SERVIÇO PÚBLICO FEDERAL
UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

MICROBIOMA DO ALIMENTO LARVAL DE ABELHAS SEM FERRÃO: DIVERSIDADE E POTENCIAL BIOTENOLÓGICO

Aluna: Ana Carolina Costa Santos

Orientador: Prof. Dr. Carlos Ueira-Vieira

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UBERLÂNDIA – MG
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Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Genética)

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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Genética e Bioquímica				
Defesa de:	Doutorado Acadêmico/PPGGB.				
Data:	Quatro de agosto de dois mil e vinte e dois	Hora de início:	14:00h	Hora de encerramento:	17:45
Matrícula do Discente:	11823GBI001				
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Título do Trabalho:	Microbioma do alimento larval de abelhas sem ferrão: diversidade e potencial biotenológico.				
Área de concentração:	Genética				
Linha de pesquisa:	Biologia Molecular e Celular				
Projeto de Pesquisa de vinculação:	Potencial biotecnológico do microbioma de abelhas brasileiras.				

Aos quatro dias do mês de agosto de dois mil e vinte e dois, às 14:00 horas, reuniu-se via web conferência pela Plataforma *Google Meet*, em conformidade com a Portaria nº 36, de 19 de março de 2020 da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Resolução de nº 06/2020 e Resolução nº 19/2022 do Conselho de Pesquisa e Pós-graduação pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Drª. Maria Elena de Lima Perez Garcia, Drª. Tatiana Amabile de Campos, Dr. Flavio Henrique da Silva, Dr. Carlos Henrique Gomes Martins e Dr. Carlos Ueira Vieira, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, Dr. Carlos Ueira Vieira apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o (a) senhor (a) presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o candidato (a):

APROVADA.

Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram

encerrados os trabalhos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

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"O sucesso nasce de querer, da determinação e persistência de chegar a um objetivo.
Mesmo não atingindo o alvo, quem busca e vence obstáculos,
no mínimo fará coisas admiráveis."
(José de Alencar)

DEDICATÓRIA

Dedico esse trabalho à minha família, ao meu noivo, Joberth, por toda paciência e dedicação, ao meu filho, Artur, pelo amor incondicional e à minha mãe, Claudia, pelo exemplo de força e inspiração! E ao meu orientador, que o idealizou e o tornou possível!

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Muito obrigada!

Sumário

APRESENTAÇÃO

Diante da importância das abelhas sem ferrão como principais polinizadores de espécies nativas do Brasil e da escassez de dados sobre a microbiota presente neste grupo, este estudo gerará conhecimento sobre a microbiota associada a esse inseto altamente social. Esta pesquisa terá como foco principal o uso biotecnológico de bactérias isoladas do alimento larval de abelhas sem ferrão.

No primeiro Capítulo desta pesquisa tratamos da importância das abelhas sem ferrão, da composição do alimento larval e sua importância para a diferenciação de castas dessas abelhas. Assim como, a importância da microbiota que compõe esse alimento larval. O potencial para uso biotecnológico dos produtos das abelhas sem ferrão e dos microrganismos associados a essas abelhas; O uso de ferramentas de NGS e de isolamento para caracterização de microrganismos; E por fim, a importância da descoberta de antimicrobianos para uso contra bactérias multirresistentes.

No Capítulo 2, apresentamos o artigo intitulado: "Microbiota associated with larval food of Brazilian stingless bees: *F. varia*, *M. quadrifasciata*, *M. scutellaris*, and *T. angustula*", disponível no formato de envio para publicação. Esta pesquisa traz resultados inéditos sobre a microbiota associada às abelhas sem ferrão *Frieseomelitta varia*, *Melipona quadrifasciata*, *Melipona scutellaris* e *Tetragonisca angustula*. Contribuindo para melhorar o entendimento sobre o papel desses microrganismos no desenvolvimento das abelhas.

No Capítulo 3, apresentamos o artigo "Antimicrobial activity of supernatants produced by bacteria isolated from Brazilian **stingless bee's larval food**"; já publicado. Neste trabalho são avaliados os efeitos antimicrobianos de diferentes sobrenadantes de bactérias isoladas do alimento larval de abelhas sem ferrão, das espécies *Melipona quadrifasciata, Melipona scutellaris* e *Tetragonisca angustula.* Nossas investigações resultaram na descoberta de bactérias com potencial de produção de moléculas antimicrobianas.

CAPÍTULO 1
FUNDAMENTAÇÃO TEÓRICA

1- ABELHAS SEM FERRÃO

1.1- Importância

As abelhas sem ferrão, pertencentes à subfamília Meliponinae (Apidae), representam o grupo de abelhas eusociais mais diverso, acomodando mais de 550 espécies em regiões tropicais e subtropicais (JOHNSON e HUBBELL, 1974; SILVEIRA, *et al.*, 2002; MICHENER, 2007). A principal característica desse grupo de abelhas é a ausência de um ferrão funcional (MELO et. al, 2020). As abelhas sem ferrão apresentam grande variação comportamental, nos sistemas de comunicação, estratégias de forrageamento, densidades populacionais e arquitetura de ninhos (BIESMEIJER, et al, 2004; MICHENER, 1974; SAKAGAMI, 1982). Pode apresentar comportamento de forrageamento oportunista ou generalista, dependendo da espécie, podendo apresentar preferências por algumas flores para otimizar o forrageamento (RAMALHO et al. 2007).

Acredita-se que as abelhas sem ferrão sejam responsáveis por 40 a 90% da polinização das árvores nativas (KERR, et al., 1996), sendo os polinizadores mais importantes para a reprodução das angiospermas e para a conservação de espécies nativas nos trópicos (ROUBIK, 1992). Ademais, apresentam papel chave em florestas tropicais e seus remanescentes e coom indicadores de qualidade ambiental (PALAZUELOS BALLIVIÁN, 2008). As abelhas sem ferrão, também são polinizadores eficientes para as culturas importantes economicamente como o morango, tomate, urucum, pimentão e guaraná (HEARD, 1999; ROSELINO, et al., 2010; SLAA, et al., 2006) e desempenham um papel chave nos processos ecossistêmicos em que se encontram engajadas (IMPERATRIZ-FONSECA, et al., 2004). Apesar disso, estima-se que aproximadamente 100 espécies de meliponíneos conhecidos no Brasil apresentem risco de extinção (PALAZUELOS BALLIVIÁN, 2008) devido à perda de hábitat e desmatamento de florestas nativas (LOPES, et

al., 2005). E além da importância como polinizadores, esses organismos são cruciais na manutenção das redes de interação entre plantas e animais (YAMAMOTO et al. 2010).

A meliponicultura no país contribui para a produção de pólen e mel para as populações do norte e nordeste do Brasil, sendo um produto bem valorizado para a exploração comercial (ALVES, et al., 2007), contribuindo para a renda familiar, elaboração de produtos medicinais, melhoria do ensino dos alunos de forma geral, visto que os meliponínios não têm ferrão e são manuseáveis, e também para a biologia, especialmente para genética e evolução dos Apidae (KERR, 1997). Além do fornecimento de mel e própolis, as abelhas produzem cera, pólen e podem gerar lucro com o comércio de colônias. Estima-se que um terço da alimentação humana dependa direta ou indiretamente da polinização realizada por abelhas (VILLAS-BÔAS, 2012).

1.2 - O Alimento Larval

As abelhas sociais têm sua alimentação baseada na coleta de néctar e pólen. O néctar é coletado e armazenado no papo de mel/intestino anterior até chegar na colônia, onde será desidratado e fermentado (KERR et al., 1996; NOGUEIRA-NETO, 1997; VIT et al., 2013). Através desses processos o néctar é transformado em mel, a principal fonte de carboidratos para essas abelhas. O pólen coletado é transportado para a colônia, onde passará por um processo de maturação após ser manipulado pelas abelhas (NOGUEIRA-NETO, 1997). O pólen maturado é a principal fonte de proteínas, lipídeos, macro e micronutrientes (ROUSTON; CANE, 2000; REBELO et al. 2016).

O pólen maturado e o mel são essenciais para a confecção do alimento larval, substância responsável por nutrir a prole da colônia. O alimento larval é uma mistura de pólen maturado, mel e secreções glandulares, sendo depositado nas células de cria, que após a ovoposição das rainhas,

são fechadas até a eclosão dos adultos (KERR et al., 1996; NOGUEIRA-NETO, 1997; VIT et al., 2013). O alimento larval tem em média 40 a 60% de água, 5 a 12 % de açúcares, 1,1 a 19,4 % de proteínas e 0,2 a 1,3% de aminoácidos (HARTFELDER, ENGELS 1989).

Figura 1- A- Alveólo de cria *Frieseomelitta varia*, seta vermelha mostrando o aliento larval *(*fonte*: https://ctresshop.com/marmelada/)*, B- Disco de cria *Melipona quadrifsciata,* seta vermelha mostrando célula de cria (fonte: Luiza Borges), C- Células de cria de *Melipona scutellaris*, evidenciando alimento larval, ovos e larvas *(*fonte: Ana Santos); D- Disco de cria de *Tetragonisca angustula (*fonte: Ana Santos).

1.2- Influência do Alimento larval na formação de Castas

O alimento larval é fundamental para o processo de diferenciação de castas nas abelhas sem ferrão (DE WILDE, BEETSMA, 1982; WHEELER, 1986). Nas espécies *Frieseomelitta*

varia e *Tetragonisca angustula* a diferenciação em rainha será determinada pela quantidade de alimento larval ingerido pela prole. Em *T. angustula* existe a diferenciação das células de cria, onde os ovos que darão origem às rainhas serão depositados em células reais (IMPERATRIZ-FONSECA, 1984). Em *F. varia* não existe a construção de células reais para a diferenciação da quantidade de alimento larval depositado nas células, e as larvas ingerem alimento de mais de uma célula de cria para se tornarem rainhas (FAUSTINO et al., 2002).

Os ovos das abelhas no gênero *Melipona* são depositadas em células de cria do mesmo tamanho e a taxa de eclosão de rainhas é de 25%, porém existe uma dependência alimentar, uma vez que larvas com genótipo para se tornar rainhas que não recebem alimentação adequada não se diferenciam em rainhas (KERR, 1946; 1948; 1950; KERR, NIELSEN, 1966; KERR ET AL., 1966; RATNIEKS). No entanto, apesar dessa diversidade para diferenciação das rainhas, esses grupos apresentam a estrutura do estoque alimentar na colmeia (potes de cerume), pote de pólen, mel, e a produção de alimentos larvais similares (PRATO, 2015).

2- MICROBIOTA ASSOCIADA A ABELHAS SEM FERRÃO

A diversidade de microrganimos que pode ser encontrada associadas às colônias de abelhas sem ferrão é ampla, assim como o papel que desempenham na saúde e vitalidade desses organismos (DILLON, 2004; HAMDI, *et al.*, 2011; VÁSQUEZ; *et al.,* 2012). Essa microbiota contribui para o desenvolvimento das abelhas e auxilia em processos fisiológicos, como a digestão (MAZMANIAN, *et al.,* 2005; MARTIN, 2008).

Os microrganismos apresentam relações complexas com diversos organismos, influenciando diretamente a sobrevivência dos seus hospedeiros. As abelhas sem ferrão podem apresentar bactérias, leveduras e fungos filamentosos associados à colônia e esses microrganismos

parecem estar associados à fermentação do néctar e maturação do pólen, além de produzirem moléculas antimicrobianas e nutritivas para as abelhas.

2.1- Bactérias associadas às abelhas sem ferrão

Há algum tempo, a diversidade bacteriana associada às abelhas sem ferrão começou a ser investigada, com destaque para o gênero *Bacillus*. No estudo de Machado (1971), foram observadas bactérias do gênero *Bacillus* associadas ao pólen e ao alimento larval de 14 espécies de abelhas sem ferrão. Gilliam e colaboradores (1990) identificaram *Bacillus* spp. associadas ao alimento larval, pólen e mel de *Melipona fasciata*. Esses microrganismos produziram várias enzimas relacionadas à conversão de alimentos em produtos digeríveis, além da produção de antibióticos e ácidos graxos, que impedem outros organismos competidores de deteriorar os alimentos armazenados (GILLIAM et al., 1985; GILLIAM; ROUBIK; LORENZ, 1990; WANG *et al.*, 2015. A análise de microrganismos presentes em *M. scutellaris* e *T. clavipes* identificou diversas espécies do gênero *Bacillus* são as mais frequentes no mel e própolis dessas abelhas (RAMOS, 1997; SANTOS, 2007).

Bactérias dos gêneros *Bacillus* e *Streptomyces* foram identificadas em colmeias de *Melipona* sp. e *Trigona* sp., onde produzem substâncias antimicrobianas contra fungos e contra o patógeno *Panaebacillus larvae*. Bactérias do gênero *Lactobacillus* e representantes da família Acetobacteraceae foram identificados no papo de mel/intestino anterior de abelhas sem ferrão, e o gênero *Lactococcus* no alimento larval de *Melipona seminigra* (CERQUEIRA *et al.*, 2021; MARÇAL, 2017). Esse grupo de bactérias desempenha um papel importante no processamento do pólen e do néctar, no armazenamento do mel, devido a seu potencial fermentativo, e na proteção dessas abelhas, produzindo substâncias antimicrobianas (KEŠNEROVÁ *et al.*, 2017; MOHAMMAD; MAHMUD-AB-RASHID; ZAWAWI, 2020; VÁSQUEZ; OLOFSSON, 2009).

Bactérias dos gêneros *Enterococcus*, *Providencia*, *Serratia* e *Vagococcus* foram encontradas associadas ao alimento larval dos gêneros de abelhas *Melipona* sp. e *Tetragonisca* sp., onde produzem substâncias antimicrobianas contra patógenos Gram positivos e Gram-negativos (SANTOS et al., 2022). A espécie *Paenibacillus polymyxa* foi identificada em simbiose com a espécie *M. scutellaris* (MENEGATTI *et al.*, 2018), produzindo moléculas antimicrobianas.

As bactérias também podem ser maléficas para as abelhas e apesar de terem poucos relatos em abelhas sem ferrão (SHANKS *et al.*, 2017). A bactéria *Lysinibacillus sphaericus* é causadora de doenças em *Tetragonula carbonária* (FÜNFHAUS; EBELING; GENERSCH, 2018; SHANKS *et al.*, 2017). Apesar dos avanços nos estudos ainda não se sabe ao certo qual o efetivo papel dessas bactérias na manutenção e no desenvolvimentos das colônias.

2.1- Fungos associados às abelhas sem ferrão

A associação de fungos com as abelhas sem ferrão é inquestionável, e estes microrganismos desempenham funções mutualísticas importantes com as colônias de meliponíneos (DE PAULA et al., 2021). A literatura demonstra que diferentes espécies de leveduras e fungos filamentosos compõem a diversidade da microbiota das abelhas (MENEZES et al.,2015; PALUDO et al., 2018; PALUDO et al., 2019). A importância das leveduras, seu papel e potencial é semelhante às funções bacterianas, ou seja, secretam enzimas que convertem substâncias de alimentos armazenados e ajudam a preservá-los (DE PAULA et al., 2021; ECHEVERRIGARAY et al., 2021; SANTOS et al., 2018; SILVA et al., 2020).

