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Research Article

# The bacterial community associated with the solitary resin bee *Megachile tosticauda* throughout its life cycle

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## Abstract

Unlike in eusocial bees where the identity, acquisition, and function of symbiotic microbes are well understood, little is known about the relationships formed between solitary bees and bacteria. Assessing the potential role of microbes in solitary bee health is important, especially in the face of global bee declines. Early evidence suggests solitary bee microbiomes differ between bee species and development stages, but the reported bacteria are often indistinguishable from environmental taxa. Here, we use metabarcoding of the 16S rRNA gene to characterize the bacterial communities associated with solitary resin bee *Megachile tosticauda*. We describe the microbiome at different life cycle stages, and within pollen provisions, and investigate indirect inheritance from nesting substrate upon eclosion. The microbiome of adult *M. tosticauda* was consistent between samples, and the bacterial composition of larval pollen supplies changed with progressing larval development. In wild adults and pollen provisions, the genus *Acinetobacter*—a common nectar associate—dominated the communities. In prepupae and frass, *Tyzzerella* dominated, a genus that has been found in a number of other immature bee systems. Intriguingly, while wild adults did not harbour *Tyzzerella*, all bees that had newly emerged from the nest did. The combined observations show that *M. tosticauda* acquire their bacterial community from the environment, and *Tyzzerella* may represent a beneficial symbiont for mature brood.

**Keywords:** 16S; horizontal transmission; insects; microbiome; microbiota; transiency

## Introduction

In the face of a global decline in bee diversity and abundance (Potts et al. 2010, Mathiasson and Rehan 2020, Zattara and Aizen 2021), a better understanding of the factors that influence solitary bee health is urgently needed. While this decline is primarily caused by changes in land cover and management, exposure to agrochemicals is considered an equally important threat (Dicks et al. 2021). Pesticides, antibiotics, and other agrochemicals can alter the bees' microbiome, causing dysbiosis, which negatively impacts bee health and survival (Kakumanu et al. 2016, Hotchkiss et al. 2022). To support and protect bee populations, more knowledge about the interactions between pollen, bacteria, and developing bee is urgently needed.

Most research on bee–bacteria relationships focuses on the eusocial European honeybee, *Apis mellifera*. Honeybees form symbiotic relationships with five host-specific gut bacteria that are essential for their health and survival (Kwong and Moran 2015, 2016). As in all holometabolous insects, during moulting and pupation the honeybee larval gut and its contents completely break down (Hammer and Moran 2019). Consequently, newly emerged honeybees are initially devoid of bacteria. However, within the first few days following eclosion (the process in which an insect emerges from their pupal case and cell), the honeybee gut bacteria establish through contact with other individuals and their shared living quarters (Martinson et al. 2011, Engel and Moran 2013, Powell et al. 2014). Such vertical transmission of beneficial microbes is also seen in other eusocial bees (e.g. stingless bees and bumble bees), and it is the reliable transmission pathway provided by social interactions that has facilitated the evolution of host-specific bacteria (Engel and Moran 2013, Kwong and Moran 2015, 2016). However, >90% of bee species are solitary (Danforth et al. 2013). Despite contributing essential pollination services (Losey and Vaughan 2006), microbiomes associated with most solitary bee species are undescribed, and the functional significance remains unknown.

Of the few solitary bee species whose microbiome has been studied, evidence suggests that the communities they harbour are species-specific (Martinson et al. 2011). In the absence of social inheritance, combined with the effect of holometabolism, most studies about the establishment of solitary bee microbiomes have focused on environmental factors (horizontal transmission) as the potential drivers for the composition (Voulgari-Kokota et al. 2019a). For example, the differences in the bacterial communities between wild-caught, laboratory-reared and newly eclosed bees of *Nomia melanderi* have highlighted the influence of external environmental exposure on microbial assembly (Kapheim et al. 2021). The observation that bees and the flowers they forage on share common microbes also supports environmental acquisition (McFrederick et al. 2014, 2017). However, bee-associated bacteria can be present on flowers independently of bee visitation (McFred-

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erick et al. 2017), suggesting that environmentally acquired bee bacteria are not strictly dependent on the bee host. This is unlike in honeybees where host-specific bacteria are rarely found outside of the bee environment (Martinson et al. 2011). In addition to horizontal transmission, it has been postulated that indirect vertical transmission could occur through direct contact with nest substrates (i.e. cell and nest walls, cell closures, frass, or cocoons) upon eclosion (McFrederick et al. 2017, Keller et al. 2021). This is seen in a social capacity in honeybees, where the inheritance of three core bacteria is from direct contact with faecal material in the nest (Powell et al. 2014). While few studies have investigated indirect vertical inheritance in solitary bees, it has been shown that newly eclosed *N. melanderi* acquire bacteria from the nest (Kapheim et al. 2021).

In this study, we characterize the microbiome in the pollen provisions and the different life stages of the widespread solitary Australian resin bee *Megachile tosticauda*. We use DNA sequencing of the V4 hypervariable 16S rRNA gene region to reconstruct the composition of bacteria associated with *M. tosticauda* throughout their development. In addition, we experimentally investigate whether adults vertically inherit their microbiome from nest substrates during eclosion. Specifically, we aim to (i) identify the bacterial communities in provisioning females, pollen provisions, larvae, and prepupae; (ii) identify changes in bacterial communities in the brood and food over time; and (iii) investigate how newly emerged adults acquire bacteria by comparing the microbiome among adults with varying degrees of environmental exposure.

## Materials and methods

### Location and biology of *M. tosticauda*

Adult *M. tosticauda* females were found nesting in paper straws and bamboo shoots in a bee hotel in a revegetated suburban garden in Eden Hills Adelaide, South Australia (−35.022, 138.597). A detailed account for the nesting behaviour, diet, and development times have been described by Williamson et al. (2025) and are summarized here. These bees build solitary linear nests in preexisting cavities using resin mixed with small grains for cell partitions and nest closures. Inside the nest, females provision a series of brood cells with *Eucalyptus* pollen, suggesting oligolecty. Larvae will begin to void and spin cocoons a few days before they finish consuming their provisions. Larvae consume their provisions within 3–4 weeks, after which prepupae will continue to spin cocoons, and the frass is weaved into the textile. Brood overwinters for up to 10 months until pupation. When metamorphosis is complete the bees eclose, using their mandibles to chew through nesting materials, such as cocoons and cell closures, and males emerge 1–2 weeks prior to females.

