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ERICK KENJI NISHIO

**AVALIAÇÃO DA ATIVIDADE ANTIBACTERIANA DE MÉIS
DE ABELHAS SEM FERRÃO**

Londrina
2015

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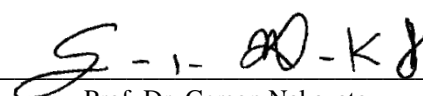
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“Nós geralmente descobrimos o que fazer percebendo aquilo que não devemos fazer. E provavelmente aquele que nunca cometeu um erro nunca fez uma descoberta”

(Samuel Smiles)

NISHIO, Erick Kenji. **Avaliação da atividade antibacteriana de méis de abelhas sem ferrão**. 2015. 94f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina. Londrina, Paraná, Brasil, 2015.

RESUMO

A apiterapia é uma forma de medicina popular e preventiva para tratamento de doenças. Apesar de diversos estudos comprovarem a atividade antimicrobiana dos méis, a ação é variável dependendo do tipo e origem do mel, além disso ainda não houveram relatos sobre o efeito da combinação entre méis. Neste estudo, a atividade antibacteriana dos méis e em combinação entre eles, produzidos por abelhas sem ferrão *Scaptotrigona bipunctata* e *Scaptotrigona postica*, foi avaliada contra cepas bacterianas Gram-positivas (*Enterococcus* spp., *Staphylococcus* spp. e *Streptococcus* spp.) e Gram-negativas (*Escherichia* spp., *Klebsiella* spp., *Pseudomonas* spp. e *Salmonella* spp.). Os ensaios para a determinação da ação foram realizados através de técnicas de poço difusão em ágar, microdiluição em caldo (tratado e não com catalase), curva de crescimento e morte, citotoxicidade e microscopia eletrônica de varredura (MEV). Os resultados mostram atividade de ambos os méis contra todas as cepas testadas, com os valores de concentração inibitória mínima (CIM) variando de 0,62 a 5% (Gram-positivas), 2,5 a 10% (Gram-negativas) e quando combinadas apresentam efeito sinérgico. Através das imagens de MEV pode-se observar diferentes formas de ação dos méis sobre as bactérias. Este estudo demonstrou que o mel possui atividade bactericida *in vitro* contra cepas Gram-positivas e Gram-negativas, incluindo cepas multirresistentes. A combinação dos méis de *S. bipunctata* e *S. postica* pode levar a um novo antimicrobiano de amplo-espectro, que poderia vir a ser utilizado como medicamento ou cosmético, com potencial de prevenir a emergência de cepas bacterianas resistentes.

Palavras-chave: Mel. Abelhas sem ferrão. Atividade antibacteriana. Sinergismo.

NISHIO, Erick Kenji. **Evaluation of antibacterial activity of honey from stingless bees**. 2015. 94 I. Dissertation (Master Science in Microbiology) – Universidade Estadual de Londrina. Londrina, Paraná, Brazil, 2015.

ABSTRACT

Apitherapy has become the attention as a form of folk and preventive medicine for treating diseases. Although several studies prove the antibacterial activity of honeys, the action is variable depending of type and origin of honey, furthermore there is no relate about the effect of honey combination. In this study, the antibacterial activity of honey isolated and in combination, produced by stingless bee *Scaptotrigona bipunctata* and *Scaptotrigona postica*, was evaluated against Gram-positive (*Enterococcus* spp., *Staphylococcus* spp. e *Streptococcus* spp.) and Gram-negative (*Escherichia* spp., *Klebsiella* spp., *Pseudomonas* spp. e *Salmonella* spp.) bacterial strains. The assays for determining the action were made through agar well difusion assay, broth microdilution (treated and not with catalase), time-kill curve, cytotoxicity and scanning electron miscroscopy (SEM). Results showed activity of both honey against all strains tested, the minimal inhibitory concentrations (MICs) values ranged from 0.62 to 5% (Gram-positive), 2.5 to 10% (Gram-negative) and when used in combination showed synergistic effect. Through SEM images could be observed different forms of action of honey on bacteria. This study demonstrated that honey has bactericidal activity *in vitro* against Gram-positive and Gram-negative strains, including multidrug-resistant strains. The combination of honey from *S. bipunctata* and *S. postica* could lead to a new broad-spectrum antimicrobials, which could be used as medicines or cosmetic, that have the potential to prevent the emergence of resistant bacterial strains.

Keywords: Honey. Stingless bee. Antibacterial activity. Synergism.

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LISTA DE ABREVIATURAS E SIGLAS

%	Porcentagem
a.C.	Antes de Cristo
Ag	Prata
ATCC	American Type Culture Colection
Au	Ouro
AWD	Agar Well Diffusion
AZ	Arizona
Ba	Bário
BHI	Brain Heart Infusion
Bi	Bismuto
Br	Bromo
Ca	Cálcio
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CC ₅₀	50% cytotoxicity concentration
CFU	Colony Forming Unit
CIP	Ciprofloxacín
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
Co	Cobalto
CO ₂	Dióxido de carbono
Cu	Cobre
DA	Clindamycin
Dr	Doutor
E	Erythromycin
ESBL	Bacilos Gram-negativos Produtores de Beta-lactamase de Espectro Estendido
Fe	Ferro
FIC	Fractioned Inhibitory Concentration
g	Gramma
Ga	Gálio
Ge	Germânio

h	horas
HEP2	Human Epidermoid Carcinoma strain 2
HMF	Hidroximetilfurfural
HU	Hospital Universitário
K	Potássio
kg	Quilograma
Li	Lítio
LZD	Linezolid
M	Molar
mEq	Miliequivalente
mg	Miligrama
Mg	Magnésio
MGO	Metilglioxal
MH	Mueller-Hinton
MIC	Minimal Inhibitory Concentration
MIC ₁₀₀	100% inhibitory concentration
min	Minuto
ml	Mililitros
mm	Milímetros
MEV	Microscopia Eletrônica de Varredura
Mn	Manganês
Mo	Molibdênio
MRSA	<i>Staphylococcus aureus</i> meticilina-resistente
MSSA	<i>Staphylococcus aureus</i> meticilina-sensível
Na	Sódio
NCCLS	National Committee for Clinical Laboratory Standards
Ni	Níquel
nº	Número
°C	Graus Celsius
°CL	Graus Clark
Os	Ósmio
OX	Oxacillin
P	Penicillin

Pb	Chumbo
PBS	Phosphate-Buffered Saline
PR	Paraná
PROAP	Programa de Apoio à Pós-graduação
RD	Rifampicin
RJ	Rio de Janeiro
ROS	Espécies Reativas de Oxigênio
RPMI	Roswell Park Memorial Institute
SB	Mel proveniente da abelha <i>Scaptotrigona bipunctata</i>
SE	Standard Errors
SEM	Scanning Electron Microscopy
Sn	Estanho
SP	Mel proveniente da abelha <i>Scaptotrigona postica</i>
SP	São Paulo
Sr	Estrôncio
SXT	Trimethoprim-sulfamethoxazole
TET	Tetracycline
TGC	Tigecycline
Ti	Titânio
TNF- α	Fator de Necrose Tumoral Alfa
UEL	Universidade Estadual de Londrina
UI	Atividade de invertase imobilizada - grama de açúcar redutor produzido por litro, por minuto, por grama de suporte
USA	United States of America
V	Vanádio
v	Volume
VRE	<i>Enterococcus vancomicina-resistente</i>
w	Weight
Zn	Zinco
α	Alfa
β	Beta
μ l	Microlitros
μ m	Micrômetro

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1. INTRODUÇÃO

Desde a descoberta e aplicação clínica dos primeiros antibióticos na década de 40 e 50, estes são a forma de terapia mais eficaz no combate as doenças infecciosas até os dias atuais. Apesar do sucesso, o uso contínuo dos antimicrobianos pode representar uma ameaça devido a dois desafios: desenvolvimento de novos antimicrobianos e combate a resistência bacteriana.

Após a descoberta da penicilina iniciou a "era de ouro" da desenvolvimento de novos antimicrobianos, porém após 1985 ocorreu uma queda na descoberta de novos antimicrobianos, devido aos poucos progressos no desenvolvimento e síntese a partir de compostos já conhecidos, desenho de estruturas e triagem de produtos naturais.

Pouco tempo após a aplicação da penicilina houveram relatos de cepas resistentes, constatando que tal resistência poderia ser natural de uma espécie ou adquirida por cepas individuais em uma população sensível.

Cada vez mais espécies bacterianas estão desenvolvendo resistência aos diversos antimicrobianos. Este fato se deve ao uso indiscriminado de antibióticos tanto no ambiente hospitalar, na comunidade, indústrias alimentícias e veterinárias. Tais antimicrobianos exercem uma pressão seletiva selecionando cepas multirresistentes. Ao mesmo tempo e de forma inversamente proporcional ao desenvolvimento de novos antimicrobianos, surgem novas cepas multirresistentes.

Dentre os microrganismos multirresistentes podemos citar *Staphylococcus aureus* metilicina-resistente (MRSA), *Enterococcus vancomicina-resistente* (VRE) e bacilos Gram-negativos produtores de beta-lactamase de espectro estendido (ESBL).

O número de óbitos causado por bactérias multirresistentes, como MRSA, vem aumentando nos últimos anos. Muitas cepas já apresentam resistência aos antimicrobianos de última geração disponíveis no mercado. Somado a isso, outros fatores como a baixa disponibilidade de antimicrobianos têm dificultado a terapia dessas infecções bacterianas.

Alguns antimicrobianos convencionais também têm apresentado efeitos indesejáveis aos hospedeiros, aumentando ainda mais a importância pela

busca de novos antibacterianos com baixa citotoxicidade ao hospedeiro. Por isso, produtos naturais como o mel vem sendo utilizados principalmente na prevenção de infecções.

Frente a estes problemas, a busca de novos compostos antimicrobianos de diferentes produtos naturais é de grande importância, tais como o mel, servindo como alternativa à antibioticoterapia convencional.

O objetivo do trabalho foi avaliar a atividade antibacteriana dos méis de abelhas indígenas sem ferrão *Scaptotrigona bipunctata* e *Scaptotrigona postica* em bactérias Gram-positivas e Gram-negativas.

2. REVISÃO BIBLIOGRÁFICA

2.1. Definição, origem e classificação do mel

O Ministério da Agricultura, por meio do Decreto-Lei nº. 214/2003 de 18 de setembro classifica o mel como uma solução açucarada natural produzida por abelhas a partir de compostos de plantas, recolhidos, transformados, depositados, desidratados e armazenados em favos das colmeias (BRASIL, 2000).

Pelo mesmo decreto, o mel pode ser classificado quanto à sua origem e modo de produção. No quesito origem é dividido em: mel de néctar (flores), quando obtido a partir do néctar das flores; e mel de melada, quando obtido principalmente a partir de excreções de insetos sugadores de plantas. No quesito modo de produção, o mel é dividido em: mel em favos, quando armazenado pelas abelhas nos alvéolos de favos construídos pelas próprias abelhas; mel com pedaços de favos, quando contém pedaços de favos; mel escorrido, quando obtido por escorrimento de favos; mel prensado, quando obtido por compressão de favos; e mel filtrado, quando é obtido por um processo de eliminação de matérias orgânicas ou inorgânicas (BRASIL, 2000).

2.2. Composição do mel

O mel é uma solução supersaturada de açúcares, sendo os principais constituintes a frutose (38%) e glicose (31%) (Tabela 1). Pode conter também outros constituintes, incluindo flavonoides, ácidos fenólicos, enzimas (glicose oxidase, catalase), ácido ascórbico, produtos da reação de Maillard (desidratação de açúcares), substâncias semelhantes a carotenoides, outros ácidos orgânicos, aminoácidos e outras proteínas (GHELDOLF; WANG; ENGESETH, 2002; GÓMEZ-CARAVACA et al., 2006). A composição do mel é variável, dependendo de inúmeros fatores como origem floral, condições ambientais e os tratamentos posteriores (AZEREDO et al., 2003; VIUDA - MARTOS et al., 2008).

Tabela 1- Principais constituintes dos méis em geral.

Constituintes	Quantidade (%)
Presentes em maior quantidade	
Frutose	38
Glicose	31
Sacarose	1
Água	17
Não identificado	11,5
Presentes em menor quantidade	
Peptídeos antimicrobianos	Variável
Flavonoides	Variável
Carotenoides	Variável
Ácidos orgânicos	Variável
Aminoácidos	Variável
Vitaminas	Variável

Fonte: Maddocks e Jenkins (2013).

2.2.1. Composição físico-química

O Ministério da Agricultura estabelece os critérios de composição para o mel quando comercializado para o consumo humano. Este deve obedecer aos seguintes critérios: teor de açúcares (> 60%), sacarose (< 6%), umidade (< 20%), sólidos insolúveis (< 0,1%), ácidos livres (< 50 mEq/kg), atividade diastásica (>8 Göthe) e hidroximetilfurfural - HMF (< 60 mg/kg). A cor pode variar de incolor a pardo-escuro, a consistência pode apresentar-se fluida, espessa ou cristalizada. O sabor e aroma variam de acordo com a origem botânica (BRASIL, 2000).

