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The role of plant–pollinator interactions in structuring nectar microbial communities

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Abstract

1. Floral nectar harbours a diverse microbiome of yeasts and bacteria that depend predominantly on animal visitors for their dispersal. Since pollinators visit specific sets of flowers and carry their own unique microbiota, we hypothesize that plant species visited by the same set of pollinators may support non-random nectar microbial communities linked together by the type of pollinator.
2. Here we explore the importance of plant–pollinator interactions in the assembly of nectar microbiome and study the role of plant geographic location as a determinant of microbial community composition. We intensively sampled the nectar of 282 flowers of 48 plant species with beetles, birds, long-tongued and short-tongued insects as pollinators in wild populations in South Africa, one of the world's biodiversity hotspots, and using molecular techniques we identified nectar yeast and bacteria taxa. The analyses provided new insights into the richness, geographic structure and phylogenetic characterization of nectar microbiome, and compared patterns of composition of bacteria and yeast communities in relation to plant and pollinator guild.
3. Our results showed that plant–pollinator interactions played a crucial role in shaping nectar microbial communities. Plants visited by different pollinator guilds supported significantly different yeast and bacterial communities. The pollinator guild also contributed to the maintenance of beta diversity and phylogenetic microbial segregation. The results revealed different patterns for yeast and bacteria; whereas plants visited by beetles supported the highest richness and phylogenetic diversity of yeasts, bacteria communities were significantly more diverse in plants visited by other insect groups. We found no clear microbial spatial segregation at

different geographical scales for bacteria, and only the phylogenetic similarity of yeast composition was correlated significantly with geography.

4. *Synthesis*. Interactions of animal vector, plant host traits and microbe physiology contribute to microbial community assemblages in nectar. Our results suggest that plants visited by the same pollinator guild have a characteristic nectar microbiota signature that may transcends the geographic region they are in. Contrasted patterns for yeast and bacteria stress the need for future work aimed at better understanding the causes and consequences of the importance of plants and pollinators in shaping nectar microbial communities in nature.

KEYWORDS

beetle, floral microbiome, insect, multi-trophic interactions, nectar bacteria, nectar yeasts, pollination, South Africa

1 | INTRODUCTION

From the root tips to the flowers and apical meristems, plants offer diverse physico-chemical below- and above-ground habitats for complex communities of micro-organisms (Andrews & Harris, 2000; Nelson, 2018; Öpik et al., 2006; Vannette, 2020). Floral nectar has until recently been one of the most neglected microbial habitats. By the secretion of this solution rich in carbohydrates, flowers obtain pollinator services (Nicolson et al., 2007; Willmer, 2011). Floral nectar is also of central importance in hosting a diverse and dynamic microbiome of yeasts and bacteria. Once established, nectar micro-organisms may act as 'ecosystem engineers,' directly altering the floral environment and consequently the relationships with their large partners. Microbial communities can reduce nectar sugar concentration (de Vega & Herrera, 2012; de Vega et al., 2009; Herrera et al., 2008) modify nectar carbohydrate and amino-acid composition (de Vega & Herrera, 2013; Herrera et al., 2008; Lenaerts et al., 2016; Vannette & Fukami, 2018), warm the flowers (Herrera & Pozo, 2010) and emit volatiles (Rering et al., 2018; Schaeffer et al., 2019) that ultimately alter pollinator foraging preferences and influence pollination success (Herrera et al., 2013; Junker et al., 2014; Schaeffer & Irwin, 2014; Schaeffer et al., 2017; Vannette & Fukami, 2016; Vannette et al., 2013; Yang et al., 2019). Nectar thus constitutes a unique aquatic microenvironment in terrestrial ecosystems that provides a link between macro and micro-organisms.

Flowers are exposed to a virtual sea of micro-organisms that can be passively dispersed by wind, rainfall or even soil among others (Sharaby et al., 2020; Zarraonaindia et al., 2015). However, most of the evidence suggests that microbial dispersal to nectar is not a stochastic, abiotic, passive process. Microbes like yeasts and bacteria depend predominantly on animal visitors, especially insect vectors, for their dispersal and establishment in nectar (Brysch-Herzberg, 2004; de Vega & Herrera, 2012; Herrera et al., 2010; Schaeffer & Irwin, 2014; Vannette & Fukami, 2017). Incoming micro-organisms undergo filtering growth processes, priority effects and microbe–microbe interactions that may further shape floral microbiota composition (Álvarez-Pérez et al., 2019; Peay et al., 2012; Tucker & Fukami, 2014). Nectar is colonized by different genera of yeasts and bacteria recovered repeatedly worldwide from this particular ecological niche (Álvarez Pérez & Herrera, 2013; Belisle et al., 2012; Brysch-Herzberg, 2004; Canto et al., 2017; Fridman et al., 2012; Jacquemyn, Lenaerts, Tyteca, et al., 2013; Pozo et al., 2011) and some general patterns have emerged. The dominance of bacterial species within the Proteobacteria, especially species of *Acinetobacter*, *Rosenbergiella* and *Pseudomonas* (Aizenberg-Gershtein et al., 2013; Álvarez-Pérez & Herrera, 2013; Álvarez Pérez et al., 2013; Morris et al., 2020; Sharaby et al., 2020), and yeast species within the genus *Metschnikowia* (Belisle et al., 2012; Brysch-Herzberg, 2004; Pozo et al., 2012; Schaeffer et al., 2015) is evident in the nectar microbiota. The repeated occurrence of the same group of species identified using either cultivation-dependent or cultivation-independent methods is indicative of an adaptive strategy and equilibrium in the nectar communities.

While the initial research focus was to discover which micro-organisms were the main inhabitants of floral nectar, the more interesting question that has arisen subsequently is why they are found in certain floral environments and not others. Individual flowers harbour diverse microbial communities that may or may not be similar to those found on other flowers. But as pollinators visit specific sets of flowers and carry their own unique microbiota (Brysch-Herzberg, 2004; da Costa Neto & de Morais, 2020; Ganter, 2006; Koch & Schmid-Hempel, 2011; Lachance et al., 2001; Martinson et al., 2011; Rosa et al., 2003), it has been suggested that flowers and pollinators share microbes (Brysch-Herzberg, 2004; Keller et al., 2020; McFrederick et al., 2017) and that host plant species visited by the same set of pollinators may support non-random or even unique nectar microbial communities that are linked together by the type of pollinator (de Vega et al., 2017). However, our understanding of how plant–pollinator assembly processes might ultimately be responsible for nectar microbial community structure remains limited. Moreover, most studies that focused on estimates of the composition and abundance of micro-organisms associated

with nectar appear to be biased toward plant species that are visited by bees and/or hummingbirds. Additionally, geographic bias is skewed towards temperate regions, with North America and Europe accounting for the majority of studies, despite preliminary evidence that tropical areas may sustain a higher nectar microbial biodiversity (Álvarez Pérez et al., 2012; Canto et al., 2017; Oliveira Santos et al., 2020). There has been little research identifying the biotic factors structuring assemblages of nectar microbial communities in tropical biodiversity hotspots with enormous functional diversity of plant–pollinator interactions. Through recent investigations in tropical regions, we are gaining a clearer perspective of the role of other floral visitors, such as beetles, in vectoring micro-organisms to nectar (de Vega et al., 2012, 2017; Lachance et al., 2001, 2003). Thus, intimate symbiotic relationships of microbes with different plants and insects have been suggested, but the fundamental nature of these interactions remains an open question.

