_4	C2	Not-impacted	Mariana - near Bento Rodrigues	14/10/2019	S 20° 23' 05.8"	W 43° 32' 27.9"
C2_Not-Impacted_M_NBR_5	C2_5	C2	Not-impacted	Mariana - near Bento Rodrigues	14/10/2019	S 20° 23' 05.8"	W 43° 32' 27.9"
C2_Not-Impacted_M_NPB_1	C2_6	C2	Not-impacted	Mariana - near Paracatu de Baixo	16/10/2019	S 20° 20' 35.5"	W 43° 17' 29.4"
C2_Not-Impacted_M_NPB_2	C2_7	C2	Not-impacted	Mariana - near Paracatu de Baixo	16/10/2019	S 20° 20' 35.5"	W 43° 17' 29.4"
C2_Not-Impacted_M_NPB_3	C2_8	C2	Not-impacted	Mariana - near Paracatu de Baixo	16/10/2019	S 20° 20' 35.5"	W 43° 17' 29.4"
C2_Not-Impacted_M_NPB_4	C2_9	C2	Not-impacted	Mariana - near Paracatu de Baixo	16/10/2019	S 20° 20' 35.5"	W 43° 17' 29.4"
C2_Not-Impacted_M_NPB_5	C2_10	C2	Not-impacted	Mariana - near Paracatu de Baixo	16/10/2019	S 20° 20' 35.5"	W 43° 17' 29.4"
C3_Not-Impacted_OP_1	C3_1	C3	Not-impacted	Ouro Preto	14/10/2019	S 19° 53' 00.7"	W 44° 25' 03.5"
C3_Not-Impacted_OP_2	C3_2	C3	Not-impacted	Ouro Preto	14/10/2019	S 19° 53' 00.7"	W 44° 25' 03.5"
C3_Not-Impacted_OP_3	C3_3	C3	Not-impacted	Ouro Preto	14/10/2019	S 19° 53' 00.7"	W 44° 25' 03.5"
C3_Not-Impacted_OP_NBR_1	C3_4	C3	Not-impacted	District of Ouro Preto - near Bento Rodgruiges	15/10/2019	S 20° 18' 24,0"	W 43° 29' 00.00"
C3_Not-Impacted_OP_NBR_2	C3_5	C3	Not-impacted	District of Ouro Preto - near Bento Rodgruiges	15/10/2019	S 20° 18' 24,0"	W 43° 29' 00.00"
C3_Not-Impacted_NOP_NBR_1	C3_6	C3	Not-impacted	Near Ouro Preto - near Bento Rodrigues	15/10/2019	S 20° 17' 31.7"	W 43° 28' 19.3"
C3_Not-Impacted_NOP_NBR_2	C3_7	C3	Not-impacted	Near Ouro Preto - near Bento Rodrigues	15/10/2019	S 20° 17' 31.7"	W 43° 28' 19.3"

Bees were collected from each colony and placed in sterile tubes containing 95% ethanol for microbial DNA preservation. Then, the abdomen of 5 bees were separated from the rest of the body at the stereoscopic microscope using sterile forceps and iris scissors, pooled together as a single sample, and submitted to total DNA extraction. The NucleoSpin® Soil (Macherey-Nagel) kit was used for this purpose following manufacturer's protocol but preceded by a proteinase K treatment for 4 hours at 56°C. Purified DNA was sent to Novogene Corporation Inc (Sacramento, CA, USA) at which library preparation, targeting the 16S rRNA V3-V4 region (341F [CCTAYGGGRBGCASCAG] – 806R [GGACTACNNGGGTATCTAAT] primer pair), and sequencing, using Illumina NovaSeq 6000 System, were performed.

Raw data were processed using DADA2 (CALLAHAN et al., 2016) R package guided by the DADA2 microbiome pipeline for 16S (available at: <https://benjjneb.github.io/dada2/tutorial.html>). In a summary, we performed filtering of low-quality sequences, trimming, de-replication, denoising and chimera removal. Afterwards, exact sequence variants (ESVs – sequences with 100% similarity) table were obtained from merged paired-end reads and classified by naive Bayesian classifier method implemented in DADA2 using trained SILVA database for bacteria. Then, we proceeded to data analysis, using the R packages “mctoolsr” (available at: <https://github.com/leffj/mctoolsr>), “phyloseq” (MCMURDIE; HOLMES, 2013), “ggplot2” (WICKHAM, 2016) and “vegan” (OKSANEN et al., [s.d.]). Initially mitochondria and chloroplasts were removed from the dataset and low abundance ESVs (with less than 100 reads in the whole dataset) were removed, to eliminate possible contaminants. Then, dataset was rarefied to the same number of reads per samples and rarefaction curves were generated to evaluate if our sampling adequately covered most of microbial diversity.

Afterwards, we calculated a Bray-Curtis dissimilarity matrix in square-root transformed whole dataset and plotted in a dendrogram to understand how samples group based on community composition. Subsequently, aimed at understanding the differences in microbial composition between the categories, we divided the dataset in two different classes: (1) bacterial ESVs found in all samples, possibly representing core gut symbionts of *Tetragonisca angustula*. By this approach we evaluated how the same bacterial ESVs change in proportion between samples, thus inferring the favoring/depletion in the relative abundance of bacterial specimens in different

categories due to environmental impact; (2) possibly transient and environmental bacteria, i.e. bacteria not commonly associated to bees gut or hive environment (considered here as non-core/"strictly environmental" bacteria), obtained by filtering out the genera of which belong the above mentioned ESVs plus bacterial genera already described as the most abundant and/or core and environmental bacteria associated to eusocial corbiculate bees (BONILLA-ROSSO et al., 2019; CARDOSO et al., 2020; KWONG et al., 2017; KWONG; MORAN, 2016; MARTINSON et al., 2011; NOWAK et al., 2021), and *Melipona* stingless bees (CERQUEIRA et al., 2021). Filtered out bacteria were the eusocial corbiculate core *Lactobacillus* (Firmicutes), *Bifidobacterium* (Actinobacteria), *Snodgrassella*, *Gilliamella*; Honeybee specific *Frischella* and *Bartonella*; eusocial corbiculate less abundant but also dominant Acetobacteraceae Alpha 2.1 - *Commensalibacter* sp. and Alpha 2.2 - *Parasaccharibacter* and *Bombella* (Proteobacteria), environmental Acetobacteraceae already associated to corbiculate bees *Saccharibacter*; possible core bacteria for *Melipona* stingless bees *Floriccoccus*; and *T. angustula* possible core bacteria *Izhakiella*, *Nguyenibacter*. This approach gave us information of how the occurrence of "strictly environmental" bacteria (not commonly found in association with eusocial corbiculate bees) is influenced by the environmental impact and change between categories.

Made that, we evaluated how the bacterial composition and diversity (alpha and beta), differs among the different sample categories (C1-C3). Barplots presented the relative abundances of core and non-core bacteria among samples. The Shannon, Richness and Simpson indexes were used to evaluate difference in diversity, number of ESVs, and the dominance of ESVs distribution among the categories (C1-C3), respectively. Beta diversity was evaluated by calculating the Bray-Curtis dissimilarity matrix after performing square-root transformation in the data and were plotted in a Non-metric Multidimensional Scaling (NMDS). Significance of differences in ESVs proportion and alpha diversity were accessed by Kruskal-Wallis test; In the Dendrogram and NMDS significance were obtained by PERMANOVA. In addition, we used the complete dataset to verify the occurrence of habitat specialist ESVs, i.e. ESVs significantly associated (p-value <0.05) to any of the categories, to understand the influence of environmental impacts in microbiome shaping, by utilization of the R package "indicspecies" (DE CÁCERES; LEGENDRE, 2009).

Results

Initially, we observed that our sequencing efforts adequately covered microbial diversity in the *Tetragonisca angustula* samples from the three categories (Figure 2).