### Identifying the bacterial communities in provisioning females, pollen provisions, larvae, and prepupae

To describe the microbiome within the nest, food, and brood, and how the bacterial community changes through time, we collected 11 nests made in paper straws in March 2022 and 2023. Nests were collected within hours after the nest entrance had been plugged by the provisioning female or soon after the female was seen to stop provisioning the nest. The nests were then randomly assigned to groups as follows: active nests ($n = 4$) that were frozen immediately after collection; month-old nests ($n = 2$), which were left to develop for 4 weeks after collection, and for the bacterial acquisition experiment; eclosion from the nest ($n = 3$) where bees were left to develop in and eclose from their nest; and controlled eclosion ($n = 2$) where bees were left to develop in their nest until pupation, after which they were moved to petri dishes for eclosion (detailed further below, Table 1). All nests were then opened inside a laminar flow hood that had been sterilized using UV light for 20 min and by spraying down the surfaces with 80% ethanol. Nests were opened by carefully making two longitudinal slits along each side of the nest with a sterile scalpel, and samples were obtained with forceps that were flame sterilized between handling each specimen.

To describe the bacterial community throughout development, and the changes through time, we used samples from active and month-old nests. From active nests, we obtained samples of pollen provisions ($n = 36$), feeding larvae ($n = 8$), and mature larvae ($n = 3$). Mature larvae were defined as brood that were still eating but had also started voiding and spinning cocoons. From one active nest collected in 2023, we only sampled pollen provisions ($n = 9$). While we found 14 eggs, we were unable to assess their microbiome because, due to their delicate nature, we were unable to reliably remove all the pollen provision substrate from their surface, and because they yielded few sequences. We therefore do not include the results from egg samples. From month-old nests, we sampled frass ($n = 13$) and prepupae ($n = 10$), which were defined as brood that have finished eating, voiding, and spinning their cocoons. To describe the bacterial community of wild bees, we collected provisioning females across both years (seven in 2022 and three in 2023). Of the 10 provisioning females collected, 7 were collected with their completed nest. All samples were stored in sterile Eppendorf tubes at −80°C until DNA extraction.

### Bacterial acquisition experiment for adult *M. tosticauda*

As seen in honeybees, nest materials can serve as a transmission route for bee bacteria (Powell et al. 2014). To investigate whether the nest contents could serve as a means of indirect vertical inheritance for *M. tosticauda*, we performed an eclosion experiment. We collected five nests in March 2022, and kept them at room temperature until after the brood had pupated, the timing of which was determined in a preliminary study on developmental times (Williamson et al. 2025). After pupation, the nests were separated into one of two treatments: (i) **eclosion from nests**, where three nests were moved into sterilized chambers in an incubator set to 26°C, and eclosing bees ($n = 14$) only had contact with the nest materials, or (ii) **controlled eclosion**, where two nest were opened in a laminar flow hood and the pupae were carefully removed from their cocoons and transferred over to a sterile 96-well plate, placing one pupa per well ($n = 3$), then moved into the same incubator until eclosion. The incubator was checked twice daily for newly eclosed bees, which were then sampled and preserved until 16S rRNA gene sequencing (see later). We then compared the 16S sequences from our three adult *M. tosticauda* groups (i.e. eclosion from nest, controlled eclosion, and wild provisioning females) to assess bacterial acquisition from the nest substrate and the outside environment.

### DNA extractions, PCR, and 16S rRNA gene sequencing

We extracted DNA in a dedicated pre-polymerase chain reaction (PCR) laboratory from whole insect homogenates using the ZymoBIOMICS DNA miniprep kit according to the manufacturer's instructions. Because our samples were suspected to have low biomass, we ensured that our protocol was conducted in ac-

**Table 1.** Four nest treatments, corresponding conditions, and samples obtained for the 11 sampled nests of *M. tosticauda*.[a]

| Year nest was sampled | Nest number | Nest treatment | Nest conditions | Samples obtained |
|---|---|---|---|---|
| 2022 | 1 | Active | Frozen immediately after collection. | Provisioning adult, pollen provisions ($n = 10$), eggs ($n = 3$), larvae ($n = 3$) |
| | 2 | Active | | Provisioning adult, pollen provisions ($n = 9$), eggs ($n = 2$), larvae ($n = 3$), mature larva ($n = 1$), frass ($n = 1$) |
| | 3 | Active | | Provisioning adult, pollen provisions ($n = 8$), eggs ($n = 2$), larvae ($n = 2$), mature larvae ($n = 2$), frass ($n = 2$) |
| | 4 | Month-old | Left to develop for 4 weeks before opening | Provisioning adult, prepupae ($n = 5$), frass ($n = 6$) |
| | 5 | Month-old | | Prepupae ($n = 5$), frass ($n = 2$) |
| | 6 | Eclosion from nest | Three nests were moved into incubators set to 26°C. The nests were placed into chambers and eclosing bees only had contact with the nest materials. | Corresponding provisioning adult, newly eclosed male adults ($n = 2$) |
| | 7 | Eclosion from nest | | Corresponding provisioning adult, newly eclosed male adults ($n = 5$), newly eclosed female adults ($n = 4$) |
| | 8 | Eclosion from nest | | Newly eclosed female adult ($n = 3$) |
| | 9 | Controlled eclosion | Left to develop for 10 months, after which pupae were carefully removed from their cocoons and transferred over to a sterile 96-well plate and placed into an incubator until eclosion | Corresponding provisioning adult, newly eclosed female adult ($n = 1$) |
| | 10 | Controlled eclosion | | Newly eclosed female adults ($n = 2$) |
| 2023 | 11 | Active | Frozen immediately after collection | Pollen provisions ($n = 9$), eggs ($n = 7$), larvae ($n = 2$, used in a separate study) |
| | | | NA | Provisioning adults ($n = 3$) |

