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Environmentally acquired gut-associated bacteria are not critical for growth and survival in a solitary bee, *Megachile rotundata*

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ABSTRACT Social bees have been extensively studied for their gut microbial functions, but the significance of the gut microbiota in solitary bees remains less explored. Solitary bee, *Megachile rotundata* females provision their offspring with pollen from various plant species, harboring a diverse microbial community that colonizes larvae guts. The *Apilactobacillus* is the most abundant microbe, but evidence concerning the effects of *Apilactobacillus* and other provision microbes on growth and survival are lacking. We hypothesized that the presence of *Apilactobacillus* in abundance would enhance larval and prepupal development, weight, and survival, while the absence of intact microbial communities was expected to have a negative impact on bee fitness. We reared larvae on pollen provisions with naturally collected microbial communities (Natural pollen) or devoid of microbial communities (Sterile pollen). We also assessed the impact of introducing *Apilactobacillus micheneri* by adding it to both types of pollen provisions. Feeding larvae with sterile pollen + *A. micheneri* led to the highest mortality rate, followed by natural pollen + *A. micheneri*, and sterile pollen. Larval development was significantly delayed in groups fed with sterile pollen. Interestingly, larval and prepupal weights did not significantly differ across treatments compared to natural pollen-fed larvae. 16S rRNA gene sequencing found a dominance of *Sodalis*, when *A. micheneri* was introduced to natural pollen. The presence of *Sodalis* with abundant *A. micheneri* suggests potential crosstalk between both, shaping bee nutrition and health. Hence, this study highlights that the reliance on nonhost-specific environmental bacteria may not impact fitness of *M. rotundata*.

IMPORTANCE This study investigates the impact of environmentally acquired gut microbes of solitary bee fitness with insights into the microbial ecology of bee and their health. While the symbiotic microbiome is well-studied in social bees, the role of environmental acquired microbiota in solitary bees remains unclear. Assessing this relationship in a solitary pollinator, the leaf-cutting bee, *Megachile rotundata*, we discovered that this bee species does not depend on the diverse environmental bacteria found in pollen for either its larval growth or survival. Surprisingly, high concentrations of the most abundant pollen bacteria, *Apilactobacillus micheneri* did not consistently benefit bee fitness, but caused larval mortality. Our findings also suggest an interaction between *Apilactobacillus* and the *Sodalis* and perhaps their role in bee nutrition. Hence, this study provides significant insights that contribute to understanding the fitness, conservation, and pollination ecology of other solitary bee species in the future.

KEYWORDS solitary bee, *Megachile rotundata*, *Apilactobacillus micheneri*, *Sodalis*, bee health and nutrition, bee microbiome, bee growth, bee survival

The insect-bacteria relationships extend from tight-knit symbiotic mutualism to commensal or parasitic interactions. Bacteria can be present inside the gut or live in specialized structures within insects, enhancing insect fitness by providing essential

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nutrients (1–3). Insects that depend on plants for food, such as bees, can benefit from microbes that digest plant tissues, facilitating the insect's access to carbohydrates, proteins, lipids, water-soluble vitamins, inorganic elements, and minerals (4, 5). Social bees serve as a model for symbiotically associated microbiome studies (6), but many microbial findings from these studies may not always be relevant to solitary bee species. In social bees, the gut microbiota is transmitted via social interactions and is beneficial to the host's health (7). Unlike social bees, solitary species lack the transmission routes to acquire gut symbionts from nest mates. Bacteria present in plants are transferred to the guts of larval and adult solitary bees through pollen consumption, but these bacteria are not necessarily mutualistic and can be neutral or detrimental to fitness (8). Considering this functional disparity between social transmission and environmental transmission of bacteria in bees, the ecological relationship between pollen-associated microbes and the fitness of solitary bees should be investigated.

The gut microbiota of social bees is species-specific, providing the host with various health benefits. Honey bee workers are predominantly inhabited by five core, highly conserved, host-specific microbes that make up 95% of the total gut microbiome. The hindgut of every adult worker across the globe contains *Snodgrassella alvi*, *Gilliamella apicola*, two species of *Lactobacillus (Lactobacillus Firm-5 and Lactobacillus Firm-4.*), and *Bifidobacterium* species (9). A few environmental bacteria that are present in pollen and hive surfaces also colonize the foregut and midgut but are not stably associated with the host (10). The gut microbiome plays a crucial role in promoting weight gain, hormone signaling (11), and immune system function (12, 13). Additionally, honey bee gut bacteria produce metabolites that promote host growth and physiology, facilitate the breakdown of toxic dietary compounds, and modulate immune functions in the gut (7). Moreover, *G. apicola* which forms a continuous lining layer over the ileum with *S. alvi*, potentially contributes to an increase in the weight of honey bee workers by enabling bees to break down pollen using genes coding for pectate lyase (11) and hydrolases (14). In larvae, bioassay studies have shown that several bacteria, including *Lactobacillus* and *Bifidobacterium*, can hamper the pathogens responsible for American and European foulbrood diseases (15, 16). The detailed functions and roles of the gut microbiota in social bees raise the question of whether gut-associated microbiota in solitary bees is of comparable significance and perform similar functions.

Solitary bee gut microbiota comprises highly diverse, fluctuating, and nonhost-specific bacterial communities that are acquired from the pollen of multiple plant species (17). The hypothesis that the environment is the main source of bacterial transmission is supported by the presence of the same bacteria on flowers and in association with multiple wild bee species (18, 19), correlations between pollen sources and specific bee-associated bacteria (20, 21), and correlations between the bacterial communities present in pollen provisions and the guts of solitary bee larvae and adults (8, 22). Based on maternal foraging preference and geographical location, the pollen provisions have a diverse and environmentally acquired microbial community from multiple bacterial families, including *Acetobacteraceae*, *Bacillaceae*, *Clostridiaceae*, *Enterobacteriaceae*, *Lactobacillaceae*, *Methylobacteriaceae*, *Moraxellaceae*, and *Sphingomonadaceae* (23). The solitary bee's gut also harbors typically endosymbiotic bacteria like *Sodalis*, *Wolbachia*, *Arsenophonus*, *Cardinium*, and *Rickettsia* (24–26). These bacteria colonize the larval gut, but are lost during metamorphosis. The gut microbiome is regained when the emerged adults start foraging for pollen (27). The pollen-borne microbiome, like yeast, has the capability to ferment the pollen mass, thereby providing insects with several nutritional benefits. These include the degradation and fermentation of complex carbohydrates, assistance in digestion, and the synthesis of essential nutrients (28–30). Moreover, based on genomic data, acidophilic bacteria present within solitary bee pollen have been suggested to safeguard against the proliferation of mold growing inside the nests (31). Other environmentally dependent bacterial strains extracted from nests of solitary bees have suggested potent bioactivity against disease-causing fungi and bacteria (32). Thus,

the entire pollen-borne microbiome may be important for growth, development, and survival in solitary bees.

