Chemical Diversity in a Stingless Bee—Plant Symbiosis

Eduardo A. Silva-Junior,‡,§ Camila R. Paludo,‡,∥ Juliano G. Amaral,‡,¶ Marília E. Gallon,‡
Leonardo Gobbo-Neto,† Fabio S. Nascimento,‡ and Norberto P. Lopes‡,†,‡

†NPPNS, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14040-903, Brazil
‡Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14040-901, Brazil
§Centro Universitário de Vale do Araguaia, Barra do Garças, MT 78600-000, Brazil
∥Campus Universitário do Araguaia, Universidade Federal de Mato Grosso, Barra do Garças, MT 78600-000, Brazil
¶Instituto Multidisciplinar em Saúde, Universidade Federal da Bahia, Vitória da Conquista, BA 45029-094, Brazil

ABSTRACT: Bees are essential pollinators on earth, supporting forest equilibrium and human agriculture. The chemistry of the stingless bee—plant symbiosis is a complex and not completely understood phenomenon. Here, we combined untargeted tandem mass spectrometry, molecular networking, and multivariate statistical analysis to investigate the chemical diversity in colonies of the stingless bee Scaptotrigona depilis. Flavonoids were the most representative and diverse group of plant metabolites detected, indicating the importance of these biologically active natural products to the bees. We unveiled the metabolome, mapped the distribution of plant metabolites in stingless bee colonies, and digitized the chemical data into a public database.

INTRODUCTION

Plants and bees engage in symbiotic relationships vital for forest equilibrium and human agriculture. One important group of these pollinators is the stingless bee tribe (Hymenoptera, Apidae, Meliponini), which resides in tropical and subtropical regions of the planet. This group is the most diverse tribe of eusocial corbiculate bees, with more than 500 species distributed in tropical and subtropical areas of the globe. Stingless bees visit several plants to collect resins, pollen, and nectar necessary for hive construction and feeding. Nectar is the main source of carbohydrates, while pollen is the protein source for these pollinators. The plants’ resins are mixed with cephalic glandular secretions to produce propolis, which is essential to seal the colony cavities and to inhibit invading insects and microorganisms.

The stingless bees’ behaviors are coordinated by an elaborate and complex chemical communication system, which is mediated mainly by nonpolar compounds. Cuticular hydrocarbons act as chemical signals to indicate the queen’s presence and to reduce the reproductive behavior of the bee workers. Although there is an increasing knowledge regarding the nonpolar compounds from stingless bees, little is known about the metabolome of the polar compounds. Recent advances in analytical techniques based on mass spectrometry (MS) can be applied to unravel the metabolome of stingless bees’ colonies. The Global Natural Products Social Molecular Networking (GNPS), for instance, has been applied by the scientific community as a valuable tool to unveil the metabolome from microorganisms, plants, animals, and humans. The GNPS molecular networking organizes the compounds according to their tandem mass spectrometry (MS/MS) features in clusters and compare the spectrums with spectral libraries. Hits to the GNPS libraries provide the level 2 annotations, which are putatively annotated compounds according to the metabolomics initiative.

The stingless bee Scaptotrigona depilis participates in a multispecies symbiosis with plants and has recently received attention due to the consumption of a brood fungus required for their larval development. This generalist pollinator can visit up to 54 plant genera over the year to collect pollen. Such a massive collection of plant substrates results in a remarkable and underexplored mixture of natural products in these bees’ colonies. The poor information regarding the chemical diversity of stingless bees’ colonies reinforces the importance to investigate and digitize the chemical data in open databases for future public multi-omics platforms. As an initiative to study the global metabolome of bees, we digitized the metabolites and mapped the chemical diversity in colonies of the fungus-growing stingless bee S. depilis.
RESULTS AND DISCUSSION

Samples of honey, fermented pollen, nurse bees, pupae, larvae, larval food from brood cells with eggs, larval food from brood cells with larvae, brood fungus, cerumen, propolis, and the colony entrance were collected from three colonies of the stingless bee *S. depilis* (Figure 1). The extracts were analyzed by untargeted liquid chromatography coupled with mass spectrometry (LC-ESI-MS/MS) and subjected to multivariate statistical analyses and molecular networking. Unsupervised principal component analysis (PCA) indicated a clustering tendency in four groups, according to the chemical similarity of the detected ions (Figure 2). Subsequently, supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA) revealed the clear separation between the four groups (Figure S1). Pollen, larval food with eggs, larval food with larvae, nurse bees, larvae, and brood fungus were clustered together (group 1), as well as the samples of cerumen, entrance, and propolis (group 2). Meanwhile, the samples of honey (group 3) and pupae (group 4) were chemically different from the other samples. Nurse bees, larvae, and brood fungus were clustered in group 1 due to the presence of compounds from pollen since nurse bees prepare larval food with pollen and larvae and brood fungus grow in intimate contact with larval food.

