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RESEARCH

Pollen Diet Diversity does not Affect Gut Bacterial Communities or Melanization in a Social and Solitary Bee Species

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Abstract

Pollinators face many stressors, including reduced floral diversity. A low-diversity diet can impair organisms' ability to cope with additional stressors, such as pathogens, by altering the gut microbiome and/or immune function, but these effects are understudied for most pollinators. We investigated the impact of pollen diet diversity on two ecologically and economically important generalist pollinators, the social bumble bee (*Bombus impatiens*) and the solitary alfalfa leafcutter bee (*Megachile rotundata*). We experimentally tested the effect of one-, two-, or three-species pollen diets on gut bacterial communities in both species, and the melanization immune response in *B. impatiens*. Pollen diets included dandelion (*Taraxacum officinale*), staghorn sumac (*Rhus typhina*), and hawthorn (*Crataegus* sp.) alone, each pair-wise combination, or a mix of all three species. We fed bees their diet for 7 days and then dissected out guts and sequenced 16S rRNA gene amplicons to characterize gut bacterial communities. To assess melanization in *B. impatiens*, we inserted microfilament implants into the bee abdomen and measured melanin deposition on the implant. We found that pollen diet did not influence gut bacterial communities in *M. rotundata.* In *B. impatiens*, pollen diet composition, but not diversity, affected gut bacterial richness in older, but not newly-emerged bees. Pollen diet did not affect the melanization response in *B. impatiens*. Our results suggest that even a monofloral, low-quality pollen diet such as dandelion can support diverse gut bacterial communities in captive-reared adults of these bee species. These findings shed light on the effects of reduced diet diversity on bee health.

Keywords Pollinator · Immunity · Gut microbiome · Diet diversity · *Bombus impatiens* · *Megachile rotundata*

Introduction

Reduced diet diversity due to landscape simplification can negatively impact wildlife populations by limiting nutrient availability and increasing foraging time. Low-diversity diets lacking protein and other nutrients can impair animal performance, particularly by weakening strength and immune function (e.g., [1]). Such effects of diet are often mediated by the gut microbiome, which can modulate host immunity, digestion, and metabolism [2]. Diet can influence gut microbial communities ("microbiota" hereafter) by introducing foodborne microbes and/or nutrients that alter dynamics of the existing community [3]. Thus, diet can influence host health directly and through its effects on the gut microbiome.

Bees are critical pollinators, and many populations have shown recent declines [4]. Reduced floral diversity has been implicated in those declines [5], yet the mechanisms behind this trend remain poorly understood for most taxa. Studies on bee nutrition, immunity, and the gut microbiota have mostly focused on the social and commonly managed taxa, such as honey bees and bumble bees (reviewed in [6]). However, the vast majority of bee species (~80%), including many important wild bee pollinators, are solitary [7].

Previous studies have found varying effects of diet diversity on bee growth, development, and immune function.

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For example, bumble bees produced smaller and fewer offspring when fed a low-protein pollen (*Cistus* sp.) compared to those fed a mixed pollen diet [8]. In the field, *Andrena* bees in more diverse, natural landscapes were larger than those in homogenous agricultural landscapes [9] and *Osmia* nests with more diverse pollen provisions produced more female offspring [10]. Diet can also affect bee immunity. For example, bumble bees fed a sunflower pollen-only diet had different immune gene expression profiles compared to bees fed a polyfloral diet [11]. These studies demonstrate links between diet diversity, longevity, and pathogen resistance in a limited number of bee species, but the mechanistic links between diet diversity and bee health remain poorly understood.

Diet can influence the bee gut microbiota, but this has only been investigated in a few studies using social bees [12–14]; dietary effects on solitary bee gut microbes have not been assessed. Social behavior strongly influences routes of acquisition of gut microbes in bees and other animals. Thus, the gut microbiota in the highly social, corbiculate bee species is made up of a relatively small and consistent group of coevolved taxa [15] that contribute to individual digestion, growth, immune modulation, and detoxification (e.g., [16]). Solitary bees, however, typically host more diverse gut microbes. While some solitary bees host a core group of bacteria [17], which can overlap with social bees [18]), their microbes are primarily acquired from the environment rather than within-nest social contact [19]. The functions of solitary bee microbiota are also less understood, although microbes on pollen provisions are important for larval development [20]. Due to their different gut microbiota, bees that differ in sociality may respond differently to changes in diet breadth, such that studies on social bees may not translate to solitary bees.

We tested the effect of one-, two-, and three-species pollen diets on immunity and the gut microbiota of the social common eastern bumble bee, *Bombus impatiens*, and the gut microbiota of the solitary alfalfa leafcutter bee, *Megachile rotundata.* We used sterilized pollen to test solely the effects of pollen nutrient input on the existing microbial community, rather than evaluating pollen as a source of novel microbial diversity. We predicted that higher diet diversity would result in higher gut microbial diversity and a stronger immune response. Further, we predicted that the gut microbiota of *M. rotundata* would reflect changes in diet more than *B. impatiens* due to *B. impatiens'* coevolution with a core group of bacterial taxa. Lastly, we predicted that dandelion pollen would result in a microbiota rich in Lactobacillaceae, since these bacteria have been associated with Asteraceae pollen in bumble bee pollen baskets [21] and *Megachile* nests [22]. Understanding the mechanisms by which diet diversity affects both social and solitary wild bees is critical for supporting their populations, particularly in agricultural settings where crop monocultures are common.

