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Maturation of honey from Uruçú-Amarela (*Melipona mondury*): Metagenomics, metabolomics by NMR ^{1}H, physicochemical and antioxidant properties

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ABSTRACT

The objective of this study was to characterize the microbiota biodiversity of Uruçú-Amarela honey through metagenomics. Furthermore, the impact of maturation temperatures (20 and 30 °C) and time (0–180 days) on the physicochemical and antioxidant properties was investigated. ^{1}H NMR was performed to verify metabolites formed during maturation. Uruçú-Amarela honey was mainly composed by lactic acid bacteria and osmophilic yeasts of genus *Zygosaccharomyces*. Maturation at 30 °C led to a higher fermentation activity, resulting in greater carbohydrate consumption, ethanol formation (0.0–0.6 %) and increased acidity (34.78–45.74 meq/kg) over the 180 days. It also resulted in honey with higher brown color (a* 0.7 to 3.89, b* 17.50–25.29) and antioxidant capacity, corroborating that the maturation is a suitable preservation technique for stingless bee honey, because it does not cause negative changes as it extends the shelf life of the stingless bee honey.

1. Introduction

Native stingless bees are social insects found in most tropical and subtropical regions, such as Australia, Africa, Southeast Asia, and South America (Chuttong et al., 2016). More than 600 species of stingless bees have been identified to date, with more than 200 species and 29 genera distributed throughout Brazil, and honey is the main product these insects supply (Lavinas et al., 2019).

Honey is a natural product derived from nectar from flowers or secretions from living parts of plants or from excretions of plant-sucking insects that bees collect, transform, combine with their specific substances, store, and allow to mature in the combs of the hive (Codex Alimentarius Commission, 2001). However, this definition is specific to honey produced by *Apis mellifera* bees. Therefore, it may not apply to stingless bee honey (Pimentel et al., 2022).

Several studies have reported that honey from different species of stingless bees does not meet the quality standards established for *Apis mellifera* honey (Bogdanov, Martin & Lüllmann, 1997), emphasizing the need for a unique standard for stingless bee honey (Biluca et al., 2016, Braghini et al., 2021, Chuttong et al., 2016, Moniruzzaman et al., 2014, Oddo et al., 2008). Stingless bee honey usually shows a higher acidity and moisture content and lower concentrations of sugar and 5-hydroxymethylfurfural (5-HMF) than *Apis mellifera* honey. In this way, it has attracted consumer attention, mainly from those looking for more acidic and less sweet honey with a unique color and aroma (Braghini et al., 2021).

One of the main concerns about stingless bee honey is preserving the quality of this product, as it has a high moisture content (on average between 25 and 30 %), which facilitates the spontaneous fermentation process after honey harvest. Dehumidification, pasteurization, and refrigeration techniques can improve the preservation of this type of honey. However, these techniques usually cause irreversible changes in the natural properties of honey, such as sugar content and enzymatic activity, in addition to undesirable sensory changes (Camargo, Oliveira, & Berto, 2017, Ribeiro et al., 2018).

Post-harvest maturation is another preservation technique that has been little studied and is already used by Brazilian meliponiculturists, especially at Northeast region of Brazil. This preservation technique was

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developed by the Native Bee Project between 2001 and 2011. It consists in put the newly harvested honey from the hive kept under temperature of 30 °C (average annual temperature from Northeast Brazil), to be fermented by the active microbiota naturally present in the matrix, being recommended from three to eight months of fermentation to obtain an accepted product, in sensory aspects (Camargo et al., 2017; Ribeiro et al., 2018, Villas-Bôas, 2018).

The maturation process is a method that, unlike those mentioned above, does not avoid the fermentation process. In the case of honey, due to its high sugar content, osmophilic yeasts of the genus *Zygosaccharomyces* seem to be the protagonists of fermentation. As these yeasts grow in honey, they compete with other microorganisms, including pathogens, providing food safety for the consumption of this type of honey. As maturation progresses, the concentration of ethanol and organic acids produced by the fermentation itself increases, turning the honey into an inhospitable environment for the yeasts themselves. At this point, fermentation stops resulting in a more acidic, less sweet, and stable honey (Villas-Bôas, 2018).

During maturation, the temperature is the most important parameter to be controlled, as it may impact the honey properties (Ribeiro et al., 2018). Furthermore, it is important to evaluate the microbial diversity of the honey, aiming to obtain information about the natural microbiota (Echeverrigaray et al., 2021). However, only one study evaluated the impact of maturation at 20 and 30 °C on the properties of stingless bee honey (Ribeiro et al., 2018). Still, the authors used Tiúba honey (*Melipona fasciculata*) and only evaluated the physicochemical and sensory properties. *Melipona mondury* (Uruçú-Amarela) is one of Brazil's stingless bee species exploited for honey (Pimentel et al., 2022). However, the intensive culture of this specie is incipient, and the physicochemical characteristics of the produced honey are scarcely reported (Alves et al., 2018). Furthermore, as far as the authors know, only one study performed a metagenomic analysis on honey produced by the bee of this species (Echeverrigaray et al., 2021), still, it evaluated only the biodiversity of yeasts.

Therefore, the objective of this study was to characterize the bacteria and yeast biodiversity of honey produced by the bee Uruçú-Amarela (*Melipona mondury*) using a metagenomic approach, and also to investigate the impact of different temperatures (20 and 30 °C) and times (0 to 180 days) of maturation over the physicochemical and color properties, bioactive compounds content and antioxidant activity, using metabolomics approach.

2. Material and methods

2.1. Honey sample

The honey sample of the stingless bee Uruçú-Amarela (*Melipona mondury*) was produced in Aracruz-ES, Brazil (19°49′13″S/40°16′36 W). A 10L sample was collected and packaged in a single bottle in May 2017 (Brazilian autumn), then it was sent directly to Londrina city (Paraná, Brazil) by airplane, where the research was carried out.

2.2. Uruçú-Amarela honey maturation

The temperatures and times chosen for the maturation of honey were based on Ribeiro et al. (2018), that applied the temperature (30 °C) indicated by the Native Bee Project (Villas-Bôas, 2012), and another one (20 °C) to contemplate other regions of the world with a milder climate.

The refrigerated honey was homogenized manually, and then 50 g were distributed in 33 sterile glass jars with screw caps. Three flasks were separated with unmatured honey (T0), 15 flasks were kept in a Bio-Oxygen Demand (B.O.D.) oven (TE-371, TECNAL, Piracicaba, Brazil) at 20 °C, and 15 flasks were kept in another B.O.D oven at 30 °C, both for 180 days. Every 36 days (20 % of the total time), 3 flasks of each temperature were removed from the ovens for analyses (T36, T72, T108, T144, and T180 days). Every 15 days, the flasks were opened and then closed again, under aseptic conditions, to release any CO_2 possibly formed during fermentation.

2.3. Physicochemical characterization of honey

Over the 180 days of maturation (T0 - T180), the moisture content, total soluble solids (TSS), free acidity, and HMF of the honey samples were evaluated (n = 9), according to the Bogdanov et al. (1997). In addition, water activity (aw) and pH values were determined according to AOAC (2012) (n = 9). Sugars of honey samples determined according to Justus, Ida and Kurozawa (2021) with modifications. The sugars were quantified in an HPAEC-PAD chromatography (ICS 5000, Dionex Canada Ltd., Oakville, Canada), equipped with a CarboPac PA-10 analytical column (250 mm × 4 mm, id, Dionex, Oakville, Canada). Samples diluted in ultrapure water and filtered (0.22 μm) were injected into the equipment (10 μl). The carbohydrates were separated in isocratic elution of 20 mM NaOH, using ultrapure water (90 % mobile phase A) and 200 mM NaOH (10 % mobile phase B) in a flow of 1 mL/min at 25 °C for 27 min (n = 9). For the quantification of carbohydrates, analytical curves of standards glucose (R^2 = 0.9992); fructose (R^2 = 0.9955), maltose (R^2 = 0.9989) and sucrose (R^2 = 0.9978) were used (Sigma-Aldrich, St. Louis, USA).

The color parameters, in the Cielab L*a*b* system, were determined at 25 °C using a colorimeter (Konica Minolta – Chroma Meter CR-4000, Osaka, Japan) with D65 illuminant (daylight) (Ribeiro et al., 2018) (n = 9).

2.4. Bioactive compounds and antioxidant activity

At all maturation times (T0 – T180), the total phenolic compounds (TPC) were determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965) and expressed in mg equivalent of gallic acid per kg of the sample (mg eq GA/kg), calculated from a standard curve of gallic acid (Sigma-Aldrich, St. Louis, USA) (0 – 100 mg/mL, R^2 = 0.9983) (n = 9). The content of total flavonoids (TF) was determined according to Arvouet-Grand et al. (1994). The results are expressed in mg equivalent of quercetin per kg of honey (mg eq QUERC/kg), calculated from the standard curve for quercetin (Sigma-Aldrich, St. Louis, USA) (0 - 100 μg/mL, R^2 = 0.9991) (n = 9). Total phenolic acids (TPA) were determined according to Bueno-Costa et al. (2016) and expressed in mg equivalent of caffeic acid per kg of honey (mg eq CA/kg), calculated from a standard curve of caffeic acid (0 - 6 μg/mL, R^2 = 0.9976) (n = 9).

