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Fungal community structure in bees: influence of biome and host species

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Abstract
Understanding the ecological relationship between fungi and insects is essential for elucidating interactions in biodiverse regions such as South American biomes. This study aimed to evaluate the influence of biome, host species, and microhabitat on the community structure of yeasts (using culture-dependent methods) and fungi (culture-independent methods) in bees and to identify the functional characteristics of isolated strains. Samples were collected from the body, hive, honey, and beebread of bees from the genera *Trigona*, *Scaptotrigona*, *Tetragona*, *Apis*, *Meliponas*, and *Tetragonisca* in the Pantanal, Amazon, and Cerrado biomes. We isolated 176 strains representing 46 yeast species, predominantly from the genera *Starmerella* (44.32%), *Hanseniaspora* (5.16%), and *Wickerhamiella* (4.38%). *Starmerella etchellsii* (32%) was the most abundant species, while *Aureobasidium leucospermi* (< 0.01%) was the least abundant. Only *S. etchellsii* and *S. apicola* (11%) were present in all bee species. The composition and abundance of yeasts were significantly influenced by biome and host species (PERMANOVA, $p < 0.05$). Alpha diversity varied significantly among microhabitats (Dunn's $p < 0.05$), bee species, and biomes (Duncan $p < 0.05$). Culture-independent methods identified 234 Ascomycota ASVs, 18 Basidiomycota ASVs, and 1 Mucoromycete ASV across 90 genera and 108 species. Saccharomycetales accounted for approximately 72% of the fungal abundance, with *S. apicola* (14.64%) and *S. meliponinorum* (11.21%) being the most abundant. Additionally, barcoding identified 100 ASVs of plants associated with bees, grouped into 22 families and 24 species, predominantly Asteraceae, Anacardiacea, Elaeocarpaceae, and Solanaceae. The functional characteristics of the yeasts showed potential for industrial applications, varying according to the strain.

Keywords Yeasts · Diversity · Biome · Stingless bee · Microbiota

1 Introduction

Bees are important because they perform the ecosystem service of pollination, which directly influences the reproduction and establishment of different plant species (Camargo and Pedro 2013), and influences agricultural production of agronomic interest, such as corn, soybeans and cotton (Pozo et al. 2018; Yun et al. 2018).

Social bees are categorized into two main groups: stinging bees (Apini) and stingless bees (Meliponini). These groups differ in biology, behavior, and ecological interactions (Drossart and Gérard 2020). Apini bees, known for their complex social organization and honey production, are found worldwide except Antarctica, with notable species like the European and Africanized honeybees (*Apis mellifera*) (Spivak and Danka 2021).

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Meliponini bees, primarily distributed in tropical and subtropical regions, also exhibit social structures and are reared for their honey and pollen (Grüter and Grüter 2020; Bustamante et al. 2022). Their unique social organization involves males contributing to hive tasks and a queen focused solely on egg-laying (Grüter and Grüter 2020; Bustamante et al. 2022). Stingless bee honey is valued for its unique sensory characteristics, resulting from microbial fermentation of complex sugars (Aljohar et al. 2018; Grüter and Grüter 2020; Li et al. 2023).

Ecological associations between bees and microorganisms span a range of interactions, from mutualism to parasitism (Malassigné et al. 2021). Mutualistic yeasts, in particular, play a vital role in bee nutrition (Vega and Dowd 2005). These unicellular fungi colonize diverse habitats, including hives, bee bodies, and intestines (Detry et al. 2020). Yeasts have been detected in various bee species, including stingless bees *Tetragonisca angustula*, *Melipona quadrifasciata* and *Frieseomelitta varia* in the Cerrado and Caatinga biomes (Guzmán et al. 2013; Rosa et al. 2003). Notably, *Starmerella meliponinorum*, *S. apicola*, *S. neotropicalis*, *S. etchellsii*, and *S. bombicola* are strongly associated with Meliponini bees (De Paula et al. 2021).

The bee-associated microbiota plays important roles through commensal or symbiotic relationships (Tauber et al. 2022; Santos et al. 2023b). Yeasts serve as probiotics, providing additional nutrients through the metabolization of complex carbohydrates. *Zygosaccharomyces* sp. promotes healthy growth and interacts with other yeasts to enhance hive health (Paludo et al. 2019). *Starmerella* species ferment low carbohydrate amounts, producing sophorolipids with antimicrobial effects (Detry 2020). In turn, bees offer a suitable environment and aid in yeast dispersal (Da Costa Neto and Benevides de Morais 2020).

The bee-associated microbiota is not limited to external surfaces but also inhabits the nest environment, including honey and beebread (Smutin et al. 2022). Despite the challenging gut environment, some microbes, including yeasts, colonize this niche and can be transferred to the hive, playing functional roles such as honeycomb preservation and potentially influencing honey properties (Khan et al. 2020; Malassigné et al. 2021; Rosa et al. 2003).

Knowledge gaps persist in understanding bee-associated yeast communities, hindering biodiversity conservation and biotechnological applications. Research has revealed new species, like *Starmerella neotropicalis* (Daniel et al. 2013). Genera like *Starmerella*, *Wickerhamiella*, *Metschnikowia*, and *Candida* are frequently associated with bees and can be acquired from floral nectar and transmitted vertically (Rosa et al. 2023; Pozo et al. 2018; Malassigné et al. 2021).

Yeast abundance and composition vary across neotropical savannas, suggesting environmental influences (Da Costa Neto and Benevides de Morais 2020). Studies have revealed specific associations between yeast and bee species, highlighting the complexity of these interactions (Costa Neto and Benevides de Morais 2020; Echeverrigaray et al. 2021).

Mato Grosso, Brazil, encompasses three biomes (Cerrado, Pantanal, and Amazon) with rich biodiversity (Da Silva Junior et al. 2019; Maia and Silva 2021). However, the microbiota of this state remains poorly understood. Existing research has focused primarily on plant-associated microbiota (Pietro-Souza et al. 2017, 2020; De Siqueira et al. 2018; Mello et al. 2019, 2020; Mariano et al. 2020; Da Silva Maciel et al. 2021), including descriptions of new species (Senabio et al. 2023) and arthropod-associated microbiota (Falqueto et al. 2022). This biodiversity faces challenges from climate change and land-use transformations (Gollnow et al. 2018; Pires 2020).

Mato Grosso harbors over 76 stingless bee species, many unidentified (Pedro 2014). Understanding bee-microbiota interactions is crucial for developing conservation measures to maintain ecosystem services (Schaeffer et al. 2023). Studies on bee-fungal interactions in Mato Grosso are limited, primarily focusing on honeybee microbiological quality (Ferreira et al. 2013; Da Silva et al. 2015). Research on regional bee species has focused mainly on diversity or biotechnological exploitation (Pietro-Souza et al. 2017; Silva et al. 2018; Costa Neto and Benevides de Morais 2020; De Paula et al. 2023a; Senabio et al. 2023).

Therefore, our hypothesis is that the structure and functionality of yeast communities associated with bees are host dependent and influenced by different biomes (Pantanal, Cerrado and Amazon Rainforests). The objectives of this work were as: I - describe the structure of the yeast communities associated with the microhabitats of different bee species in the Pantanal, Cerrado and Amazon biomes; II - determine the functionality of the cultivable species and the chemical characterization of the honey and beebread collected from the hives; and III - analyze the floral association of bees studied.

2 Materials and methods

2.1 Sample collection and molecular identification of bees

Bee species were collected at random as long as they were present in the defined areas in three biomes in the state of Mato Grosso, Brazil (Table S1). *Trigona cf. fuscipennis* (Supplementary Material Fig. S1), *Melipona (Melipona) orbignyi* (Supplementary Material Fig. S2), *Tetragonisca fiebrigi* (Supplementary Material Fig. S3) (assigned *T. fiebrigi* I), and *Apis mellifera* (Supplementary Material Fig.

S4) were obtained in areas of native vegetation in the Pantanal biome during the dry season (between the months of June and July) in the Poconé region, which has a hot subhumid tropical climate with a low flood pulse (De Moraes et al. 2022). *Scaptotrigona xanthotricha* (Supplementary Material Fig. S5), *T. fiebrigi* (assigned *T. fiebrigi* II) *and Tetragonisca angustula* (Supplementary Material Fig. S6) (assigned as *T. angustula* I) were collected in an urban area of Cuiabá, with a predominance of the Cerrado biome, characterized by a predominantly tropical seasonal climate, dry winters and rainy summers, savannah vegetation and mixed urban vegetation (Sano et al. 2019; Rodrigues Silva et al. 2022). *Scaptotrigona cf. polysticta* (Supplementary Material Fig. S7), *Melipona seminigra pernigra* (Supplementary Material Fig. S8), *Scaptotrigona cf. nigrohirta* (Supplementary Material Fig. S9) *and T. angustula* (assigned as *T. angustula* II) were captured in a meliponary in the rural region of the city of Sinop, whose climate is characterized as humid tropical, with Cerrado/Amazon transition vegetation predominated by forest, savannah fields and pastures (Cassettari et al. 2019).

The individuals were collected between 6:00 and 9:00 a.m. with a properly sterilized M-type entomological aspirator (Amado de Santis and Chacoff 2020). Biological samples from the hives were obtained with a sterile swab and transported in 0.8% saline solution until processing. Honey and beebread were collected only from domestic hives in the Amazon region. When collecting honey, the nest lid was removed, and a small opening was made in the honey pot using a sterile spatula. The honey was collected with tips and kept in sterilized microtubes. The beebread was obtained using a steel spatula and stored in tubes. The samples were kept in an ice bath until processing, which took place no more than 24 h after collection (Detry et al. 2020).

With bees from the Cerrado and Pantanal biomes, it was not possible to collect samples of honey and beebread because when they were identified, they were in structures that made it impossible to open the nest.