Methods

Overview

We tested the effect of pollen diet diversity on gut bacterial communities in *Bombus impatiens* and *Megachile rotundata*, and on the melanization immune response in *B. impatiens* (Fig. 1). For all experiments, we placed bees in individual containers with assigned pollen diets (sterilized) and 30% sucrose solution (not sterilized). Sucrose was accessible to the bee through a cotton wick. We replaced sucrose and pollen every other day and measured consumption over a 48-hr period. After 7 d, we anesthetized bees on ice and froze them at -80 °C for later processing. We collected the right forewing and measured marginal cell length

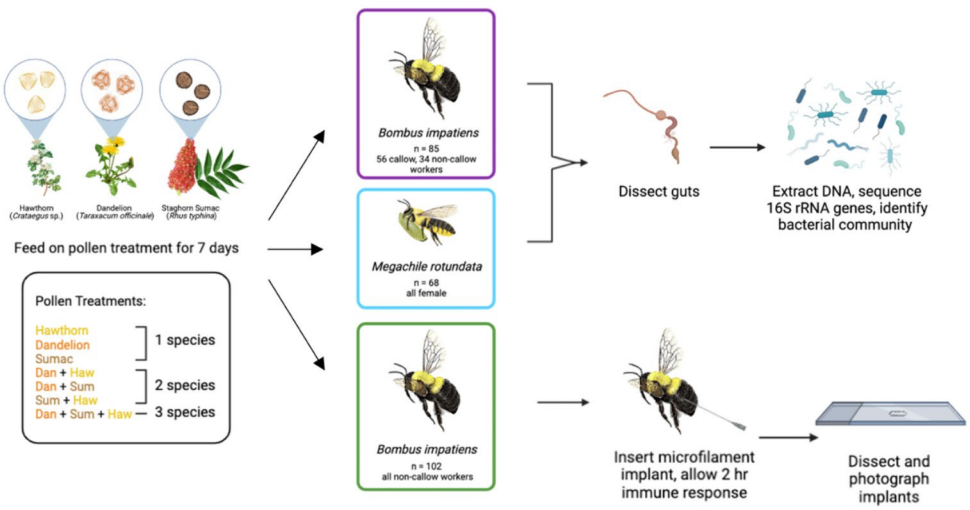

Fig. 1 Experimental design diagram. We tested the effect of pollen diet on the gut microbiome in *B. impatiens* and *M. rotundata* (top portion) and the effect of pollen diet on the melanization response in *B. impatiens* (bottom portion)

as a proxy for bee size. Additional details for all methods can be found in the supplement.

Pollen Diets

We used pollen from dandelion (*Taraxacum officinale*, Asteraceae), staghorn sumac (*Rhus typhina*, Anacardiaceae), and hawthorn (*Crataegus* sp., Rosaceae). Pollens were honey-bee collected from Quebec, Canada and confirmed to be > 95% one species by microscopic assessment of morphology (using fuchsin dyed glycerin jelly). We chose these species in part because they are abundant bee-collected resources in an area where *B. impatiens* is common, and in part because we could obtain a sufficient quantity from these species of single-species pollen for feeding trials. These pollens differ in nutritional content (see Supplement for references), and *Taraxacum* pollen may have chemical or mechanical defenses that reduce performance in *B. terrestris* [23]. Pollens were sterilized by ethylene oxide (USDA, Logan, Utah [24]; see Supplement for protocol), ground by mortar and pestle, and sorted into seven treatments: each species alone (three 1-species treatments), each pair-wise combination (three 2-species treatments), and one combination of all three species. We made multifloral treatments by combining the pollen species in equal parts by weight and then mixed each treatment with deionized water to make a paste (see Table S2 for pollen:water ratios). We measured pollen and sucrose consumption per bee in the first 48 h by weighing before and after delivery.

Bombus impatiens Gut Microbiota

We used *Bombus impatiens* (Apidae) workers derived from wild-caught queens collected in Amherst, Massachusetts in spring of 2021. *Bombus impatiens* are generalist foragers, and workers consume pollen in addition to foraging for pollen for the colony (personal observations). We reared queens in the laboratory until colonies were large enough to remove workers without affecting colony survival. Colonies were fed non-sterilized, honey bee-collected wildflower mix pollen (Koppert Biological Systems, Howell, Michigan). All queens were screened for *Crithidia* sp. infections via microscopy of feces, and only uninfected queens were used.

We used 90 workers from six colonies, of which 56 were newly emerged, also known as callows (identified by silver body hair, which turns black after about 1 day post-eclosion). We placed callows in individual vials for 2 h, and hand-inoculated them with 10–15 µL of pooled feces from five nestmates mixed with 50% sucrose. Similar inoculation protocols result in microbiomes indistinguishable from those of mature, in-colony workers [25, 26]. We initially only used callows to control for worker age and starting microbiome. However, callows were reluctant to consume fecal inoculum, and we switched to non-callow adults for the last 34 bumble bees. The non-callows were taken straight from their colony and did not receive fecal inoculum; we presume they were inoculated with nestmate feces prior to removal (bumble bees' gut microbiomes stabilize within 4 days of eclosion; [27]). We included age (callow/non-callow) in analyses. Bees were housed in 16-oz deli cups in darkness with access to 30% sucrose and their pollen diet. Bumble bees were placed in the experiment on ten start dates between August 11–26, 2021. Five bees died, resulting in 85 samples (Table S1).

Megachile rotundata Gut Microbiota

We used commercial *Megachile rotundata* (Megachilidae; masonbeesforsale.com, Deweyville, Utah). Bees arrived as pupae, which we kept at 7 °C until use. We then placed pupae in darkness inside a mesh cage at 27 °C until individuals emerged (based on methods from [28]. *Megachile rotundata* is a solitary bee that nests in cavities and lines brood cells with leaves. Females provision cells for a single egg, then seal the cell and provide no additional care to the developing larva. *Megachile rotundata* are highly valued and managed in North America for alfalfa pollination, but they are also generalist foragers [29].