The impact of maturation on the antioxidant activity of honey was evaluated through the Ferric Reducing Ability of Plasma (FRAP) assay, determined according to Benzie and Strain (1996) and expressed in mM Fe^{2+}/g (n = 9) and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity, determined according to Re et al. (1999) and expressed in Trolox micromolar equivalent (μM TE/g) determined from a Trolox curve (0 - 500 μM, R^2 = 0.9911) (n = 9).

2.5. Metagenomics analysis

2.5.1. DNA extraction and amplification of 16S and ITS rRNA regions

For DNA extraction, honey was diluted in distilled water (1:3, w/v), followed by oven incubation at 55 °C (TE-394/2, TECNAL, Piracicapa, Brazil) for 30 minutes. The sample was separated into smaller volumes and centrifuged at 10,000 × g for 10 minutes in a microcentrifuge (Heraeus Pico 21, Thermo Fisher Scientific, Waltham, USA), the supernatant was removed, and the pellet obtained was resuspended in PBS; then the DNA from microbiota in the honey was extracted using a Quick-DNA™ Fungal/Bacterial Miniprep (Zymo Research, Orange, USA) assay kit, according to the manufacturer's protocol. DNA concentration was evaluated by fluorimetry, and quality was analyzed by electrophoresis (1 % w/v agarose) and polymerase chain reaction (PCR), using specific primers for 16S and ITS rRNA genes, as suggested by Weisburg et al. (1991). PCR reactions were conducted with 20 μL final volume, with 10

μL of GoTaq® Colorless Master Mix 2x (Promega Co., Madison, USA), 0.3 μM of the reverse oligonucleotide, 1 μL of genomic DNA, and sterile ultra-pure water to reach 20 μL. For the amplification of V4 region of the 16S rRNA gene were used the primers 515F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA-3) and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT-3'). For the ITS regions the primers ITS-86F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGAATCATCGAATCTTTGAA-3') and ITS-4R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-3') were used (Jo, Hong, & Unno, 2019).

The amplification was performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, USA). The program for the 16S (V4) region consisted of initial denaturation at 94 °C for 3 min, followed by 29 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The program for the ITS region consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 40 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min. After each sample amplification, the procedure was verified in electrophoresis (2 % w/v agarose) stained with UniSafe Dye 0.03 % (v/v) (Uniscience, Osasco, BR) (Weisburg et al., 1991). Amplification products were purified with magnetic beads Agencourt AMPure XP (Beckman Coulter, Indianapolis, USA) and quantified with KAPA® Fast Universal kit (Merck, Darmstadt, DE) according to the supplier protocols.

2.5.2. Next-Generation sequencing and data analysis

In this step, indexers were inserted in the common adapters necessary for generating clusters and sequencing the samples. The indexation reaction was performed following the kit protocol Nextera XT Index (Illumina, San Diego, USA). The amplification program consisted of incubation at 72 °C for 3 min, initial denaturation at 95 °C for 30 s, followed by 12 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 5 min conducted in a Veriti 96- Well Thermal Cycler (Applied Biosystems, Carlsbad, USA). The generated libraries were purified and quantified with the same protocol described in the amplification step. An equimolar pool of DNA was obtained with sample normalization utilized to the NGS in the MiSeq system (Illumina, San Diego, USA) (Jo, Hong, & Unno, 2019). Sequences with 97 % similarity were assigned to the same operational taxonomic units (OTUs). Phylogenetic trees for 16S and ITS were obtained using the web tool phyloT, based on NCBI taxonomy.

2.6. ^{1}H NMR spectroscopy for metabolites identification during maturation of Uruçú-Amarela honey

The preparation of honeys samples and in ^{1}H NMR analysis parameters were performed according to Del Campo et al. (2016), with slightly modifications. The total sugar percentage of each honey sample was previously measured by refractometry (Metler Toledo - LiquiPhysics™ Excellence RM40, Greifensee, Switzerland). Next, 20 g of honey was weighed and mixed with 5 mL of water. The pH of the resulting solution was adjusted to 1.0 by adding HCl 3.0 M. Then, the total sugar percentage was reduced to 40.0 % (w/w) by dilution with acidified water at pH 1.0. Finally, the solution was filtered through a 0.45 μm nylon membrane (Cameo, Scharlab, Spain). A total of 600 μL of the samples (above pretreated honey solution) was placed into a 5 mm diameter NMR tube (Sigma Aldrich, St. Louis, USA), with 100 μL of a solution containing 70 % (v/v) D_2O and 10.0 g/L of sodium trimethylsilyl propionate (TSP) (Sigma Aldrich, St. Louis, USA). TSP was added as an internal standard that supplied a reference for the chemical shift. One-dimensional spectra were recorded on a Bruker spectrometer (Mod. Ascend 400, Karlsruhe, Germany) at 400.13 MHz for a ^{1}H set with a 5 mm multinuclear probe. To obtain the spectra of the samples, 128 scans of 32 K data points were acquired at 25 °C using a spectral width of 6393 Hz (15 ppm), the acquisition time of 4.0 s, recycle delay of 2.0 s, and a 90° flip angle, requiring approximately 14 min per sample. Water suppression was achieved using the one-dimensional nuclear Overhauser effect spectroscopy (NOESY 1D) pulse sequence. The spectra were referenced to the TSP singlet peak at 0.0 ppm. The resulting spectra were phased, baseline corrected manually to attain reliable results using TopSpin software version 3.6.3 (Bruker Biospin, Karlsruhe, Germany), and converted to CSV file format (spectra region ranging from 10.5 - 0 ppm). Data values were entered into Microsoft Excel spreadsheets for further processing.

2.6.1. NMR data processing and multivariate statistical analysis

The resulting data set from NOESY 1D NMR experiments were imported into the web-based metabolomics data processing tool MetaboAnalyst platform (version 5.0) for multivariate statistical analysis. The data were then filtered in interquartile range (IQR) and row-size normalized by the sum of the intensities to reduce systematic bias during sample collection (Worley & Powers, 2016). Finally, principal component analysis (PCA) was used for a synopsis of the grouping trend within the data set and outlier detection.

2.7. Experimental design and statistical analysis

The experiments followed a completely randomized design with three replications. The split-plot scheme was used, with the maturation time (T0 – T180) being the main treatment and the maturation temperature the secondary. The physicochemical and color determinations were carried out in triplicate for each repetition (n = 9). The data were submitted for analysis of variance (ANOVA) and mean comparison test, using the Tukey test to compare the results over time and the t-test to compare the results between the two maturation temperatures. Both statistical tests were performed at the 5 % significance level using the Sisvar 5.6.86 software (DES/UFLA).

For global analysis of results, PCA was performed on average data from physicochemical characterization, color properties, bioactive compounds, and antioxidant activity of honey over 180 days of maturation at both temperatures. The principal components (PC) with eigenvalues equal to or greater than 1 were chosen to interpret the PCA, following the Kaiser criterion (Kaiser, 1958). To know the importance of each variable in each PC, the numerical correlations between them were obtained, with minimum correlations of 0.7 being considered important in interpreting the PC. Pearson's correlation was also evaluated to verify significant correlations between the analyzed variables. The PCA for NMR data was performed using the MetaboAnalyst platform (version 5.0). This web application performs statistical computing and visualization operations using functions from R packages (Chong, Wishart, & Xia, 2019). For the physicochemical and antioxidant properties of Uruçú-Amarela honey, PCA was performed using the XLSTAT software for Windows (version 2021; Addinsoft, New York, NY).

3. Results and discussion

3.1. Bacteria and yeast biodiversity of Uruçú-Amarela honey

It is noteworthy that this is the first study to identify the bacterial microbiota and the second to identify the yeast biodiversity of Uruçú-Amarela honey. The first report about the yeast biodiversity of this honey was made by Echeverrigaray et al. (2021).

The high concentration of sugars represents a challenge for the extraction of DNA from honey. In addition to carbohydrates, polyphenols can also interfere on the enzymatic reactions that are involved in the evaluation of extraction quality, such as in PCR reactions. According to the manufacturer of the extraction kit used in the present study, with previous scientific corroboration by Lalhmangaihi et al. (2014), the steps of dilution in water and pre-incubation of honey help

to minimize these interferents.

The metagenomic analysis generated 21,991 reads in the sequencing of the 16S rRNA gene and 13,516 reads in the ITS fungal region, with 54.3 OTUs assigned for bacterial diversity and 27 OTUs for yeasts. Figure 1 presents the microbial community identified in honey from Uruçú-Amarela. Bacteria were distributed in six different phyla, predominately Firmicutes (71 % of bacterial diversity) and Proteobacteria (28 %) (Figure 1A). These phyla were also reported as predominant in other taxonomic characterization studies of other honeys (Bovo et al., 2020; Ngalimat et al., 2019), gastrointestinal tract of stingless bees (Kwong et al., 2018), and pollen (Casalone et al., 2020).