[a]From 'active' and 'month-old' treatments, we sampled pollen provisions, larvae, prepupae, and frass, and from 'eclosion from nest' and 'controlled eclosion' treatments, we sampled newly emerged adults for the bacterial acquisition experiment. Where possible we also sampled the corresponding provisioning adult with the collected nest.

cordance with the RIDE checklist (Eisenhofer et al. 2019), which stands for Report methodology, Include negative controls, Determine the level of contamination, and Explore contamination downstream. For each batch of DNA extractions, we included an extraction blank control ($n = 22$), which was also included in all downstream molecular processing and analysis to assess contamination. PCR targeted the V4 region of the 16S rRNA gene, using the primer pair 515F 5′-GTGCC AGCMGCCGCGGTAA-′3 and 806R 5′-GGACTACHVGGGTWTCTAAT-′3 (Caporaso et al. 2010, 2011), which has also been used in other bee microbiome studies (e.g. Holley et al. 2022, Kwong et al. 2017). PCRs were performed with the following parameters: single reactions of 2.5 $\mu$l X10 HiFi buffer, 0.1 $\mu$l Platinum™ Taq DNA Polymerase (ThermoFisher), 19.2 $\mu$l $dH_2O$, 0.2 $\mu$l 100 mM deoxynucleotide triphosphate mix, 0.5 $\mu$l each of 10 $\mu$M forward and uniquely barcoded 806R reverse primer, and 1 $\mu$l DNA. DNA was amplified using an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1 min, elongation at 68°C for 90 s, with final adenylation for 10 min at 68°C. The barcoded amplicons were pooled and sequenced on an Illumina MiSeq (v2, 2 × 150 bp) at the SAGC (South Australian Genomics Centre).

## Quantitative PCR and measuring the limit of detection

We selected DNA extractions of six pollen provisions, adults per treatment (six wild, six nest eclosion, and three controlled eclosion), four negative controls, and three honeybees for quantitative PCR (qPCR). In addition, we included single reactions of ZymoBiomics bacterial DNA standards of pure strains of *Escherichia coli* at varying concentrations (E2006-2, ZymoBiomics, USA). The qPCR used the same 515F-806R primers targeting the V4 region of the 16S rRNA gene with the following parameters: single reactions of 7 $\mu$l PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, USA), 1 $\mu$l each of 5 $\mu$M forward and reverse primer, and 1 $\mu$l DNA. The DNA was amplified and analysed on a QuantStudio™ 6 Flex Real-Time PCR System, using an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 1 min and elongation at 72°C for 45 s. qPCR of each sample was done in triplicate. Using the bacterial standards, the total 16S rRNA gene copies per standard were calculated based on *E. coli*'s genome size (bp) and known 16S rRNA copy number of 7 (Zymobiomics). We made a standard curve by plotting the threshold cycle (CT) scores from each standard against the 16S rRNA gene copy number (log10), and then used the equation of the line to measure the total gene copies for all samples (calculations provided in the supplementary materials). The limit of detection (LoD) was determined using the average total gene copies from the amplified negative control samples, which was then used for comparison with the total gene copies for each of the biological samples.

## Bioinformatics pipeline and statistical analysis

Demultiplexed sequences were analysed using QIIME 2's pipeline software (v2-2022.8) (Bolyen et al. 2019). After importing the sequence data using a manifest file, the forward and reverse reads were joined using VSEARCH (Rognes et al. 2016). Quality control was applied to the joined sequences using the default quality-filter q-score command, and sequences were denoised using the deblur algorithm (Amir et al. 2017) and trimmed to 250 bp. Taxonomy was assigned to the representative sequences using the SILVA