To ensure that the leafcutter bees were exposed to bacterial cells for an initial microbiome, we created an ecologically-relevant microbial inoculum using deionized water sonicated with flowers collected from Amherst, Massachusetts on the first trial date and frozen at -80 °C in a glycerol solution. The flower species included *Solidago* sp. (Asteraceae), *Cosmos* sp. (Asteraceae), *Impatiens capensis* (Balsaminaceae), *Lobelia siphilitica.* (Campanulaceae), *Trifolium repens* (Fabaceae), *Pycnanthemum virginianum* (Lamiaceae), *Satureja hortensis* (Lamiaceae), and *Allium tuberosum* (Amaryllidaceae). We prepared inoculum for each trial by mixing 1:1 flower water and 50% sucrose (see Supplement for additional details). While we are unaware of previous studies that have used such a floral inoculum, the concept is similar to inoculating individual *Bombus* with feces from nestmates (as in [25, 26]); our goal here was to simulate exposure to a floral microbiome that *M. rotundata* might encounter from flowers. We placed the bees in their individual containers (60 mm diameter petri dish; with drilled holes) and pipetted 10 µL of the flower inoculum directly onto a cotton wick, which was placed in a 30% sucrose solution. We entered 74 bees in the experiment on September 14, 15 and 16, 2021. Four bees died and two samples could not be PCR amplified, resulting in 68 samples (Table S1).

Microbiome Processing and Analysis

Sample Processing and Sequencing

We stored each bee at -80 °C until gut dissection and DNA extraction. We dissected out the gut of each bee under sterile conditions and placed each (excluding the crop and rectum) into a tissue collection plate (Qiagen, Germantown, Maryland). We included four blank extractions as no-template controls in all downstream procedures and analyses. To characterize bacterial communities, we prepared amplicon libraries using the 799 F (CMGGATTAGATACCCKGG) and 1115R (AGGGTTGCGCTCGTTG) 16S rRNA gene primers (e.g., [30]). The Genomics Core at the University of California, Riverside checked DNA quality and concentration using the 2100 Bioanalyzer (Agilent, Santa Clara, California) and then sequenced the libraries in a single run on the MiSeq (Illumina, San Diego, California) using the V3 2×300 reagent kit.

Bioinformatics

We used QIIME2 to process the Illumina fastq files [31]. We removed the barcodes and concatenated them into a separate file to be compatible with QIIME2, and then demultiplexed the sequences. To trim low quality sequences and bin reads into amplicon sequence variants (ASVs), we ran DADA2 with default parameters and read trimming of 253 bases for forward reads and 211 bases for reverse reads [32]. We assigned taxonomy to genus level by using the QIIME2 sklearn classifier trained to the 799 to 1115 region of the SILVA 16S rRNA gene database [33, 34]. We additionally used the NCBI database to conduct local BLAST searches for ASVs that required further classification. We used R version 4.2.1 for decontamination and QIIME2 for final filtering, including the removal of singletons [35]. We identified contaminants using the "prevalence" method in the decontam package [36], with a conservative threshold of 0.5, which identifies ASVs that were more prevalent in negative controls than in samples. We then filtered out the 22 identified contaminants as well as mitochondria and chloroplast ASVs. We rarefied to 8000 reads per sample.

Statistics

To assess community diversity, we used the vegan [37] and phyloseq packages [38] in R [31]. We first ran models to assess community metrics in response to host bee species. After finding significant effects of host species (see Results), we analyzed effects of diet within each host. Models for each species included diet composition (7 levels) or diet diversity (3 levels; 1-, 2- or 3-species), but not both.

To investigate alpha diversity, we modeled the number of ASVs ("ASV richness" hereafter) and Shannon index by diet treatment (both composition and diversity in separate models). For *B. impatiens*, full models included age (callow/non-callow) and bee size (estimated by wing marginal cell length) as additional fixed effects and start date and colony as random effects. For *M. rotundata*, full models included bee size as an additional fixed effect, and start date and PCR plate ID as random effects. All final models (except those with host as a predictor) retained diet treatment as a fixed effect since it was our variable of interest. To analyze pollen consumption, we used a similar approach, using a linear model with a normal distribution (lme4 package) [39] and analyzed survival using the coxme function (coxme package [40]), survfit, and Surv functions (survival package [41]). For pollen consumption and survival models, we used pollen diet composition (not diversity) as the treatment predictor. We ran full linear mixed models (lme4 package) [42] and then used Akaike information criterion (AIC) for model selection [43]. We report results from the best-fitting models in the tables. We tested the significance of terms using likelihood ratio χ^2 tests. We validated models using the simulateResiduals function [44] and produced figures with emmeans [39] and ggplot [45].

To investigate beta diversity, we calculated Bray-Curtis distance matrices (results using unweighted UniFrac, and weighted UniFrac were generally similar and are reported in Supplement Tables S4, S5). We then analyzed three community metrics: (1) community dispersion among treatment groups using the betadisper function, (2) overall community composition between treatment groups using the adonis function, and (3) similarity percentages to identify taxa that contributed most to the dissimilarity between groups using the simper function. Betadisper models are limited to one predictor variable at a time, therefore we ran dispersion models for each predictor. For the *B. impatiens* adonis models, we included diet composition, age, and colony as predictors. We did not include start date as a block since it was confounded with age. For *M. rotundata*, we included diet as a predictor and start date as a block. We visualized beta diversity using Principal Coordinates Analysis (PCoA) ordinations.