The mass spectrometry data were submitted to the Global Natural Products Social Molecular Networking platform (GNPS) to generate the molecular networks and to perform the spectral library search. A total of 129129 spectra from positive and negative LC-ESI-MS/MS analyses was merged by GNPS into 3124 nodes, which represent the chemical entities. The nodes were connected based on the MS/MS spectral similarity, resulting in 5018 pairs in the molecular networks (Figure 3 and Figure S2). The large number of nodes revealed the complex chemical composition of samples from *S. depilis* colonies, while the clusters evidenced the diversity of molecular families. The spectral library search resulted in 724 hits, and the majority of MS/MS spectra (76.8%) were not annotated, indicating the potential of these stingless bee colonies as sources of new compounds.

Primary metabolites, such as amino acids, nucleotides, and vitamins, were identified predominantly in pollen, honey, larval food, larvae, pupae, nurse bees, brood fungus, and propolis (Figure S4 and Table S1). Flavonoids were detected in all *S. depilis* samples and were the most diverse class of natural products identified (Figure 4, Figure S5, Table S1). Flavonoids were detected in all *S. depilis* samples and were the most diverse class of natural products identified (Figure 4, Figure S5, Table S1). Flavonoids were grouped in clusters of aglycones, biflavonoids, and flavonoid glycosides. The glycoside attachments were diverse, occurring on carbons or oxygens from rings A, B, and C. Glucose, xylose, and rhamnose were the major glucosides attached. Aglycones were common in all samples, while biflavonoids were restricted to the propolis, cerumen, and colony entrance. Flavonoids were also detected in colonies...
of other stingless bees’ species and the honey bee *Apis mellifera*.19−21

Such a diversity of flavonoids, combined with their notable biological activities, indicates their importance to the bees. The antioxidant activities of flavonoids can contribute to neutralizing the oxidant effects of honey, which presents antibiotic activity mostly due to the presence of hydrogen peroxide (H$_2$O$_2$).22,23 The oxidizing effects of H$_2$O$_2$ can be harmful to the bees, and thus, these pollinators produce enzymes to eliminate H$_2$O$_2$, for example, catalase and peroxidase.24 However, this agent (H$_2$O$_2$) can oxidize compounds from honey originating compounds that cannot be neutralized by these enzymes,25 and flavonoids can play an important role in neutralizing the remaining oxidizing agents.

Flavonoids can also contribute to the chemical protection of bee colonies against invading microorganisms since these compounds have cytotoxic and antimicrobial activities.25 Despite their protective role, flavonoids can be harmful to bees by chelating metals and binding proteins and carbohydrates.26−29 Insects produce surfactants to prevent the injurious effects of polyphenols, such as the lysophospholipids.30,31 We found that *S. depilis* nurse bees, pupae, and larvae also contain lysophospholipids, such as 1-palmitoylglycerylphosphocholine (XIII) and 1-stearoylglycerophosphocholine (XIV), which could protect them against the side effects of flavonoids. Interestingly, the lysophospholipid precursor glycerophosphorylcholine (XII) was detected in pollen and propolis, indicating that plants are also a source of these surfactant precursors for the bees.

The phenolamide spermidine-diferuloyl (XV) was detected in pollen and larval food with larvae, while the spermidine-p-coumaroyl-feruloyl (XVI) was detected in pollen, larval food with eggs and with larvae, brood fungus, larvae, bees, cerumen, and entrance. The absence of spermidine-p-coumaroyl-feruloyl (XVI) in propolis and the presence in cerumen indicate that this compound can be diffused from pollen-derived samples to cerumen. Although containing a high number of plant metabolites, the bacterial antibiotic lincomycin (XVII) was annotated in the *S. depilis* honey. Studies reported the isolation of antibiotic-producing bacteria from bees’ colonies, and lincomycin could be produced by such symbiont bacteria.32,33

The tandem mass spectrometry data of the four groups were also organized in a Venn diagram (Figure 2). This diagram revealed that most of the detected compounds (67.7%) were unique to each group and that 30.8% were shared between two or three groups, while just 1.6% were common to all samples. Flavonoids, riboflavin (X) and adenosine (XI) were detected in all samples, indicating that these compounds can play an important ecological role for the bees. The compounds common to all samples could be investigated in future studies to verify their biological roles in stingless bees’ colonies.

Bees manipulate and consume the plant-derived samples, and consequently, the collected compounds can be metabolized. As we applied untargeted mass spectrometry analysis, these putative metabolites produced by bee metabolism can also be included in the networks. In the case of *S. depilis*, the chemical diversity in pupae was lower than those in the larval food and larva samples (Figures S6−S11), indicating that the
pupae can metabolize the ingested compounds, but the resulting metabolites remain unknown. Flavonoid metabolizing enzymes were found in the honey bee; however, the produced metabolites were not identified.\textsuperscript{34,35} The metabolism of plant metabolites by these pollinators merits to be further investigated, and our data open perspectives to study the metabolism of plant metabolites by bees.