Teixeira e colaboradores (2003) observaram 32 linhagens da levedura *Staremella melponinorum* associadas às espécies *Tetragonisca angustula*, *Melipona quadrifasciata*, *Melipona rufiventris* e *Trigona fulviventris*. Barbosa e colaboradores (2016) identificaram 12 espécies de leveduras, especialmente o gênero *Candida*, em mel de espécies dos gêneros *Melipona*,

Scaptotrigona e *Partamona*. Echeverrigaray e colaboradores (2021) observaram 16 espécies de leveduras em mel de 17 espécies de abelhas sem ferrão, sendo *Starmerella* e *Zygosaccharomyces* as mais abundantes.

A espécie *Scaptatrigona depilis* apresenta relação mutualista com fungos dos gêneros *Monascus* sp. e *Zygosaccharomyces* sp., que influenciam o desenvolvimento dessas abelhas (MENEZES *et al.*, 2015; PALUDO, CAMILA R. *et al.*, 2018; PALUDO, CAMILA RAQUEL *et al.*, 2019). Rosa e colaboradores (2013) identificaram as leveduras C*andida apicola* e *Starmerella meliponinorum* em pólen e em mel de *M. quadrifasciata* e *Tetragonisca angustula,* indicando relação mutualista. Massaro e colaboradores (2018) identificaram mais de 140 isolados do filo Ascomycota e Basidiomycota em pote-pólen, potássio-mel e ninhada nas espécies de *Austroplebeia Australis, Tetragonula carbonaria* e *Tetragonula Hockingsi.*

3- USO BIOTECNOLÓGICO DOS PRODUTOS DAS ABELHAS SEM FERRÃO

O mel, o pólen maturado e a própolis produzido por abelhas sem ferrão tem sido alvo de estudos para descoberta de biomoléculas com potencial de uso biotecnológico. Pesquisas demonstram que estes produtos possuem efeitos principalmente para uso farmacológico, com grande potencial (AL-HATAMLEH et al., 2020; DE PAULA et al., 2021; SANTOS et al., 2022; RAO et al., 2016).

O pólen maturado de abelhas sem ferrão apresenta efeito antimicrobiano, anticâncer, antidiabetes, anti-inflamatório e antinoceptivo (MALIHAH MOHAMMAD *et al.*, 2021). A geoprópolis (resina vegetal adicionada a barro) desses organismos pode ter efeito anticâncer, anti-inflamatório, antioxidante e antimicrobiano (AL-HATAMLEH et al., 2020; CHOUDHARI et al, 2013; DUTRA et al., 2014; KOTHAI & JAYANTHI, 2015; KUSTIAWAN et al., 2015; MARTINOTTI & RANZATO, 2015; SANCHES; PEREIRA; SERRÃO, 2017). Estudos

demonstram que a própolis e o pólen maturado de *Heterotrigona itama* apresentaram efeitos antioxidante e antimicrobiano contra bactérias Gram-negativas e Gram-positivas (AKHIR; BAKAR; SANUSI, 2017) e um estudo recente observou que o extrato de própolis das abelhas *Melipona quadrifasciata* e *Tetragonisca angustula* possuem efeito antimicrobiano contra bactérias e leveduras patogênicas (VALCANAIA *et al.*, 2022).

O mel da espécie *Melipona marginata* apresentou efeito anti-inflamatório (BORSATO *et al.*, 2014) e o mel de algumas abelhas sem ferrão do Amazonas tiveram efeito antimicrobiano *in vitro* (DOMINGOS et al., 2021). O mel da espécie *Melipona compressipes manaosensis* apresentou efeito antimicrobiano contra bactérias patogênicas (PIMENTEL et al., 2013). O potencial antimicrobiano dos produtos das abelhas sem ferrão pode representar alternativas eficientes para o tratamento de infecções causadas por bactérias resistentes aos antibióticos atualmente disponíveis (SANTOS et al., 2022).

Além de aplicações farmacológicas, outras aplicações biotecnológicas podem ser exploradas a partir dos microrganismos isolados das abelhas sem ferrão. Novas cepas de *Saccharomyces cerevisiae* isoladas de abelhas sem ferrão tem sido alvo de estudos biotecnológicos, devido ao maior potencial fermentativo para produção de bioetanol (SILVA *et al.*, 2020).

4- MÉTODOS DE ESTUDO DE DIVERSIDADE MICROBIANA

O uso de metodologias de Sequenciamento de Nova Geração (do inglês, NGS) tem sido relevante para o estudo da composição e da estrutura de comunidades microbianas. Além de terem menor custo e serem mais rápidos, trazem novas perspectivas em relação às metodologias baseadas em cultivo, uma vez que a diversidade de organismos não-cultiváveis chega a 90% (SUENAGA, 2015; RAPPÉ; GIOVANNONI, 2003; HANDELSMAN, 2004; SINGH et al., 2009).

As tecnologias NGS são baseadas no preparo de bibliotecas usando o DNA, possibilitando a análises de milhares de fragmentos de DNA. Dentre as metodologias utilizadas, a Illumina tem sido muito empregada no sequenciamento das regiões variáveis do rRNA 16S e espaçador transcrito interno (ITS) (Fig. 2) e o sequenciamento por síntese, utilizado nessa metodologia, possibilita a identificação de microrganismos menos abundantes. A utilização das regiões conservadas do gene rRNA para a montagem de primers permite a amplificação de regiões variáveis do DNA desses microrganismos. O gene codificador rRna 16S, componente da subunidade menor do ribssomo, é utilizado para a identificação de bactérias e arquea, e o ITS, um espaçador transcrito interno entre os genes rRNA 18S e 26S, tem sido utilizado para a identificação de fungos (SULAIMAN; JACOBS; SIMPSON, 2019).

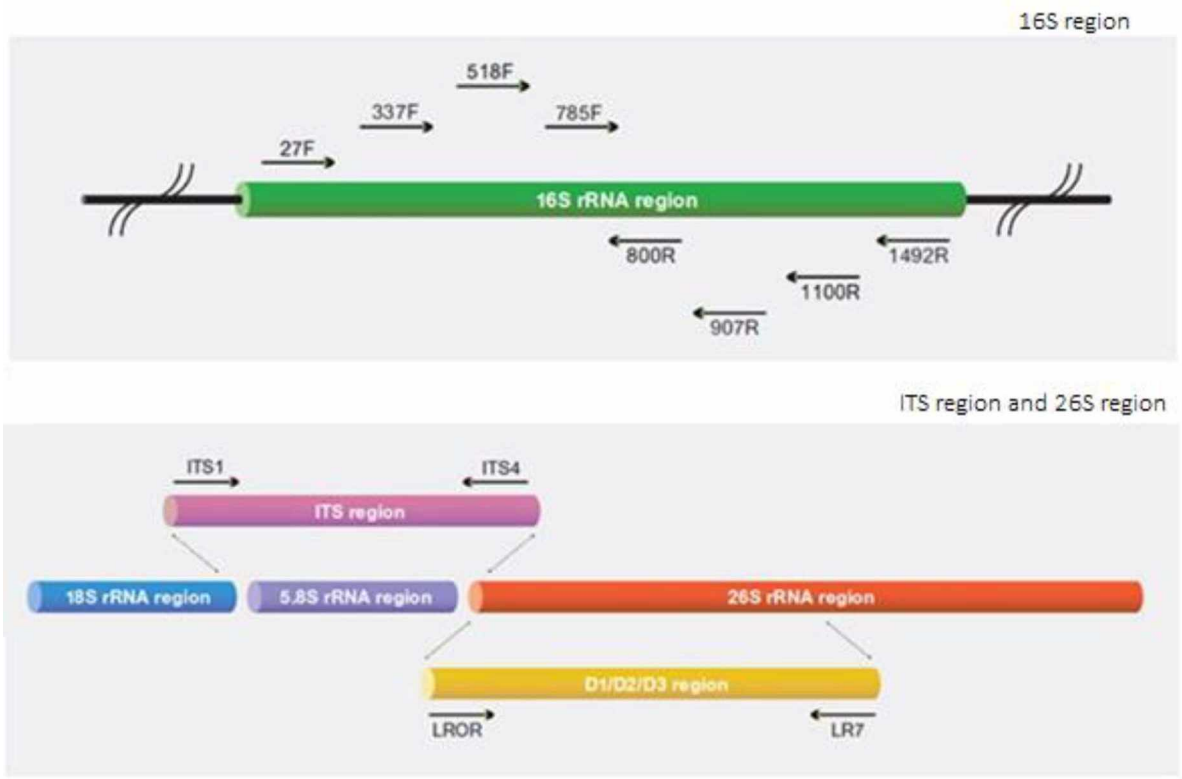

Figura 2- Regiões variáveis do gene 16S e Região ITS (fonte: https://www.barcodebiosciences.com/genomics-services/microbial-identification/).

O sequenciamento de fragmentos das regiões rRNA 16S e ITS possibilita caracterizar melhor a diversidade da comunidade microbiana presente no ambiente, porém, para acessar o potencial biotecnológico de produtos microbianos, o uso de técnicas baseadas em cultivo e isolamento se tornam necessárias.

5- ANTIMICROBIANOS NA ATUALIDADE

O uso indiscriminado de antibióticos na agricultura, saúde humana e veterinária é o principal fator para o desenvolvimento e disseminação de resistência bacteriana, contribuindo para o problema global de infecções bacterianas causadas por patógenos com resistência à maioria dos antibióticos comercialmente disponíveis (HOSAIN, *et al.*, 2021, VARELLA, et al., 2021). A disseminação de resistência bacteriana é definida como o surgimento de cepas que conseguem se desenvolver, mesmo na presença de concentrações de antibióticos nas quais eram inicialmente sensíveis (ACAR, ROSTEL, 2001). As bactérias podem desenvolver resistência naturalmente, por mutação ou pela aquisição de material genético exógeno. Essa aquisião de material genético se dá por 3 mecanismos pror transformação e/ou conjugação, ou adquirir genes de resistência como forma, principalmente, conjugação, transformaão e transdução (BLAIR et al., 2015; CHRISTAKI et al., 2020).

Os antimicrobioanos atualmente disponíveis possuem diversos tipos de ação na célula bacteriana, sendo que os antibióticos das classe β-lactamicos os mais utilizados, por atuarem a nível de síntese da parede celular (peptideoglicano), apresentando menor toxicidade (LIMA et al., 2020). Outros antibióticos podem atuar a nível de membrana citoplasmática, ácidos nucleicos e de síntese proteica (LOUREIRO et al., 2016). Apesar dos diversos mecanismos de ação antimicrobiana, as bactérias resistentes apresentam mecanismos de escape aos efeitos antimicrobianos, incluindo a produção de enzimas inativadoras do fármaco, alterações em sítios de ligação dos antibióticos, alterações da permeabilidade celular e produção de bombas de efluxo,

que eliminam o fármaco do interior da célula microbiana (BLAIR et al., 2015; VARELA et al., 2021).

Dentre as bactérias patogênicas resistentes encontram-se espécies Gram-negativas e Gram-positivas(Fig. 3) que contribuem para o sério problema de saúde pública no mundo, atingindo tanto os países em desenvolvimento como os países desenvolvidos (BANIN et al., 2017). Dentre as Gram-positivas, os gêneros *Staphylococcus* e *Enterococcus* destacam-se por possuírem diversos mecanismos naturais de resistência antimicrobiana, além de mecanismos genéticos adquiridos (DE OLIVEIRA et al., 2020; KOS et al., 2012; JOHNSON et al., 2010). Dentre as bactérias Gram-negativas destacam-se espécies não-fermentadoras, como *Pseudomonas aeruginosa* e *Acinetobacter baumannii*, além de várias espécies da familia Enterobacteriacea, incluindo *Klebsiella pneumoniae*, *Escherichia coli* e gêneros como *Enterobacter* sp., *Salmonella* sp., entre outros (SANTAJIT et al., 2016; DE OLIVEIRA et al., 2020; ENG et al., 2015).

Bactérias patogênicas, tais como *Staphylococcus aureus* e *Eschechiria coli*, podem demonstrar-se resistente a uma ampla gama de antibióticos. A Gram-positiva *S. aureus* pode apresentar mecanismos de resistência a penicilina, sulfonamidas,meticilina e, mais recentemente, à vancomicina (BANIN et al., 2017). A Gram-negativa *E. coli* pode ser resistente a amplimicina, tetraciclina, cefalocetim, dentre outros antibióticos (DE OLIVEIRA, 2008; SILVA, *et al.,* 2017; REINTHALER, *et al.,* 2003).

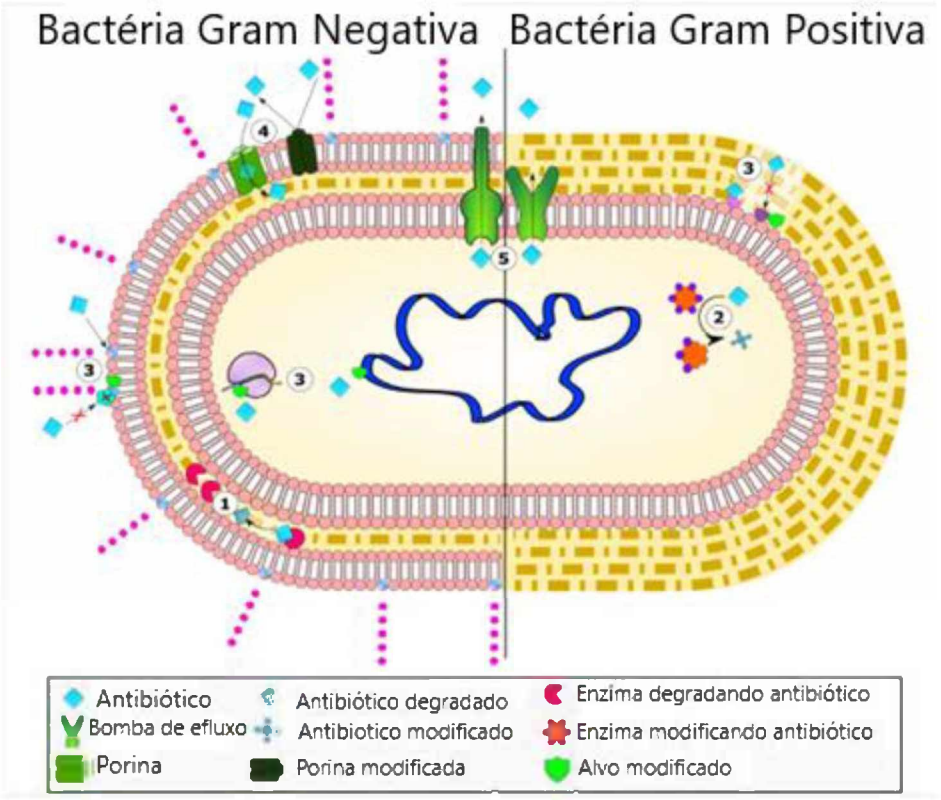

Figura 3- Diferenças da parede e mecanismos de ação de antibióticos em bactérias Gram positivas e Gram negativas (fonte: VARELA,2021)

O aumento de casos de espécies multirresistentes tem se tornado frequente tanto pelo tratamento ineficaz de antibióticos existentes, quanto pela dificulade de descoberta de novos agentes antimicrobianos (MOELLERING JR, 2011). O tratamento de doenças causadas por essas bactérias está relacionada ao emprego de técnicas que visam identificar ou modificar molecúlas antimicrobianas já existentes (FARHA; BROWN, 2019; MOELLERING JR, 2011). Dentre as estratégias utilizadas estão: a modificação de antibioticos exitentes ou seu uso sinérgico com moléculas que contornem a resistência bacteriana, a descoberta e peptídes antimicrobianos, inibidores de fatores de virulência, o uso de nanopartículas, utilização de oligonucleotídeos "antisensee a descoberta de antibióticos com novos mecanismos de ação (DURAND et al., 2019; HOBSON et al., 2021;VILA et al., 2020).