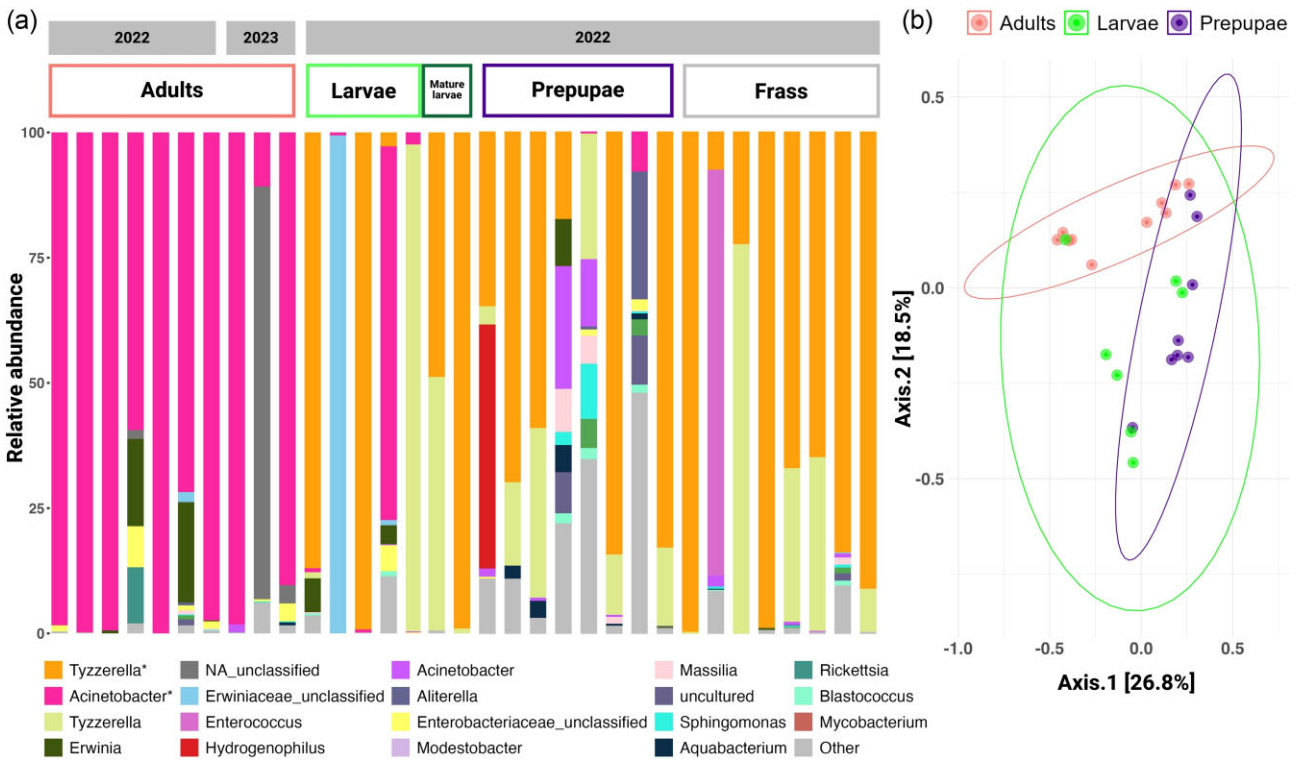


**Figure 1.** (a) The relative abundance (%) of the top 20 genera of bacteria from sequences at the V4 region of the 16S rRNA gene in provisioning adults, feeding larvae, mature larvae, prepupae, and frass samples. The top 20 genera make up 94.5% of the total sequences for these samples. Each bar represents one sample with larvae ordered by length (left to right samples go from smallest to largest) and prepupae by cell (left to right is from the newest to oldest cell) as a proxy for relative age. (b) Principal coordinate analysis (PCoA) plot showing the distribution of samples based on unweighted UniFrac distances. Each point represents one sample and ellipses represent the 95% confidence interval around each development stage.

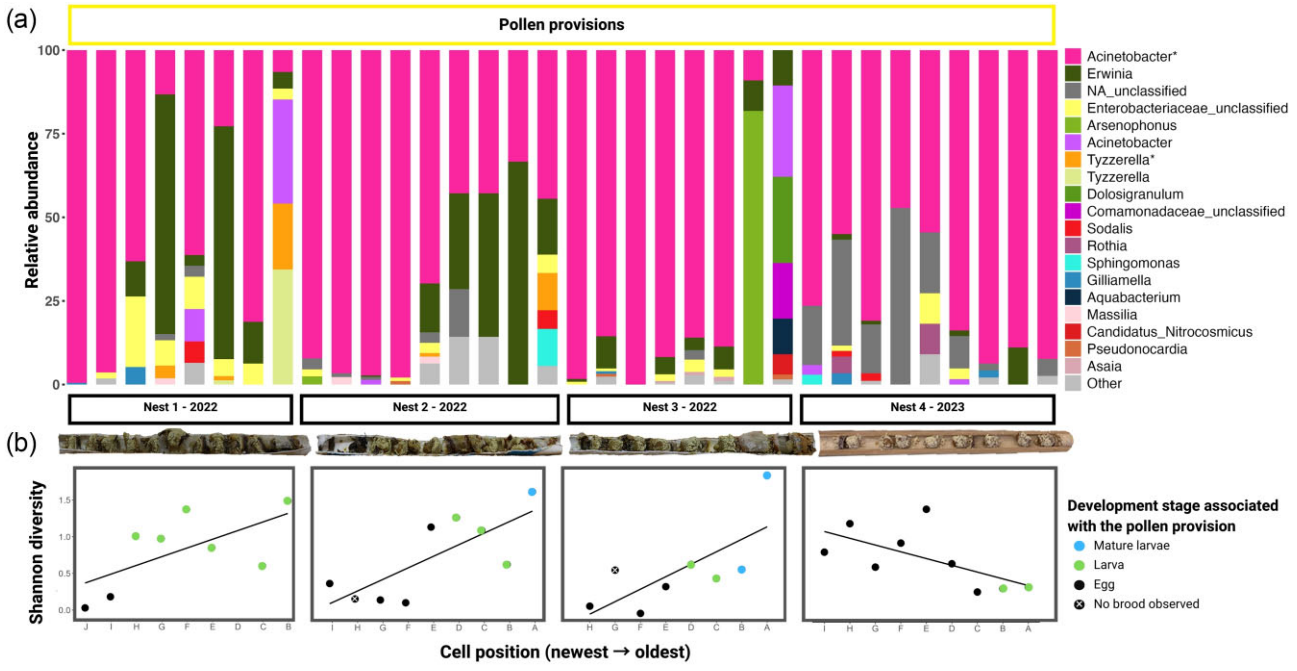


**Figure 2.** (a) The relative abundance (%) of the top 20 genera of bacteria from sequences at the V4 region of the 16S rRNA gene in the pollen provisions of four active nests. The top 20 genera represent 97.8% of the total sequences for these samples. Each bar represents one sample and is ordered by cell location (from left to right samples go from the newest to oldest cell) as a proxy for relative age. (b) Shannon $\alpha$-diversity of pollen provisions, ordered by cell. From left to right the provisions go from newest to oldest. Colours denote the development stage of the brood corresponding with each pollen provision.