Bombus impatiens Melanization

Experimental Design

We measured melanization of an implant in the bee abdomen, a commonly used method for estimating immune function in bumble bees (e.g., [46]). We used 135 *B. impatiens* workers from three commercial colonies (Koppert Biological Systems, Howell, Michigan), reared in the lab. We

Table 1 Statistics from final models predicting alpha diversity (Shannon index and ASV richness) and beta diversity (community dispersion and composition) in microbial communities based on host species. Beta diversity metrics are based on Bray-Curtis distance. Italics and bold indicate $P < 0.05$

Top model	F or χ^2	d.f.	*P* value
Shannon ~ Host	9.699	1	***0.002***
ASV richness ~ Host	32.212	1	***< 0.0001***
Community dispersion ~ Host	11.345	1	***0.001***
Community composition ~ Host	41.93	1	***< 0.0001***

placed non-callow workers into individual deli cups with their pollen diet (same as previous experiments) and 30% sucrose. On day 7, we anesthetized bees by placing them in the freezer for 3–5 min, punctured the pleural membrane between the 3rd and 4th abdominal tergite using a sterile needle, and then inserted a sterile, transparent 1-mm long microfilament (Sufix Elite) into the abdominal cavity using forceps. The bee then recovered in its container for 2 h in a dark room with access to sucrose and its pollen diet. After 2 h, bees were frozen at -20 °C. We later dissected abdomens to recover implants, mounted implants to microscope slides using clear nail polish, and photographed implants using a dissecting microscope at 20X magnification. Bees initiated the experiment on five dates between November 29-December 13, 2021. Twelve bees died, 7 escaped, 9 died after being anesthetized, and we could not find the implants in 5 bees, resulting in 102 samples (Table S1). We imported photographs of each implant to ImageJ [47] and estimated percent of the implant covered by melanin (see Supplement for further methods and discussion).

Statistics

We modeled proportion melanization with a generalized linear mixed model with a beta error distribution [48]. Initial models included diet treatments (composition or diversity) and bee size as fixed effects and start date and natal colony as random effects. We built separate models using diet composition or diversity as predictors and performed model selection using AIC.

Results

Gut Microbiomes in Both Host Species

After quality control and removing contaminants, we retained an average of 25,177 reads per sample across 153 samples. We identified 334 ASVs. Top models and results assessing effect of host species are in Table 1. The host species differed significantly in community composition (Fig. 2C). Compared to *M. rotundata*, *B. impatiens* had higher alpha diversity (Fig. 2A and B) and wider dispersion among replicates (Fig. 2C). The bacterial taxa that appear to drive dissimilarities between hosts, based on the simper function, were *Neokomagataea, Rosenbergiella, Pantoea*, and ASVs that matched to genera within the Enterobacteriaceae, which were prominent in *M. rotundata* guts, while *B. impatiens* harbored more *Candidatus Schmidhempelia* and *Snodgrassella*, which are common members of their core microbiota.

Bombus impatiens Gut Microbiome

Bumble bee guts contained previously-documented core members, including *Snodgrassella, Bifidobacterium, Lactobacillus*, and *Candidatus Schmidhempelia* (Fig. 3A). However, they were missing a commonly dominant member, *Gilliamella* [15]. Communities also included other genera previously associated with bees and flowers, *Acinetobacter, Asaia, Pantoea* [19], *Neokomagataea* [49]) and soil bacteria (ASVs in Enterobacteriaceae, multiple of which shared 100% sequence identity to *Citrobacter, Enterobacter*, and *Pantoea* species).

For *B. impatiens*, top models and results are reported in Table 2. We found a significant diet composition by age interaction on ASV richness (Fig. 2D), and subsequently assessed effect of diet within callows and non-callows using the joint_tests function. Diet composition affected ASV richness in non-callows, but not callows; non-callows fed the sumac + hawthorn diet had particularly low ASV richness, with an average of 16.2 ASVs (all pairwise comparisons were non-significant except dandelion; $z = -3.05$, $P = 0.035$). When diets were combined by levels of diversity, we found no significant effects of diet diversity, bee size, or the diet by age interaction on ASV richness (Fig. 2E). Diet (whether composition or diversity) did not affect Shannon diversity.

Beta diversity was significantly affected by colony, but not diet (composition or diversity), or age (Table S4, Figure S3). Community composition was affected by colony, but not diet composition (Fig. 3A), diversity, or age (Figure S3). One bacterial genus, *Bombella*, was only present in bees from one colony (detected in 11 out of 18 bees from this colony; seven of which were callows and four non-callows; Figure S4). We also note that *Bifidobacterium* was detected in 18 bees, however was only found at abundances > 1% in non-callows (Figure S4).

Table 2 Statistics from final best-fit models predicting alpha diversity (Shannon index and richness) and beta diversity (community dispersion and composition) in microbial communities in *Bombus impatiens*. Beta diversity metrics are based on Bray-Curtis distance. "Diet composition" refers to the seven diet treatments, while "diet diversity" refers to the number of pollen species (i.e., one, two, or three); each model included diet composition or diversity as a predictor but not both. Italics indicates $P < 0.1$ and bold indicates $P < 0.05$