The bacterial biodiversity of Uruçú-Amarela honey was mainly composed of lactic acid bacteria, with 70 % of the identified bacteria belonging to the Lactobacillaceae family, containing 64 % of bacteria of the genus *Lactobacillus*, considered the dominant bacterial genus in honey from stingless bee (Rosli et al., 2020), 5 % of *Pediococcus* and 1 % of *Fructobacillus* (Figure 1C). Among Proteobacteria, mainly enterobacteria were identified (14 % of the total diversity), in addition to bacteria of the genus *Acinetobacter* (4 %), *Saccharibacter* (4 %), and *Pseudomonas* (2 %) (Figure 1C). The bacterial genera often associated with stingless bee species are *Lactobacillus*, *Bacillus*, *Streptomyces*, *Clostridium*, *Staphylococcus*, *Streptococcus*, *Enterobacter*, *Ralstonia*, *Pantoea*, *Pseudomonas*, *Fructobacillus*, *Lysinibacillus* and *Neisseria* (Paula et al., 2021). Our results demonstrate the most prevalent species in Uruçú-Amarela honey.

The Firmicutes phylum was also dominant in the honey biodiversity of *Heterotrigona itama* from Malaysia, analyzed by Ngalimat et al. (2019), with a predominance of bacteria of the genus *Bacillus*, a genus that was not identified in the Uruçu-Amarela honey that we analyzed. These authors also reported that the phylum Proteobacteria was the second most abundant, with a predominance of the genus *Enterobacter*, as we observed in Uruçu-Amarela honey, which demonstrates a high occurrence of these bacteria from the gastrointestinal tract of stingless bees in honey, regardless of the species and region where the honey was produced. Beux et al. (2022) also reported a predominance of *Bacillus* sp. in *Tetragonisca angustula* (Jataí) honey, produced in Curitiba, Brazil.

Several bacteria isolated from stingless bee honey can use different carbohydrates, amino acids, and carboxylic acids. In addition, species such as *Lactobacillus* sp. may be associated with the antimicrobial properties of honey by producing bacteriocins and organic acids during the fermentation process (Ngalimat et al., 2019; Paula et al., 2021). Gammaproteobacteria of the Enterobacteriaceae family originates from bees' pollen and intestinal tract (Casalone et al., 2020). Likewise, bacteria of the genus *Acinetobacter* come from the soil and pollen. Alphaproteobacteria of the Acetobacteraceae family (acetic acid bacteria) come from the intestinal tract of sugar-eating insects (Siozios et al., 2019). In the analyzed honey from Uruçú-Amarela, the main acetic acid bacteria identified were *Saccharibacter floricola*.

Regarding the ITS rRNA region, there was a predominance of microorganisms from the phylum Ascomycota (92.5 % of the total diversity of eukaryotes, Figure 1B), with 92 % of the microorganisms identified in the ITS region belonging to the genus *Zygosaccharomyces* (Figure 1D). The species *Z. mellis* (51.3 %) and *Z. pseudorouxii* (40.7 %) were the only two species of yeast identified in the Uruçú-Amarela honey from Aracruz, Brazil. Yeasts of this genus act as symbionts in the development of bees by providing ergosterol to larvae that ingest this yeast, allowing their pupation (Paludo et al., 2018), which explains the predominance of this genus among the microorganisms identified in the ITS region.

Echeverrigaray et al. (2021) evaluated the yeast biodiversity in honey from Uruçú-Amarela produced in southern Brazil (Nova Petropolis, RS) and verified only the presence of yeast of the *Starmerella* genus, but not *Zygosaccharomyces* sp. as in this study. The region affects the biodiversity of yeasts in honey produced by the same species of stingless bee, since the presence of *Zygosaccharomyces mellis* was identified in the honey of other species of stingless bee produced in the same region. Beux et al. (2022) identified the presence of the osmophilic yeast *Zygosaccharomyces bailli* in the honey of *T. angustula* from Brazil, in addition to yeasts of the genus *Starmerella* and *Candida*.

The abundance of the genera *Lactobacillus* and *Zygosaccharomyces* in honey from Uruçú-Amarela may be associated with the fact that stingless bees collect food close to the hive, which causes a small variety of microorganisms from the environment to be collected (Rosli et al., 2020).

3.2. Characterization of the physicochemical properties, color, and antioxidant activity of Uruçú-Amarela honey over 180 days of maturation

The stabilization of the maturation process was visually identified when the foam collar, formed during fermentation, adhered to the flask containing the honey and it did not move with the inclination of the flask, as recommended in the Technical Manual for the Full Use of Native Stingless Bee Products (Villas Bôas, 2012). This behavior was observed at the end of the 180 days of evaluation.

The PCA's first three components had eigenvalues greater than 1, indicating that they should be interpreted according to the Kaiser Criterion (Kaiser, 1958). The first principal component (PC1) explained

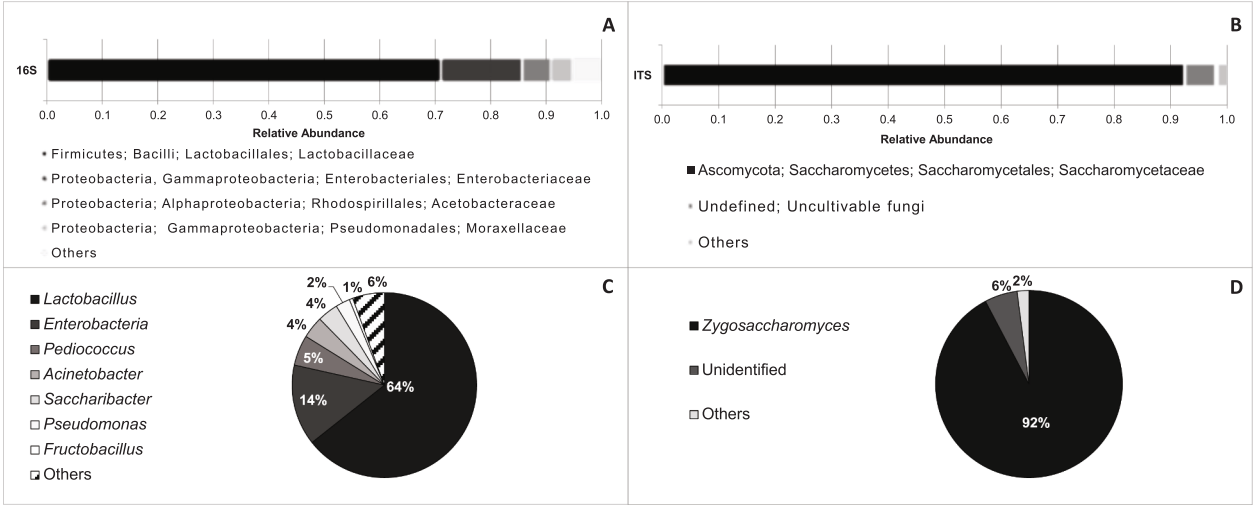

Fig. 1. Microbial community associated with Uruçú-Amarela honey from Aracruz-ES, Brazil. A: Relative abundance of bacterial taxonomic diversity (Phylum; Class; Order; Family) identified in the 16S region. B: Abundance of identified bacterial genera. C: Relative abundance of taxonomic diversity of fungi and yeasts (Phylum; Class; Order; Family) identified in the ITS region. D: Abundance of yeast and fungal genera identified in honey.

62.69 % of the variability contained in the original variables, whereas the second (PC2) and third (PC3) principal components explained 16.14 and 11.19 %, respectively, explaining 90.02 % of the total variability.

To simplify the interpretation of the different dimensions of the PCA, the factors were orthogonally rotated following the VARIMAX transformation with the Kaiser normalization method (Marchi et al., 2012, Figures 2A and B). As a result, a new distribution of the total variance explained by each component was obtained (D1 = 34.57 %, D2 = 27.87 % and D3 = 27.58 %), maintaining the accumulated variance for the first three components (90.02 %). Although the variance accumulated by the first three components remained the same, the distribution among the components was more homogeneous (Pimentel et al., 2015).

On each axis (D1, D2, or D3), the attributes that showed a correlation coefficient with the component higher than 0.7 (absolute values) were considered important (Marchi et al., 2012). In D1, HMF, ethanol, and a* and b* color components were positively correlated with this axis, while pH and L* were negatively correlated. The TSS, fructose, TPA, and FRAP antioxidant capacity were positively correlated with D2, while moisture and TF were positively correlated with D3 (Figure 2A and B).

The first principal component (D1) separated the samples in terms of the maturation temperature, with samples matured at 20 °C grouped on the left side and samples matured at 30 °C on the right side of the axis. The samples T0, T36, and T72 matured at 30 °C. They were located in the center of this axis, showing that for the maturation at 30 °C, the physicochemical properties of honey had major changes after 72 days of maturation. The second principal component (D2) also separated the samples based on the maturation temperature, as the samples matured at 30 °C were above the axis (except T180). The Uruçú-Amarela honey matured at 30 °C and was characterized by having higher contents of HMF and ethanol and higher values for a* and b* color components. In comparison, the honey matured at 20 °C with higher fructose contents and higher FRAP antioxidant capacity, especially at the T36. Finally, it is noteworthy that the third principal component (D3) represents the maturation effect on the Uruçú-Amarela honey since this axis separated the non-matured honey sample from the matured ones, regardless of the temperature applied, confirming that alterations in the physicochemical and antioxidant properties of the Uruçú-Amarela honey occurred since the early days of maturation.