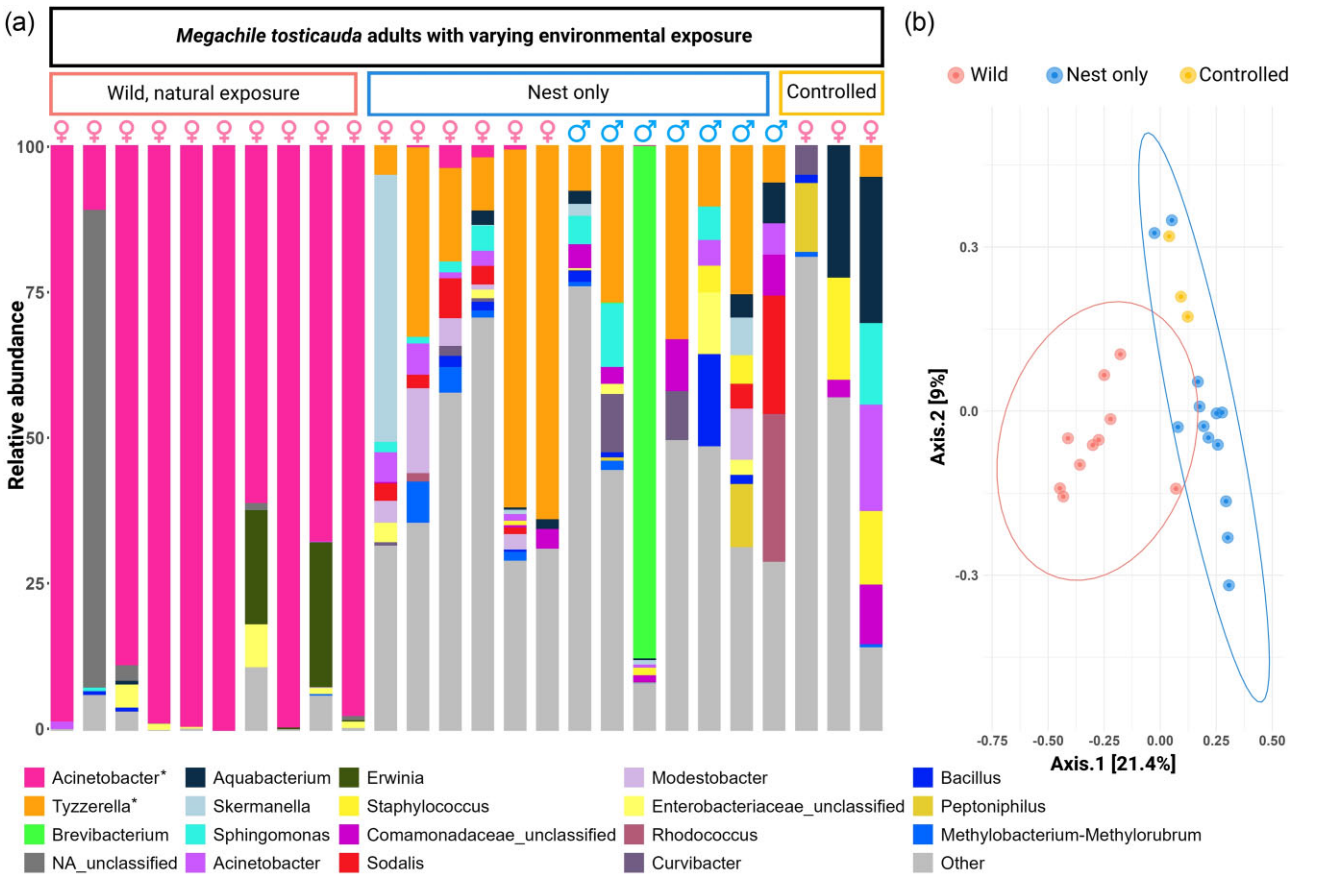


**Figure 3.** (a) The relative abundance of the top 20 genera from sequences at the V4 region of the 16S rRNA gene from adult bees in our eclosion experiment to assess the source of bacterial sequences. The top 20 genera represent 71.9% of the total sequences for these samples. Each bar represents one bee, and the sex is indicated above the bar. The 'wild' group is females that were caught provisioning nests. The 'nest only' group is bees that eclosed from their nest in a sterilized container in the incubator. The 'controlled' group is the bees that eclosed in sterile well plates and had no interaction with the nest or the external environment. (b) Principal coordinate analysis (PCoA) plot showing the distribution of samples based on unweighted UniFrac distances between adults with different levels of environmental exposure. Ellipses represent the 95% confidence interval around each development stage.

reference classifier (silva-138-99-515-806-nb-classifier.qza), using a trained classifier on the SILVA 138 reference database at a 99% similarity threshold, and at the 515F-806R primer region (Quast et al. 2013). A phylogenetic tree was created using SATé-enabled phylogenetic placement (Janssen et al. 2018).

Analysis of bacterial communities at the genus level was done in R (v.4.1.2) and visualized using the ggplot2 package (v.3.4.0). If genera were unclassified, they were elevated to family and denoted as 'family_unclassified'. The Decontam package (v.1.14.0) (Davis et al. 2018) was used to call contaminants with the prevalence approach, using a threshold of 0.5. For bacterial community analysis, i.e. relative abundances and alpha diversity, we removed putative contaminants identified using Decontam, as well as reads classified as chloroplasts and mitochondria, and rarefied sequences at a depth of 1500 (rarefaction curve provided in the supplementary materials, Fig. S1).

As described earlier, as an indicator for microbial biomass, the 16S rRNA gene copy number per sample was measured using bacterial standards of *E.coli* with a known 16S copy number. Using the total 16S gene copy number per sample, we scaled the relative abundance to generate plots that show both the community composition and the approximate microbial biomass; however, this assumes that the number of 16S genes in *E. coli* is representative of all taxa in the samples. While absolute biomass was not calculated by accounting for copy number variation between microbes, the total 16S gene copy number is still informative as an indication of biomass differences between sample types and controls.

To analyse the 16S data, we used phyloseq (v.1.38.0) (Lozupone and Knight 2005, McMurdie and Holmes 2013) and tidyverse (v. 1.3.1) packages. We ensured that all major honeybee bacteria were detected in our honeybee samples, which verified the success of our protocol and greenlit the analysis of our *M. tosticauda* samples. To explore $\beta$-diversity, we used principal coordinate analysis (PCoA) and plotted the ordination of unweighted UniFrac distances (Lozupone and Knight 2005). We assessed the homogeneity of multivariate dispersion among the wild adults, larvae, and prepupal groups, using the vegan (v 2.6-4) betadisper function, which confirmed that dispersion was not significantly different among communities. We therefore proceeded to test whether bacterial communities differed between developmental stages we used phyloseq's PERMANOVA. Two amplicon sequence variants (ASVs) were observed as prevalent across most samples and present in high relative abundances, and so to distinguish between these ASVs and others in the same genus, they are labelled with an asterisk, '*', in the plots and in the discussion.

## Results and discussion

### Bacterial communities dependent on development stage

Our raw sequencing results returned 1 360 779 reads from 1089 ASVs. One of our prepupal samples failed to return any reads. Our samples had a high amount of off-target amplification of chloroplasts and mitochondria, which contributed 69.6% of our total reads. After filtering out chloroplasts, mitochondria, and putative contaminants, a total of 349 077 reads from 961 ASVs remained for analysis. The quality assessment, filtering, and rarefaction resulted in the loss of two pollen provisions, three feeding larvae, one mature larva, three prepupae, and one nest eclosed adult samples due to insufficient reads.