Top model	Predictor	F or χ^2	d.f.	*P* value
Shannon ~ Diet composition	Diet composition	0.239	6	0.962
Shannon ~ Diet diversity	Diet diversity	0.529	2	0.591
ASV richness ~ Diet composition * Age + Wing size + (1|Colony) + (1|Date)	Diet composition	8.439	6	0.208
	Age	2.015	1	0.156
	Wing size	0.786	1	0.375
	Diet * Age	19.541	6	***0.003***
Post-hoc test by age for above model:				
Non-callow	Diet composition	2.835	6	***0.017***
Callow	Diet composition	1.365	6	0.243
ASV richness ~ Diet diversity * Age + Wing size + (1|Colony) + (1|Date)	Diet diversity	4.894	2	*0.087*
	Age	1.483	1	0.223
	Wing size	3.117	1	*0.077*
	Diet diversity * Age	5.926	2	*0.052*
Community dispersion ~ Diet composition	Diet composition	1.003	6	0.430
Community dispersion ~ Diet diversity	Diet diversity	1.824	2	0.168
Community dispersion ~ Age	Age	0.129	1	0.720
Community dispersion ~ Colony	Colony	2.714	5	***0.026***
Community composition ~ Diet comp + Age + Colony	Diet composition	1.073	6	0.363
	Age	1.785	1	*0.068*
	Colony	1.433	5	***0.050***
Community composition ~ Diet div + Age + Colony	Diet diversity	1.458	2	0.101
	Age	1.567	1	0.123
	Colony	1.497	5	***0.035***

Megachile rotundata Gut Microbiome

Leafcutter bees guts contained flower-associated bacteria, including *Neokomagataea, Rosenbergiella, Acinetobacter*, and *Asaia*, as well as soil-associates, including multiple genera in the Enterobacteriaceae (*Klebsiella*, *Kluyvera*, and ASVs that matched *Citrobacter, Enterobacter, Klebsiella*, and *Pantoea*; Fig. 3B; [19]). *Megachile rotundata* contained other genera at lower abundances that were also in bumble bee guts, including *Acinetobacter* and *Asaia* (Fig. 3B) as well as *Snodgrassella* and *Candidatus Schmidhempelia* (found at < 1% abundance and therefore not included in Fig. 3).

For *M. rotundata*, top models and results are reported in Table 3. Neither ASV richness nor Shannon diversity was affected by diet composition (Fig. 2F), diversity (Fig. 2G), or bee size (Table S5). Community dispersion and composition were not affected by diet composition, diversity, or start date (Fig. 3B, Figures S5, S6, Table S5).

Bombus impatiens Melanization

Most implant samples (93.2%) showed evidence of melanization. On average, bees melanized about 11% (±0.984 SE) of the implant surface area. The top models included diet (composition or diversity in separate models) as the predictor and start date and colony as random effects. Proportion melanization was not affected by diet composition ($\chi^2 = 2.693$, d.f. = 6, $P = 0.846$; Fig. 4) nor diversity ($\chi^2 = 1.219$, d.f. = 2, $P = 0.544$).

Discussion

We predicted that a low-diversity pollen diet would result in less diverse gut microbial communities due to lower nutritional diversity, but our results did not support this prediction. We found no obvious relationship between pollen diversity and gut bacterial diversity, suggesting that initial bacterial communities were relatively stable despite nutritional change. Even single-species diets still contributed adequate resources for the persistence of diverse communities over seven days. We used sterile pollen to rule out effects of foodborne microbes,