O mel é rico em açúcares sendo encontrados: glicose, frutose, sacarose, maltose, isomaltotetraose, maltulose, isomaltulose, nigerose, turanose, cojibiose, neotrealose, gentiobiose, laminaribiose, leucrose, melesitose, rafinose, isopanose, isomaltetraose, arabogalactomanose, erlose, dectrantiose, maltotriose, isomaltopentose, centose, 1-cestose, panose, isomaltotriose e 3- α -isomaltosilglicose.

Os açúcares redutores são as frações dominantes, os quais apresentam capacidade de reduzir alguns íons metálicos em solução alcalina. A glicose apresenta pouca solubilidade tendendo à cristalização, já a frutose que apresenta alta higroscopicidade possibilita a doçura (DE CARVALHO et al., 2005).

O teor de sacarose está relacionado ao tempo de colheita do mel. Quando o mel é colhido prematuramente, a sacarose do néctar ainda não foi totalmente convertida em glicose e frutose pela ação da enzima invertase. Além disso, a sacarose também pode ser utilizada como controle de qualidade para detecção de adulteração do mel, devido a adição de sacarose diretamente no mel ou superalimentação de abelhas com solução açucarada. Porém néctares de origens florais distintas apresentam diferenças em sua composição química de carboidratos (CRANE, 1975; PUSCAS; HOSU; CIMPOIU, 2013).

O teor de umidade é um critério de qualidade que determina a capacidade do mel permanecer estável e livre de fermentação (CERESER CAMARA; LAUX, 2010). O elevado teor de umidade do mel pode facilitar o processo de fermentação, além de dificultar o armazenamento, tornando-se importante diferentes estratégias, tais como pasteurização e refrigeração para uma melhor conservação (BOGDANOV et al., 1999; LIBERATO et al., 2013).

A acidez livre pode ser explicada pela presença de ácidos orgânicos, ésteres e alguns íons orgânicos. Uma alta acidez pode ser indicativo de fermentação de açúcares em ácidos orgânicos (GOMES et al., 2010).

O HMF é um composto furânico, formado como um produto intermediário da reação de Maillard, pela desidratação de açúcares em condições acídicas ou durante tratamentos térmicos e devido à longos períodos de estocagem, dependendo também do tipo de açúcar, pH, atividade da água e concentração de cátions divalentes no meio (BOGDANOV et al., 1999; RIZELIO et al., 2012)

O mel contém uma pequena quantidade de enzimas, sendo as mais importantes, a diastase (α e β -amilase), invertase (α -glicosidase), glicose oxidase, catalase e fosfatase ácida. Tais enzimas são provenientes de fontes nectaríferas, fluidos salivares e secreções da glândula faríngea das abelhas (PIAZZA; PULCINI, 1999). A atividade das enzimas decresce após um longo tempo de estocagem e tratamentos térmicos (BOGDANOV; RUOFF; PERSANO ODDO, 2004).

O teor de cinzas expressa os minerais presentes, que podem variar dependendo da abelha, apicultor, clima, solo e origem botânica. Diversos elementos químicos já foram identificados como: K, Na, Ca, Mg, Mn, Ti, Co, Mo, Fe, Cu, Li, Ni, Pb, Sn, Zn, Os, Ba, Ga, Bi, Ag, Au, Ge, Sr, Br, e V. Esses elementos são interessantes, pois são encontrados na forma assimilável (DE CARVALHO et al., 2005).

A cor do mel depende de diversos fatores como origem floral, processamento, armazenamento, condições climáticas e temperatura. Além disso, a quantidade de minerais também interfere na coloração, ou seja, quanto menor o teor de cinzas mais claro é o mel (AL et al., 2009).

2.3. Abelhas indígenas sem ferrão

Atualmente existe cerca de 20.000 espécies de abelhas distribuídas mundialmente, com diversos comportamentos, tamanhos, formas e habitats. A maioria apresenta hábitos solitários, e alguns possuem uma organização social (PRONI; MACIEIRA, 2002).

Dentre as espécies de abelhas mais conhecidas, destacamos *Apis mellifera* (Linnaeus), introduzida nas Américas pelos conquistadores e missionários espanhóis e portugueses, durante o período colonial. *A. mellifera* se propagou por todo o país rapidamente habitando todos os ambientes em qualquer estado. A presença desta espécie exótica sempre causou muita discussão sobre os possíveis impactos que causariam sobre as espécies nativas, estas últimas conhecidas como abelhas indígenas sem ferrão (MINUSSI; ALVES-DOS-SANTOS, 2007).

As abelhas indígenas sem ferrão encontram-se distribuídas nas regiões tropicais e clima temperado subtropical. Uma das principais características dessas abelhas é o fato delas apresentarem um ferrão atrofiado, o que explica a denominação “sem ferrão” (PRONI; MACIEIRA, 2002).

De acordo com a classificação estabelecida por Silveira e colaboradores (2002), as abelhas indígenas sem ferrão pertencem à superfamília Apoidea, família Apidae, tribo Apini e sub-tribo Meliponina que contém mais de 400 espécies já reconhecidas. Essas abelhas também são conhecidas por Meliponíneos,

portanto a criação dessas abelhas é conhecida como Meliponicultura (MOLAN, 1992; TEMARU et al., 2007).

As abelhas sem ferrão são encontradas em regiões tropicais e subtropicais, ocorrendo nas Américas do Sul e Central, África, Sudoeste da Ásia e Austrália. No Brasil encontram-se distribuídas por todo o país, porém as espécies diferem em cada região (CORTOPASSI-LAURINO et al., 2006; RASMUSSEN; CAMERON, 2010).

Os ninhos são geralmente construídos em cavidades de árvores mas podem nidificar em fendas de rochas e buracos no chão ou locais abertos como galhos de árvores. A parte interna do ninho é constituído geralmente de uma mistura de cera e própolis conhecido como cerume, em forma de potes, onde é guardado o mel. O barro é bastante utilizado para construção da entrada, e o geoprópolis (mistura de própolis com barro) é usado para vedar frestas (VILLAS-BÔAS, 2012).

No Brasil, diversas espécies de abelhas indígenas sem ferrão estão ameaçadas de extinção devido às alterações no habitat por desmatamentos, queimadas, agrotóxicos, urbanização entre outros. Essas abelhas são ótimas polinizadoras, tanto que já foi verificado que a extinção de uma espécie de abelha sem ferrão implica na extinção de espécies vegetais (PRONI; MACIEIRA, 2002). Existem grupos de abelhas que estão presentes essencialmente em áreas conservadas como florestas primárias, servindo como bioindicadores da qualidade ambiental (SILVA; PAZ, 2012).

A importância dessas abelhas na preservação ambiental é nítida. São responsáveis por cerca de 30% da polinização de espécies da Caatinga, Pantanal, e até 90% das espécies da Mata Atlântica (DE MELLO PEREIRA; DE ALMEIDA SOUZA; DO RÊGO LOPES, 2010). Portanto, sua importância está além dos aspectos sociais e econômicos pois interfere diretamente nos processos ecológicos. São necessárias medidas de conscientização da população e conservação dessas abelhas, o que acaba refletindo diretamente na conservação da flora e fauna (SILVA; PAZ, 2012).

2.3.1. Mel de abelhas indígenas sem ferrão

O relato sobre o consumo de mel de abelhas indígenas data desde os períodos pré-colombianos no continente Americano (DE CARVALHO et al., 2005). Este mel possui muitos apreciadores, até mais que o mel de *Apis* spp., em algumas regiões do planeta (NOGUEIRA-NETO, 1997).

A produtividade pode variar muito dependendo da espécie, pasto apícola e manejo. As abelhas sem ferrão produzem entre 1 a 10 kg de mel ao ano, dependendo da espécie e da região. Este valor é menor em comparação à produção de mel por abelhas *A. mellifera* (15 kg de mel ao ano), porém como o número de abelhas indígenas sem ferrão por ninho é menor que o das abelhas africanizadas, portanto a mesma quantidade de ninhos em uma mesma área não leva ao esgotamento do pátio apícola. Devido ao valor do mel dessas abelhas ser maior, menor custo de produção e manejo mais simples, tais características tornam a meliponicultura uma atividade vantajosa (DE MELLO PEREIRA; DE ALMEIDA SOUZA; DO RÊGO LOPES, 2010; VENTURIERI, 2004). Frente às vantagens apresentadas, a meliponicultura tem se mostrado como uma atividade de grande importância agropecuária, sendo uma fonte de renda alternativa, além de contribuir para a conservação dessas abelhas (BRITO et al., 2013).

De acordo com De Carvalho e colaboradores (2005), no Brasil os principais gêneros de abelhas indígenas sem ferrão potencialmente produtivas são: *Melipona*, *Scaptotrigona* e *Trigona*. É importante que sejam feitas análises mais cuidadosas quanto às propriedades físico-químicas dos méis desses três grupos, permitindo a valorização de determinados parâmetros na caracterização do mel.

Como as abelhas sem ferrão são dependentes do ambiente onde vivem, ou seja, possuem uma íntima relação com os recursos florais característicos de cada região e climas específicos, a escolha da espécie para criação deve ser de uma espécie naturalmente existente na região de implantação da meliponicultura (VILLAS-BÔAS, 2012).

2.3.2. Composição físico-química do mel de abelhas indígenas sem ferrão

Souza e colaboradores (2006) compilaram dados de estudos sobre a composição de 152 amostras de méis de abelhas indígenas sem ferrão. Em seus estudos encontraram os seguintes parâmetros: umidade (19,9 a 41,9%), pH (3,15 a 4,66), acidez livre (5,9 a 109,0 mEq/kg), cinzas (0,01 a 1,18%), atividade diastásica (0,9 a 23,0 Göthe), HMF (0,4 a 78,4 mg/kg), atividade da invertase (19,8 a 90,1 UI), nitrogênio (14,34 a 144 mg/100g), açúcares redutores (58,0 a 75,7%) e sacarose (1,1 a 4,8%).

As características físico-químicas acima mencionadas são utilizadas como parâmetro de qualidade (BRASIL, 2000). Geralmente, o mel de abelhas indígenas sem ferrão apresenta valores médios dos parâmetros físico-químicos dentro dos pré-requisitos estabelecidos pela legislação brasileira e internacional, com exceção do teor de umidade (Tabela 2) (ALFREDO LOPES DE CARVALHO, 2009). A umidade determina a capacidade do mel permanecer estável e livre de fermentação (LIBERATO et al., 2013). O teor de umidade acima de 20% pode facilitar o processo de fermentação, além de dificultar o armazenamento, tornando-se importante para diferentes estratégias, tais como pasteurização e arrefecimento para uma melhor conservação (DRUMMOND, 2013).

Tabela 2 - Comparação da composição físico-química entre méis de abelhas européia (comercial) e indígena.

Composição	Quantidade	
	Abelha européia	Abelha indígena
Teor de açúcares	> 60%	58 - 75,7%
Sacarose	< 6%	1,1 - 4,8%
Umidade	< 20%	19,9 - 41,9%
Ácidos livres	< 50 mEq/kg	5,9 - 109 mEq/kg
Atividade diastásica	>8 Göthe	0,9 - 23 Göthe
HMF	< 60 mg/kg	0,4 - 78,4 mg/kg
Cinzas	< 0,6%	0,01 - 1,18%

2.4. Propriedades medicinais do mel

O mel tem sido utilizado como alimento desde os tempos mais antigos, e considerado como parte da medicina tradicional. A primeira referência escrita sobre o mel data 2000 anos a.C. na Suméria, mencionando o uso do mel como remédio e pomada. A crença de que o mel é um nutriente, remédio e pomada tem continuado até os dias atuais. A apiterapia tornou-se uma forma de medicina preventiva e popular para o tratamento de doenças e promoção da saúde global (Tabela 3) (ALVAREZ-SUAREZ et al., 2010; GÓMEZ-CARAVACA et al., 2006; MOLAN, 1992).

A presença de radicais livres e outros agentes oxidantes são de grande importância no mecanismo de ação de várias toxinas. Os radicais livres induzem danos em biomoléculas como carboidratos, lipídios, proteínas e ácidos nucleicos provocando alterações celulares e causando até morte celular (VIUDA-MARTOS et al., 2008).

O mel e outros produtos de abelhas são usados como alimentos funcionais devido ao seu alto potencial antioxidante natural. Dentre as substâncias antioxidantes podemos citar aminoácidos e proteínas, carotenos, ácido ascórbico, ácidos orgânicos, produtos da reação de Maillard, porém os principais são os compostos fenólicos e flavonoides (VIUDA-MARTOS et al., 2008).

Tabela 3 - Principais propriedades terapêuticas e benefícios de méis.

Propriedades	Fatores responsáveis
Antioxidante	Ácido fenólicos e flavonoides
Anti-inflamatória	Flavonoides
Antimicrobiana	Alta osmolaridade, acidez, peróxido de hidrogênio e outros componentes

Fonte: Christy e Anna (2011).