The community composition of wild adults was consistent between sample types and between years (Figs 1 and 2), suggesting that *M. tosticauda* may be associated with a specific microbiome. The different developmental stages (wild adults, larvae, and prepupae) differed with respect to their bacterial communities (PERMANOVA on unweighted UniFrac distance: $F = 2.7256$, $P < .05$; Fig. 1). Pollen provisions contained bacterial communities that were similar to those of wild adults, and larvae and prepupae exhibited some overlap in composition but greater variability. While further verification is required due to our small sample size, and the fact that sampling was limited to a single location, our results support previous findings showing that solitary bee bacteria are dependent on development stage and host species (McFrederick et al. 2014, Rothman et al. 2019, Fowler et al. 2024). However, in all our bee samples, the communities were dominated by two ASVs, with *Acinetobacter** accounting for most sequences in adults and feeding larvae, and *Tyzzerella** in older brood and frass (Figs 1 and 2).

### *Tyzzerella* prevalent in older brood and frass

As shown in Fig. 1a, some larvae and most prepupae harboured a high relative abundance of a strain of *Tyzzerella**. The presence of *Tyzzerella* in older larvae and prepupae has been found in other bee systems, and the closest match to our nucleotide sequence was from larval gut samples of the megachilid bee *Osmia bicornis* (Mohr and Tebbe 2006), with a percentage identity of 99.6% and coverage of 98%. *Tyzzerella* has also been identified as a core bacterium in larvae of *Osmia excavata* (Wang et al. 2024), and honeybee larvae (Mohr and Tebbe 2006, Maigoro et al. 2024). Similar to our results, in the latter two studies *Tyzzerella* was only present in the developing brood, and not in adults. Immature bee microbiomes can provide key benefits such as pathogen protection and cold tolerance during overwintering (Christensen et al. 2024), and this may explain why the bacterial communities differ so greatly between brood and adult *M. tosticauda*.

While the function of *Tyzzerella* in bee systems requires further investigation, the genus has been positively correlated with detoxifying genes PaCYP6GF1 and PaCYP9HL1 in antibiotically challenged *Diptera* larvae (Sun et al. 2023) and we can therefore speculate that it may play a part in detoxification. Interestingly, *Osmia*, *M. tosticauda*, and honeybees collect resin, and these materials often contain terpenoids and phenolic compounds, which can have insecticidal action. Thus, it is possible that *Tyzzerella* aids larvae in detoxification of these resin compounds in the nest environment. It is also possible that the *Tyzzerella* is derived from resin, and it has been documented that the internal microbiome of bee nests reflects the materials used in its construction (Voulgari-Kokota et al. 2019b).

### High relative abundance of *Acinetobacter* in wild adults and pollen provisions

The samples of wild, nest provisioning female *M. tosticauda* returned bacterial communities with a high relative abundance of the same ASV of *Acinetobacter** (Fig. 3). This genus has been frequently found in other bee studies, is a common nectar associate, and a common environmental taxon (Cohen et al. 2020, Vannette 2020, Holley et al. 2022, Nguyen and Rehan 2023). Our results add to the growing body of literature indicating that while the microbiome may depend on the bee species, its taxonomic composition overlaps with that of common environmental taxa (McFrederick et al. 2014, Voulgari-Kokota et al. 2019a, Dew et al. 2020).

The pollen provisions contained a bacterial community that was like that of adult females. The relative abundance of *Acinetobacter** in the pollen provisions of most nests decreased with progressing larval development, accompanied by an increase in the $\alpha$-diversity of the community (Fig. 2). Similar shifts in the diversity of bacteria in larval pollen supplies that correlate with progressing larval development have also been described for *Osmia cornifrons* and *O. caerulescens* (Voulgari-Kokota et al. 2018, Kueneman et al. 2023). However, one active nest (nest 4) does not show a decrease in relative abundance of *Acinetobacter** in older cells. All brood in this nest were very young, containing eggs, and two young feeding larvae, whereas the brood in the other active nests consisted mostly of feeding larvae and some young prepupae (Fig. 2 and Table 1). Therefore, if composition changes over time, or in correlation with larval feeding, perhaps not enough time had passed in this nest.

While the cause for the decline in the relative abundance of *Acinetobacter** and increase in $\alpha$-diversity in the pollen provisions is unknown, we offer a few possible explanations. There could be a proliferation of bacteria other than *Acinetobacter**, which thereby gain a higher representation in the proportional abundance. Specifically, the common plant associate and pathogen *Erwinia* (Kado 2006) increased in relative abundance over time and may have been degrading pollen and multiplying as a result. Alternatively, *Acinetobacter** may die off because the pollen provisions are not conducive to its survival.

We also found that the relative abundance of *Acinetobacter** decreased in older larvae and was mostly absent in voided prepupae and in frass-containing samples (Figs 1 and 2). It is therefore likely that *Acinetobacter**, along with other bacteria, pollen, and nectar, is digested. Possibly, ingested *Acinetobacter** is transiently passing through larvae, supported by its absence in prepupae. Similar observations have been described in *M. rotundata* (Brar et al. 2024), and in caterpillars (Hammer et al. 2017), and in booth these cases the brood microbiomes were indistinguishable from their diet, similar to *M. tosticauda* and their pollen provisions. Interestingly Brar et al (2024) also found that removal of bacteria from the pollen provisions did not negatively impact prepupal weight or survival, and suggest the environmentally acquired, non-host-specific bacteria are non-essential, further supporting the possibility of transiency.