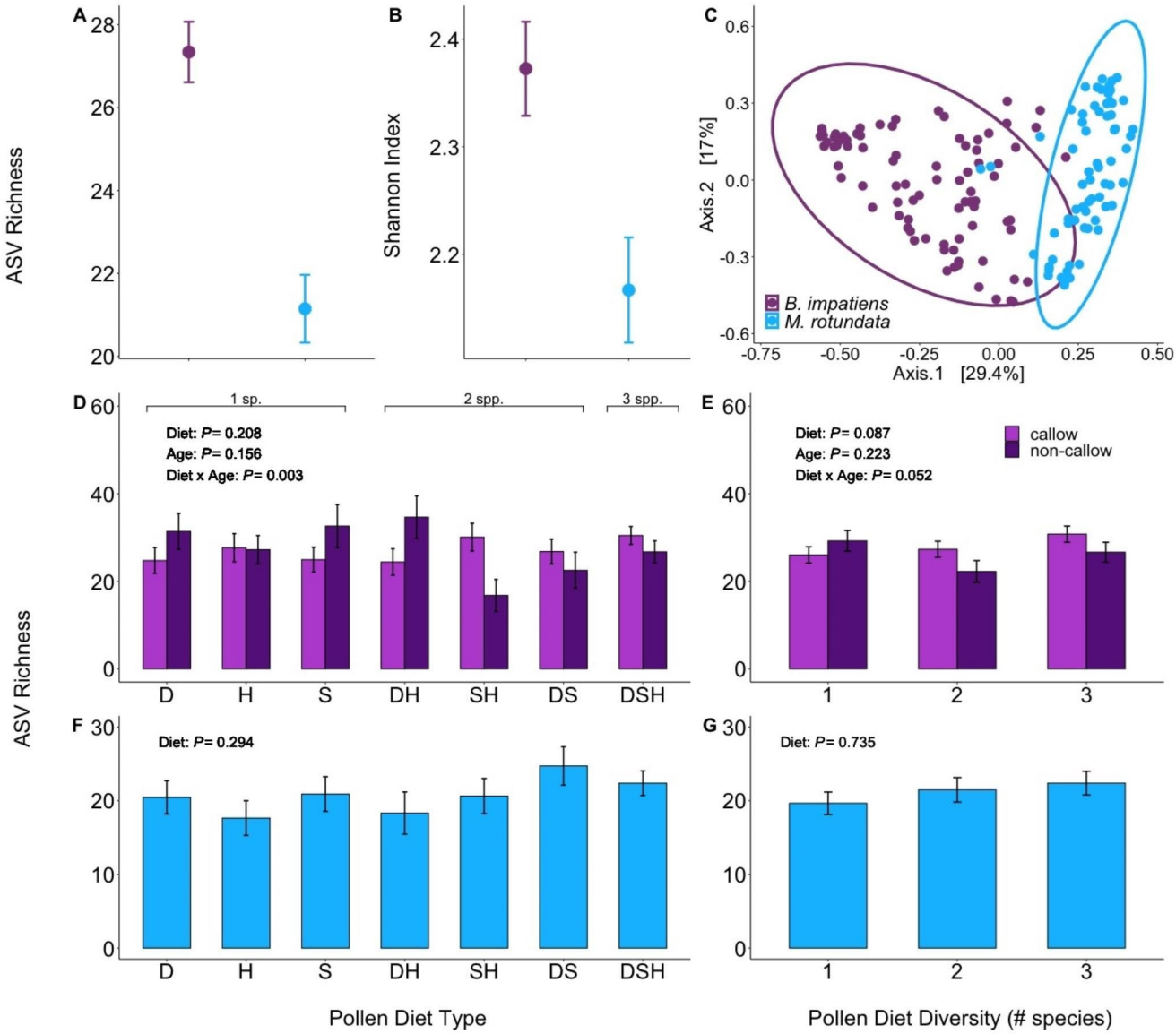

Fig. 2 Bee species differed in their gut bacterial communities. Compared to *M. rotundata*, *B. impatiens* had higher **(A)** ASV richness, **(B)** Shannon diversity, and **(C)** community variation, visualized as a PCoA ordination of Bray-Curtis dissimilarities. Ellipses are 95% confidence intervals. The effect of diet on bacterial ASV richness in *B. impatiens* depended on worker age **(D, E).** Diet did not significantly affect bacterial ASV richness in *M. rotundata***(F, G)**. (Diets are D = Dandelion, H = Hawthorn, S = Sumac.)

thereby allowing us to attribute any effects of diet on microbial communities to nutritional differences. We recognize this may not be reflective of nature, since pollen commonly harbors its own microbial community [50], but nonetheless sheds light on diet-microbiome dynamics. The stability of these communities suggests that priority effects and drift play important roles in long-term assemblages. Additionally, pollen diet may not affect gut microbiota if these microbes are more dependent on carbohydrates, such as pectin of the pollen wall [51] or nectar sugars, than the proteins and lipids inside pollen. Indeed, nectar qualities such as the concentration of different sugars can affect bumble bee gut bacterial composition [13]. While the relevance of these results may be limited for wild bees, our results suggest that simple, low-diversity diets of sterile pollen can still support established microbial communities.

Our results are consistent with previous studies that found little to no correlation between diets and host microbial diversity. For example, multiple studies found weak or no correlation between landscape, pollen and microbial diversity in pollen provisions of several bee species [22, 30, 52]. Conversely, our results contradict studies that found a positive relationship between diet and host microbial diversity, for example in antelope [53]. These contrasting patterns may be due to host taxa differing in levels of microbial filtering, which can also vary by ecological context [54]. While correlations between dietary and microbial diversity vary, short-term dietary changes can