In this study we focus on South Africa, containing three of the world's biodiversity hotspots—the Cape Floristic Region, the Succulent karoo and the Maputaland-Pondoland-Albany hotspot—to explore how plant–pollinator assembly processes may ultimately influence nectar microbial community structure. Since nectar microbes, plants and pollinators can be inextricably linked, we hypothesize that plants visited by the same pollinator guild possess a unique nectar microbiota signature that could transcend the geographic region that they inhabit. We analyse a diverse set of plants with birds, Hymenoptera, Lepidoptera, Diptera and Coleoptera as main pollinator groups in the Cape and KwaZulu-Natal regions. The study of similar habitats in ecologically disparate settings scenarios is particularly relevant to test the hypothesis that variation of nectar microbial communities is correlated with differences in pollinator composition. Our specific goals were: (a) to explore broad-scale differences in nectar microbial communities among plant species visited by different pollinator groups, (b) to ascertain whether the resulting patterns are similar for bacterial and yeast communities and (c) to test whether geographic location is one of the main determinants of nectar microbial community composition. Our findings may have broad implications for a better understanding of the distribution of nectar inhabitants in different plant species visited by different pollinator guilds worldwide, and provide a framework for identifying areas for future research.

2 | MATERIALS AND METHODS

2.1 | Study site and study species

The composition and diversity of microbial communities naturally occurring in the floral nectar of animal-pollinated plants were studied in the KwaZulu-Natal province and in the Cape Region of South Africa (Figure S1). Nectar samples were collected from 282 flowers (from 282 different individual plants) of 48 plant species belonging to 16 angiosperm families (Table S1). The study was conducted in 10 ecologically diverse localities separated by 20 to 1,250 km. Localities differed in climatic conditions, plant species composition and soil type, covering sites near the coast to high altitudes in the Drakensberg mountains (up to 1,800 m a.s.l.; Table S2).

Our previous investigations in the study area suggested that microbes carried by beetles can differ from those transported by other insects (de Vega et al., 2012, 2017). The major groups of floral visitors were grouped as nectar-feeding birds (mainly Nectariniidae sunbirds; henceforth BI), Coleoptera (mainly in the subfamilies Cetoniinae and Hopliinae and also the families Scarabaeidae, Chysomelidae, Melyridae, Nitidulidae, Staphylinidae and Tenebrionidae; BE) and other insects (IN), both short-tongued (ST) insects (including Hymenoptera and Diptera mainly visiting flowers with easily accessible pollen and nectar) and long-tongued (LT) insects (including Hymenoptera, Lepidoptera and Diptera usually in tubular flowers; Figure 1; Table S1). Plants were classified into main floral visitor groups using a combination of literature review, floral trait-based assignment and personal observations of visitors in the field. See Table S1 for detailed information.

2.2 | Nectar sampling and microbial cultures

Open flowers exposed to pollinator visitation were collected in natural populations ($M \pm SE$ nectar samples per species = 5.9 ± 0.6). The mean number of nectar samples per species is similar in other nectar microbial surveys throughout the world (Brysch-Herzberg, 2004; Canto et al., 2017; Fridman et al., 2012; Jacquemyn, Lenaerts, Tyteca, et al., 2013; Pozo et al., 2011). The main criteria used for selecting flowers were that no recently opened flowers were sampled, flower collection should be done with good weather conditions, and whenever possible flowers should have floral visitors or signs of have been visited. Flowers were carried immediately to the laboratory in a cooler and nectar collection was done within a few hours (<4 hr) after sampling. Nectar was carefully extracted with sterile glass microcapillaries (Drummond Scientific Co.), immediately diluted in 500 μl of sterile, ultrapure deionized water (Purite Select; Purite Ltd), and stored at 4°C.

Twenty-five microlitres of nectar dilutions were streaked on YGC (yeast extract glucose chloramphenicol) agar plates (Fluka, Sigma Aldrich) and trypticase soy agar (TSA; Panreac) for yeast and bacterial isolation, respectively. Cultures were incubated at room temperature for 7 days. A representative colony of each different morphotype was purified by repeated streaking on solid medium (Álvarez-Pérez & Herrera, 2013). Yeasts were preserved at −80°C in 10% glycerol (Sigma-Aldrich) or using the Microbank system (Pro-Lab diagnostics), and bacterial isolates were stored at −20°C in lysogeny broth (LB; Difco) containing 25% v/v glycerol.

It can be argued that our study relies only on culturable microorganisms identified by Sanger sequencing of purified cultures. However, most nectar microbial communities worldwide have been studied using culture-dependent methods (79.5% of the published manuscripts; C. de Vega, unpubl. review), and more importantly

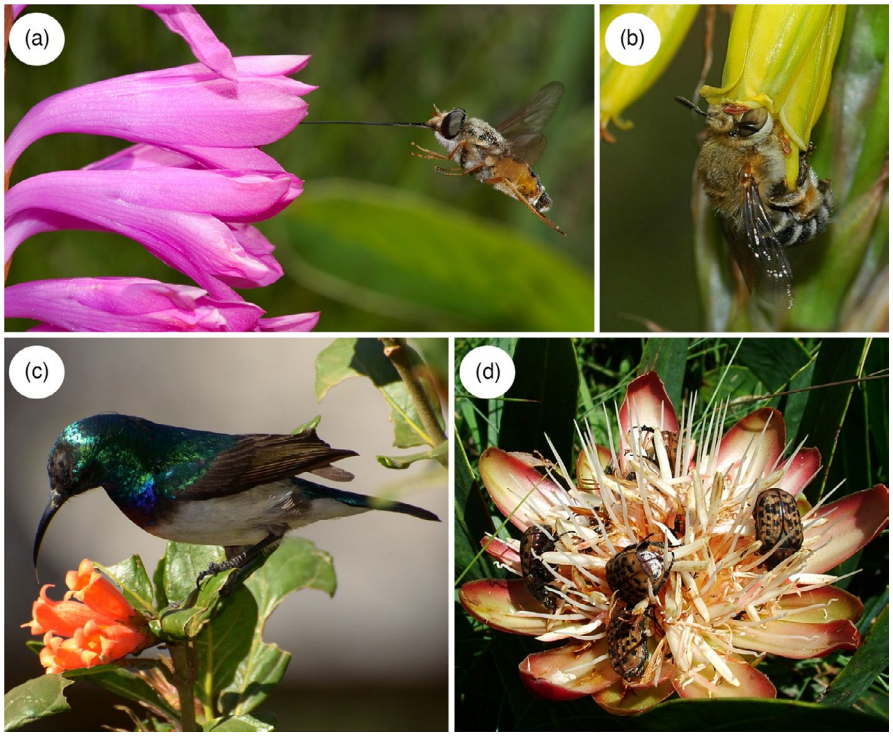

FIGURE 1 Examples of flowers visited by different pollinator guilds in South African plants. (a) Long-tongued tabanid fly *Philoliche aethiopica* on *Watsonia lepida*; (b) Short-tongued bee on *Aloe dominella*; (c) Malachite sunbird on *Burchellia bubalina*; (d) *Atrichelaphinis tigrina* on *Protea simplex*

studies based on massive DNA sequencing have only extended the tail of rare species recovered by culturing. The diversity of the nectar inhabitants is dominated by a few culturable yeast and bacterial taxa (Morris et al., 2020; C. de Vega, unpubl. review).

2.3 | DNA extraction and sequencing

Yeast genomic DNA was extracted by freezing in liquid nitrogen a loopful of cells suspended in 500 μl of sterile MilliQ water for 5 min, followed by a 5-min heat shock at 100°C (Baleiras Couto et al., 2005). For every yeast isolate, the divergent D1/D2 domain of the large-subunit rRNA gene was amplified by PCR using primers NL-1 and NL-4, following methods of Kurtzman and Robnett (1998). Reaction mixtures and amplification conditions were as described in de Vega and Herrera (2012).

Genomic DNA of bacteria was isolated by boiling bacterial colonies in 500 μl of ultrapure deionized water at 100°C for 20 min. Cell debris was removed by centrifuging at 8,000 g for 2 min. The bacterial 16S rRNA gene was amplified using the universal primer 1492R (Reysenbach et al., 2000) and the eubacterial-specific primer 27F (Braker et al., 2001) as described in Álvarez-Pérez et al. (2012). The amplified DNA was cleaned with ExoSAP-IT (USB). Sequencing was performed by Macrogen (The Netherlands) and at 'Estación Biológica de Doñana' in an ABI PRISM 3130xl DNA automatic sequencer (Applied Biosystems). Sequences were assembled and edited using Sequencher 4.9 (Gene Codes Corporation, Inc.). Consensus sequences were aligned using MAFFT online service (Katoh et al., 2019). Analyses were carried out for yeast and bacteria separately.