Apilactobacillus species dominate the brood provisions of most solitary bee species (27). Comparative genomic studies have shown that the *Apilactobacillus micheneri* has a pectate lyase gene that may play a crucial role in helping the larva digest pollen (33), similar to the role of *Gilliamella* sp. in honey bee workers (11). Additionally, *A. micheneri* can thrive in acidic environments, potentially inhibiting opportunistic pathogens (33). However, these findings are limited to genomic data and *A. micheneri* may have detrimental effects on its host because *Lactobacilli sensu lato* have a tendency to produce harmful metabolites like histamines and tyramines (34). The direct effect of the microbiome on the biology of solitary bees must be investigated through microbial bioassays, empirically testing these genomic findings.

In this study, we conducted fitness bioassays and metabarcoding of microbial communities in *Megachile rotundata* larvae. We reared larvae on pollen provisions containing environmentally collected microbial communities (Natural pollen) and pollen provisions lacking natural microbial communities (Sterile pollen). To study the role of the *Lactobacillus* clade in bee nutrition, *A. micheneri* was added to the natural pollen and sterile pollen. As reported in a prior study, wherein *A. micheneri* was determined to be the most abundant bacterial species in larval gut of *M. rotundata*, a control group was established through the use of pollen provisions treated with a mixture of antibiotics (35). We used 16S rRNA gene amplicon sequencing to identify the bacterial communities present inside *M. rotundata* larvae from each pollen treatment to correlate the phenotype outcomes with the absence, presence, and changes in abundance of bacterial genera. We hypothesized that, (i) the absence of gut microbiota will deteriorate the overall health and survival and (ii) adding *A. micheneri* to the larval provisions will have synergistic effects on the physiology of *M.rotundata*. While we found evidence that the natural gut microbiome was beneficial in some respects, *A. micheneri* appeared to be not beneficial in all contexts and was pathogenic when present in abundance.

MATERIALS AND METHODS

Animal collection

Alfalfa leafcutter bee nesting boxes were set up in a single location in the university alfalfa field plots near North Dakota State University (Cass County, Fargo, ND) where adult females foraged and collected pollen and nectar from surrounding vegetation. Each nest box was constructed with a commercial Styrofoam nesting block placed inside a wooden box (Northstar Seed, Canada), providing a total of 360 nesting cavities. Mothers laid eggs on the top of pollen provision inside a cavity (paper straws) provided in nesting boxes. Straws having freshly laid eggs and pollen inside brood cells were collected on a daily basis. Straws were cut open using sterilized razor blades and eggs were sexed based on the position of the brood cell inside the straws, as *M. rotundata* assign the front cell positions to male offspring (36). To avoid sex-specific differences in growth rate and development, we used males by only collecting eggs from the first two cells. Eggs were collected from pollen provisions using size zero paint brush (#1 Camel Hair Bristle, Wooster) and were distributed randomly across the treatments.

Pollen treatments

M. rotundata provisions are a mixture of pollen and nectar made of 33%–36% pollen, and 64%–67% nectar by weight, but the majority of the mass is pollen containing 1.3 million pollen grains and is 47% sugar by weight (37). Fresh larval provisions were collected in the first 14 days of July 2021 and pooled to avoid floral and microbial variation. Field-collected provisions were divided in half: one half was set aside (natural pollen:control) keeping the microbial community intact, and the other half was sterilized using gamma-irradiation (28 kGy for 12 h) at an off-site facility (VPT Rad – Radiation Lab

& Test Services, Chelmsford, MA). However, it is important to note that this process may have some bacterial DNA fragments, as indicated in the results. The presence of bacterial genome does not make the sample unsterile. The sterility of pollen was confirmed by plating on different agar plates [Lactobacillus MRS (de Man, Rogosa, and Sharpe agar) agar, trypticase soy agar + 5% defibrinated sheep blood, and LB agar] and observing for growth after 3 days (Fig. S1). Treatments were made using natural pollen (N) and sterilized pollen (S) placed in 96-well plates (150 mg of pollen/cell) (Fig. 1; Table 1). Pure culture of *A. micheneri* was purchased from McFrederick lab at the University of California, Riverside and was grown overnight in MRS broth with 2% fructose at 25°C. Our hypothesis was that *A. micheneri* was a beneficial microbe, so we increased the load to determine whether there would be improved growth and survival. The addition of *A. micheneri* was done on pollen provisions having an intact microbiome (NAm) and was also added to sterile provision to test whether *A. micherenri* would be beneficial when acting alone (SAm). Approximately 50,000 cells of *A. micheneri* were added to 150 mg of pollen in each cell of a 96-well plate in two doses. The number of bacterial cells was counted using a hemocytometer under a microscope (Agilent BioTek). The first dose was given before eggs were placed on the provision, and the second dose was 10 days afterward. The same number of bacterial cells were embedded in 150 mg of sterile pollen (SAm) to know the effect of single bacterial species on the growth and development of *M. rotundata*. An antibiotic cocktail (AC) treatment was made according to McFrederick (2014) consisting of 3 µg/µL each of rifampicin, tetracycline, ampicillin, chloramphenicol, and erythromycin that was fed 3 µL every other day for 8 days (35). McFrederick (2014) showed that *A. micheneri* was the most abundant bacterial species in *M. rotundata* gut, and that it was resistant to a combination of antibiotics. This antibiotic cocktail from the previous study served as another treatment group to determine if we could obtain reproducible results. All treatments consisted of three replicates of 96-well plates, with the treatments mixed into the pollen provisions before feeding. The sample sizes for