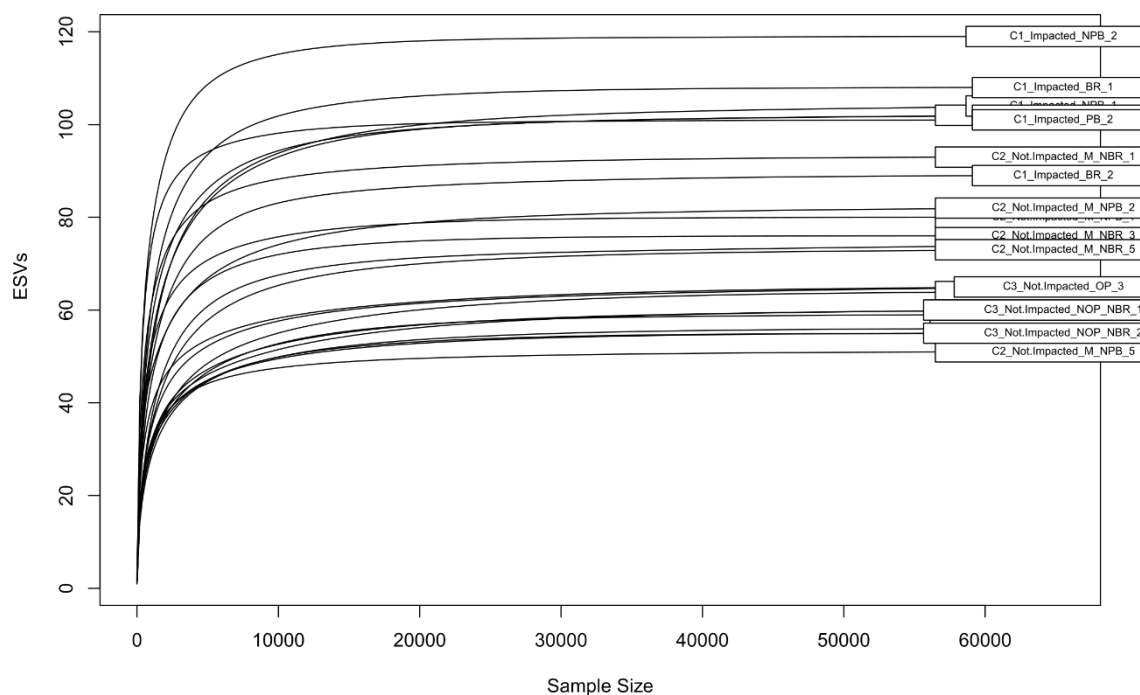


Figure 2 – Rarefaction curves for all the samples in the dataset. We observed plateaus for all samples, indicating adequate cover of microbial diversity.

Then, proceeding the data analysis, we found that microbial community composition of samples tend to be less dissimilar among the two Not-impacted areas compared to impacted areas (Figure 3). In this sense, Not-impacted C2 and C3 categories formed a clade comprising most of their samples, except for the two C2 samples collected closer to the C1 impacted area of Bento Rodrigues that, interestingly, clustered with impacted areas. There was also a tendency of clustering by sampling location, except for the samples C3 collected Near Ouro Preto – Near Bento Rodrigues and C1 collected in Paracatu de baixo (Figure 3).

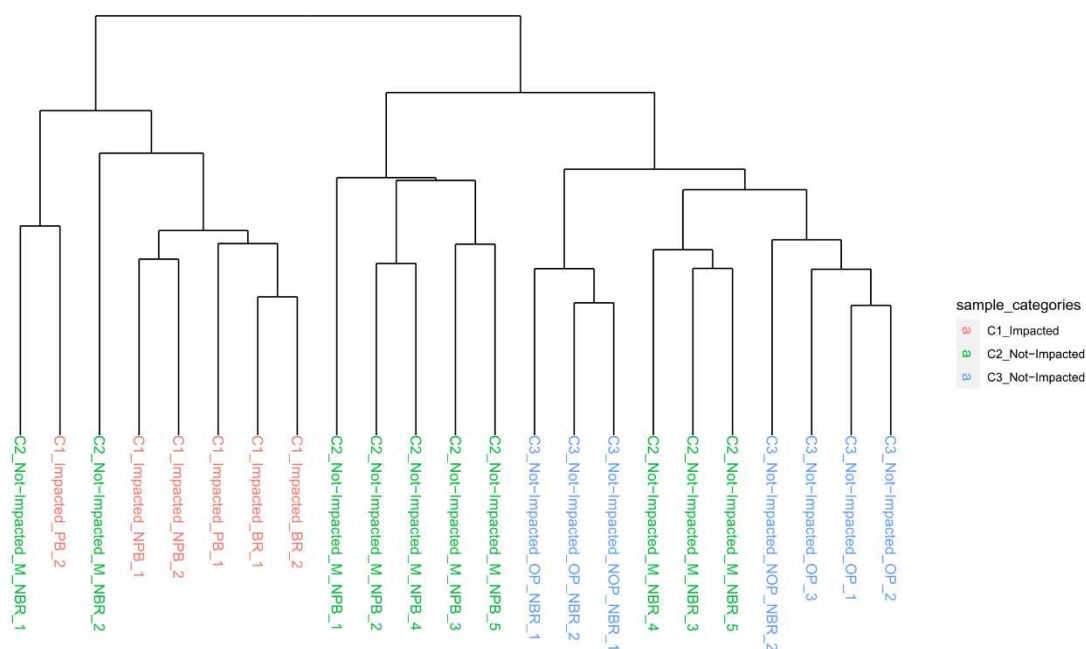


Figure 3 - Sample grouping by community composition, based on a Bray-Curtis dissimilarity matrix calculated on square-root transformed dataset.

Since samples grouped based on category, we evaluated the differences in core (present in all samples) and non-core (exclusion of core microorganisms – see methods) microorganisms between the categories. Considering core ESVs only we observed a slight tendency of grouping by category; lower dissimilarity was observed between the C2 and C3 Not-impacted areas in relation to C1 impacted areas in the axis MDS2 (Figure 4). Also, in the C1 impacted area most samples (except one), despite being collected in three different locations (Table 1), grouped close to each other, indicating less dissimilar core ESVs composition between replicates, compared to C2 and C3 Not-impacted areas (Figure 4). However, in relation to MDS1 most samples, regardless of the category, tended to be close to the positions 0.0 to 0.2.