2.4 | Phylogenetic analyses

Phylogenetic trees were constructed for yeast and bacteria to test whether the microbial communities recovered from plants visited by distinct floral visitors were phylogenetically different (see below in phylogenetic characterization of microbial communities). Phylogenetic reconstructions were carried out using Bayesian inference and maximum likelihood (ML) approaches. For the Bayesian inference we used MrBayes v.3.2.7 (Ronquist et al., 2012) on XSEDE via the CIPRES Science Gateway (http://www.phylo.org/). We performed two independent runs for 5×10^6 generations with four chains each and trees sampled every 1,000 generations. Parameter values from each run and convergence of runs was assessed with Tracer v.1.6 (http://tree.bio.ed.ac.uk/software/tracer/). We allowed MrBayes to sample across the general time reversible (GTR) nucleotide substitution model space using reversible jump Markov chain Monte Carlo (rjMCMC) with the function nst = mixed (Huelsenbeck et al., 2004). The first 25% of trees of each run were discarded as burnin and a 50% majority-rule consensus tree was constructed.

Maximum likelihood analyses were implemented in PhyML v.3.0 (Guindon et al., 2010) using the Smart Model Selection (http://www.atgc-montpellier.fr/phyml/) based on the Akaike Information Criterium (Lefort et al., 2017). Trees were estimated with the GTR nucleotide substitution model. Branch support was assessed by 1,000 bootstrap resamples. *Mucor mucedo* (GenBank accession number JN206480) and *Deinococcus radiodurans* (Y11332) were used as outgroups for yeasts and bacteria, respectively. The resulting trees were drawn and edited with iTOL 5.6.3 (Letunic & Bork, 2016).

Bayesian and ML approaches provided very similar and well-resolved trees, and only Bayesian results are shown.

2.4.1 | Operational taxonomic units (OTUs) designation

Bacterial and yeast alignments were clustered into operational taxonomic units (OTUs) at 97% sequence similarity with MOTHUR v. 1.44.0 (Schloss et al., 2009). OTUs were assigned to a taxonomic identity by querying sequences against the GenBank database using the BLAST algorithm (Altschul et al., 1990) and the EzBioCloud server (https://www.ezbiocloud.net; last accessed 01 April 2020). Yeast and bacterial taxonomic information was obtained from the fungus nomenclature database Mycobank (http://www.mycobank.org) and NCBI's microbial database, respectively.

2.5 | Statistical analyses

All analyses were conducted using R version 3.6.3 unless otherwise stated.

2.5.1 | Microbial incidence and co-occurrence

Presence–absence of each OTU in each nectar sample (1/0 matrix) was used in this analysis to describe microbial incidence. To assess whether the incidence of yeasts and bacteria differs in nectar samples by pollinator type we fitted GLMMs with a binomial error distribution and including plant species identity as random factor. Models were fitted with the packages LME4 (Bates et al., 2015) and CAR (Fox & Weisberg, 2019). Additionally, in a subsequent analysis plant family was included in the same model as above as a fixed factor.

Pearson correlations were used to examine relationships between yeast and bacteria co-occurrence in flowers using presence/absence matrices (coded as categorical binary variable 1/0). This analysis informed about the co-occurrence of yeast and bacteria in the same nectar sample. We included both all nectar samples and only those containing any microbe.

2.5.2 | Structure and phylogenetic characterization of microbial communities

Permutational multivariate analysis of variance (PERMANOVA) was conducted to test for differences in microbial communities among plant species visited by different pollinator groups.

Colony-forming unit (CFU) counts of nectar bacteria could not be performed to estimate the number of viable microbial cells of each particular OTU in each nectar sample because of the high morphological similarity between the colonies of different OTUs. For this and subsequent analyses input data are based on counts of different OTUs recovered (confirmed by sequencing) in each set of replicate nectar samples for each plant species (replicated incidence data). We transformed the replicated incidence OTU table to a relative proportional data table with the *decostand* function (method = 'total') of the VEGAN package (v. 2.5-7; Oksanen et al., 2019). Analysis of microbial community composition was conducted with the *decostand* and *vegdist* functions of this package. Two different analyses, including and excluding unique OTUs (those that appeared in a single nectar sample across all plant species) were carried out. The number of permutations was set at 9,999 and all other arguments used the default values. Pairwise comparisons were performed with the package RVAIDEMEMOIRE (v. 0.9-79; Hervé, 2020) and correcting significance values for multiple testing with the 'fdr' option.

Long-tongued and short-tongued insect-associated communities were populated by virtually the same microbial taxa; yeast communities associated to plants visited by LT and ST insects were compositionally similar ($p = 0.441$), and the same arose for bacteria ($p = 0.776$). Consequently, the LT insects and ST insects categories were then merged in the same group (IN) for further analyses. Although our data suggest that as a group, plants visited by non-beetle insects share similar nectar microbial communities, this does not mean that the degree of specialization of the plant species associated with these insects is similar. In South Africa, many of the plant species visited by non-beetle insects, whether ST or LT, have highly specialized pollination systems involving a single species of insect pollinator (see Table S1).

Beta diversity was measured using the unweighted UniFrac (uwUniFrac) distance to test whether the microbial communities recovered from plants visited by distinct floral visitors were phylogenetically different. Unweighted UniFrac distance considers presence/absence of OTUs (see McMurdie & Holmes, 2013 for details), and uses both the tree topology and the branch lengths, to test the hypothesis that there has been more unique evolution within each environment than would be expected by chance (Lozupone et al., 2006; Lozupone & Knight, 2005). The distance between communities is measured as the fraction of the branch length of the phylogenetic tree that is unique to one of the communities.

The analyses were done separately for yeasts and bacteria. To calculate the uwUnifrac distance, two files are used as input: (a) the rooted OTU phylogenetic tree obtained for yeasts and bacteria using MrBayes as explained above, and selecting one representative of each OTU, and (b) a file linking presence/absence of OTUs to the environment information, using the three pollinator guilds (BE, BI, IN) as environment information. Distances were calculated with the *UniFrac* function of the package PHYLOSEQ (v. 1.34.0; see McMurdie & Holmes, 2013 for details).

PERMANOVA analyses were conducted as above to test whether the microbial communities inhabiting the nectar of plants visited by different pollinator guilds were phylogenetically different using the uwUniFrac distance matrix. Pairwise dissimilarities between microbial communities were evaluated by comparisons adjusted for multiple testing (via 'fdr').

2.5.3 | Rarefaction, richness and (phylo) diversity assessment

Rarefaction methods were applied to evaluate the adequacy of sampling by assessing whether the cumulative number of OTUs reached

an asymptote. Species richness and evenness measurements can only be meaningfully compared among different communities when sample sizes are equal and thus sequences were extrapolated at the double sample size of the highest reference sample (N = 206; see results; Colwell et al., 2012; Chao et al., 2014). Sample-based rarefaction curves were computed for pollinator groups (BE, BI, IN, see above) using 10,000 randomizations without replacement. Microbial alpha diversity in each group was calculated as the number of OTUs (OTU richness), rarefied Chao richness, Simpson (1/D) and Shannon observed and estimated diversity indices with the package iNext v.2.0.20 (Hsieh et al., 2016). *iNext* allows incidence-frequency data for diversity calculations. Input data for each assemblage consist of OTUs sample incidence frequencies (row sums of each incidence matrix). For each diversity measure, *iNext* uses the observed incidence data to compute diversity estimates and the associated 95% confidence intervals for rarefaction and extrapolation (Hsieh et al., 2016).