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CAPÍTULO 2
ARTIGOS CIENTÍFICO A SER SUBMETIDO PARA REVISTA MICROBIOME

MICROBIOTA ASSOCIATED WITH LARVAL FOOD OF BRAZILIAN STINGLESS BEES: *F. VARIA*, *M. QUADRIFASCIATA*, *M. SCUTELLARIS,* AND *T. ANGUSTULA*

Microbiota associated with larval food of Brazilian stingless bees: *F. varia*, *M. quadrifasciata*, *M. scutellaris*, and *T. angustula*

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Abstract

Background – Stingless bees represent a diverse group with a relevant role in pollinating native species. Its diet is rich in carbohydrates and proteins, by collecting pollen and nectar provides the development of its offspring. Fermentation of these products is associated with the presence of microorganisms in the colony. However, the constitution of microorganisms that make up this Microbiome and the fundamental role in colony development is still unclear.

Results- A of 129 Amplicon Sequence Variant (ASVs) were classified from the sequencing of the V3/V4 region of the 16S gene. Were found specimens of the three phyla: Firmicutes, Proteobacteria, and Actinobacteria. Twenty-six (26) bacterial genera were classified, with *Lactobacillus* being the most abundant in the 4 bee species. In the sequencing of the ITS1 (Internal

transcribed spacer) region, 300 ASVs were classified among the phyla Ascomycota, Basidiomycota, Mucoromycota, and Mortierellomycota. Forty-eight genera of fungi were classified, divided into 58 species. Diversity analyses showed that *F. varia* presented greater diversity of bacteria in its microbiota, and *T. angustula* greater diversity of fungi. The isolation technique allowed the isolation of 189 bacteria and 75 fungi.

Conclusion- In summary, this research showed bacteria and fungi associated with *F. varia*, *M. quadrifasciata*, *M. scutellaris,* and *T. angustula* species and that they may play an essential role in the survival of these organisms.

Key-words- Microbiota, Stingless bees, ITS rRNA, 16S rRNA, Larval food

Background

Stingless bees (Apidae, Meliponini) represent the most diverse group of eusocial bees, with more than 550 species distributed in tropical and subtropical regions [1–3]. Stingless bees are largely economically explored in Brazil's north and northeast regions due to the production of honey, wax, geopropolis, pollen, and the colonies commercialization. However, this main socio-economic contribution is the pollination of native species [4–8].

The nectar and the pollen collected by stingless bees are stored in cerumen pots, which are used to ferment them to provide the necessary nutrients for the development of larvae and survival of adults [9]. Through these processes, the nectar, transformed into honey, will be the primary source of carbohydrates for these bees. Fermented pollen is the source of proteins, lipids, and other nutrients [10–13]. Larval food (LF) results from a mix of pollen fermented, honey, and glandular secretions of nurse bees and is richly associated with microbial communities[14–18].

Microorganisms contribute to the development of the bee's immune system, aid in the food digestion process, and defend the hives against pathogens [19–21]. Bacteria of the genera *Bacillus* and *Streptomyces* were identified in beehives of the genera *Melipona* and *Trigona*. These

organisms are known to produce antimicrobial substances against fungi and *Paenibacillus larvae*, besides presenting fermentative potential [22–25].

Scaptotrigona depilis stingless bees have a mutualist relationship with fungi of the genera *Monascus* and *Zygosaccharomyces,* which influence the development of these bees [26–28]. The frequency that C*andida apicola* and *Starmerella meliponinorum* yeasts are found in pollen and honey of *Melipona quadrifasciata* and *Tetragonisca angustula* suggests a mutualistic relationship between them [29].

In this context, knowing the microbial community present in the LF of species *Frieseomelitta varia*, *Melipona quadrifasciata*, *Melipona scutellaris*, and *Tetragonisca angustula* becomes necessary. They are Brazilian stingless bee species that present behavioral differences and in the determination of caste. However, present the structure of food stock in the colony (cerumen pots), pollen pot, honey, and the production of LF [30].

Despite the recent advances in the microbiota associated with stingless bee colonies research, the composition of the larval food microbiome of these bees is still unclear. The role of the microorganisms in the feeding and manutention of bee colonies remains an open field to be investigated. In this study, the microbial community was characterized using next-generation sequencing techniques and culture-based techniques.

Methods

Sample collection

The larval food samples were collected from four stingless bees colonies (*Frieseomelitta varia*, *Melipona quadrifascita*, *Melipona scutellaris,* and *Tetragonisca angustula*) and processed to obtain bacteria isolated by the culture technique (Figure 01). Biological material was collected from beehives in an urban meliponary in Uberlândia city, Minas Gerais, Brazil.

Larval food collection

Brood cells were collected from beehives and stored in sterile Petri dishes. In the laminar flow cabinet, the cell brood was cleaned with sterilized distilled water and rinsed with 70% ethanol. Then, the brood cells were opened with a sterile pipette tip, and the LF was collected. Three grams of LF were collected from larvae brood cells provision of each colony.

Molecular characterization of the Microbiome

DNA extraction

For DNA extraction, 1 g of LF was collected from larvae brood provision cells of each colony. According to the manufacturer, bacterial and fungal DNA extraction was performed using the DNeasy® Blood & Tissue (Qiagen) commercial kit. The DNA quality was confirmed using NanoDrop 2000 Spectrophotometer, and agarose gel run.

Amplicon Sequencing and Data analysis

For the construction of libraries, 30 ng of DNA were used as input to amplify the V3/V4 region of 16S rRNA gene with primers 341F (CCTACGGGRSGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) [31,32], and ITS1 region with the primers IST1(GAACCWGCGGARGGATCA) e ITS2 (GCTGCGTTCTTCATCGATGC) [33,34] on the Illumina MiSeq platform. The amplicons were sequenced in the paired-end reads of 500 cycles. The raw data was processed using the package DADA2 in R version 4.0.2 [35] to generate the Amplicon Sequence Variants.

The reads of the V3/V4 region of the 16S rRNA gene were filtered to trim the primers using the parameter *trimLeft* and the values 17 and 20 for forward and reverse reads, respectively. The forward reads were truncated at position 290 and reverse reads at position 200. Reads

containing more than two (2) expected errors were removed from further analysis. The Amplicon Sequence Variants (ASV) were classified using Silva 138 database [36].

The amplicons of the ITS1 region followed the same steps as the rRNA gene amplicons, with the distinction ofthe parameters *trimLeft* (default 0), *truncLen* (default 0) and, *minLen*, removing reads with lengths less than 50. The ASVs were classified using UNITE ITS database using pre-trained.

The alfa and beta diversity analyses were performed with phyloseq and vegan packages in R [36].

Microbiome analysis by cultivation-based techniques

Microorganisms Isolation

Three grams of LF were collected from larvae brood provision cells of each colony. One (1) gram of sample was resuspended in 10 ml of saline solution 1%, 1 g in 10 ml of peptone water, and 1 g in 10 ml of 15GF broth [26], as shown in Figure 1[37]. The second and third solutions are incubated for 24 hours at 32ºC±0,5 (Incoterm® Dual Sensor Digital Thermo-Hygrometer). From the mother solution, the serial solution was made to the order 10^{-4}, and 100 µL of each one was plated on the surface in different culture media and incubated in a bacteriological incubator at 32° C and monitored for 48 hours for bacterial growth and seven days for fungal growth.

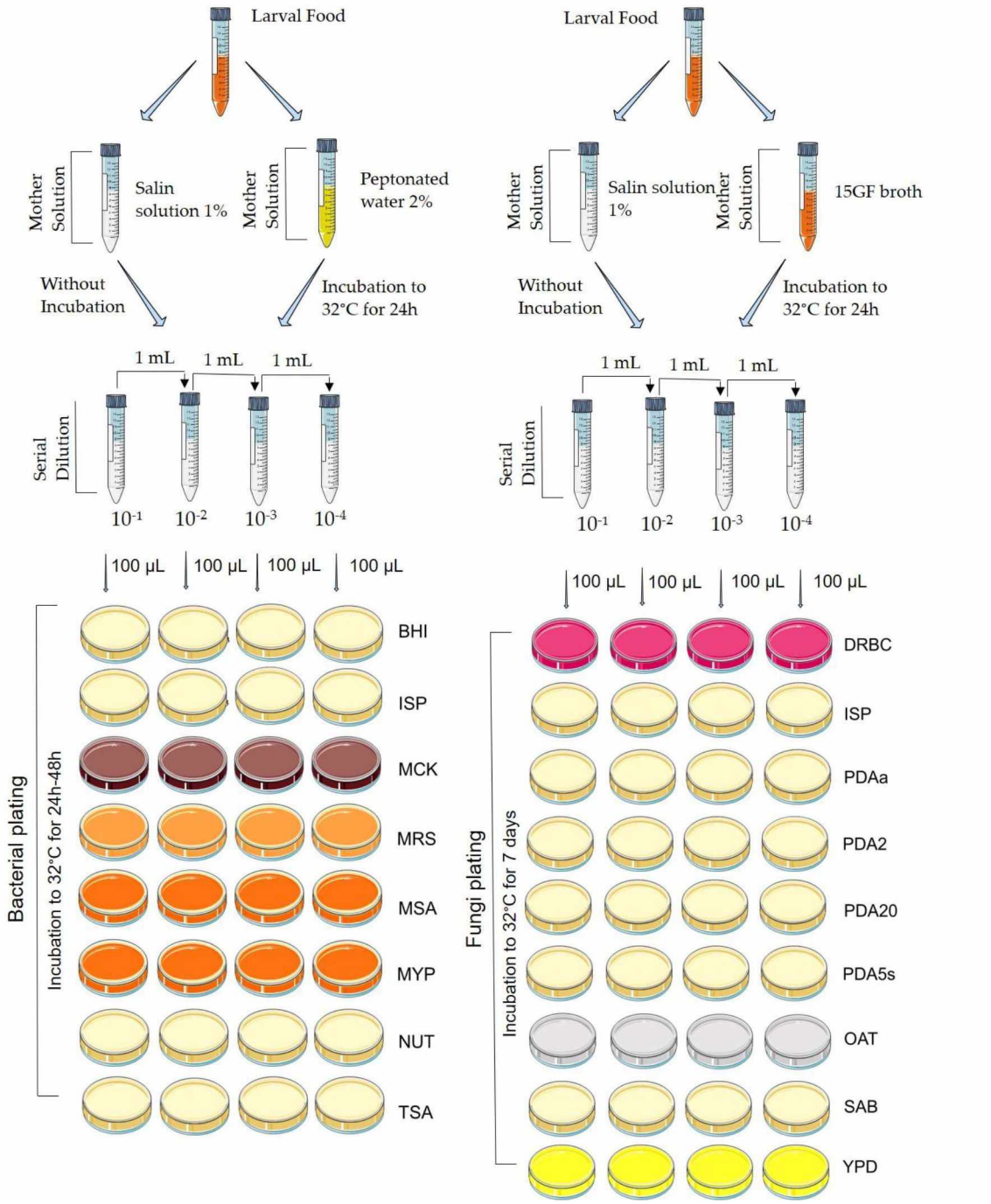

Figure 4- Methodology for cultivating larval food from four stingless bee species with different dilutions and culture mediums. Brain Heart Infusion (BHI), Nutrients (NUT), Triptina soy agar (TSA), Man, Rogosa and Sharpe (MRS), Mac Conkey (MCK), Mannitol Salgado (MAS), Yeast Malt Agar (ISP), Potato Dextrose Agar 2% (PDA2) and 20% (PDA20) glucose, PDA acidified with tartaric acid (PDAa), PDA NaCl 5% (PDA5s), Aveia (OAT), Yeast extract-Peptone-Dextrose

(YPD), DRBC (Dicloran Rosa Bengala Cloranfenicol) e Sabouraud (SAB). *Images of representative methodology from 'Smart Servier Medical Art' (https://smart.servier.com/).*

After the incubation, the colonies were isolated on BHI agar for bacteria and PDA agar for fungi. Macroscopic morphological differences select the colonies, and the isolated microorganisms were preserved in LB broth (Luria Bertani) plus 20% glycerol and kept in an ultra-freezer at -80°C.

Identification by MALD-TOF of some cultivable microorganisms

The bacteria were plated on BHI for taxonomic identification and incubated at 37°C±1 for 24h. An isolated colony of each strain was collected from the agar using an inoculation loop and inactivated with absolute ethanol. This colony underwent MALDI-TOF mass spectrometry using the MALDI Biotyper version 3 (Bruker Daltonics), according to the manufacturer's suggested settings using automated collected spectra. The biomolecular identification of the bacteria was analyzed according to the score values proposed by the manufacturer [37].

Results

Bacterial Community Sequencing output

One hundred and twenty-nine (129) ASVs were classified for bacterial composition analysis (Table S1). The most abundant phyla observed in the LF of the four bee species were Firmicutes and Proteobacteria (Fig. 2A). The phylum Actinobacteria were not represented in the species *M. scutellaris* and *T. angustula*.

The ASVs were divided into 26 genera; the most abundant genus in all bees was *Lactobacillus* (Fig. 2C). No ASVs were found simultaneously in the LF of the 4 bee species. Sixty-three (63) exclusive ASVs were found in *F. varia* LF, 23 in *M. quadrifasciata*, 25 in *M.*

scutellaris, and 16 in *T. angustula* (Fig.2B). Fifteen (15) exclusive bacteria genera was shown in *F. varia*, 5 in *M. quadrifasciata*, and only the genus *Anthococcus in M. scutellaris*. No exclusive genus was identified in *T. angustula* and the genus *Micrococcus* is common to LF of *F. varia* and *M. quadrifasciata*. Only 5 ASVs were classified at a species level, including *Bombella intestine, Corynebacterium lipophiloflavum, Serratia symbiotica* in *F. varia*, and *Acinetobacter junii* and *Variovorax paradoxes* in *M. quadrifasciata*.

Figure 2-(A) Abundance of bacterial phyla observed in *F. varia*, *M. quadrifasciata*, *M. scutellaris,* and *T. angustula* larval food.; (B) Venn Diagram of ASVs classified for stingless bees; (C) Genus x Family histogram of 15 genera most abundant.