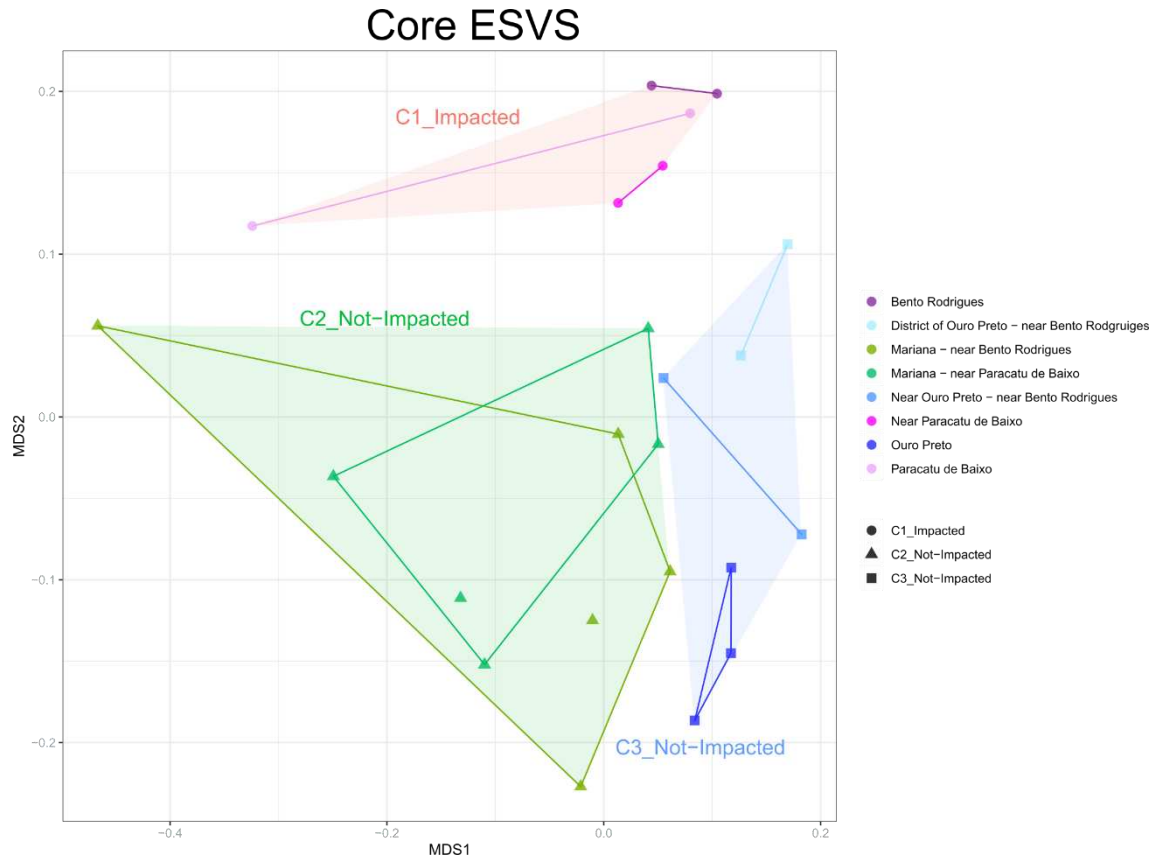


Figure 4 – Non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix calculated on square-root transformed core ESVs dataset.

In general, core ESVs belonged to genera already described as dominant gut/hive associated bacteria for eusocial corbiculate bees, such as *Bifidobacterium*, *Gilliamella*, *Lactobacillus*, *Snodgrassella* and *Bombella* and to less common genera *Izhakiella* (Erwiniaceae) and *Nguyenibacter* (Acetobacteraceae) genus (Figure 5). However, as indicated by NMDS (Figure 4), the proportion of ESVs between categories is different (Figure 5). We highlight significantly higher prevalence of *Snodgrassella* ESV_2 in the impacted areas (Kruskall-wallis p-value <0.05) and of *Snodgrassella* ESV_4 in Not-impacted areas (significantly higher in C2). *Izhakiella* ESV_6 was statistically higher in C3, comprising around 26% of the dataset in this category. In C2 correspond to 6% of the dataset and only 0.1% in C3. Thus, this ESV is more associated to Not-impacted areas.

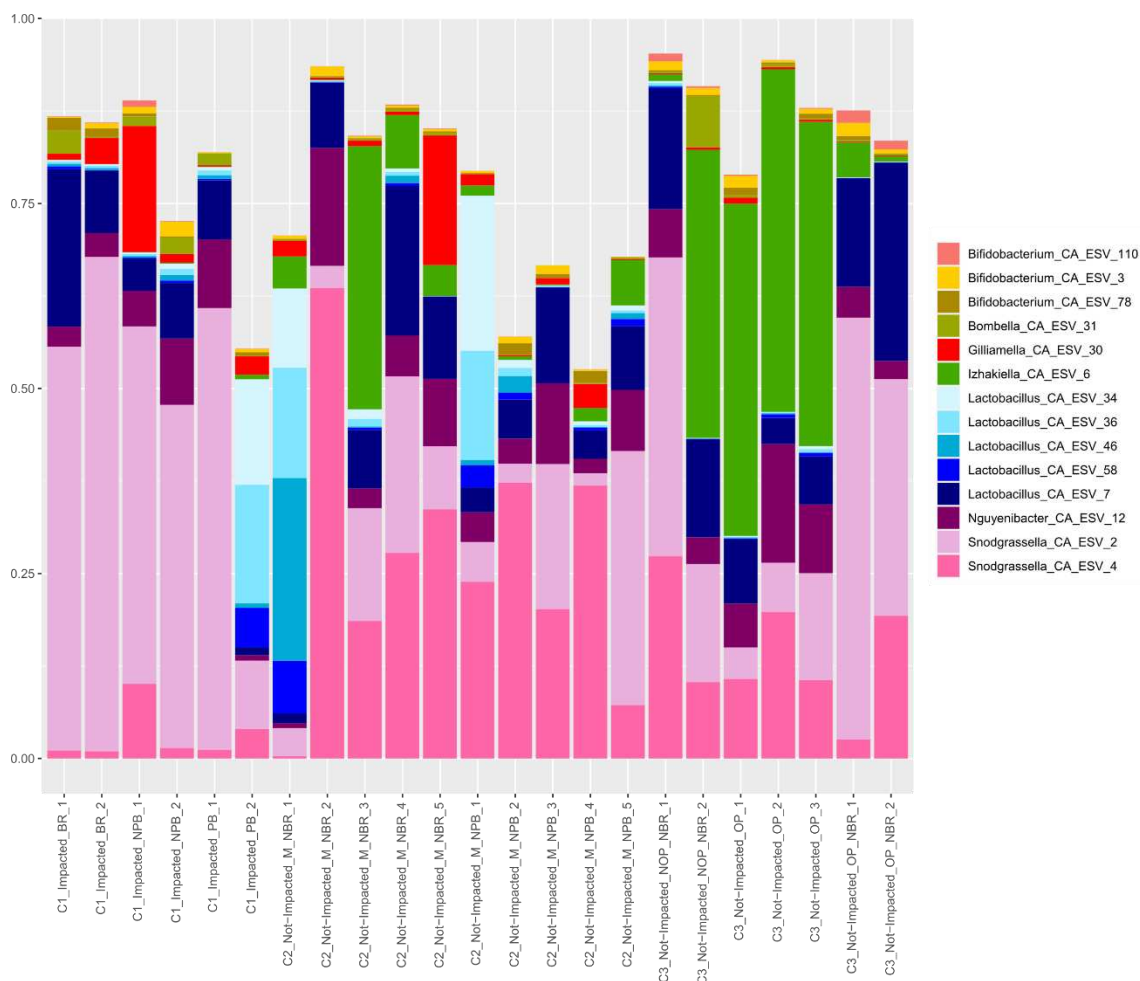


Figure 5 - Relative abundance of the core ESVs found in the dataset.

In addition, impacted areas tended to have higher dominance of core ESVs than Not-impacted areas, i.e., lower overall Simpson diversity, corroborating the favoring of some specimens possibly due to the environmental impact (Figure 6).

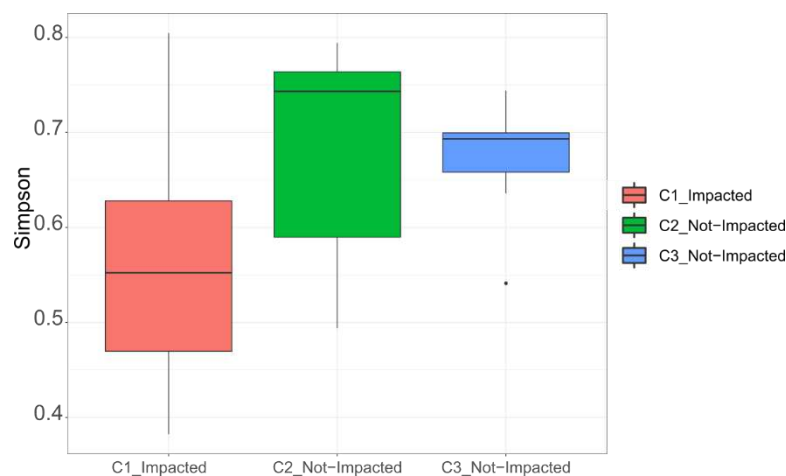


Figure 6 – Mean Simpson diversity index values for the three categories. Higher Simpson index (1-D) represent lower dominance. The x-axis represent the index values and y-axis the categories. The

interquartile ranges (IQR) between the first and third quartiles are represented by top and bottom boxes boundaries, respectively. The horizontal lines inside boxes represent the median diversity values. The lowest and highest diversity values are shown in the ends of the vertical lines.

