**Title:**

A case study of the diet-microbiota-parasite interplay in bumble bees

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**Short running head**

Study of a diet-microbiota-parasite interplay

## Abstract

*Aims:* Diets and parasites influence the gut bacterial symbionts of bumble bees, but potential interactive effects remain overlooked. The main objective of this study was to assess the isolated and interactive effects of sunflower pollen, its phenolamides and the widespread trypanosomatid *Crithidia* sp. on the gut bacterial symbionts of *Bombus terrestris* males.

*Methods and results:* Bumble bee males emerged in microcolonies fed on either (i) willow pollen (control), (ii) sunflower pollen or (iii) willow pollen spiked with phenolamide extracts from sunflower pollen. These microcolonies were infected by *Crithidia* sp. or were pathogen-free. Using 16S rRNA amplicon sequencing (V3-V4 region), we observed a significant alteration of the beta diversity, but not of the alpha diversity, in the gut microbial communities of males fed on sunflower pollen compared to males fed on control pollen. Similarly, infection by the gut parasite *Crithidia* sp. altered the beta diversity, but not the alpha diversity, in the gut microbial communities of males irrespective of the diet. By contrast, we did not observe any significant alteration of the beta or alpha diversity in the gut microbial communities of males fed on phenolamide-enriched pollen compared to males fed on control pollen. Changes in the beta diversity indicate significant dissimilarities of the bacterial taxa between the treatment groups, while the lack of difference in alpha diversity demonstrates no significant changes within each treatment group.

*Conclusions:* Bumble bees harbour consistent gut microbiota worldwide, but our results suggest that the gut bacterial communities of bumble bees are somewhat shaped by their diets and gut parasites as well as by the interaction of these two factors. This study confirms that bumble bees are suitable biological surrogates to assess the effect of diet and parasite infections on gut microbial communities.


## Impact Statement

Our findings underscore the pivotal role of diet-parasite interactions in shaping bumble bee gut microbiota and indicate more variation of the gut microbiota than generally thought, emphasizing the significance of diet-parasite interactions in understanding pollinator health.

**Keywords**

Bumblebee; Microbiota; Parasite; Phenolamides; Pollen diets

## 1. Introduction

'There has never been any natural animal or plant free of microorganisms' (Zilber-Rosenberg and Rosenberg, 2008), later reiterated and emphasised as 'We [multicellular eukaryotes] have never been individuals' (Gilbert et al., 2012), has been an outstanding concept (i.e., holobiont concept) tightly merging micro- and evolutionary biology (Rosenberg and Zilber-Rosenberg, 2016). Microbial symbionts play vital roles with regards to their hosts' anatomy, physiology, immunology, development and overall fitness, to such an extent that, when considering the human holobiont, there are as many bacterial as human cells in any individual (Sender et al., 2016), and there are ~390 times more bacterial than human genes (Yang et al., 2009). Because microbial symbionts are crucial for their hosts, a disruption of their communities and functionality (i.e., dysbiosis) may have drastic – sometimes lethal – consequences for the hosts (Levy et al., 2017).

Owing to their undeniable function in wild and domesticated flowering plant pollination (Hristov et al., 2020), and due to their worldwide decline (Koh et al., 2016; Mathiasson and Rehan, 2019), bees (Hymenoptera: Apoidea: Anthophila) and their associated microbiota have been increasingly studied (Maebe et al., 2021). Social bee species (i.e., Apini, Bombini, Meliponini; Anthophila: Apidae: Corbiculata) primarily acquire their microbiota within the colony from their nestmates (Kwong and Moran, 2016). They harbour a rather constant core microbiota across the globe, consisting of <10 bacterial phylotypes (Kwong et al., 2017). Particularly, bumble bees (Bombini: *Bombus* spp.) hold seven core gut microbial symbiont phylotypes which consist of ~ 30 million cells (Table 1; *sensu* Hammer et al., 2021 and Kwong et al., 2017). Multiple benefits from bees' microbial symbionts have been described, such as assisting nutrient assimilation (Bonilla-Rosso and Engel, 2018), inhibiting gut parasite growth (Mockler et al., 2018), neutralising dietary toxins (Rothman et al., 2019) and stimulating the innate immune system (Näpflin and Schmid-Hempel, 2016). Recently, an outstanding paper

demonstrated that the bumble bees' resident gut microbiota was necessary to deglycosylate a dietary phytochemical and trigger its antiparasitic activity against the gut parasite *Crithidia bombi* (Euglenozoa: Kinetoplastea: Trypanosomatidae; Koch et al., 2022). Despite these pioneering studies, the diet-microbiota-parasite interplay is barely described and understood in bees.