To estimate observed and extrapolated sample-based phylogenetic diversity, Faith's phylogenetic diversity estimator was calculated with the iNextPD v.0.3.1 package (Hsieh et al., 2016). Estimators were considered significantly different among floral visitor groups whenever the 95% confidence interval created by resampling did not overlap.

2.5.4 | Geographic structure

The correlation between community dissimilarities and geographic distances matrices were tested with Mantel tests in vegan. As community distance measure we used both the Euclidean distances and the uwUnifrac distances which considers the phylogenetic relatedness.

Additionally, patterns of geographic segregation were also assessed by grouping samples into two geographic origins (Cape and Drakensberg; see Table S2 and Figure S1) and further analysed with a PERMANOVA in vegan. Analyses were conducted including and excluding unique OTUs.

3 | RESULTS

3.1 | Incidence of microbial taxa and OTUs designation

Nectar microbial communities occurred frequently in the study area, with 85.4% and 60.4% of plant species harbouring detectable yeasts and bacteria, respectively (Figure 2). For five plant species, we did not detect either yeast or bacterial communities (*Albuca nelsonii*, *Aloe dominella*, *Cyrtanthus contractus*, *Dipcadi viride* and *Tulbaghia natalensis*). For these species, the observation of nectar drops under the microscope confirmed the absence or extremely infrequent presence of microbial cells.

The incidence of microbes in nectar samples differed between floral pollinator assemblages both for yeasts (Wald's χ^2 = 23.542, p < 0.001) and bacteria (Wald's χ^2 = 6.740, p = 0.034), with plant species visited by Coleoptera showing the highest percentage of flowers containing micro-organisms. However, when we include

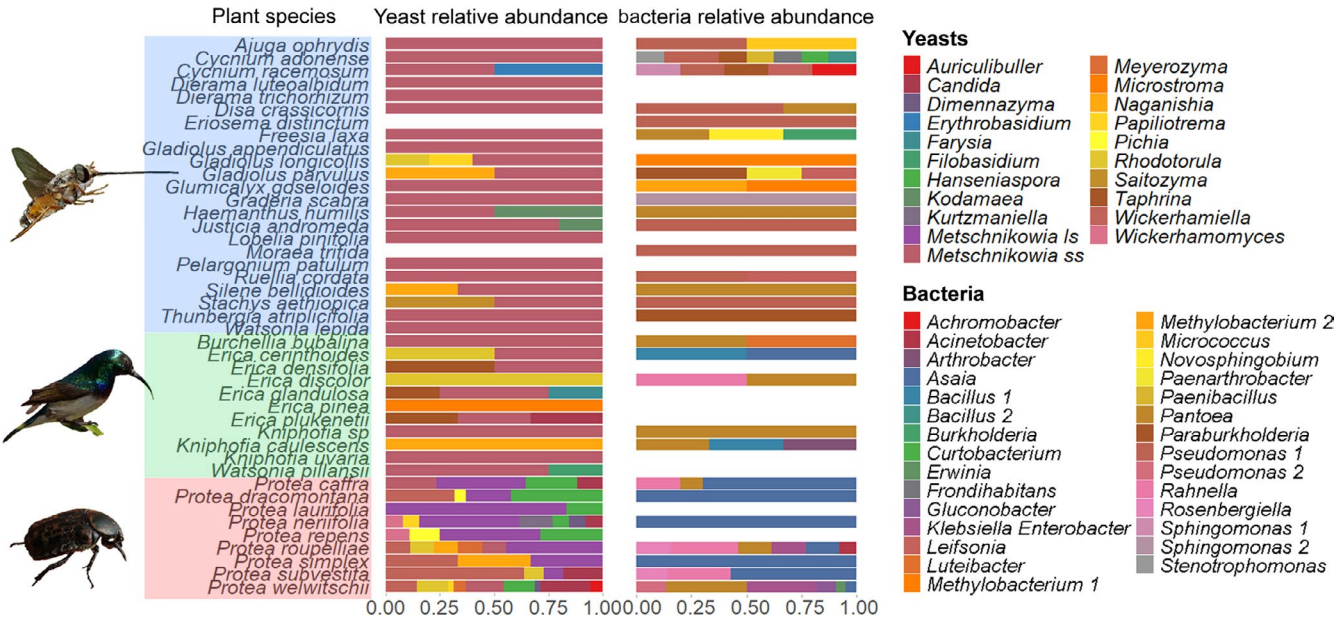

FIGURE 2 Diversity and composition of nectar yeast and bacteria genera across the plant species visited by different pollinator guilds sampled in the Kwazulu-Natal and Cape region of South Africa. White bars indicate absence of yeast/bacteria communities in the nectar of a particular plant species. Five plant species that did harbour neither yeast nor bacterial communities are not included in the figure (*Albuca nelsonii*, *Aloe dominella*, *Cyrtanthus contractus*, *Dipcadi viride* and *Tulbaghia natalensis*). The yeast genera *Metschnikowia* is separated in ls (large-spored, including *M. caudata*, *M. drakensbergensis* and *M. proteae*) and ss (small-spored, including *M. koreeensis*, *M. rancensis* and *M. reukaufii*)

plant family in the models the differences remained significant for yeast (Wald's $\chi^2 = 9.648$, $p = 0.008$), but not for bacteria (Wald's $\chi^2 = 0.050$, $p = 0.975$), suggesting that plants visited by different pollinator guilds have a similar probability of incidence of bacterial communities in nectar once the plant family is accounted for.

Yeasts and bacteria coexisted only in 17% of the nectar samples. The frequency of co-occurrence of yeasts and bacteria across all nectar samples was not significant ($N = 282$, $R = 0.082$, $p = 0.171$). However, when only nectar samples containing microbes were considered, the regression showed a highly significant negative correlation ($N = 182$, $R = -0.526$, $p < 0.001$).

DNA sequencing yielded a total of 217 yeast and 113 bacterial sequences which clustered into 35 and 29 OTUs, respectively (Table S3). Across plant species, we recorded 1–13 OTUs ($M \pm SE = 2.58 \pm 0.37$; Figure 2). Individual nectar samples were colonized by 1–5 OTUs.

3.2 | Nectar microbial communities structure by pollinator type

Pollinator guilds help predict the composition of nectar microbial communities. Plants visited by either birds, beetles or by other insects supported significantly different yeast ($F = 3.667$, $p < 0.001$) and bacterial communities ($F = 5.104$, $p < 0.001$; Table 1). Pairwise comparisons of nectar microbial communities of plants visited by different pollinators were all significant (Table 1). Analyses including unique OTUs yielded similar results (Table S4).

The pollinator guild also contributed to the maintenance of beta diversity and nectar-associated phylogenetic microbial segregation. Significant *p*-values for Unifrac analyses were found when testing yeast ($F = 5.305$, $p < 0.001$) and bacteria ($F = 5.769$, $p < 0.001$) communities indicating that microbial communities are significantly clustered phylogenetically by pollinator type (Table 1; Figures 3 and 4). Pairwise comparisons performed with uwUnifrac distance both for yeast and for bacterial communities revealed significant differences for the comparisons of BE-IN communities ($p = 0.001$ and $p = 0.003$ for yeast and bacteria) for BE-BI communities ($p = 0.001$ and $p = 0.023$ for yeast and bacteria, respectively) and for BI-IN communities ($p = 0.023$ and $p = 0.024$ for yeast and bacteria, respectively). Analyses including unique OTUs yielded similar results (Table S4).

3.3 | Yeast communities richness and diversity assessment

Rarefaction curves of yeast OTU richness were close to reaching an asymptote for BE and IN groups (Figure 5) with samples having a Good's coverage value close to 1 (Table 2), indicating that our sampling effort was sufficient. Based on the Chao estimator of estimated richness, we detected in average 95.7% of expected OTUs in BE yeast communities (25 OTUs), 61.9% in BI communities (10 OTUs) and 69.4% in IN communities (9 OTUs; Table 2). The group of plants visited by beetles supported a significantly higher level of OTU richness and phylogenetic diversity than found in the other pollinator groups (Table 2; Figure 5). Estimated Shannon and Simpson indices showed an increase in diversity from the IN group (5.526 ± 1.058; 3.508 ± 0.619) to the BI group (10.896 ± 3.313; 6.948 ± 2.276) and the BE group (16.243 ± 1.218; 10.935 ± 1.092; Table 2).