Table 1 shows the physicochemical, color parameters, bioactive compounds, and antioxidant activity of the Uruçú-Amarela honey. Uruçú-Amarela honey showed moisture content of 27.76-32.83 g/100 g, glucose content of 22.37-25.53 g/100 g, fructose content of 26.07-25.53 g/100 g, free acidity of 34.78-43.89 meq/kg, and HMF of 0-18.81 mg/kg. Considering the standards for honey (Bogdanov et al., 1997), Uruçú-Amarela honey presented HMF and free acidity in the preconized values (<40 mg/kg for HMF and < 50 meq/kg for free acidity). However, it presented higher moisture and lower sugar contents than the established (<20 g/100 g for moisture and > 60 g/100 g of fructose + glucose). Our results emphasize the need for a standard for stingless bee honey, as suggested in previous studies (Biluca et al., 2016, Braghini et al., 2021, Chuttong et al., 2016, Moniruzzaman et al., 2014, Oddo et al., 2008).

The honey matured at 30 °C showed lower moisture content, sugar content (glucose and fructose), and pH values, and higher TSS and ethanol contents compared to the honey matured at 20 °C ($p < 0.05$). On the other hand, no effects on protein, ash, and aw were observed ($p > 0.05$). These results are mainly associated with the fermentative metabolism of yeasts and lactic acid bacteria in honey from Uruçú-Amarela (Figure 1). Their activities were more favored at 30 °C than at 20 °C, Ribeiro et al., 2018 observed the same phenomena for maturation of *M. fasciculata* honey. The increased acidity is essential in preserving this honey, preventing the development of pathogenic and spoilage microorganisms that can negatively alter its properties. Furthermore, the increased acidity and the lower sugar content may attract more consumers, mainly those looking for more acid and less sweet honey (Gonçalves et al., 2018).

Since there was water loss during the maturation at both temperatures (Table 1) and considering the sugar content in dry basis, is possible to note that glucose, after 180 days decreased from 38.01 % to 32.28 % and 30.97 % for the maturation at 20 °C and 30 °C, respectively. Fructose decreased from 42.86 % to 36.76 % and 36.09 %, for maturation at 20 and 30 °C, respectively.

Zygosaccharomyces sp. are fructophilic yeasts (Escott et al., 2018) and are capable to produce ethanol from fructose, so is possible to infer that this sugar was fermented to ethanol throughout maturation, while suggesting glucose was converted into gluconic acid by the enzyme glucose oxidase, which led increasing total acidity (Table 1), showing that this enzyme plays an important role during the stingless bee honey maturation.

In addition, may have occurred a fermentation of trehalulose, an oligosaccharide recently discovered by Fletcher et al. (2020) in the honey of different species of stingless bees from different localities (*Tetragonula hockingsi* and *T. carbonaria* from Australia, *Geniotrigona*

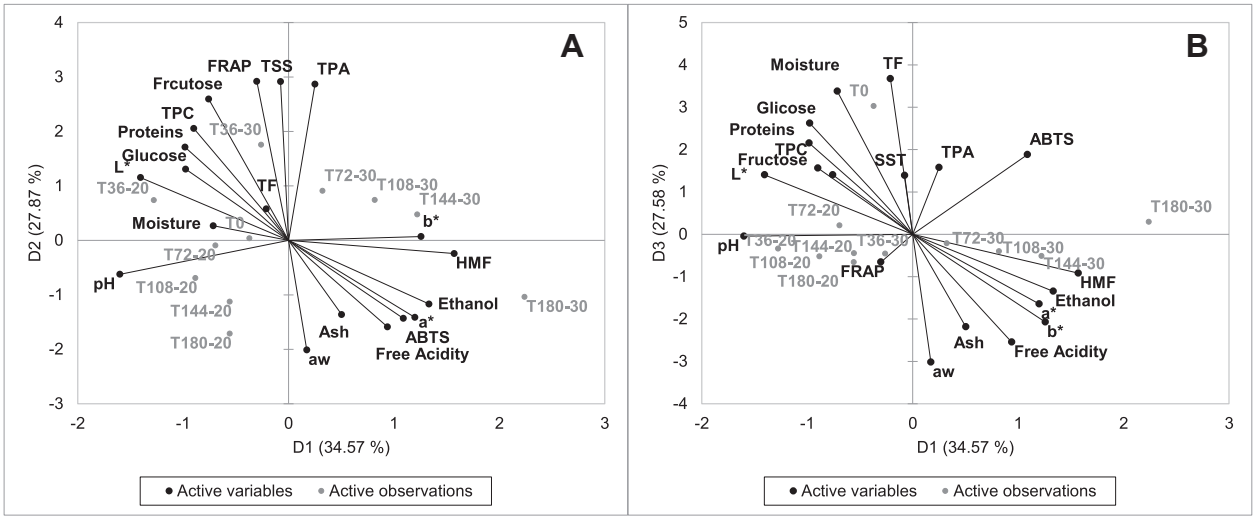

Fig. 2. Projections of physicochemical parameters, color and antioxidant activity of Uruçú-Amarela honey over 180 days of maturation at 20 and 30 °C on factorial plans (A: D1 × D2 and B: D1 × D3) of the PCA. TSS: Total Soluble Solids; aw: Water activity; HMF: Hydroxymethylfurfural; L*: Luminosity; a*: Red/green component; b*: yellow/blue component; TPC: Total Phenolic Compounds; TF: Total Flavonoids; TPA: Total Phenolic Acids; ABTS: ABTS radical scavenging; FRAP: Iron ion reducing power. T0-20 – T180-20: different maturation times (days) at 20 °C; T0-30 – T180-30: different maturation times (days) at 30 °C.

Table 1
Characterization of physicochemical properties, color, bioactive compounds and antioxidant activity of Uruçú-Amarela honey over 180 days of maturation at 20 and 30 °C.