The total DNA of the bees was extracted using an Axygen Biosciences kit (Axygen, Canada). The mitochondrial cytochrome oxidase subunit 1 gene was amplified from the samples by PCR using the oligonucleotides LCO1490 (5'-GGT CAACAAATCATAAAGATATTGG-3') and HCO2198 (5' TAAACTTCAGGGTGACCAAAAAAAATAC-3') (Alabdali et al. 2021). The PCR mixture was 25 µL and was composed of 1 µL of DNA (50 ng), 10 mM of each primer, 150 mM dNTPs, 25 mM $MgCl_2$, 2.5 µL of 10X PCR-Buffer and 1.5 U of Taq DNA Polymerase (Invitrogen, Thermo Fisher). The reaction conditions were as follows: initial denaturation at 94 °C (2 min); 38 cycles of denaturation at 94 °C (30 s), 46 °C (45 s) and 72 °C (60 s); and a final extension at 72 °C (10 min). The amplification products were confirmed by electrophoresis in a 1.2% agarose gel. The PCR products were enzymatically purified (ExoSap-it, GE Healthcare) and sequenced by the Sanger method (BigDye Terminator Cycle Sequencing). The sequencing products of both DNA strands were grouped, aligned and corrected contiguously using the software Chromas 2.6.6 and BioEdit 7.2.5. The sequences obtained were compared with existing sequences in the GenBank database using the nBLAST tool (http://www.ncbi.nlm.nih.gov). The sequences were deposited in GenBank with the accession numbers shown in Supplementary Table S1.

Specimens of bees from the study were deposited in the Entomology Section of the Zoological Collection (CEMT) of the Federal University of Mato Grosso (UFMT) under the number CEMT 0000045028 after confirmation of genetic identification by a taxonomist.

2.2 Isolation of yeasts by culture-dependent methods

The bees were superficially rinsed and agitated for 1 min in PT solution (0.8% peptone and 0.1% tween 20), after which the supernatant was diluted in sterile PT solution. The bees were superficially disinfected with 70% alcohol and 0.1% neutral detergent for 1 min and then rinsed three times in sterile distilled water. After disinfection of the external part, the GIT region (gastrointestinal tract) was removed using tweezers and a scalpel for subsequent maceration in a PT solution, and the mixture was serially diluted. Honey, beebread and swab samples from the hives were serially diluted in PT. All the serially diluted samples were inoculated (in triplicate) into Petri dishes containing YMA medium (yeast extract 0.3%, malt extract 0.3%, peptone 0.5%, glucose 1% and agar 2%) supplemented with chloramphenicol (100 mg/L). The plates were kept at 25 °C for 7 days and observed daily to count, morphotype and isolate the colonies. After purification, the yeasts were grouped by their morphology (color, texture, shape and size of colonies) and microscopic characteristics (Kurtzman et al. 2011). The isolates were preserved in 20% glycerol at -80 °C.

2.3 Identification of cultivable yeasts

The different morphotypes were subjected to molecular identification. DNA was extracted according to the methods of Aldrete-Tapia et al. (2020). The genotyping of the morphotypes was determined by the PCR mix amplification profile using the oligonucleotide $(GACA)_4$ (Meyer et al. 1993) under the following reaction conditions: initial denaturation at 94°C (5 min); 30 amplification cycles at 94°C (30 s), 56°C (45 s), and 72°C 56°C (45 s); and 8 amplification cycles at 94°C (30 s), 53°C (45 s), and 72°C (45 s), with a

final extension of 72°C (10 min). The amplification products were analyzed by 1.4% agarose gel electrophoresis using 0.01% ethidium bromide and observed under a transilluminator. Morphotypes with identical amplicon patterns were grouped together and considered to be the same species (Lopes et al. 2018). Subsequently, one strain representing each morphotype was subjected to molecular identification by amplifying the D1/D2 domain of the large subunit of the 26S ribosomal gene (LSU rDNA) using the primers NL1 (5′ GCATATCAATAAGCGGAGGAAAAG-3′)/NL4 (5′ GGT CCGTGTTTCAAGACGG-3′) or ITS1 (5'TTCCGTAG-GTGAACCTGCGG3')/ITS4 (5′-TCCTCCGCTTATTGAT ATGC-3′) (Kurtzman e Robnett 1998). The PCR mixture consisted of 2 µl of yeast DNA. The reaction conditions for ITS1/ITS4 were initial denaturation at 95 °C (5 min); 35 cycles of denaturation at 94 °C (45 s), 55 °C (45 s) and 72 °C (60 s); and a final extension at 72 °C (10 min). The following steps were used for the NL1/NL4 primers: initial denaturation at 95 °C (2 min); 35 cycles of denaturation at 95 °C (1 min), 55 °C (1 min) and 72 °C (2 min); and a final extension at 72 °C (10 min). The PCR products were enzymatically purified (ExoSap-it, GE Healthcare) and sequenced using the Sanger method (BigDye Terminator Cycle Sequencing). The sequencing products of both DNA strands were edited using Chromas 2.6.6 and BioEdit 7.2.5 software. The sequences obtained were compared in GenBank using the nBLAST tool (www.ncbi.nlm.nih.gov) considering identity at the species level above 97% similarity with the sequences deposited on the platform (Kurtzman and Robnett 1998). The sequences were deposited in GenBank with the accession numbers shown in Table S2.

2.4 Eukaryotic community analysis by the independent cultivation method

The total DNA of the samples used for the isolation described above was extracted using the DNeasy Power Soil Kit (Qiagen) according to the manufacturer's instructions. The nRLSU-U (nuclear ribosomal large subunit rDNA) region of the eukaryotes (fungi and plants) 28 S rDNA gene was amplified using primers U1 and U2 (Huang et al. 2014) and sequenced on the Illumina MiSeq platform (2 × 300 bp) at GenOne Biotech © (Rio de Janeiro, Brazil).

The quality of the sequencing data was assessed using the "FastQC" program (v.0.11.9; Andrews 2010). To check the quality, size and error distribution of the sequences, the libraries were analyzed using the "fastq_eestats2" functions of the "USEARCH" program (v.11.0.667; Edgar 2010). The "search_oligodb" function of the USEARCH program was used with the ITS region primer sequences (ITS3F '5-GCATCGATGAAGAACGCAGC-3'; ITS4R '5-TCC TCCGCTTATTGATATGC-3') to detect the presence and position in the reads. From the identified primer sequences, we removed them along with any possible upstream bases using the "Atropos" program (v.1.1.31; Didion et al. 2017). Reads without the presence of primers were also removed (--discard-untrimmed). Sequences with a total average of less than Q25 ("--average_qual 25") were removed using the "Fastp" program (v.0.23.2; Chen et al. 2018). Finally, the library pairs (forward and reverse) were joined by overlapping using the "PEAR" program (v.0.9.11; Zhang et al. 2014) with a minimum overlap of 10 bases ("--min-overlap 10").

The merged readings were processed using the "DADA2" pipeline (Callahan et al. 2016) via the "dada2" package (v.1.22.0) for the "R" statistical program (v.4.1.2; R Core Team 2020). Initially, the readings were filtered using the "filterAndTrim" function with a maximum expected error value of 3 ("maxEE = 3"). Next, the base error probabilities were estimated ("learnErrors"), and the sequences were corrected based on the model obtained ("dada"). The resulting amplicon sequence variants (ASVs) were checked for chimeric sequences, which were then filtered using "removeBimeraDenovo". Eukaryotic ASVs were taxonomically annotated using the full UNITE database (v.9.0; Abarenkov et al. 2022). The sequence datasets of ASVs identified as fungi and plants were then processed separately. In addition, ASVs present in only one sample were excluded. The sequences have been deposited in GenBank under the accession number PRJNA977197.

2.5 Functional profile of the isolated yeasts

The isolated yeasts were previously activated in YMA broth at 28 °C under agitation at 100 rpm for 24 h. The strains were characterized for their ability to secrete hydrolytic enzymes such as esterases, cellulases, proteases, amylases, and lignases and for phosphate solubilization according to the methodologies described by Carrim et al. (2006) and Mello et al. (2019). The growth and production of halos (or precipitates, in the case of esterase) were assessed as absent or present (Soares et al. 2015).

2.6 Growth under different pH conditions and in beer wort

The ability of the isolates to grow at different pH values was determined in liquid YMA media inoculated into 96-well plates at 28 °C for 48 h. The pH of the medium was adjusted to 3, 7 and 9 by adding HCL (1 N) or NaOH (1 N). Growth was estimated by optical density in a spectrophotometer at 570 nm (Silva et al. 2020). The growth capacity of the beer wort was determined under the same conditions as above by replacing the YMA medium with Pilsen wort (malt, water,

and hops, 14.1° plateau, pH 5.45) previously sterilized by continuous boiling for 15 min (Coulibaly et al. 2022).

2.7 Chemical characteristics of the hive's nutrient sources

Honey and beebread samples were analyzed for pH, soluble solids content (Brix), and moisture content using standard methods (Adaškevičiūtė et al. 2019; Alves et al. 2005). Sugar content in honey was determined using high-performance liquid chromatography (HPLC) following the method described by Alghamdi et al. (2020). Beebread samples underwent methanolic extraction prior to HPLC analysis (Lu et al. 2022). Sugar identification and quantification were performed by comparing chromatograms and peak areas with standard solutions, calculated the concentration of the samples used in the respective dilutions (Alghamdi et al. 2020).

2.8 Data analysis

The alpha-abundance, Shannon, evenness, Margalef, Fisher, and Chao-1 estimated richness and indicator species analysis (IndVal) diversity estimators were obtained using Past software version 4.03 (Hammer et al. 2001). The distribution of the data was assessed using the Shapiro–Wilk test, and for normally distributed data, the difference in means was assessed using ANOVA with Dunnet's post hoc test, while for nonparametric data, the differences in means were assessed by the Kruskal–Wallis test with Dunn's post hoc test using SPSS version 17.0 (IBM Corp, Armonk, NY, USA). Beta diversity was estimated using the Jaccard (species composition) and Bray–Curtis (abundance) indices, and data dispersion was determined via NMDS (nonmetric multidimensional scaling analysis). The differences between the groups were estimated by permutational MANOVA (PERMANOVA) using Past software version 4.03 (Hammer et al. 2001). The species composition of the communities within the sampled groups was visualized in a Venn diagram built using Draw Venn software (http://bioinformatics.psb.ugent.be/webtools/Venn/). The metabarcoding data were analyzed using the Microbiome Analyst platform (version: 4.0.2; software Microbiome Analyst, https://www.microbiomeanalyst.ca). For the chemical characteristics and composition of the honey and bee bread samples, an unpaired t test was performed, and for the types of bees, ANOVA was performed, followed by the Tukey test when applicable. The samples were analyzed using the biplot and principal component analysis (PCA) method (Zawawi et al. 2022), considering the chemical characteristics of the samples and the diversity of yeasts associated with the bee species, with the Euclidean distance and the UPGMA (unweighted pair-group average) method. For the functional characteristics of the strains, we analyzed similarity using the Gower index, followed by PERMANOVA for biomes, bees and microhabitats.