Fungal Community Sequencing output

A total of 300 ASVs were classified for fungal composition analysis in bees LF (Table S2). Fungi of the phyla Ascomycota, Basidiomycota, Mucoromycota, and Mortierellomycota were sampled (Fig.3A). The only phylum present in the four species of stingless bees was the Ascomycota. *F. varia* had 39 exclusive ASVs, *M. quadrifasciata* 68, *M. scutellaris* 45 and *T. angustula* 128. No common ASVs were found for the four stingless bee species (Fig. 3B). Stingless bees of the genus *Melipona* shared three exclusive ASVs. Forty-eight (48) fungal genera were observed in the LF of all bee species analyzed here, six in *F. varia*, 31 in *M. quadrifasciata*, nine *in M. scutellaris*, and 24 *in T. angustula*. The family emphasizing *M. quadrifasciata* and *F. varia* were Claridosporiaceae, and *for T. angutula,* Erysiphaceae (Fig. 3C) stood out. Forty-eight (48) genera of fungi were identified, 11 were yeast genera, and 37 were filamentous fungi.

Fifty-eight (58) fungal species were identified. The most abundant species in *F. varia* LF were *Cladosporium exasperatum* and *Saccharomyces cerevisiae*. In *M. quadrifasciata* were *Cladosporium delicatulum* and *Penicillium citrinum*. In *M. scutellaris, Cladosporium delicatulum, Bipolaris shoemakeri* and *T. angutula, Erysiphe quercicola* and *Saccharomyces cerevisiae* (Fig. 3D). The *F. varia* LF showed three exclusive species, *M. quadrifasciata,* 21, *M. scutellaris,* five and *T.angustula,* 11.


A
Stingless Bees
T.angustula
M.scutellaris
M.quadrifasciata
F.varia
Abundance
Phylum
Ascomycota
Basidiomycota
Mortierellomycota
Mucoromycota
B
M.quadrifasciata
M.scutellaris
F.varia
T.angustula
68 (22.7%)
45 (15%)
2 (0.7%)
3 (1%)
3 (1%)
39 (13%)
0 (0%)
1 (0.3%)
128 (42.7%)
0 (0%)
1 (0.3%)
6 (2%)
0 (0%)
0 (0%)
4 (1.3%)
C
F.varia
M.quadrifasciata
M.scutellaris
T.angustula
Abundance
Family
Genus
Antennariella
Aspergillus
Bipolaris
Candida
Cercospora
Cladosporium
Didymella
Erysiphe
Fusarium
Meyerozyma
Penicillium
Peniophora
Rhodotorula
Saccharomyces
Starmera
Starmerella
Symmetrospora
Tilachlidium
Toxicocladosporium
Wickerhamiella
D
F.varia
M.quadrifasciata
M.scutellaris
T.angustula
Abundance
Genus
Species
A.placitae
A.sydowii
A.unguis
B.shoemakeri
C.apicola
C.delicatulum
C.exasperatum
C.halotolerans
C.orthopsilosis
C.paczoskii
C.parapsilosis
C.zeylanoides
E.quercicola
M.guilliermondii
P.citrinum
R.mucilaginosa
S.cerevisiae
S.etchellsii
S.stellimalicola
T.brachiatum
T.irritans
W.versatilis

Figure 3- (A) Abundance of fungal phyla observed in *F. varia*, *M. quadrifasciata*, *M. scutellaris,* and *T. angustula* larval food.; (B) Venn Diagram of ASVs classified for stingless bees; (C) Genus x Family histogram of 20 genera most abundant; (D) Species x Genus histogram of 22 species most abundant.

Diversity index

For Bacterial Community Sequencing (Fig. 4A), the diversity and richness indexes were higher in the samples of *F. varia* LF. Regarding the number of bacterial species, the larval food of *T. angustula* showed the lowest diversity. *M. scutellaris* presented the lowest species diversity indexes. *M. quadrifasciata* showed a lower Shannon and Simpson diversity index, despite a higher number of species than *T. angustula* and *M. scutellaris*.

For Fungal Community Sequencing (Fig. 4B), the higher richness of fungi species was found in *T. angustula* and the lowest in *F. varia*. *M. quadrifasciata* and *T. angustula* LF had the highest Simpson and Shannon indices, and *M. scutellaris* had the lowest indices.

We observed that *F.varia* had a higher richness of bacteria species but had lower diversity rates of fungal species. Unlike *T. angustula*, which presented low species richness rates for bacteria and a higher rate for fungi.

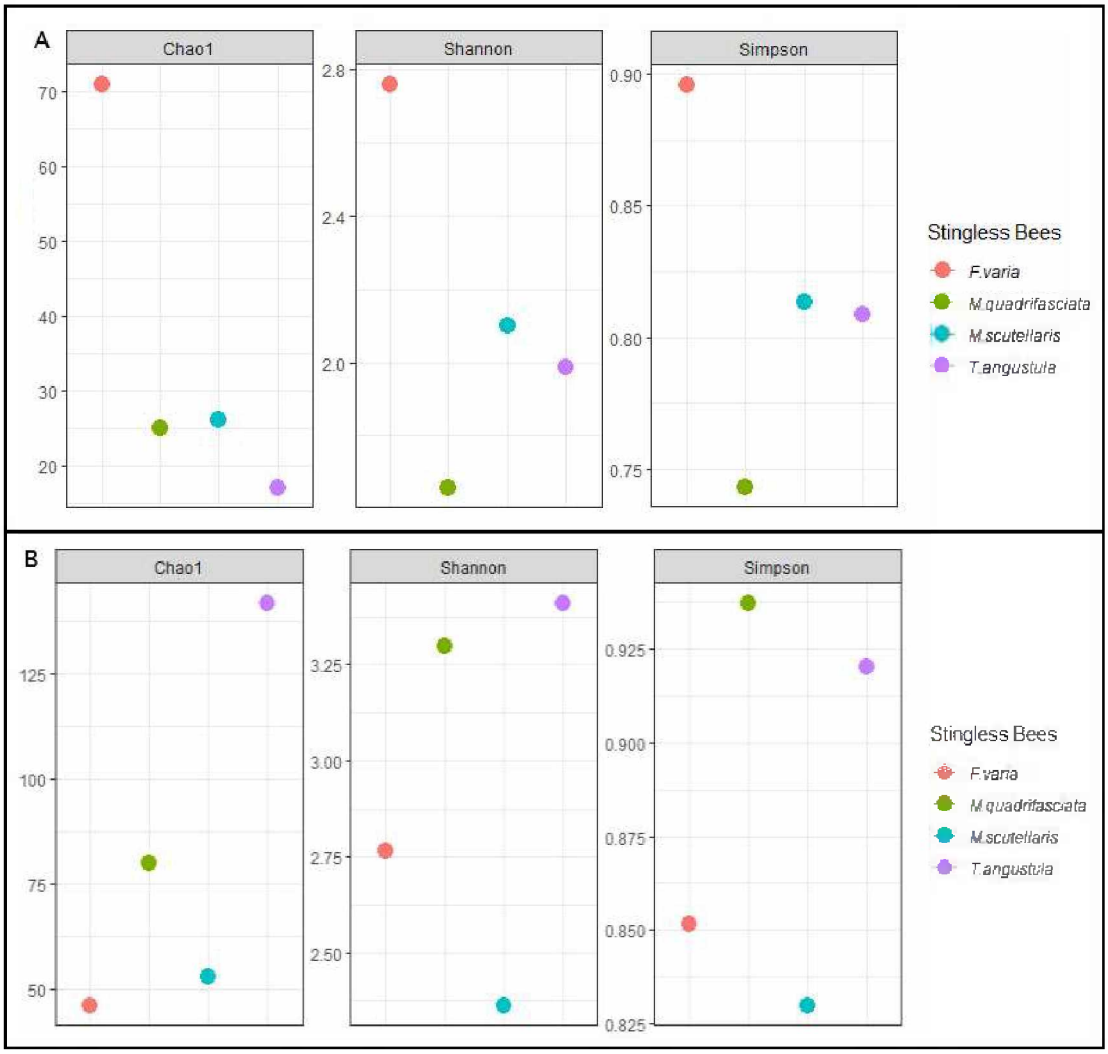

Figure 4- Alpha diversity index generated from the data of Bacterial(A) and Fungal(B) comumnity sequencing

The Principal Coordinate Analysis (PcoA) (Fig. 5A) of bacteria comumnity suggests different microbial patterns for *M. quadrifasciata* and *M. scutellaris* compared with *F. varia* and *T.angustula* LF. The patterns of *F. varia* and *T. angustula* presented similar compositions and were grouped about bees of the genus Melipona, suggesting that they present a similar species composition.

The PCoA analysis (Fig. 5B) of fungal community suggests different microbial among between samples.

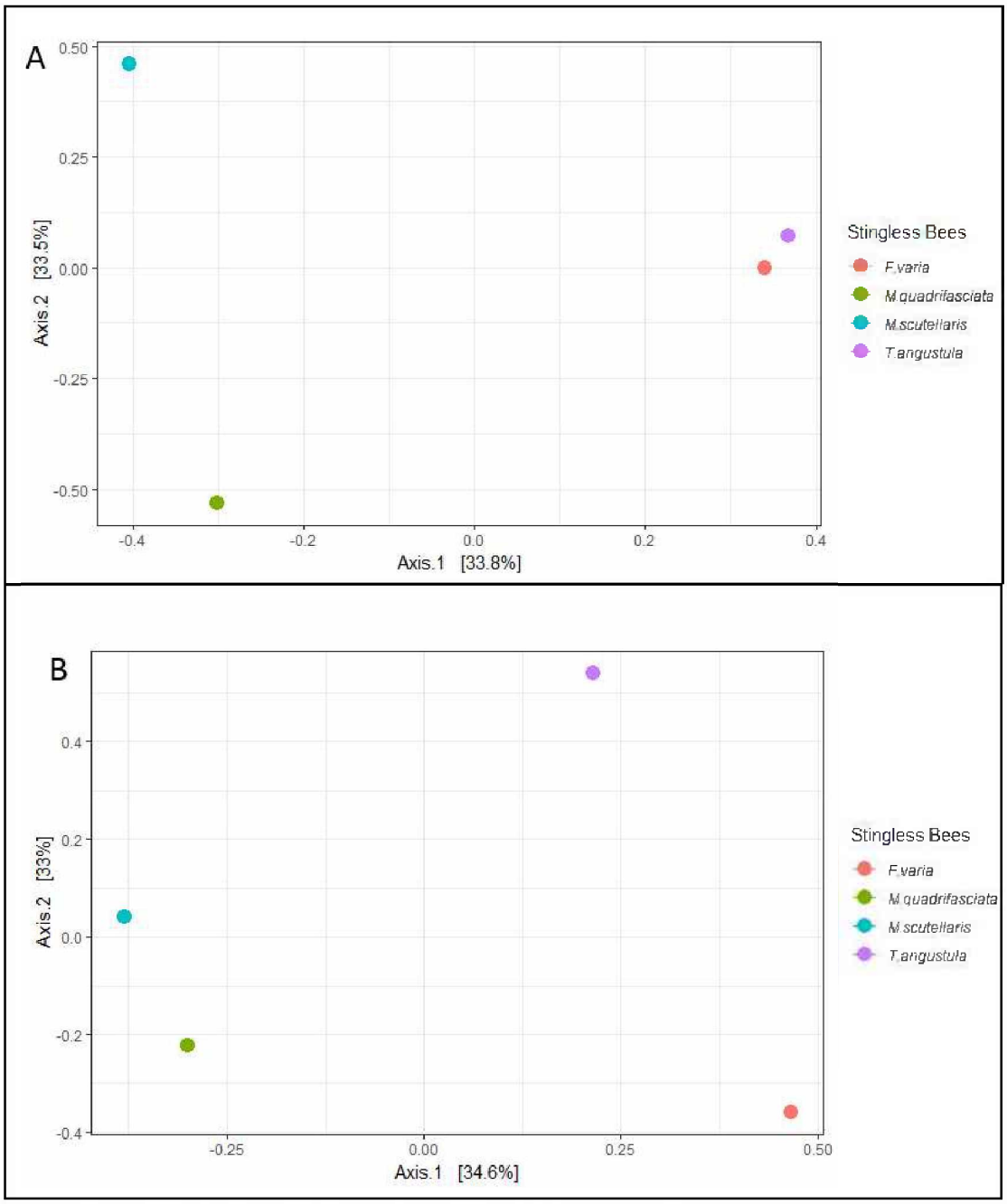

Figure 5- PCoA analsys Bacterial(A) and Fungal(B) comumnity sequencing

Cultivable fraction of the Microbiome

A total of 189 bacteria were isolated in culture medium, 41 from *F. varia* LF, 41 of *M. quadrifasciata*, 71 of *M. scutellaris* and 36 of *T. angustula*. Seventy-five (75) fungi specimens

were isolated from bees LF, 10 from *F. varia*, 18 from *M. quadrifasciata*, 29 from *M. scutellaris*, and 18 from *T. angustula*. The isolated microorganisms were preserved in the Collection of Microorganisms Isolated from Stingless Bee of the Laboratory of Genetics of Biotechnology of UFU (CoMISBee, Table I). It was possible to identify bacteria by the MALDI-TOF method, 42 bacteria individuals from the larval food of the four stingless bees were grouped into 12 genera (Fig. 6A).

Table I- Number of microorganisms isolated and preserved in the CoMISBee

Stingless bees	**Number of bacteria isolated**	**Number of fungi isolated**
Frieseomelitta varia	41	10
Melipona quadrifasciata	41	18
Melipona scutellaris	71	29
Tetragonisca angustula	36	18

Stingless bees of the genus *Melipona* had the highest number of organisms identified for MALDI. In *M. scutellaris* were identified 12 bacteria specimens, *M. quadrifasciata,* eight, *F. varia* and *T. angustula* had only two bacteria identified (Figure 6B).