Concerning the non-core bacteria, we observed that most C1 impacted samples had lower richness of ESVs and Shannon diversity indexes than the Not-impacted areas (Figure 7, Figure 8).

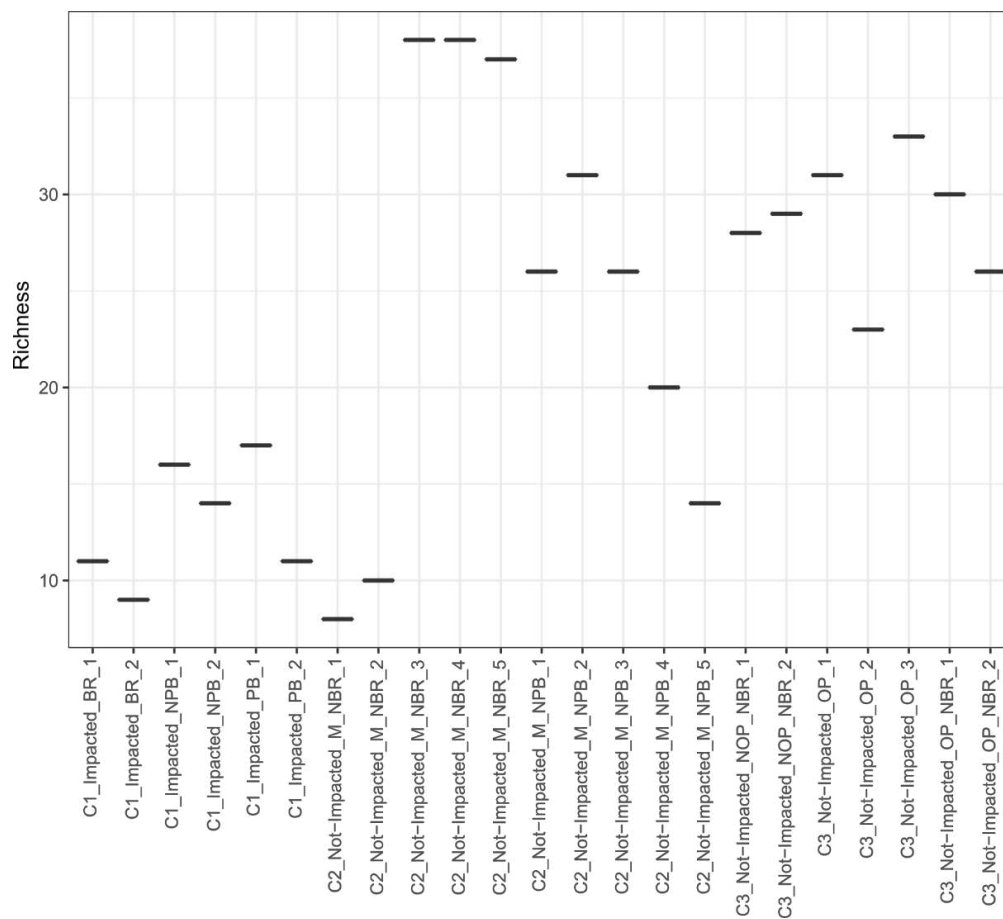


Figure 7 - ESV richness for each sample.

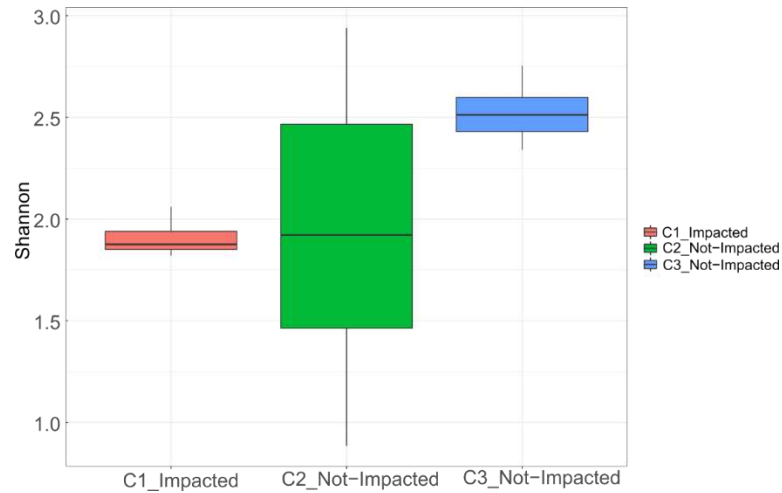


Figure 8 - Shannon diversity index for each of the sample categories. The x-axis represent the index values and y-axis the categories. The interquartile ranges (IQR) between the first and third quartiles are represented by top and bottom boxes boundaries, respectively. The horizontal lines inside boxes represent the median diversity values. The lowest and highest diversity values are show in the ends of the vertical lines.

Concerning beta diversity, dissimilarity among C1 impacted and C2/C3 Not-impacted samples increased in the non-core dataset (except for two C2 samples); most C1 impacted samples are at MDS1 position -0.6 to -0.4, while Not-impacted C2/C3 are located between 0.2 to 0.4 (Figure 9). Also, dissimilarity decreased between the samples of two Not-impacted categories in the non-core dataset in relation to the core dataset, except for two C2 samples (Figure 9).