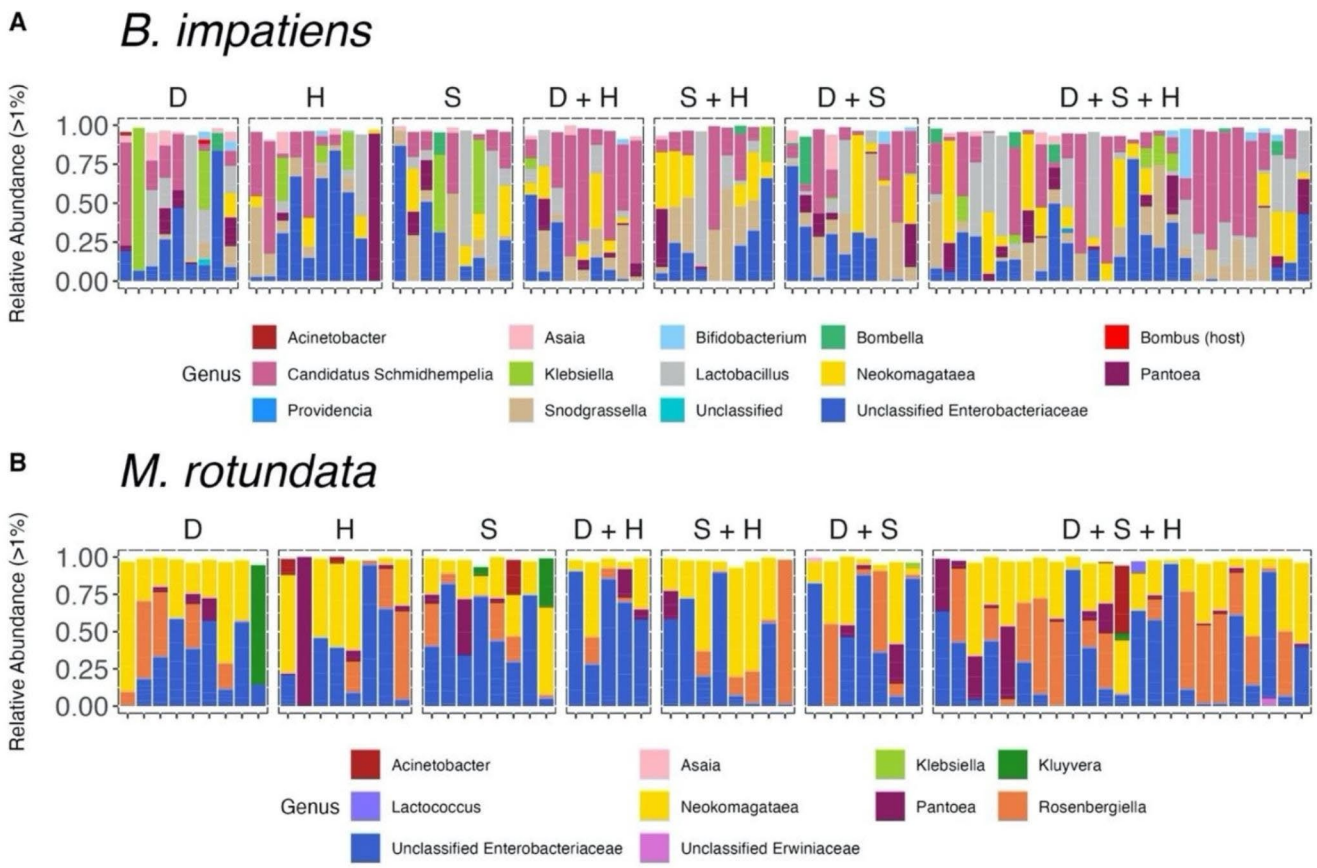

Fig. 3 Relative abundance of genera (with > 1% abundance) across the seven diet treatments in *B. impatiens***(A)** and *M. rotundata***(B)**. Plots are ordered by diet treatments (D = Dandelion, H = Hawthorn, S = Sumac). Each column represents one bee

Table 3 Statistics from final models predicting alpha diversity (Shannon index and richness) and beta diversity (community dispersion and composition) in microbial communities in *Megachile rotundata*. Beta diversity metrics are based on Bray-Curtis distance matrices. "Diet composition" refers to the seven diet treatments, while "diet diversity" refers to the number of pollen species (i.e., one, two, or three); each model included diet composition or diversity as a predictor but not both

Top model	Predictor	F or χ^2	d.f.	*P* value
Shannon ~ Diet composition	Diet composition	1.104	6	0.371
Shannon ~ Diet diversity	Diet diversity	0.953	2	0.391
Richness ~ Diet composition + Wing + (1|Plate ID)	Diet composition	7.294	6	0.294
	Wing size	0.0032	1	0.954
Richness ~ Diet diversity + Wing + (1|Plate ID)	Diet diversity	2.470	2	0.291
	Wing size	0.114	1	0.735
Community dispersion ~ Diet composition	Diet composition	0.612	6	0.720
Community dispersion ~ Diet diversity	Diet diversity	0.224	2	0.800
Community composition ~ Diet composition + (1|Start date)	Diet composition	0.751	6	0.826
Community composition ~ Diet diversity	Diet diversity	0.944	2	0.514

alter gut microbiome composition, demonstrating that certain diets are associated with certain bacterial taxa. For example, in humans, a shift to meat-eating increased clusters of bile-resistant bacteria [3]. In nests of *Ceratina* bees, *Acinetobacter, Lactobacillus, Pantoea*, and *Sodalis* bacteria were positively correlated with pollen from multiple plant genera [30]. *Lactobacillus* was also positively correlated with Asteraceae pollen in *Megachile* nests and *Bombus* corbicula [21, 22]. We thus predicted that dandelion diet would be relatively rich in *Lactobacillus*, but our results did not support this (Fig. 3, S4), suggesting that gut-inhabiting *Lactobacillus* does not preferentially feed on Asteraceae pollen. *Lactobacillus* may be associated with Asteraceae resources in the wild; we would not have observed this relationship due to sterilizing pollen.

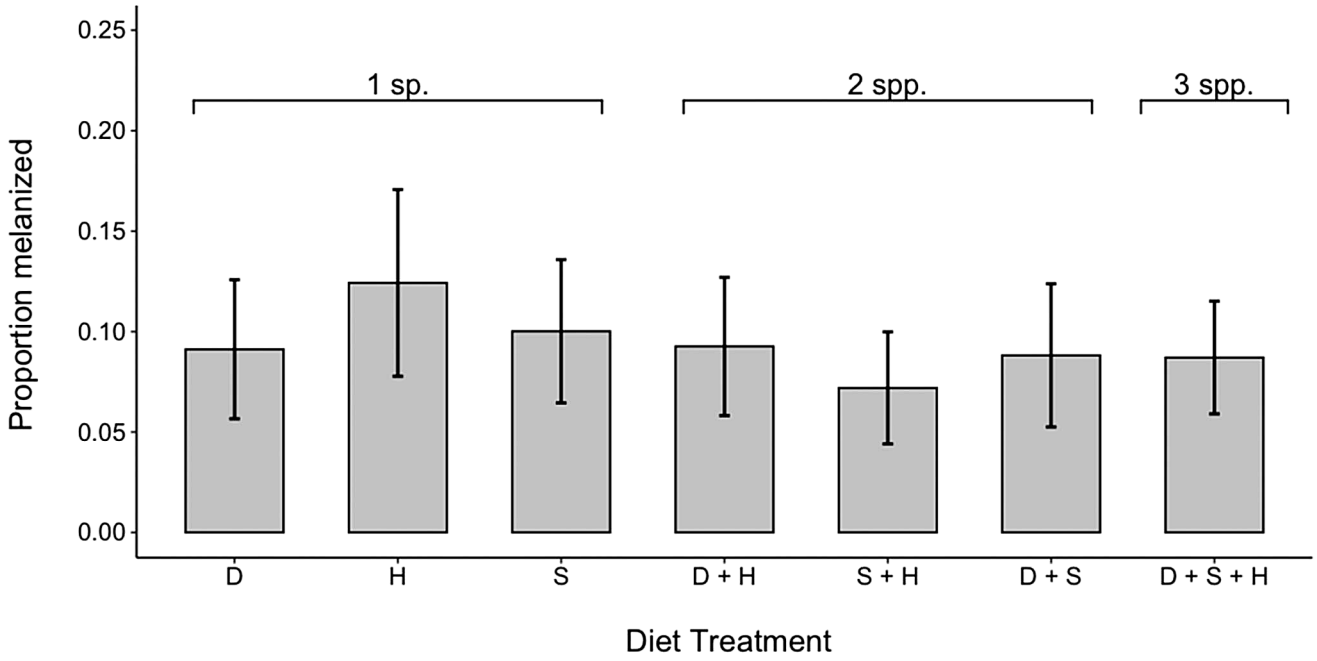

Fig. 4 Pollen diets did not significantly affect the melanization response in *B. impatiens*, as measured by proportion of the implant melanized. Means are model estimates, produced with emmeans; error bars are standard error. (Diets are D = Dandelion, H = Hawthorn, S = Sumac.)