Parameter¥	T °C	Days of Maturation					
		0	36	72	108	144	180
Moister (g.100 g^{-1})	20 °C	32.83 ± 0.81^{Aa}	30.27 ± 0.33^{Ab}	29.06 ± 0.05^{Ac}	28.54 ± 0.15^{Acd}	28.30 ± 0.09^{Ad}	28.28 ± 0.09^{Ad}
	30 °C	32.83 ± 0.81^{Aa}	28.19 ± 0.24^{Bb}	28.16 ± 0.09^{Bb}	28.01 ± 0.05^{Bb}	27.86 ± 0.12^{Bb}	27.76 ± 0.05^{Bb}
TSS (°Brix)	20 °C	70.79 ± 0.34^{Aa}	70.55 ± 0.02^{Bb}	70.08 ± 0.11^{Bc}	69.70 ± 0.05^{Bd}	69.46 ± 0.06^{Be}	68.38 ± 0.15^{Bf}
	30 °C	70.79 ± 0.34^{Aa}	70.68 ± 0.02^{Ab}	70.58 ± 0.11^{Ab}	70.42 ± 0.05^{Ac}	70.32 ± 0.06^{Ac}	69.32 ± 0.15^{Ad}
aw	20 °C	0.73 ± 0.01^{Ab}	0.76 ± 0.01^{Aa}	0.76 ± 0.01^{Aa}	0.78 ± 0.01^{Aa}	0.78 ± 0.01^{Aa}	0.78 ± 0.01^{Aa}
	30 °C	0.73 ± 0.01^{Ab}	0.76 ± 0.01^{Aa}	0.76 ± 0.01^{Aa}	0.76 ± 0.01^{Aa}	0.77 ± 0.01^{Aa}	0.77 ± 0.01^{Aa}
Proteins (g.100 g^{-1})	20 °C	0.39 ± 0.01^{Aa}	0.37 ± 0.01^{Aa}	0.34 ± 0.01^{Ab}	0.30 ± 0.03^{Ac}	0.28 ± 0.03^{Ac}	0.28 ± 0.01^{Ac}
	30 °C	0.39 ± 0.01^{Aa}	0.34 ± 0.01^{Bb}	0.30 ± 0.01^{Bc}	0.30 ± 0.01^{Ac}	0.27 ± 0.01^{Ad}	0.26 ± 0.02^{Ad}
Ash (g.100 g^{-1})	20 °C	0.26 ± 0.01^{Aa}	0.28 ± 0.02^{Aa}	0.28 ± 0.01^{Aa}	0.28 ± 0.03^{Aa}	0.28 ± 0.05^{Aa}	0.28 ± 0.04^{Aa}
	30 °C	0.26 ± 0.01^{Ab}	0.26 ± 0.01^{Ab}	0.28 ± 0.02^{Aba}	0.28 ± 0.02^{Aba}	0.28 ± 0.01^{Aba}	0.29 ± 0.01^{Aa}
pH	20 °C	3.87 ± 0.02^{Ac}	3.90 ± 0.01^{Ab}	3.90 ± 0.01^{Aba}	3.90 ± 0.01^{Aba}	3.90 ± 0.01^{Aba}	3.91 ± 0.01^{Aa}
	30 °C	3.87 ± 0.02^{Aa}	3.86 ± 0.01^{Ba}	3.83 ± 0.01^{Bb}	3.78 ± 0.01^{Bc}	3.74 ± 0.02^{Bd}	3.72 ± 0.02^{Bd}
Free Acidity (meq.Kg^{-1})	20 °C	34.78 ± 0.63^{Ad}	39.29 ± 0.75^{Ac}	39.50 ± 0.73^{Bc}	41.91 ± 0.20^{Ab}	42.30 ± 0.59^{Bb}	43.89 ± 0.81^{Ba}
	30 °C	34.78 ± 0.63^{Af}	38.75 ± 0.00^{Be}	40.33 ± 0.23^{Ad}	41.54 ± 0.84^{Ac}	44.07 ± 0.00^{Ab}	45.74 ± 0.27^{Aa}
HMF (mg.Kg^{-1})	20 °C	0.00 ± 0.00^{Af}	1.82 ± 0.06^{Be}	1.93 ± 0.02^{Bd}	2.07 ± 0.02^{Bc}	3.17 ± 0.08^{Bb}	4.57 ± 0.13^{Ba}
	30 °C	0.00 ± 0.00^{Af}	3.96 ± 0.72^{Ae}	6.52 ± 0.12^{Ad}	9.45 ± 0.97^{Ac}	15.12 ± 0.33^{Ab}	18.81 ± 0.38^{Aa}
Ethanol (%)	20 °C	0.00 ± 0.00^{Ad}	0.10 ± 0.01^{Ac}	0.10 ± 0.01^{Bc}	0.20 ± 0.02^{Bb}	0.2 ± 0.01^{Bb}	0.30 ± 0.02^{Ba}
	30 °C	0.00 ± 0.00^{Ae}	0.00 ± 0.00^{Be}	0.20 ± 0.04^{Ad}	0.40 ± 0.12^{Ac}	0.5 ± 0.10^{Ab}	0.60 ± 0.01^{Aa}
Glucose	20 °C	25.53 ± 0.14^{Aa}	24.12 ± 0.24^{Ab}	23.52 ± 0.87^{Abc}	23.42 ± 0.21^{Ac}	23.32 ± 0.11^{Ac}	23.15 ± 0.06^{Ac}
	30 °C	25.53 ± 0.14^{Aa}	24.20 ± 0.09^{Ab}	23.86 ± 0.21^{Ac}	23.08 ± 0.02^{Bd}	22.84 ± 0.34^{Bd}	22.37 ± 0.10^{Be}
Fructose	20 °C	28.79 ± 0.21^{Aa}	28.75 ± 0.37^{Aa}	28.12 ± 0.80^{Aab}	27.20 ± 0.77^{Ab}	26.95 ± 0.44^{Ab}	26.34 ± 0.04^{Ac}
	30 °C	28.79 ± 0.21^{Aa}	28.66 ± 0.20^{Aab}	28.39 ± 0.61^{Aab}	27.95 ± 0.54^{Ab}	27.03 ± 0.29^{Ac}	26.07 ± 0.16^{Bd}
L*	20 °C	49.52 ± 0.27^{Aa}	49.32 ± 0.26^{Aa}	48.47 ± 0.28^{Ab}	47.24 ± 012^{Ac}	45.79 ± 0.31^{Ad}	45.71 ± 0.21^{Ad}
	30 °C	49.52 ± 0.27^{Aa}	47.31 ± 0.56^{Bb}	46.49 ± 0.21^{Bc}	45.83 ± 0.19^{Bd}	44.42 ± 0.31^{Be}	41.18 ± 0.42^{Bf} Continuation
a*	20 °C	0.70 ± 0.03^{Ae}	1.40 ± 0.04^{Bd}	1.44 ± 0.04^{Bd}	1.66 ± 0.04^{Bc}	2.03 ± 0.08^{Bb}	2.61 ± 0.19^{Ba}
	30 °C	0.70 ± 0.03^{Ad}	1.68 ± 0.09^{Ac}	2.12 ± 0.15^{Ab}	2.22 ± 0.12^{Ab}	2.21 ± 0.19^{Ab}	3.89 ± 0.19^{Aa}
b*	20 °C	17.50 ± 0.13^{Ae}	17.64 ± 0.39^{Be}	20.42 ± 0.12^{Bd}	21.25 ± 0.19^{Bc}	22.40 ± 0.22^{Bb}	22.96 ± 0.28^{Ba}
	30 °C	17.50 ± 0.13^{Ad}	23.71 ± 0.27^{Ac}	24.41 ± 0.25^{Ab}	24.83 ± 0.33^{Aab}	25.27 ± 0.34^{Aa}	25.29 ± 0.19^{Aa}
TPC (mg eq GA.Kg^{-1})	20 °C	515.11 ± 1.15^{Aa}	507.11 ± 0.50^{Ab}	505.28 ± 0.27^{Ac}	504.75 ± 0.51^{Ac}	470.82 ± 1.50^{Bd}	469.39 ± 0.15^{Ad}
	30 °C	515.11 ± 1.15^{Aa}	504.57 ± 0.76^{Bb}	504.39 ± 0.31^{Bb}	486.89 ± 1.00^{Bc}	482.96 ± 1.42^{Ad}	463.5 ± 0.25^{Be}
TF (mg eq QUERC.Kg^{-1})	20 °C	35.43 ± 0.24^{Aa}	26.86 ± 0.01^{Bb}	26.24 ± 0.02^{Bc}	26.04 ± 0.40^{Acd}	26.16 ± 0.43^{Acd}	25.34 ± 0.72^{Ad}
	30 °C	35.43 ± 0.24^{Aa}	27.49 ± 0.60^{Ab}	27.47 ± 0.14^{Ab}	26.34 ± 0.01^{Ac}	26.25 ± 0.32^{Ac}	26.24 ± 0.43^{Ac}
TPA (mg eq CA.Kg^{-1})	20 °C	4.91 ± 0.07^{Aa}	4.46 ± 0.02^{Bb}	4.39 ± 0.02^{Bc}	4.01 ± 0.18^{Bd}	3.96 ± 0.18^{Bd}	3.75 ± 0.03^{Be}
	30 °C	4.91 ± 0.07^{Aa}	4.90 ± 0.05^{Aa}	4.90 ± 0.13^{Aa}	4.84 ± 0.01^{Aa}	4.46 ± 0.05^{Ab}	4.19 ± 0.14^{Ac}
FRAP (mM Fe^{2+}.g^{-1})	20 °C	4.04 ± 0.06^{Ab}	4.72 ± 0.05^{Ba}	4.08 ± 0.18^{Ab}	3.96 ± 0.07^{Bb}	3.83 ± 0.02^{Bc}	3.80 ± 0.05^{Ac}
	30 °C	4.04 ± 0.06^{Ad}	5.19 ± 0.06^{Aa}	4.33 ± 0.09^{Ab}	4.30 ± 0.06^{Ab}	4.19 ± 0.02^{Ac}	3.83 ± 0.03^{Ae}
ABTS (TEAC mM.g^{-1})	20 °C	8.50 ± 0.00^{Aa}	7.71 ± 0.17^{Ac}	8.14 ± 0.35^{Ab}	8.11 ± 0.06^{Ab}	8.12 ± 0.15^{Ab}	8.22 ± 0.29^{Bab}
	30 °C	8.50 ± 0.00^{Ab}	7.98 ± 0.27^{Ac}	8.19 ± 0.00^{Ac}	8.24 ± 0.13^{Ac}	8.11 ± 0.17^{Ac}	8.89 ± 0.08^{Aa}

¥TSS: total soluble solids; aw: water activity; HMF: hydroxymethylfurfural; L*: luminosity; a*: red/green coordinate; b*: yellow/blue coordinate; TPC: total phenolic compounds (equivalent milligrams of gallic acid/kg); TF: total flavonoids (equivalent milligrams of quercetin/kg); TPA: total phenolic acids (equivalent milligrams of caffeic acid/kg); FRAP: iron ion reducing power (millimolar Fe^{2+}); ABTS: capacity to scavenge ABTS radicals (millimolar trolox equivalent capacity). Results are expressed as mean values ± standard deviation (n = 9). Capital letters in the same column means differences between maturation temperatures ($p < 0.05$). Lowercase letters in the same line means differences during maturation time for the same maturation temperature ($p < 0.05$).

thoracica and *Heterotrigona itama* from Malaysia, and *Tetragonisca angustula* from Brazil), but it was not measured in the present study.

Honey from Uruçú-Amarela did not present HMF at T0, and during the maturation process, this compound was gradually formed at both temperatures, mainly at 30 °C ($p < 0.05$). HMF is a product of the Maillard reaction's degradation of simple sugars (mainly fructose). Therefore, its formation is influenced by inadequate heating or extended storage periods (Ávila et al., 2018, Del Campo et al., 2016, He et al., 2020). Nevertheless, despite the formation of this compound during the maturation process, the quality of this honey was not compromised since the Codex Alimentarius establishes that after processing, the HMF content in honey should not be greater than 40 mg/kg or 80 mg/kg in cases of tropical countries (Codex Alimentarius Commission, 2001).