Compostos fenólicos como tocoferóis, flavonoides e ácidos fenólicos são potentes antioxidantes contra radicais peroxil. A ação dos compostos fenólicos está relacionada com a capacidade de reduzir e quelar íons férricos, os quais catalisam a peroxidação lipídica. O plasma sanguíneo é protegido pelo mel devido ao fato de seus componentes antioxidantes serem hidrossolúveis (FERREIRA et al., 2009).

A inflamação de tecidos podem ser resultado de infecção de uma ferida ou devido às queimaduras, envolvendo um processo de extravasamento de plasma do sangue e formando edemas. A ação anti-inflamatória do mel foi observada pela análise microscópica de tecidos lesados que mostram uma diminuição no número de glóbulos brancos recrutados, além da remoção de bactérias que desencadeiam o processo de inflamação (MOLAN, 1999; SUBRAHMANYAM et al., 2003).

Sabe-se que durante um processo de inflamação ocorre liberação de TNF- α e recrutamento de neutrófilos para a defesa do organismo. Porém os neutrófilos produzem e liberam espécies reativas de oxigênio (ROS) que acabam causando lesões no tecido. Kassim e colaboradores (2010) observaram que extratos de méis têm capacidade de sequestrar radicais livres, conseqüentemente diminui a citotoxicidade do processo inflamatório induzido pelo TNF- α .

Outros autores apontam a capacidade dos flavonoides, presentes em méis, inibirem o desenvolvimento da inflamação provocada por agentes como a enzima ciclo-oxigenase 2 que catalisa a transformação do ácido araquidônico em prostaglandina (VIUDA-MARTOS et al., 2008).

No estudo de Leong e colaboradores (2012), que trabalharam com mel de abelha indígena de Nova Zelândia, mostrou uma grande capacidade destes méis na supressão da produção de superóxidos pelos neutrófilos além de supressão de edemas e infiltração de leucócitos sugerindo um possível uso tópico do mel para tratamento de feridas.

2.4.1. Ação antimicrobiana

Existem diversos trabalhos avaliando a atividade antibacteriana, antifúngica e antiviral de diferentes méis. Apesar de tantos trabalhos, poucos levam em conta a grande variação da atividade antimicrobiana de diferentes méis (AGGAD; GUEMOUR, 2014; AL-WAILI et al., 2013; MOLAN, 2001).

O primeiro relato sobre a atividade antibacteriana do mel de *Apis* spp. data 1892 (BOGDANOV, 1997a). De acordo com Nogueira-Neto (1997) o primeiro relato sobre a atividade antimicrobiana do mel de abelhas indígenas sem ferrão foi feito no ano de 1964. Desde então intensos estudos vêm sendo realizados na tentativa de elucidarem as propriedades antibacterianas, inclusive de abelhas indígenas sem ferrão (Tabela 4).

Alguns pesquisadores acreditam que o peróxido de hidrogênio produzido pela enzima glicose oxidase presente no mel, seja o principal agente antibacteriano (BOGDANOV, 1997b; DUSTMANN, 1979; MADDOCKS; JENKINS, 2013; WHITE JR; SUBERS; SCHEPARTZ, 1963).

Um dos primeiros estudos para avaliar a atividade antibacteriana do mel foi feito por Dold e colaboradores (1937), nesse estudo testaram o mel frente à bactérias e fungos constatando uma atividade inibitória principalmente contra bactérias. Além disso, em seu trabalho os autores compararam o mel puro, com mel processado, ou seja, mel passado por diferentes tratamentos. Observaram que a atividade antibacteriana do mel é termolábil, apresenta certa fotossensibilidade e pode ser influenciada pela filtração. Este comportamento foi semelhante ao encontrado em outras substâncias de origem natural e foi nomeada substância responsável pela atividade antibacteriana como “inibina”.

Em 1941, Gauhe demonstrou que na glândula faríngea das abelhas estava presente a enzima glicose-oxidase, produtora de ácido glucônico e peróxido de hidrogênio (BIENENARBEITERIN; HALBERSTADT; FRAGESTELLUNG, 1966). Em 1963 os pesquisadores White Jr e colaboradores relataram a presença de peróxido de hidrogênio no mel, e quando este era tratado com catalase permitia o crescimento bacteriano, comprovando então a atividade antibacteriana do peróxido de hidrogênio (inibina) presente no mel.

Tabela 4 - Lista dos estudos sobre atividade antibacteriana de méis de abelhas indígenas, indicando a espécie de abelha, faixa de Concentração Inibitória Mínima e bactérias utilizadas.

Abelha/Mel	CIM (%)	Bactérias	Referência
<i>Melipona quadrifasciata</i> , <i>Melipona scutellaris</i> , <i>Melipona subnitida</i> , <i>Pleibeia</i> sp., <i>Tetragonisca angustula</i> , <i>Tetragona clavipes</i>	14,00 - 52,00	<i>Bacillus subtilis</i> , <i>Bacillus stearothermophilus</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i>	Cortopassi-Laurino e Gelli (1991)
<i>Trigona</i> spp.	1,00 - 20,00	<i>B. subtilis</i> , <i>Bacillus megaterium</i> , <i>Bacillus brevis</i> , <i>E. coli</i> , <i>Micrococcus luteus</i> , <i>Pseudomonas syringae</i>	Garedew et al. (2003)
<i>Tetragonisca angustula</i>	12,28 - 21,43	<i>S. aureus</i>	Miorin et al. (2003)
<i>T. angustula</i>	*	<i>B. cereus</i> , <i>P. aeruginosa</i>	DeMera e Angert (2004)
<i>Nannotrigona testaceicornis</i>	*	<i>Enterobacter aerogenes</i> , <i>Enterobacter cloacae</i> , <i>E. coli</i> , <i>Proteus mirabilis</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> , <i>Streptococcus pyogenes</i>	Gonçalves et al. (2005)
<i>Friesiometita nigra</i> , <i>Melipona beecheii</i> , <i>Melipona solani</i> , <i>M. quadrifasciata</i> , <i>Melipona beecheii</i> , <i>Scaptotrigona bupunctata</i> , <i>Scaptotrigona mexicana</i> , <i>Scaptotrigona pectoralis</i> , <i>Trigona australis</i> , <i>Trigona biroi</i>	*	<i>E. coli</i> , <i>Enterococcus faecalis</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	Temaru et al. (2007)
<i>Trigona carbonaria</i>	17,50 - 32,10	<i>S. aureus</i>	Irish (2008)
<i>T. carbonaria</i>	4,00 - 16,00	<i>Acinetobacter baumannii</i> , <i>B. cereus</i> , <i>B. subtilis</i> , <i>Citrobacter freundii</i> , <i>E. cloacae</i> , <i>E. faecalis</i> , <i>E. coli</i> , <i>Khocuria rhizophila</i> , <i>K. pneumoniae</i> , <i>Listeria monocytogenes</i> , <i>S. aureus</i> , <i>Staphylococcus epidermidis</i> , <i>Staphylococcus xylosum</i> , <i>P. aeruginosa</i> , <i>Salmonella Typhimurium</i> , <i>Serratia marcescens</i> , <i>Shigella sonnei</i> , <i>Yersinia enterocolitica</i>	Boorn et al. (2010)

Meliponinae	0,001 - 0,1	<i>E. coli</i> , <i>E. faecalis</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	Guerrini et al. (2009)
<i>M. beecheii</i>	4,00 - 16,00	<i>E. coli</i> , <i>E. faecalis</i> , <i>S. aureus</i>	Chan-Rodríguez et al. (2012)
<i>Apis mellipodae</i>	6,25 - 12,50	<i>E. coli</i> , <i>Salmonella</i> sp., <i>Salmonella typhi</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>Streptococcus pneumoniae</i> , <i>Shigella flexneri</i> , <i>Shigella dysenteriae</i> , <i>Proteus vulgaris</i>	Andualet (2013a)
<i>Trigona iridipennis</i>	6,25 - 12,50	<i>E. coli</i> , <i>Salmonella</i> sp., <i>S. typhi</i> , <i>L. monocytogenes</i> , <i>S. aureus</i> , <i>S. pneumoniae</i> , <i>S. flexneri</i> , <i>S. dysenteriae</i> , <i>P. vulgaris</i>	Andualet (2013b)
<i>Melipona bicolor</i> , <i>Melipona marginata</i> , <i>M. quadrifasciata</i> , <i>Melipona rufiventris</i> , <i>S. bipunctata</i> , <i>Scaptotrigona xantotricha</i> , <i>Tetragona clavipes</i> , <i>T. angustula</i>	1,56 - 50,00	<i>E. coli</i> , <i>S. aureus</i>	Borsato et al. (2013)
<i>Friseomelita doederleinei</i> , <i>Melipona asilvai</i> , <i>M. quadrifasciata anthidioides</i> , <i>Plebeia</i> sp., <i>T. angustula</i>	1,90 - 25,40	<i>E. coli</i> , <i>S. aureus</i>	Peraltai et al. (2013)
<i>Melipona compressipes manaosensis</i> , <i>M. subnitida</i>	11,10 - 50,00	<i>E. coli</i> , <i>Klebsiella</i> sp., <i>P. vulgaris</i> , <i>Salmonella paratyphi</i> , <i>S. sonnei</i> , <i>S. aureus</i>	Pimentel et al. (2013)
<i>Trigona</i> spp.	5,00 - 50,00	<i>B. cereus</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>S. aureus</i>	Zainol et al. (2013)
<i>S. bipunctata</i> , <i>Scaptotrigona postica</i>	0,62 - 2,50	<i>S. aureus</i>	Nishio et al. (2014)

Banco de dados: Hal Archives-ouverts, CNKI, IDOSI, Pubmed, Scielo, Science Direct, Levantamento no período de 2013 a 2015.

*Sem dados de CIM, os autores utilizaram outro método para determinação da atividade antibacteriana dos méis.

CIM: Concentração inibitória mínima

A atividade bactericida do peróxido de hidrogênio está relacionada com a acumulação de danos oxidativos irreversíveis causados na membrana, proteínas, enzimas e DNA (BIZERRA et al., 2012).

Outros pesquisadores observaram atividade antibacteriana em ausência de peróxido de hidrogênio e creditam o efeito osmótico causado pelos açúcares presentes no mel como o principal responsável por esta atividade antibacteriana (WHITE JR; SUBERS; SCHEPARTZ, 1963). Outros estudos descreveram que o baixo pH, junto à alta osmolaridade do mel são responsáveis pela atividade antibacteriana (BOGDANOV, 1997b). Em contrapartida, alguns autores elaboraram um mel artificial, constituído pelos principais açúcares, para simular a alta osmolaridade. Porém essa solução não apresentou atividade antibacteriana, indicando que somente esta propriedade não é responsável pela atividade (CHEN et al., 2012; COOPER; MOLAN; HARDING, 2002; NISHIO et al., 2014).

No estudo de Bogdanov (1997b) foi constatado que mesmo modificando os tipos de açúcares presentes no mel não houve grande diferença na atividade antibacteriana. Nishio e colaboradores (2014) separaram os açúcares dos méis de *Scaptotrigona bipunctata* e *Scaptotrigona postica*, por meio de sedimentação dos açúcares totais com álcool, e avaliaram a atividade antibacteriana, constatando que somente os açúcares não foram capazes de inibir o crescimento bacteriano.

Outros autores destacam a presença de compostos fenólicos como flavonoides (BOGDANOV, 1989; CUSHNIE; LAMB, 2005). Estes compostos não só apresentam atividade antibacteriana, mas também antifúngica e antiviral (GÓMEZ-CARAVACA et al., 2006). Os principais flavonoides encontrados em méis são: *apinegin*, *genistein*, *pinocembrin*, *tricetin*, *chrysin*, *luteolin*, *quercetin*, *kaempferol*, *galangin*, *pinobanksin* e *myricetin* (ALVAREZ-SUAREZ et al., 2010).

Foi comprovada a presença de altas quantidades de metilglioxal (MGO) em méis provenientes de Manuka (ADAMS et al., 2008; MAVRIC et al., 2008). O MGO é um componente antimicrobiano formado pela conversão da di-hidroxiacetona, proveniente do néctar, de forma não enzimática por reação com aminoácidos como arginina ou lisina (ADAMS;

MANLEY-HARRIS; MOLAN, 2009). Já foi descrito a ação antibacteriana do MGO contra bactérias multirresistentes como *S. aureus* metilina-resistente e *Pseudomonas aeruginosa* (HAYASHI et al., 2014; JENKINS; BURTON; COOPER, 2011). Pesquisadores acreditam que o MGO é o principal agente antimicrobiano do mel na ausência do peróxido de hidrogênio, já que estudos demonstram perda da atividade de méis quando este componente é neutralizado (KWAKMAN; ZAAT, 2012).

