Microbial diversity in stingless bee gut is linked to host wing size and influenced by the environment

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Abstract

Stingless bees are important social corbiculate bees, fulfilling critical pollination roles in many ecosystems. However, their gut microbiota, particularly the fungal communities associated with them, remains inadequately characterised. This knowledge gap hinders our understanding of bee gut microbiomes and their impacts on the host fitness. We collected 121 samples from two species, *Tetragonula carbonaria* and *Austroplebeia australis* across 1,200 km of eastern Australia. We characterised their gut microbiomes and investigated potential correlations between bee gut microbiomes and various geographical and morphological factors. We found their core microbiomes consisted of the abundant bacterial taxa *Snodgrassella*, *Lactobacillus* and Acetobacteraceae, and the fungal taxa Didymellaceae, *Monocilium mucidum* and *Aureobasidium pullulans*, but variances of their abundances among samples were large. Furthermore, gut bacterial richness of *T. carbonaria* was positively correlated to host forewing length, an established correlate to body size and fitness indicator in insects relating to flight capacity. This result indicates that larger body size/longer foraging distance of bees could associate with greater microbial diversity in gut. Additionally, both host species identity and management approach significantly influenced gut microbial diversity and composition, and similarity between colonies for both species decreased as the geographic distance between them increased. We also quantified the total bacterial and fungal abundance of the samples using qPCR analyses and found that bacterial abundance was higher in *T. carbonaria* compared to *A. australis*, and fungi were either lowly abundant or below the threshold of detection for both species. Overall, our study provides novel understanding of stingless bee gut microbiomes over a large geographic span and reveals that gut fungal communities likely not play an important role in host functions due to their low abundances.

Key words

Austroplebeia australis; bacterial and fungal communities; core microbiome; forewing size; geographic variation; stingless bee; *Tetragonula carbonaria*

Introduction

Insect guts harbour many microorganisms across the three primary regions; foregut, midgut and hindgut (Chapman and Chapman, 1998). These microorganisms have various host functions that include aiding nutrient extraction from foods (Engel and Moran, 2013), detoxification of harmful compounds (Ceja-Navarro et al., 2015) and protection against parasites and pathogens (Endt et al., 2010). Social corbiculate bees in particular, are known to possess characteristic gut microbiomes. Honey bee (*Apis* spp.) guts, for example, consist of a core bacterial community including *Snodgrassella*, *Gilliamella*, *Lactobacillus* Firm-4 and -5 and *Bifidobacterium* (Koch and Schmid-Hempel, 2011; Kwong et al., 2017), which is acquired mostly through social transmission and from the hive environment (e.g. the hive surface) (McFrederick et al., 2017; Liu et al., 2019). Increasing evidence shows that, like other insects, corbiculate bees may have formed mutualistic relationships with their microbial gut symbionts. The bees benefit from the gut microbiome primarily through defence against enemies and regulation of growth and development (Vásquez and Olofsson, 2009; Koch and Schmid-Hempel, 2011; Zheng et al., 2017). Conversely, changes to the gut microbiome composition of social bees, such as those caused by antibiotic exposure, can lead to dysregulated immune systems and reduced ecological fitness (the overall health and well-being of a bee colony or individual bee) (Liu et al., 2019).

Among the corbiculate bees, stingless bees (Apidae: Meliponini) comprise >500 species globally, of which 11 recognised species occur in Australia, under two genera: *Austroplebeia* and *Tetragonula* (Dollin and Dollin, 1997; Dollin et al., 2015). They are important pollinators of natural plants and crops (Heard, 1999; Hall et al., 2020), and can be harnessed by beekeepers either through rescuing colonies from felled trees, or propagation in man-made hives (Halcroft et al.,

2013). In contrast to the managed stingless bees that are kept and cared for by humans in hives, wild stingless bees are those living and building nests in natural settings, such as forests, jungles, or other types of undisturbed habitats. *Austroplebeia* and *Tetragonula* spp. are similar in body size and colour and occur along the east coast of Australia. However, they belong to different phylogenetic clades, and *Austroplebeia* tends to occur further inland into semi-arid habitats (Heard, 1999). Their behaviour also differs; for example, *T. carbonaria* is more active in flight and evidently collects more resin and pollen than *A. australis* (Leonhardt et al., 2014). In contrast, *A. australis* colonies are more likely to focus on collecting high-quality nectar (e.g., of high sugar concentrations) (Leonhardt et al., 2014). Such distinct behaviour, along with differences in available floral resources within their habitats can thus shape different gut microbiomes (Vásquez et al., 2012). Previous studies of Australian stingless bees have identified a novel clade of host-specific lactic acid bacteria (*Lactobacillus*) (Leonhardt and Kaltenpoth, 2014) and showed that bacterial communities can change rapidly with site movement (Hall et al., 2021). However, these studies used relatively few samples and to date there is limited comparison of gut microbial communities across species and geographic ranges. Additionally, like other animal gut microbiome studies, fungal communities in the guts of insects, including stingless bees, have received little attention (de Paula et al., 2021). Insect-associated fungi, including moulds and yeasts, can contribute to host nutrient provision (Menezes et al., 2015). For instance, the intracellular symbiotic fungi of beetles, *Symbiotaphrina* spp., can both aid in food digestion and detoxify a variety of plant materials (Dowd and Shen, 1990). Despite their importance, fungal community composition and diversity, interactions with the host and drivers of fungal community assembly remain poorly understood. One of our primary objectives in this study was to characterize the gut

fungal community of stingless bees. By doing so, we aimed to gain a more comprehensive understanding of the stingless bee gut microbiome, beyond just the gut bacterial communities.

Wing size in insects is an essential functional trait for flight performance (flying ability for foraging, mating, and finding new nesting sites) (Wootton, 1992), foraging, dispersal and migration (Johansson et al., 2009). Maximum flight distances of stingless bees were highly correlated with wing size in six stingless bee species, suggesting that flight capacity is a function of their wing size, and thus, bees with larger wings may be able to fly further to forage on more diverse plant resources, resulting a greater number of microbial species in their gut (Casey et al., 1985; Byrne et al., 1988; Araújo et al., 2004). Similarly, it was found that bees with larger body size (as indicated by intertegular span) had larger foraging distances than smaller bees (Greenleaf et al., 2007). However, to date no link has been found between insect morphological traits, such as wing, tibia (where the pollen basket is found) and body sizes, and gut microbial diversity. Despite a correlation between morphological traits and gut microbial diversity does not necessarily imply a causal relationship, understanding the potential relationship in stingless bees could help to shed light on the factors contributing to microbial diversity in different insect species. It could also have implications for agriculture and other areas where insect populations play a critical role. Given that microbiomes are evidently linked to the health and vitality of insect species, we then aimed to determine whether there is a correlation between bee gut microbiomes and morphological traits, such as wing and body size. We hypothesized that a larger gut area or increased foraging distance in stingless bee may support a greater number of gut microbial species.