Ascomycetes were the largest phylum of yeasts recorded, representing 87.6% of the total yeast isolates and 21 OTUs (Figure 3), while Basidiomycetes represented only 12.4% of the isolates. Seventy three percent of Ascomycetous OTUs were unique to BE plants, including all species of *Wickerhamiella*, *Hanseniaspora*, *Wickerhamomyces* and *Candida* species in the *Lodderomyces* clade. Interestingly phylogenetic clades of the nectar specialist *Mestchnikowia*, the dominant genus in nectar, were separated by pollinator visitor type. Distinct clades were evident for beetle-visited flowers (large-spored species: *Metschnikowia drakensbergensis*, *M. caudata* and *M. proteae*) and insect/birds-visited flowers (small-spored species *M. koreeensis*, *M. reukaufii* and *M. rancencis*; Figures 2 and 3). *Taphrina inosithophila* was exclusively isolated from BI plants. Species of Basidiomycetes did not show a clear relationship to any pollinator group.

TABLE 1 Differences between nectar microbial communities of plants visited by different pollinator guilds, using Euclidean and unweighted Unifrac distances. 'Insects' = insects other than beetles

	Euclidean distance			uwUnifrac distance		
	F	*df*	*p*	*F*	*df*	*p*
Yeast						
Floral pollinators	3.667	2	<0.001	5.305	2	<0.001
Beetle versus insects			0.001			0.001
Beetle versus birds			0.001			0.001
Birds versus insects			0.042			0.023
Bacteria						
Floral pollinators	5.104	2	<0.001	5.769	2	<0.001
Beetle versus insects			<0.001			0.003
Beetle versus birds			0.014			0.023
Birds versus insects			0.023			0.024

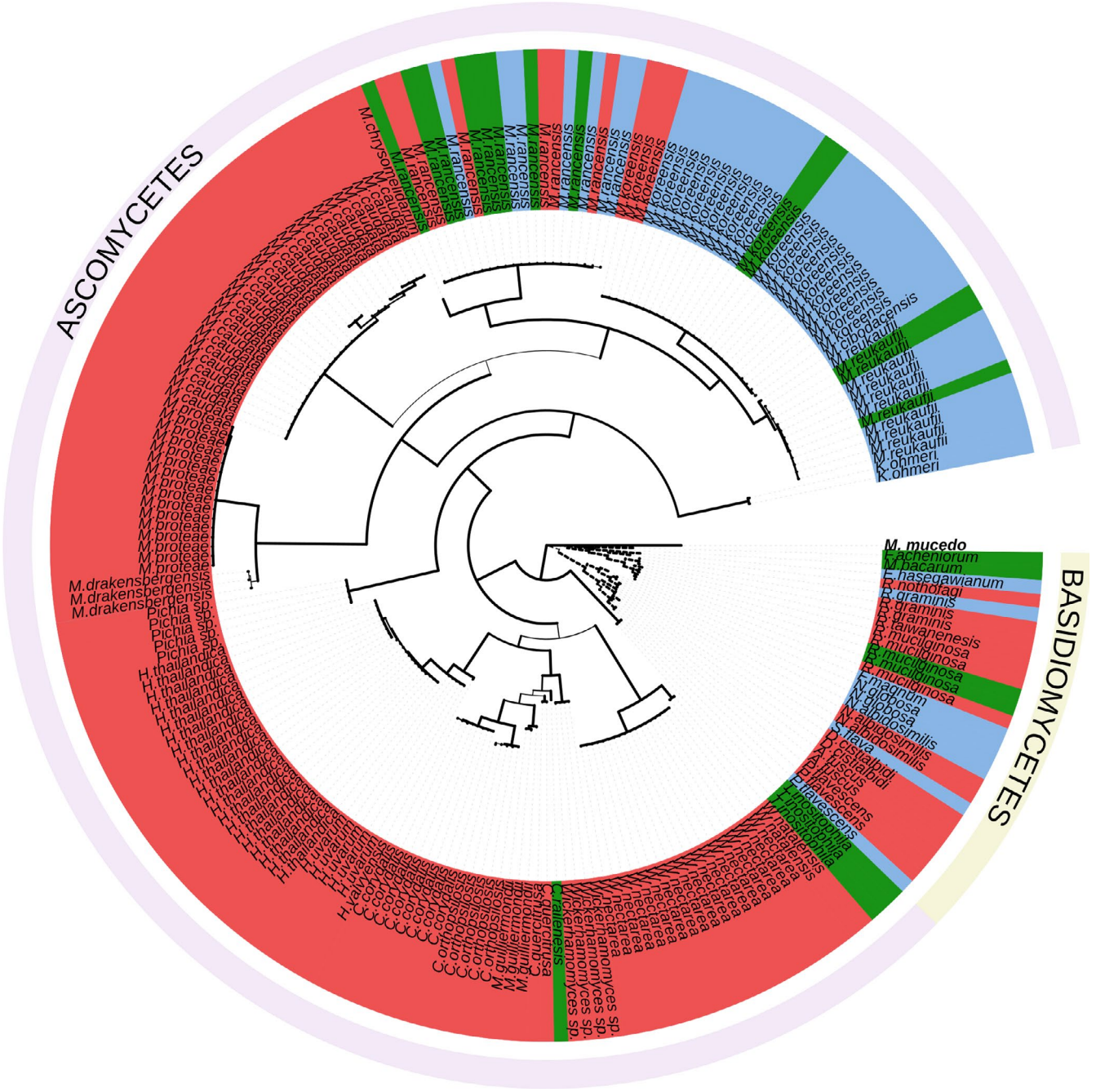

FIGURE 3 Bayesian phylogenetic tree with the 217 yeast strains isolated from floral nectar in our study system in South Africa, based on the analysis of the D1/D2 domain of the large-subunit rRNA gene. Inner circle represents leaves labelled with colours corresponding to their plant pollinator guilds; isolates from beetle-visited plants (red), insect-pollinated (other than beetle) plants (blue) and bird-pollinated plants (green). The outer ring represents taxonomic class. Branch width is proportional to the posterior probability

3.4 | Bacterial community richness and diversity assessment

In bacteria, the OTU richness estimators only reached the plateau phase for the BE group (Figure 5). This suggests that additional sampling efforts would be required to cover total bacterial species richness in the BI and IN groups, as pointed by the Chao estimator (Table 2).

Rarefaction curves for bacterial communities differed from those for yeast communities. The IN group showed the highest Shannon and Simpson estimated indices (23.747 ± 9.401; 7.971 ± 2.941), followed by the BI group (10.045 ± 5.842; 6.357 ± 5.248) and the BE group (6.579 ± 0.788; 5.126 ± 0.644; Table 2). Floral nectar of plants visited by non-beetle insects have the highest phylogenetic diversity of bacteria (Table 2).