Core microbial communities in the gut of social bees may however be skewed and disrupted following exposure to environmental stressors, thereby leading to a deleterious dysbiosis (Anderson and Ricigliano, 2017). First, some pollen and nectar diets, containing specific profiles of nutrients and phytochemicals, may shape core symbiont communities. For instance, in honey bees, a high-fat diet increased *Gilliamella apicola* and decreased *Bartonella apis* abundances (Wang et al., 2021) while four pollen- or nectar-derived phytochemicals induced short-term increases in gut microbiota diversity and abundance (Geldert et al., 2020). Second, gut-colonising bee pathogens and parasites may influence gut symbiont communities through competition for nutritional and spatial niches as well as production of metabolites that target, or are metabolised, by the hosts' symbionts. For example, *Vairimorpha ceranae* (Fungi: Microsporidia) infection was positively associated with several core bacteria of the honey bee gut, thereby presumably enabling to maintain host homeostasis and subsequently to sustain the host survival and benefit the pathogen (Castelli et al., 2021; Zhang et al., 2021). In bumble bees, *Crithidia bombi* infection was negatively associated with the core bacterium *Gilliamella apicola* and positively associated with non-core bacteria, while *Vairimorpha bombi* infection was positively associated with the core bacterium *Snodgrassella alvi* (Cariveau et al., 2014). These previous studies show that interactions between gut symbionts and parasites are complex and species-specific.

In human-modified landscapes (e.g., monofloral crops), bees may suffer from reduced food quality/quantity and are exposed to specific phytochemicals (Aizen et al., 2019; Parreño

et al., 2022). Human activities are also responsible for the disturbance of natural host–pathogen dynamics (Meeus et al., 2018), notably through global pathogen spill-over and spill-back between domesticated and wild bee populations (Martin et al., 2021; Pereira et al., 2021). Moreover, monoculture farming was associated with amplification of bees' parasite prevalence, thereby linking these two human-driven pressures (Cohen et al., 2021). However, despite the key roles of diets and parasites on wild bees' microbial symbionts in the Anthropocene context, it remains an hitherto overlooked aspect in bee conservation plans (Maebe et al., 2021). No study has ever addressed potential interactive effects of diet and parasite infections on the gut symbiont communities of bumble bees.

In one of our previous studies, we found that both diet (i.e., sunflower pollen and their phenolamides) and parasite infections (i.e., *Crithidia* sp.) impact bumble bee fitness. We also found that diet shaped the bumble bee susceptibility to infections (Gekière et al., 2022). Here, we address the diet-microbiota-parasite interplay by assessing how pollen diet and parasite infections shape the gut microbial community of bumble bees. Using Illumina MiSeq 16S rRNA amplicon sequencing (V3-V4 region), we tested the influence of pollen from a mass-flowering crop (i.e., sunflower; *Helianthus annuus*; Asterids: Asteraceae) and its phenolamides – a major class of phenylpropanoid phytochemicals – as well as infection by the highly prevalent gut parasite *Crithidia* sp. on the intestinal bacterial composition of indoor-reared bumble bee males (*Bombus terrestris* L. 1758). We used sunflower pollen as it has been repeatedly found to reduce *Crithidia* sp. load in bumble bees (Giacomini et al., 2018; Fowler et al., 2022), most likely because of its exine spines (Figueroa et al., 2023), and thus could also alter the gut bacterial communities. We focussed on its phenolamides since these phytochemicals have been widely found in flowering plants (Roumani et al., 2021), have been found to harbour antimicrobial properties (Kyselka et al., 2018), and have been found to upregulate genes facilitating excretion – and hypothetically gut symbiont flushing – in insects

(Chahine and O'Donnell, 2011). We used the trypanosomatid *Crithidia* sp. as this gut parasite is widespread in wild bumble bee populations (Tripodi et al., 2018) and its impacts on the gut microbiota of its hosts differed among studies (Mockler et al., 2018; Näpflin and Schmid-Hempel, 2018; Straw et al., 2023). We expected the gut microbiota would be shaped by specific pollen diets and parasite infections. We also expected diet-specific effects of the parasite infections – and *vice-versa* – on the bumble bee males' gut microbial communities.