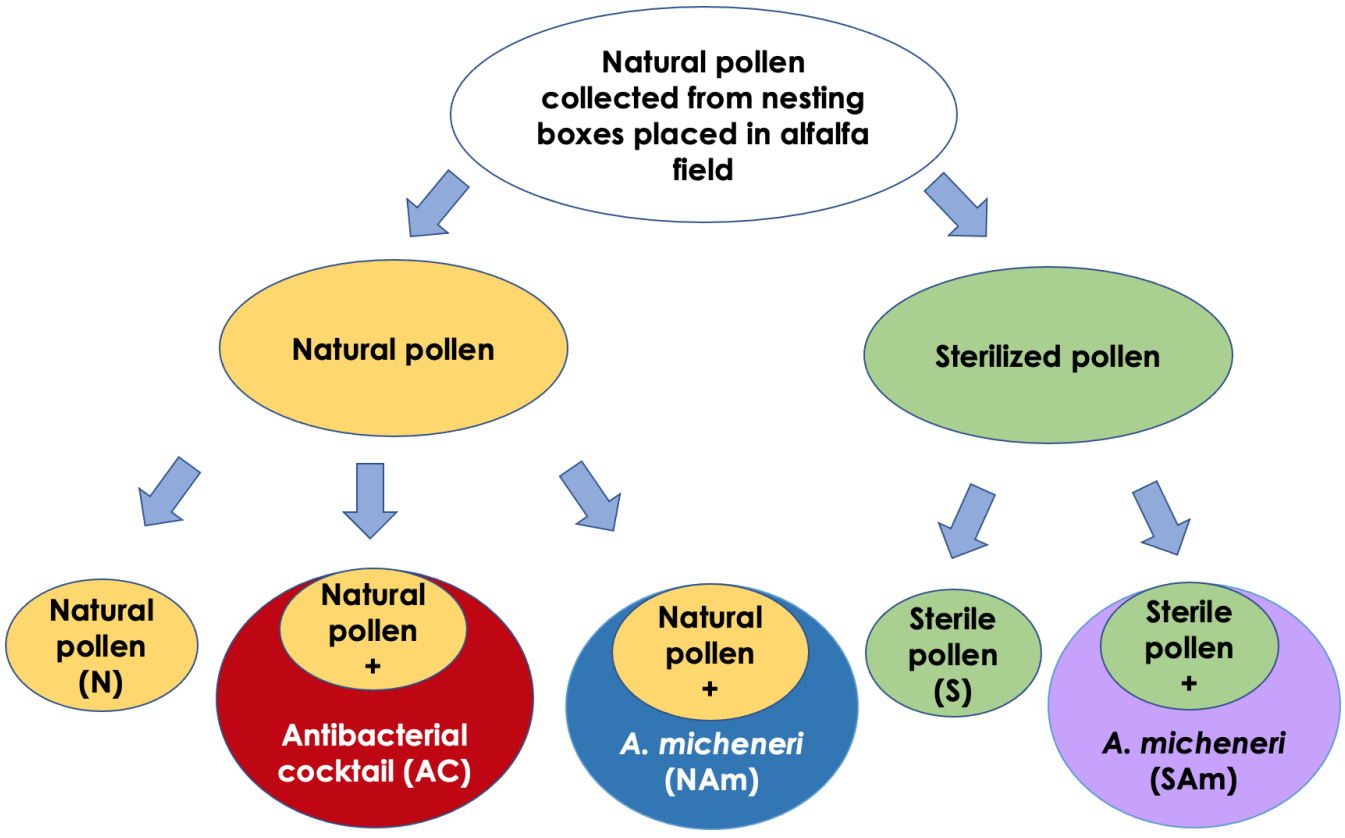

FIG 1 Illustration of different treatments used to feed *Megachile rotundata* larvae for growth and survival study.

TABLE 1 Pollen treatments fed to *Megachile rotundata* larvae

Treatment	Description	Dose
Natural pollen (N)	Pollen having naturally occurring intact microbiome	3 µL of PBS
Natural pollen + *A. micheneri* (NAm)	*Apilactobacillus micheneri* added to natural pollen	~50,000 cells in PBS fed twice (1st day and 10th day)
Sterile pollen (S)	Sterilized pollen using gamma-irradiation (28 kGy for 12 h)	3 µL of PBS
Sterile pollen + *A. micheneri* (SAm)	*Apilactobacillus micheneri* added to sterile pollen	~50,000 cells in PBS fed twice (1st day and 10th day)
Antibacterial cocktail (AC)	3 µg/µL each of rifampicin, tetracycline, ampicillin, chloramphenicol, and erythromycin (in PBS) added to natural pollen (35)	3 µL of antibacterial cocktail was fed every other day for 8 days

each treatment are illustrated in Fig. 2. Several factors contributed to the reduction in sample size, including mortality during the initial handling of eggs and significant mortality during the larval stages. We have collected and recorded mortality data for these stages, which is reflected in the reduced sample sizes presented in Fig. 2. Thus, the final sample sizes in Fig. 2 represent the individuals that survived both the initial handling and the larval mortality.

Freshly laid eggs were transferred from field-collected straws on top of the pollen placed in 96-well plates using a fine point round brush and were allowed to develop until they were fifth instar larvae. A different approach was used to place eggs on a sterile diet to maintain their sterility. Surface sterilization of eggs was done using two washes of contact lens wash solution (BioTrue) and three washes of sterilized water (38). To avoid sinking into pollen, eggs were placed on black autoclaved filter paper placed in sterilized petri-plate. When eggs started showing first instar emergence, they were transferred to sterile pollen inside 96-well plates. No surface sterilization was done to

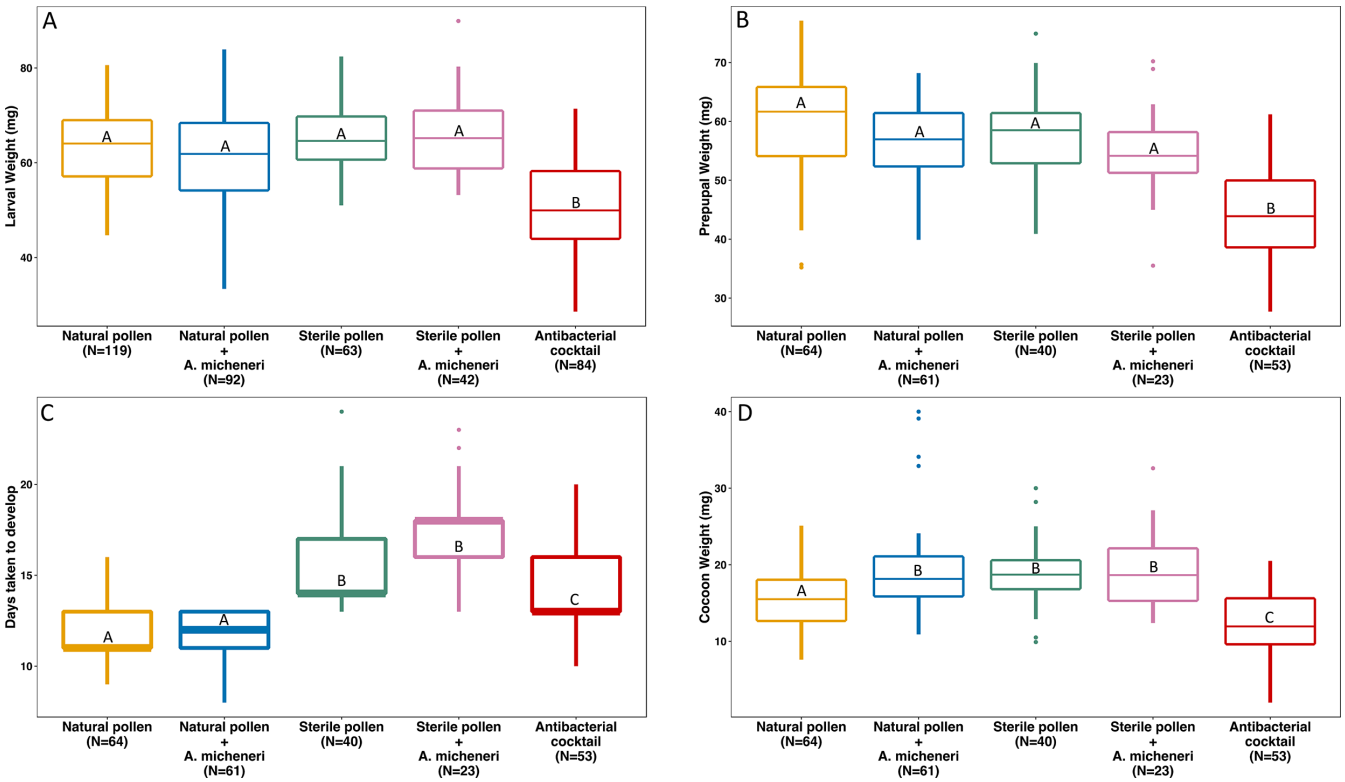

FIG 2 Larval weight (A), prepupal weight (B), days taken to develop from first instar to fifth instar (C), and cocoon weight (D) after feeding on pollen treatments. The central line of the boxplot represents the medians, and boxes comprise the 25th–75th percentiles and whiskers denote the range. Data were analyzed using Dunn's test (*P* values adjusted with the Bonferroni method). Different letters indicate significant differences between treatments.