Alternatively, *Acinetobacter** may not be transient but digested as a food source. Kueneman et al. (2023) found that the presence of larvae influenced the bacterial composition over time. Furthermore, investigations with regards to the trophic level based on amino acid and fatty acid trophic biomarker analyses of native bees spanning 6 families and 12 genera (Steffan et al. 2019), as well as specifically for *Osmia ribifloris* (Dharampal et al. 2019), have suggested that bees are omnivorous. This is supported by experiments where solitary bee larvae are fed sterilized pollen, which

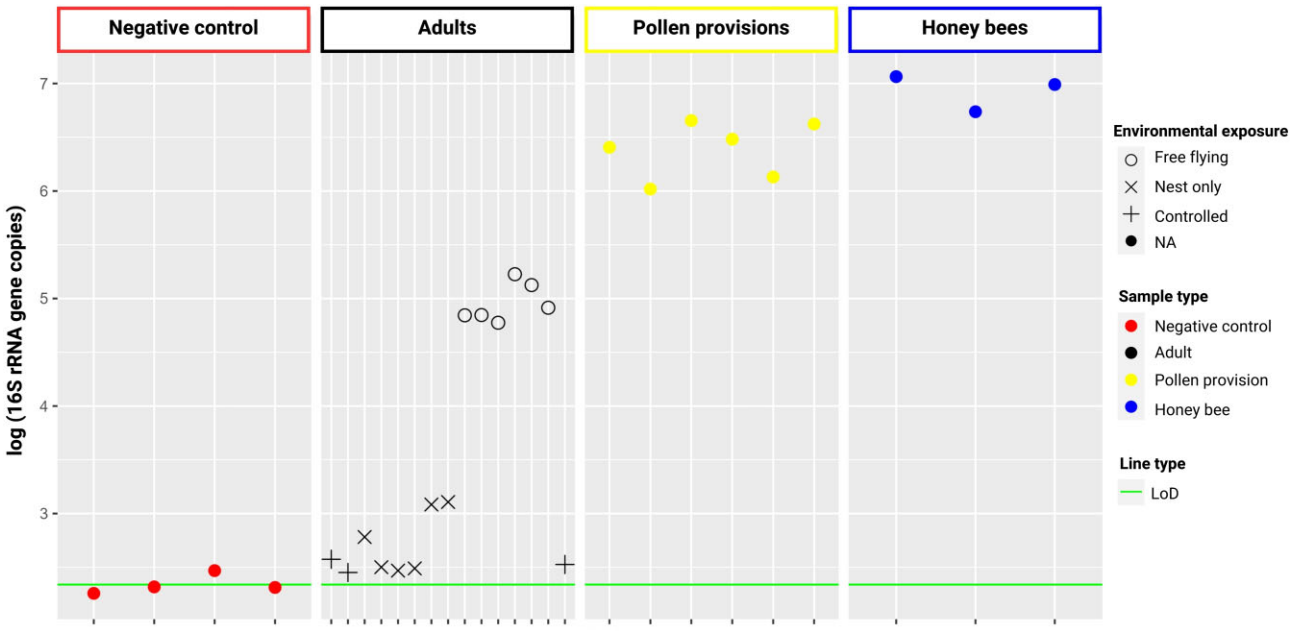


**Figure 4.** The total log (16S gene copy number) in adult *M. tosticauda*, pollen provisions, and honeybees from qPCR of the V4 region of the 16S rRNA gene, compared to limit of detection (LoD; green line) as determined by the average abundance from the extraction blank controls (negative controls).

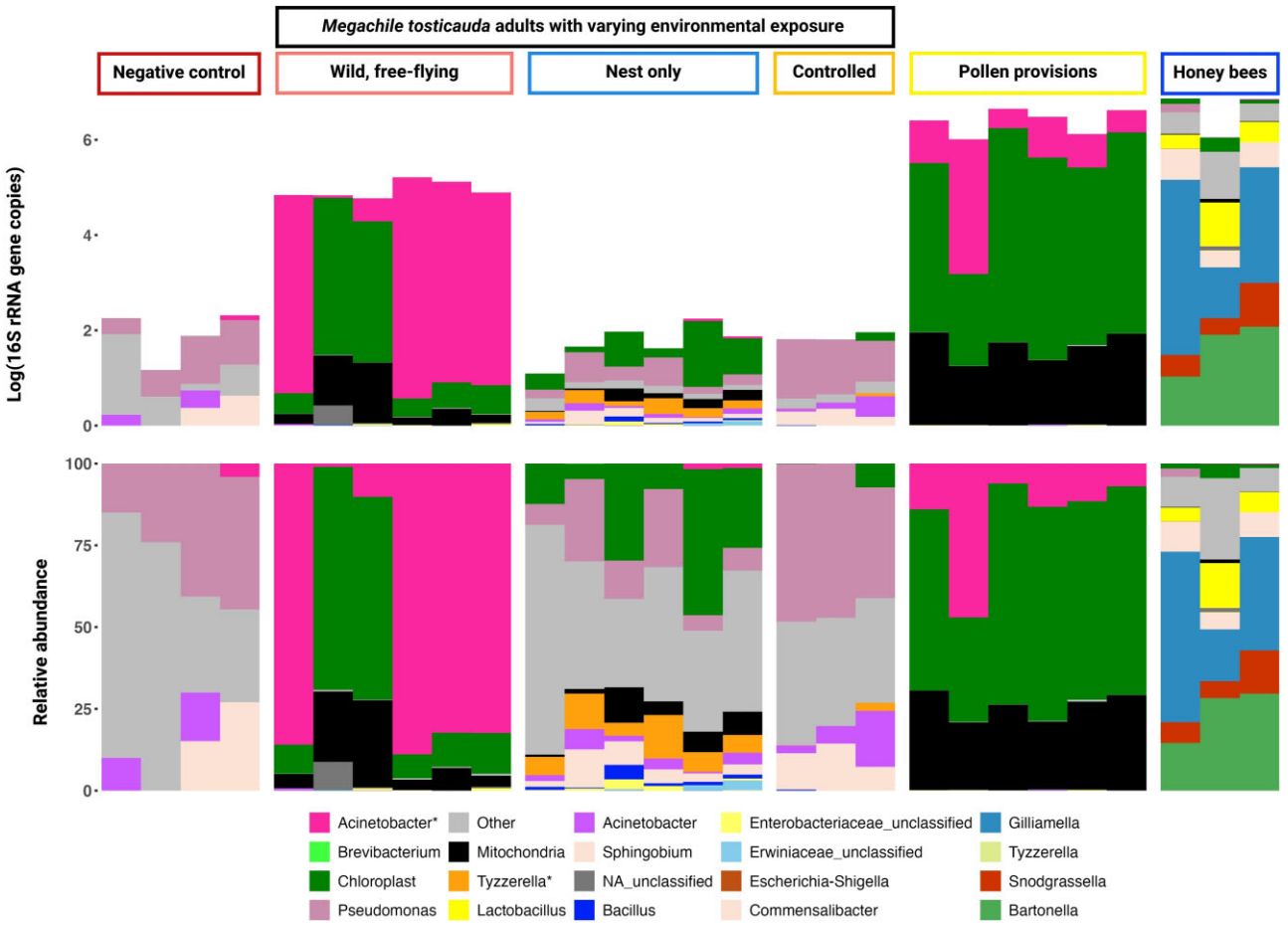


**Figure 5.** The unfiltered (i.e. including reads from mitochondrial, chloroplast, and contaminant DNA) abundance (top) and relative abundance (bottom) of the 20 most abundant sequences at the V4 region of the 16S rRNA gene for a subset of samples of negative controls, adult *M. tosticauda* with varying environmental exposure, pollen provisions, and honeybees as a positive control. The abundance is the log (16S rRNA gene copy number). The top 20 genera represent 90.1% of the total sequences for these samples.