At T0 Uruçú-Amarela honey was dark brown (L*=49.52, a*=0.70, b*=17.50). During maturation, L* values gradually decreased, and a* and b* values increased, with more pronounced changes for honey matured at 30 °C ($p < 0.05$). Pure honey becomes naturally darker over time (Ávila et al., 2018, Can et al., 2015). Therefore, the results of color parameters could be associated with the greatest Maillard reaction at 30 °C, as observed for the formation of HMF.

The bioactive compounds were degraded over 180 days of maturation at both temperatures compared to not matured honey ($p < 0.05$), resulting in a reduction of antioxidant activity (FRAP, $p < 0.05$). Furthermore, there was a significant negative correlation between the bioactive compounds and the free acidity of honey (-0.85; -0.84 and -0.71 for TPC, TF, and TPA, respectively; $p < 0.05$) and a significant positive correlation between the glucose and bioactive compounds (+0.80, +0.92 and + 0.80, for TPC, TF, and TPA, respectively, $p < 0.05$). In other words, the production of organic acids through the fermentation of glucose by the active microbiota of honey was the leading cause of the decrease of these compounds in honey over 180 days of maturation. The increase in acidity in the honey matrix can result in changes in the structure of the phenol group of phenolic compounds through hydroxylation, methylation, dimerization, and glycosylation reactions, as well as in the formation of phenolic derivatives by partial degradation of combined forms or by loss of fractions between phenols and sugars (Chen et al., 2016). However, the honey that matured at 30 °C showed higher concentrations of TPA and increased antioxidant activity (ABTS, $p < 0.05$). Furthermore, ABTS radical scavenging activity showed a positive correlation with HMF and a negative correlation with L* (0.56 and -0.84, respectively; $p < 0.05$). These results allow us to infer that during the Maillard reaction, HMF is formed, and the L* parameter is

reduced, resulting in the generation of intermediate compounds that promote the antioxidant activity of honey throughout maturation at 30 °C.

3.3. Metabolomic and multivariate analysis of the ^{1}H NMR of Uruçú-Amarela honey during 180 days of maturation

In the Figure 3, divided into three regions (A-C), are presented the ^{1}H NMR spectra of the samples of Uruçú-Amarela honey before (T0) and after 180 days of maturation at 20 and 30 °C. The different spectral regions are characterized by the resonance of specific compounds, such as the regions of aliphatic compounds (3.00 – 0.00 ppm), carbohydrates (6.00 – 3.00 ppm), and aromatic compounds (10.00 - 6.00 ppm) (Del Campo et al., 2016).

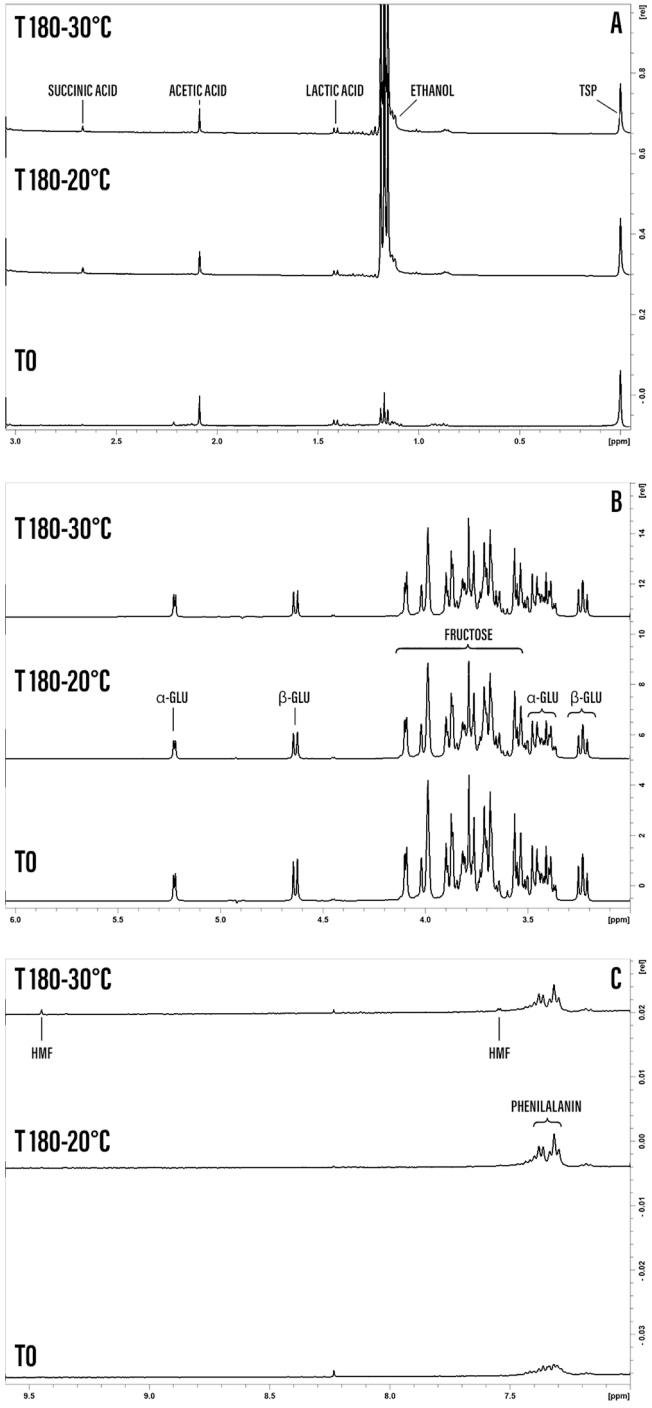

Fig. 3. ^{1}H NMR spectrum of honey from Uruçú-Amarela before (T0) and after (T180) maturation at 20 and 30 °C. A: region of aliphatic compounds. B: region of carbohydrates. C: region of aromatic compounds. TSP: Sodium trimethylsilyl propionate; α-GLU: α-Glucose; β-GLU: β-Glucose; HMF: hydroxymethylfurfural.

In the aliphatic region (Figure 3A), it is possible to verify the presence of ethanol (triplet at 1.15 ppm) in the three spectra. There was an increase in the signal intensity of this compound over the 180 days of maturation at both temperatures. This increase is related to osmophilic yeasts identified in the metagenomic analysis, which showed fermentative activity at 20 and 30 °C. In the same spectrum region, it is possible to verify the presence of lactic acid (double in 1.42 ppm) produced by lactic acid bacteria in the honey microbiota. It is noteworthy that there was no increase in the signal intensity of this compound in the spectra of honey matured for 180 days at both temperatures, showing that the activity of these bacteria was lower than those of yeasts. This can be explained by the optimal fermentation temperature of these bacteria being around 35-37 °C, higher than the maturation temperatures used in this study (25 or 30 °C).

In the aliphatic region, acetic acid (singlet at 2.10 ppm) and succinic acid (singlet at 2.67 ppm) were also identified (Figure 3A). It is possible to infer that most of the acetic acid present in Uruçú-Amarela honey was produced before the maturation since the signal intensity of this compound remained constant after 180 days at both temperatures and may be associated with the metabolism of *Saccharibacter floricola* bacteria identified in the microbiota of this honey. An increase in the signal intensity of succinic acid from T0 to T180 was observed, indicating an increase in its concentration at the two maturation temperatures. The production of succinic acid during maturation is associated with the fermentative activity of yeasts of the genus *Zygosaccharomyces* (Li et al., 2021) and the metabolism of lactic acid bacteria identified in honey (Özcelik et al., 2016).

The region with the highest signal intensity in the ^{1}H NMR spectrum (5.30 - 3.20 ppm) corresponds to carbohydrates, mainly glucose and fructose (Del Campo et al., 2016; He et al., 2020). During fermentation, the active microbiota consumed part of the carbohydrates in honey, mainly glucose. As a result, it is possible to verify that there was a decrease in the intensity of some signals in this region by comparing the T0 spectrum with the T180 spectra at both temperatures. For example, in the signals of α-glucose at 3.42 ppm (double-double) and 5.23 ppm (double), fructose at 4.00 ppm and 4.10 ppm (multiplet), and β-glucose at 3.25 ppm (double-double) and 4.65 ppm (double), as shown in Figure 3B. Gluconic acid, main responsible for the increase on total acidity over the 180 days of maturation, could not be identified through ^{1}H NMR analysis because the signals attributed to this organic acid were overlapped by the signals of sugars, since them are in the same region of the spectrum.

In the spectrum's aromatic region (10.0 – 5.5 ppm), the amino acid phenylalanine was identified (multiplet at 7.32 ppm) in the three spectra presented, showing the stability of this compound during maturation at both temperatures. On the other hand, HMF (double at 7.54 ppm and a singlet at 9.45 ppm) was identified only in honey matured for 180 days at 30 °C, as shown in Figure 3C. This result suggests that the Maillard reaction occurred at higher rates in honey matured at higher temperatures.

In the Figure 4A are presented the projection of honey samples over 180 days of maturation at 20 and 30 °C on the PC1 X PC2 of the PCA. Figure 4B shows the projection of metabolites identified in honey through ^{1}H NMR superimposed on the evaluated honey samples. The first two PC explained 98.6 % of the total variance contained in the data. PC1 explained 97.7 % of this variance and separated the samples as a function of maturation time, regardless of the applied temperature. Samples matured up to T72 were grouped to the left of PC1, and samples from T108 to T180 were grouped to the right. In this way, the most remarkable changes in the metabolites profile of Uruçú-Amarela honey occurred after 72 days of maturation, both at 20 and 30 °C.