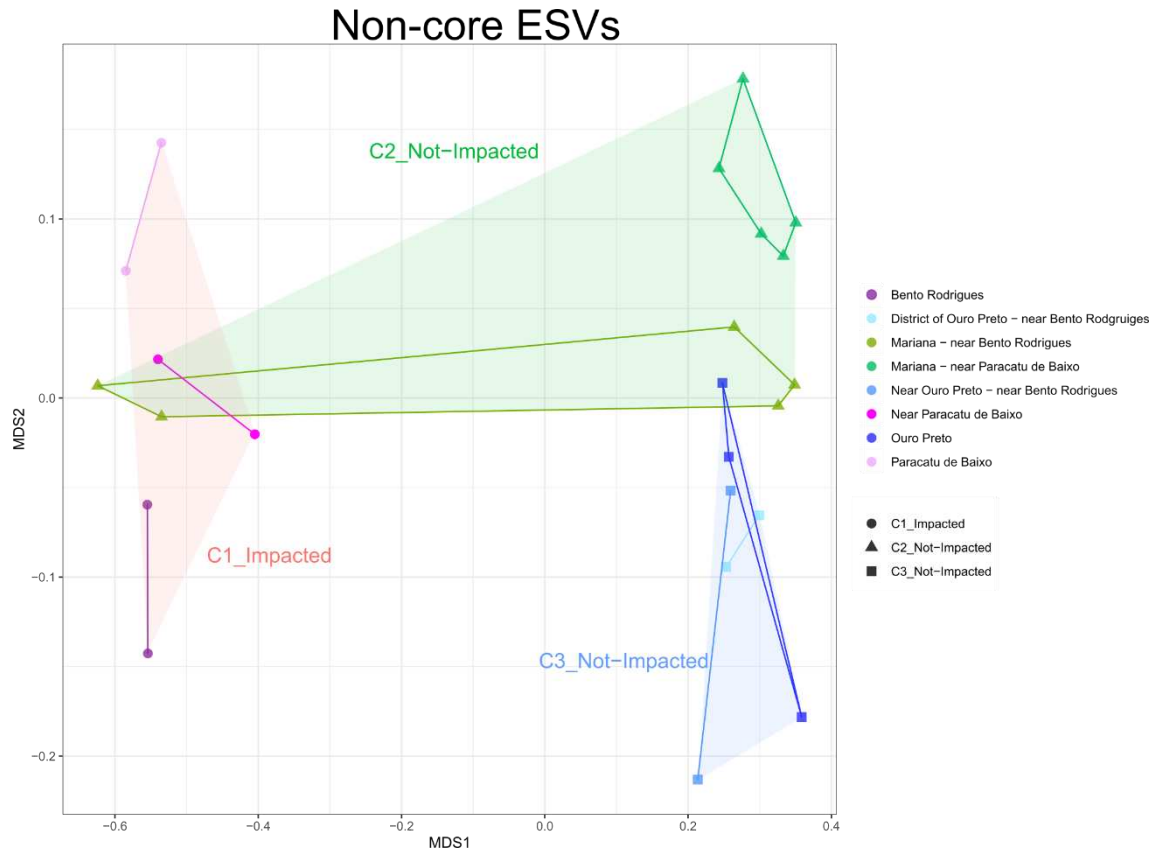


Figure 9 - Non-metric multidimensional scaling (NMDS) based on a Bray-Curtis dissimilarity matrix calculated on square-root transformed core ESVs dataset.

Finally, we searched for habitat specific ESVs (indicator ESVs) for each sample category. We observed a higher number of indicator ESVs for the C1 impacted area (71) in relation to C2/C3 not impacted areas, 13 and 15 respectively (Figure 10). Few ESVs are shared among impacted and not impacted areas, but 12 are shared among C2/C3 not impacted areas.

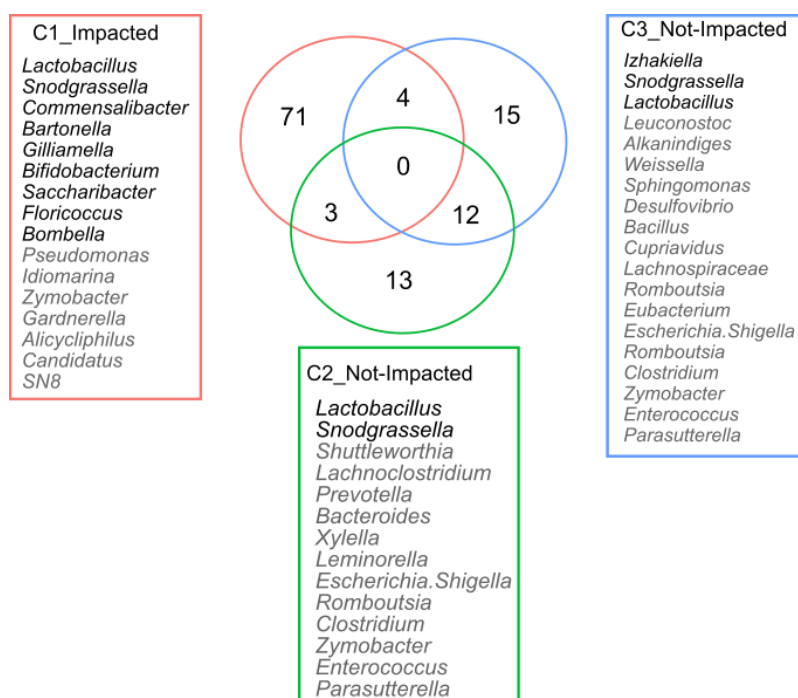


Figure 60 - Venn diagram showing the number and boxes showing the genus level classification of the indicator ESVs found in each sample category. Genera written in black letters are those, belonging to the ones considered as high abundant/core genera in eusocial corbiculate bees, *Melipona*, and/or genera of which our core ESVs belong (genera filtered out in the non-core dataset – see methods). Remaining genera are written in gray letters.

Interestingly, most of these C1 core ESVs belonged to some of the genera considered as high abundant/core genera in eusocial corbiculate bees, *Melipona*, and/or genera of which our core ESVs belong (genera filtered out in the non-core dataset – see methods), such as *Lactobacillus*, *Snodgrassella*, *Commensalibacter*, *Bartonella*, *Gilliamella*, *Bifidobacterium*, *Saccharibacter*, *Floricoccus* and *Bombella*. Some of the most abundant indicator ESVs found in our dataset are represented in Figure 11, including *Commensalibacter*, *Bartonella* and *Bifidobacterium* commonly associated to eusocial corbiculate bees and indicator for impacted areas (Figure 11 - red colored ESVs). Otherwise, most abundant indicator ESVs for C2 and C3 Not-impacted areas do not belong to genera not commonly associated to eusocial corbiculate bees (Figure 10 – green and blue ESVs).