## 2. Materials and Methods

### *2.1. Bioassays*

Bumble bee males were sampled in microcolonies (i.e., small queenless colonies) from an experiment described in Gekière et al. (2022). Briefly, five *Bombus terrestris* L. colonies were ordered from Biobest *bvba* (Westerlo, Belgium) and fed on willow pollen (*Salix* sp.; Rosids: Salicaceae) and Biogluc® (fructose 37.5%, glucose 34.5%, sucrose 25%, maltose 2%, oligosaccharides 1%, preservatives potassium sorbate (E202) 0.15% and citric acid (E330) 0.06%) (Wäckers et al., 2017) *ad libitum* in laboratory conditions (26 ± 1 °C; 60 ± 10% relative humidity, red light). Ninety microcolonies (i.e., plastic boxes 10 × 16 × 16 cm) of five workers were implemented from these five foundress colonies (i.e., 18 microcolonies per colony). These microcolonies were allocated to one of six treatments (i.e., three microcolonies per colony per treatment = 15 microcolonies per treatment). Microcolonies were fed on either (i) willow pollen, (ii) sunflower pollen or (iii) willow pollen supplemented with sunflower pollen phenolamides for 35 days (see Appendix 1 for phenolamide extraction and Table S1 for diet preparation). Workers in microcolonies were either (i) uninfected or (ii) initially infected with the gut parasite *Crithidia* sp. (see Appendix 2 for *Crithidia* sp. inoculation). In these queenless microcolonies, one or several workers increased their dominance and laid haploid eggs that brought forth male individuals ~25 days after microcolony establishment. At the end of the experiment, in every treatment, five males were sampled randomly, each one from different

156 microcolonies (i.e., 30 males in total; Appendix 3). We made sure not to take callow (i.e., one-
157 day old) individuals, because freshly emerged individuals require contacts with their nestmates'
158 faeces and hive materials to build up their microbiota (Kwong and Moran, 2016). We also
159 confirmed *Crithidia* sp. infection in males by screening their faeces at the end of the experiment.
160 Honey bee-collected willow and sunflower pollen loads were purchased from the company
161 'Ruchers de Lorraine' (Nancy, France) and provided by the INRAE (Paris, France),
162 respectively. No ethical and /or legal approval was required for this study.

163 *2.2. Gut dissection and DNA extraction*

164 The gut dissection was conducted under a laminar airflow cabinet and bees were
165 surface-sterilised with 2.5% Umonium$^{38®}$. The whole guts of the males (i.e., fore-, mid- and
166 hindgut) were dissected with sterile instruments and placed in 250 µL physiological saline. The
167 guts were then crushed with 1.5-mL Eppendorf tube mini-pestles to obtain a homogeneous
168 suspension, centrifuged (7,800 g, 20 min, 4 °C) and stored at -20 °C until DNA extraction. Total
169 bacterial DNA was extracted following a procedure modified from Snauwaert et al. (2016).
170 Thawed cell pellets were washed with 1 mL TES buffer (6.7% sucrose, 50 mM Tris–HCl, pH
171 8.0, 1 mM EDTA), centrifuged (27,700 g, 20 min, 4 °C) and resuspended in 275 µL STET
172 buffer (8.0% sucrose, 5.0% Triton X-100, 50 mM Tris–HCl, pH 8.0, 50mM EDTA). Ninety
173 microliter of enzymatic lysis buffer (TES buffer containing 1667 U/mL mutanolysin, 33 mg/mL
174 lysozyme, and 2.73 mg/mL proteinase K) was added and the suspensions were incubated at 37
175 °C for 1 h. Forty microliter of TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) + 20% SDS buffer
176 was preheated at 37 °C and added along with a spatula of 0.1 mm glass beads. The solutions
177 were vortexed for 1 min, incubated at 37 °C for 10 min and then at 65 °C for 10 min. Lysed cell
178 suspensions were centrifuged (20,800 g, 2 min, 4 °C) and the supernatants were transferred to
179 new 2-mL Eppendorf tubes before protein precipitation with 250 µL ammonium acetate on ice
180 for 10 min. One hundred microliter of TE buffer was added and DNA was extracted using 755