In this study, we collected 121 stingless bee samples from the two species, *T. carbonaria* and *A. australis* in Australia. We investigated the bee gut microbial abundances and diversity using qPCR analyses and amplicon sequencing (targeting the 16S rRNA and ITS genes). We measured bee morphological traits and tested their correlations with the gut microbial diversity. As research has shown, both bacteria and fungi reside in the gut of insect pollinators, but in honey bees, for example, the abundance of fungal communities is typically lower than that of bacterial communities (Engel and Moran, 2013). Although this pattern may also exist for stingless bees, there is currently a lack of studies investigating the fungal community and its level of abundances. We therefore tested the hypotheses that (i) the bee gut microbiome structure is influenced by host species, geographic location and by whether they are wild or managed (cultivated in hives); (ii) characteristics associated with flight and foraging capacities, such as forewing size, positively correlate to host gut microbiome diversity; and (iii) gut fungal communities are of lower abundances relative to bacterial communities.

Material and methods

Specimen collection, measurement and gut dissection

We collected 121 samples (one sample per nest/hive) from the two most common and widespread Australian stingless bee species, *T. carbonaria* and *A. australis,* within their distributional ranges in QLD and NSW, Australia (Fig.1A, Table S1). We collected 3~12 bees per sample so that we had ample bee materials for investigation. Stingless bee foragers were collected from individual managed hives or wild locations (separated by at least 1 km). The *T. carbonaria* samples (n= 80) were collected between September 2018 and January 2020 across a range of 1,200 km in eastern Australia. They comprised 43 samples collected from managed hives and 37 samples collected

from the wild (e.g., national parks). The wild and managed bee populations were geographically separated by more than 20 km, making it unlikely that they directly influenced each other through contact or pathogen spillover. The *A. australis* samples (n= 41) had been collected from wild tree-living colonies across 250 km within their natural distributional range (Fig.1A). The geographic range and management types (wild/managed) of species were considered in analyses when comparing between samples. Consequently, *T. carbonaria* was used for analysing management effects on gut microbiomes, and wild bees of both species were compared to investigate species effects. During sampling, geographic coordinates (longitude and latitude) were recorded, and all collected specimens were immediately preserved in 70% ethanol and stored at -20 °C prior to gut dissection and morphological measurement. Three individuals from each sample were used for morphometric measurements that may infer bee flight performance/fitness including forewing length, forewing area (forewing length × width), hind tibia length and total body length (Wootton, 1992) (Fig.1B). These morphological traits were measured using a digital microscope (Leica EZ4W, Leica Microsystems, Buffalo Grove, IL), by the same observer. Digital images of the whole bees were taken for measurement of body length, and forewings and hind tibia were removed, mounted under a cover slip, photographed and measured (Fig.1B). The whole gut of each individual was then dissected on a sterile Petri dish under a microscope using sterilized forceps, and the gut materials of the three bees were pooled and transferred to a 1.5 mL sterile centrifuge tube and preserved at -20 °C prior to DNA extraction for the analyses of gut microbiomes. We decided to use a pooling approach to obtain sufficient DNA for molecular analyses as we found using individuals could be insufficient to achieve this (Hall et al., 2021).

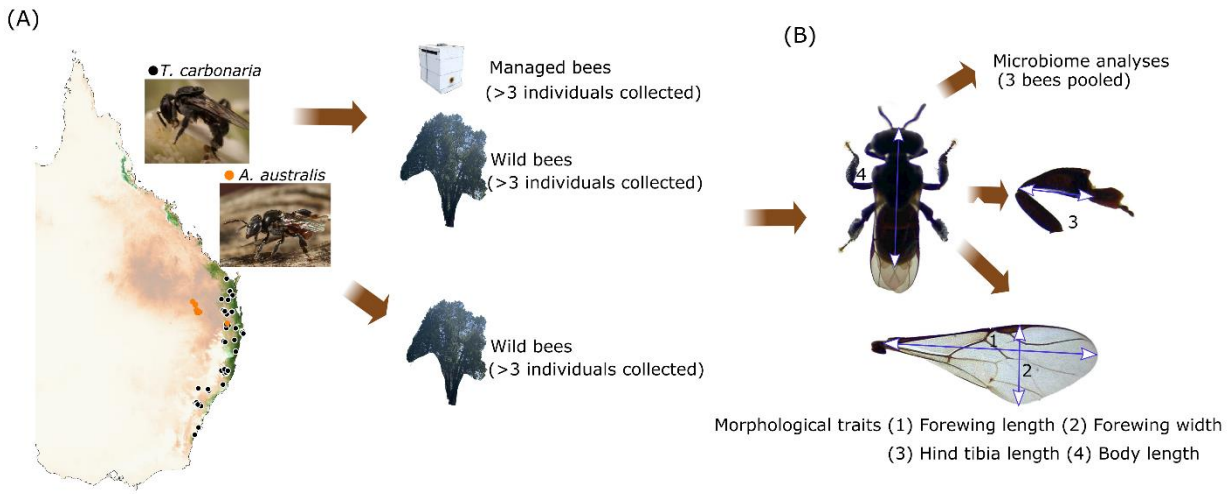

Fig.1 Sampling locations of stingless bees across eastern Australia and the morphological traits measured. (A) Sampling locations; and (B) morphological traits measured for the two stingless bee species.

DNA extraction from gut material and library preparation for high throughput amplicon sequencing

DNA was extracted from pooled gut materials using the DNeasy Blood and Tissue Kit (Qiagen) as per the manufacturer's recommendations. DNA samples were quality checked and quantified using a Nanodrop 2000 (Thermofisher Scientific, USA) and Qubit (Thermofisher Scientific, USA) respectively, before being stored at -20°C. Library preparation and bacterial and fungal amplicon sequencing were then carried out at the Next Generation Sequencing Facility (Western Sydney University, Australia). The 16S rRNA gene (V3-V4 region) primers 341F (CCT ACG GGN GGC WGC AG) and 805R (GAC TAC HVG GGT ATC TAA TCC) (Herlemann et al., 2011) and the fungal ITS2 primers fITS7 (GTG ART CAT CGA ATC TTT G) and ITS4 (TCC GCT TAT TGA TAT GC) (White et al., 1990; Ihrmark et al., 2012) were used for the amplification and subsequent sequencing. For library preparation of the Miseq sequencing, the PCR was carried out using the Eppendorf Master Cycler Pro S system. The thermal conditions comprised an initial denaturation

step at 95°C for 3 minutes, followed by 25 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The final extension step was at 72°C for 5 minutes. After amplification, the amplicons were purified using Agencourt AMPure XP beads from Beckman Coulter, Inc. Dual indexing was performed on the purified amplicons using the Nextera XT v2 Index Kit from Illumina, following the manufacturer's instructions. The indexed amplicons were quantified using the PicoGreen dsDNA Quantification method. The samples were then pooled at equimolar ratios and sequenced on an Illumina MiSeq instrument at the Western Sydney University's Next-Generation Sequencing facility in Richmond, NSW. The sequencing run included 15% PhiX Control v3 from Illumina and used a MiSeq Reagent Kit v3 (600 cycle kit), as per the manufacturer's instructions. For both the bacterial and fungal sequencing, negative (no template) and positive (a Zymo mock community) controls were sequenced, which worked as expected and were removed from further analyses. All the samples were run on the MiSeq (Illumina) platform, generating 300 bp paired end reads.