eggs transferred to natural pollen. Data were collected for various parameters, including the number of days taken for development from egg to fifth instar larvae, the weights of fifth instar larvae and prepupa (recorded 4 days after cocoon spinning was completed), the weight of cocoons spun by fifth instar larvae, and the percent mortality of larvae. Larval weight was measured at the end of the feeding period and therefore is a measure of the maximum weight prior to pupation. We also measured prepupal weight, which is the weight of the bee after initiating metamorphosis and spinning the cocoon, but prior to molting into the pupal stage. All individuals in this study were reared at 25°C and 70% relative humidity. For comparing the effect of different treatments on larval weight, prepupal weight, cocoon weight, and days taken to develop from first instar to fifth instar, the non-parametric Kruskal-Wallis test and Dunn's test with BONFERRONI-type adjustment were used at $\alpha = 0.05$.

16S rRNA gene sequencing

Twenty-five instar larvae of *M. rotundata* from each treatment were snap-frozen for microbial analysis. The larvae were surface sterilized using 1% sodium hypochlorite followed by three washes using sterilized water in sterile conditions. Whole larvae were used for extraction of DNA using tissue collection plates (Qiagen, Germantown, MD), followed by bead beating the samples on a Qiagen Tissue Lyser for 6 min at 30 Hz for recalcitrant bacterial cell lysis. Samples for cell lysis were prepared by adding two 3 mm chromium steel beads and ~50 µL of 0.1 mm zirconia beads (Biospec, Bartlesville, OK) in 180 µL of Qiagen buffer ATL and 20 µL of proteinase K. A second round of bead beating was done by rotating plates for 6 min at 30 Hz, followed by incubation at 56°C for an hour. Qiagen DNeasy Blood and Tissue protocol was used for the rest of the DNA extraction process and three blank extractions were included as a no template control for further downstream analysis.

PCR amplification and Illumina Miseq analysis

16S rRNA gene libraries for paired-end reads were prepared using previously described protocol by references (20, 21).

We used the 16S rRNA gene primers (799F mod3, CMGGATTAGATACCCKGG and 1115R, AGGGTTGCGCTCGTTG) having unique barcode sequence (39) to amplify V5-V6 region of the 16S rRNA gene. To amplify this region, we performed PCRs using 4 µL of DNA, 10 µL of 2 × Pfusion High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), 10 µL of ultrapure water, and 0.5 µL of 10 µM 799F-mod3, 0.5 µL of 10 µM 1115R primers with an annealing temperature of 52°C for 25 cycles. To complete the Illumina adapter sequence, we first cleaned the PCR product with exonuclease and shrimp alkaline phosphatase to remove excess primers and deoxynucleoside triphosphates (dNTPs), respectively, then we used the cleaned PCR products as the template for a second PCR. We performed the second PCR with 1 µL of cleaned PCR product as a template with primers PCR2F (CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC) and PCR2R (CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGC) (39) under identical conditions to the initial PCR. About 18 µL of PCR product was normalized using SequalPrep Normalization plates (Thermo Fisher Scientific, Waltham, MA) and 5 µL of normalized product from each sample was pooled into a single sample. In order to perform Ultraclean sequencing, the pooled library was cleaned with AMPure XP beads (Beckman Coulter, Brea, CA) to remove primer-dimers and excess master mix components. Finally, library quality was assessed using 2100 Bioanalyzer (Agilent, Santa Clara, CA) and multiplexed libraries were sequenced using Miseq Reagent Kit with MiSeq sequencer (Illumina) with 2 × 300 cycles, at the IIGB Genomics Core, UC Riverside.

Bioinformatics

QIIME 2-2019 (40) was used to visualize and trim the low-quality ends of reads from raw 16S rRNA sequence libraries. DADA2 (41) was used to assign sequences to amplicon

sequence matches (ASVs; 16S rRNA gene sequences that are 100% matches) followed by removing chimeras and reads with more than two expected errors. Taxonomy was assigned to the ASVs using the sklearn classifier trained to the 799–1,115 region of the 16S rRNA gene with the SILVA database (42, 43). We also conducted local BLASTn searches against the NCBI 16S microbial database (accessed June 2022). Features were filtered out from the resulting ASV table that corresponded to contaminants as identified in our blanks and R package decontam (version 1.10.0) (44) at a conservative threshold at 0.5 (method = "prevalence") to identify contaminants along with removal of ASVs identified as chloroplast and mitochondria. Alpha rarefaction in QIIMME2 was used to normalize the number of sequences per library and 12,000 reads per sample were selected to retain samples and still capture the majority of the diversity. Alpha diversity was analyzed using the Pielou eveness index using the Kruskal-Wallis test in QIIME2. Additional analysis for Observed, Shannon, and Simpson indices was conducted. Beta diversity was tested using Adonis Bray—Curtis distance dissimilarities and nonmetric multidimensional scaling (NMDS) ordination in R v4.3.0 (45) with the package vegan (46). Betadisper function in the vegan package was used to check for differences in dispersion between treatment groups. Permutational multivariate analysis of variance (PERMANOVA) analysis based on rarefied Bray–Curtis matrices (pairwise BH-FDR correction) was performed in QIIME2 to determine the statistical significance of differences in bacterial communities between treatments.

RESULTS

Growth and survival

We predicted that sterile pollen would have a negative effect on larval and prepupal weight, and that the addition of *A. micheneri* would increase weight. Neither of these predictions were supported. We found an overall effect of treatments on larval weight (Kruskal-Wallis X^2 = 110.22, df = 4, P < 2.2e−16) where only antibiotic treatment had a significant (P < 0.05), negative effect on larval and prepupal weight in pairwise comparisons to natural pollen. There was no significant effect of treatment on larval weight when individuals were fed on natural pollen + *L. micheneri* (P = 0.22), sterile pollen (P = 0.60), and sterile pollen + *A. micheneri* (P = 0.62) as compared to natural pollen (control) fed individuals (Fig. 2A). Pairwise comparisons showed no significant effect of the addition of *A. micheneri* on prepupal weight as compared to control (Fig. 2B; natural pollen + *A. micheneri* (P = 0.60), sterile pollen (P = 1.0), sterile pollen + *A. micheneri* (P = 0.104). Prepupae developed on the antibiotic cocktail also weighed significantly less as compared to all other treatments and controls (P < 0.05). There was an overall effect of treatment on prepupal weight (Kruskal-Wallis X^2 = 81.765, df = 4, P < 2.2e−16), primarily due to individuals feeding on the antibiotic treatment weighing less than those in the control group. None of the other treatments were significantly different from control, indicating that *A. micheneri* and gut microbes in general do not influence the final weight gained at the end of the larval stage.