µL of phenol:chloroform:isoamyl alcohol (49.5:49.5:1.0) solution (Sigma-Aldrich, St. Louis, MO, USA). Phases were separated by centrifugation (20,800 g, 10 min, 4 °C) and the upper aqueous phase was transferred to a new 2-mL Eppendorf tube. DNA precipitation was conducted on ice for 15 min after the addition of 70 µL 5 M NaCl and 1 mL ice-cold 70% ethanol. DNA was collected by centrifugation and the pellets were washed twice with 150 µL ice-cold 70% ethanol (centrifugation in-between: 20,800 g, 1 min, 4 °C). DNA pellets were left to dry under a laminar airflow cabinet and then solubilised overnight in 50 µL TE buffer. To remove RNA materials, 2.5 µL RNase (2 mg/L) were added to the nucleic acid solutions which were incubated at 37 °C for 60 min. The purity of the DNA samples was evaluated using 0.7% (w/v) agarose gels stained in ethidium bromide and by spectrophotometric measurements at 280, 260, and 234 nm (Nanodrop). The concentration of the DNA samples was measured using a Quantus Fluorometer (Promega) and diluted up to 20 ng/µL with TE buffer (Appendix 3). DNA samples were kept at -20 °C until sequencing. No blank negative sample was sent to sequencing. Yet, because we did not find any aberrant or uncommon bacterial taxa in our results, we are confident that contamination did not occur.

*2.3. 16S rRNA amplicon sequencing and processing*

The V3-V4 region of the 16S rRNA gene was amplified using forward primer 341F (5'-CCTACGGGNGGCWGCAG-3') and reverse primer 785R (5'-GACTACHVGGGTATCTAATCC-3'). Library preparation and paired-end sequencing using MiSeq Illumina was performed at BaseClear B.V. (Leiden, the Netherlands). Upon retrieving the sequencing results, raw reads were processed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) package (B. J. Callahan et al., 2016) with the default pipeline parameters (B. Callahan, 2020a). First, based on the read quality profiles, we trimmed the forward and reverse reads at 260 and 230 nucleotides, respectively, and primers were removed from the respective reads using the trimLeft parameter (21 nucleotides). Afterwards, data were filtered

by removing the trimmed forward and reverse reads with more than two expected errors. Then, data were dereplicated and paired reads were merged, to allow amplicon sequence variants (ASVs) table construction. After removal of chimeras, taxonomy was assigned based on the RDP v18 database (B. Callahan, 2020b) and SILVA SSU r138.1 database (McLaren, 2020) with the IDTAXA algorithm developed in the DECIPHER package (Wright, 2016). Since the RDP v18 database was found to be more accurate, we used it for the analyses (Appendix 4). ASVs belonging to mitochondria, chloroplast and eukaryotes as well as ASVs that were not assigned at least at class level were discarded. Data analyses – except analyses of alpha diversity – were restricted to ASVs found in minimum two samples (i.e., singleton removal).

*2.4. Statistical analyses*

To compare alpha diversity (i.e., Shannon and Gini-Simpson indices; Jost, 2006) among treatments, we used the data including singletons, as the latter should be considered when measuring alpha diversity among treatments. We ran models including diet, parasite and their interaction as fixed effects and colony as random effect. The normality and homoscedasticity of the residual assumptions were violated so we performed aligned rank transformation (art function, ARTool package; Kay et al., 2021) on our models as it allowed non-parametric testing of interactions and main effects using standard ANOVA (Wobbrock et al., 2011). For significant models ($p < 0.05$), custom multiple pairwise comparisons (i.e., sunflower *vs.* control and supplemented *vs.* control) were conducted and the error was controlled using the one stage false discovery rate (FDR) method (contrast function, emmeans package; Lenth, 2022). Then, after singleton removal, we tested differences in bacterial communities across treatments (i.e., beta diversity) using a perMANOVA (adonis2 function, vegan package; Oksanen et al., 2019) based on Bray-Curtis dissimilarities. Diet, parasite and their interaction were used as fixed effects. Because perMANOVA does not allow random effects, we also specified the colony as the first term in the formula, and used the sequential sums of squares. Doing so enabled to

explain as much variation as possible with the first (random) term (i.e., colony) before the other factors (i.e., diet, parasite and their interaction) were evaluated (Bakker, 2022). Also, because we focussed on comparing the sunflower and supplemented diets against the willow diet (and not against each other), we used a custom contrast formula in the adonis2 function. This was the only way to run custom contrasts in a perMANOVA since the pairwiseAdonis function (Martinez Arbizu, 2020) does not have custom contrasts implemented yet. Homogeneity of variances among treatments was tested using the betadisper function (i.e., multivariate version of Levene's test for homogeneity of variances; vegan package; Oksanen et al., 2019). Differences in bacterial community composition were visualised through a NMDS plot of Bray-Curtis dissimilarities (plot_ordination function, phyloseq package; McMurdie and Holmes, 2013). As a follow-up to analysing differences at the community level, we also assessed whether there were differences in the relative abundance of different ASVs among treatments (diet*parasite) using the DESeq function (DESeq2 package; Love et al., 2014) and volcano plots (i.e., plotting –Log Bonferroni-adjusted p-values on the y-axis and Log2 fold-change values on the x-axis; EnhancedVolcano function, EnhancedVolcano package; Blighe et al., 2021). Only volcano plots with significant results are shown. All data were analysed in R version 4.1.3 (R Core Team, 2020).