Recentemente foi identificada a presença do peptídeo antimicrobiano *Bee-defensin-1* no mel. Este peptídeo já havia sido identificado na hemolinfa, glândulas da cabeça e tórax, na comida e larvas de abelha rainha. O peptídeo *Bee-defensin-1* é secretado pela glândula hipofaríngea da abelha e essas secreções são utilizadas na produção do mel (KWAKMAN; ZAAT, 2012). Esse peptídeo apresenta atividade contra bactérias Gram-positivas como, *B. subtilis* e *S. aureus* (KWAKMAN et al., 2010). Sabe-se que esses peptídeos podem agir formando poros na membrana celular, reagir com o material genético e também interferir na síntese da parede celular (JENSSEN; HAMILL; HANCOCK, 2006).

Cortopassi-Laurino e Gelli (1991) mostraram maior efeito antibacteriano dos méis de abelhas indígenas comparado ao mel de *A. mellifera*. Estudos de DeMera e Angert (2004) confirmam que o mel das abelhas indígenas é mais ativo do que mel de abelhas *Apis* spp.. Ainda nesse mesmo estudo, descobriram que méis de diferentes origens entomológicas e mesma região fitogeográfica não diferem em sua atividade antimicrobiana. No entanto, a atividade antimicrobiana varia em méis de diferentes regiões fitogeográficas e origem floral (LEE; CHUREY; WOROBO, 2008).

Todos estes estudos comprovam o potencial do mel para prevenção e tratamento de infecções bacterianas. Embora seja evidente a capacidade antibacteriana do mel, o mecanismo de ação não está totalmente esclarecido (AGGAD; GUEMOUR, 2014). Alguns autores sugerem a existência de um possível efeito sinérgico entre os componentes, contribuindo para a atividade antibacteriana (VIUDA - MARTOS et al., 2008).

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4. RESULTADOS E DISCUSSÃO

Os resultados e discussão, juntamente com a metodologia referente a cada trabalho, da presente dissertação foi apresentado na forma de artigos (Artigo I e Artigo II) cada qual seguindo as normas de formatação da revista para qual foram enviados.

ARTIGO I

**Antistaphylococcal effect of honey from stingless bees
Scaptotrigona bipunctata Lepeletier, 1836 and *Scaptotrigona
postica* Latreille, 1807 (Hymenoptera: Apidae: Meliponinae)**

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ABSTRACT

The antistaphylococcal effect of honey produced by the stingless bees *Scaptotrigona postica* (SB) and *Scaptotrigona bipunctata* (SP) was evaluated against reference strains and clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and reference strains of methicillin-sensitive *S. aureus* (MSSA) strains. Evaluation of the antibacterial activity of honey samples was determined by agar well diffusion assay, calculation of minimum inhibitory concentrations (MICs) and construction of time-kill curves. The inhibition zones generated by the honey samples ranged from 20 ± 0.21 to 27 ± 0.46 mm (SB) and 20 ± 0.46 to 24 ± 0.51 mm (SP), while MIC values ranged from 0.62 ± 0.11 to $1.25\pm 0.27\%$ (SB) and 1.25 ± 0.27 to $2.50\pm 0.27\%$ (SP). According to the time-kill curves, all MRSA and MSSA strains were completely eliminated. Honey cytotoxicity was evaluated and the CC_{50} values were found to be 13% (SB) and 7.3% (SP), which were significantly higher when compared to the MIC values. Both honey SB and SP can be utilized as antistaphylococcal substances against MSSA and MRSA strains and this effect is probably due to a synergistic action of different components such as hydrogen peroxide, sugars and other compounds not identified in this study. The application of natural products is important for the control of MRSA infections.

Keywords: *Scaptotrigona postica*, *Scaptotrigona bipunctata*, *Staphylococcus aureus*, minimum inhibitory concentration, antibacterial activity, honey.

1. INTRODUCTION

The introduction of antibiotics in the early 1930s was thought to herald the end of infectious diseases. However, not long after this, *Staphylococcus aureus* began to develop resistance to antimicrobial agents, such penicillin in the 1944 and finally the first strains of methicillin-resistant *S. aureus* (MRSA) emerged (Jevons, 1961; Ippolito *et al.* 2010).

Since the first report of MRSA, it became an important pathogen worldwide and represents a serious public health problem. MRSA strains have occurred in diverse geographic regions due to its capacity to widely disseminate (Harris *et al.* 2010; Lowy, 2013

A large number of infections are caused by multiresistant bacteria including MRSA which has become one of the most studied (Lucet *et al.* 2010). Multiple and costly infection control interventions are often used to reduce MRSA transmission, supported by patient, staff, and environmental screening programs (Harris *et al.* 2010).

There are few effective antimicrobials against MRSA strains, and these antimicrobials are often associated with high costs and multiple side effects for patients (Cardozo *et al.* 2013). Furthermore, the rising use of complementary medicine has prompted increased interest in traditional and non conventional medical treatments. One treatment that has been the subject of much interest is honey (Chauhan; Desai, 2012).

Honey has been used as a food since the earliest days of human civilization and is considered a component of traditional medicine. Apitherapy has gained attention as a form of folk and preventive medicine for the treatment of diseases and to promote overall health (Molan 1992; Gómez-Caravaca *et al.* 2006). A study from Johnson *et al.* (2005) demonstrated that the application of honey (Medihoney) to the exit sites of

tunneled, cuffed hemodialysis catheters is safe and effective compared with topical mupirocin.

Gonnet *et al.* (1964) described the antimicrobial activity of stingless bees honeys. Cortopassi-Laurino and Gelli (1991) showed that the antimicrobial activity of these honeys is higher than *Apis mellifera* honeys.

Such studies reinforce the potential of honey as an antimicrobial, replacing conventional antibiotic therapy. Furthermore, the search for new antimicrobial compounds derived from different natural products is of high importance in the face of indiscriminate and consequently selective pressures on microorganisms (Garedew *et al.* 2004; Cardozo *et al.* 2013).

In this study it was evaluated the antibacterial activity of the honeys from stingless bees (*Scaptotrigona bipunctata* e *S. postica*) against methicillin-sensitive *S. aureus* (MSSA) and MRSA strains as a possible means to control MRSA.

2. MATERIAL AND METHODS

2.1. Honey

The honey samples used in this study were collected from Meliponary of Universidade Estadual de Londrina (Londrina-PR, Brazil) and Unidade de Conservação Monte Sinai (Mauá da Serra-PR, Brazil), during all 2013. The samples of honey called SB and SP were obtained from the stingless bees *Scaptotrigona bipunctata* (Lepeletier, 1836) and *Scaptotrigona postica* (Latreille, 1807), respectively. The honey samples were diluted in equal volumes of water to 50% (v v⁻¹), and sterilized by filtration through a 0.22- μ m filter (Millipore®).

2.2. Bacterial strains

Fifty-three MRSA strains from the bacterial collection of the hospital of Londrina State University (Londrina-PR, Brazil) were used in this study. These MRSA strains were isolated in 2010 from secretion, trachea, urine and blood cultures. Two standard MSSA strains, *S. aureus* ATCC 25923 (MSSA 1) and *S. aureus* ATCC 29213 (MSSA 2) and three standard MRSA strains were also used. The MRSA strains N315 (MRSA 1) (Kuroda *et al.* 2001), BEC9393 (MRSA 2) (Soares *et al.* 2001) and rib1 (MRSA 3) (Cury *et al.* 2009) were provided by Dr. Elsa Masae Mamizuka (Universidade de São Paulo, São Paulo, São Paulo-SP, Brazil), Dr. Agnes Marie Sá Figueiredo (Universidade Federal do Rio de Janeiro, Rio de Janeiro-RJ, Brazil), and Dr. Wanderley Dias da Silveira (Universidade Estadual de Campinas, Campinas-SP, Brazil), respectively. All strains were stored in Brian Heart Infusion (BHI – Oxoid®) broth at -80 °C in stocks containing glycerol (2.5M).

2.3. Identification of MRSA with an antimicrobial susceptibility assay

Clinical strains of *S. aureus* were subjected to antimicrobial susceptibility testing using an automated method with a Microscan device (Siemens®). We tested the following antimicrobials: penicillin (P), oxacillin (OX), trimethoprim-sulfamethoxazole (SXT), clindamycin (DA), erythromycin (E), ciprofloxacin (CIP), gentamicin (CN), tetracycline (TET), rifampicin (RD), tigecycline (TGC) and linezolid (LZD). These tests were performed in triplicate and conducted prior to this study in the Department of Microbiology, Pathology Laboratory, Clinical and Toxicological Analysis, University Hospital, Universidade Estadual de Londrina (UEL-HU) in accordance with the standards required by the Clinical and Laboratory Standards Institute (CLSI, 2012).

2.4. Agar well diffusion assay (AWDA)

The agar well diffusion assay was performed in triplicate based on the work of Holder and Boyce (1994), with modification. Bacteria were first grown in Mueller-Hinton (MH) agar (Difco, USA), and incubated at 37 °C for 24 h. Isolated colonies for each bacterial strain was suspended in sterile saline and adjusted to 0.5 on the McFarland scale, which corresponds to 1.5×10^8 CFU ml⁻¹ (Colony-forming units per milliliter). This suspension was inoculated by streaking the swab over surface of MH agar media plates (thickness of approximately 4 mm). Following inoculation, a sterile glass borer was used to cut 6-mm wells into the surface of the agar. The wells were filled with 50 µL of honey 50% (v v⁻¹). All plates were incubated at 37°C for 24 h. After incubation, the diameters of inhibition zones were measured.

2.5. Broth microdilution assay

Minimal inhibitory concentrations (MIC) were determined in triplicate by microdilution assays in 96-well plates according the CLSI (2012). Bacteria were first grown in MH agar incubated at 37 °C for 24 h. The bacterial suspension was prepared in the same way described previously. These suspensions were diluted in MH broth (Difco®, USA) and plated in 96-well plates at a density of 5.0×10^5 CFU well⁻¹. Finally, different concentrations of honey (0.15 to 20%) solutions were added to each well to determine MIC₁₀₀ values. The plates were incubated at 37 °C for 18 h, and then optical density values at 600 nm were determined using a Bio-Rad Microplate Reader (model 3550).

2.6. Time-kill assay

To evaluate the effect of honey samples on bacterial growth, a time-response growth curve was constructed according to NCCLS (1999). First, a single colony

forming unit for each MSSA and MRSA strain was inoculated in MH broth and grown for 18 h at 37°C with constant shaking at 200 rpm. Then, each culture was adjusted to 0.5 on the McFarland scale and inoculated at a cell density of 10^6 CFU ml⁻¹ in three tubes each containing 1 ml of MH broth. One culture received SB (1x MIC), the second SP (1x MIC), and the last culture did not receive honey (control). These cultures were incubated at 37 °C with constant shaking (200 rpm). Broth aliquots were collected at different time points, serially diluted in saline solution, plated on MH agar media and grown for 18 h at 37 °C to determine the total CFU of each culture.

2.7. Non-peroxide activity

For non-peroxide antibacterial activity assay, the catalase solution was prepared by dissolving 11,000 units mg⁻¹ solid catalase from bovine liver (Sigma) in distilled and sterilized water, and this solution was added to the honey to a concentration of 2,860 units ml⁻¹ (TEMARU *et al.* 2007). The honey with catalase was used to determine the MIC value according CLSI (2012). The honey concentration ranged 0.15 to 20% in MIC assay.

2.8. Induction of sugar crystallization

The separation of sugar from other components from honey was based on Siddiqui (1965), with slight modification. The sugars were precipitated by adding 1 ml of ethanol at 0.5 g of honey. The mixture was homogenized and incubated for 24 h at 4 °C. The precipitate was separated from supernatant and allowed evaporate ethanol, both were dissolved with sterile water to concentration 50% w v⁻¹ and were used to determine MIC in triplicate (CLSI, 2012). An artificial honey (75% w v⁻¹), which was used as a control, was prepared by dissolving fructose (32 g), glucose (31 g), maltose

(12 g) and sucrose (0.1 g) in 100 ml of distilled water, followed by sterilizing at 121 °C for 15 min. This formulation reflects the approximate sugar composition of most stingless bees honeys (Bogdanov *et al.* 1996).

2.9. Cytotoxicity assay

The HEP-2 cell line was cultured in 96-well culture plates, in RPMI medium, at a density of 2.5×10^5 cells well⁻¹ and incubated for 24 h. When the cells were confluent, non-adherent cells were removed by washing with sterile 0.01 M phosphate-buffered saline (PBS). RPMI medium containing different concentrations of SB and SP honey (2.5 to 20%) was added to each well containing cells, and the plates were incubated for 24 h at 37 °C in 5% CO₂. For controls, cells were cultured in growth medium alone. Cell viability was determined in triplicate by the dimethylthiazol diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) method according to the manufacturer's recommendation. The concentrations of the honey samples necessary to inhibit cell viability by 50% (CC₅₀) were determined by regression analysis.

2.10. Statistical analysis

Data were analyzed by one-way ANOVA, and the difference among means was determined using Tukey's post-hoc test ($p < 0.05$). The Student's *t*-test ($p < 0.05$) was also used for the analysis of means. All tests were performed with the statistical program BioEstat version 5.3.