3 Results

3.1 Cultivation-dependent yeast community associated with bees

The field collections obtained bee specimens from the three biomes, representing nine distinct species that were identified through the application of both morphological and molecular methods (Table S1 and Supplementary Material Figures S1 to S9). The chemical characteristics of honey and beebread from Amazonian bee nests were analyzed (Table S3). No significant differences were observed in the water content and pH values of the honey and beebread samples among the different bee species, just Brix ($p < 0{,}05$). However, beebread from *S. cf. polysticta* and *S. cf. nigrohirta* exhibited the highest total soluble sugar content.

Fructose and glucose were present in all honey and beebread samples. Except for *M. seminigra*, honey samples exhibited greater variability in sugar composition than beebread samples, with *T. angustula* samples displaying the highest variegate. Chemical profiles of honey and beebread differed significantly (PERMANOVA, $p = 0.0287$), with a clear separation evident in the PCoA analysis (Supplementary Material Fig. S10).

We isolated 176 strains, selecting 116 based on morphological and fingerprinting patterns (Table S2). These strains represent 46 species across 27 genera and 10 orders, belonging to the phyla Ascomycota and Basidiomycota (Table S4). The genera *Starmerella*, *Candida*, *Hanseniaspora*, and *Wickerhamiella* comprised more 90% of yeast abundance across the Amazon, Cerrado, and Pantanal biomes (Fig. 1 and Table S5), distributed among the species *S. etchellsii*, *C. orthopsilosis*, *Candida* sp., *S. apicola*, *Hanseniaspora opuntiae* and *Wickerhamiella versatilis* (Fig. 1). Notably, *S. etchellsii* was prevalent in the Cerrado and Pantanal, while *S. apicola* was abundant in the Amazon. *Starmerella etchellsii* and *S. apicola* were the only species shared among bee species across all three biomes, with most other species being biome-specific (Fig. 2a).

The composition of yeast communities exhibited variation among bee species within each biome. In the Amazonian environment, four bee species (*M. seminigra pernigra*, *S. cf. nigrohirta*, *S. cf. polysticta*, and *T. angustula II*) shared only six yeast species, with distinct dominant yeasts characterizing each species (Fig. 2b, Table S6). For instance, *H. opuntiae* was most abundant in *S. cf. nigrohirta* and *T. angustula II*, while *Pseudozyma hubeiensis* and *S. apicola*

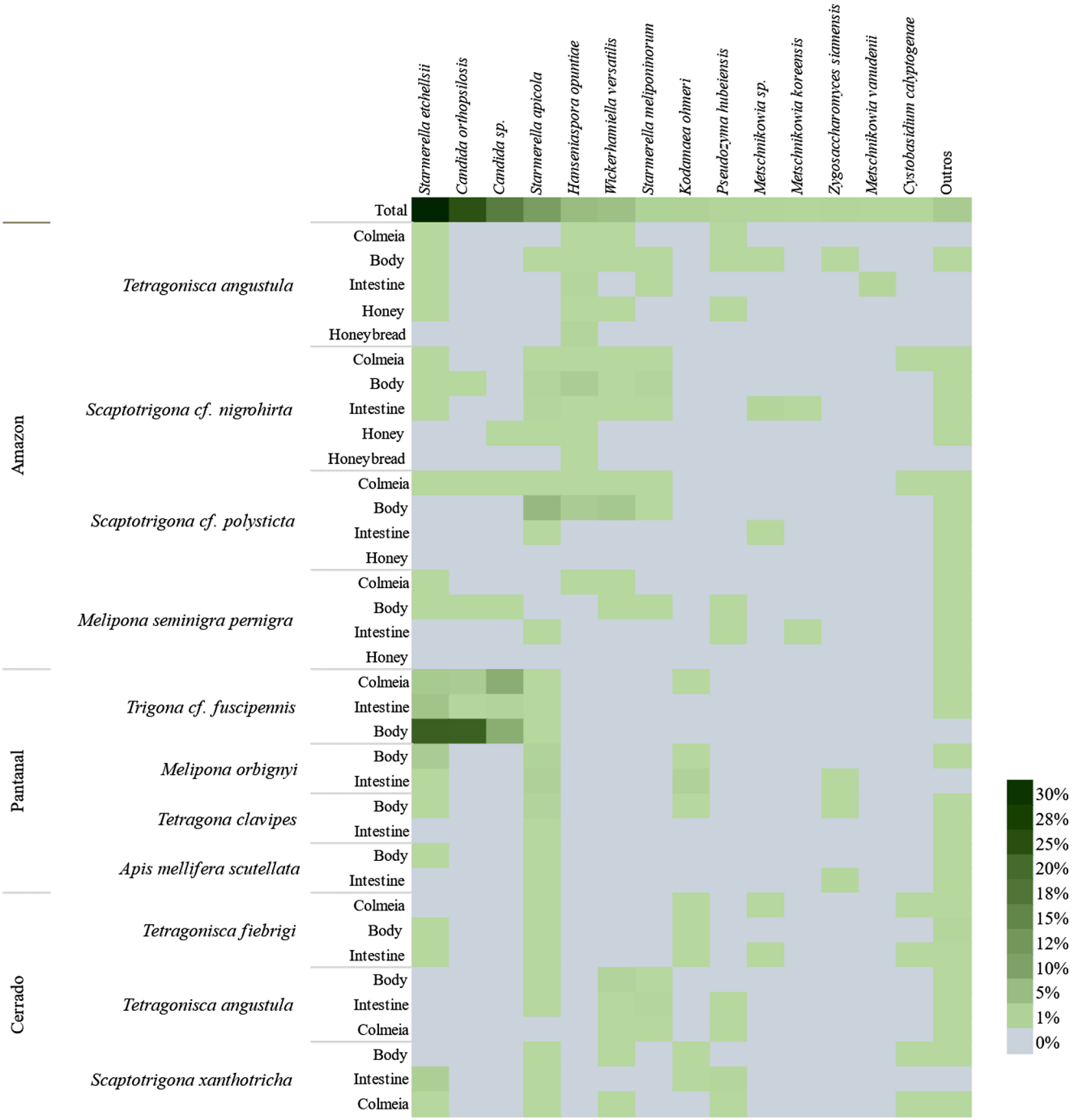

Fig. 1 Relative frequency of yeast species isolated from different bee microhabitats in the biomes studied

were predominant in *M. seminigra pernigra* and *S. cf. polysticta*, respectively. Additionally, each bee species, except for *S. cf. nigrohirta*, harbored unique yeasts, further emphasizing species-specific associations.

Similarly, the bees collected in the Cerrado (*T. fiebrigi II*, *T. angustula*, and *S. xanthotricha*) shared only two yeast species (*Aureobasidium pullulans* and *S. apicola*) (Fig. 2c, Table S7). *Starmerella etchellsii* dominated the yeast community associated with *S. xanthotricha*, while *W. versatilis* and *Rhodotorula* sp. were prevalent in *T. angustula* II and *T. Fiebrigi* II, respectively. Notably, all Cerrado bee species exhibited exclusive yeasts, further highlighting the specificity of yeast-bee associations in this biome.

In the Pantanal, the bees *A. mellifera*, *M. orbignyi*, *T. fiebrigi* I, and *Trigona cf. fuscipennis* shared only *S. apicola* and *S. etchellsii* (Fig. 2d, Table S8). The dominant

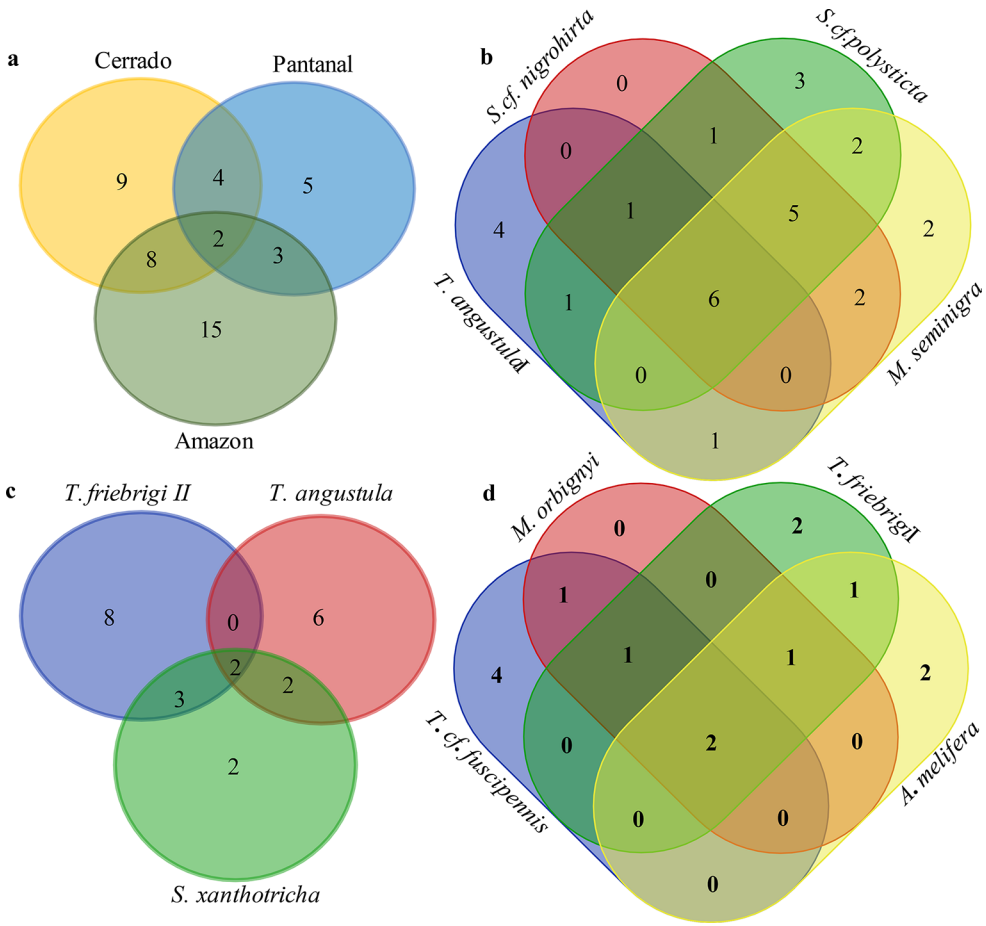

Fig. 2 Crop-dependent richness of yeast species associated with bees. (**a**) Species shared between biomes; (**b**), (**c**) and (**d**) yeast species shared between bees collected in the Amazon, Cerrado and Pantanal biomes, respectively

yeast varied among bee species, with *S. apicola* being more abundant in *T. fiebrigi* I and *M. orbignyi*, while *S. etchellsii* was predominant in *T. cf. fuscipennis* and *M. orbignyi*. As observed in other biomes, each bee species, except for *M. orbignyi*, also hosted exclusive yeasts, underscoring the influence of host specificity on yeast community composition.