Gut bacterial communities did not show distinct clusters by diet treatments in either species (Figure S3, S5), however this was dependent on worker age in bumble bees. Consistent with previous work [13], diet influenced bacterial diversity in older, but not newly emerged workers (Fig. 2D). A recent study found that bumble bee gut microbiomes exhibit a predictable successional trajectory, growing logistically in both abundance and diversity, and plateauing around 4 days old [27]. Given the non-callow group is a much wider age range (any age > 24 h), and therefore has opportunity for more variation, it is surprising that we found a significant effect of diet on diversity in this group and not callows. However, because the callows received a fecal inoculum pooled from multiple non-callows, they did not experience this succession process and may have developed a more diverse and therefore stable microbiome than expected naturally. Future studies using germ-free bees inoculated with controlled microbiomes could assess how bee gut microbiomes are influenced by diet at different life stages and why certain diets, such as sumac and hawthorn, yielded relatively low ASV richness in non-callows.

We found that bumble bees hosted many core bacterial taxa, including *Snodgrassella, Lactobacillus, Candidatus Schmidhempelia*, and *Bifidobacterium*, which have been shown to play important roles for the bee host including digestion, detoxification, and pathogen defense (reviewed in [6, 55]). We also found multiple flower-associated bacteria including *Neokomagataea, Acinetobacter, Asaia*, and *Pantoea* in both host species [14, 19, 49]. This is interesting given that we did not directly feed floral microbes to the bumble bees. They may have acquired floral microbes through their wild-caught queen and/or the non-sterilized honey bee-collected pollen they were fed prior to the experiment. Colony had a significant effect on community composition in bumble bees, and one bacterial genus, *Bombella*, which is an acetic acid bacteria with antifungal effects [56], only occurred in workers from one colony. This suggests that the first microbes that arrive in the gut, likely originating from the queen during spring foraging, become established and then remain stable in the face of varying nutritional input. This significant effect of colony on bumble bee gut communities contrasts with recent work showing that honey bees from different locations around the United States harbored similar gut bacteria down to the strain level [57], suggesting that environmental factors play a stronger role in shaping the gut microbiota of *B. impatiens* compared to *A. mellifera*. *Megachile rotundata*, which were dominated by nectar-inhabiting taxa, may have acquired floral microbes from the flower inoculum that we provided, or from larval pollen provisions (although bees shed their larval microbiome during metamorphosis), the nest cavity, or leaves lining the nest. This is consistent with the hypothesis that flowers are transmission hubs for pollinator microbes [19].

Captive rearing likely affects the bee gut microbiota. In our study, individuals from both species were provisioned by wild mothers, but were then exposed to a subset of the microbes they would normally encounter as adults since they had no direct contact with the natural environment. Although we did not compare our bees to wild-caught bees, captive rearing likely limits the diversity of the adult gut microbiome. This

may affect the health of captive-reared bees, especially if bees are multiple generations removed from the wild or exposed to stressors, such as agrochemicals, that can further disrupt their native microbiota. There is new research on potential probiotics for honey bees (reviewed in [58]) that could be considered by bee rearing companies, although further studies are needed to assess their efficacy and in more bee species.

We predicted that a low-diversity pollen diet would result in reduced melanization in bumble bees, but our results did not support this prediction (Fig. 4). Although we only assessed a small portion of the bee immune system, our results are consistent with previous studies that found that monofloral and polyfloral pollen diets resulted in similar immune function in bumble bees (e.g., [59]). However, bumble bees with no access to pollen had reduced immune gene expression [60] and immunity was positively correlated with amount of pollen consumed [59]. This suggests that pollen is important for immune function in adult bees, but the type or diversity of pollen may be less so. However, in nature certain pollen types are correlated with certain microbes, and these microbes likely have important functions for training the bee immune system and/or inhibiting pathogens through other mechanisms [16].

Our results suggest that even single-species, low-protein pollen diets such as dandelion and sumac provided adequate nutrition to support diverse bacterial communities in adults of two distantly related bee species. However, older bumble bee workers may be more vulnerable to dietary quality than callows, since their gut microbiome was more sensitive to diet composition. This study is the first to assess the gut microbiome in a solitary bee species in an experimental setting and contributes to our understanding of the composition, variation, and drivers in solitary bee gut microbes. Our results further demonstrate that variation in bee gut microbiomes in the wild are not likely driven by pollen nutritional resources, and may be more strongly influenced by other factors such as the introduction of new microbes from flowers, nests, and social interactions. This study improves our understanding of wild bee gut microbiomes and biology, as well as drivers of bee health in the face of reduced biodiversity and emergent pathogens.

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Author Contributions AEF and LSA designed the study. AEF conducted the experiments in the lab of LSA and the dissections, DNA extractions, and library preparations in the lab of QSM. AEF analyzed the data with guidance from QSM and wrote the first draft of the manuscript. All authors reviewed the manuscript.

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Data Availability All data and code are available at https://github.com/aefowler/bee-microbiome-diet-diversity and on UMass ScholarWorks.

Declarations

Competing interests The authors declare no conflict of interest.

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