The composition of the microbiome influenced the duration of the larval stage (Kruskal-Wallis X^2 = 245.5, df = 4, P < 2.2e−16). Larval development (number of days until cocoon spinning started) was significantly delayed when individuals were fed on sterile pollen (P < 0.05), sterile pollen + *A. micheneri* (P < 0.05), and the antibiotic cocktail (P < 0.05) as compared to control. There was no significant difference in developmental days when larvae grew on natural pollen + *A. micheneri* compared to control (P = 1.0) (Fig. 2C). After cocoon spinning was over, weight of cocoon spun by larvae in different treatments was measured. Larvae spun significantly heavier cocoons when fed with sterile pollen (P = 0.001), sterile pollen + *A. micheneri* (P = 0.02), and natural pollen + *A. micheneri* (P < 0.05) as compared to control. Antibiotic cocktail-fed individuals spun significantly lighter cocoons as compared to all other treatments and control (P = 0.007) (Fig. 2D). A potential explanation is that the prolonged developmental time of larvae, in either of sterile pollen provided the larvae with more spinning duration, leading to significant differences in

cocoon weights compared to the antibiotic group, where larval developmental time was significantly shorter.

The difference in microbiome significantly affected the survival of the larvae (Kruskal-Wallis X^2 = 11.9009, df = 4, P = 0.02). Pairwise comparisons showed those individuals fed on sterile pollen + *A. micheneri* had significantly higher mortality than individuals fed on natural pollen (P = 0.0077, α = 0.05) or sterile pollen (P = 0.0060, α = 0.05). Antibacterial cocktail fed (P = 0.0313, α = 0.05) and natural pollen + *A. micheneri* (P = 0.0384, α = 0.05) fed individuals have significantly higher mortality than individuals fed on natural pollen. No other pairwise comparison for mortality showed a significant difference. The Schneider-Orelli formula (corrected % mortality ±SE) was used to calculate corrected percentage mortality (47). Sterile pollen + *A. micheneri* pollen feeding caused the highest mortality (36.98 ± 7.37%), followed by the antibiotic cocktail (14.45 ± 4.3%), natural pollen + *A. micheneri* fed (6.30 ± 1.6%), sterile pollen fed (3.75 ± 1.17%), and natural pollen fed (0.82 ± 1.04%) (Table 2). These results suggest that the high *A. micheneri* isolate loads inoculated in the pollen provisions seemed to have negative effect on larval survival.

Genetic analysis

Bacterial taxonomic profile and relative abundance

Following demultiplexing using QIIME, we obtained a total of 9,669,732 paired-end 16S rRNA gene amplicon reads with an average of 94,801 reads per sample and an average quality of 38 (minimum quality score = 25). Across the 102 samples, the lowest number of reads was 32,441 and the greatest was 186,025 (Data available; fastq_read_data.csv). The taxonomic profile shows that samples were dominated by the members of *Firmicutes*, *Proteobacteria*, and *Actinobacteriota* at the phylum level (Data available; level 7_taxonomy.csv).

Bees fed on natural pollen (N) had microbiota dominated by *Lachnospiraceae* family (Clostridia), *Sodalis*, and *A. micheneri*, with highly variable abundances between individuals. We predicted that bees fed on Natural pollen + *A. micheneri* (NAm) would be dominated by the *A. micheneri* because this bacterial species was fed to them in abundance. Surprisingly, these bees were dominated by the *Sodalis*, although *A. micheneri* was found in all samples. In the treatment where *A. micheneri* was added into sterile pollen (SAm) we detected *A. micheneri,* but the samples were dominated by a diversity of bacterial species that represent <2.5% of the relative abundance of the total microbiome (Fig. 3). Overall, an average reads per sample of *A. micheneri* across treatments were N—14,409 (SE ± 4,253. 75), NAm—13,587 (SE ± 3398.26), S—276 (SE ±86.84), SAm—3,595 (SE ± 1173.67), and AC—38,853 (SE ± 6,002. 89) . We observed that larva fed on sterile pollen still had 89,932 reads on average, which indicates that individuals had fragments of bacterial DNA. Although the most prevalent bacteria, found in other treatments, decreased in sterile pollen, species with less than 2.5% relative abundance were still detected. Prior to transferring eggs to the pollen, we tested that sterilized pollen was still sterile by plating on multiple agars. We observed no growth of any microbe after 3 days (Fig. S1). Therefore, bacterial present in these samples likely came from other environmental sources besides the pollen. A similar trend was seen in

TABLE 2 Mortality rates after feeding larvae on pollen treatments using Schneider-Orelli's formula[a]

Treatment	Corrected % mortality
Natural pollen (N)	0.82
Natural pollen + *A. micheneri* (NAm)	6.30
Sterile pollen (S)	3.75
Sterile pollen + *A. micheneri* (SAm)	36.98
Antibacterial cocktail (AC)	14.45

[a]Significantly higher mortality was observed when individuals were fed with sterile pollen with additional *Apilactobacillus micheneri*, compared to natural pollen (P = 0.0077) or sterile pollen (P = 0.0060).