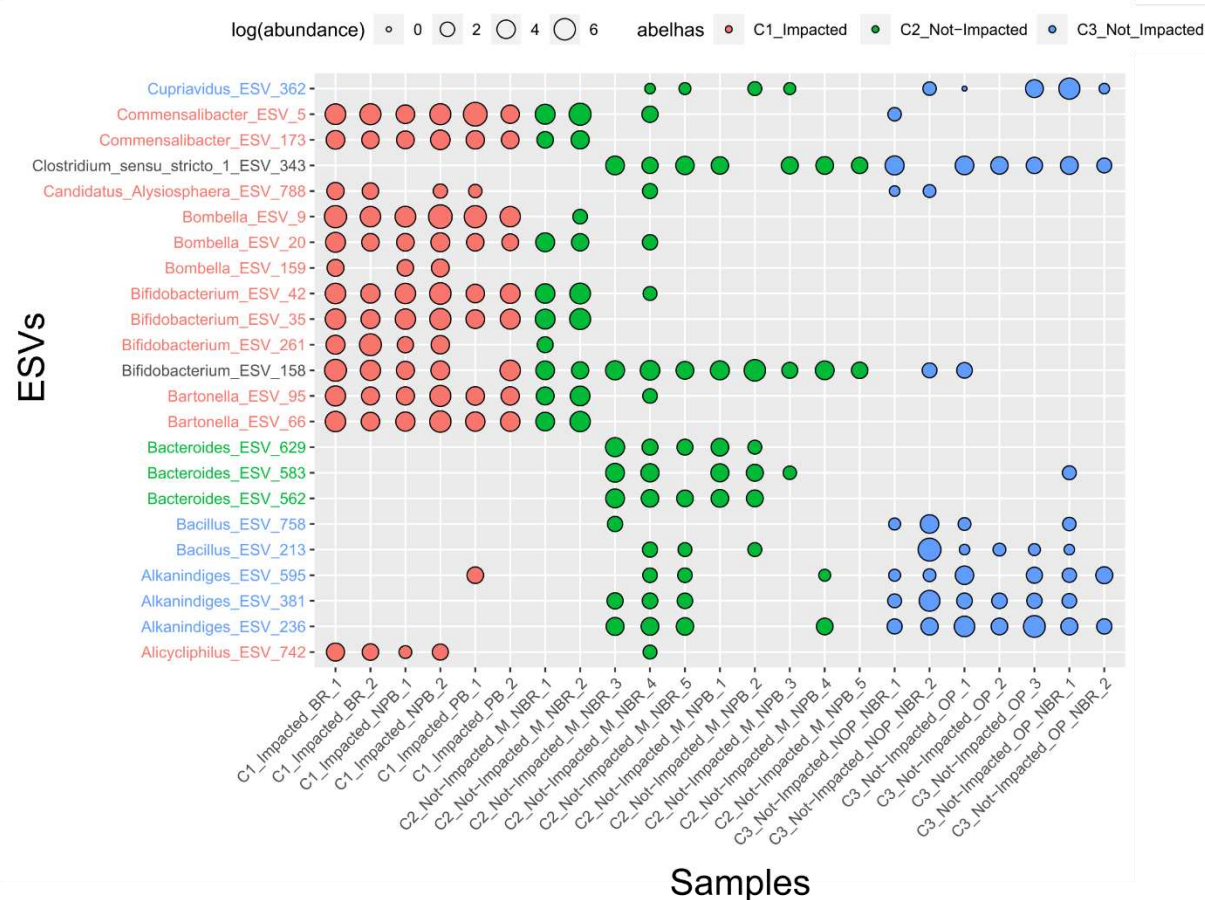


Figure 11 - Most abundant indicator ESVs in the complete dataset. Red colored ESVs are habitat specific for C1- Impacted areas; green and blue colored ESVs, are indicator for C2 and C3 Not-impacted areas, respectively. Bubbles represent log(abundance). *Clostridium_sensu_stricto_1_ESV_343* is indicator for C2 and C3 Not-impacted areas. *Bifidobacterium_ESV_158*, although occur in C3, is only indicator for C1 and C2 areas.

Discussion

Our study adequately covered gut associated microbial diversity of *T. angustula* samples (Figure 2) in the different studied categories, thus the results are not biased by underestimation of microbial community. Overall, environmental stressors altered microbial community, since most samples from impacted areas and not impacted areas grouped separately (Figure 3).

We then evaluated differences in microbial community composition among core ESVs and “strictly environmental” ESVs (see methods). The community composition of core ESVs, i.e., those found in all samples, is influenced by environmental impact, as shown by the differences among C1-impacted and C2/C3 not-impacted categories in relation to axis MDS2 (Figure 4). Even samples from different C1-impacted localities (Table 1, Figure 4) grouped close to each other, showing that environmental impact tended to have a similar effect on microbial community, even in different areas.

Afterwards, we described the taxonomy of the core ESVs associated to *T. angustula*. The occurrence of ESVs from the eusocial corbiculate core *Lactobacillus*, *Bifidobacterium*, *Snodgrassella*, and *Gilliamella* genera in all the 23 evaluated samples (Figure 5), regardless of sample categories, can be explained their transmission by insect sociality (KOCH; SCHMID-HEMPEL, 2011; KWONG et al., 2017; KWONG; MORAN, 2016; MARTINSON et al., 2011; POWELL et al., 2014). However, the proportion of ESVs from these genera differed among categories, especially the prevalence of *Snodgrassella* ESV_2 in the impacted areas (Kruskall-Wallis p-value <0.05), and of *Snodgrassella* ESV_4 in Not-impacted areas (significantly higher in C2) indicating that some strains can possibly be favored/decreased in impacted areas. Increase and decrease of *Snodgrassella* ESVs has been related in cadmium and selenate treated honeybees, respectively (ROTHMAN et al., 2019). Similar tendency was observed in an experiment that compared the microbiome of bumblebees between control and samples treated with field-realistic doses of selenate, copper, and cadmium. Treatments did not affect the occurrence of the same core bacteria (*Lactobacillus*, *Bifidobacterium*, *Snodgrassella*, *Gilliamella*), but alteration in their ESVs proportions among the treatments show that some ESVs are more tolerant/susceptible to the chemicals, and that the treatments possibly affect bees health (ROTHMAN et al., 2020). Indeed, *T. angustula* bees are

capable of accumulating contaminants (BARBOSA et al., 2021) which might affect microbial composition. Although we did not evaluate the levels of pollutants in our bees, increased levels of metals, including cadmium content above the levels of the National Environment Council, were observed in sediments of Rio Doce after the mining ore tailings deposition (DUARTE et al., 2021). Increased copper and other metals has also been observed in soils and plants located in areas affected by the Fundão dam residues (COELHO et al., 2020).

Other genera such as *Bombella* (Acetobacteraceae Alpha 2.2) usually less prevalent but also corbiculate associated (BONILLA-ROSSO et al., 2019; KWONG et al., 2017; KWONG; MORAN, 2016; NOWAK et al., 2021), *Nguyenibacter* and *Izhakiella* have also core ESVs in *T. angustula*, the latter being statistically higher in C3 (*Izhakiella* ESV_6). *Nguyenibacter* and *Izhakiella* are not commonly described in association to eusocial corbiculate bees, but the occurrence of core ESVs of these genera points toward a possible stable association with *Tetragonisca angustula*. Nevertheless, more extensive sampling in different areas is needed to confirm such relationship. In general, concerning core ESVs, dominance is higher in impacted areas (Figure 6), i.e., considering the same dataset the proportion of ESVs is more even in non-impacted samples, which is certainly due to the high dominance of *Snodgrassella* ESV_2 in the impacted areas (Figure 5).

Non-core bacteria were considered strictly environmental, because the most associated bacteria to eusocial corbiculate bees, including stingless bees, and core ESVs found in our dataset were filtered out from the data (see methods). Remaining bacteria were those usually not associated to bees. The non-core dataset showed lower richness of ESVs and Shannon diversity in the C1 impacted samples in comparison to not impacted C2/C3 (Figure 7, Figure 8). Environmental transmission is an important source of acquired bacterial, being more important than social transmission in facultatively social *Megalopta* bees (MCFREDERICK et al., 2014). Environmental bacteria are also important sources of microbes in eusocial bees, possibly being acquired during foraging or from hive material (KWONG et al., 2017). The lower richness and diversity of environmental bacteria in impacted areas may be due to compromised foraging, because of the altered availability and diversity of plant resources and the presence of metals in areas impacted by the Fundão dam mining ore tailings. In this sense, predominance of pollen from herbs and sub-shrubs in the

impacted areas of Bento Rodrigues and Paracatu de Baixo in bee-bread at nests of *T. angustula* has been detected, instead of pollen from secondary forests species was observed (VIEIRA et al., 2020). This is a consequence of the force of the mud that uprooted many trees in these impacted areas as well as of the introduction of herbaceous species in affected areas to prepare the soil for further plantation of other species (VIEIRA et al., 2020). In addition, bees have already been reported to reduce the foraging time in nectar contaminated plants with metals, such as Ni (nickel), but are not capable to discriminate Al (aluminum) contaminated nectar (MEINDL; ASHMAN, 2013). Al (Aluminum) can affect bees foraging abilities, compromising pollination (CHICAS-MOSIER et al., 2017). Increased metals concentration (including Al) were detected in sediments impacted by the Fundão mud (GOMES et al., 2017). Taken together, we infer that alteration in plant composition due to forest destruction and introduction of herbaceous plants, possible avoidance of some contaminated plants but contact with other plants with contaminants non-distinguishable for bees – affecting bee foraging, might have contributed to reduction of richness and diversity of environmental bacteria, usually acquired by foraging.

The same factors might explain the higher dissimilarity among C1 impacted and C2/C3 not impacted and the reduced dissimilarity among C2/C3 samples in the non-core dataset, compared to core dataset (Figure 9, Figure 4). Bees from not impacted areas, find more similar floral resources and less contaminants than impacted areas, do not suffering impact in forage-derived microbial acquisition, thus having closer bacterial composition among them, and higher dissimilarity compared to impacted areas (Figure 9). In the core dataset, otherwise, most bee bacterial species are socially transmitted, and, although some strains are favored/decreased, similar bacteria are found in the different categories.