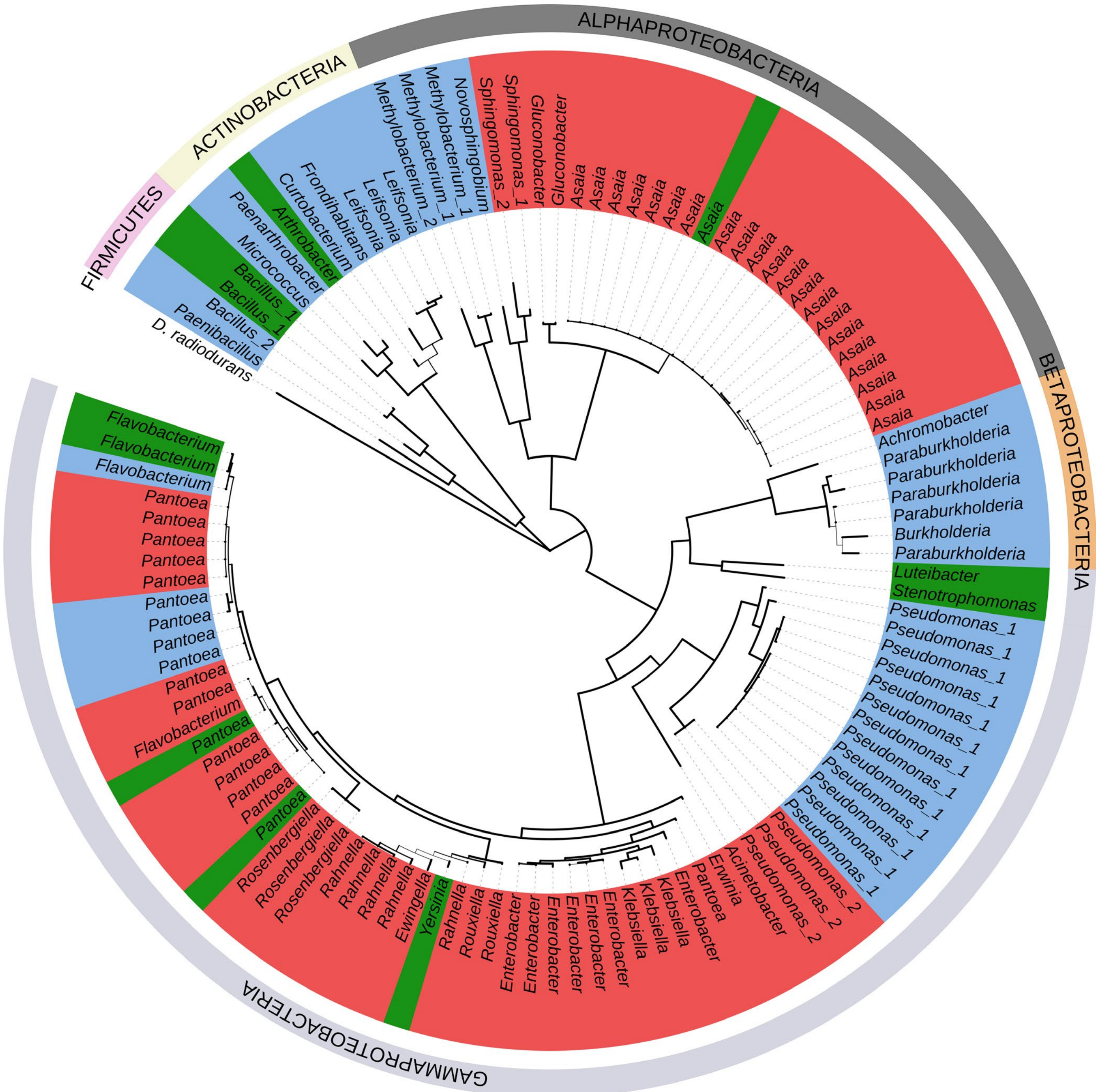

FIGURE 4 Bayesian phylogenetic tree with the 113 bacterial strains isolated from floral nectar, in our study system in South Africa based on of 16S rRNA gene sequences. Inner circle represents leaves labelled with colours corresponding to their plant pollinator guilds; isolates from plants visited by beetle (red), insect-pollinated (other than beetle) plants (blue) and plants visited by birds (green). The outer ring represents taxonomic class. Branch width is proportional to the posterior probability

Nectar-associated bacteria fell into three phyla as follows: Proteobacteria (89.4% of isolates, including α-, β- and γ-Proteobacteria, 20 OTUs), Actinobacteria (7.1%, 6 OTUs) and Firmicutes (3.5%, 3 OTUs; Table S3). The most frequently isolated genera were *Pantoea*, *Asaia*, *Pseudomonas* and *Enterobacter*, accounting for 60% of total isolates (Figures 2 and 4). The type of floral visitor clearly structured the nectar bacterial communities. All bacterial isolates recovered from BE flowers belonged to the α- and γ-Proteobacteria and several genera as *Asaia*, *Enterobacter* or *Rahnella* were exclusively isolated from BE flowers. In the genus *Pseudomonas*, a well-supported phylogenetic clade was exclusively isolated from BE flowers (*P. psychrotolerans*) and another clade was isolated exclusively from IN flowers (*P. palleroriana* and *P. extremorientalis*; called *Pseudomonas* sp.1 and sp.2 in Figures 2 and 4).

All genera in the β-Proteobacteria and almost all genera in the Actinobacteria were isolated exclusively from IN flowers, including among others the genera *Paraburkholderia*, *Methylobacterium* or *Leifsonia*, while the phylum Firmicutes was isolated both from IN and

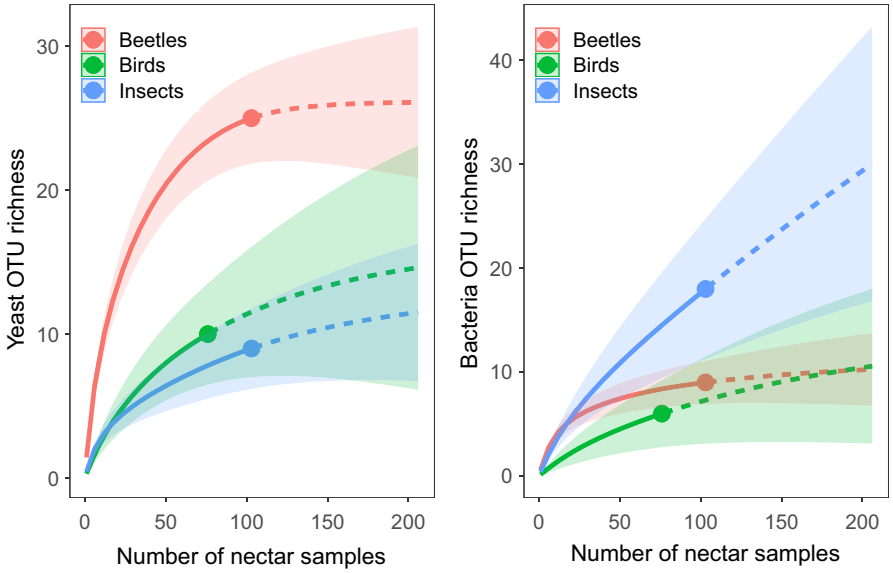

FIGURE 5 Sample-based rarefaction curves (solid line) and 95% confidence intervals (shaded areas) of microbial species richness estimated by the Chao richness estimator (dash-dotted lines) for yeast (left panel) and bacteria (right panel) grouped by pollinator assemblage. Plotted values are means of 9,999 randomizations. Note that *y*-axes for yeast and bacterial communities are not in the same scale. 'Insects' = insects other than beetles

TABLE 2 Mean of observed and rarefied OTU richness, Chao richness estimator, Shannon and Simpson indices, coverage and phylogenetic diversity estimator of yeast and bacterial microbial communities in plants visited by different groups of pollinators