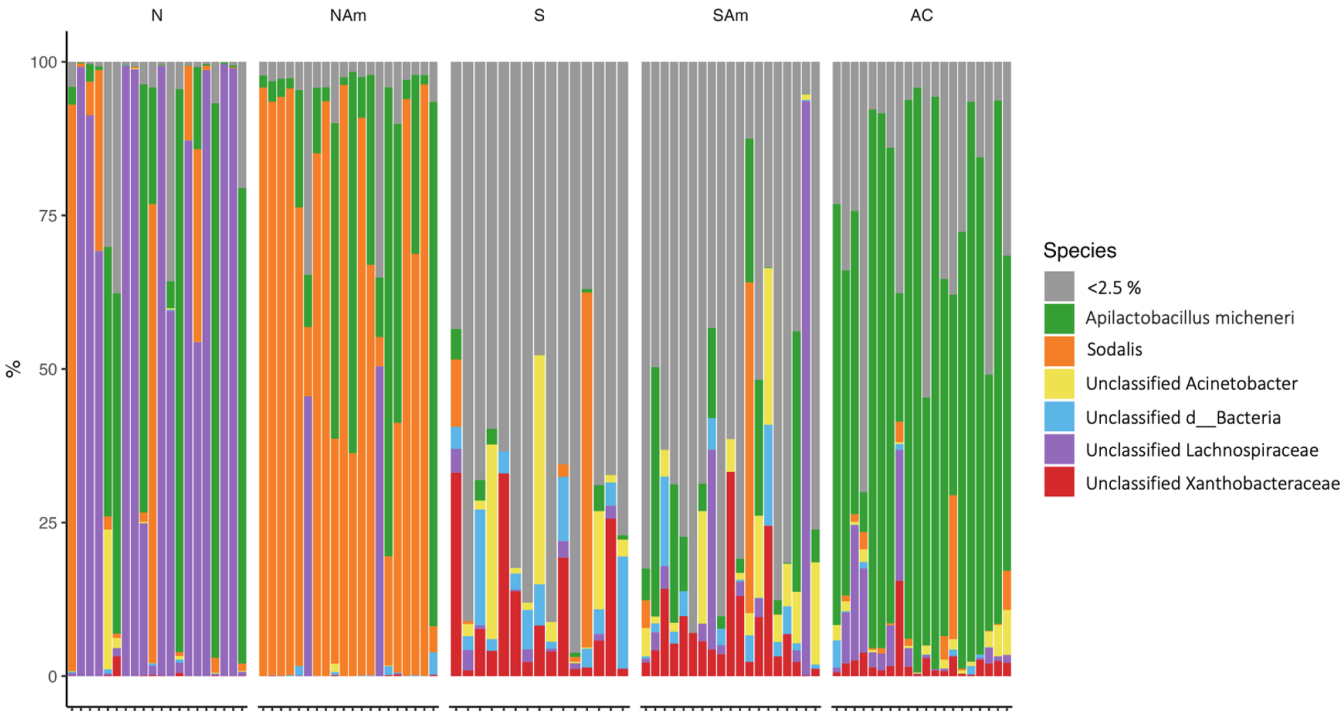

FIG 3 Relative abundances of the top six bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen. Treatments were: natural pollen (N), natural pollen with added *A. micheneri* (NAm), Sterile pollen (S), sterile pollen with *A. micheneri* (SAm), and natural pollen with an antibacterial cocktail (AC). Less than 2.5% is a category of low abundance species that made up less than 2.5% of the median number of reads. Each column represents an individual bee. The relative abundance, represented in percentages, is shown on the y-axis.

mason bees, where sterile pollen fed to larvae of *O. bicornis* showed low count bacterial reads in the bees (48). *A. micheneri* was dominant in larvae fed on pollen treated with antibiotics (AC), reproducing results found by McFrederick et al. (35), which showed selection for a higher relative abundance of this bacterial species.

Diversity analysis

Alpha diversity of bacteria, as calculated by Pielou evenness, was significantly different between Natural pollen +*A. micheneri* (NAm) compared to control and all other treatments. The Pielou evenness was higher in the sterile pollen +*A. micheneri* (SAm) and sterile pollen (S) treatment followed by subsequent decline in evenness in antibiotic cocktail (AC) treated pollen and natural pollen (N). There were no significant differences obtained in the values of diversity between Natural pollen (N) and antibiotic cocktail treatment (AC) and sterile pollen (S) and sterile + *A. micheneri* (SAm) treatment (Kruskal–Wallis test at $P > 0.05$) (Fig. 4). Additional analysis for Observed, Shannon, and Simpson indices was conducted, revealing similar statistical patterns as observed in Pielou evenness (Fig. S2). Bray Curtis dissimilarities showed that beta diversity of bacteria differed by the treatments (Bray Curtis bacteria, adonis $F = 3.66$, $R^2 = 0.141$, df = 4, $P = 0.001$). Beta dispersion analysis revealed significant clustering of bacterial communities in antibacterial cocktail treatment (AC) and natural pollen + *A. micheneri* (NAm) (Fig. 5, adonis $F = 9.58$, df = 4, $P = 0.001$ and betadisper $F = 14.69$, df = 4, $P < 0.001$). Pairwise comparisons using PERMANOVA show the significant difference between pollen with antibiotics (AC), sterile pollen (S), and sterile pollen with added *A. micheneri* (SAm) as compared to Natural pollen (N) (Table S1). The introduction of an antibiotic cocktail to pollen (AC) resulted in a significant alteration of the bacterial community, with selecting *A. micheneri*. Furthermore, irrespective of the pollen sterility in NAm, the addition of *A. micheneri* did not induce a change in the composition of the larval bacterial community when compared to sterile pollen.

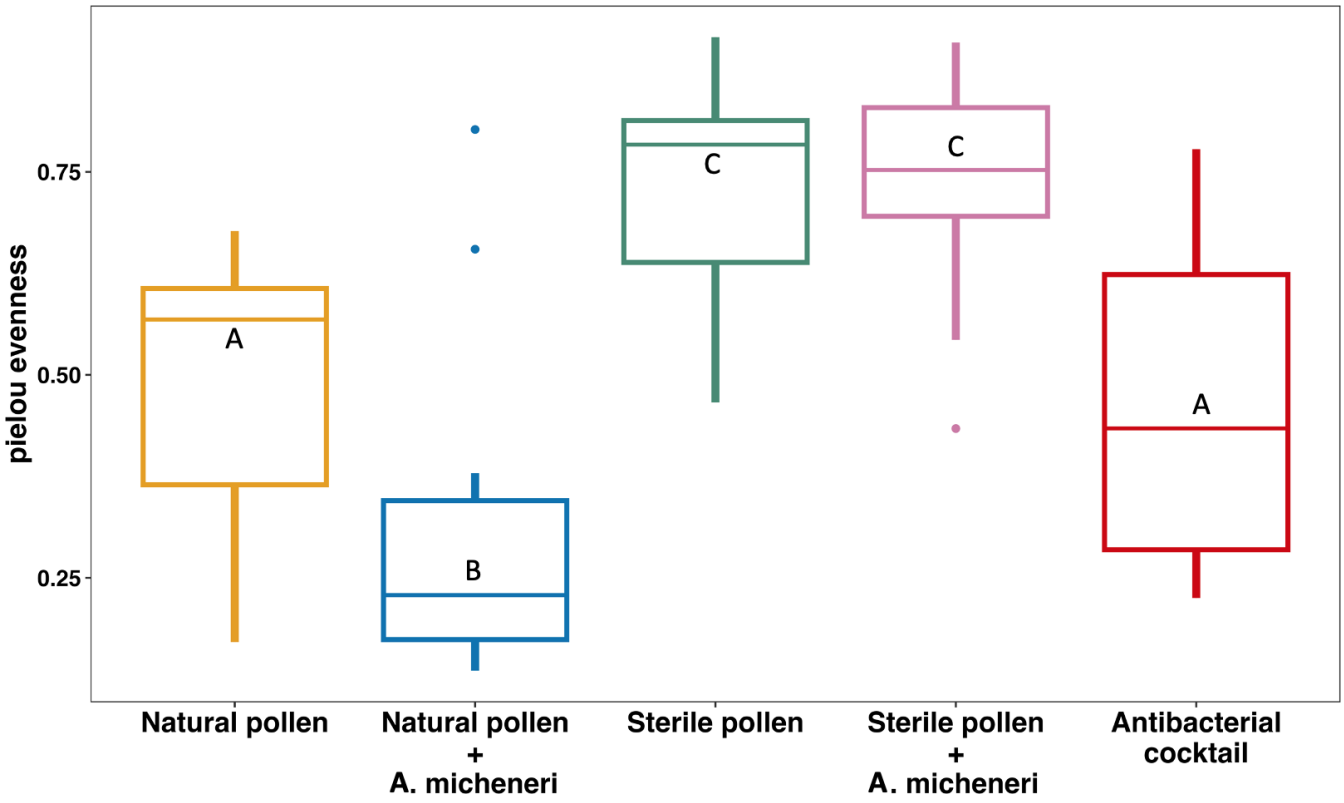

FIG 4 Pielou evenness indices using bacterial ASVs. Bacterial diversity is reduced in Natural pollen with added *A. micheneri* (NAm), as compared to Sterile pollen (S), Sterile pollen with added *A. micheneri* (SAm), and Natural pollen treatment (N; control). The central line of the boxplot represents the median, and boxes comprise the 25th–75th percentiles and whiskers denote the range. Different letters indicate significant differences detected between treatments using a pairwise Kruskal-Wallis test.