qPCR analyses for the identification of total bacteria in stingless bee gut

To compare total bacteria between stingless bee gut samples, quantification of 16S rRNA genes was performed relative to the host bee DNA abundance. All DNA samples were normalised to 5.0 ng μL^{-1}. 16S rRNA gene was amplified using universal 16S rRNA gene primers 16S-f (AGG ATT AGA TAC CCT GGT AGT CC) and 16S-r (YCG TAC TCC CCA GGC GG) (Kešnerová et al., 2017) while stingless bee actin gene was amplified using primers act-f (CCT GGA ATC GCT GAC AGA ATG C) and act-r (AAG AAT AGA TCC ACC GAT CCA TAC) (Hall et al., 2021). Reactions were carried out in a 10 µL system containing 5.0 µL 1× LightCycler 480 SYBR Green I Master mix, 1.5 µL (15 pmol) primer mix, 2.5 µL nucleotide free water, and 1 µL DNA template,

and reactions without adding DNA templates were used as negative controls. Thermal cycling conditions included an initial denaturation of 95°C for 5 min, 35 cycles of 95°C for 15 s, 53 °C for 15s and 72°C for 30s, followed by a melting curve analysis (Hall et al., 2021). The bacterial abundance for each sample was calculated using the formula below,

$$Total\ 16S\ rRNA\ copy\ numbers = \frac{(Eff.Actin)^{Ct(Actin)}}{(Eff.16S\ rRNA\ )^{Ct(16S\ rRNA)}}$$

where *Eff.* is the PCR amplification efficiency calculated using LinRegPCR (version 2021.2) (Ruijter et al., 2013). We attempted to determine the total fungal abundance for the samples but found very low fungal abundance (Ct values mostly >35), which made accurate evaluation of the total fungi infeasible.

Bioinformatics and statistics

Sequencing files (FASTQ format) were processed using QIIME2 software and its plugins (version 2019.7; http://qiime2.org/) (Bolyen et al., 2019). Sequencing quality was first assessed using FastQC (Andrews, 2010), then QIIME2 implementation of cutadapt v2019.7.0 was used for removal of primer sequences, and DADA2 v2019.7.0 (Callahan et al., 2016) was used for error-correction, quality filtering, chimera removal and constructing feature tables and final sequence files. DADA2 shows several advantages over other methods including (i) improved accuracy in amplicon sequence variant (ASV) calling and better resolution of closely related ASVs, (ii) higher accuracy compared to methods relying on reference databases, and (iii) DADA2 resolves closely related ASVs with accuracy, which is particularly important for gut microbiome analysis where a high degree of microbial diversity exists (Callahan et al., 2016). Sequencing reads were truncated at 260 bp and 240 bp for forward and reverse reads, respectively, resulting in sequence quality Q>20. The ASVs obtained were summarised and then assigned with taxonomic information using

the q2-feature-classifier, a QIIME2 plugin. For the bacterial data, a Naïve Bayes classifier pre-trained on full length Silva sequences (99%) was used to assign taxonomy to each representative sequence. Bee- and plant-associated mitochondria and/or chloroplast sequences were removed from the feature table to retain microbial features only. For the ITS fungal dataset, the classifier was trained to UNITE v8.0 database (99%) (UNITE Community, 2019) (DeSantis et al., 2006). The number of reads for the bacterial and fungal sequencing data was rarefied to 7,125 and 944 sequences, respectively, per sample by re-sampling the feature table. The mean number of observed ASVs, Chao1, Simpson's, Shannon and Evenness diversity index values were calculated using QIIME2.

Statistical analyses

R version 4.0.3 (2020-10-10) was used for analyses unless otherwise stated. Correlations between stingless bee traits and gut microbial alpha diversity (the diversity of microbial species within a sample, calculated in QIIME2) were examined using multiple linear regression and visualised in R. The effect of stingless bee species and management types on gut microbial community composition and diversity were investigated using permutational multivariate analysis of variance (PERMANOVA, permutation=9999), and visualised with principal component analysis (PCA) using the Vegan package (v.2.5-6) (Oksanen et al., 2013). Fitting bee traits onto PCA ordination was then performed using function *envfit* in Vegan (v.2.5-6). The ggplot2 package (version 3.3.3) (Ginestet, 2011) was used to produce the stacked graph at phylum level.

For core microbiome analysis and random forest test, we used an online microbiome analyses tool (MicrobiomeAnalyst, https://www.microbiomeanalyst.ca/) following recommended parameters

(Chong et al., 2020). Some previous studies identified those members occurring in >20% hosts at abundance of >0.1% within a defined host population as their core microbiomes (Bereded et al., 2020). In this study we calculated core microbiomes in stingless bees using 20% threshold but also analysed with a 40% and 60% threshold to increase the likelihood of biological relevance of the gut microbiome. For analysing gut microbial community variation over spatial gradients (latitude and longitude of each sample), we constructed geographic and environmental distance-decay relationships based on our spatially highly resolved set of samples (Soininen et al., 2007). This analysis reveals how the similarity in host microbiome composition between communities varies with geographic distance. The R package geosphere (1.5-10) (Hijmans, 2019) was used to calculate distance (km) between locations based on geographic coordinates for each sample. The *vegdist* function in the Vegan package (v.2.5-6) was used to calculate Bray-Curtis similarity (1-Bray-Curtis dissimilarity). Distance-based multivariate analysis for a linear model was then performed to investigate correlations between the Bray-Curtis similarity and geographic distance between samples.

Lastly, structural equation models (SEM) were used to evaluate the effects of morphometric traits and management approaches of bees on the bacterial richness (the number of different types of microorganisms present in a sample) in their gut, which was conducted using AMOS17.0 (AMOS IBM, USA). The measurement of richness can provide insights into the overall health and stability of the gut microbiome, as well as the availability of different types of microbes that may impact the bee's health and fitness. The maximum-likelihood estimation was fitted to the SEM modelling, and Chi-square and approximate root mean square error were calculated to examine model fit. Adequate model fits were determined according to a non-significant chi-square test ($P > 0.05$),

high goodness fit index (GFI) (> 0.90), low Akaike value (AIC) and root square mean error of approximation (RMSEA) (< 0.05) as previously described (Delgado-Baquerizo et al., 2016).