The yeast isolates from the microhabitats exhibited compositional variation across the bee species sampled. The following compositions were identified in bees from the Amazon biome:

Tetragonisca angustula II is colonized by thirteen yeast species, distributed in the different microhabitats. Seven exclusive species were detected on its body, while *H. opuntiae* was present in all microhabitats and was the most abundant species (~62% abundance). *Wickerhamiella versatilis* was more abundant in the hive and honey (Supplementary Material Fig. S11a and Table S9).

Scaptotrigona cf. nigrohirta is colonized by sixteen yeast species across five microhabitats. The only species shared among all microhabitats was *H. opuntiae*, which constituted the most abundant species (~38% of abundance) in the bee (Fig. S12b). *Metschnikowia spp.* (~44%) were the most abundant in the gut (Table S9).

Scaptotrigona cf. polysticta harbors nineteen species of yeast, primarily in its hive and body. No yeasts were observed on this bee's beebread. *S. apicola* was the most abundant species (~52%), being the only species shared between the body, gut, and hive. *Z. rouxii* was exclusively observed in honey samples (Fig. S11c and Table S9).

M. seminigra pernigra was associated with eighteen yeast species. *P. hubeiensis* was the most abundant (~31%), particularly in the body and intestine. This bee also exhibited several exclusive species across different microhabitats (Fig. S11d and Table S9).

The composition of yeast species in the microhabitats of Cerrado bees also exhibited notable differences. *Tetragonisca fiebrigi II* harbors thirteen yeast species, with *K. ohmeri* and *S. apicola* shared among the hive, body, and gut. *Rhodotorula* sp. was the most abundant species (~22%) regardless of the microhabitat. The gut and body share one exclusive species (Fig. S12a and Table S10).

The yeast species identified in *Tetragonisca angustula* included ten distinct species. The yeast species *W. versatilis* and *S. meliponinorum* were observed to be present in all three microhabitats, namely the body, hive, and gut. However, each microhabitat also exhibited exclusive species. The most abundant species overall was *W. versatilis*,

representing approximately 60% of the total (Fig. S12b and Table S10).

Finally, *S. xanthotricha* was colonized by nine yeast species. *S. apicola* was shared among the body, gut, and hive, while the intestine had exclusive species. *Starmerella etchellsii* was the most abundant species (~63%) overall (Supplementary Material Fig. S12c and Table S10).

In the Pantanal, *T. cf. fuscipennis* hosted eight yeast species across three microhabitats (Fig. S13a). *Starmerella etchellsii* was the most abundant overall (~40%), while *Candida* spp. dominated the hive (Table S11). *Melipona orbignyi* harbored five species, with *S. etchellsii* and *S. apicola* being most abundant (~39% each) (Fig. S13b and Table S11). These two, along with *K. ohmeri*, were shared between body and gut, with each microhabitat also having an exclusive species.

Tetragonisca fiebrigi I hosted seven species, with *S. apicola* shared between body and gut (Fig. S13c). The gut harbored five exclusive species, the body one. *Starmerella apicola* was most abundant (~87%) (Table S11). *Apis mellifera* was colonized by seven species, with *S. apicola* shared between body and gut (Fig. S13d). Each microhabitat had three exclusive species. *Zygosaccharomyces siamensis* was most abundant (~34%) overall and in the gut, while *Sympodiomycopsis* sp. dominated the body.

A comparison of the various biomes revealed that the yeast communities associated with bees in the Amazon exhibited higher Fisher diversity and estimated Chao-1 richness (Duncan, $p < 0.05$) than those in other regions. The evenness indices differed between the Amazon and Pantanal, but not the Cerrado (Duncan, $p < 0.05$) (Table S12a).

The alpha diversity of Amazonian bee-associated yeasts exhibited slight differences (Duncan, $p < 0.05$), whereas no differences were observed for Cerrado and Pantanal bees (ANOVA, $p < 0.05$) (Table S12b to S12d).

Alpha diversity exhibited variation between microhabitats, contingent on the bee species in question. In *Tetragonisca angustula* II, significant differences were observed for evenness, Margalef, Fisher, and Chao_1, but not for Shannon diversity (Table S12e). No differences were observed in the case of *S. cf. nigrohirta*. The yeast diversity observed in the hive was greater for *S. cf. polysticta* than in other microhabitats (Table S12e).

In *M. seminigra pernigra*, the alpha diversity of honey and beebread was found to be similar but differed from that of the hive and body. The estimated richness of gut-associated yeast in *A. mellifera* was found to be greater than in other microhabitats. In *S. xanthotricha*, the hive exhibited greater yeast biodiversity (Shannon), and Margalef, Fisher, and Chao1 richness were greater in the hive than in the body and gut. No significant differences were identified among microhabitats of *T. cf. fuscipennis*, *M. orbignyi*, *T. Fiebrigi* II, and *T. angustula I* (Table S12e).

The structure of yeast communities associated with bees exhibited a significant influence from the biome, microhabitat, and bee species (PERMANOVA, $p < 0.05$) (Fig. 3 and Table S13). The beta diversity analysis further demonstrated that the composition and abundance of yeast are significantly impacted by the species of bee (PERMANOVA = 0.001) (Supplementary Fig. S14 and Table S14).

For certain bee species (*M. seminigra*, S. cf. polysticta, S. cf. nigrohirta, T. angustula I, *T. angustula* II, and *S. xanthotricha*), the microhabitat was found to significantly influence yeast composition and abundance (PERMANOVA, $p < 0.05$) (Table S13). However, for other bee species, microhabitat did not have a significant impact on yeast community structure (PERMANOVA, $p > 0.05$) (Table S13).

The biome, bee species, and microhabitat were found to be significant indicators of yeast species ($p < 0.05$) (Table S14). Specifically, 16 indicator species were identified across the three biomes, with two in the Cerrado, seven in the Pantanal, and seven in the Amazon (Table S14). Moreover, 23 indicator yeast species were identified in association with the analyzed bees, with a range of one to five species per bee. In addition, eleven significant yeast species were identified as indicators for characterizing the microhabitats, with a range of one to four species per microhabitat (Table S14).

3.2 Cultivation-independent yeast community associated with bees

In our culture-independent analysis of bee-associated eukaryotic diversity, we generated a total of over five million raw ITS sequences, with 89% of these successfully assigned to amplicon sequence variants (ASVs). Of these, 69.76% were identified as fungal species, while 19.25% were identified as plant species (Table S15). This approach yielded approximately twice the diversity compared to culture-dependent methods, with 15 genera and species identified by both (Fig. S15).

A total of 253 fungal ASVs were obtained, grouped into 108 known and 115 unclassified species, distributed across various taxonomic levels (Table S16). The genera *Starmerella*, *Metschnikowia*, *Clavispora*, and *Cladosporium* collectively constituted approximately 61% of the ASVs abundance. The most abundant species were *Metschnikowia koreensis*, *Saccharomyces apicola*, *Saccharomyces meliponinorum*, *Saccharomyces etchellsii*, *Heterosporosopsis pseudoguilliermondii*, and *Wickerhamomyces versatilis*, collectively representing approximately 58.5% of ASVs abundance. The fungal *S. apicola* was particularly prevalent in the Amazon and Pantanal regions, whereas *S. etchellsii*

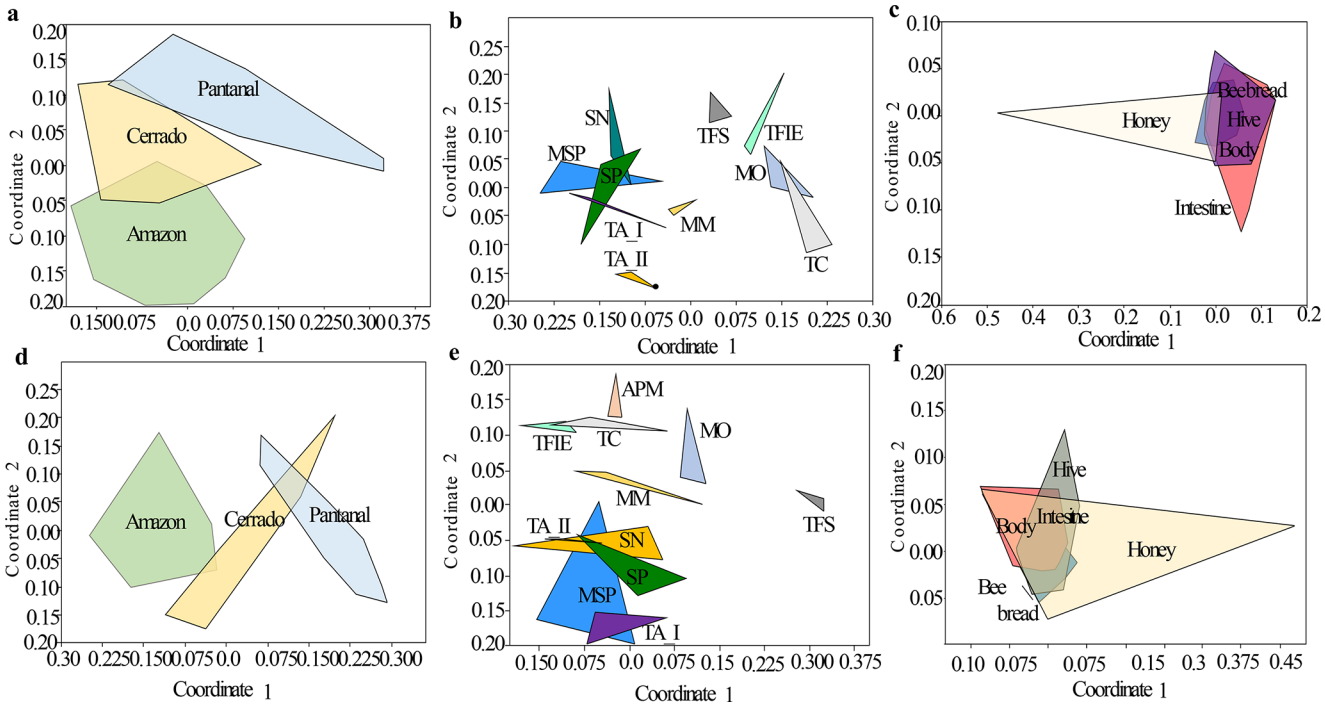

Fig. 3 Nonmetric multidimensional scaling (NMDS) calculated from Jacard (top figures) and Bray–Curtis (bottom figures) distances for biome, (**a** and **d**), bee species (**b** and **e**) and microhabitats (**c** and **f**) of the yeast communities. *Apis mellifera* (AMP), *S. xanthotricha* (MM), *M. orbignyi* (MO), *M. seminigra pernigra* (MSP), *S. cf. nigrohirta* (SN), *S. cf. polysticta* (SP), *Tetragona clavipes* (TC), *T. angustula* I (TA_I), *T. angustula* II (TA_II), *T. fiebrigi* I (TFIE) and *T. fiebrigi* II (TFS)

was abundant in the Amazon. While 34 ASVs were shared among the different biomes, the majority of fungals diversity was biome-specific (Fig. 4a, Table S16).