3. RESULTS

3.1. Antimicrobial susceptibility assay for clinical MRSA strains

Clinical MRSA strains exhibited resistance to penicillin, oxacillin, ciprofloxacin and erythromycin. For other antimicrobials, the resistance varied among strains, and different resistance patterns could be observed (data not shown).

3.2. Agar well diffusion of honey samples against *S. aureus* standard strains

As an initial screen to evaluate the antimicrobial activity of the honey samples SB and SP, we measured the diameters of the inhibition zones generated by the honey samples against the *S. aureus* standard strains MSSA and MRSA (Table 1). When the diameters of the inhibition zones generated by SB and SP were compared, there were no significant differences according to the Student's *t*-test test ($p > 0.05$). Although N315 strain is a multi-resistant strain compared with the MSSA strain, there was no significant difference in inhibition zone size ($p > 0.05$).

3.3. MICs of standard strains and clinical strains of *S. aureus*

The MICs for the honey samples SB and SP against MRSA standard and clinical strains fell into the range of 0.62 to 1.25% (Table 2). The MIC values of SB and SP against *S. aureus* standard strains were in the range of 0.62 to 2.5% (Table 1). Some clinical MRSA strains exhibited MIC values similar to each other and could be grouped based on their MIC patterns (Table 2).

3.4. Time-kill curve of *S. aureus* against honey SB and SP on MIC concentration

The MSSA standard strain ATCC 25923 and MRSA standard strain N315, which were grouped in patterns 3 and 4, were selected to perform growth and viability tests. For each strain, we used the 1xMIC respectively values for SB and SP, and in all cases there was complete inhibition of bacterial growth. Overall, *S. aureus* strains exhibited similar behaviors in response to SB and SP.

We observed a significant difference (ANOVA, $p < 0.05$) between the treatments (SB and SP) and control after 2 h of incubation in MSSA strain. Comparison of the treatments revealed a significant difference at 2 h and 4 h incubation for the N315 strain and MSSA strain (Figures 1A and 1B).

The strain N315 demonstrated total growth inhibition after 10 h of incubation with both honey samples. The growth of the MSSA strain was completely inhibited after 7 h of incubation with both honey samples.

3.5. Non-peroxide activity

The MICs values for honey samples SB and SP treated with catalase against MRSA and MSSA strains fell into the range of 10 to 20% (Table 1). When MIC with catalase (10 to 20%) were compared with the MIC without catalase values (0.62 to 2.5%) for SB and SP, respectively, both honey samples exhibited significant differences ($p < 0.05$).

3.6. Effect of precipitate, supernatant and artificial honey

The MIC values for precipitate, supernatant from both honeys and artificial honey against three MRSA and two MSSA standard strains were $> 20\%$ (Standard Error

± 1.78), 20% (SE ± 0.89) and $> 20\%$ (SE ± 1.78) respectively. These results showed that the supernatant have low effect, meanwhile the precipitate and artificial honey have no effect.

3.7. Cytotoxicity of honeys

The CC_{50} values calculated for the honey samples SB and SP by cytotoxicity assay were 13% and 7.3%, respectively (Table 3). When CC_{50} values (13% and 7.3%) were compared with the highest MIC values (1.25% and 2.5%) for SB and SP, respectively, both honey samples exhibited significant differences ($p < 0.05$). Although when honey was treated with catalase the MICs values were higher than CC_{50} values, the CC_{50} values of honey treated with catalase was not determined.

4. DISCUSSION

The first report concerning the antimicrobial properties of *Apis mellifera* honey was made in 1892 by Van Ketel (Dustmann, 1979). The first report of the antibacterial activity of honey from stingless honeybees was in 1964 by Gonnet, Lavie and Nogueira-Neto.

Since then, several studies have attempted to elucidate the activities and antimicrobial properties of honey from stingless honey bees, including Vit *et al.* (1997), Garedeu *et al.* (2003), Miorin *et al.* (2003), DeMera and Angert (2004), Temaru *et al.* (2007), Boorn *et al.* (2009) and Sgariglia *et al.* (2010). In addition, the physico-chemical and microbiological properties of honey from stingless bees have been studied by Bijlsma *et al.* (2006), Souza *et al.* (2006), Almeida-Anacleto *et al.* (2009), and De Moura Oliveira *et al.* (2013).

Temaru *et al.* (2007) showed that the majority of the honey tested exhibited antibacterial activity against Gram-positive and Gram-negative bacteria, but *S. aureus* appeared to be more susceptible to honey than the other test strains. Our results corroborate to this study due to honey from *S. bipunctata* and *S. postica* were effective against *S. aureus*.

With the AWD test it was possible to observe that both honey samples possessed antibacterial activity, as evidenced by inhibition zones showed no significant difference was observed between SB and SP in terms of inhibition zone size for the same bacterial strain or between different bacterial strains for the same honey sample. Comparing our study with Temaru *et al.* (2007) that also tested the *S. bipunctata* honey against *S. aureus* ATCC 25923, a small difference was observed. Their results showed size of inhibition zone of 16 mm, and in the present study for the same strain it was 23 mm.

The MIC values reported by Boorn *et al.* (2009) for *Trigona carbonaria* honey ranged from 6 to 10% for standard *S. aureus* MRSA and MSSA strains. George and Cutting (2007) found that the MIC value of Medihoney was approximately 4% for *S. aureus* ATCC 25923 and clinical isolates of MRSA. In Anthimidou and Mossialos (2013) study, the MICs values from *Abies* Honey against MRSA was 6.25% (without catalase) and 50% (with catalase). In our study, the MIC values of SB and SP without catalase, ranged from 0.62 to 1.25% against MRSA clinical and standard strains, ranged from 0.62 to 2.5% for MSSA strains, and both ranged from 10 to 20% when treated with catalase, showing that SB and SP are most efficient than the honey mentioned previously.

Clinical MRSA strains were grouped into four patterns according to sensitivity to honeys, the pattern 1 group the most sensitive strains and pattern 4 group the most resistant strains. Fourteen clinical MRSA strains, corresponding to the third pattern,

were more sensitive to SB (0.62%) than SP (1.25%), similar to pattern of MRSA strain BEC9393. Thirty-three strains exhibited the same MIC values of SB (1.25%) and SP (1.25%), which corresponded to the fourth pattern, similar to MRSA strains N315 and rib1. Such patterns can be related to clonality and the propagation of these strains, which can either be the same types or part of the same clonal groups.

Analysis of the time-kill curves indicated an increased sensitivity of the MSSA strain (Figure 1B) compared to the MRSA strains, achieving complete inhibition after 7 h of incubation. However, among MRSA strains, the standard strain N315 (Figure 1A) appeared to be sensitive achieving complete inhibition after 10 h of incubation.

In general, bacteria exhibited differential behavior in response to the different honey samples. Only after 2 and 4 h of incubation was significant difference observed between the honey samples; at subsequent time points, this difference was no longer noticeable.

In the study by Jenkins *et al.* (2011), who tested Manuka honey against standard strains of MRSA, obtained a difference of approximately 5-log_{10} between control and treated cultures after 24 h of incubation with a 5% honey solution but did not achieve complete growth inhibition. In our study, after 24 h of incubation with 0.62% SB occur total elimination of MRSA, showing a higher antibacterial activity of this honey, however the *in vitro* effect may be different when it compared to *in vivo* assay.

There have been reports of honey that exhibits bacteriostatic activity at higher concentrations and bactericidal activity at more dilute concentrations. There are several factors that contribute to this effect, but one in particular is hydrogen peroxide (Bogdanov, 1997; Taormina *et al.* 2001; Viuda-Martos, 2008). The honey hydrogen peroxide exert oxidative damage causing bacterial growth inhibition and DNA degradation (Brudzynski *et al.* 2011; Brudzynski; Lannigan, 2012). Our results showed

that the presence of hydrogen peroxide contributes to the antibacterial activity, however these honeys have other inhibitory substances.

Furthermore the high sugar concentration and osmolarity of honey are also responsible for the inhibition of bacterial growth (Molan, 1992; Chan-Rodríguez *et al.* 2012). However our results showed that the sugars only cannot inhibit bacterial growth, MIC of precipitate was >20%, reinforcing the presence of other substances with antibacterial activity.

According DeMera and Angert (2004) found that honey samples with different entomological origins but of the same phytogeographic region did not differ in their antimicrobial activity. However, antimicrobial activity varied between honey samples from different phytogeographic regions.

Physicochemical analysis is important to understand the sources of antibacterial activities in honey. Factors such as pH, sugar levels, moisture, and osmolarity can influence bacterial growth (Chan-Rodríguez *et al.* 2012). Although there have been studies that performed physicochemical analysis of honey from stingless bees, there is little information on the composition of honey, which is a very important quality control (Vit *et al.* 2006).

Generally, honey from stingless bees presents average physico-chemical values for most prerequisites established by the Brazilian government and international regulations, with the exception of moisture content (Brasil, 200; Borsato, 2013).

In addition to physical and chemical factors that contribute to the antibacterial activity of honey, phytochemicals factors and a number of other compounds are also important. Other antibacterial factors in addition to hydrogen peroxide have been found; this occurred when it is observed that the antibacterial activity of honey persisted after heating (to inactivate glucose oxidase) or after treatment with catalase (to remove

hydrogen peroxide) (Bogdanov, 1997; Taormina *et al.* 2001; Viuda-Martos, 2008). In this study although the MIC values has increased after treatment with catalase (10 to 20%) the antibacterial activity remained, which suggests the presence of other antibacterial compounds.

Fauzi *et al.* (2011) tested Taulang Honey against cell line HeLa, the CC_{50} obtained was 2.8%. In our study the CC_{50} against HEP-2 was 13% (SB) and 7.3% (SP), showing a lower cytotoxicity when compared with Fauzi *et al.* (2011).

There are few effective antimicrobials against multi-resistant bacteria, including MRSA strains. These antimicrobials are often associated with high costs and serious patient side effects. Many different natural antimicrobials have been studied as alternatives to control these infections (Cardozo *et al.* 2011; Darabpour *et al.* 2012). This study demonstrated that, *in vitro*, the natural product honey possessed antistaphylococcal activity against the *S. aureus* MSSA and MRSA organisms that were tested. Further studies are required to determine the mechanism and components involved in this activity and whether it has clinical applications for the treatment of MRSA infections.

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7. CAPTIONS

Table 1- MICs and diameters of inhibition zones (mm) generated by honey samples SB and SP in well diffusion assay against standard *S. aureus* strains grown on agar.

Table 2- MICs of the honey samples SB and SP against clinical MRSA strains based on pattern distribution and number of strains per pattern and resistance percentage to antimicrobial susceptibility test.

Table3- Comparison of CC₅₀ and MICs generated by honey samples SB and SP against HEP-2 cell and standard *S. aureus* strains.

Figure 1- Time-kill curves of *Staphylococcus aureus* strains exposed to honey in 1xMICs concentrations. **A:** MRSA N315 strain exposed to SB (1.25%) and SP (1.25%); **B:** MSSA ATCC 25923 strain exposed to SB (0.62%) and SP (1.25%); **C:** clinical MRSA (pattern 4) strain exposed to SB (1.25%) and SP (1.25%); **D:** clinical MRSA (pattern 3) strain exposed to SB (0.62%) and SP (1.25%). Control indicates bacterial growth without honey.

TABLE 1

Table 1- MICs and diameters of inhibition zones (mm) generated by honey samples SB and SP in well diffusion assay against standard *S. aureus* strains grown on agar.

<i>S. aureus</i> strains	AWD zone (mm)*		MIC (%)*			
			Without catalase		With catalase	
	SB	SP	SB	SP	SB	SP
MRSA N315	27±0.46	24±0.21	1.25±0.11	1.25±0.11	10±1.78†	10±1.78†
MRSA BEC9393	24±0.67	24±0.51	0.62±0.11	1.25±0.11	10±1.78†	10±1.78†
MRSA Rib1	20±0.21	21±0.29	1.25±0.27	1.25±0.27	20±2.19†	20±2.19†
ATCC 25923	23±0.52	22±0.29	0.62±0.11	1.25±0.11	20±2.19†	20±2.19†
ATCC 29213	20±0.21	20±0.46	1.25±0.27	2.50±0.27	10±1.78†	10±1.78†
Mean ± SE	23±0.13	22±0.08	0.99±0.34	1.50±0.37	30±0.39†	30±0.39†

*Standard errors

† $p < 0.05$

AWD: Agar Well Diffusion

MIC: Minimal Inhibitory Concentration

MRSA: Methicillin-resistant *Staphylococcus aureus*

ATCC: American Type Culture Collection

TABLE 2

Table 2- MICs of the honey samples SB and SP against clinical MRSA strains based on pattern distribution and number of strains per pattern

Patterns	MIC (%)*		Number of strains
	SB	SP	
1	0.62±0.11	0.62±0.11	4
2	1.25±0.27	0.62±0.11	2
3	0.62±0.11	1.25±0.27	33
4	1.25±0.27	1.25±0.27	14
Mean ± SE	0.81±0.36	1.18±0.17	53

*Standard errors.