resulted in negative health outcomes, suggesting an essential role of the food microbiome during feeding (Dharampal et al. 2019, 2020, 2022). Future experimental studies could elucidate whether *Acinetobacter* is actively broken down by the larvae, and therefore serves as a food source, or whether these bacteria are simply not surviving.

### The microbiome of *M. tosticauda* is environmentally acquired

By comparing bees with varying degrees of environmental exposure after eclosion (wild, nest only, and controlled eclosion in well plates), we aimed to determine whether bacteria are indirectly vertically inherited from the nest or solely acquired horizontally from the environment. While the sample size of the controlled eclosion group is small, as only three survived the transfer to the well plates, the bacterial communities of the controlled and nest-eclosed bees differed from each other and both groups were different to that of the wild adults. Several bacterial genera present in wild females were also picked up in bees exposed to the nest only, including *Acinetobacter*, *Enetrobacteracae*_unclassified, and *Bacillus* (Fig. 3).

Interestingly, *Tyzzerella** was present in all nest-only exposed adults, but not in the adults that were wild or eclosed outside the nest. Therefore, bees likely pick up bacteria from the nest during emergence, either by chewing the substrate or passively on their surface. The finding that *Tyzzerella** was absent in wild adults indicates transiency in the system (Fig. 3). It is possible that *Tyzzerella** is brought to newly founded nests by the nest founding females soon after eclosion, but it is also possible that it is present in the nesting material (e.g. resin) or associated with the area the bees were nesting in. To distinguish between these possibilities requires further study of *M. tosticauda*'s microbiome at different locations.

As shown in Fig. 3, there were five cases where the same ASV of *Acinetobacter** prevalent across our samples of wild adults and pollen provisions were also present in the nest-only-exposed bees. However, because not all bees eclosed with *Acinetobacter**, this observation should not be considered evidence of indirect vertical inheritance. It is intriguing that most of these cases were eclosed females, but this could be explained by their position at the back of the nest, which may result in greater contact with the nest material or residual floral substrates during eclosion. Importantly, it should also be noted that the low biomass nature of these samples means many represented genera have a very low number of reads; specifically, the counts of *Acinetobacter** in nest-only bees ranged from 2 to 27 reads (read counts for *Acinetobacter** and *Tyzzerella** in adults are provided in the supplementary materials, Figs S2 and S3).

Flowers are hubs for microbial transmission, and bees are important vectors that enable microbes to hitchhike between ephemeral floral habitats (Keller et al. 2021). Outside of flowering, bee nests could serve as a reservoir for floral microbes (potentially staying dormant or surviving on leftover larval food or in frass), and therefore, the *Acinetobacter* on the newly eclosed bees could be hitchhikers (Vannette 2020). Interestingly, it has been demonstrated that *Acinetobacter* induces pollen germination to gain access to nutrients, and their resulting proliferation has been postulated to increase their chance of being transmitted by bees (Christensen et al. 2021).

The qPCR revealed that the nests and controlled eclosed bees had a lower microbial abundance as measured by 16S gene copy number than the wild bees (Fig. 4). While still above the LoD, a deeper look at the composition of the amplified 16S genes reveals that it is largely attributed to off-target DNA from chloroplasts, mitochondria, and contamination (Fig. 5). Together, the finding that newly eclosed bees harbour low microbial abundance, and that the community of bacteria was dependent on the substrates they encountered, provides strong evidence for the environmental acquisition of bacteria in *M. tosticauda*. However, as indicated earlier, indirect vertical inheritance of *Tyzzerella** cannot be excluded as a possibility.

## Conclusion

The life stages of *M. tosticauda* hosted distinct bacterial communities, with *Acinetobacter* and *Tyzzerella* as predominant taxa. The decline of *Acinetobacter* in the pollen provisions over time likely indicates an inability to survive in the pollen provisions. The source of *Tyzzerella* may be the nesting substrate, such as in brood cell walls or hardened resin, as *Tyzzerella* was only found in older brood and newly eclosed adults. The role of *Acinetobacter* and *Tyzzerella* for the health and development of brood should be tested experimentally. We provide strong evidence that the adult microbiome is environmentally acquired after eclosion, likely from the nest and from floral resources such as nectar and pollen. Our findings highlight the dynamic and likely transient nature of bacterial communities in this solitary bee system, where bees are as much microbial vectors as they are hosts. Further research on the functional importance of these bacteria is warranted, to help elucidate the forces that underpin the formation of these bacterial communities in solitary bees, as well as the broader ecological implications.

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## Author contributions

Elisabeth Williamson (Data curation, Formal analysis, Methodology, Visualization, Investigation, Writing—original draft), Kelly Hill (Formal analysis, Methodology, Supervision, Writing—review & editing), Katja Hogendoorn (Conceptualization, Data curation, Project administration, Resources, Investigation, Writing—review & editing), and Raphael Eisenhofer (Data curation, Formal analysis, Methodology, Project administration, Writing—review & editing)

## Supplementary data

Supplementary data is available at *FEMSMC Journal* online.

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## Data availability

All sequence data code used to analyse the data is available on Github at: https://github.com/hi-its-lisou/Megachile_tosticauda_bacterial_community.

All DNA sequences have been uploaded to the NCBI sequence read archive under bioproject PRJNA1146331.

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