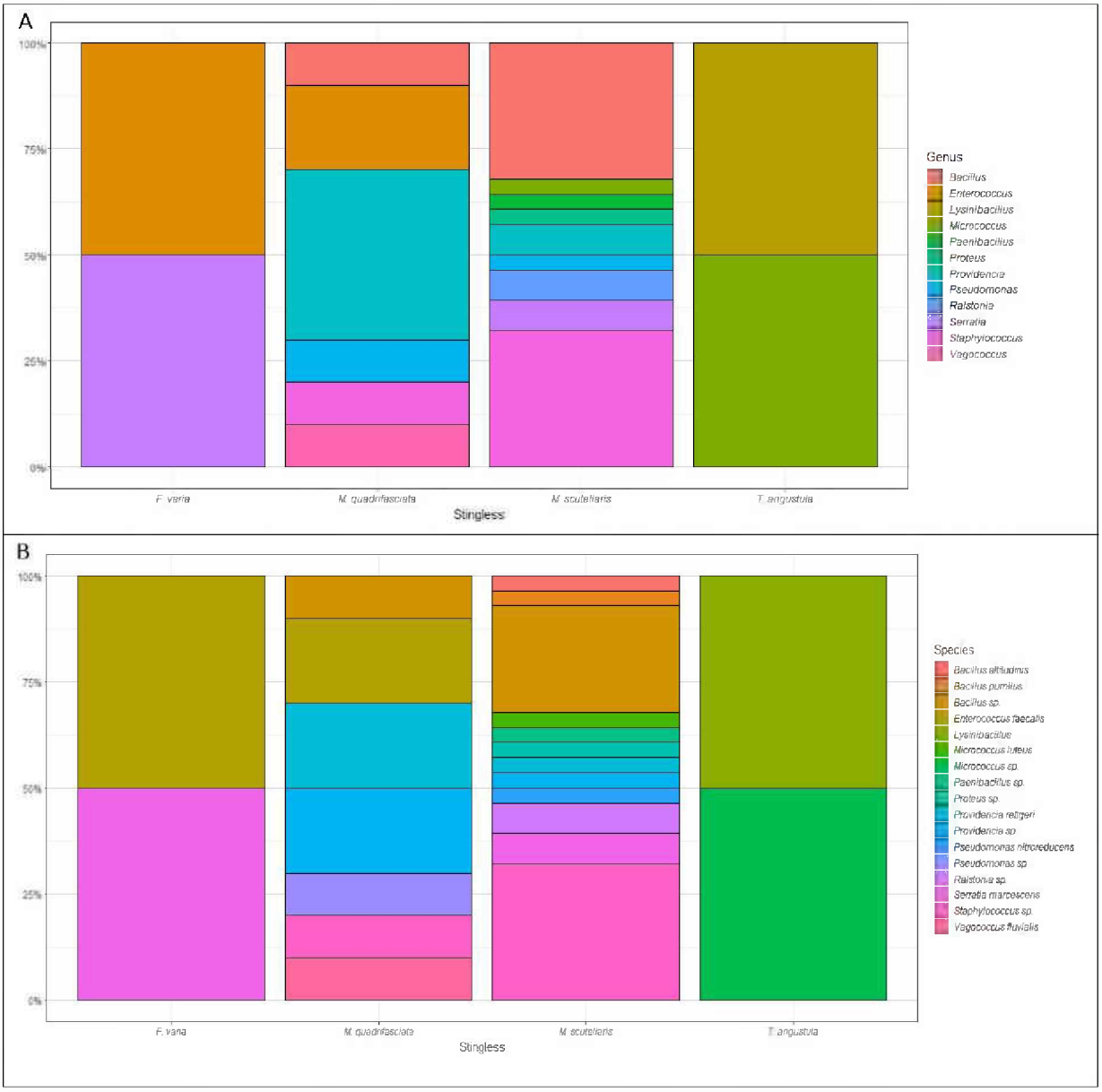

Figure 6-(A) Abundance of genera bacterial isolated from larval food of stingless bees. (B) An abundance of species was identified from the larval food of stingless bees.

Discussion

Stingless bees are the primary pollinators in Brazil, and the hive structure is entirely different from other social bee. The honey and pollen are stored in cerumen pots which are used to mature the food by fermentation from microorganisms[11,16]. After this process, the food is

mixed with glandular secretion to produce larval food [15,17,38,39]. Despite the great importance of stingless bees for pollination, little is known about the microbiological community present inside the hive. In this work, we describe a little about the Microbiome present in larval food of four species of Brazilian stingless bee rearing in the urban area of the Cerrado (Brazilian Savanna) biome, one of the most import important and large biomes in Brazil little-studied[40,41].

This work found bacteria, fungi, and yeasts associated with larval food of *F. varia*, *M. quadrifasciata*, *M. scutellaris*, and *T. angustula*. These microorganisms are important for maintaining colonies and crucial for pollen maturation and fermentation of honey from stingless bees [14–17,38]. Differences were found in the LF microbiota of the four stingless bees, demonstrating high variability and richness of composition in species of other groups of bees [42].

Sequencing of the V3/V4 region of the 16S, a region of great taxonomic coverage to genus level [31,32], showed that the most abundant bacteria in the LF of the stingless bees investigated were those of the *genus Lactobacillus* and the family Acetobacteraceae. The abundance *of Lactobacillus* and Acetobacteraceae has also been reported in the honey stomach/anterior intestine of *Melipona seminigra* [43,44] this may explain their presence in food, as the nurse transports fermented honey and pollen by ingestion, storage in the honey stomach, and subsequent regurgitation into the brood cells [4,45–47]

Bacteria of the genus Lactobacillus play an essential role in pollen and nectar processing, honey storage, and protection of these bees [17,47,48]. The genus *Bacillus*, although to a lesser extent, is related to nectar and pollen processing by the production of enzymes such as amylase, esters, lipases, proteases, phosphatases, and glycosidases [16,49]. Such enzymes had been described as present in larval food of *M. scutellaris*[14]. The presence of this genus in the colonies of *M. quadrifasciata* may be related to the fermentation of pollen and honey and may also protect against pathogens by producing antimicrobial molecules[17,50–52].

Bacteria of the *genus Paenibacillus* were isolated in *M. scutellaris*. Some species of this genus are producers of antimicrobials and various enzymes [53]. *Paenibacillus polymyxa* was identified in the bee *M. scutellaris*, probably producing antimicrobial compounds protecting the bees against pathogens [54]. However, although *Paenibacillus* harbors species are beneficial to bees, some species of the genus are disease-causing honey bees [53,55].

There is only one report of pathogens associated with stingless bees, the bacterium *Lysinibacillus sphaericus* disease-causing in *Tetragonula carbonaria*. [56,57]. Moreover, *Serratia marcences* identified in stingless bees LF, is an opportunistic disease-causing species in *Apis melifera* [57,58] and it was found in this work in *F. varia* and *M. scutellaris* through the isolation technique. The discovery of bacteria of the genuera *Lysinibacillus* and *Serratia* in the colonies may be a warning to the health of these bees. However, it was not identified in sequencing. It may be indicated that the microorganisms present in the larval food prevent the growth of *S. marcescens* and keep it so low that the extraction and sequencing technique could not reach the species. Critical report here that all colonies used for collecting larval food in this work were heath.

The sequencing of 16S regions makes it possible to identify the microbial community present in larval food of stingless bees and new species related to stingless bees [59]. This is the first report of the bacterial species bees *Acinetobacter junii, Bombella intestine, Corynebacterium lipophiloflavum*, *Serratia symbiotica,* and *Variovorax paradoxus*. Nonetheless, isolation in a culture medium is essential because it gives access to these microorganisms and allows their biotechnological use [59].

In this study, 42 bacteria isolated by the culture-dependent method have been identified. No species of the genus *Lactobacillus* were found in these isolates identified. In *F. varia* were identified the genus *Bacillus* by sequencing, *in M. scutellaris* and *M. quadrifasciata*, by isolation. This fact reinforces the need for molecular techniques to elucidate the Microbiome's diversity

better. In addition, the identification of microorganisms by cultivation that was not present in sequencing highlights the importance of using both methodologies for the knowledge of the microbiota of stingless bees.

In addition to having an intimate relationship with bacteria, stingless bees establish symbiont relationships with fungi [3,26,28]. The diversity of fungi in the LF of stingless bees was visualized only with the sequencing of the ITS1 region found different species of fungi and yeasts in the larval food of *F. varia*, *M. quadrifasciata*, *M. scutellaris*, and *T. angustula*. They indicated that filamentous fungi and yeasts are closely related to stingless bees. Fungi of the genus *Monascus* and yeasts of the genus *Zygosaccharomyces* establish a close relationship with the species *S. depilis* [26–28].

The diversity of yeast species found in the larval food of stingless bees varied among the bee species studied, *Saccharomyces*, *Starmerella,* and *Candida* are the most representative genera in this research. The yeasts species *Candida* bee, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae,* and *Starmerella meliponinorum* were observed in pollen and honey *of T. angustula*, *Nannotrigona testaceicornes* and *F. varia* stingless bees [60]. The species *Saccharomyces cerevisiae*, *Starmerella meliponinorum, and Rhodotorula mucilaginosa* were observed in the *species T. angustula*, as well as in our work, suggesting that they may be endemic yeasts in hives of this bee species. Also observed was that the pollen of F. varia presented little yeast diversity and none was in honey, and in our work, the yeast diversity was low [60].

The yeast *Saccharomyces cerevisiae* was observed in the larval food of all bee species in this study. Moreover, observing these organisms in stingless bees paves the way to find new strains with more significant biotechnological potential[60]. *Starmerella is* a genus of yeast common in stingless bees [61–63] This study identified it in the larval food of 3 species of bees. The species *Candida apicola* (=*Stamerella apicola*), *Starmerella etchellsii* e *Starmerella meliponinorum* foram

observed in the stingless bees *F. varia, M. quadrifasciata* e *T. angustula* [61]. This group of yeasts seems to be related to pollen and honey fermentation [3,29,61,64].

Bees of the genus *Trigona* collect spores of the fungi *Dictyophora*, *Phallus,* and *Rhizopus* as a source of protein for the colony or as a complement to the colony diet [65,66] The protein value of fungi may indicate why bees collected fungi and contamination by other organisms such as aphids and plants near hives[13]. *T. angustula* presented the most remarkable diversity of filamentous fungi and is considered a specialist specie in colonizing diverse niches. It can use fungi as a source of proteins to maintain colonies[61].

The genera *Cladosporium* and *Epicoccum*, filamentous fungi most representative in the species *F. varia*, are described as phytopathogens [67,68]. However, they are used as a biological control for various pathogens, *Epicoccum dendrobii* produces biomolecules capable of inhibiting phytopathogens [69]. Twenty-one (21) species, 21 of *Penicillium* and 6 of *Talaromyces,* were observed in *M. scutellaris* [70]. The genus *Penicilium* were observed in the larval food of *F. varia* and *M. quadrisfaciata* and *Talaromyces* in *M. quadrifasciata*.

The diversity in bacteria and fungi in stingless bees follows the diversity indices evaluated. The diversity of bacterial species suggests that the greater the richness of bacteria species, the lower the richness of fungal species and vice versa, as occurred in *F. varia* and *T. angustula.* The same yeast diversity was observed in the honey of *the species F. varia, M. quadrifasciata,* and *T. angustula* [62], indicating that the diversity of yeasts is related to the honey of these species. The data shown here reinforces the theory that bees are not herbivores. As the larvae consume a large number of microorganisms and they eat pollen, so they are metazoan in trophic hierarch. Thus, stingless bees are omnivores [71].

Our findings on the microbiota of larval food reinforce the importance of vertical transmission of the microbiota and social contact between individuals in colonies [72]. Suggesting

that part of the microbiota associated with LF be passed between bees in the nectar collection process and during the transfer of honey and matured pollen to the offspring cells [44,47].

This work opens a perspective for knowledge of Brazilian stingless bees' microbiomes, which is still little explored. In addition, microorganisms isolated in this work can be an essential source for discovering and prospecting novel bioactive compounds. Recently our group showed the biotechnological potential of some isolated bacteria in this work (genera *Providência, Serratia,* and *Vagococcus*) in producing antimicrobial activity against multiresistant hospital bacteria [73]. In addition to biotechnological prospecting, this work can prove how the presence of these bacteria in the larval food of stingless bees can confer protection through the production of antibiotics, reducing the proliferation of pathogens in colonies.

Conclusion

This research describes the microbiota associated with the species *F. varia*, *M. quadrifasciata*, *M. scutellaris,* and *T. angustula* and may play a role in the maintenance and protection of the colonies. This is the first research with microbiota of the larval food of stingless bees, and this work opens the way for studies that elucidate the role of bacteria and fungi in the survival of these organisms. Stingless bees larval food showed to be a rich source for the prospection of bioactive peptides with potential as drugs for the treatment of a wide range of diseases. The next step in this group will be developing new methods of isolating microorganisms and their use for biotechnological development.

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Authors' contributions
CUV, RCCD and ACCS conceived and supervised the project. ACCS and LDFB collected the larval food and performed analyzes. VACA and NDCR performed the MALDI-TOF analyzes for

assembly. GRF, ARS and ACCS performed the statistical analyses. CUV, AMB and ACCS wrote the draft. All authors discussed the results and commented on the manuscript. All authors approved its final version.

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Availability of data and materials

The files have been deposited in the SRA (Bioproject: PRJNA860336).

Declarations

Ethics approval and consent to participate. Biological material of the *F. varia, M. quadrifasciata, M. scutellaris,* and *T. angustula* was obtained under Brazilian laws. The species does not fall under the IUCN Red List categories as a threatened species. But the specie *M. scutellaris* endangered on the official list of species of the Brazilian fauna of the ICMBio

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest related to the results reported in this study.

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Table S1 - List of ASVs classified of bacterial community.

Stingless Bees	ASV	Phylum	Class	Order	Family	Genus	Species
F. varia	ASV19	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV77	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV32	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV113	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Saccharibacter*	NA
F. varia	ASV85	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV83	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV52	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV87	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV51	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV57	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV90	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV23	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV81	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV88	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV100	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV78	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV22	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV79	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV18	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV16	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV46	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*NA*	NA
F. varia	ASV80	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV20	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV49	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*NA*	NA
F. varia	ASV21	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV94	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV43	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Serratia*	*Serratia symbiotica*
F. varia	ASV86	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV17	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV122	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV104	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV84	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA

F. varia	ASV127	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV76	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV82	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV114	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV128	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV59	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV123	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
F. varia	ASV112	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Saccharibacter*	NA
F. varia	ASV70	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Saccharibacter*	NA
F. varia	ASV102	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV109	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	*Zymobacter*	NA
F. varia	ASV71	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Bombella*	*Bombella intestine*
F. varia	ASV119	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
F. varia	ASV115	Firmicutes	Bacilli	Lactobacillales	NA	*NA*	NA
F. varia	ASV48	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	*Zymobacter*	NA
F. varia	ASV124	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	NA
F. varia	ASV108	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	*Zymobacter*	NA
F. varia	ASV75	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	NA
F. varia	ASV44	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	*Micrococcus*	NA
F. varia	ASV47	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*NA*	NA
F. varia	ASV42	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Providencia*	NA
F. varia	ASV118	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Enterococcus*	NA
F. varia	ASV69	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*SN8*	NA
F. varia	ASV62	Actinobacteria	Actinobacteria	Pseudonocardiales	Pseudonocardiaceae	*Saccharopolyspora*	NA
F. varia	ASV61	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	NA
F. varia	ASV64	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	NA
F. varia	ASV45	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Tyzzerella 3*	NA
F. varia	ASV129	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Burkholderia-Caballer*	NA
F. varia	ASV67	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	*Corynebacterium 1*	*Corynebacterium lipophiloflavum*
F. varia	ASV105	Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	*Bifidobacterium*	NA
F. varia	ASV72	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Neisseriaceae	*Snodgrassella*	NA
F. varia; M. quadrifasciata	ASV106	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia/Shigella*	NA

F. varia; T. angustula	ASV58	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
M. quadrifasciata	ASV39	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV101	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV96	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV99	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV26	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV40	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV55	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
M. quadrifasciata	ASV4	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV5	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV63	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Rhodocyclaceae	*Dechloromonas*	NA
M. quadrifasciata	ASV111	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*	NA
M. quadrifasciata	ASV8	Actinobacteria	Actinobacteria	Micrococcales	Micrococcaceae	*Micrococcus*	NA
M. quadrifasciata	ASV60	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	NA
M. quadrifasciata	ASV97	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV50	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	*Acientobacter junii*