Finally, using the whole dataset we detected a high number of habitat specific (indicator) ESVs for the C1 impacted category, and most of these belonged to commonly bee associated genera (Figure 10, Figure 11). C2/C3 impacted areas, otherwise, had lower number of indicators ESVs and most of them belonging to genera not dominant or undescribed in bees. Together with previous results, these observations indicate that bees living in impacted areas might have altered foraging activity and do not obtain many bacteria from the environment. On the other side, socially transmitted bacteria predominate in these bees in such a way that habitat

specific strains, possibly tolerant to stress conditions, are selected in these bees. Bees living in different not impacted areas otherwise, may share anciently associated core strains not specialized for stress conditions, and most habitat specific ESVs are those from the surrounding environment they live, because of occurrence in natural environmental conditions.

Social bees have been described as bioindicators of environmental pollution, such as occurrence of heavy metals (GIZAW et al., 2020) accumulated from the environment (plants, soil and water) during foraging (ZARIC et al., 2018). Among them, *Tetragonisca angustula* is an important tool for environmental monitoring studies in Atlantic Forest (BARBOSA et al., 2021), and here we observed that their associated microorganisms might also respond to environmental impacts, corroborating our hypothesis that bees living in impacted areas have altered microbial community in relation to not-impacted areas. Thereby, *T. angustula* microbiota can be included in the monitoring of environmental impacts of the Fundão dam collapse, since its alteration might affect bee health and consequently pollination services, compromising recovery of impacted areas. Studies on stingless bee's microbiome will clarify the importance of associated microorganisms for *T. angustula* survival and might open perspectives on the isolation and use of putative resistant strains as probiotics for bees living in impacted areas.

Acknowledgments

We acknowledge the economic and structural support of the Federal University of Viçosa. The financial support was provided by the CNPq, CAPES - finance code 001 and FAPEMIG. We thank Helder Resende for providing bees.

References

- BARBOSA, M. DE M. et al. Effects of native forest and human-modified land covers on the accumulation of toxic metals and metalloids in the tropical bee *Tetragonisca angustula*. **Ecotoxicology and environmental safety**, v. 215, p. 112147, 2021.
- BONILLA-ROSSO, G. et al. Acetobacteraceae in the honey bee gut comprise two distant clades with diverging metabolism and ecological niches. **bioRxiv**, v. 41, n. 0, p. 1–50, 2019.
- BROWN, J. C.; ALBRECHT, C. The effect of tropical deforestation on stingless bees of the genus *Melipona* (Insecta : Hymenoptera : Apidae : Meliponini) in central Rondonia , Brazil. **Science**, v. 28, n. 5, p. 623–634, 2001.
- CALLAHAN, B. J. et al. DADA2 : High-resolution sample inference from Illumina amplicon data. **Nat Methods**, v. 13, n. 7, p. 581–583, 2016.
- CARDOSO, E. B. et al. Composition and diversity of prokaryotes at an iron ore post-mining site revealed the natural resilience 10 years after mining exploitation. **Land Degrad Dev.**, p. 1–14, 2020.
- CERQUEIRA, A. E. S. et al. Extinction of anciently associated gut bacterial symbionts in a clade of stingless bees. **ISME Journal**, p. 2–5, 2021.
- CHICAS-MOSIER, A. M. et al. The effects of ingested aqueous aluminum on floral fidelity and foraging strategy in honey bees (*Apis mellifera*). **Ecotoxicology and environmental safety**, v. 143, n. February, p. 80–86, 2017.
- COELHO, D. G. et al. Evaluation of Metals in Soil and Tissues of Economic - Interest Plants Grown in Sites Affected by the Fundão Dam Failure. **Integrated Environmental Assessment and Management**, v. 00, n. 00, p. 1–12, 2020.
- DE CÁCERES, M.; LEGENDRE, P. Associations between species and groups of sites : indices and statistical inference. **Ecology**, v. 90, n. 12, p. 3566–3574, 2009.
- DOLEZAL, A. G.; TOTH, A. L. Feedbacks between nutrition and disease in honey bee health. **Current Opinion in Insect Science**, v. 26, p. 114–119, 2018.
- DUARTE, E. B. et al. Trace metals in Rio Doce sediments before and after the collapse of the Fundão iron ore tailing dam, Southeastern Brazil. **Chemosphere**, v. 262, p. 127879, 2021.
- ENGEL, P.; MARTINSON, V. G.; MORAN, N. A. Functional diversity within the simple gut microbiota of the honey bee. **Proceedings of the National Academy of Sciences**, v. 109, n. 27, p. 11002–11007, 2012.
- FERREIRA, G. W. D. et al. Assessment of iron-rich tailings via portable X-ray fluorescence spectrometry: the Mariana dam disaster, southeast Brazil. **Environmental Monitoring and Assessment**, v. 193, n. 4, p. 1–19, 2021.
- GIANNINI, T. C. G. et al. Crop pollinators in Brazil : a review of reported interactions. **Apidologie**, v. 46, p. 209–223, 2015.
- GIZAW, G. et al. Effect of environmental heavy metals on the expression of detoxification-related genes in honey bee *Apis mellifera*. **Apidologie**, v. 51, n. 4, p. 664–674, 2020.
- GOMES, L. E. DE O. et al. The impacts of the Samarco mine tailing spill on the Rio Doce estuary, Eastern Brazil. **Marine Pollution Bulletin**, v. 120, n. 1–2, p. 28–36, 2017.
- HEARD, T. A. The role of stingless bees in crop pollination. **Annual Review of Entomology**, v. 44, n. 131, p. 183–206, 1999.
- HLADUN, K. R. et al. Metal contaminant accumulation in the hive: Consequences for whole-colony health and brood production in the honey bee (*Apis mellifera* L.).

- Environmental Toxicology and Chemistry**, v. 35, n. 2, p. 322–329, 2016.
- IBAMA. Laudo Técnico Preliminar: Impactos ambientais decorrentes do desastre envolvendo o rompimento da barragem de Fundão, em Mariana, Minas Gerais. v. 34, n. 1, p. 1–9, 2015.
- KATSNELSON, A. The puzzle in a bee 's gut. **Nature**, v. 521, n. 2014, p. S56, 2015.
- KOCH, H.; SCHMID-HEMPEL, P. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. **Proceedings of the National Academy of Sciences of the United States of America**, v. 108, n. 48, p. 19288–19292, 2011.
- KWONG, W. K. et al. Dynamic microbiome evolution in social bees. **Science Advances**, v. 3, n. 3, p. 1–17, 2017.
- KWONG, W. K.; MANCENIDO, A. L.; MORAN, N. A. Immune system stimulation by the native gut microbiota of honey bees Subject Category : Subject Areas : **R. Soc. open sci**, v. 4, p. 1–9, 2017.
- KWONG, W. K.; MORAN, N. A. Gut microbial communities of social bees. **Nature Reviews Microbiology**, v. 14, p. 374–384, 2016.
- MARTINSON, V. G. et al. A simple and distinctive microbiota associated with honey bees and bumble bees. **Molecular Ecology**, v. 20, n. 3, p. 619–628, 2011.
- MCFREDERICK, Q. S. et al. Host species and developmental stage, but not host social structure, affects bacterial community structure in socially polymorphic bees. **FEMS Microbiology Ecology**, v. 88, n. 2, p. 398–406, 2014.
- MCMURDIE, P. J.; HOLMES, S. phyloseq : An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. **PLoS ONE**, v. 8, n. 4, p. e61217, 2013.
- MEINDL, G. A.; ASHMAN, T. L. The effects of aluminum and nickel in nectar on the foraging behavior of bumblebees. **Environmental Pollution**, v. 177, p. 78–81, 2013.
- MORAIS, C. R. DE et al. Ecotoxicological effects of the insecticide fi pronil in Brazilian native stingless bees *Melipona scutellaris* (Apidae : Meliponini). **Chemosphere**, v. 206, p. 632–642, 2018.
- MORÓN, D. et al. Survival, reproduction and population growth of the bee pollinator, *Osmia rufa* (Hymenoptera: Megachilidae), along gradients of heavy metal pollution. **Insect Conservation and Diversity**, v. 7, n. 2, p. 113–121, 2014.
- MOTTA, E. V. S.; RAYMANN, K.; MORAN, N. A. Glyphosate perturbs the gut microbiota of honey bees. **Proceedings of the National Academy of Sciences**, v. 115, n. 41, p. 10305–10310, 2018.
- NOWAK, A. et al. Characterization of *Apis mellifera* Gastrointestinal Microbiota and Lactic Acid Bacteria for Honeybee Protection-A Review. **Cells**, v. 10, n. 3, p. 1–29, 2021.
- OKSANEN, J. et al. **vegan: Community Ecology Package**. [s.l: s.n.].
- OLOFSSON, T. C.; VÁSQUEZ, A. Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. **Current Microbiology**, v. 57, n. 4, p. 356–363, 2008.
- OMACHI, C. Y. et al. Atlantic Forest loss caused by the world's largest tailing dam collapse (Fundão Dam, Mariana, Brazil). **Remote Sensing Applications: Society and Environment**, v. 12, p. 30–34, 2018.
- POWELL, J. E. et al. Routes of Acquisition of the Gut Microbiota of the Honey Bee *Apis mellifera*. **Appl. Environ. Microbiol.**, v. 80, n. 23, p. 7378–7387, 2014.
- POWELL, J. E. et al. Genome-wide screen identifies host colonization determinants in a bacterial gut symbiont. **Proceedings of the National Academy of Sciences**, v. 113, n. 48, p. 13887–13892, 2016.
- PRONI, E. A.; MACIEIRA, O. J. D. Ritmo circadiano da taxa respiratória de