Results

Core microbiome analyses of the stingless bee gut microbial communities

The gut microbial communities of the two bee species were characterised using high throughput amplicon sequencing. At the phylum level, gut bacterial communities of both species were dominated by Proteobacteria, Firmicutes and Actinobacteria, along with less abundant Bacteroidetes, Verrucomicrobia, Tenericutes, Acidobacteria, Gemmatimonadetes and other unidentified taxa (Fig.S1A). The fungal community was dominated by Ascomycota and Basidiomycota, with Chytridiomycota, Mucoromycota and other unidentified taxa also common (Fig.S1B). The stingless bee gut microbiome composition appears to be highly variable within host species and colonies, as shown by the heatmaps (Figs.2 and 3). When the presence threshold was set at 20% for defining the core microbiome, we found seven (four *Lactobacillus* spp., two Acetobacteraceae and a *Snodgrassella* sp.) core bacterial ASVs for *T. carbonaria* and nine (four *Lactobacillus* spp., a *Lachnoclostridium* sp., a *Bombella* sp., a Snodgrassella sp., a *Carnimonas* sp. and a *Gilliamella* sp.) for *A. australis* (Fig.2). When further increased to 60%, only three (two *Lactobacillus* spp., and an Acetobacteraceae) and one (*Snodgrassella* sp.) core taxa were observed for *T. carbonaria* and *A. australis* (Fig.2). We also identified the core fungal species; with the 20% threshold, six (a Didymellaceae, an *Alternaria* sp., a *Neophaeomoniella* sp., a *Monocillium* sp., a Basidiomycota and a *Malassezia* sp.) core fungal taxa were identified for *T. carbonaria* and five (an *Aaureobasidium* sp., a Didymellaceae, an *Alternaria* sp., a Zygosaccharomyces, and a

Malassezia sp.) for *A. australis* (Fig.3). Only two fungal taxa were detected for both the bee species when the threshold increased to 40% and no fungal taxa were detected at a 60% threshold (Fig.3).

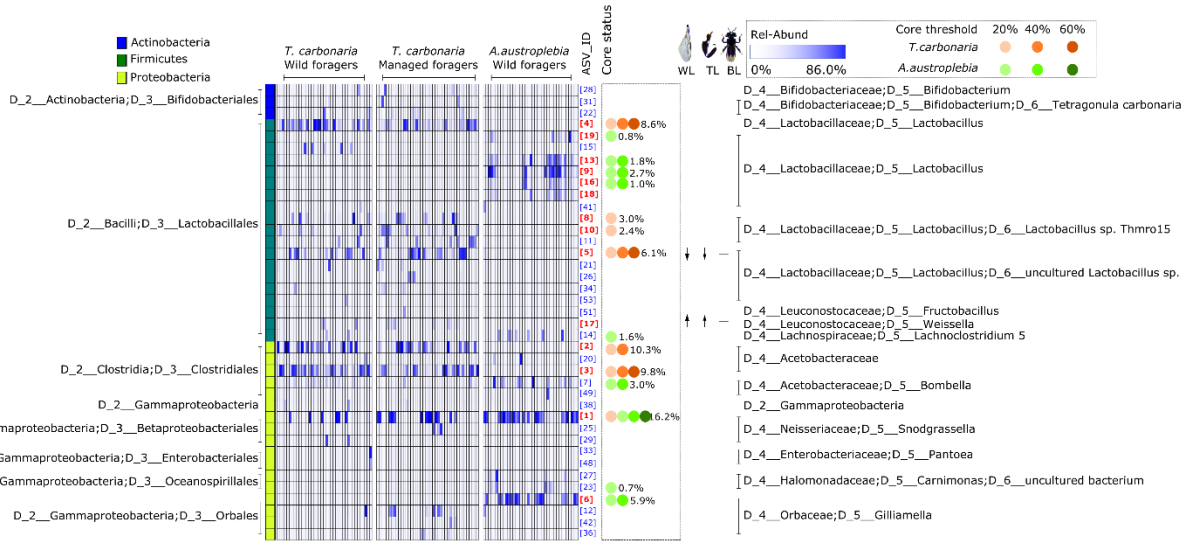

Fig.2 Heatmap summarising variation in the composition of bacterial communities in the stingless bee gut. Each amplicon sequence variant (ASV) has a unique numeric identifier shown in square brackets that is consistent with the main text and those shown in other figures. ASVs that were present at ≥10% relative abundance in any sample were included. ASVs highlighted in red significantly differed in relative abundance between the two bee species; up and down arrows besides ASVs marked significant correlations between the ASV with the bee morphological trait ($P < 0.01$). The dots on the right summarise the core conditions of that ASV in the stingless bee gut bacterial communities. In this case, 20%, 40% and 60% occurrences across samples were tested. The phylum of each ASV is indicated by the colours on the left of the heatmap, and the percentage besides dots indicates its contribution to the total number of reads obtained from sequencing. The text on the left shows bacterial taxonomy at class (D_2) and order (D_3) level, while text on the right shows bacterial taxonomy at the family (D_4), genus (D_5) and species (D_6) level.

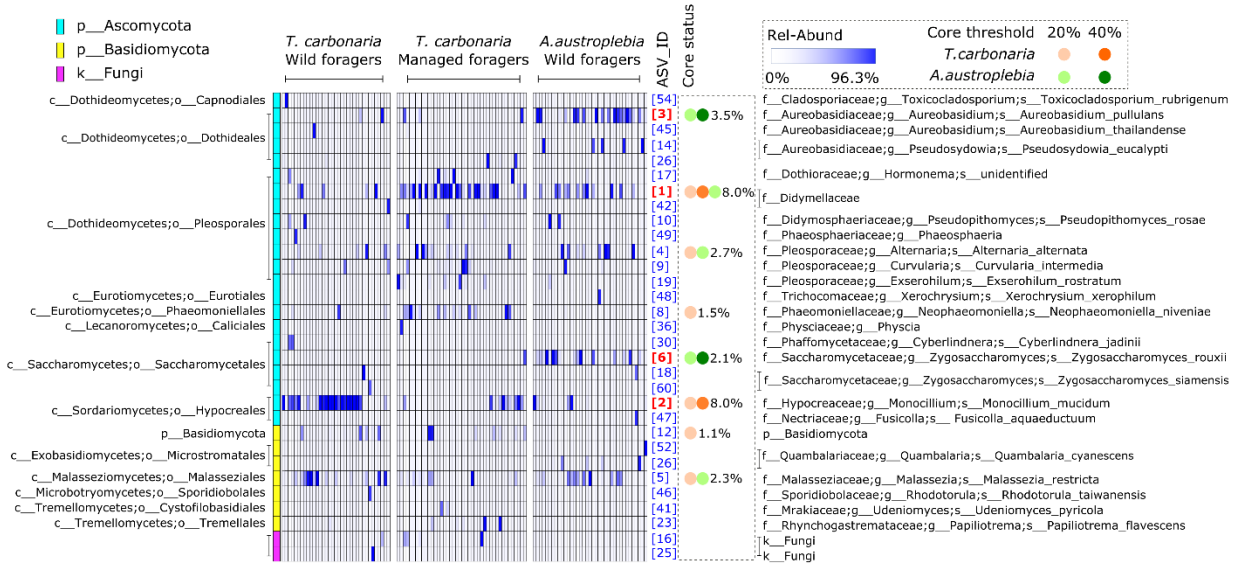

Fig.3 Heatmap summarising variation in the composition of fungal communities in the stingless bee gut. Each amplicon sequence variant (ASV) has a unique numeric identifier shown in square brackets that is consistent with the main text and those shown in other figures. ASVs that were present at ≥10% relative abundance in any sample were included. ASVs highlighted in red significantly differed in relative abundance between the two bee species. The dots on the right summarise the core conditions of that ASV in the stingless bee gut bacterial communities. In this case, 20%, 40% and 60% occurrences across samples were tested. The phylum of each ASV is indicated by the colours on the left of the heatmap, and the percentage besides each ASV indicates its contribution to the total number of reads obtained from sequencing. The text on the left shows bacterial taxonomy at class (c_) and order (o_) level, while text on the right shows bacterial taxonomy at the family (f_), genus (g_) and species (s_) level.