TABLE 3

Table 3- Comparison of CC₅₀ and MICs generated by honey samples SB and SP against HEp-2 cell and standard *S. aureus* strains.

	CC ₅₀ (%)*	MIC (%)*	<i>p</i> value**
SB	13±0.32	0.62 - 1.25±0.11	< 0.05
SP	7.3±0.25	1.25 - 2.50±0.27	< 0.05

*Standard errors.

**Significance level 5%.

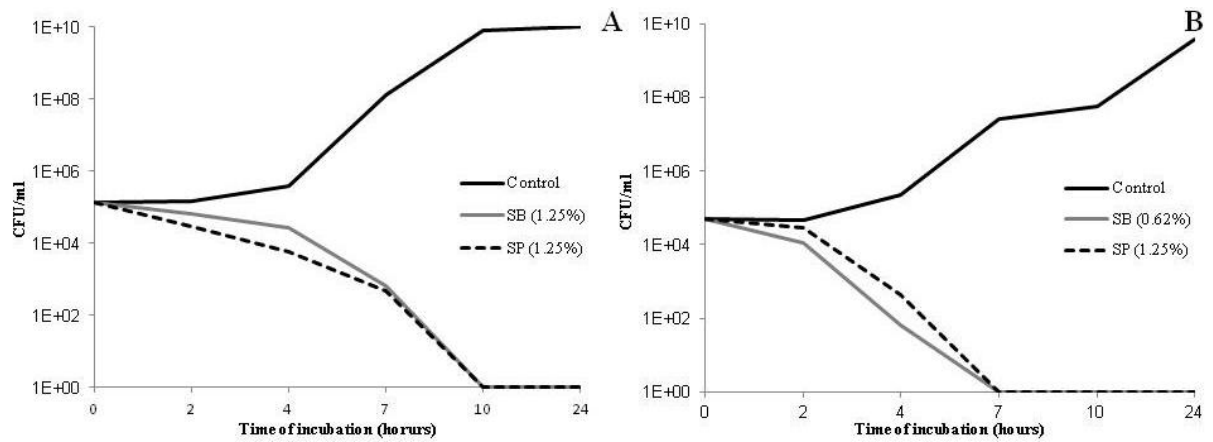
FIGURE 1

Figure 1- Time-kill curves of *Staphylococcus aureus* strains exposed to honey in 1xMICs concentrations. **A:** MRSA N315 strain exposed to SB (1.25%) and SP (1.25%); **B:** MSSA ATCC 25923 strain exposed to SB (0.62%) and SP (1.25%);

ARTIGO II

Synergistic antibacterial effect between honeys from two stingless bees *Scaptotrigona bipunctata* Lepeletier, 1836 and *Scaptotrigona postica* Latreille, 1807 (Hymenoptera: Apidae: Meliponinae)

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ABSTRACT

Recently has been a sharp fall-off in new antibiotic drug discovery and multidrug-resistant bacteria have occurred, and are anticorrelated with the introduction of new anti-infectives. Front of this problem, the search for new antimicrobial compounds from different natural products is of high importance, such as honey, replacing the conventional antibiotic therapy. In this study we tested a combination of two stingless bees honeys against Gram-positive and Gram-negative strains. The antibacterial activity of honey produced by the stingless bees *Scaptotrigona bipunctata* (SB) and *Scaptotrigona postica* (SP) was evaluated by agar well diffusion assay, broth microdilution assay, time-kill curves and scanning electron microscopy (SEM). Interaction of two honeys was evaluated by checkerboard assay. Inhibition zones ranged from 8 ± 0.67 to 22 ± 0.84 mm, minimal inhibitory concentration values of individual honeys ranged 0.62 ± 0.11 to 10 ± 0.79 % ($v v^{-1}$) and when combined decreased 1/4 to 1/32. SEM images showed division inhibition (SB) and cell wall disruption (SP), and when used in combination both alterations were observed in same field. This study demonstrated that, *in vitro*, the natural product honey possessed bactericidal activity against Gram-positive and Gram-negative, including multi-resistant strains. Combinations of honey SB and SP could lead to a new broad-spectrum antimicrobials that have the potential to prevent the emergence of resistant bacterial strains.

Keywords: Antibacterial, honey, stingless bee, synergism, *Scaptotrigona*.

1. INTRODUCTION

The emergence of multidrug-resistant bacteria and the rapid global spread has been a growing threat to public health. In most cases, antibiotic-resistant infections require prolonged and/or costlier treatments, extend hospital stays, healthcare use, and result in prolonged illness and increased mortality compared with infections that are easily treatable with antibiotics^{1,2}.

Recently has been a sharp fall-off in new antibiotic drug discovery, the reasons are mainly few progressive clues of new antibiotic screening. During this period of stagnation in the new antibiotics development, multidrug-resistant bacteria have occurred and are anticorrelated with the introduction of new anti-infectives³.

Honey has been used as a food since the earliest times and considered as part of traditional medicine. Apitherapy has become the attention as a form of folk and preventive medicine for treating diseases and promoting overall health.^{4,5}

Although the use of honey as antibacterial is a folk medicine, this practice has been replaced by synthetic and semisynthetic antimicrobials.^{6,7} The use of these antibiotics indiscriminately exerts a selective pressure on microorganisms selecting progressively most resistant.⁸ Front of this problem, the search for new antimicrobial compounds from different natural products is of high importance, such as honey, replacing the conventional antibiotic therapy.⁹⁻¹²

Several studies have tested combinations of honey and various substances including conventional antibiotics,¹³⁻¹⁵ plant extracts,¹⁶⁻¹⁸ royal jelly¹⁹ and propolis.^{20,21} Although there are various combinations of honey and other compounds, it is still not known the effect of the combination of different honeys on bacteria. In this study we tested a combination of two stingless bees honeys against various bacterial strains. These combinations could lead to a new broad-spectrum antimicrobials that have the potential to prevent the emergence of resistant bacterial strains.

2. RESULTS

2.1. Agar well diffusion of honey samples against Gram-positive and Gram-negative bacteria

As an initial screen to evaluate the antimicrobial activity of honey samples against all bacteria strains, the AWD assay was carried out and inhibitions zones were measured (Table 1). Inhibition zones of Gram-positive strains treated with honey SB had an average of 18.30 ± 1.07 mm whereas for Gram-negative was 10.28 ± 0.56 mm indicating that Gram-positive bacteria were more sensitive ($p < 0.05$). The same was observed when treated with honey SP, Gram-positive had an average of 13.90 ± 1.17 mm and Gram-negative 8.14 ± 0.24 mm ($p < 0.05$) (Table 1). But when comparing the inhibition zones generated by honey SB with SP showed no statistical difference for both Gram-positive and negative ($p > 0.05$).

2.2. Minimal inhibitory concentration of honey samples

The mean MIC values (Table 2) for Gram-positive bacteria was $1.87 \pm 0.39\%$ and $2.50 \pm 0.81\%$ while for Gram-negative was $5.36 \pm 0.78\%$ and $6.07 \pm 0.99\%$ for honeys SB and SP, respectively, with no significant differences between the honeys ($p > 0.05$) (Table 3). The MIC data corroborate which we observed in AWD assay, and Gram-positive bacteria seem to be more sensitive compared to Gram-negative strains ($p < 0.05$).

2.3. Non-peroxide activity

To evaluate non-peroxide activity, honeys were treated with catalase for degradation of hydrogen peroxide. The MIC average values were $13.00 \pm 1.73\%$ and $14.00 \pm 1.85\%$ for Gram-positive, $18.57 \pm 1.32\%$ and $18.57 \pm 1.32\%$ for Gram-negative bacteria, for SB and SP respectively (Table 2). Artificial honey showed not bacterial inhibition, $MIC > 50\%$.

Comparing the MICs values of treated and not treated with catalase ($p < 0.05$) we can observe a large contribution of hydrogen peroxide to the honey antibacterial activity.

2.4. Interaction of honey

To determine the type of interaction of SB and SP honeys when combined, checkerboard assay was performed. A synergistic antibacterial effect was observed against all bacterial strains when both honeys, SB and SP, were combined. The MIC values of combinations reduced 1/4 to 1/32 in relation to the MIC values for honeys alone (Table 3).

2.5. Time-kill curve of *S. aureus* for both honey

The evaluation of the kinetics of antibacterial effect of honey against MRSA N315 strain was performed by constructing a time-kill curve. We can observe a decrease of up to $4\log_{10}$, compared to control, in treatment after 4 hours of incubation, but between treatments there was no statistical difference ($p > 0.05$) (Figure 1). The treatment containing combined honey decreased the time needed for elimination of all cell (Figure 1).

2.6. Scanning electron microscopy of *S. aureus* against both honey

The analysis of the SEM electron micrograph for the control cells (Figure 2A) showed a large amount of cells covering the entire field, with intact structure, all uniform size (0.5 a 1.0 μm). After 3 hours of incubation in presence of honey, we observed a large reduction of the bacterial population in all treatments (Figure 2B, 2C and 2D) compared with control (Figure 2A). When treated with honey SB, it can be seen in various cells the presence of septum but without cell division (Figure 2B arrowhead). When treated with honey SP cells appear to increase in size and cytoplasmic leakage (Figure 2C arrow). But when treated with

both honeys can observe all these events described above, happening in the field, cell division septum (Figure 2D arrowhead), cells enlarged (Figure 2D arrow) and cytoplasmic leakage.

3. DISCUSSION

Based in AWD assay to evaluate the antibacterial activity of stingless bee honeys, Chan-Rodríguez *et al.*²⁹ and Temaru *et al.*²⁷ showed that Gram-positive bacteria were more sensitive to these honeys, compared to Gram-negative bacteria. Same results was observed in our work to both honey, SB and SP, showing be more effective to Gram-positive bacteria. Although AWD assay is widely used to evaluate the antibacterial activity of honey there are several variables that can influence the results.³⁰

In study of Garedew *et al.*,³¹ working with honey from *Trigona* spp., and Boorn *et al.*,³⁰ working with honey from *Trigona carbonaria*, found MICs values ranged 1 to 32% (Gram-positive) and 4 to 32% (Gram-negative). In our study the MICs values were lower compared to the cited authors, ranging 0.63 to 10% (Gram-positive) and 2.5 to 10% (Gram-negative). Determination of MICs from different stingless bee honeys reaffirm data obtained from AWD assay, showing that Gram-positive were more sensitive than Gram-negative bacteria. Among the Gram-positive strains, *S. aureus* presented the lowest MIC values.

Different studies evaluated the interaction of honey with other compounds derivated from bees, like propolis,²⁰ and royal jelly.¹⁹ In both studies there have a positive interaction but it can be considered additive according to Chin *et al.*²⁵ Our study showed that the combination of honey SB and SP have a synergistic interaction, decreasing expense and obtaining an equal or even better effect.

Kinetics evaluation of the antibacterial effect of stingless bees honeys, SB and SP, showed bactericidal action, similar result observed by Temaru *et al.*²⁷ with three stingless bees honeys (*Melipona beecheii*, *Trigona biroi* and *Scaptotrigona pectoralis*). When honey SB and

SP are used in combination the time required to eliminate any bacteria is shorter than when applied separately, showing the advantage of use honey in combination.

Several authors attribute the high osmolarity, acidity (low pH) and especially hydrogen peroxide as the main honeys antimicrobial factors.^{4, 32, 33} In our study, artificial honey did not inhibit bacterial growth, these data show that only high osmolarity (simulated by artificial honey) is unable to inhibit bacterial growth.

Evaluation of non-peroxide activity of honey by Kuncič *et al.*³⁴ showed that Slovenian honey when treated with catalase increased 20 fold the MICs values against Gram-positive and Gram-negative bacteria. In our study, when honey was treated with catalase we observed increase of 5 fold in MIC values. These data show the importance of hydrogen peroxide to honey antibacterial activity used in this work, but other components present in honey can inhibit bacterial growth.

Pimentel *et al.*³⁵ testing *Melipona compressipes manaosensis* honey against Gram-positive and Gram-negative bacteria observed difference in the antibacterial action of honey collected in different seasons (wet and dry) showing that honey collected in the dry season have higher activity. This difference was not observed in our study because the honeys used were collected throughout the year and there were no variations in the antibacterial activity. Studies indicate that the antibacterial activity is related significantly to the floral source, varying the action if the honey are monofloral or multifloral, beyond action vary according to the phytogeographic region.^{36, 37} Furthermore new studies indicate the presence of other antimicrobial components such as methylglyoxal,³⁸ antimicrobial peptide bee-defensin 1,³⁹ hydroxymethylfurfural⁴⁰ and phenolic compounds such as flavonoids.⁴¹

Analysis of SEM micrographs allow us observe morphological alterations caused by the honeys placed in contact with *S. aureus*. When we observe images of cells treated with honey SB noted a large amount of cells with septa, suggesting that effects on the cell make

unfeasible the ability to complete the process of cell division. Studies from Jenkins *et al.*⁴² exposed *S. aureus* MRSA to manuka honey and observed similar results, by TEM micrographs. These authors suggested that honey may be acting on the murein hydrolase by interfering the post-translational modification since the expression of the gene responsible for encoding the enzyme was enhanced. It is known that the murein hydrolase enzyme, also called autolysins, is component of the family of hydrolytic enzyme, which targeted cell wall components,⁴³ therefore a decrease in enzyme activity would lead to failure in cell separation. Jenkins *et al.*⁴² further stated that methylglyoxal (MGO) synergistically with other components present in honey may be responsible for effects caused on bacteria.