DISCUSSION

The goals of this study were to determine whether the absence of environmentally acquired microbiome negatively affects the larval growth and development of *M. rotundata*. Additionally, the study aimed to investigate whether providing the excess amount of *A. micheneri* enhances the fitness of *M. rotundata*. We found that developmental time was delayed when larvae ingested sterilized pollen provisions compared to the control group. However, there was no significant difference in body weight at the end of larval period across all the treatments except antibiotic-fed larvae. This suggests that *M. rotundata* larvae are able to attain full body weight in the absence of an intact pollen microbiome, possibly compensating by increasing developmental time. Similar trends have been observed in other invertebrates where axenic insects exhibit phenotypic differences and delayed development when compared to gnotobiotic groups (having intact microbial community). A delay in development has been observed in axenic individuals of *Aedes aegypti* (49), *Drosophila* (50), and *Caenorhabditis elegans* (51). This implies that eliminating the pollen microbiome primarily impacts developmental time, suggesting a role for the microbiome in facilitating growth, but not being required for weight gain.

Our results show that the pollen microbial community is not essential for weight gain in *M. rotundata* larvae. Larvae reared on Sterile pollen did not show any reduced effect on larval and prepupal weight compared to pollen with intact bacterial communities. Similar to our study, previous studies have shown that there was no significant difference in larval development or prepupal weight when *M. rotundata* individuals were reared on a sterile pollen using gamma irradiation as compared to the natural pollen having all the environmental bacteria (52). Moreover, another study showed bees weighed more

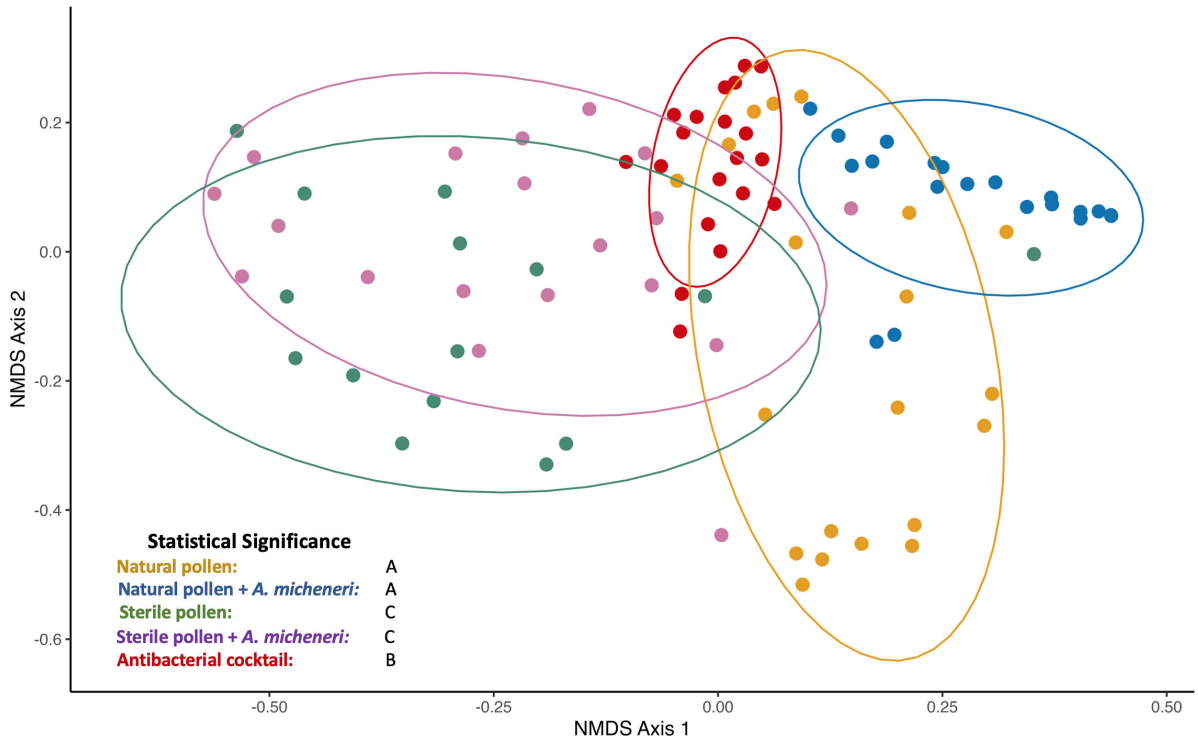

FIG 5 Nonmetric multidimensional scaling (NMDS) ordination of Bray—Curtis distance matrices representing bacteria from different treatments. Letters indicate significant differences detected between treatments. Overall, antibacterial cocktail treatment samples were the most significantly different from control and other treatments. Ellipses denote 95% confidence intervals.

when fed a sterile diet compared to pollen that had naturally occurring bacteria (53). Pollen sterilized using propylene oxide to get rid of all microbes had no differences in mortality of *M. rotundata* larvae when compared to pollen having natural microbiota (53). A previous study in *M. rotundata* demonstrated that larvae fed on pollen mixed with antibiotics had reduced weight and increased mortality (35). However, based on the antibiotic treatment in this study, that observed pattern may be due to direct toxicity of antibiotics or may be due to a dysfunctional microbiota. Our results both support (survival and development) and contrast (weight) a previous study conducted on the solitary bee *Osmia ribifloris* where natural pollen with intact microbial communities was mixed with sterile pollen and fed to larvae to determine the effect of the microbiome on host weight (53). *O. ribifloris* larvae fed an increasingly sterile diet exhibited a significant decrease in wet weight, growth rate, and survival. The study in *O. ribifloris* is the only report of a negative impact of sterile pollen on the weight of the host in solitary bees (54). While sterile pollen may not consistently influence bee weight, it remains essential to delve deeper into the microbial interactions for a holistic understanding of their effects on solitary bee biology and health.