Total bacterial and fungal abundance, and alpha diversity of the stingless bee gut microbiomes

We compared wild (*T. carbonaria* & *A. australis*) and managed (*T. carbonaria* only) samples within their geographic range to detect species and management effects on gut microbiomes. We found that the total gut bacterial abundance was significantly higher in *T. carbonaria* and was correlated to their geographic locations, including both the latitude (R=-0.21, *P*=0.0009) and longitude (R=0.43, *P*<0.0001) (Fig.4A). However, we found no evidence that gut bacterial abundance correlated to any of the phenotypic traits of stingless bees (Table 1). In contrast, total fungi within bee gut samples were very low and we could not detect effective amplifications in >80%

samples using qPCR method (Ct>35). Multiple alpha indices were then compared among samples to provide detailed insight into the gut microbial diversity (Fig.4B,C). There was a significantly higher bacterial diversity in the gut of *A. australis* than *T. carbonaria* in terms of the observed ASVs, Chao1 and Shannon (Table 1, Fig.4B). Alpha diversity of the gut fungal community of *A. australis* was also significantly higher than that of *T. carbonaria* in terms of Evenness and Simpson but not the observed ASVs (Table 1, Fig.4C). For bacterial communities of *T. carbonaria*, wild bees had higher diversity than managed bees (Observed ASVs, Chao1 and Evenness) while diversity of the fungal community did not differ between management approaches (Table 1).

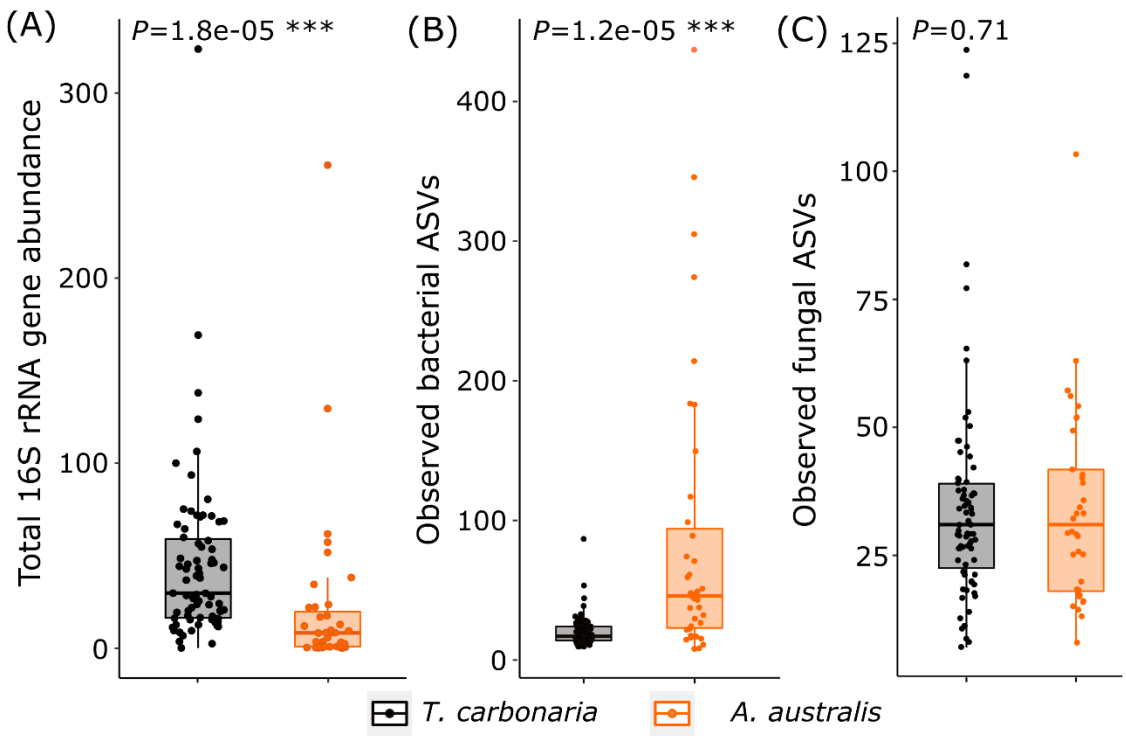

Fig.4 Alpha diversity and total bacterial abundance of the stingless bee gut microbiomes. Total bacterial abundance measured by qPCR analyses (A). Observed amplicon sequence variants (ASVs) in the stingless bee gut (B: bacteria, C: fungi). Boxplots indicate the first and third quartiles with the median value indicated by a horizontal line. Asterisks denote statistically significant differences between the two species.

Table 1 Differences of the microbial community diversity and composition among samples and their correlations to stingless bee phenotypes, latitude and longitude. Significant *P* values are highlighted in bold (*P*<0.05).

	Management (Wild ~Managed)		Species (*T. carbonaria* ~ *A. australis*)		Wing length		Tibia length		Body length		Wing size		Latitude		Longitude	
	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
Total bacterial abundance	0.024	0.88	24.31	**1.75e-05**	0.68	0.41	0.17	0.68	1.56	0.22	0.14	0.71	28.67	**0.0009**	11.70	**4.75e-07**
Bacterial community (alpha-diversity)																
Observed ASVs	7.23	**0.014**	57.29	**2.93e-10**	0.019	0.89	0.21	0.64	3.87	0.054	0.78	0.38	0.93	0.34	41.42	**3.07e-09**
Chao1	6.70	**0.004**	35.68	**1.43e-07**	2.49	0.12	1.01	0.12	1.10	0.30	1.48	0.23	3.29	0.072	20.50	**1.48e-05**
Shannon	0.21	0.78	7.12	**0.01**	0.0004	0.98	0.16	0.69	0.42	0.52	0.004	0.95	0.70	0.40	6.28	**0.014**
Evenness	5.31	**0.03**	1.64	0.21	0.001	0.97	0.19	0.67	0.056	0.94	0.29	0.59	0.18	0.67	4.26	**0.041**
Simpson	2.10	0.26	0.93	0.34	0.14	0.71	0.001	0.99	0.052	0.82	0.096	0.92	0.44	0.51	0.17	0.68
Fungal community (alpha-diversity)																
Observed ASVs	1.54	0.22	1.29	0.26	0.81	0.37	0.72	0.40	0.087	0.77	0.64	0.43	0.063	0.80	1.68	0.20
Shannon	0.81	0.37	4.71	**0.034**	0.50	0.48	0.21	0.65	0.24	0.63	0.22	0.634	1.34	0.25	2.30	0.13
Evenness	0.0009	0.98	11.38	**0.0014**	0.059	0.81	2.33	0.13	0.071	0.79	0.14	0.71	1.40	0.24	7.14	0.63
Simpson	0.37	0.55	5.26	**0.025**	0.58	0.45	3.02	0.087	0.011	0.97	0.12	0.73	1.10	0.30	2.90	0.091
Bacterial community beta-diversity																
Community composition	2.40	**0.0097**	22.31	**0.0001**	-	**0.0001**	-	**0.001**	-	0.20	-	**0.004**	-	**0.0001**	-	**0.0001**
Unifrac distance (weighted)	1.55	0.20	26.53	**0.0001**	-	**0.001**	-	**0.001**	-	0.73	-	**0.002**	-	**0.001**	-	**0.001**
Unifrac distance (unweighted)	5.42	**0.011**	41.26	**0.0001**	-	**0.001**	-	**0.001**	-	**0.022**	-	**0.010**	-	**0.001**	-	**0.001**
Fungal community beta-diversity																
Community composition	4.22	**0.0001**	4.13	**0.0001**	-	**0.001**	-	**0.001**	-	0.54	-	0.073	-	0.70	-	**0.001**