Cells treated with honey SP showed be enlarged compared to adjacent cells and to the control. This enlargement of the cell may be occurring due to the partial disruption or degradation of bacterial cell wall, leading to water entering and eventually cell lysis and cytoplasmic leakage. We suggest that some compound present in honey is acting to prevent the formation of bacterial cell wall, leading to the formation of spheroplasts therefore cell lysis. A large number of flavonoids can be found in honey and are known to have antimicrobial effects. Cushnie and Lamb⁴⁴ tested the action of Galangin (flavonol present in honey) against *S. aureus* and found that the cells have lost large amounts of potassium, similar when treated with penicillin G. This occur due to the inhibition of cell wall synthesis reducing the mechanical strength of the cell wall. According to the authors, Galangin may be damaging the cytoplasmatic membrane directly or weakening the cell wall and thereby causing osmotic lysis.

When we observe SEM micrography of cells treated with both honeys, we noted alterations caused by honey SB and SP, individually, occurring in the same field. Lorian and Fernandes⁴⁵ worked with a combination of two semi-synthetic derivatives of pristinamycin, quinupristin/dalfopristin against *S. aureus*. These authors observed that when used

individually, bacterial cells appeared larger and stained more intensely than control, and when used in combination had increases in cell size, thickness of the cell wall, breaks in the wall and ghosts lysed cells. The honeys effects cover cells in different stages of cell division cycle, since peptidoglycan synthesis, an initial stage prevented (totally or partially) by honey SP, until the septa formation to separate dividing cells, proving honeys synergistic effect.

Although studies increasingly seek the elucidation of the active principles of honey, is known they act synergistically, may be that a synergy of all ingredients will bring about the maximum of therapeutic efficacy, making the honey as a whole a product of great interest.

There are few effective antimicrobials against multi-resistant bacteria, including MRSA strains. These antimicrobials are often associated with high costs and serious patient side effects. Several studies have shown combination of honey with commercial antimicrobial drugs,^{14, 15} vegetable origin natural products,⁴⁶ animal origin.^{19, 20} Antibiotic combinations are advantageous and commonly used for treatment. In many cases, these combinations are used to provide a broad spectrum of activity by multitarget effects or to delaying or suppressing the emergence of drug-resistant population.^{47, 48} This study demonstrated that, *in vitro*, the combination of honey possessed antimicrobial activity against Gram-positive and Gram-negative, including multi-resistant strains, and this combination generated a patent.⁴⁹ Further studies are required to determine whether it has clinical applications for the treatment of several infections.

3. MATERIALS AND METHODS

3.1. Honey

The honey samples used in this study were collected from Meliponary of Universidade Estadual de Londrina (Londrina-PR, Brazil) and Unidade de Conservação Monte Sinai (Mauá da Serra-PR, Brazil), during 2013. The samples of honey called SB and SP were obtained

from the stingless bees *Scaptotrigona bipunctata* (Lepeletier, 1836) and *Scaptotrigona postica* (Latreille, 1807) respectively. The honey samples were diluted in equal volumes of water (50% v v⁻¹) and sterilized by filtration through a 0.22- μ m filter (Millipore®).

3.2. Bacteria strains

Reference strains of Gram-positive and Gram-negative bacteria were used: *Staphylococcus aureus* ATCC25923 (strain 1) and ATCC29213 (strain 2), *Staphylococcus epidermidis* ATCC12228, *Enterococcus faecalis* ATCC29212, *Enterococcus faecium* ATCC6569, *Streptococcus mutans* UA159, *Streptococcus pyogenes* ATCC19615, *Escherichia coli* ATCC25922 (strain 1) and ATCC8739 (strain 2), *Salmonella enterica* serovar Enteritidis ATCC13076, *Klebsiella pneumoniae* ATCC700603 and *Pseudomonas aeruginosa* ATCC27853 (strain 1) and ATCC9027 (strain 2).

Furthermore, other bacterial strains were used, MRSA N315 (strain 1) and BEC9393 (strain 2), which were provided by Dr. Elsa Masae Mamizuka (São Paulo University, São Paulo, São Paulo-SP, Brazil), Dr Agnes Marie Sá Figueiredo (Rio de Janeiro Federal University, Rio de Janeiro-RJ, Brazil), respectively and *Salmonella enterica* serovar Typhimurium UK1 provided by Dr. Roy Curtiss (Arizona State University, Tempe, AZ). All strains were stored at - 80 °C in stocks containing glycerol (2.5M).

3.3. Agar well diffusion assay (AWDA)

The agar well diffusion assay was performed in triplicate based on the work of Holder and Boyce,²² with modification. Bacteria were first grown in Mueller-Hinton (MH) agar (Difco, USA), and incubated at 37 °C for 24 h. Each bacterial strain was suspended in sterile saline and adjusted to 0.5 on the McFarland scale, which corresponds to 1.5×10^8 CFU ml⁻¹. This suspension was spread on MH agar media plates (thickness of approximately 4 mm). Following inoculation, a sterile glass borer was used to cut 6-mm wells into the surface of the agar. The wells were filled with 50 µL of honey 50% (v v⁻¹). All plates were incubated at 37 °C for 24 h. After incubation, the diameters of inhibition zones were measured.

3.4. Broth microdilution assay

Minimal inhibitory concentrations (MIC) were determined in triplicate by microdilution assays in 96-well plates according the CLSI.²³ The bacterial suspension was prepared in the same way described previously. These suspensions were diluted in MH broth (Difco®, USA) and plated in 96-well plates at a density of 5.0×10^5 CFU well⁻¹. Finally, different concentrations of honey solutions were added to each well to determine MIC₁₀₀ values. The plates were incubated at 37 °C for 18 h, and then optical density values at 600 nm were determined using a Bio-Rad Microplate Reader (model 3550).

3.5. Non-peroxide activity

For testing non-peroxide antibacterial activity the catalase solution was prepared by dissolving 11000 units mg⁻¹ solid catalase from bovine liver (Sigma) in distilled water, and this solution was added to the honey to a concentration of 2860 units ml⁻¹.²⁷ The honey containing catalase was used to determine the MIC value.²³ An artificial honey (75% w v⁻¹), which was used as a control, was prepared by dissolving 32 g of fructose, 31 g of glucose, 12

g of maltose and 0.1 g of sucrose in 100 ml of distilled water, followed by sterilizing at 121 °C for 15 min. This formulation reflects the approximate sugar composition of most stingless bees honeys.²⁸

3.6. Checkerboard assay

To evaluate the antibacterial effects and interactions of honey SB combined with SP against bacteria strains, assays of microdilution in double-antimicrobial gradient were used. The microdilution was made in 96-well plates in triplicate according Kelly and Matsen.²⁴ To evaluate the interaction between both antimicrobials, the fractioned inhibitory concentration (FIC) index was used as described by Chin *et al.*:²⁵

$$FIC = \frac{MIC_{SB_c}}{MIC_{SB_a}} + \frac{MIC_{SP_c}}{MIC_{SP_a}}$$

Where MIC_{SB_c} is the MIC of honey SB used combined with the honey SP, MIC_{SB_a} is the MIC of honey SB used alone, MIC_{SP_c} is the MIC of honey SP used combined with the honey SB, MIC_{SP_a} is the MIC of honey SP used alone.

3.7. Time-kill assay

To evaluate the effect of honey samples on bacterial growth, a time-response growth curve was constructed according to NCCLS.²⁶ First, a single colony forming unit for each MSSA and MRSA strain was inoculated in MH broth and grown for 18 h at 37 °C with constant shaking at 200 rpm. Then, each culture was adjusted to 0.5 on the McFarland scale and inoculated at a cell density of 10^6 CFU ml⁻¹ in two tubes each containing 1 ml of MH broth. One culture received honey and other culture did not receive honey (control). These cultures were incubated at 37 °C with constant shaking (200 rpm). Broth aliquots were

collected at different time points, serially diluted in saline solution, plated on MH agar media and grown for 18 h at 37 °C to determine the total CFU of each culture.

3.8. Scanning electron microscopy (SEM)

Scanning electron microscopy was used to observe the cell morphology after treatment with honeys. Colonies from *S. aureus* MRSA N315 culture grown in MH agar (24 h, 37 °C) were transferred to MH broth and the cell density were adjusted to 10^8 CFU ml⁻¹. One milliliter of the cell suspension was distributed in 3 tubes and honeys (SB, SP and SB+SP) were added at MIC concentrations. Culture in absence of the honey was considered as the control. The cultures were incubated at 37 °C; 150 rpm for 3 h. After incubation, 20 µl of each culture was collected and spotted onto polylysine-coated glass slides. Each slide was fixed by immersion in 1 ml of 2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) solution for 20 h, following post-fixation in 1% OsO₄ for 2 h. The fixed samples were dehydrated in an ethanol gradient (70, 80, 90 and 100 °CL) and then were critical point dried in CO₂ (BALTED CPD 030 Critical Point Dryer). Finally, the slides were taped onto stubs, coated with gold (BALTEC SDC 050 Sputter Coater) and observed under a FEI Quanta 200 scanning electron microscope to analyze alterations in the cell.

3.9. Statistical analysis

All data presented represent mean values from three replicate experiments. Data were analyzed by one-way ANOVA, and the difference among means was determined using Qui-square test ($\alpha = 5\%$). All tests were performed with the statistical program BioEstat version 5.3.

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7. CAPTIONS

Table 1 - Diameters inhibition zones (mm) generated by honey samples SB and SP in agar well diffusion assay against Gram-positive and Gram-negative bacteria.

Table 2 - MICs values of honey samples SB and SP with and without catalase against Gram-positive and Gram-negative bacteria.

Table 3 - Antibacterial effect of honeys SB and SP against Gram-positive and Gram-negative bacteria: MIC values of SB and SP alone or in combination, and FIC values.

Figure 1 - Time-kill curves of *Staphylococcus aureus* MRSA N315 strain exposed to honey in 1xMICs concentrations; SB used alone (1.25%); SP used alone (1.25%); SB/SP (0.31/0.31%) used in combination; Control indicates bacterial growth without honey.

Figure 2 - Scanning electron microscopy images of the antibacterial effect of honey SB, SP and SB/SP against MRSA N315 strain in 3 h of incubation. A: negative control (without honey); B: treated with honey SB (1.25%) showing cells formed septa (arrowhead); C: treated with honey SP (1.25%) showing spheroplast (arrow); D: treated with both honey, SB (0.31%) and SP (0.31%) showing cells formed sept (arrowhead) and spheroplast (arrow). Different morphological alterations can be observed in treatment. Inset images show detail of morphological alterations.

TABLE 1

Table 1 - Diameters inhibition zones (mm) generated by honey samples SB and SP in agar well diffusion assay against Gram-positive and Gram-negative bacteria.

Bacterial strains	Mean (\pm SE) of inhibitory zone size (mm)	
	SB	SP
<i>Enterococcus faecalis</i>	22 (\pm 0.84)	18 (\pm 0.34)
<i>Enterococcus faecium</i>	10 (\pm 0.84)	7 (\pm 0.34)
<i>Escherichia coli</i> strain 1	9 (\pm 0.67)	8 (\pm 0.31)
<i>E. coli</i> strain 2	8 (\pm 0.67)	8 (\pm 0.31)
<i>Klebsiella pneumoniae</i>	11 (\pm 0.84)	7 (\pm 0.34)
<i>Pseudomonas aeruginosa</i> strain 1	12 (\pm 0.67)	8 (\pm 0.31)
<i>P. aeruginosa</i> strain 2	11 (\pm 0.67)	9 (\pm 0.31)
<i>Samonella enterica</i> Enteritidis	12 (\pm 0.31)	9 (\pm 0.63)
<i>S. enterica</i> Typhimurium	9 (\pm 0.31)	8 (\pm 0.63)
<i>Staphylococcus aureus</i> strain 1	19 (\pm 0.52)	15 (\pm 0.30)
<i>S. aureus</i> strain 2	20 (\pm 0.52)	16 (\pm 0.30)
<i>S. aureus</i> methicillin-resistant strain 1	20 (\pm 0.45)	16 (\pm 0.21)
<i>S. aureus</i> methicillin-resistant strain 2	19 (\pm 0.45)	15 (\pm 0.21)
<i>Staphylococcus epidermidis</i>	20 (\pm 0.45)	18 (\pm 0.21)
<i>Streptococcus mutans</i>	19 (\pm 0.52)	11 (\pm 0.30)
<i>Streptococcus pyogenes</i>	14 (\pm 0.31)	8 (\pm 0.63)
Mean total	14.69 (\pm 1.19)	11.31 (\pm 1.01)

SE: Standard error

ATCC: American Type Culture Colection

SB: Honey from *Scaptotrigona bipunctata*

SP: Honey from *Scaptotrigona postica*

TABLE 2

Table 2 - MICs values of honey samples SB and SP with and without catalase against Gram-positive and Gram-negative bacteria.