M. quadrifasciata	ASV125	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas*	NA
M. quadrifasciata	ASV10	Proteobacteria	Gammaproteobacteria	Betaproteobacteriales	Burkholderiaceae	*Variovorax*	*Variovorax paradoxes*
M. quadrifasciata	ASV110	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	NA
M. quadrifasciata	ASV103	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV98	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV126	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. quadrifasciata	ASV116	Firmicutes	Bacilli	Lactobacillales	NA	*NA*	NA
M. quadrifasciata	ASV68	Actinobacteria	Actinobacteria	Propionibacteriales	Nocardioidaceae	*Nocardioides*	NA
M. scutellaris	ASV33	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV27	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV89	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV34	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV56	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*NA*	NA
M. scutellaris	ASV53	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
M. scutellaris	ASV91	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV2	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV120	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV6	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV3	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV29	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV35	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV28	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV92	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV9	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*NA*	NA
M. scutellaris	ASV65	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV93	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA

M. scutellaris	ASV24	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV54	Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	*Ameyamaea*	NA
M. scutellaris	ASV11	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Anthococcus*	NA
M. scutellaris	ASV117	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV66	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia/Shigella*	NA
M. scutellaris	ASV73	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
M. scutellaris	ASV74	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV36	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV30	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV41	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV37	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV15	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV25	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV95	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV121	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV38	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV12	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV31	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV13	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV1	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV14	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	*Lactobacillus*	NA
T. angustula	ASV7	Firmicutes	Bacilli	Lactobacillales	NA	*NA*	NA
T. angustula	ASV107	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia/Shigella*	NA

Table S2- List of ASVs classified of fungal community.								
Stingless Bees	ASV	Kingdom	Phylum	Class	Order	Family	Genus	Species
F. varia	ASV8	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV46	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV54	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium exasperatum*
F. varia	ASV56	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV62	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Wickerhamiella*	*Wickerhamiella versatilis*
F. varia	ASV65	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV66	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV70	Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	NA	*NA*	*NA*
F. varia	ASV74	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV80	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV83	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida apicola*
F. varia	ASV84	Fungi	Ascomycota	Eurotiomycetes	NA	NA	*NA*	*NA*
F. varia	ASV88	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV94	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV106	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV107	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV131	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida jaroonii*
F. varia	ASV157	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV166	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV171	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV176	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV184	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV190	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV203	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV207	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV209	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*	*Epicoccum dendrobii*
F. varia	ASV217	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV234	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV245	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV252	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV257	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV263	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV279	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV280	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida jaroonii*
F. varia	ASV281	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV282	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV285	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV286	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia	ASV296	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia; M. quadrifasciata	ASV12	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*NA*
F. varia; M. quadrifasciata	ASV47	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Starmerella*	*Starmerella etchellsii*
F. varia; M. scutellaris	ASV40	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia; T. angustula	ASV20	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*Saccharomyces cerevisiae*
F. varia; T. angustula	ASV26	Fungi	NA	NA	NA	NA	*NA*	*NA*

F. varia; T. angustula	ASV59	Fungi	NA	NA	NA	NA	*NA*	*NA*
F. varia; T. angustula	ASV95	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV6	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium citrinum*
M. quadrifasciata	ASV10	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV11	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium delicatulum*
M. quadrifasciata	ASV18	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	NA	*NA*	*NA*
M. quadrifasciata	ASV19	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida apicola*
M. quadrifasciata	ASV22	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida zeylanoides*
M. quadrifasciata	ASV25	Fungi	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rhodotorula mucilaginosa*
M. quadrifasciata	ASV27	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Meyerozyma*	*Meyerozyma guilliermondii*
M. quadrifasciata	ASV34	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Toxicocladosporium*	*Toxicocladosporium irritans*
M. quadrifasciata	ASV36	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV37	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida apicola*
M. quadrifasciata	ASV38	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV39	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Tilachlidiaceae	*Tilachlidium*	*Tilachlidium brachiatum*
M. quadrifasciata	ASV41	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida parapsilosis*
M. quadrifasciata	ASV43	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida orthopsilosis*
M. quadrifasciata	ASV48	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*Saccharomyces cerevisiae*
M. quadrifasciata	ASV49	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida parapsilosis*
M. quadrifasciata	ASV52	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Phaffomycetaceae	*Starmera*	*Starmera stellimalicola*
M. quadrifasciata	ASV55	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	NA	*NA*	*NA*
M. quadrifasciata	ASV58	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus unguis*
M. quadrifasciata	ASV60	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus sydowii*
M. quadrifasciata	ASV64	Fungi	Basidiomycota	Agaricomycetes	Russulales	Peniophoraceae	*Peniophora*	*NA*
M. quadrifasciata	ASV68	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Candida*	*Candida apicola*
M. quadrifasciata	ASV69	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*NA*
M. quadrifasciata	ASV73	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium paczoskii*
M. quadrifasciata	ASV75	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV81	Fungi	Basidiomycota	Cystobasidiomycetes	Cystobasidiomycetes_ord_Incertae_sedis	Symmetrosporaceae	*Symmetrospora*	*NA*
M. quadrifasciata	ASV85	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*NA*
M. quadrifasciata	ASV91	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium delicatulum*
M. quadrifasciata	ASV97	Fungi	Ascomycota	Dothideomycetes	Dothideales	NA	*NA*	*NA*
M. quadrifasciata	ASV102	Fungi	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Hannaella*	*Hannaella sinensis*
M. quadrifasciata	ASV110	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Diutina*	*Diutina catenulata*
M. quadrifasciata	ASV112	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV113	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV116	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Curvularia*	*NA*
M. quadrifasciata	ASV121	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium parvulum*
M. quadrifasciata	ASV123	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Wickerhamiella*	*Wickerhamiella pararugosa*
M. quadrifasciata	ASV124	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV125	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Toxicocladosporium*	*Toxicocladosporium banksiae*
M. quadrifasciata	ASV128	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium aurantiogriseum*
M. quadrifasciata	ASV129	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*NA*
M. quadrifasciata	ASV132	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus penicillioides*
M. quadrifasciata	ASV133	Fungi	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*	*Malassezia restricta*
M. quadrifasciata	ASV135	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV138	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*Saccharomyces cerevisiae*
M. quadrifasciata	ASV147	Fungi	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	*Phellinus*	*Phellinus gilvus*
M. quadrifasciata	ASV150	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreales_fam_Incertae_sedis	*Acremonium*	*Acremonium hyalinulum*
M. quadrifasciata	ASV164	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium bilaiae*

M. quadrifasciata	ASV168	Fungi	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	*Trametes*	*Trametes hirsuta*
M. quadrifasciata	ASV169	Fungi	Ascomycota	Sordariomycetes	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV196	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces*	*Talaromyces wortmannii*
M. quadrifasciata	ASV202	Fungi	Ascomycota	Sordariomycetes	Hypocreales	NA	*NA*	*NA*
M. quadrifasciata	ASV205	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*Saccharomyces cerevisiae*
M. quadrifasciata	ASV212	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus sydowii*
M. quadrifasciata	ASV213	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV215	Fungi	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	*Trametes*	*Trametes hirsuta*
M. quadrifasciata	ASV218	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus waynelawii*
M. quadrifasciata	ASV221	Fungi	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Blumeria*	*NA*
M. quadrifasciata	ASV223	Fungi	Ascomycota	Sordariomycetes	Xylariales	Bartaliniaceae	*Bartalinia*	*Bartalinia pondoensis*
M. quadrifasciata	ASV225	Fungi	Mortierellomycota	Mortierellomycetes	Mortierellales	Mortierellaceae	*Mortierella*	*NA*
M. quadrifasciata	ASV230	Fungi	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*	*Malassezia restricta*
M. quadrifasciata	ASV239	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*NA*	*NA*
M. quadrifasciata	ASV243	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV259	Fungi	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*	*Malassezia globosa*
M. quadrifasciata	ASV270	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata	ASV274	Fungi	Basidiomycota	Agaricomycetes	Hymenochaetales	Hymenochaetales_fam_Incertae_sedis	*Resinicium*	*Resinicium saccharicola*
M. quadrifasciata	ASV290	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus tubingensis*
M. quadrifasciata	ASV298	Fungi	Ascomycota	Eurotiomycetes	Chaetothyriales	Chaetothyriales_fam_Incertae_sedis	*Strelitziana*	*Strelitziana eucalypti*
M. quadrifasciata; M. scutellaris	ASV2	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata; M. scutellaris	ASV44	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Didymella*	*NA*
M. quadrifasciata; M. scutellaris	ASV23	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*Saccharomyces cerevisiae*
M. quadrifasciata; M. scutellaris; T. angustula	ASV3	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium delicatulum*
M. quadrifasciata; T. angustula	ASV5	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium halotolerans*
M. quadrifasciata; T. angustula	ASV53	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Mycosphaerellaceae	*Cercospora*	*NA*
M. quadrifasciata; T. angustula	ASV67	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*NA*
M. quadrifasciata; T. angustula	ASV109	Fungi	Mucoromycota	Mucoromycetes	Mucorales	Mucoraceae	*Mucor*	*Mucor circinelloides*
M. quadrifasciata; T. angustula	ASV224	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. quadrifasciata; T. angustula	ASV100	Fungi	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*Malassezia*	*Malassezia restricta*
M. scutellaris	ASV16	Fungi	NA	NA	NA	NA	*NA*	*NA*

M. scutellaris	ASV21	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV31	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV76	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV77	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Bipolaris*	*Bipolaris shoemakeri*
M. scutellaris	ASV86	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV96	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Didymella*	*NA*
M. scutellaris	ASV105	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV117	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV118	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV119	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV120	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV136	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV137	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV140	Fungi	Basidiomycota	Agaricomycetes	NA	NA	*NA*	*NA*
M. scutellaris	ASV142	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV143	Fungi	Basidiomycota	Cystobasidiomycetes	Erythrobasidiales	Erythrobasidiaceae	*Bannoa*	*Bannoa ogasawarensis*
M. scutellaris	ASV144	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV148	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV156	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV161	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV163	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus pseudodeflectus*
M. scutellaris	ASV179	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV180	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV181	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV187	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV193	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV194	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV197	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV210	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV211	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Xeromyces*	*Xeromyces bisporus*
M. scutellaris	ASV227	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV240	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV249	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV254	Fungi	Ascomycota	Sordariomycetes	NA	NA	*NA*	*NA*
M. scutellaris	ASV268	Fungi	Basidiomycota	Malasseziomycetes	Malasseziales	Malasseziaceae	*NA*	*NA*
M. scutellaris	ASV272	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV273	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV277	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Toxicocladosporium*	*Toxicocladosporium immaculatum*
M. scutellaris	ASV284	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV289	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV293	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV294	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV295	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris	ASV299	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris; T. angustula	ASV9	Fungi	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Erysiphe*	*Erysiphe quercicola*
M. scutellaris; T. angustula	ASV4	Fungi	NA	NA	NA	NA	*NA*	*NA*
M. scutellaris; T. angustula	ASV15	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV1	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV7	Fungi	NA	NA	NA	NA	*NA*	*NA*

T. angustula	ASV13	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV14	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV17	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV24	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV28	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV29	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV30	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV32	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV33	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV35	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	*Saccharomyces cerevisiae*
T. angustula	ASV42	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV45	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV50	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV51	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV57	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV61	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium citrinum*
T. angustula	ASV63	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV71	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV72	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV78	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV79	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV82	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Capnodiaceae	*Antennariella*	*Antennariella placitae*
T. angustula	ASV87	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV89	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV90	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV92	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV93	Fungi	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Podosphaera*	*Podosphaera astericola*
T. angustula	ASV98	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV99	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV101	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV103	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV104	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV108	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV111	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetales_fam_Incertae_sedis	*Starmerella*	*Starmerella meliponinorum*
T. angustula	ASV114	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV115	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV122	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV126	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV127	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV130	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV134	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV139	Fungi	Ascomycota	Sordariomycetes	Ophiostomatales	Ophiostomataceae	*Cornuvesica*	*Cornuvesica acuminata*
T. angustula	ASV141	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
T. angustula	ASV145	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV146	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Penicillium*	*Penicillium citrinum*
T. angustula	ASV149	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	*Pseudopithomyces*	*Pseudopithomyces karoo*
T. angustula	ASV151	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV152	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV153	Fungi	Ascomycota	Leotiomycetes	Helotiales	Helotiaceae	*Articulospora*	*NA*
T. angustula	ASV154	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV155	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV158	Fungi	NA	NA	NA	NA	*NA*	*NA*

T. angustula	ASV159	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV160	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV162	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV165	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV167	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV170	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV172	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV173	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*NA*
T. angustula	ASV174	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV175	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV177	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV178	Fungi	Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	*Nigrospora*	*Nigrospora oryzae*
T. angustula	ASV182	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreales_fam_Incertae_sedis	*Hapsidospora*	*Hapsidospora irregularis*
T. angustula	ASV183	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV185	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV186	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV188	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV189	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV192	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV191	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Cucurbitariaceae	*Pyrenochaeta*	*NA*
T. angustula	ASV195	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	*Claviceps*	*Claviceps chloridicola*
T. angustula	ASV198	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV199	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV200	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV201	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV204	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV206	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV208	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV214	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV216	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Epicoccum*	*Epicoccum dendrobii*
T. angustula	ASV220	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV219	Fungi	Ascomycota	Dothideomycetes	Capnodiales	Capnodiaceae	*Scorias*	*Scorias mangiferae*
T. angustula	ASV222	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV226	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	*Trichoderma*	*NA*
T. angustula	ASV228	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV229	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV231	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV232	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV233	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Zygosaccharomyces*	*Zygosaccharomyces mellis*
T. angustula	ASV235	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV236	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV238	Fungi	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Erysiphe*	*NA*
T. angustula	ASV237	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV241	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV242	Fungi	Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	*Erysiphe*	*Erysiphe quercicola*
T. angustula	ASV244	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV246	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV247	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV248	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV250	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV251	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV253	Fungi	NA	NA	NA	NA	*NA*	*NA*

T. angustula	ASV255	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV256	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV258	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV260	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV261	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV262	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV264	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV265	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV266	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV267	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV269	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV271	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV275	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV276	Fungi	Ascomycota	NA	NA	NA	*NA*	*NA*
T. angustula	ASV278	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV283	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV287	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV288	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV291	Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus flavus*
T. angustula	ASV292	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV297	Fungi	NA	NA	NA	NA	*NA*	*NA*
T. angustula	ASV300	Fungi	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Bipolaris*	*NA*

CAPÍTULO 3
ARTIGOS CIENTÍFICO PUBLICADO NA REVISTA: BMC Microbiology

ANTIMICROBIAL ACTIVITY OF SUPERNATANTS PRODUCED BY BACTERIA ISOLATED FROM BRAZILIAN STINGLESS BEE'S LARVAL FOOD

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Antimicrobial activity of supernatants produced by bacteria isolated from Brazilian stingless bee's larval food

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Abstract

Background: The discovery of new molecules with antimicrobial properties has been a promising approach, mainly when related to substances produced by bacteria. The use of substances produced by bees has evidenced the antimicrobial action in different types of organisms. Thus, the use of bacteria isolated from larval food of stingless bees opens the way for the identification of the new molecules. The effect of supernatants produced by these bacteria was evaluated for their ability to inhibit the growth of bacteria of clinical interest. Furthermore, their effects were evaluated when used in synergy with antibiotics available in the pharmaceutical industry.