Factors correlated to the beta-diversity of the stingless bee gut microbiomes

Bacterial and fungal community composition (beta-diversity, differences in microbial community composition between samples) were influenced by both host species identity (Bacterial: R^2=0.23, P=0.0001, fungal: R^2=0.054, P=0.0001) and management approach (managed/wild, assessed in *T. carbonaria* only) (Bacterial: R^2=0.031, P=0.0097, fungal: R^2=0.053, P=0.0001) (Table 1, Fig.5A,B). When both stingless bee species were considered together, the gut bacterial community composition was closely associated with the host forewing length, forewing size, tibia length as well as latitude and longitude (Table 1, Fig.5A). However, none of these correlations were significant when the two species were analysed independently, indicating that phenotype differences between the two species may have driven the occurrence of such correlations. Similarly, the fungal community composition also significantly correlated to the host forewing length, tibia length, latitude and longitude when the two species were analysed together (Table 1, Fig.5B). When examining species separately, only body length was associated with the fungal community composition of *A. australis* (R^2=0.16, P=0.021).

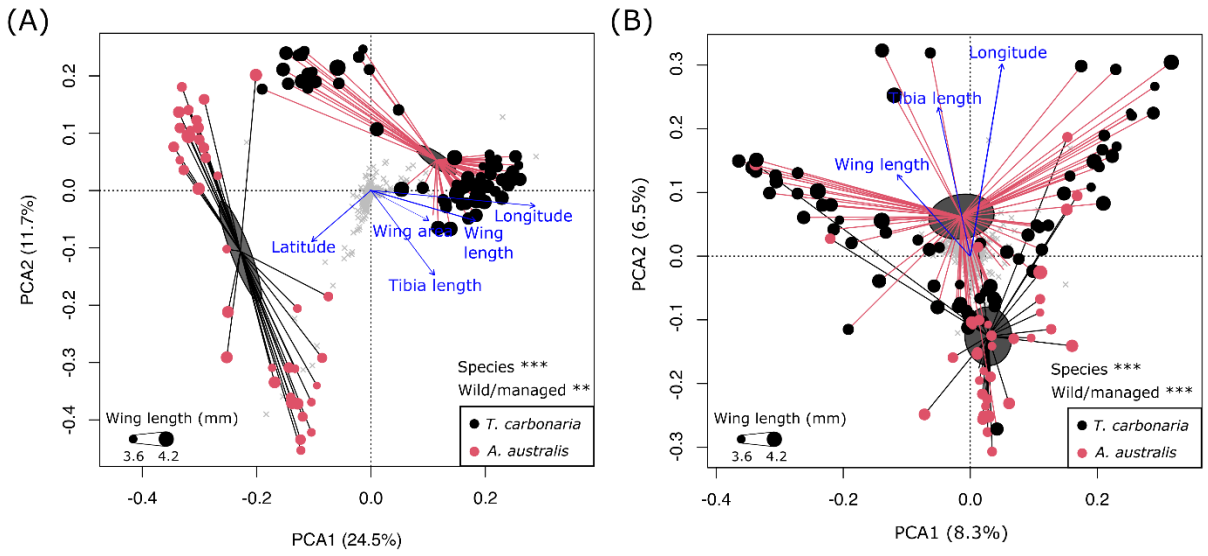

Fig.5 Principal component analyses (PCA) of the gut bacterial (A) and fungal (B) microbial community composition. Ellipses show the standard error of the mean. Blue arrows represent the direction of environmental gradients, with length proportional to strength of the correlation.

We then used Bray-Curtis similarity index to determine the influence of geographic distance (km) between samples on gut bacterial and fungal community composition (Fig.S2). A significant linear decrease in composition similarity with increasing geographic distance was seen between samples for *T. carbonaria* gut bacterial communities (R^2=0.005, *P*<0.0001). In addition, there was a similarity decrease with increasing geographic distance for fungal communities for both *T. carbonaria* (R^2=0.006, *P*<0.0001) and *A. australis* (R^2=0.01, *P*=0.002) (Fig.S2A,C,D). These results show that the microbiome composition varies more between samples from geographically distant locations. However, the adjusted R^2 for each correlation was small (0.005~0.01), indicating only a weak link. No significant correlation was seen for gut bacterial communities in *A. australis* (Fig.S2B). The *A. australis* samples covered a relatively smaller area, and so the power to detect small changes may have been reduced compared to *T. carbonaria*.

Correlation of stingless bee gut microbial richness with host morphological traits and geography

Interestingly, gut bacterial richness (as indicated by observed ASVs, R=0.38, F=11.89, *P*=0.00078) and Chao1 diversity index (an estimate of the total number of bacterial species, R=0.38, F=11.91, *P*=0.00094) in *T. carbonaria* showed a significant positive correlation with host forewing length (Fig.6A); a similar trend was also observed for the forewing area (observed ASVs, R=0.34, F=8.06, *P*=0.0059; Chao1, R=0.30, F=7.43, *P*=0.008). Total body length, hind tibia length, latitude and longitude showed no correlation with gut bacterial richness or any other alpha diversity indices. The forewing area/body length ratio, which is believed to determine flight capacity, also correlated with gut bacterial richness in *T. carbonaria* (R=0.24, F=5.66, *P*=0.021). SEM analysis that

considered morphological traits, geographical factors and management effects together showed consistent results with a dominant effect of forewing length on gut bacterial richness in *T. carbonaria* being revealed (Fig.6B). Lastly, linear models showed that forewing length and area, tibia length and body length were all positively correlated with one another (e.g. $R_{\text{wing length–wing area}}$=0.85; $R_{\text{wing length–body length}}$=0.38; $R_{\text{wing length–tibia length}}$=0.72; P<0.0001 in all cases), indicating that larger bees possess longer wings (Fig.S3A,B,C).