The composition of ASVs associated with Amazonian bees demonstrated a greater degree of specificity than overlap. Each bee species exhibited a high degree of specificity in its fungal microbiome, with the presence of numerous exclusive ASVs (Fig. 4b and Table S16). *Diutina rugosa* was the most prevalent species in *S. cf. nigrohirta*(~30%) and *S. cf. polysticta*(~37%), while *Zygosporium. Oscheoides*(82%) was the most abundant in *T. angustula*II (Table S17). The highest abundance of *S. bombicola* (~27%) was observed in *M. seminigra pernigra*.

The Cerrado bees exhibited a greater degree of shared ASVs and orders than exclusive ones (Fig. 4c and Table S15). *Tetragonistica angustula* I and *S. xanthotricha* were found to harbor 29 and 28 ASVs, respectively, with 37 being shared between the two. The highest abundance of *D. rugosa* and *Z. oscheoides*, whit ~ 12% both, was observed in *S. xanthotricha*, while *W. versatilis* (43,9%) and *M. koreensis* (19,32%) were the most prevalent in *T. angustula* I. Both species exhibited elevated relative abundances of *M. koreensis* and *Starmerella spp* (Table S17).

Pantanal bees, *A. mellifera* and *M. orbignyi* I, shared 84 ASVs but also exhibited 32 and 41 exclusive ones, respectively (Fig. 4d and Table S15). The most prevalent species in *A. mellifera* was *C Cladosporium velox* (~52%), while *D. rugosa* (29,5%) was the most abundant in *M. orbignyi*. All fungal ASVs were derived from the body.

The order Saccharomycetales was the most abundant in all biomes, accounting for approximately 72% of the total, followed by the orders Unclassified, Hypocreales and Capnodiales, which together accounted for approximately 96% of the total (Table S17). Each biome had a different set of dominant orders (Fig. 4a). In the Amazon, the most dominant orders were Saccharomycetales (81.99%), Hypocreales (9.11%) and Capnodiales (2.45%). In the Cerrado, the dominant orders were Saccharomycetales (16.86%), Eurotiales (0.21%) and Capnodiales (0.19%). In the Pantanal, the most abundant orders were Capnodiales, Botryosphaeriales, Saccharomycetales and GlomerellalesCapnodiales (33.99%), Botryosphaeriales (24.25%), Saccharomycetales (18.98%) and Glomerellales (16.13%). A total of fifteen orders were shared between the three biomes, and the sum of twelve orders were observed as exclusively and distributed among him (Fig. 4a).

The number of fungal orders hosted by Amazonian bees exhibited variation (Fig. 4b and Table S16). The order Saccharomycetales was the most abundant in all species except *T. angustula* II. Seven orders were observed to be shared among all Amazonian bees (Table S17).

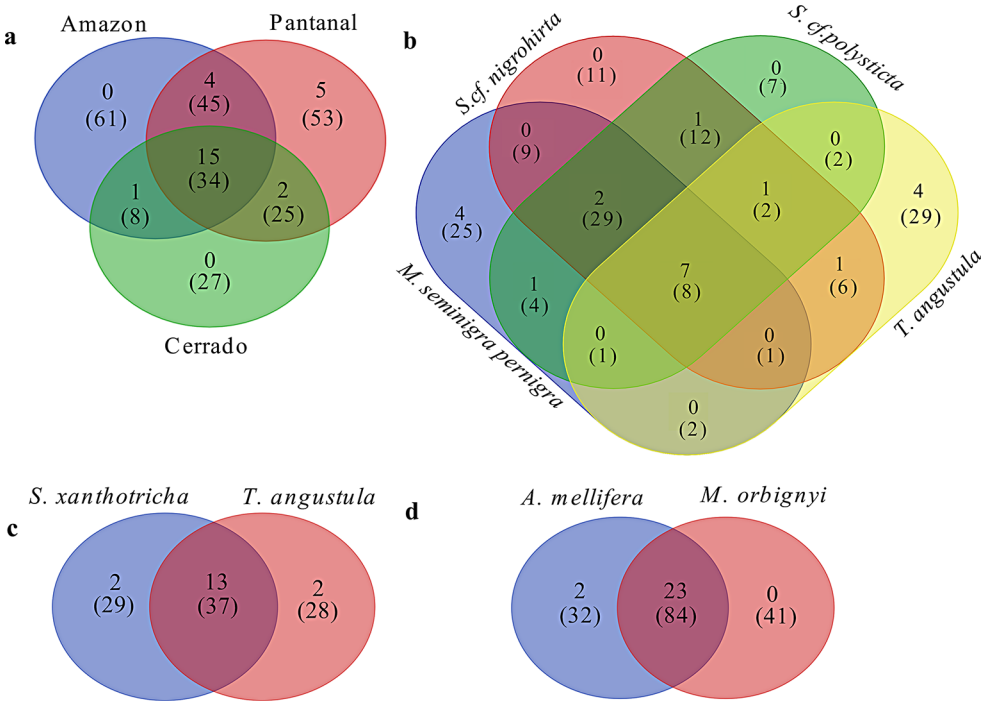

Fig. 4 ASVs richness (among relatives) and crop-independent fungal orders shared by **a** - biomes, **b** - Amazonian bees, **c** - Cerrado bees, and **d** - Pantanal bees

In the Cerrado, both *T. angustula* I and *S. xanthotrichae*xhibited the presence of 2 fungal orders each (Fig. 4c and Table S16). The unclassified orders were the most abundant in both cases (more 75% in the ambas bee), followed by Saccharomycetales (Table S17). A total of 13 orders were shared by the two species.

The fungal orders identified in the samples from the Pantanal region included 25 in the total, two of which were exclusive to *A. melifera* (Fig. 4d). The Saccharomycetales were the most prevalent fungal order in *M. orbignyi* (~39%), while the Botryosphaeriales (43.59%) and Capnodiales (43.62%) were the most common in *A. mellifera* (Table S17).

Furthermore, the composition of ASVs varied across microhabitats. In *S. cf. nigrohirta*, 23 ASVs were identified as being shared among the body, honey, and gut, with each having exclusive species (Fig. S16a and Table S15). The most abundant shared species were *Starmerella spp*. and *W. versatilis* (Table S17). In *M. seminigra pernigra*, eight ASVs were shared among the honey, intestine, and body, with each having unique ASVs (Fig. S16b). The most abundant shared species were *M. guilliermondii* and *S. apicola* (Table S17).

Tetragonisca angustula was found to harbor 35 and 12 fungal ASVs in the body and gut, respectively, with 14 being shared (Fig. S16c). The most abundant species was *M. koreensis* across the various microhabitats (Table S16). In *S. cf. polysticta*, 35 ASVs were identified as being shared between the body and the gut, with each having exclusive ASVs (Fig. S16d). The most abundant shared species were *C. tropicalis*, *S. apicola*, and *S. meliponinorum* (Table S17).

Furthermore, fungal order diversity exhibited variation across microhabitats. In *S. cf. nigrohirta*, seven orders were identified as being shared among the body, honey, and gut, with each having exclusive orders (Fig. S16a). *Melipona seminigra* pernigra exhibited a notable degree of microhabitat specificity, with six orders being shared among the three microhabitats (Fig. S16b). *Tetragonisca angustula* and *S. cf. polysticta* exhibited the sharing of six fungal orders between the body and gut (Fig. S16c and Fig. S16d).

Considering each sampling unit within the biomes, fungal ASVs alpha diversity indices (Shannon, Simpson, Fisher's alpha, and Chao1-estimated richness) did not differ significantly among biomes (ANOVA, $p > 0.05$) (Table S18a). However, at the order level, the Pantanal exhibited higher diversity across all indices (Tukey, $p < 0.05$) 9Table S18b).

Similarly, fungal ASVs alpha diversity did not differ significantly among bee species, regardless of biome (ANOVA, $p > 0.05$) (Table S18c). However, Fisher's alpha diversity was lower in *S. xanthotricha* and *T. angustula II* compared to other bees. Chao1-estimated richness was higher in *S. cf. nigrohirta* and *S. cf. polysticta*.

At the order level, *M. orbignyi* showed no significant differences in Shannon and Simpson indices compared to other species, except for *M. seminigra pernigra*. (Table S18d). Fisher's alpha diversity was higher in *A. mellifera* and *M. orbignyi*. Chao1-estimated richness was similar and higher

in *M. orbignyi*, *S. cf. nigrohirta*, and *S. cf. polysticta* compared to other bees.

Regardless of bee species, no significant differences were found in ASVs alpha diversity among microhabitats (ANOVA, $p < 0.05$) (Table S18e). At the order level, only Fisher's alpha diversity differed, with the body showing higher diversity than honey and gut (Tukey, $p < 0.05$) (Table S18f).

The structure of yeast communities (composition and abundance) differed significantly mong Microhabitat, bee species and biome, for both ASVs and fungal orders (PERMANOVA, $p < 0.05$) (Fig. 5a and f; Fig. S17 to Fig. S17e and Table S19).