Results: A few supernatants showed an inhibitory effect against susceptible and multiresistant strains in the PIC assay and the modulation assay. Emphasizing the inhibitory effect on multidrug-resistant strains, 7 showed an effect on multidrug-resistant *Escherichia coli* (APEC), *Klebsiella pneumoniae* carbapenemase (KPC), multidrug-resistant *Pseudomonas aeruginosa*, and multidrug-resistant *Staphylococcus aureus* (MRSA) in the PIC assay. Of the supernatants analyzed, some presented synergism for more than one species of multidrug-resistant bacteria. Nine had a synergistic effect with ampicillin on *E. coli* (APEC) or *S. aureus* (MRSA), 5 with penicillin G on *E. coli* (APEC) or KPC, and 3 with vancomycin on KPC.

Conclusion: In summary, the results indicate that supernatants produced from microorganisms can synthesize different classes of molecules with potent antibiotic activity against multiresistant bacteria. Thus, suggesting the use of these microorganisms for use clinical tests to isolate the molecules produced and their potential for use.

Keywords: Antibiotics, Pathogen, Antibacterial effect, Microorganisms, Multiresistant bacteria, Stingless bees, Larval food

Background

Faced with the global concern with public health, which has been facing difficulties in combating resistant pathogens, the search for new drugs with antimicrobial function becomes an emergency. Indiscriminate use of antibiotics results in the selection of resistance to most commercially available antimicrobials [1–3]. Among the classic pathogens, some have a high capacity for the acquisition and dissemination of resistance genes, becoming a public health problem worldwide [4–6]. In this context, bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* stand out, which present multidrug resistance genotypes [2, 4–7].

The antimicrobial effect of products generated by stingless bees has contributed to discovery of biomolecules

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capable of inhibiting the growth of microorganisms. The pollen of *Melipona compressipes manaosensis* [8], the geopropolis extract of the bee *Melipona quadrifasciata* and *Tetragonisca angustula* [8–10] , as well as the propolis and honey, named by authors as bee bread of *Heterotrigona itama* [11] have shown action against pathogenic microorganisms.

The diversity of microorganisms associated with stingless bee colonies are broad, even as their role in the health and vitality of these organisms [12–16] Microorganisms can contribute to the development of the immune system of bees, assist in food digestion and defend the hive against pathogens [17–19]. *Scaptotigona depilis* bees are related to a mutualistic relationship with fungi of the genus *Zygosaccharomyces*, which influence larval development, survival rate, and differentiation of queens [20–22]. Despite the progress in studies with the microbiota associated with stingless bee colonies, it is still unknown its effective role in the maintenance and development of the colonies. Bacteria associated with stingless bees have biotechnological purposes, such as probiotics, disease biocontrol agents, producers of enzymes, and antimicrobial substances [12, 23].

In this context, studies related to the discovery of new molecules with antimicrobial function obtained from by-products generated by stingless bees may represent an alternative to the global public health problem about antibiotic resistance and treatment of infections caused by multidrug-resistant pathogens. The present investigation aims to evaluate the capability of microorganisms isolated from larval food of stingless bees to generate biomolecules with antimicrobial potential.

Methods

Sample collection

This study used 14 bacteria's isolated from *Melipona quadrifasciata, Melipona scutellaris,* and *Tetragonisca angustula* (Table 1) from the Collection of Microorganisms Isolated from Stingless Bee from the Laboratory of Genetics of Biotechnology of UFU (CoMISBee).

Table 1 CoMISBee Bacteria and Bee Code

CoMISBee Code	Bees Larval Food
Mq-ISP-1A	*Melipona quadrifasciata*
Mq-ISP-1B	*Melipona quadrifasciata*
Mq-MCK-7	*Melipona quadrifasciata*
Mq-MRS-9A	*Melipona quadrifasciata*
Mq-MRS-9BI	*Melipona quadrifasciata*
Mq-TSA-12	*Melipona quadrifasciata*
Ta-TSA-14	*Tetragonisca angustula*
Mq-BHI-20	*Melipona quadrifasciata*
Mq-OAT-27	*Melipona quadrifasciata*
Ms-BHI-39A	*Melipona scutellaris*
Ms-BHI-39B	*Melipona scutellaris*
Ta-NUT-45	*Tetragonisca angustula*
Mq-BHI-47	*Melipona quadrifasciata*
Mq-NUT-54B	*Melipona quadrifasciata*

Supernatant production

For production of supernatant, a bacterial suspension was prepared in 5mL of BHI and incubated at 37°C for 24 hours. A 200μL rate of the suspension was inoculated in 50mL of LB broth (Luria-Bertani) and incubated at 31°C±1 for 48h under shaker agitation at 200 rpm. The broth obtained was centrifuged at 10,000 g for 4 minutes for bacterial cell sedimentation [24] and the supernatant was separated from the precipitated and filtered at 0.22 μm (Figure 1). The 14 supernatants was stored in a -20°C freezer for later use in antimicrobial activity and resistance modulation tests.

Bacterial identification

For a taxonomic identification at the level of genus and species, the precipitate generated in the centrifugation was inoculated on LB agar plate, and incubated at 37°C±1 for 24h. An isolated colony of each strain was harvested from the agar using an inoculation loop and inactivated with absolute ethanol. This strain was also submitted to MALDI-TOF mass spectrometry using the MALDI Biotyper version 3 (Bruker Daltonics), according to manufacturer´s suggested settings using automated collected spectra. The biomolecular identification of bacteria were analyzed according to the score values proposed by the manufacturer [25].

Antimicrobial activity assay

The potential of supernatants to inhibit bacterial growth was evaluated using the Plaque Inhibition Concentration (PIC) method, evaluating for the capacity to kill or inhibit the growth of bacteria with antimicrobial-sensitive and antimicrobial-resistant genotypes. Gram-positive (1) bacteria were used: *Staphylococcus aureus* (sensitive genotype), *Staphylococcus aureus* MRSA (Methicillin-resistant *S. aureus*, multidrug-resistant genotype) and (2) Gram-negative bacteria *Escherichia coli* (ATCC 8739, sensitive genotype), *Escherichia coli* (APEC, multiresistant genotype), Sensitive *Klebsiella pneumoniae*, KPC (Carbapenem-resistant *Klebsiella pneumoniae*, multidrug-resistant genotype) and *Pseudomonas aeruginosa* (sensitive genotype, PAO-1) and *Pseudomonas aeruginosa* (multiresistant genotype). The pathogenic bacteria used were obtained from culture collections or isolated from clinical samples, provided by the Laboratories of

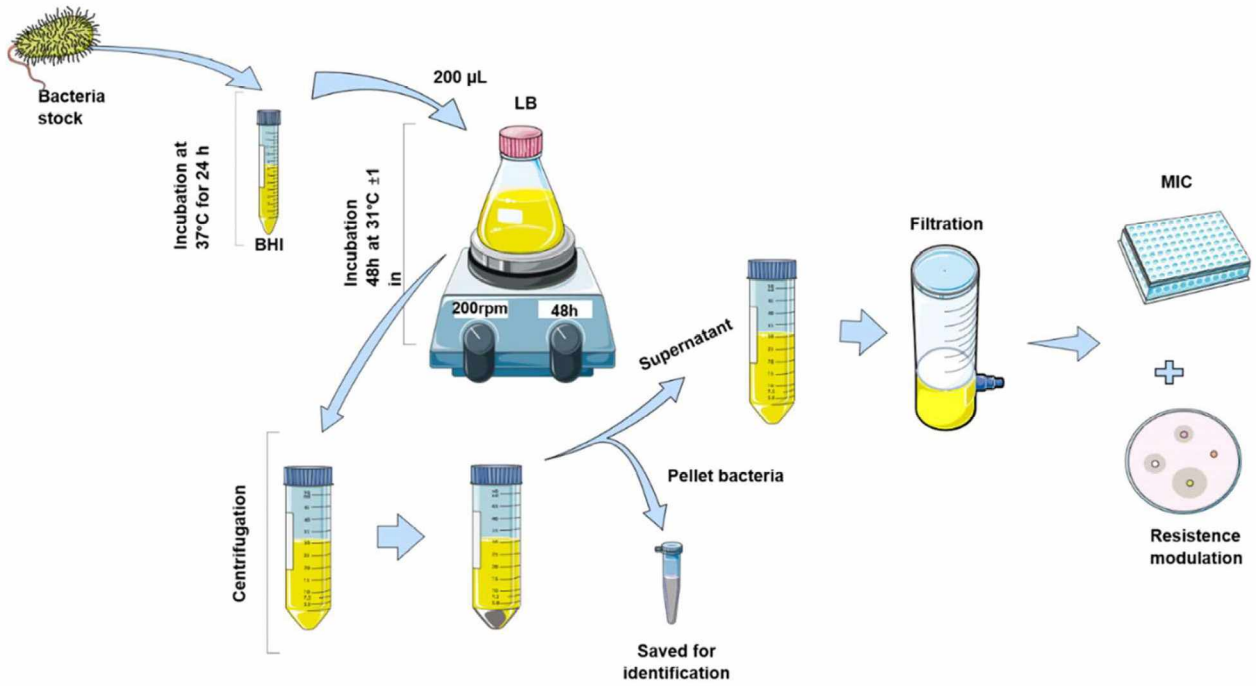

Fig. 1 Methodology for supernatant production from microorganisms Collection of Microorganisms Isolated from Stingless Bee (CoMISBee) from the Laboratory of Genetics of UFU. Images of representative metodology from 'Smart Servier Medical Art' (https://smart.servier.com/)

Molecular Microbiology (MICROMOL) and Animal Biotechnology Laboratory (LABIO) of the Federal University of Uberlândia.

For the assay, a bacterial suspension was prepared in LB broth at 37°C±0.5 for 24 hours and diluted to 10^4 cells/mL. Fifty microliters of bacterial suspension was transferred to a 96-well plate containing 50 µL of supernatant to assess whether would reduce microbial growth or kill microorganisms. Wells containing 100 µL of bacteria and LB were used as control of the experiment, positive and negative for bacterial growth, respectively. The plaque was incubated for 24 hours at 37°C±0.5 and bacterial growth was evaluated in microtiter plate reader at 595 nm at 0; 6; 12 and 24 hours.

Resistance modulation assay

The supernatants were evaluated for the ability to modulate bacterial resistance when used in synergy with antibiotics that pathogenic bacteria are resistant. Ampicillin (10 Mcg) was tested for strains of *E. coli* (APEC) and *S. aureus* (MRSA), Gentamicin (10 Mcg) for *P. aeruginosa* (multiresistant), Penicillin-G (10 U) for *E. coli* and *K. pneumoniae*, and Vancomycin (30 Mcg) for *K. pneumoniae*. The antimicrobial effect was determined using the modified Kirby-Bauer disk diffusion test (Bauer et al., 1966). The plates were drilled with wells of 6mm inoculated 50 µL of supernatant, along with one antibiotic disc per well. The diameter of the microbial growth halo was evaluated for each supernatant-antibiotic association and compared with the control, containing only the antibiotic disc. The target bacterium was considered sensitive when inhibition zone formation occurred.

Statistical analysis

Data are expressed as arithmetic means ± standard error of the mean and were analyzed by analysis of variance for two-way (ANOVA), followed by Dunnet post-test using GraphPad Prism software version 8.0.2 (available http://www.graphpad.com/scientific-software/prism/). Statistical significance was considered when $p < 0.05$.

Results

Bacterial identification

Of the 14 strains analyzed, six (42.85%) had a probable species identification, two (14.3%) obtained genus identification and six (42.85%) did not obtain reliable identification (Table 2).

Antimicrobial activity assay

The PIC (Plate Inhibitory Concentration) values of the supernatant for sensitive and resistant pathogenic bacteria are shown in Figure 2. All analyzed supernatants showed an inhibitory effect on more than one bacterium. Sensitive *E. coli*, multidrug-resistant *E. coli*, sensitive *K.*

Table 2 Identification for MALDI-TOF of bacteria isolated

Supernatant Code	Organism (best match)	Score Value	Organism (second match)	Score Value
1A[b]	*Staphylococcus epidermidis*	1.775	not reliable identification	1.672
1B[c]	not reliable identification	1.413	not reliable identification	1.379
7[b]	*Providencia rettgeri*	1.787	not reliable identification	1.691
9A[a]	*Enterococcus faecalis*	2.413	*Enterococcus faecalis*	2.354
9BI[a]	*Providencia rettgeri*	2.065	*Providencia rettgeri*	2.056
12[c]	not reliable identification	1.438	not reliable identification	1.379
14[c]	not reliable identification	1.399	not reliable identification	1.326
20[a]	*Vagococcus fluvialis*	2.272	*Vagococcus fluvialis*	2.247
27[c]	not reliable identification	1.518	not reliable identification	1.485
39A[a]	*Serratia marcescens*	2.041	*Serratia marcescens*	2.01
39B[a]	*Providencia rettgeri*	2.026	*Providencia rettgeri*	1.924
45[c]	not reliable identification	1.335	not reliable identification	1.332
47[c]	not reliable identification	1.56	not reliable identification	1.512
54B[a]	*Providencia rettgeri*	2.05	*Providencia rettgeri*	2.028

[a] secure genus identification, probable species identification, [b]probable genus identification, [c]not reliable identification

pneumoniae, *carbapenemase K. pneumoniae*, sensitive *S. aureus*, methicillin-resistant *S. aureus*, or multiresistant *P. aeruginosa*.

For sensitive *E. coli*, the supernatant 1A inhibited bacterial growth in 12 and 24 hours of treatment (p<0,01) (Figure 2A). The supernatant 39B showed an inhibiting effect on the growth of *E. coli* multidrug-resistant in the 24 hours of treatment (Figure 2B). And the supernatants 9A showed significant effect only 12 hours of treatment (p<0,01) and 9BI within 6 (p<0,05) and 12 hour of treats (p<0,001).

For the species *K. pneumoniae* was observed significant effect by supernatant 07 (p<0,01) in 24 hours, (Figure 2C), and 27, in 6 (p<0,01) and 12 hours (p<0,01). Similarly, several supernatants had an inhibiting effect on KPC in 6 hours, and1A, 1B, 9A and 54 B reduced growth in 12 hoursbut only the 39B (p<0,05) supernatant showed a reduction in optical density in 24 hours of bacterial growth (Figure 2D).

Regarding *P. aeruginosa* sensitive, we can highlight the action of the supernatants 12, with an inhibiting effect between the times 6 and 24 hours of growth (Figure 2E). For the multidrug-resistant microorganism, none of the supernatants showed significant effect at the end of growth observed in 24 hours, but the supernatants 1A (p<0,01), 1B(p<0,05), 9BI (p<0,05), 20 (p<0,05) and 54B (p<0,05) showed significant effect up to 12 hours (figure 2F).

For *S. aureus* sensitive, no antimicrobial effects of the supernatants were observed in 24 hours, and only samples 1A, 1B, 20, and 27 reduced microbial growth to 12 hours, but without significant reduction at the end of 24 hours. For the resistant microorganism (*S. aureus* MRSA), it was possible to verify growth reduction by the growth reducing the effect of supernatants 12, 14 and 54B until the end of the 24 hours of observation. The supernatants 1B (p<0,05), 9A (p<0,01), 12 (p<0,01), 14 (p<0,05) and 39A (p<0,05), showed a significant effect in the time of 12 hours, although they did not lead to a reduction in bacterial growth in 24 hours of treatment.