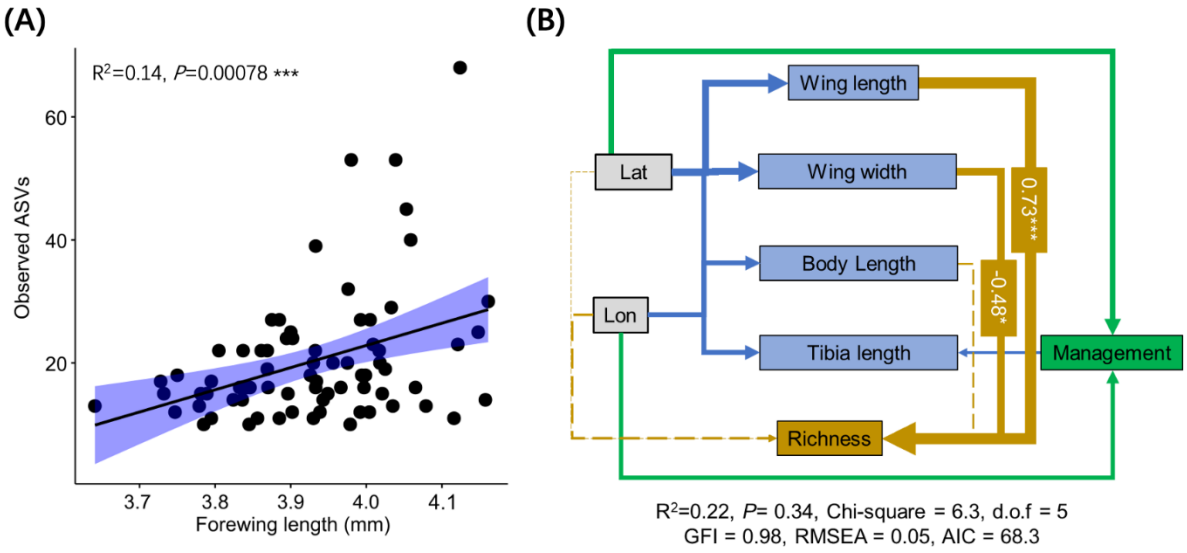

Fig.6 A significant linear correlation between gut observed ASVs with host forewing length in *T. carbonaria*. Person correlation (A) and structural equation modelling (SEM) summarising correlations of the bee morphometric trait, geographical factor and management approach with the observed ASVs in the gut of *T. carbonaria* (B). In A, the black line shows regression with shading area representing 95% confidence intervals, and data points were shown with black dots. In B, solid arrows indicate significant effect sizes ($P < 0.05$, dashed lines $P > 0.05$) and width of the arrow represents the strength of the relationship. The colour of the arrows corresponds to each targeted factor. Standardised path coefficient values are shown besides the significant pathways. Lon: longitude; Lat: latitude.

Discussion

We characterised the whole gut microbiome of two stingless bee species, *T. carbonaria* and *A. australis*, from 121 locations spanning a large geographic range in eastern Australia. We quantified total gut bacterial and fungal abundance using qPCR and found that bacterial abundance was higher in *T. carbonaria* (45.11±46.15) compared to *A. australis* (21.64±46.48), and fungi were either lowly abundant or below the threshold of detection. We also tested whether gut microbiomes are linked to host traits, geographic location (longitude and latitude), host species and management type (wild vs managed) to understand the factors that shape the gut microbiome. This understanding may provide insights into how hosts and their microbiomes have evolved in response to different environmental conditions. For *T. carbonaria*, we observed a positive correlation between both forewing length and area with host gut bacterial richness (the number of bacterial species in bee gut). For both species, microbiomes consistently became more distinct from one another in their composition with increasing geographic distance between samples, which suggests a role of geographic factors in shaping stingless bee gut microbiomes. Additionally, variations in climate (e.g., temperature) across the 1200km range where samples were collected have also likely contributed to the differences in microbiomes observed between samples. Overall, by combining analyses of both the bacterial and fungal communities with host traits and geography, we provide novel understandings of the stingless bee gut microbiomes.

Stingless bee gut microbial diversity correlates with host forewing size

Our findings revealed that *Lactobacillus* spp., Acetobacteraceae, and *Snodgrassella* spp. were consistently present in both bee species. The core fungal species identified were less consistent among samples than bacteria, but they likely included a *Neophaeomoniella* sp., a *Monocillium* sp.,

a Basidiomycota, and a *Malassezia* sp. Further investigations are needed to determine their origins, such as whether they were acquired from the environment or inherited from parents, or from interactions with other bees. We observed a positive correlation between gut bacterial species richness and host forewing length and size of *T. carbonaria*, as indicated by using a linear model and SEM approach. Insect wing sizes are closely related to their flight capacity (DeVries et al., 2010); longer wings favour wider variation in speed and increase capacity for longer flight duration and energy saving (DeVries et al., 2010). These factors potentially increase the capacity of bees to collect diverse floral resources. Furthermore, insects with larger wings are more successful in host-seeking and their location of oviposition sites (Berwaerts et al., 2002; Davis and Holden, 2015). Therefore, there are two possible drivers of the link between wing length and bacterial richness: (i) bees with larger wings have larger bodies (supported by our data), so may have a larger gut area for bacteria to colonize, and (ii) bee foragers with larger wings are to encounter more bacterial species when accessing more diversified floral resources across an area.

As found in this study, stingless bee guts contained bacterial genera such as *Pantoea, Sphingomonas, Stenotrophomonas, Gilliamella* spp. (Graystock et al., 2017), *Saccharibacter* spp. (McFrederick et al., 2012), *Massilia* spp. (Graystock et al., 2017) and *Acinetobacter* spp. (Graystock et al., 2017), which are commonly found on all parts of flowering plants, suggesting plant visits might be key to the microbial acquisition by stingless bees and support the second hypothesis. But additional research is needed to test the above hypotheses in detail, and to determine whether the findings here apply to other insects. Gaining this knowledge should aid a better understanding of microbial ecology in insect pollinators. The composition and distribution of gut microbiomes in stingless bees are likely to vary between gut regions, similar to

other insect species such as honeybees. The stingless bee foregut or crop may exhibit more variability due to exposure to the environment and diverse food sources, but the exact patterns of gut microbiome distribution in stingless bees are not yet fully characterized.