The core microbiome exhibited variability according to the biome, bee species, and microhabitat under analysis (Fig. S18a). The core microbiome was composed of *S. apicola*, S. *meliponinorum*, *S. etchellsii*, and *W. versatilis*, and was observed in all sampled bees. Similarly, these yeast species are present in the core microbiomes of the bees *S. cf. nigrohirta* (Fig. S18b), *M. seminigra pernigra* (Supplementary Material Fig. S18c), *M. orbignyi* (Fig. S18e), and *S. xanthotricha* (Fig. S18g).

LEfSe analysis identified taxa discriminating fungal populations. *H. pseudoguilliermondii* and Saccharomycetales were strongly associated with the Amazon biome. *M. koreensis*, *S. apicola*, and *S. meliponinorum* were associated with specific bee species, and Saccharomycetales with *T. angustula II*. No significant taxa were identified for microhabitats.

3.3 Plant community associated with bees

Environmental DNA analysis identified 100 plant ASVs associated with the collected bees, representing 24 known and 55 unclassified species (Table S20). The genera *Vernonia*, *Astronium*, *Sloanea*, and *Solanum* accounted for ~93% of total ASV abundance across the three biomes (Table S21). Unclassified plant species were most abundant (~51%), followed by *Vernonia echioides*. *Brassica carinata* was prominent in the Amazon, while *V. echioides* dominated in the Cerrado and Pantanal. Eight ASVs were shared among all biomes, with additional biome-specific ASVs observed (Fig. 6a and Table S20).

Plant family composition varied among bee species and biomes (Fig. 6b-d). Regardless of biome, bees were associated with seven families, with Anacardiaceae and Asteraceae being predominant. Exclusive families were detected in each biome. In the Amazon, Combretaceae and Anacardiaceae were prevalent; Anacardiaceae dominated in the Cerrado; and Asteraceae, Elaeocarpaceae, Solanaceae, and Fabaceae were prevalent in the Pantanal.

Amazonian bees showed no shared plant ASVs and families (Fig. 6b and Table S20). Each species had exclusive

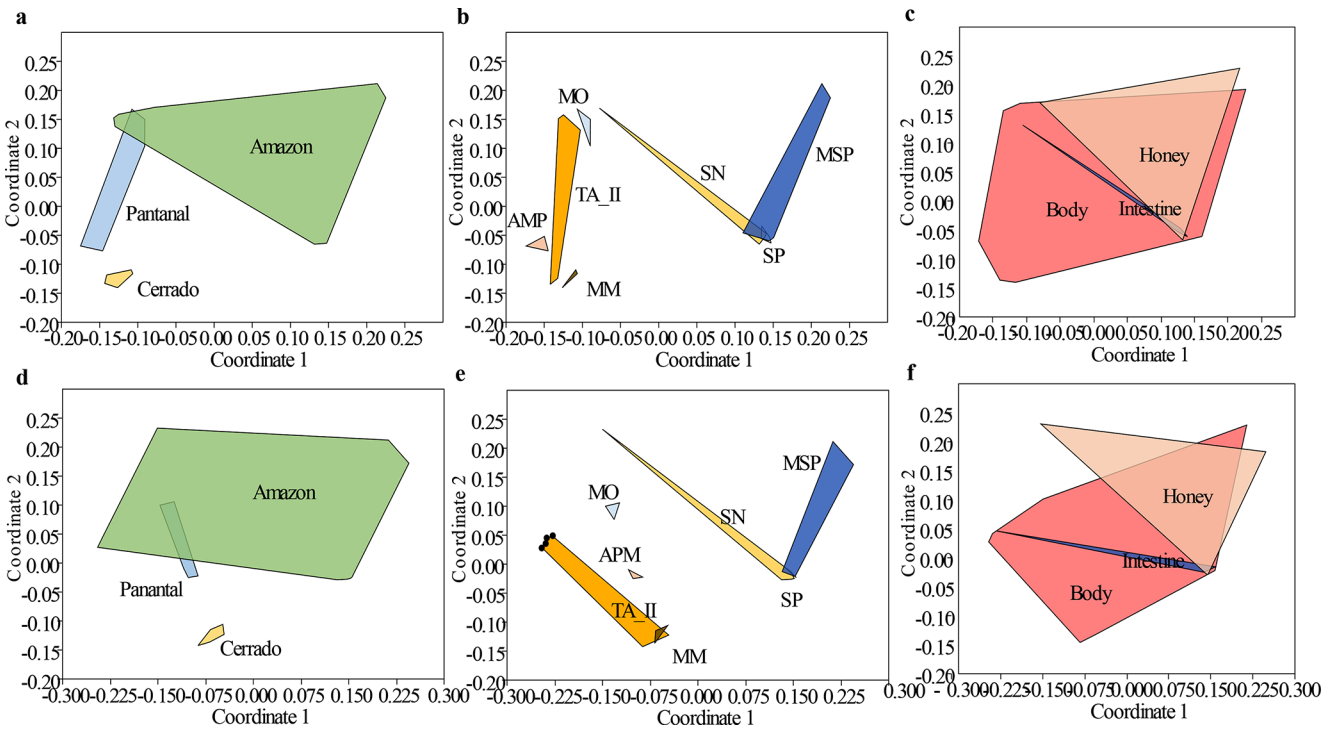

Fig. 5 Nonmetric multidimensional scaling (NMDS) calculated from Jaccard (top figures) and Bray–Curtis (bottom figures) distances for biomes (**a** and **d**), bees (**b** and **e**) and microhabitats (**c** and **f**) in fungal ASVs. *Apis mellifera* (AMP), *S. xanthotricha* (MM), *M. orbignyi* (MO), *M. seminigra pernigra* (MSP), *S. cf. nigrohirta* (SN), *S. cf. polysticta* (SP), *Tetragona clavipes* (TC), *T. angustula* I (TA_I), *T. angustula* II (TA_II), *T. fiebrigi* I (TFIE) and *T. fiebrigi* II (TFS)

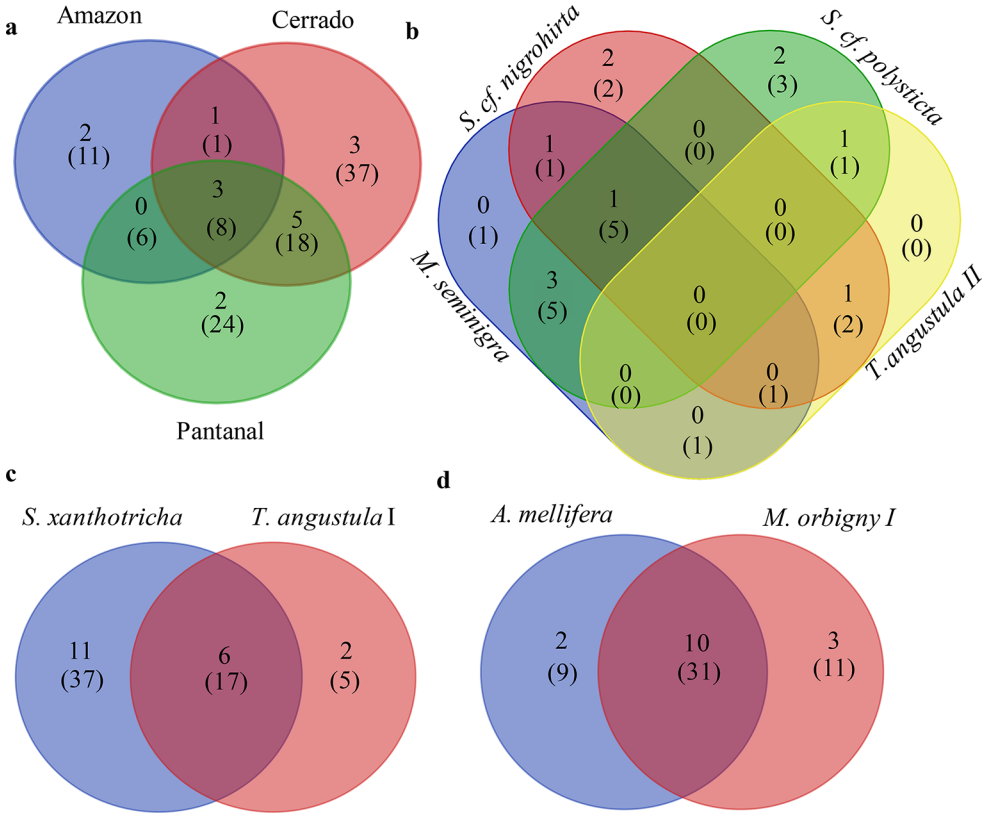

Fig. 6 Richness of (ASVs) and plant families associated with the bees studied: a - biomes, b - Amazonian bees, c - Cerrado bees, d - Pantanal bees

associations. In the Cerrado, *S. xanthotricha* and *T. angustula I* shared 17 plant ASVs, with Anacardiaceae representing ~94% of associated ASVs (Fig. 6c and Table S21).

A. mellifera and *M. orbignyi* in the Pantanal shared 31 plant ASVs, with *V. echioides* being most abundant for both (Fig. 6d). They were associated with ten plant families, with Asteraceae predominant (~73% of ASVs) (Table S21). Each bee species also had exclusive plant associations.

Considering each sampling unit, the biome significantly influenced the alpha diversity of plant ASVs associated with bees (ANOVA, $p < 0.05$) (Table S22a). Amazonian bees were associated with greater plant biodiversity, while Pantanal bees showed greater ASV richness (Tukey, $p < 0.05$). Regarding plant families, Pantanal bees exhibited higher diversity, whereas Cerrado and Amazonian bees showed higher richness (Tukey, $p < 0.05$) (Table S22b and Table S22d).

Plant ASV diversity also varied among bee species (ANOVA, $p < 0.05$). *A. mellifera* had the highest ASV diversity, while *A. mellifera*, *M. orbignyi*, and *S. xanthotricha* exhibited greater ASV richness. *S. xanthotricha* showed the greatest richness of associated plant families, followed by *M. orbignyi* and *A. mellifera* (Table S22 c).

Among microhabitats, no significant differences were found in floral ASV diversity (Shannon and Simpson) (ANOVA, $p > 0.05$). However, Chao1-estimated richness differed significantly, with the body showing the highest richness for both ASVs and floral families (Tukey, $p < 0.05$) (Table S22 e and Table S22 f).