The projection of the variables on the PCA factorial plane (Figure 4B)

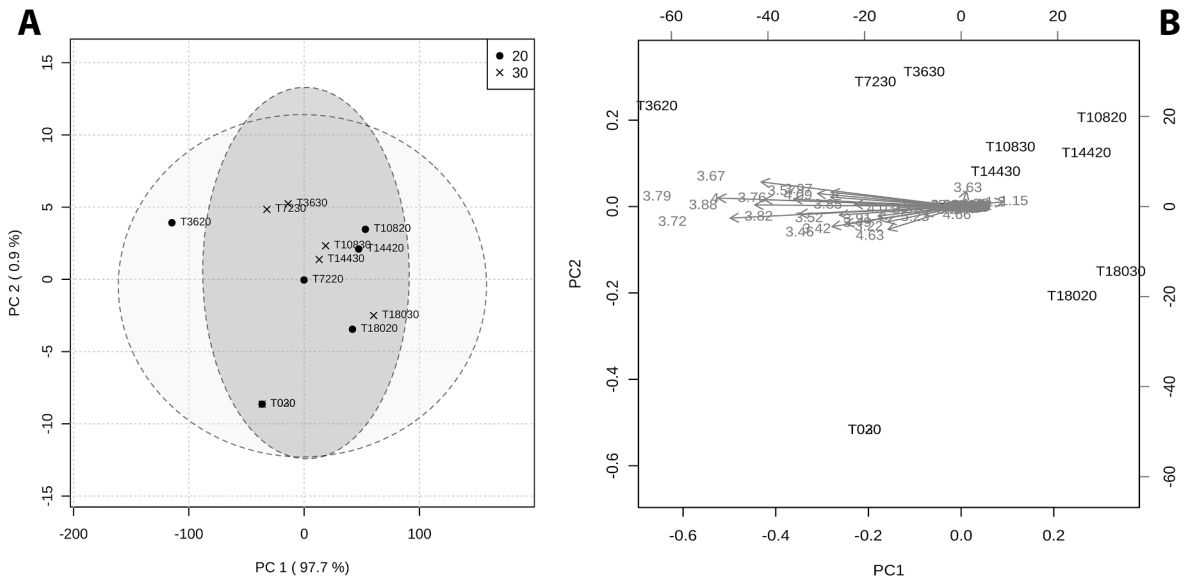

Fig. 4. Projections of matured honey samples (A) and metabolites identified in honey throughout maturation (B) on the factorial plane (PC1 × PC2) of the PCA. 20: honey matured at 20 °C. × 30: honey matured at 30 °C. T020 – T18020: different maturation times (days) at 20 °C; T030 – T18030: different maturation times (days) at 30 °C.

shows that the samples grouped to the left (T0 – T72) were mainly characterized by having higher carbohydrate concentrations (5.3 - 3.2 ppm); the samples grouped on the right are mainly characterized by higher concentrations of ethanol (triplet at 1.15 ppm), and the expression of this compound was more accentuated from T108 at both temperatures evaluated. Thus, the longer the maturation time, the greater the consumption of carbohydrates, mainly by osmophilic yeasts (*Zygosaccharomyces* sp.) that make up the honey microbiota, which resulted in the more significant formation of ethanol in Uruçú-Amarela honey during maturation at both 20 and 30 °C.

NMR provided important information about the changes during maturation. However, physicochemical, color and antioxidant properties were quantified to complement the data.

4. Conclusion

The microbiota biodiversity of Uruçú-Amarela honey produced in Aracruz-ES, Brazil, was mainly composed by bacteria of the genus *Lactobacillus* and yeasts of the genus *Zygosaccharomyces*, which are the main active microorganisms associated with the fermentation of this stingless bee honey. Ethanol is the main metabolite produced by the active microbiota of this honey, in addition to organic acids that increased the total acidity of the matured honey.

At least 72 days of maturation is advised, as products with greater carbohydrate consumption (glucose and fructose), more significant ethanol formation, and increased acidity were obtained. The maturation process does not prevent the degradation of phenolic compounds, being the degradation of phenolic acids higher at 20 °C and for flavonoids degradation was higher at 30 °C. Greater changes on physicochemical properties occurred when honey was fermented at 30 °C, this temperature also led to a honey with higher antioxidant activity.

Even within 180 days of maturation, honey showed moisture and sugar contents not complying with the legislation for *Apis mellifera* honey, demonstrating the urgency for specific legislation for stingless bee honey. Our results demonstrate that maturation is suitable preservation technique for stingless bee honey, resulting in a honey with improved physicochemical properties and biological activity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

Alves, R. M. O., Viana, J. L., Sousa, H. D. A. C., & Waldschmidt, A. M. (2018). Physicochemical parameters of honey from Melipona mondury Smith, 1863 (Hymenoptera: Apidae: Meliponini). *Journal of Agricultural Science, 10*, 196–205. https://doi.org/10.5539/jas.v10n7p196

Aoac. (2012). *AOAC official methods of analysis* (18th ed.). Gaithersburg: AOAC international.

Arvouet-Grand, A., Vennat, B., Pourrat, A., & Legret, P. (1994). Standardisation d'un extrait de propolis et identification des principaux constituants. *Journal de Pharmacie de Belgique, 49*, 462–468.

Ávila, S., Beux, M. R., Ribani, R. H., & Zambiazi, R. C. (2018). Stingless bee honey: Quality parameters, bioactive compounds, health- promotion properties and modification detection strategies. *Trends in Food Science & Technology, 81*, 37–50. https://doi.org/10.1016/j.tifs.2018.09.002

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Analytical Biochemistry, 239*, 70–76. https://doi.org/10.1006/abio.1996.0292

Beux, M. R., Surek, M., Bordin, K., Barbieri, F., Ferreira, S. M. R., & Rosa, E. A. (2022). Microbial biodiversity in honey and pollen pots produced by *Tetragonisca angustula* (Jataí). *Brazilian Archives of Biology and Biotechnology, 54*, e22210440.

Biluca, F. C., Braghini, F., Gonzaga, L. V., Costa, A. C. O., & Fett, R. (2016). Physicochemical profiles, minerals and bioactive compounds of stingless bee honey (Meliponinae). *Journal of Food Composition and Analysis, 50*, 61–69. https://doi.org/10.1016/j.jfca.2016.05.007

Bogdanov, S., Martin, P., & Lüllmann, C. (1997). Harmonised methods of the European Honey Commission. *Apidologie, 28*, 1–59.

Bovo, S., Utzeri, V. J., Ribani, A., Cabbri, R., & Fontanesi, L. (2020). Shotgun sequencing of honey DNA can describe honey bee derived environmental signatures and the honey bee hologenome complexity. *Scientific Reports, 10*, 9279. https://doi.org/10.1038/s41598-020-66127-1

Braghini, F., Biluca, F. C., Schulz, M., Gonzaga, L. V., Costa, A. C., & Fett, R. (2021). Stingless bee honey: A precious but unregulated product - reality and expectations. *Food Reviews International, 1–30*. https://doi.org/10.1080/87559129.2021.1884875

Bueno-Costa, F. M., Zambiari, R. C., Bohmer, B. W., Chaves, F. C., Da Silva, W. P., Zanusso, J. T., & Duta, I. (2016). Antibacterial and antioxidant activity of honeys from the state of Rio Grande do Sul. *LWT – Food Science and Technology, 65*, 333–340. https://doi.org/10.1016/j.lwt.2015.08.018

Camargo, R. C. R., Oliveira, K. L., & Berto, M. I. (2017). Mel de abelhas sem ferrão: Proposta de regulamentação. *Brazilian Journal of Food Technology, 20*, 1–6. https://doi.org/10.1590/1981-6723.15716

Can, Z., Yildiz, O., Sahin, H., Turumtay, E. A., Silici, S., & Kolayli, S. (2018). An investigation of Turkish honeys: Their physico-chemical properties, antioxidant capacities and phenolic profiles. *Food Chemistry, 180*, 133–141. https://doi.org/10.1016/j.foodchem.2015.02.024

Casalone, E., Cavalieri, D., Daly, G., Vitali, F., & Perito, B. (2020). Propolis hosts a diverse microbial community. *World Journal of Microbiology and Biotechnology, 36*, 1–11. https://doi.org/10.1007/s11274-020-02827-0

Chen, G. L., Chen, S. G., Chen, F., Xie, Y. K. F., Han, M. D., Luo, C. X., … Gao, Y. Q. (2016). Nutraceutical potential and antioxidant benefits of selected fruit seeds subjected to an in vitro digestion. *Journal of Functional Foods, 20*, 317–331. https://doi.org/10.1016/j.jff.2015.11.003

Chong, J., Wishart, D., & Xia, J. (2019). Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocol in Bioinformatics, 68*, 1–128. https://doi.org/10.1007/978-1-0716-0239-3_17

Chuttong, B., Chanbang, Y., Sringarm, K., & Burgett, M. (2016). Physicochemical profiles of stingless bee (Apidae: Meliponini) honey from South East Asia (Thailand). *Food Chemistry, 192*, 149–155. https://doi.org/10.1016/j.foodchem.2015.06.089

Codex Alimentarius Commission. (2001). *Codex standard 12* (p. 11). Standards and Standard Methods: Revised Codex Standard for Honey.