	Floral pollinators		
	Beetles	**Birds**	**All insects excluding beetles**
Yeast			
Number of nectar samples	103	76	103
Observed OTU richness	**25 (a)**	10 (b)	9 (b)
Estimated OTU richness (Chao)	**26.132 ± 1.611 (a)**	16.168 ± 7.458 (ab)	12.961 ± 5.244 (b)
Observed Shannon diversity	**14.826 (a)**	7.427 (b)	4.739 (b)
Estimated Shannon diversity	**16.243 ± 1.218 (a)**	10.896 ± 3.313 (ab)	5.526 ± 1.058 (b)
Observed Simpson diversity	**10.332 (a)**	5.568 (b)	3.348 (b)
Estimated Simpson diversity	10.935 ± 1.092 (a)	6.948 ± 2.276 (a)	**3.508 ± 0.619 (b)**
Sample coverage	0.974 (a)	0.785 (a)	0.914 (a)
Rarefied coverage	0.999 (a)	0.946 (a)	0.968 (a)
Faith's phylogenetic diversity	**5.462 (a)**	3.190 (b)	2.989 (b)
Rarefied phylogenetic diversity	**5.625 ± 0.248 (a)**	4.320 ± 0.711 (ab)	3.389 ± 0.366 (b)
Bacteria			
Number of nectar samples	103	76	103
Observed OTU richness	9 (b)	6 (b)	**18 (a)**
Estimated OTU richness (Chao)	10.981 ± 3.708 (b)	13.895 ± 11.513 (ab)	**101.608 ± 96.128 (a)**
Observed Shannon diversity	6.005 (a)	5.000 (a)	10.952 (a)
Estimated Shannon diversity	6.579 ± 0.788 (b)	10.045 ± 5.842 (ab)	**23.747 ± 9.401 (a)**
Observed Simpson diversity	4.841 (a)	4.167 (a)	6.861 (a)
Estimated Simpson diversity	5.126 ± 0.644 (b)	6.357 ± 5.248 (ab)	**7.971 ± 2.941 (a)**
Sample coverage	0.968 (b)	0.603 (ab)	**0.683 (a)**
Rarefied coverage	0.988 (b)	0.832 (ab)	**0.729 (a)**
Faith's phylogenetic diversity	1.319 (b)	1.236 (ab)	**2.257 (a)**
Rarefied phylogenetic diversity	1.435 ± 0.213 (b)	2.178 ± 0.688 (ab)	**10.833 ± 4.474 (a)**

Note: All values: *M* ± *SE*. Rarefied at double the sample size. Different letters denoted by bold font within rows indicate significant difference between microbial communities associated to plants visited by different pollinator groups.

BI flowers. Only the genus *Pantoea* was isolated from plants visited by all different pollinator guilds (Figure 4).

3.5 | Geographic patterns

We found a relative microbial spatial segregation at geographical scales depending on the method used. Mantel tests revealed no correlation between geographic distance and community dissimilarity when using Euclidean distances for yeasts ($r = 0.138$, $p = 0.102$) and bacteria ($r = -0.060$, $p = 0.376$). When the phylogenetic component was considered by using the uwUnifrac distance, similar results were found for bacteria ($r = -0.019$, $p = 0.413$), although significant correlations were observed for yeast communities ($r = 0.283$, $p < 0.001$).

Mantel test results were supported by PERMANOVA tests. When microbial communities were clustered in two geographic regions (Cape/Drakensberg) this categorical geographic factor was not significant for bacteria ($F = 0.296$, $p = 0.961$ with Euclidean distances; $F = 0.700$, $p = 0.656$ with uwUnifrac distances) but significant for yeast with uwUnifrac distances ($F = 1.760$, $p = 0.109$ with Euclidean distances; $F = 4.020$, $p = 0.003$ with uwUnifrac distances). Similar results were obtained when unique OTUs were included in the analyses (see Table S5).

4 | DISCUSSION

Plant–pollinator interactions contribute to the maintenance of nectar-associated microbial diversity. Floral nectar of plants visited by different types of pollinators harbours different, and, in some instances, unique microbial communities. Of particular interest were the results that revealed that yeast and bacterial communities showed contrasting patterns of diversity and richness depending on the pollinator type.

4.1 | Factors affecting the structure of nectar microbial communities

The structure and diversity of nectar microbial communities can be affected by processes such as limited dispersal and restricted biotic interactions of flowers with their animal vectors (Belisle et al., 2012; Morris et al., 2020; Vannette, 2020; Vannette & Fukami, 2017), as well as other factors such as microbial–microbial interactions. To reach the nectar, yeasts and bacteria share animal vectors, but once there, our results reveal that the occurrence of yeasts and bacteria is negatively related and their assemblages seem to be dominated by competitive species interactions, as has been observed previously (Álvarez-Pérez et al., 2019; Vannette, 2020). Potential mechanisms of yeast–bacterium interactions in floral nectar include antibiosis (Álvarez-Pérez et al., 2019). Accordingly, an explanation for non-random co-occurrence patterns in this study could be the production of antibacterial or antifungal substances by certain micro-organisms resulting in the exclusion of other micro-organisms. However, microbial–microbial interactions may act at the level of individual flowers and hardly explain global patterns of non-overlapping niches linked to pollinator type as observed here.

Another factor that may be important for nectar microbial community assembly is the strong habitat filtering imposed by the chemical properties of nectar (Adler, 2000; Adler et al., 2020; Carter & Thomburg, 2004; Herrera et al., 2010; Nepi, 2014). Sugars may be one of the determinants of the ability of plant species to filter microbes, as the evidence suggests that only microbes possessing certain physiological abilities like osmotolerance will successfully exploit nectar (Álvarez-Pérez et al., 2019; Jacquemyn, Lenaerts, Tyteca, et al., 2013; Jacquemyn, Lenaerts, Brys, et al., 2013; Pozo et al., 2012). Accordingly, osmotolerant yeast and bacterial species physiologically able to overcome high osmotic pressure and the presence of secondary compounds (Álvarez-Pérez et al., 2012; Lenaerts et al., 2014; Morris et al., 2020; Starmer & Lachance, 2011) were frequently isolated from nectar in this study. However, whether nectar sugars may help establish differences in the composition of microbial communities among plants visited by different pollinator guilds is not known.

It was originally postulated that nectar chemistry (based mainly on sugar ratios) is correlated with pollination syndromes (Baker & Baker, 1983, 1990). If so, sugars would be determinant in attracting specific sets of pollinators and therefore could be potentially important in the establishment of different microbial communities in nectar. However, Baker and Baker's conclusions have been partly refuted by subsequent authors, due in part to the enormous variation between plant species that potentially should share characteristics, as occured in our study within the group of plants visited by birds (Barnes et al., 1995; Brown et al., 2009; Goldblatt et al., 1999), within the group of plants visited by non-beetle insects (Goldblatt & Manning, 2002; Goldblatt et al., 1998; Johnson, 2000; Johnson & Raguso, 2016; Msweli, 2018; Springer, 2019), and within the plants visited by beetles (Nicolson & Thornburg, 2007; Nicolson & Van Wyk, 1998; Steenhuisen & Johnson, 2012). Therefore, nectar sugars may not explain exclusively differences in microbial community composition among plants visited by different pollinator guilds.

It is important to point out that nectar is much more than a dilute sugar solution. Other nectar features such as amino acids, proteins, alkaloids and other secondary compounds may also impact floral visitation (Nicolson & Thornburg, 2007), and therefore microbial communities, but this information is lacking for our studied plant species. Antimicrobial substances secreted by plants may also affect microbial communities. If so, it could be possible that antimicrobial substances in some of the study plant species (Amoo et al., 2014; Aremu & Van Staden, 2013; Buwa & Van Standen, 2006; Ncube et al., 2015) may partially explain the restricted microbial survival in their nectar, although pollinator limitation could also explain absence of microbes in flowers. With the available data, we can only suggest that the combination of animal visitation and nectar features should be the main determinants of nectar microbial communities.

Interestingly, our study has shown that a plant nectar microbiota was on average more similar to that of other plants visited by the same pollinator guild living elsewhere than to microbial communities of plant species living in the same location but visited by other pollinators. Our results do not provide support for geographical clustering for bacteria or yeasts when using Euclidean distances, but the phylogenetic similarity of yeast composition was significantly correlated with geography. We found yeast OTUs with clearly cosmopolitan distributions mostly associated to non-beetle insects, while many groups of phylogenetically related OTUs associated to beetles showed restricted distributions. This significance may result from historical contingencies including dispersal limitation, environmental selection or that yeast endemism parallels beetle endemism as previously suggested (de Vega et al., 2014; Lachance et al., 2005). The relatively low significant effect of geography highlights the fundamental importance of plant–animal interactions in shaping nectar microbial communities over spatial factors. These results suggest that plants visited by the same pollinator guild have a characteristic nectar microbiota signature that may transcend in many instances the geographic region or environment they are in.