The structure (composition and abundance) of the diversity of plants associated with bees differed between the biomes and bee species analyzed for both ASVs (Fig. 7a, b, d and e) and families (Fig. S20a - S20b and Fig. S20d - S20e) (PERMANOVA $p < 0.05$) (Table S23). The microhabitat was not influenced by the composition or abundance of the plants related to the bees collected in the different biomes, either at the level of ASVs (Fig. 7c and e) or families (Fig. S20c and S20e) (PERMANOVA $p < 0.05$) (Table S23).

We applied linear discriminant analysis effect size (LEfSe) analysis to explore the plant families and ASVs that are most strongly associated with biomes, bees and microhabitats. Among the biomes, the plant *Vernonia echioides* (Supplementary Material Fig. S21a) and the Asteraceae family (Supplementary Material Fig. S21c) are notable taxa associated with bees in the Pantanal. Among the bees, the significance cutoff made it possible to evaluate only *A. mellifera* and *M. orbignyi* for plant species, with discrimination of *V. echioides* and *Solanum tuberosum*, respectively (Supplementary Material Fig. S21b).

For the discrimination of plant families, Anacardiacea discriminates *S. xanthotricha*, Asteraceae and Elaeocarpaceae and describes *A. mellifera*; Solanaceae discriminates *M. orbignyi;* and Combretaceae discriminates S. cf. polysticta

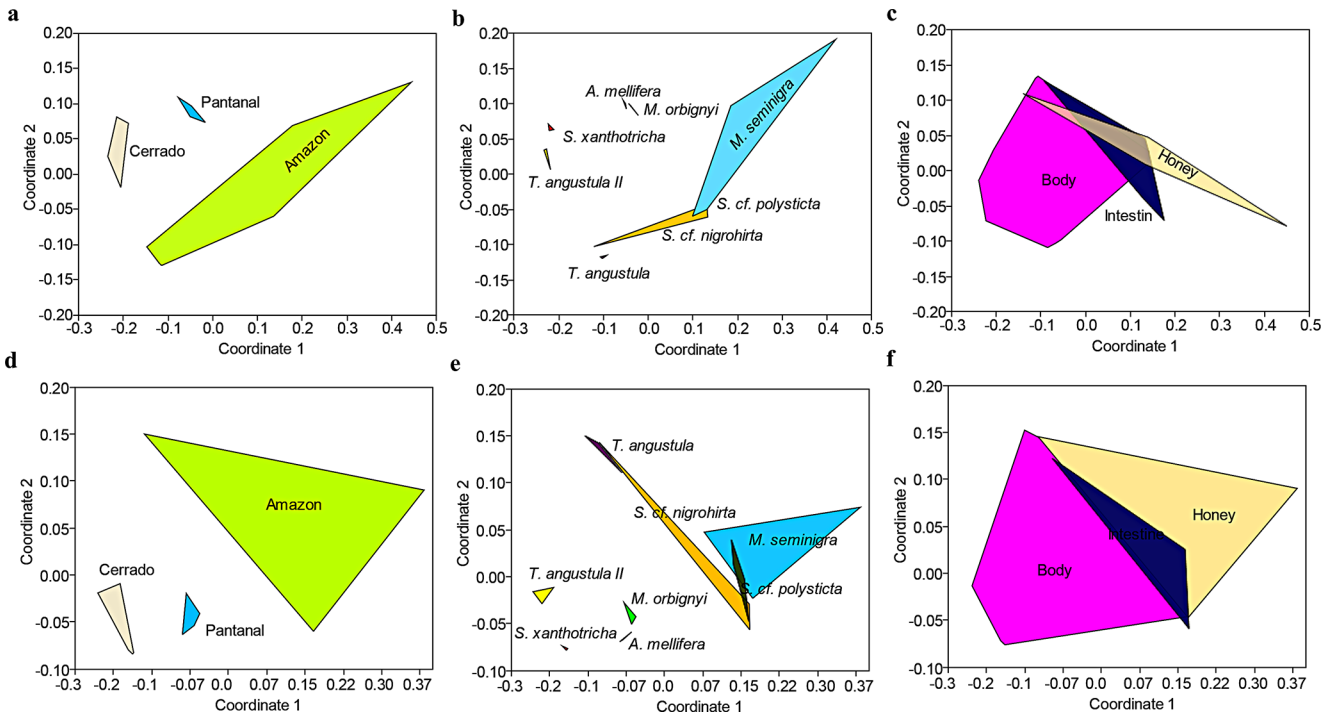

Fig. 7 Nonmetric multidimensional scaling (NMDS) calculated from Jaccard (top figures) and Bray–Curtis (bottom figures) distances for floral ASVs in different biomes (**a** and **d**), bees (**b** and **d**) and microhabitats (**c** and **e**)

(Supplementary Material Fig. S21d). Among the microhabitats, only one species, *V. echioides*, and two families, Asteraceae and Elaeocarpaceae, had LDA scores for both species and plant families.

3.4 Yeast function

The cultivable strains showed diverse secretion of hydrolytic enzymes, phosphate solubilization and pH tolerance (Table S2). *Aureobasidium melanogenum* and *A. pullulans* showed the ability to secrete all the enzymes analyzed. Phosphate solubilization was observed in about 25% of the strains (Fig. S22). Growth at neutral pH (pH 7) was predominant ($\Delta OD > 0.3$), but also in registered acidic (pH 3) and alkaline (pH 9) conditions. Notably, several strains showed robust growth over a wide pH range, including in Pilsen beer wort.

Biome of origin significantly influenced yeast functionality (PERMANOVA, $p = 0.0026$), with distinct profiles observed between yeasts associated with the Cerrado, Pantanal and Amazon. Bee species also slightly influenced functional similarity (PERMANOVA, $p = 0.05$). In addition, functional groups varied significantly between yeasts isolated from different bee species, especially between *M. orbignyi* and others (*T. angustula*, *S. xanthotricha*, *T. fiebrigi*). Interestingly, the microhabitat did not influence the analyzed characteristics (PERMANOVA, $p = 0.63$).

4 Discussion

We collected samples from eleven bee species across the Amazon, Cerrado, and Pantanal biomes, utilizing both molecular and traditional identification methods. Barcoding (COI gene) proved effective for molecular identification, corroborating morphological findings. This combined approach enhances accuracy and efficiency in bee identification, minimizing the need for extensive specimen collection (Köhler et al. 2007; Smith et al. 2024).

While *Apis mellifera* is an exotic species widespread in Brazil, our study observed it solely in the Pantanal. *Tetragonisca angustula* was common to both the Cerrado and Amazon, whereas *T. fiebrigi* was found in the Pantanal and Cerrado. These distributions align with previous reports (Costa Neto and Benevides de Morais 2020; Engel et al. 2023; Camargo et al. 2023). The endemic Amazonian subspecies *M. seminigra pernigra* was also encountered, consistent with its expected range (Francini et al. 2022). The diversity and distribution of stingless bees in Mato Grosso underscore the Brazilian rainforest's significance as a genetic reservoir for bees, capable of interacting with a wide array of plant species (Marconi et al. 2022).

We observed no significant differences in honey chemical composition (water content, pH, °Brix) among Amazonian bee species. However, both honey and beebread exhibited complex sugar compositions, with up to 8

different saccharides detected. This variability aligns with previous findings on stingless bee honey, which often differs from honey produced by Africanized bees like *A. mellifera* (Silva Macêdo et al. 2023; Ngaini et al. 2023). Glucose and fructose were consistently present in high concentrations, reflecting their central role as primary honey sugars. Our data also revealed considerable variation in sugar concentration between honey and beebread, echoing findings from a systematic review by Nordin et al. (2018).

The honey of *M. seminigra*, *S. cf. polysticta*, and *S. cf. nigrohirta* consisted mainly of glucose and fructose. While these monosaccharides predominate, other sugars are commonly detected in honey and beebread (Aljohar et al. 2018), consistent with our observations. Interestingly, *S. cf. polysticta* and *S. cf. nigrohirta* exhibited similar honey chemical compositions despite differing floral preferences, potentially due to their phylogenetic relationship (Sponsler et al. 2022), which needs to be validated in the future.

This study represents the first examination of yeast distribution in bees across diverse Brazilian biomes. We accessed a substantial yeast collection, ranging from 14 species in the Pantanal to 28 in the Amazon, with *S. etchellsii* being the most abundant overall (32%). Notably, *S. etchellsii* and *S. apicola* were the only yeast species shared by all bee species, regardless of biome. These findings reinforce the established notion that bees harbor diverse microbiota dominated by yeasts (De Paula et al. 2023b). Neotropical bees, fulfilling crucial ecological, sociocultural, and economic roles, provide various ecosystem services, including pollination and microorganism vectoring, thereby contributing to overall biodiversity (Elizalde et al. 2020).

In the Amazon, among the 32 isolated yeasts, *M. seminigra pernigra* exhibited the highest richness (18) and *T. angustula II* the lowest (13), with *S. apicola* being the most abundant (36%). This aligns with previous work on cultivable fungi from *M. seminigra pernigra* in the same biome (Tiago et al. 2022). In the Cerrado, *T. angustula* displayed the highest richness (13), consistent with findings by Januário da Costa Neto and Benevides de Morais (2020). In the Pantanal, *T. cf. fuscipennis* had the highest richness (8). Salomón et al. (2024) reported a similar trend in *T. fiebrigi* from Argentina. *Starmerella etchellsii* was more abundant in the Cerrado (33%) and Pantanal (40%), supporting the strong association between *Starmerella* species and stingless bees in the Cerrado (Januário da Costa Neto and Benevides de Morais 2020).

Our studies revealed that while all sampled bee species shared some yeast species, such as *S. apicola*, they also harbored a significant number of exclusive yeasts, sometimes exceeding the number of shared ones. This observation aligns with previous findings by Da Costa Neto and Benevides de Morais (2020), who reported *S. apicola* as a common species among several stingless bee species in northeastern Brazil. The frequent occurrence of this yeast in flowers, honey, and pollen further suggests the role of bees as vectors for microorganisms (Santos et al. 2018; Vu et al. 2016).

Our work identified shared yeast species among bees within each biome, suggesting strong associations between specific genera and tropical bees. The predominant genera, *Starmerella*, *Candida*, *Hanseniaspora*, and *Wickerhamiella*, align with those found in other studies on bee-associated yeasts (Oliveira Scoaris et al. 2021).