Del Campo, G., Zuriarrain, J., Zuriarrain, A., & Berregi, I. (2016). Quantitative determination of carboxylic acids, amino acids, carbohydrates, ethanol and hydroxymethylfurfural in honey by ^{1}H NMR. *Food Chemistry, 96*, 1031–1039. https://doi.org/10.1016/j.foodchem.2015.10.036

Echeverrigaray, S., Scariot, F. J., Foresti, L., Schwarz, L. V., Rocha, R. K. M., Da Silva, G. P., … Delamare, A. P. L. (2021). Yeast biodiversity in honey produced by stingless bees raised in the highlands of southern Brazil. *International Journal of Food Microbiology, 347*, Article 109200. https://doi.org/10.1016/j.ijfoodmicro.2021.109200

Escott, C., Del Fresno, J. M., Loira, I., Morata, A., & Suárez-Lepe, J. A. (2018). *Zygosaccharomyces rouxii*: Control Strategies and Applications in Food and Winemaking. *Fermentation, 4*, 69. https://doi.org/10.3390/fermentation4030069

Fletcher, M. T., Hungerford, N. L., Webber, D., Carpinelli de Jesus, M., Zhang, J., Stone, I., … Zawawi, N. (2020). Stingless bee honey, a novel source of trehalulose: A biologically active disaccharide with health benefits. *Scientific reports, 10*, 12128. https://doi.org/10.1038/s41598-020-68940-0

Gonçalves, J., Ribeiro, I., Marçalo, J., Rijo, P., Faustino, C., & Pinheiro, L. (2018). Physicochemical, antioxidant and Antimicrobial properties of selected Portuguese commercial monofloral honeys. *Journal of Food and Nutrition Research, 6*, 645–654. https://doi.org/10.12691/jfnr-6-10-5

He, C., Liu, Y., Liu, H., Zheng, X., Shen, G., & Feng, J. Compositional identification and authentication of Chinese honeys by ^{1}H NMR combined with multivariate analysis. *Food Research International, 130*, 1-9. 10.1016/j.foodres.2019.108936.

Jo, H., Hong, J., & Unno, T. (2019). Investigation of MiSeq reproducibility on biomarker identification. *Applied Biological Chemistry, 62*, article 60. https://doi.org/10.1186/s13765-019-0467-8

Justus, A., Ida, E. I., & Kurozawa, L. E. (2021). Microencapsulation of okara protein hydrolysate by spray drying: Physicochemical and nutritive properties, sorption isotherm, and glass transition temperature. *Drying Technology, 40*, 2116–2127. https://doi.org/10.1080/07373937.2021.1920031

Kaiser, H. F. (1958). The varimax criterion for analytic rotation in factor analysis. *Psychometrika, 23*, 187–200. https://doi.org/10.1007/BF02289233

Kwong, W., Medina, L. A., Koch, H., Sing, K., Soh, E. J., Ascher, J., … Moran, N. (2017). Dynamic microbiome evolution in social bees. *Science Advances, 3*(1–16), 2017. https://doi.org/10.1126/sciadv.1600513

Lalhmangaihi, R., Ghatak, S., Laha, R., Gurusubramanian, G., & Kumar, N. S. (2014). Protocol for optimal quality and quantity pollen DNA isolation from honey samples. *Journal of biomolecular techniques, 25*, 92–95. https://doi.org/10.7171/jbt.14-2504-001

Lavinas, F. C., Macedo, E. H. B. C., Sá, G. B. L., Amaral, A. C. F., Silva, J. R. A., Azevedo, M. M. B., … Rodrigues, I. A. (2019). Brazilian stingless bee propolis and geopropolis: Promising sources of biologically active compounds. *Revista Brasileira de Farmacognosia, 29*, 389–399. https://doi.org/10.1016/j.bjp.2018.11.007

Li, C., Ong, K. L., Cui, Z., Sang, Z., Li, X., Patria, R. D., Qi, Q., Fickers, P., Yan, J., & Lin, C. S. K. Promising advancement in fermentative succinic acid production by yeast hosts. *Journal of Hazardous Materials, 401*, 1-16. 10.1016/j.jhazmat.2020.123414.

Marchi, R., Montes-Villanueva, N. D., McDaniel, M. R., & Bolini, H. M. A. (2012). *Sensory profile and stability of a new ready-to-drink passion fruit juice beverage with different sweetener systems*. *CENTRUM Catolicas Working Paper Series*, Aticle 2012-09-0016.

Moniruzzaman, M., Rodríguez, I., Rodríguez-Cabo, T., Cela, R., Sulaiman, S. A., & Gan, S. H. (2014). Assessment of dispersive liquid-liquid microextraction conditions for gas chromatography time-of-flight mass spectrometry identification of organic compounds in honey. *Journal of Chromatography A, 1368*, 26–36. https://doi.org/10.1016/j.chroma.2014.09.057

Ngalimat, M. S., Rahman, R. N. Z. R. A., Yusof, M. T., Syahir, A., & Sabri, S. Characterisation of bacteria isolated from the stingless bee, *Heterotrigona itama*, honey, bee bread and propolis. *PeerJ, 7*, 1-20. 10.7717/peerj.7478.

Oddo, L. P., Heard, T. M., Rodríguez-Malaver, A., Pérez, R. A., Fernández-Muiño, M., Sancho, M. T., … Vit, P. (2008). Composition and antioxidant activity of *Trigona carbonaria* honey from Australia. *Journal of Medicinal Food, 11*, 789–794. https://doi.org/10.1089/jmf.2007.0724

Özcelik, S., Kuley, E., & Özogul, F. (2016). Formation of lactic, acetic, succinic, propionic, formic and butyric acid by lactic acid bacteria. *LWT - Food Science and Technology, 73*, 536–542. https://doi.org/10.1016/j.lwt.2016.06.066

Paludo, C. R., Menezes, C., Silva-Junior, E. A., Vollet-Neto, A., Andrade-Dominguez, A., Pishchany, G., … Pupo, M. T. (2018). Stingless Bee Larvae Require Fungal Steroid to Pupate. *Scientific Reports, 8*, 1–10. https://doi.org/10.1038/s41598-018-19583-9

Paula, G. T., Menezes, C., Pupo, M. T., & Rosa, C. A. (2021). Stingless bees and microbial interactions. *Current Opinion in Insect Science, 44*, 41–47. https://doi.org/10.1016/j.cois.2020.11.006

Pimentel, T. C., Madrona, G. S., & Prudencio, S. H. (2015). Probiotic clarified apple juice with oligofructose or sucralose as sugar substitutes: Sensory profile and acceptability. *LWT –. Food Science and Technology, 62*, 838–846. https://doi.org/10.1016/j.lwt.2014.08.001

Pimentel, T. C., Rosset, M., de Sousa, J. M. B., de Oliveira, L. I. G., Mafaldo, I. M., Pintado, M. M. E., Souza, E. L., & Magnani, M. (2022). Stingless bee honey: An overview of health benefits and main market challenges. *Journal of Food Biochemistry, 46*, Article e13883. 10.1111/jfbc.13883.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine, 26*, 1231–1237. https://doi.org/10.1016/s0891-5849(98)00315-3

Ribeiro, G. P., Villas-Bôas, J. K., Spinosa, W. A., & Prudencio, S. H. (2018). Influence of freezing, pasteurization and maturation on Tiúba honey quality. *LWT - Food Science and Technology, 90*, 607–612. https://doi.org/10.1016/j.lwt.2017.12.072

Rosli, F. N., Hazemi, M. H. F., Akbar, M. A., Basir, S., Kassim, H., & Bunawan, H. (2020). Stingless Bee Honey: Evaluating Its Antibacterial Activity and Bacterial Diversity. *Insects, 11*, 1–13. https://doi.org/10.3390/insects11080500

Singleton, V. L., & Rossi, J. A. (1965). Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American Journal of Enology and Viticulture, 16*, 144–158.

Siozios, S., Moran, J., Chege, M., Hurst, G. D. D., & Paredes, J. C. (2019). Complete reference genome assembly for *Commensalibacter* sp. strain AMU001, an acetic acid bacterium isolated from the gut of honey bees. *Microbiology Resource Announcements, 8*, 1–2. https://doi.org/10.1128/MRA.01459-18

Villas-Bôas, J. (2012). *Manual Tecnológico Mel de Abelhas Sem Ferrão* (1st ed.). População e Natureza (ISPN): Instituto Sociedade.

Villas-Bôas, J. (2018). *Manual Tecnológico de Aproveitamento Integral dos Produtos das Abelhas Nativas Sem Ferrão* (2th ed.). População e Natureza (ISPN): Instituto Sociedade.

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S Ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology, 173*, 697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Worley, B., & Powers, R. (2016). PCA as a practical indicator of OPLS-DA model reliability. *Current Metabolomics, 4*, 97–103. https://doi.org/10.2174/2213235x04666160613122429