Bacterial communities

In this study, Proteobacteria and Firmicutes were found to be the dominant bacterial phyla in the gut of both stingless bee species, followed by Actinobacteria; a pattern also observed in honey bees and bumble bees (Kakumanu et al., 2016; Wang et al., 2019). However, it is unclear whether the composition and diversity of gut microbiomes are comparable across bee species in different gut regions. Such microbial similarity among species supports a strong host selection of the microbial environment by eusocial bees. The core bacterial phylotype, *Lactobacillus*, has important functions in the host, such as protection against pathogens and food digestion, as has been demonstrated for honey bees in previous studies (Engel and Moran, 2013; Liu et al., 2019; Kwong and Moran, 2016; McFrederick et al., 2012). They are common in the gut system of bumblebees worldwide (Kwong and Moran, 2016), suggesting that mutualisms with *Lactobacillus* exist throughout the eusocial bees across different geographic regions. *Lactobacillus* spp. are the main indicator taxon distinguishing the stingless bee gut microbiomes of the two species, which suggests a great variance in phylogeny and abundance of the *Lactobacillus* genus at species/strain levels between the two species. *Snodgrassella* spp., another core bacterial genus in the stingless bee gut microbiome, also features in the core microbiome of both honey bees and bumble bees (Koch and Schmid-Hempel, 2011). *Snodgrassella* spp. are saccharolytic fermenters and have been implicated in the protection of bumble bees against *Crithidia bombi* (Koch and Schmid-Hempel, 2011). Laboratory studies indicated that glyphosate (the primary herbicide used worldwide) can perturb the strain abundance of core gut *Snodgrassella alvi* in honey bees, which led to higher rates

of mortality when glyphosate-treated bees were exposed to the opportunistic pathogen *Serratia marcescens* (Motta et al., 2018), highlighting the importance of this bacterium in the maintenance of host health. As with *Lactobacillus*, the relevance of the species/strain diversity of *Snodgrassella* spp. in the stingless bee gut is not yet understood but may correspond to differences in host metabolic capabilities. Interestingly, a recent study surveyed gut microbiomes of Brazilian stingless bees by sampling multiple species within the genus *Melipona,* and showed that stingless bees can lose the core symbioses of *Snodgrassella* (Cerqueira et al., 2021). This suggests that strong ecological shifts or functional replacements in the stingless bee gut microbiome can occur.

The gut bacterial species richness of *A. australis* was significantly higher than that of *T. carbonaria*. Such microbial difference may relate to the distinct foraging behaviour of the two species. *T. carbonaria* evidently collects more protein enriched food (e.g. pollen) than *A. australis* which likely focuses on high-quality nectar (carbohydrate enriched) (Leonhardt et al., 2014). This higher level of carbohydrate foraging may be linked with the higher bacterial richness seen in *A. australis*. Hive managed bees seem likely to possess less gut bacterial diversity than wild bees, which may indicate a less diverse food composition. A previous study also found that gut bacterial diversity of fruit fly (*Bactrocera tryoni*) larvae was significantly lower in laboratory populations compared with field populations (Deutscher et al., 2018). We observed that stingless bee gut microbiomes vary greatly among samples. This aligns with a previous study on whole-body bacterial and fungal communities of managed *T. carbonaria* (Hall et al., 2021). Temporal and spatial changes of the bee microbiome composition observed in that study may also, to some extent, explain the high variability of microbiome composition we saw across a geographic gradient. For example, Hall et al. (2021) saw dramatic increases in the relative abundances of *Bombella* and *Zymobacter* and

493 almost complete depletion of *Snodgrassella* when colonies were moved from a florally resource-
494 rich site to a resource-poor site. All the above findings, along with previous studies (5, 6, 8, 19),
495 suggest that stingless bees are prone to compositional shifts, putatively influenced by food
496 resources, both spatially and temporally, physiological status, origin of the colony and climate at
497 different geographic locations.
498
499 *Fungal communities*
500 The dominant fungal phyla observed, Ascomycota and Basidiomycota, usually fulfil a
501 decomposing role in most land-based ecosystems, by breaking down organic materials such as
502 large molecules of cellulose or lignin, and in doing so play important roles in carbon and nitrogen
503 cycling (Dighton, 2016). We identified core fungal taxa in *T. carbonaria* and *A. australis*.
504 Interestingly, two of these, *Malassezia restricta* and *M. globosa*, are also among the most abundant
505 fungal species in the human gut (as indicated by their large presence in faecal samples and
506 intestinal mucosa), and have been identified in association with gut diseases including colorectal
507 cancer (Coker et al., 2019). Another core fungal genus, *Monocillium* spp. has previously been
508 isolated from soil, dead leaves and wood and some species (e.g., *M. curvisetosum*) originate from
509 aphids. There is evidence that *Monocillium* spp. are able to antagonise a plant parasitic nematode
510 by colonising their cysts (Ashrafi et al., 2017). The detected *Alternaria alternata* can be an
511 opportunistic fungal pathogen on plants causing leaf spots, rots and blights (Tsuge et al., 2013);
512 however, its function in the bee gut and whether it may be vectored between plants by bees is
513 currently unknown. Further investigations are needed to determine how gut-colonising fungi
514 interact with co-occurring bacteria, and the implications for host nutrition and fitness. The low

amount of the total fungi in the stingless bee gut demands future investigations into whether these fungi have functional roles in the fitness of stingless bees.

Distance-decay relationship between stingless bee gut microbiomes and geographic distance of bee sampling

Our data showed that microbial biogeographic patterns (a distance-decay relationship) could be applied to stingless bee gut microbiomes on a geographic scale of 250~1,200 km. While we predicted decreasing community similarity with greater distance due to dispersal limitation of stingless bee microbiomes (Soininen et al., 2007; Nemergut et al., 2013), evidence for such a relationship in bee microbiomes was previously lacking. An analysis of relative abundances of *Snodgrassella* and *Gilliamella* across *Bombus* and *Apis* hosts found poor correlation with geography (Koch et al., 2013). Previous studies also found limited effects of geographic location on microbiota composition probably due to small sample sizes and/or geographic distances (Kwong et al., 2017). However, the bacterial and fungal distance-decay relationship detected in our study, although significant, explained only a small amount (R^2=0.005~0.01) of observed variation, perhaps smaller than those typically observed for plants and other animals. The number of samples, geographic area covered and sequencing depth all could affect the differences, highlighting the need to couple high-throughput sequencing methods with wide geographical coverage.

Conclusions

We characterised the gut microbiomes of two stingless bee species from different genera across 1,200 km, spanning large parts of their geographic ranges in eastern Australia. We found the gut

538 microbial richness of *T. carbonaria* correlated to key host morphological traits, namely forewing
539 length and area, that can affect foraging behaviour. In addition, total fungi in the stingless bee gut
540 appears to be in low abundance and were hardly detected in most of the bee samples. Overall, our
541 findings, esp. the observed correlation between gut microbiomes and bee fitness traits may provide
542 a novel framework to test functional interactions between insect pollinators and their gut
543 microbiome.
544
545 **Data availability**
546 The data that supports the findings of this study are available in the supplementary material of this
547 article. The raw sequencing data have been deposited in the NCBI Sequence Read Archive (SRA)
548 under Bioproject code PRJNA698658.
549
550 **Author contributions**
551 HL, BKS, JC, RS, MR and LB conceived the idea; Mark H, Megan H, SN and HL collected wild
552 and managed bee samples along eastern Australia; HL conducted bee gut dissections, bee
553 measurement and DNA extraction, and analysed the sequencing data; Mark H drew Fig.1a and JW
554 conducted the SEM analyses; HL led writing of the manuscript, and all authors contributed to
555 manuscript edits and approved final version for submission.
556
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Conflict of interests

The authors declare that there is no conflict of interests.

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