Hanseniaspora opuntiae, a yeast species studied for the biocontrol of aspergillosis in food (Gimenes et al. 2023) and for the aggregation of phenolic compounds in wine (Filippousi et al. 2024), is common among the microhabitats of *T. angustula* II and *Scaptotrigona cf. nigrohirta*, making it an indicator species for bees in the Amazon biome. On the other hand, *S. apicola*, a yeast widely studied in cereal and beverage fermentation (Kasegn et al. 2024), was common among the microhabitats of *T. fiebrigi* I, *S. xanthotricha*, *S. cf. polysticta*, *M. orbignyi*, *T. fiebrigi* II, *A. mellifera* and *T. cf. fuscipennis.*

Some yeast species can serve as indicators of environmental quality and bee community health (Stefanini 2018; Pozo et al. 2020). Thus, the identified yeasts may play crucial roles in honey, beebread, and hive environments. *Pseudozyma hubeiensis*, known for its xylose metabolism and lipid accumulation capabilities (Qvirist et al. 2022), showed a strong association with *M. seminigra pernigra*. *Wickerhamiella versatilis*, recognized for its osmotolerance (Wang et al. 2024), was predominant in *T. angustula I* and an indicator species for Amazonian bees. *Starmerella etchellsii*, commonly found in *T. cf. fuscipennis* and *M. orbignyi*, serves as an indicator for Pantanal bees.

Our results partially corroborate those of Echeverrigaray et al. (2021), who observed a high abundance of certain yeast species in honey from *Tetragonisca* and *Scaptotrigona* bees. The shared presence of yeasts across microhabitats suggests both dispersal and potential functional roles in different bee body regions (Klaps et al. 2020; Agarbati et al. 2024; Cubillos et al. 2019).

The Shannon diversity index of cultivable yeasts varied among bee species within each biome, with no significant differences observed within the Cerrado and Pantanal. Similar patterns were reported by Santos, Borges, and Rocha (2023) for larval yeasts. Our data emphasize the complexity of bee-fungal interactions across ecosystems, with potential implications for bee ecology, pollination, and honey production. Assessing species richness aids in understanding community diversity and ecosystem dynamics (Hillebrand et al. 2018).

Microhabitat-specific differences in Shannon diversity were observed in some bee species, indicating the influence

of microhabitat on yeast community structure. The occurrence or absence of yeasts in honey might be attributed to its composition, as its high sugar content and other constituents can favor or inhibit microbial growth (Rao et al. 2016; Fernandes et al. 2018). Further in-depth studies across different time periods could elucidate these interactions and the associated microbial biodiversity.

Finally, our culture-independent method detected twice the fungal diversity compared to cultivable yeasts, aligning with the findings of Prosdocimi et al. (2015), who highlighted the advantages of molecular methods in revealing a broader range of microorganisms in ecological studies of insect symbionts.

Metabarcoding analysis also revealed that most fungal ASVs were specific to bees within each biome. *Metschnikowia koreensis* was prominent among the 34 ASVs shared by bees across all three biomes. Notably, while Santos, Borges, and Rocha (2023) classified 300 fungal ASVs in stingless bee larval food, they observed no shared ASVs among the bee species studied, contrasting with our findings.

The order Saccharomycetales represented a major portion (~82%) of non-cultivable fungi associated with bees across biomes. However, Capnodiales (~34%) was particularly abundant in the Pantanal. Each biome harbored unique fungal orders, and *S. xanthotricha* and *T. angustula* showed a high abundance of unclassified fungal orders. The combination of culture-dependent and -independent methods allowed for a more comprehensive assessment of bee-associated fungal communities, enabling inferences about ecological relationships (Bairoliya et al. 2022; Jones et al. 2018).

Our results highlight the vast unexplored territory regarding fungal groups associated with bees. We identified numerous unclassified ASVs at higher taxonomic levels, suggesting bees may be a reservoir for novel fungal species. Some of these unclassified fungi might be pathogenic, impacting hive health (Wirta et al. 2021).

The abundance of fungal ASVs, predominantly yeasts (Ascomycota), underscores the fungal richness associated with bees in these ecosystems. This aligns with findings by Shell and Rehan (2022), who reported a significant fungal presence in the microbiota of *Ceratina australensis*. Our study further emphasizes the importance of the Metschnikowiaceae family in bee microbiomes.

Diutina rugosa was common among bees in the Amazon, Cerrado, and Pantanal. Other yeasts, such as *Z. oscheoides*, showed high abundance in specific bee species. The shared presence of *W. versatilis* among Amazonian bees and the high abundance of *Z. oscheoides* in Cerrado bees suggest potential roles in host adaptability. *Starmerella apicola*, a core microbiome member, was commonly observed, consistent with its widespread presence in bees and its considerable genetic polymorphism (Lachance et al. 2010).

Pantanal bees shared more ASVs than those from the Cerrado or Amazon. This might be attributed to common plant preferences, as shared plant ASVs were also higher in this biome. Our results partially agree with those of Shell and Rehan (2022), who found similar taxonomic cores associated with wild bees, but we also identified a significant presence of the Metschnikowiaceae family.

We observed distinct alpha and beta diversity among fungal orders in bee microhabitats, emphasizing the body's role and the similarity between gut and honey. Fungal diversity can impact bee health, with some species conferring resistance to pathogens or stress (Khan et al. 2020), while others, like *Aspergillus*, can be pathogenic (Becchimanzi and Nicoletti 2022).

Our metabarcoding approach also detected plant species, allowing identification of 100 plant ASVs associated with bees. The interplay between bees, floral preferences, and microorganism vectorization can influence nutritional sources and bee health (Rutkowski et al. 2023; Schaeffer et al. 2023). Metabarcoding aids in assessing bee floral preferences, informing beekeeping practices and conservation strategies (Hawkins et al. 2015; Creer et al. 2016; Pimentel et al. 2021).

Eight plant families were common to bees across biomes. Despite Fabaceae having the highest species richness, Asteraceae showed greater ASV abundance. Exclusive families were observed in each biome. *Vernonia echioides*, *B. carinata*, and *S. tuberosum* were shared among bees. Pimentel et al. (2021) reported similar floral preferences for *Melipona* bees in the Amazon, emphasizing the importance of diverse plant families.

Our findings suggest that certain bee species exhibit preferences for specific plant groups, even across different biomes. This observation indicates that some plants may be particularly attractive to multiple bee species or to specific generalist species. This aligns with the findings of Hawkins et al. (2015), who observed similar foraging habits among bees on specific plant groups, suggesting their key role in pollination, even in areas with high floral diversity.

Among all plants identified, *Vernonia echioides* had the highest relative abundance of ASVs (~50%), while *Euphorbia maculata* had the lowest (<0.01%). Alpha and beta diversity of associated plant species differed among biomes, bee species, and microhabitats, with ASV richness being particularly high in the Pantanal and Cerrado. Interestingly, *T. angustula* from the Amazon and Cerrado biomes was associated with different plant species, highlighting the potential influence of biome-specific plant availability on floral associations. A more robust sampling approach, considering seasonal variations and a comprehensive floristic

survey, would be beneficial for further elucidating these relationships (Pimentel et al. 2023).

Our study revealed that different bee species harbor distinct yeast communities, regardless of cultivation method. This distribution appears to be influenced by plant-bee associations, specific floral interactions, and geographical location. PERMANOVA analysis confirmed significant differences in fungal community composition between biomes, particularly between the Amazon and Pantanal, suggesting the influence of environmental factors and vegetation on these communities. These findings contribute to our understanding of bee ecology and pollinator-plant relationships.

The isolated yeasts exhibited diverse functional capabilities, including hydrolytic enzyme secretion, phosphate solubilization, and growth under varied pH and nutrient conditions. These traits suggest potential industrial applications for these microorganisms (Amadi et al. 2020). Notably, unconventional yeast genera displayed robust growth under specific conditions, highlighting their potential for biotechnological exploitation (Wagner and Alper 2016). *Kodamaea ohmeri W5*, isolated from the acidic gut environment of *M. orbignyi*, demonstrated marked growth at acidic pH and excelled in phosphate solubilization. *Starmerella meliponinorum W128* exhibited proficiency in multiple functional tests, underscoring the functional diversity of these yeasts and their potential for diverse biotechnological applications (Lopes et al. 2018; Silva et al. 2020; Tamang and Lama 2023; Filippousi et al. 2024). However, this response will depend on more in-depth studies according to the functional profile of the strains.

Furthermore, the functional traits of yeasts varied across biomes, suggesting that the environment of origin significantly influences yeast properties. This functional variation can be leveraged for selecting strains adapted to specific biotechnological processes and environmental conditions (Li et al. 2023; Reineke and Schlömann 2023). Thus, both the macroenvironment (biome) and microenvironment (bee species) appear to play roles in shaping yeast characteristics.

Finally, our study reaffirms that different tropical bee species exhibit distinct chemical compositions in their honey and beebread. This variation has direct implications for bee ecology, nutrition, and associated microbiota (Gruneck et al. 2021).

5 Conclusion

Our study demonstrates that biome and bee species are key determinants of the structure of both cultivable and non-cultivable yeast communities associated with bees. We identified 48 yeast species across 11 bee species from three distinct biomes. The number of yeast species associated with individual bee species ranged from 5 to 18, highlighting the variability in yeast community richness. *Starmerella etchellsii* was found to be ubiquitous across all host species, while *Meyerozyma* sp. and *Hanseniaspora opuntiae* were strongly associated with honey and bee bread, respectively. Several yeast species were also found to be exclusive to particular bee hosts, suggesting species-specific interactions.

Furthermore, our barcoding approach enabled the identification of 100 plant ASVs associated with the bees, revealing that Asteraceae and Anacardiaceae were the most abundant plant families across the sampled bees. Notably, *Vernonia echioides*, *B. carinata*, and *S. tuberosum* were associated with all bee species, suggesting their importance as floral resources.

The distinct functionalities of yeast communities associated with different biomes suggest that environmental factors significantly influence yeast properties. This functional variation can be harnessed for selecting strains tailored to specific biotechnological processes and environmental conditions.

Overall, our study highlights the complex interplay between bees, their associated yeast communities, and their environment. These findings contribute to a deeper understanding of bee ecology, plant-pollinator interactions, and the potential of bee-associated yeasts for biotechnological applications. Further research exploring the functional roles of these yeasts and their impact on bee health and pollination services is warranted.

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Data availability All data are available in the manuscript (supplementary material) or deposited in a public database (NCBI) indicated in the manuscript. The contribution of each author was specified.

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

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