\section*{CONCLUSIONS}

In summary, untargeted LC-ESI-MS/MS, multivariate analyses, GNPS molecular networking, and spectral library searches unveiled the chemical diversity of \textit{S. depilis} colonies. Flavonoids were the most representative group of plant secondary metabolites detected, indicating the importance of these compounds to the bees. The chemical data of our analysis are publicly available from GNPS and can contribute to forthcoming research regarding stingless bees. Our results highlight the importance of plants as sources of primary and secondary metabolites for stingless bees and can also contribute to planning future strategies to protect these threatened pollinators.

\section*{METHODS}

\textbf{Sample Collection.} Samples were collected from three colonies of \textit{S. depilis} maintained at the Department of Biology, University of São Paulo, Ribeirão Preto City, São Paulo State,
Chemical Extraction and Sample Preparation. Samples were first mixed with liquid nitrogen and triturated using a mortar and pestle. Aliquots of 50 mg of larvae and brood cell fungus and 100 mg of the other samples were extracted with 1 mL of methanol for 10 min in an ultrasonic bath followed by 60 min at room temperature. The solvent was evaporated using a SpeedVac, and the resulting crude extracts were cleaned using C$_{18}$ (100 mg) SPE cartridges. The cartridges were activated with pure methanol (1 mL) and conditioned with water (3 mL). Crude extracts were dissolved with 1 mL of 1:9 methanol/water (v/v) and passed through the cartridges, which were washed three times with 1 mL of water. Aliquots of honey (200 μL) were mixed with water (1800 μL) and passed through the cartridges, which were washed three times with 1 mL of water. The samples were eluted with 1 mL of 9:1 methanol/water (v/v) and concentrated to dryness. All samples were dissolved with methanol and filtered (0.22 μm) into 1 mL HPLC vials before the chemical analyses.

Chemical Analyses. Tandem mass spectrometry analyses were carried out on a Shimadzu UFLC system (two LC20AD solvent pumps, a CTO20A column oven set to 40 °C, a SIL20A autosampler, and a CBM20A controller) coupled to an ion trap mass spectrometer (AmaZon SL). The samples (20 μL) were analyzed using a C$_{18}$ Phenomenex column (250 mm × 4.6 mm; 5 μm particle) eluted with a mixture of water (solvent A) and acetonitrile (solvent B), both of which contained 0.1% formic acid, at a flow rate of 1 mL min$^{-1}$, as follows: 0–5 min (10% of B), 5–60 min (10–100% of B), 60–70 min (100% of B), 70–75 min (100–10% of B), and 75–85 (10% of B).

The ion trap mass spectrometer was operated in positive and negative ionization modes with the following parameters: capillary of 3.5 kV; end plate offset of 500 V; nebulizer of 60 psi, dry gas (N$_2$) with a flow of 10 L min$^{-1}$; dry temperature of 330 °C; auto MS/MS acquiring data between m/z 50 and 1300, average of 3 spectra; enhanced resolution for scan mode and UltraScan mode for MS/MS; spectral rate acquisition three spectra per second; exclusion of a particular ion after basis on parameters previously used in GNPS by di Flavio and UltraScan mode for MS/MS; spectral rate acquisition 1300, average of 3 spectra; enhanced resolution for scan mode 1.0 × 10$^3$; chromatogram builder (minimum time span in minutes, 0.2); minimum height, 1.5 × 10$^3$; m/z tolerance, 0.5 m/z or 10 ppm; chromatogram deconvolution using the baseline cut-off (minimum peak height, 1.5 × 10$^3$; peak duration, 0.2 to 1.0; baseline level, 1.0 × 10$^3$); isotopic peak grounder (m/z tolerance, 0.5 m/z or 10 ppm; retention time tolerance in minutes, 0.2; maximum charge, 2; representative isotope, most intense); and alignment using the join aligner (m/z tolerance, 0.5 m/z or 10 ppm; weight for m/z, 20; retention time tolerance, 5%; weight for retention time, 20). After individual preprocessing, the data matrices of the positive and negative ionization modes were combined into a single spreadsheet, and the peaks detected in the controls were removed from each sample.

Unsupervised statistical analysis (principal component analysis, PCA) and supervised statistical analysis (orthogonal projections to latent structures discriminant analysis, OPLS-DA) were carried out with SIMCA 13.0.3.0 (Umetrics, Sweden). The OPLS-DA classes were determined in accordance with the samples: pollen, larval food with eggs, larval food with larvae, brood fungus, larvae, and bees (group 1); propolis, cerumen, and entrance (group 2); honey (group 3); and pupae (group 4). Discriminant variables were assessed by the variable influence on projection (VIP) generated through OPLS-DA. Discriminant compounds were identified based on the GNPS spectral library searches.
ACKNOWLEDGMENTS

The authors thank the São Paulo Research Foundation (FAPESP) (grants #14/50265-3 and #15/05453-9), National Council for Scientific and Technological Development (CNPq) (grant 439498/2016-3), and Coordination for the Improvement of Higher Education Personnel (CAPES Finance Code 001).

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