Bacterial strains	MIC (%)			
	Without catalase		With catalase	
	SB	SP	SB	SP
<i>Enterococcus faecalis</i>	1.25	1.25	10.00	20.00
<i>Enterococcus faecium</i>	5.00	10.00	20.00	20.00
<i>Escherichia coli</i> strain 1	5.00	5.00	20.00	20.00
<i>E. coli</i> strain 2	5.00	5.00	20.00	20.00
<i>Klebsiella pneumoniae</i>	10.00	10.00	20.00	20.00
<i>Pseudomonas aeruginosa</i> strain 1	2.50	2.50	10.00	10.00
<i>P. aeruginosa</i> strain 2	5.00	5.00	20.00	20.00
<i>Samonella enterica</i> Enteritidis	5.00	5.00	20.00	20.00
<i>S. enterica</i> Typhimurium	5.00	10.00	20.00	20.00
<i>Staphylococcus aureus</i> strain 1	0.62	1.25	20.00	20.00
<i>S. aureus</i> strain 2	2.50	2.50	10.00	10.00
<i>S. aureus</i> methicillin-resistant strain 1	0.62	1.25	10.00	10.00
<i>S. aureus</i> methicillin-resistant strain 2	1.25	1.25	10.00	10.00
<i>Staphylococcus epidermidis</i>	2.50	2.50	10.00	10.00
<i>Streptococcus mutans</i>	2.50	2.50	10.00	10.00
<i>Streptococcus pyogenes</i>	1.25	1.25	10.00	10.00
Mean total*	3.44	4.14	15.00 [†]	15.63 [†]
	(± 0.59)	(± 0.79)	(± 1.25)	(± 1.24)

*Standard error

[†] $p < 0.05$

MIC: Minimal Inhibitory Concentration

ATCC: American Type Culture Colection

SB: Honey from *Scatotrigona bipunctata*

SP: Honey from *S. postica*

TABLE 3

Table 3 - Antibacterial effect of honeys SB and SP against Gram-positive and Gram-negative bacteria: MIC values of SB and SP alone or in combination, and FIC values.

Bacterial strains	MIC alone (%)		MIC combined (%)		FIC*
	SB	SP	SB	SP	
<i>Enterococcus faecalis</i>	1.25	1.25	0.31	0.31	0.50
<i>Enterococcus faecium</i>	5.00	10.00	0.63	2.50	0.38
<i>Escherichia coli</i> strain 1	5.00	5.00	1.25	1.25	0.50
<i>E. coli</i> strain 2	5.00	5.00	1.25	0.63	0.38
<i>Klebsiella pneumoniae</i>	10.00	10.00	2.50	0.31	0.28
<i>Pseudomonas aeruginosa</i> strain 1	2.50	2.50	0.63	0.63	0.50
<i>P. aeruginosa</i> strain 2	5.00	5.00	1.25	0.63	0.38
<i>Samonella enterica</i> Enteritidis	5.00	5.00	1.25	1.25	0.50
<i>S. enterica</i> Typhimurium	5.00	10.00	1.25	0.63	0.31
<i>Staphylococcus aureus</i> strain 1	0.62	1.25	0.16	0.31	0.50
<i>S. aureus</i> strain 2	2.50	2.50	0.63	0.63	0.50
<i>S. aureus</i> methicillin-resistant strain 1	0.62	1.25	0.16	0.31	0.50
<i>S. aureus</i> methicillin-resistant strain 2	1.25	1.25	0.31	0.16	0.38
<i>Staphylococcus epidermidis</i>	1.25	1.25	0.31	0.31	0.50
<i>Streptococcus mutans</i>	2.50	2.50	0.62	0.62	0.50
<i>Streptococcus pyogenes</i>	2.50	2.50	0.63	0.63	0.50

* FIC \leq 0.5: synergic interaction; 0.5 < FIC \leq 1.0: additive interaction; 1.0 < FIC \leq 4.0: no interaction and FIC > 4.0: antagonist interaction.

MIC: Minimal Inhibitory Concentration

ATCC: American Type Culture Colection

FIC: Fractioned Inhibitory Concentration

SB: Honey from *Scaptotrigona bipunctata*

SP: Honey from *Scaptotrigona postica*

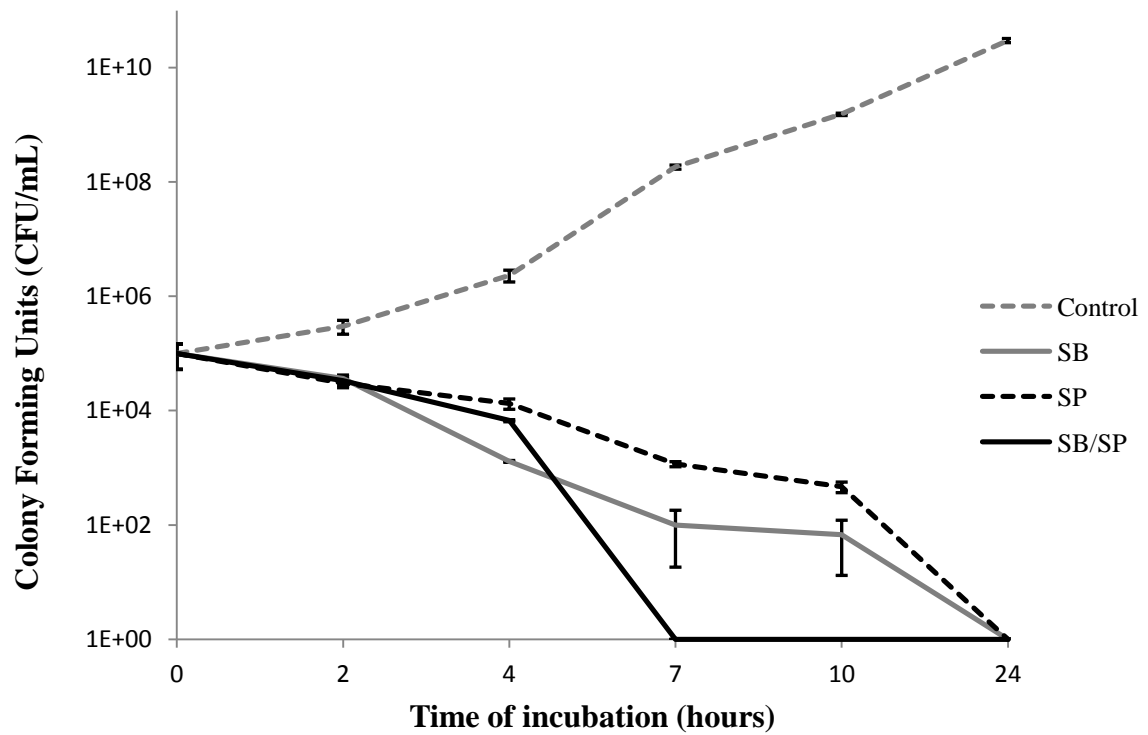
FIGURE 1

Figure 1 - Time-kill curves of *Staphylococcus aureus* MRSA N315 strain exposed to honey in 1xMICs concentrations; SB used alone (1.25%); SP used alone (1.25%); SB/SP (0.31/0.31%) used in combination; Control indicates bacterial growth without honey.

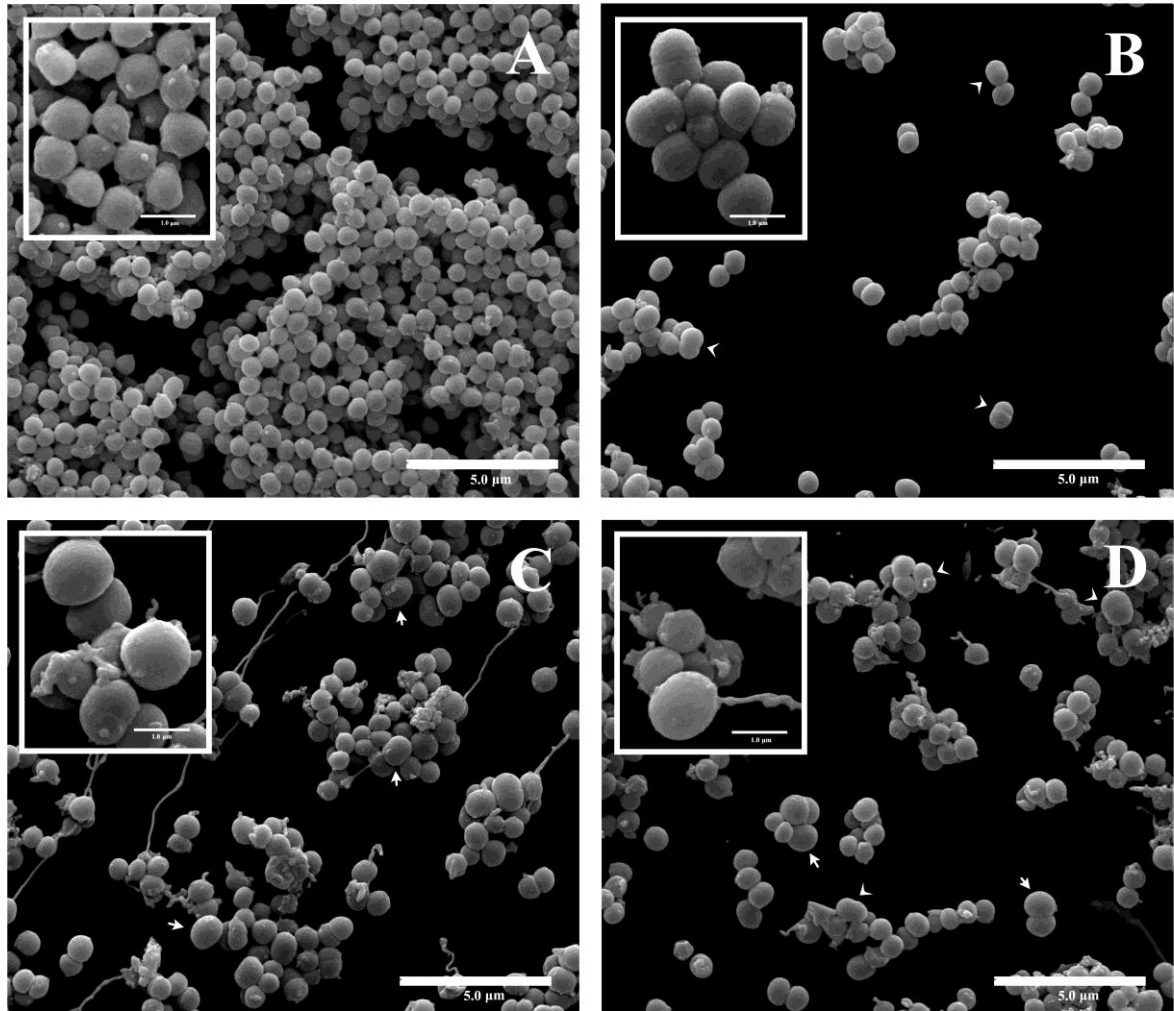
FIGURE 2

Figure 2 - Scanning electron microscopy images of the antibacterial effect of honey SB and SP against MRSA N315 strain after 3 h of incubation. A: negative control (without honey); B: treated with honey SB (1.25%) showing cells with septa (arrowhead); C: treated with honey SP (1.25%) showing spheroplast (arrow); D: treated with both honey, SB (0.31%) and SP (0.31%) showing cells with septa (arrowhead) and spheroplast (arrow). Different morphological alterations can be observed in treatment. Inset images show detail of morphological alterations.

5. CONCLUSÃO

Os méis das abelhas sem ferrão *Scaptotrigona bipunctata* e *Scaptotrigona postica* apresentam atividade antibacteriana contra todas as cepas testadas, tanto Gram-positivas quanto Gram-negativas, sendo todas agentes etiológicos de algum tipo de infecção. Quando usados em combinação o efeito é sinérgico.

A ação deve-se em grande parte devido a presença de peróxido de hidrogênio, existindo outros componentes presentes nos méis com ação antibacteriana.

Os méis estudados causam alterações morfológicas diferentes, sugerindo que cada mel possui mecanismo de ação diferente.

A partir dos resultados do presente trabalho podemos sugerir que compostos naturais, apresentam um grande potencial para serem utilizados como uma boa alternativa terapêutica para a antibioticoterapia convencional principalmente para tratamento e prevenção de infecções relacionadas a assistência à saúde causadas por micro-organismos multirresistentes. A aplicação dos méis poderiam ser realizadas na forma de medicamentos de uso tópico ou como produtos cosméticos.