Resistance modulation assay

Sixteen supernatants were evaluated in the resistance modulation assay with multidrug-resistant pathogenic bacteria, which did not present antibiotic inhibition zone. After the addition of the supernatant near the well containing the antibiotic disc, it demonstrated the formation of the inhibition zone for some microorganisms (Table 3).

The resistance modulation assay showed a synergistic effect of six supernatants on multidrug-resistant *E. coli*, with halos ranging from 9.3 to 11.18 mm. We observed 3 supernatants with synergistic effect with Vancomycin and 1 with Penicillin G effective against KPC, with emphasis on Vancomycin associated with the S54B supernatant (inhibition zone of 16.01 mm), and 6 for *Staphylococcus aureus* (all with Ampicillin) and supernatant S54B also stood out in this microorganism (inhibition zone of 16.01 mm). No effect was observed for multidrug-resistant *Pseudomonas aeruginosa*.

In total, ten supernatants had a synergistic effect with antibiotics tested, some presented synergism for more than one species of multidrug-resistant bacteria, especially: supernatant S1B, effective on *E. coli* when in synergy with Ampicillin (9.78 ±0.87) and Penicillin G (9.72 ±0.61). *K. pneumoniae* when in synergy with Vancomycin (13.87 ±1.60); S9BI supernatant, effective on *E. coli*

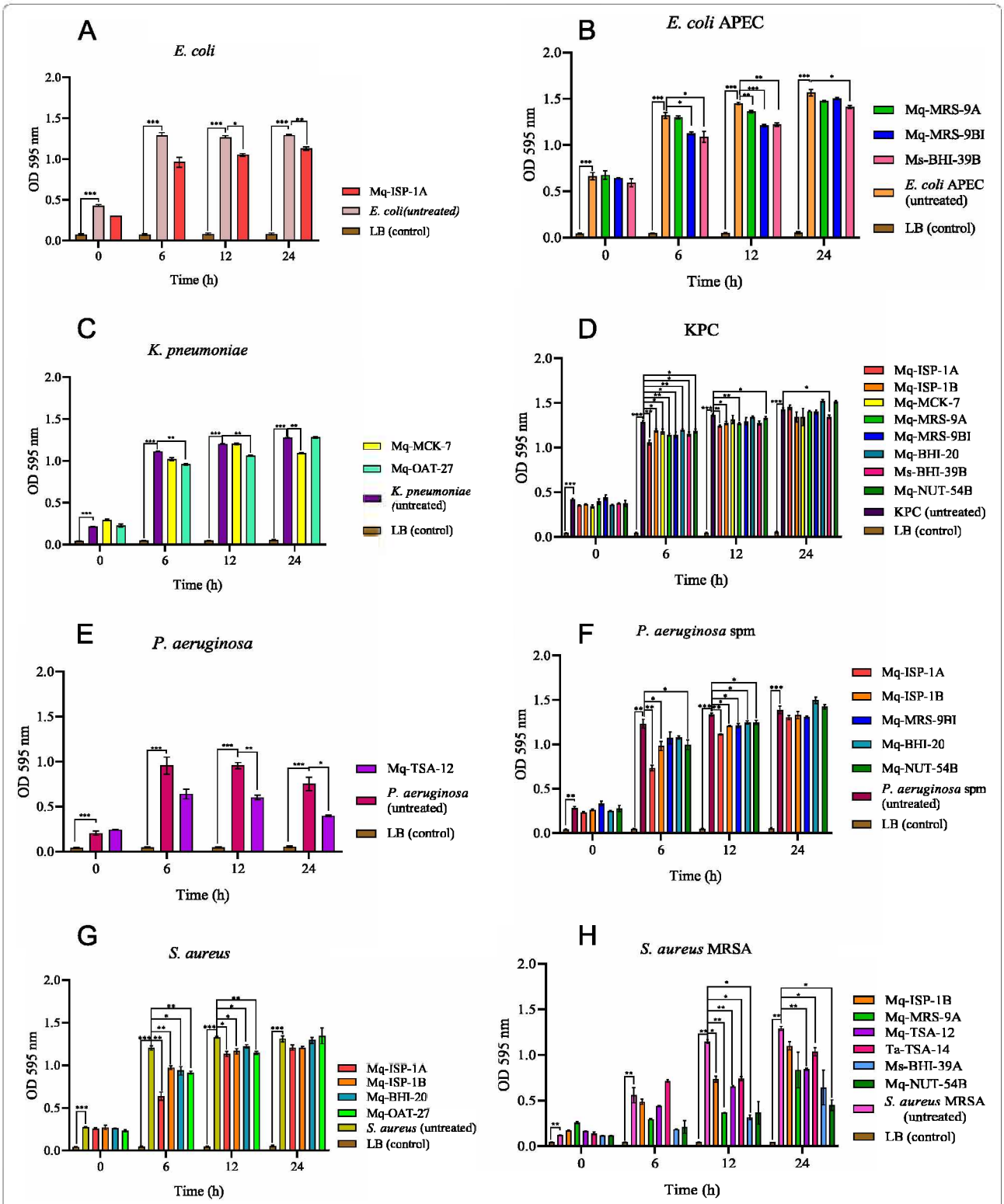

Fig. 2 Antimicrobial activity of supernatants produced by microorganisms isolated from stingless bee larval food on sensitive and resistant pathogenic bacteria. The antimicrobial properties of the supernatants were evaluated against strains of sensitive and resistant bacteria. The figure shows only the supernatants that showed a statistically significant difference between the experimental and control groups (bacteria incubated only with LB) in some period of the treatment. Data show the average _ SEM of three independent replicates. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

Table 3 Effect of resistance modulation assay using the antibiotic alone and in combination with supernatant on multidrug-resistant pathogenic bacteria

Multiresistant bacteria	Antibiotic disc (+ supernatant)	Inhibition zone (mm)
Escherichia coli	Ampicillin	Absent
	Ampicillin (+ S1B)	9,78 ±0,87
	Ampicillin (+ S9BI)	21,87 ±3,04
	Penicillin G (+ S1B)	9,72 ±0,61
	Penicillin G (+ S9BI)	11,18 ±0,20
	Penicillin G (+ S45)	9,3 ±0,13
Staphylococcus aureus	Ampicillin	Absent
	Ampicillin (+ S9A)	15,62 ±1,28
	Ampicillin (+ S20)	16,63 ±0,73
	Ampicillin (+ S39B)	16,19 ±1,27
	Ampicillin (+ S47)	16,25 ±0,74
	Ampicillin (+ S54B)	20,74 ±0,69
Klebsiella pneumoniae	Penicillin G	Absent
	Vancomycin	Absent
	Penicillin G (+ S9BI)	11,84 ±0,96
	Vancomycin (+ S1B)	13,87 ±1,60
	Vancomycin (+ S9A)	14,25 ±1,24
	Vancomycin (+ S54B)	16,01 ±1,93

when in synergy with Ampicillin (21.87 ±3.04) and Penicillin G (11.18 ±0.20) and *K. pneumoniae* when in synergy with Penicillin G (11.84 ±0.96); S54B, effective on *S. aureus* when in synergy with Ampicillin (20.74 ±0.69) and *K. pneumoniae* when in synergy with Vancomycin (16.01 ±1.93).

Discussion

With the advent of antibiotics, the excessive use and inadequate consumption of these drugs led to the rapid emergence of multidrug-resistant pathogens. Among these, Gram-positive bacteria, such as *Staphylococcus aureus*, and Gram-negative, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, stand out for their high ability to develop multiple mechanisms of antimicrobial resistance, which makes them several global public health problems [1, 26]. With this, treating a bacterial infection in modern medicine has overwhelmed researchers and pharmaceutical companies to develop new effective antimicrobials against these multidrug-resistant pathogens of arduous treatment [27, 28].

Several authors suggest that the only way to contain the current antimicrobial resistance crisis will be to develop entirely new strategies to combat these pathogens. Such as the combination of antimicrobial drugs with other agents that neutralize and obstruct the mechanisms of antibiotic resistance expressed by the pathogen [5, 6, 28–30]. In this sense, different studies have sought new bioactive substances with antimicrobial effects from microorganisms isolated from the environment. And the production of biomolecules by bacteria isolated from by-products of stingless bees represents considerable potential for bioprospecting of new compounds with antimicrobial effect [8–11, 31–34].

In the larval food of stingless bees, several species of microorganisms provide digestive enzymes, which participate in the pre-digestion of food stocks, as well as organic acids and antibiotics, which start the development of concurrent microorganisms [35, 36] Our study showed that bacteria isolated from the larval food of *Melipona quadrifasciata*, *Melipona scutellaris* and *Tetragonisca angustula* had antimicrobial activity against Gram-positive and Gram-negative bacteria, including multidrug-resistant strains. However, in general, by the antimicrobial activity assay through the PIC, it was possible to verify that the supernatants had a higher antimicrobial effect on the sensitive pathogenic bacteria when compared to their application in resistant strains. This can be explained by the fact that multidrug-resistant bacteria have several mechanisms of escape of antimicrobial molecules, including not only the production of enzymes but also the production of flow pumps and changes in membrane permeability, which prevent the accumulation of bactericidal substances inside the microbial cell [37]. The modulation assay showed that some supernatants reestablished the effect of antibiotics tested on resistant strains Gram-positive, *S. aureus* (MRSA) and Gram-negative- *E. coli* (APEC) and *K. pneumoniae* (KPC), with no effect on *P. aeruginosa*.

Many studies portray the difficulty in finding effective substances to inhibit the growth of gram-negative bacteria. Carneiro et al. [32] evaluated the antimicrobial potential of pollen extract and propolis extract of *M. compressipes manaosensis* (jupará) in *E. coli* and did not obtain significant results in the analyses performed. Similarly, Tenorio et al. [38] did not visualize the inhibiting action of *Melipona fasciculata* honey for *E. coli* and *P. aeruginosa*. In a recent investigation, Torres et al. [10] demonstrated significant inhibition of *E. coli* and *K. pneumoniae* with geopropolis extract in *Melipona quadrifasciata quadrifasciata* and *Tetragonisca angustula*, but with more effect in Gram-positive bacteria.

This study demonstrated a higher bactericidal effect of supernatants on methicillin-resistant *S. aureus* (MRSA) strains than against sensitive strains, different from that found in Gram-negative bacteria. Several studies have demonstrated a possible bactericidal action against MRSA from by-products of stingless bees or biomolecules produced by the associated microbiota [10, 32, 39–42]. Jenkins et al. [43] found that the expression of MRSA

genes decreased virulence due to exposure to different concentrations to Manuka honey and that, although the antimicrobial effect has it found, the mode of inhibition of quorum sensing of these bacterial cells have not yet it found, indicating the need for further studies.

The research by Torres et al. [10] investigated the antibacterial action of the ethanol extracts of geopropolis (EEP) of *Melipona quadrifasciata quadrifasciata* and *Tetragonisca angustula*. Finding greater efficacy of the EEPs of *M. quadrifasciata quadrifasciata* against gram-positive strains than gram-negative, especially against Methicillin-resistant *S. aureus* and *S. aureus* compared to *T. angustula* extract, by a mechanism that involves disturbance of the integrity of the bacterial cell membrane. In the study by Nishio et al. [41], the antibacterial activity of honey produced by stingless bees *Scaptotrigona postica* and *Scaptotrigona bipunctata* against methicillin-resistant *Staphylococcus aureus* (MRSA) and sensitive *S. aureus* strains was verified. A recent study demonstrated broad inhibiting activity against MRSA strains by the supernatant of *Bacillus velezensis* isolated from stingless bees [44]. However, contrary to our study, most authors also report relevant antimicrobial action on Sensitive *S. aureus* (RRR). Since MRSA is a strain of *S. aureus* with a mutation of the antibiotic action site, that is, the penicillin-binding protein (PBP), which is now called PBP2a [45], we can suggest that this mutated protein has been the target of the antimicrobial action of supernatants, making the resistant microorganism more vulnerable.

And all identified bacteria are related to the intestinal tract of bees or some insects. *Serratia marcescens* and *Providencia rettgeri* were isolated from the intestine of bees, the former being recognized as an opportunistic pathogen [46]. Furthermore, metabolites produced by *S. marcescens*, such as serrawettins, have the capacity and inhibition of gram-positive and gram-negative bacteria that present an antimicrobial resistance profile [47]. *Enterococcus faecalis* has been described colonizing the surface of nests of *Melipona quadrifasciata* and the species *Alcaligenes faecalis* is a fecal coliform found in the species *Trigona spinipes* [48]. Secondary metabolites of *Vagococcus fluvials* have been described as inhibiting the growth of bacteria such as *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, and *Aeromonas hydrophila* [49], as well as in our study, where they inhibited the growth of multiresistant *P. aeruginosa* and KPC. Indicating that these organisms have great potential for discovering new molecules with antibiotic activity.

In this context, necessary researches seek biomolecules that act in synergy with antimicrobials used to treat infections by Gram-negative and Gram-positive pathogens. Currently, combination therapy is a growing study strand because of its potential to reduce the resistance of bacteria to antibiotics and have fewer adverse effects [50]. The resistance modulation assay showed that some supernatants had a synergistic effect against resistant bacteria, indicating the existence of molecules that act together with the antibiotic to inhibit microbial growth. Of the antibiotics tested, ampicillin had satisfactory results against *S. aureus* MRSA when combined with six supernatants, which strengthens the hypothesis of the vulnerability of this strain to the action of the antibiotic together with antimicrobial biomolecules from the supernatant, which can act on PBP2a [51]. Gram-negative bacteria were also sensitive to the joint action of antibiotics associated with supernatants, indicating the existence of molecules responsible for binding to the penicillin-binding site in the case of resistance to penicillin and ampicillin. The vancomycin resistance is positively regulated by the VanS kinase receptor that may be interacting with antimicrobial peptides and allowing vancomycin to bind to receptors [52]. Effects were not found in *Pseudomonas aeruginosa*, which can be explained by the multiple intrinsic and plasmid resistance mechanisms that this pathogen can exhibit [53, 54].

Conclusion

This research, all data obtained and the analyses we perform pave the way for further studies on the molecules produced by these microorganisms to be used as antibiotics alone or in synergy with antibiotics already established in the market. It has been shown here that larval food bacteria from stingless bees produce supernatants with bioactive molecules that have the potential to inhibit the growth of antibiotic-resistant microorganisms. The next step is the identification and characterization of molecules that have an antimicrobial effect.

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Authors' contributions

CUV, RCCD and ACCS conceived and supervised the project. ACCS and SMM performed analyses the PIC and modulation assay. VACA and NDCR performed the MALDI-TOF analyses for assembly. ACCS wrote the draft and revisions of this manuscript and all authors approved its final version.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Biological material of the ***F. varia, M. quadrifasciata, M. scutellaris*** and ***T. angustula*** was obtained in accordance with Brazilian laws. The species does not fall under the IUCN Red List categories as a threatened species.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interest related to the results reported in this study.

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