- Tetragonisca angustula fiebrigi (Schwarz), T. a. angustula (Latreille) e Trigona spinipes (Fabricius) (Hymenoptera, Apidae, Meliponinae). **Revista Brasileira de Zoologia**, v. 21, n. 4, p. 987–993, 2004.
- RASMUSSEN, C.; CAMERON, S. A. Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. **Biological Journal of the Linnean Society**, v. 99, p. 206–232, 2010.
- ROMIGUIER, J. et al. Phylogenomics controlling for base compositional bias reveals a single origin of eusociality in corbiculate bees. **Molecular Biology and Evolution**, v. 33, n. 3, p. 670–678, 2016.
- ROTHMAN, J. A. et al. Cadmium and Selenate Exposure Affects the Honey Bee Microbiome and Metabolome, and Bee-Associated Bacteria ABSTRACT. **Applied and Environmental Microbiology**, v. 85, n. 21, p. e01411-19, 2019.
- ROTHMAN, J. A. et al. The direct and indirect effects of environmental toxicants on the health of bumblebees and their microbiomes. **Proc. R. Soc. B.**, v. 287, 2020.
- SEIDE, V. E. et al. Glyphosate is lethal and Cry toxins alter the development of the stingless bee *Melipona quadrifasciata*. **Environmental Pollution**, v. 243, p. 1854–1860, 2018.
- SLAA, E. J. et al. Stingless bees in applied pollination: Practice and perspectives. **Apidologie**, v. 37, n. 2, p. 293–315, 2006.
- TOMÉ, H. V. V et al. Imidacloprid-Induced Impairment of Mushroom Bodies and Behavior of the Native Stingless Bee *Melipona quadrifasciata anthidioides*. **PLoS ONE**, v. 7, n. 6, p. 1–10, 2012.
- VANENGELSDORP, D. et al. Colony collapse disorder: A descriptive study. **PLoS ONE**, v. 4, n. 8, 2009.
- VÁSQUEZ, A.; OLOFSSON, T. C. The lactic acid bacteria involved in the production of bee pollen and bee bread. **Journal of Apicultural Research and Bee World**, v. 48, n. 3, p. 189–195, 2009.
- VIEIRA, K. I. C. et al. Floral resources used by *Tetragonisca angustula* (Latreille 1811) in areas under the influence of the breach of the Fundão Dam in Mariana (Minas Gerais, Brazil). **Grana**, v. 59, n. 4, p. 273–303, 2020.
- WICKHAM, H. **ggplot2: Elegant Graphics for Data Analysis**. [s.l.] Springer-Verlag New York, 2016.
- ZARIC, N. M. et al. Assessment of spatial and temporal variations in trace element concentrations using honeybees (*Apis mellifera*) as bioindicators. **PeerJ**, v. 2018, n. 7, 2018.
- ZHENG, H. et al. Honey bees as models for gut microbiota research. **Lab Anim**, v. 47, p. 317–325, 2019.

Conclusões gerais

Em relação as abelhas sem ferrão do gênero *Melipona*, conclui-se que:

- Abelhas possuem maior diversidade alfa do que o mel, possivelmente devido às altas propriedades antimicrobianas do mel. Além disso, a microbiota das abelhas tende a ser mais espécie-específica que a do mel - mais influenciada por microrganismos ambientais.
- Fungos filamentosos predominam nas abelhas, sendo adquiridos do ambiente/plantas durante o forrageio ou são associados às colmeias. As leveduras são bem difundidas, mas são mais prevalentes em amostras de mel e próximas a isolados anteriormente encontrados no ambiente de colmeias e associados ao metabolismo do mel. As leveduras abundantes nas abelhas são ambientais.
- Em relação às bactérias, o mel apresenta grande abundância de espécies próximas às transientemente associadas a abelhas, espécies derivadas de plantas e espécies ambientais frutofílicas. As abelhas estão mais associadas a bactérias simbióticas intestinais e bactérias derivadas de plantas/ambientais.
- *Melipona* não possuem *Snodgrassella* e *Gilliamella*, bactérias simbiontes com ampla distribuição em outras abelhas sociais corbiculadas sociais. Em contraste, espécies de *Melipona* possuem maior quantidade de bactérias ambientais, incluindo *Lactobacillus*, *Acetobacteraceae*, *Floricoccus* e possivelmente *Pectinatus* e leveduras específicas de abelhas do gênero *Starmerella*. A perda de *Snodgrassella* e *Gilliamella* pode ser resultado de mudanças ecológicas em *Melipona* ou da aquisição de novos simbiontes como substitutos funcionais.

Em relação às diferenças na composição microbiana entre *Tetragonisca angustula* habitantes de áreas impactadas e não impactadas pelo rejeito da barragem de Fundão, Mariana, conclui-se que:

- O impacto ambiental possivelmente causou alteração na composição microbiana, tais como o favorecimento/dano de algumas linhagens "core", a exemplo de linhagens de *Snodgrassella* que embora presente em ambas as

amostras, compõem uma proporção diferente entre áreas impactadas e não impactadas, indicando a possível ocorrência de linhagens mais resistentes aos impactos ambientais.

- Houve também redução da riqueza de bactérias "estritamente" ambientais em áreas impactadas, possivelmente pela redução do forrageio em função da alteração da composição vegetal, e danos causados pelos contaminantes nos ambientes. Como resultado, menor diversidade e riqueza bactérias ambientais foram obtidas.
- Microrganismos do intestino das abelhas sem ferrão são alterados em áreas atingidas pelo desastre de Mariana. Portanto, alterações no microbioma destes animais podem ser consideradas em estudos de impactos ambientais, visto que potencialmente afetam a saúde e, conseqüentemente, os serviços de polinização, comprometendo a recuperação das áreas impactadas.

Esta tese contribuiu para o conhecimento da microbiota associada às abelhas sem ferrão e abrem perspectivas para o desenvolvimento desta linha de pesquisa. Os resultados obtidos podem nortear estudos sobre o microbioma destas abelhas que, aliados ao isolamento e identificação de microrganismos, podem elucidar as suas funções e importância para o hospedeiro, abrindo campo para aplicação destes microrganismos, incluindo possíveis linhagens resistentes ao estresse, para a preservação e saúde das abelhas.