A second goal of our study was to determine whether *A. micheneri* is beneficial to the development of *M. rotundata* larvae. Previous genomic studies have suggested that the presence of *A. micheneri* might be beneficial to its host. It could potentially optimize the absorption of nutrients in the gut, leading to improved growth and survival (33). To test the role of *A. micheneri*, we added it to both control pollen and sterile pollen, with the expectation that it would colonize the gut. However, neither treatment had an overabundance of *A. micheneri* in the gut, although *A. micheneri* was present in the gut of larvae fed on natural pollen. Our sequencing results reveal that when *A. micheneri* was added to sterilized pollen (SAm), instead of *A. micheneri* being dominant, we observed an increase in the presence of rare bacteria. This indicates that the larva fed on SAm was not exclusively consuming *A. micheneri*, but rather a combination of

rare bacteria and *A. micheneri*. Ingesting pollen having a mixture of rare taxonomical bacteria and *A. micheneri* (SAm) had more severe consequences on larval survival than ingesting sterilized pollen that was dominated by rare bacteria (S). Adding an excess of *A micheneri* to natural pollen did not have an effect on larval weight, development time, or survival. Whether *A. micheneri* is beneficial or detrimental to the bee host may therefore depend on context. For example, *A. micheneri* might not be directly detrimental to bees but may lead to a pollen microbiome that increases larval mortality in the right context. In a different context, *A. micheneri* may even be beneficial. This idea is supported by the mortality data from pollen provisions that received no treatment (N). *A. micheneri* was present in all samples and dominated eight of those samples in the natural pollen treatment, and the survival rate of those bees was 99%. *Apilactobacillus* clade bacteria was the most abundant bacteria in larval gut when fed on antibacterial cocktail, which is similar to what has been demonstrated previously. Additionally, the earlier study illustrated the resistance of the *Apilactobacillus* clade to a combination of rifampicin, tetracycline, ampicillin, chloramphenicol, and erythromycin (35). However, the poor performance of larvae on the antibacterial cocktail can be explained by either a negative effect of *A. micheneri*, or the toxic effects of the antibiotics themselves. Thus, the effects of an excessive load of *Apilactobacillus* bacteria on survival in solitary bees still remain unclear.

Interestingly, when *A. micheneri* was added to pollen with the natural microbiota (NAm), *Sodalis* dominated the bacterial communities in the larvae. Our taxonomical data shows that when *Sodalis* dominated the overall microbiome, *A. micheneri* was present in minimal quantities (Table S2). Similar patterns were observed in *Osmia aglaia*, *Lactobacillus* was absent when *Sodalis* dominated at 96% prevalence (55). In the halictid bees *Halictus ligatus* and *Lasioglossum pilosum*, *Sodalis* was absent when *Lactobacillus* dominated the microbiome at 94% and 9%, respectively (55). Similar trend was also seen in *Osmia excavate*, where dominance of *Sodalis* decreased the abundance of *A. micheneri* and vice-versa (56). In several Hymenopterans, *Sodalis* is maternally inherited and can potentially compromise reproductive compatibility (57). Symbiosis between halictid bees and *Sodalis* appears to be in its early life stages of evolution; *Sodalis* strains are vertically transmitted and found at higher prevalence in solitary versus social halictids (57). This suggests the prevalence of *Sodalis* differs among bee species. It remains unclear whether *Sodalis* resides within the lining of the host gut or is present inside cells. *Sodalis* is best studied in the tsetse fly *Glossina morsitans* and rice weevil *Sitophilus oryzae*, and it functions differently in these two insect species. In weevils, *Sodalis pierantonius* plays an important role in exoskeleton development (58) whereas no clear function has been documented for *S. glossinidius* in tsetse flies (59).

Our data raise the question of whether *Sodalis* plays a role in determining the total microbial composition and diversity in solitary bees and performs a specific function in bee biology. Abundance data of *Apilactobacillus* clade and *Sodalis* in this study and other bees indicates potential crosstalk between the gut microbiome and bee-associated potential symbionts (55, 57). Overall, this crosstalk may keep one or another bacterium in check, influencing symbiotic function that might provide solitary bee species with specific nutritional components that are important for their reproduction, development, and survival. When *A. micheneri* was introduced to sterilized pollen (SAm), neither *Sodalis* nor *A. micheneri* emerged as dominant members of the microbiome. This observation suggests that *A. micheneri* may require the presence of other bacteria or a complete microbial environment to sustain itself and engage in interactions with *Sodalis*. Hence, our work paves the way for future studies aimed at locating *Sodalis* via histological analysis and understanding the crosstalk of overall microbiome-*Sodalis*-host physiology interactions.

DNA metabarcoding using 16S provides relative abundance data, but not absolute abundances. This limits our ability to make specific conclusions regarding the role of *Sodalis* in determining the total microbial composition and diversity in *M. rotundata*. For example, *Sodalis* might be repressing *A. micheneri* in one treatment versus another,

but this is hard to definitively demonstrate without the measuring absolute abundance of both species across all treatments. Analyzing absolute abundance using qPCR or detecting bacterial species using shotgun sequencing metagenomics can identify potential crosstalk between the *Apilactobacillus* clade and *Sodalis*. Moreover, when *A. micheneri* was introduced to sterilized pollen (SAm), neither *Sodalis* nor *A. micheneri* emerged as dominant members of the microbiome. This raises the question whether *A. micheneri* requires the presence of other bacteria to sustain itself and engage in interactions with *Sodalis*. Future work should focus on these specific interactions to better understand the microbial dynamics that support their functions.

Conclusion

This study highlights that the dependency for the growth and survival on gut-associated bacteria is not a universal phenomenon across bee species. Environmentally acquired nonhost-specific bacteria might not shape solitary bee fitness in all host species. More phenotypic traits like adult reproduction, flight performance, and overwintering survival should be included to better understand the functions performed by gut microbiomes in solitary bees. Furthermore, this research opens new avenues for understanding interactions between gut microbiomes and typical endosymbionts. A future goal arising from our study would be to study the functional role of *Sodalis* bacteria in solitary bees using histological, immunological, and network analyses. This understanding will provide deeper insights into the significance of environmentally acquired microbiomes for the survival, growth, and development of solitary bees.

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AUTHOR CONTRIBUTIONS

Gagandeep Brar, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing | Madison Floden, Methodology, Writing – review and editing | Quinn McFrederick, Data curation, Funding acquisition, Supervision, Validation, Writing – review and editing | Arun Rajamohan, Methodology, Supervision, Validation, Visualization, Writing – review and editing | George Yocum, Supervision, Writing – review and editing | Julia Bowsher, Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Validation, Visualization, Writing – review and editing

DATA AVAILABILITY

Data are available in NCBI under BioProject accession PRJNA1138008 and in Dryad at https://doi.org/10.5061/dryad.0gb5mkm90.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figure S1 (AEM02076-23-S0001.docx). Confirmation of sterility after plating pollen on different agar (A) sterile pollen using gamma-irradiation (28 kGy for 12 h) and (B) natural pollen.

Figure S2 (AEM02076-23-S0002.docx). Observed, Shannon, and Simpson indices using bacterial ASVs.

Table S1 (AEM02076-23-S0003.xlsx). Pairwise permanova comparisons show the significant difference between pollen with antibiotics (AC), sterile pollen (S), and sterile pollen with added *A. micheneri* (SAm) as compared to Natural pollen (N).

Table S2 (AEM02076-23-S0004.csv). Relative abundances of the top six bacterial species present in *Megachile rotundata* larvae after feeding on treated pollen.

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