4.2 | Microbial association with different plants and pollinators

Our data suggest that different clades of nectar bacteria and yeasts tend to be associated with different plants and animal vectors. Our results agree with previous findings showing that whereas bees and other insects as butterflies tend to vector to nectar yeasts with affinities in the small-spored *Metschnikowia* species (Brysch-Herzberg et al., 2004; Herrera et al., 2010; Pozo et al., 2011), beetles carry not only structurally different but also phylogenetically distinct yeast species (de Vega et al., 2014, 2017; Lachance et al., 2001). Since in our study system all plants visited by beetles belonged to the genus *Protea*, it could be argued the problem of distinguishing between effects of beetles against the *Protea* lineage on the composition of the microbial assemblages. However, previous studies of the association of beetles with other plant taxa (e.g. in Convolvulaceae, Malvaceae or Cactaceae; Lachance et al., 2001, 2003, 2006) support our suggestions that beetles, and not *Protea* characteristics, drive these specific associations. These studies have shown that different beetles carry in the Neotropical, Nearctic and Australian biogeographic regions an unusual array of yeasts in the large-spored *Metschnikowia* clade as well as *Kodamaea* and *Wickerhamiella* species with which a strong symbiotic relationship has been suggested (Starmer & Lachance, 2011). Interestingly, *Protea* visited by beetles observed in our study system harbour related microbial taxa to those carried to flowers by beetles in other Regions, and those were not present neither in other insect-pollinated nor in bird-pollinated plants. This similarity of microbial communities of beetles on diverse plants in different regions of the world emphasize the crucial role of beetles in the composition of nectar microbial communities.

The gut of beetles is an extraordinary source of novel yeast species (Suh et al., 2005; Urbina et al., 2013). An important characteristic of yeasts associated with beetles that interact with ephemeral flowers is that most species are endemics and confined to certain biogeographic regions, small areas or islands, their distribution being attributed largely to the prevalence of particular species of host beetles in each area (Lachance et al., 2001, 2003; Oliveira Santos et al., 2020; Starmer & Lachance, 2011). In fact, most yeast species isolated from beetle-pollinated plants in this study have not been found in other regions or anywhere else in nature. These patterns contrast with the biogeography of yeast associated to other insect-pollinated plants (specially associated with bees). Most yeasts isolated from the nectar of bee-pollinated plants show broad geographical distribution, as for instance *M. reukaufii*, invariably present in floral nectar worldwide, and other small-spored widespread species as *M. koreensis* or *M. rancensis* (Brysch-Herzberg, 2004; Golonka & Vilgalys, 2013; Mittelbach et al., 2015).

Nectar occupancy by bacteria was also clearly shaped by interactions with their animal vectors and host plants. Whereas plants visited by beetles harboured exclusively α- and γ-proteobacteria, other insect-pollinated plants only yielded bacteria of the β-proteobacteria and Firmicutes. At lower taxonomic levels, or even at the genus level, a segregation based on pollinator type was evident. While *Asaia*, *Enterobacter* or *Rahnella* were exclusively isolated from plants visited by beetles, *Paraburkholderia*, *Methylobacterium* or *Leifsonia* were only encountered in nectar of other insect-pollinated plants, in contrast to other studies of nectar bacterial communities in other regions of the world, in which *Rosenbergiella* and *Acinetobacter* prevailed in bee-pollinated plants (Aizenberg-Gershtein et al., 2013; Álvarez-Pérez & Herrera, 2013; McFrederick et al., 2017). The existence of a well-supported clade of *Pseudomonas* living exclusively in beetle-associated plants and another clade that inhabit the nectar of plants pollinated by other insects adds interesting complexity to our understanding of the importance of plant–animal interactions in structuring microbial assemblages. What drives these specific associations is uncertain, but it is likely that both vertical transmission among pollinators (from one generation to the next) and horizontal transmission (acquired from their environment) of microbes is involved.

4.3 | Contrasting patterns of diversity for nectar yeast and bacterial communities

Yeast communities were highly diverse in beetle-associated plants, while plants pollinated by other insects harbour a higher diversity of bacteria. Insects require a wide variety of nutrients, and their associated micro-organisms can modify a wide range of carbon and nitrogen-containing, breakdown toxic chemicals and supplement the insect diet with several metabolites that afford beneficial effects to insect health (Dillon & Dillon, 2004; Ganter, 2006; Vásquez et al., 2012). The current day association of many beetles with fungi might reflect a strong symbiotic interaction (Starmer &

Lachance, 2011) and phylogenetic history (Ganter, 2006). In fact, the larvae of many beetles depend on fungi for their development, suggesting a high degree of mutual dependence (Six, 2012). Although the longevity and composition of yeast-floricolous beetle associations are still not well defined, well-established and highly specialized associations may explain the high diversity of yeast species in plants visited by beetles. The functional diversity of yeasts and bees is also known, but most of the species encountered in bee crops, bee breads and nests are not encountered in nectar, except for a few dominant species.

Bacteria are known to expand the capacity of bees and butterflies to consume poorly digestible components of flowers and confer protection against parasites, with beneficial effects for insect health (Dillon & Dillon, 2004; Ravenscraft et al., 2019; Vásquez et al., 2012). Interestingly, floral diet may shape the gut flora of butterflies by serving as a source pool of bacteria specialized on sugar catabolism, although dietary fungi hardly colonize the gut (Ravenscraft et al., 2019). Bees and butterflies may acquire bacteria horizontally through contact with flowers or other insect species (McFrederick et al., 2012; Ravenscraft et al., 2019), while vertical transmission of gut bacteria in social bees allows for co-evolution of insect-gut microbiota (Martinson et al., 2011) and strong evidence for a correlation between the phylogenies of bees and bacteria has been observed (Koch et al., 2013). Whether, as suggested by our data, the higher diversity of nectar yeasts associated with beetles and the higher diversity of bacteria in nectar associated to bees and other insects can extend to other regions is presently uncertain, but the topic certainly deserves further study.

5 | CONCLUSIONS

Wildflowers and nectar have been described as an unexplored, rich reservoir of specialist yeast and bacterial diversity with many species described in recent years, supporting the view that our knowledge of nectar microbial communities is still in its infancy. Interactions between animal vectors, plant host traits and microbe physiology may help explain microbial community assembly in nectar. We found that much of the variation in nectar microbial communities is accounted for by the plant's main floral visitor, with the least amount of variation explained by geographical distance. The consistency of these patterns across both yeast and bacterial communities lends support to the observed pattern. The novel observation of a higher yeast diversity in nectar communities of plants visited by beetle and a higher diversity of bacteria in bee- and other insect-pollinated plants demonstrates the need for future work aimed at better understanding the causes and consequences of the animal vector in shaping nectar microbial communities in nature.

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AUTHORS' CONTRIBUTIONS

C.d.V. and C.M.H. conceived the study and designed the research; C.d.V., R.G.A. and S.-L.S. conducted field sampling; C.d.V., M.-A.L. and S.Á.-P. performed labwork; C.d.V. and R.G.A. performed statistical analyses; M.-A.L., S.D.J., C.M.H. and C.d.V. contributed reagents and materials; C.d.V. wrote the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Sanger sequences are accessible at NCBI GenBank under the accession numbers MW433986–MW434042, KF876187–KF876189 and JN872496–JN872548 for bacteria and MW437767–MW437947, JN935026, JN935028, JN935035, JN935036, JN935043, JN935044, JN935049, JN935054–JN935056, KJ736786–KJ736791, KT158582, KT158583, KT158587–KT158599, KT158615–KT158619 for yeasts, respectively. Further data associated with this article is available from the Dryad Digital Repository https://doi.org/10.5061/dryad.mgqnk9901 (de Vega et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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