## 3. Results

### *3.1. Bacterial community composition*

We conducted an analysis of gut bacterial communities in indoor-reared male bumble bees that were subjected to various pollen diets and were either uninfected or initially infected by the gut parasite *Crithidia* sp. We present a list of identified bacterial taxa along with their prevalence in our experimental setup, facilitating a discussion on the presence or absence of core and non-core taxa. A total of 569,563 demultiplexed paired-end reads of the 16S rRNA V3-V4 regions were obtained. DADA2 analyses retained 483,131 reads which clustered into

139 amplicon sequence variants (ASVs). After curation, 42 ASVs were retained, of which 35 could be assigned up to genus level. The remaining ASVs were assigned up to family level (n = 6) or order level (n = 1).

All the ASVs belonged either to the phylum Pseudomonadota (~55%; 23 ASVs; synonym Proteobacteria), Bacillota (~31%; 13 ASVs; synonym Firmicutes), Actinomycetota (~12%; 5 ASVs; synonym Actinobacteria) or Bacteroidota (~2%; 1 ASV; synonym Bacteroidetes) (Figure 1). In the phylum Pseudomonadota, the ASVs were assigned to the genera *Gilliamella* (~30%; 7 ASVs), *Snodgrassella* (~9%; 2 ASVs), *Acidocella* (~4%; 1 ASV), *Asaia* (~4%; 1 ASV), *Achromobacter* (~4%; 1 ASV), *Arsenophonus* (~4%; 1 ASV), *Serratia* (~4%; 1 ASV), *Pseudomonas* (~4%; 1 ASV) and *Stenotrophomonas* (~4%; 1 ASV), or to the families Enterobacteriaceae (~22%; 5 ASVs) and Yersiniaceae (~4%; 1 ASV), or to the order Rhizobiales (~4%; 1 ASV). In the phylum Bacillota, the ASVs were assigned to the genera *Lactobacillus* (~31%; 4 ASVs), *Apilactobacillus* (~23%; 3 ASVs), *Staphylococcus* (~15%; 2 ASVs), *Bacillus* (~8%; 1 ASV), *Bombilactobacillus* (~8%; 1 ASV), *Pediococcus* (~8%; 1 ASV) and *Lactococcus* (~8%; 1 ASV). In the phylum Actinomycetota, the ASVs were assigned to the genera *Bifidobacterium* (40%; 2 ASVs), *Cutibacterium* (40%; 2 ASVs) and *Bombiscardovia* (20%; 1 ASV). In the phylum Bacteroidota, the only ASV was assigned to the genus *Apibacter*.

The five most abundant ASVs across all samples belonged to three genera, namely *Bombiscardovia* (phylum Actinomycetota, family Bifidobacteriaceae), *Gilliamella* (phylum Pseudomonadota, family Orbaceae) and *Snodgrassella* (phylum Pseudomonadota, family Neisseriaceae). Altogether, these three genera represent >75% of bacterial reads across all samples (Figure 2).

*3.2. Diversity across treatments*

Our study aimed to investigate the impact of different pollen diets, parasite infections, and their combined interaction on the diversity and abundance (i.e., alpha diversity) of gut

bacterial taxa in indoor-reared bumble bee males. The Shannon index differed among diets ($F_{2,27} = 4.36$, $p = 0.027$) but custom contrasts showed that males that emerged in microcolonies fed on the sunflower or supplemented diets did not differ from males that emerged in microcolonies fed on the willow diet (Figure 3A). There was no significant effect of the parasite infections ($F_{1,28} = 0.27$, $p = 0.610$) (Figure 3B) or the diet*parasite interaction ($F_{2,24} = 0.09$, $p = 0.913$) on the Shannon index. In addition, we found no impact of the diet ($F_{2,27} = 1.28$, $p = 0.298$) on the Gini-Simpson index (Figure 3C). Parasite infections ($F_{1,28} = 0.003$, $p = 0.951$) (Figure 3D) or the diet*parasite interaction ($F_{2,24} = 0.07$, $p = 0.933$) had no impact on the Gini-Simpson index.

We also explored the variance in gut bacterial taxa composition (i.e., beta diversity) influenced by diverse pollen diets, parasite infections, and their combined interplay in indoor-reared bumble bee males. A permutation test with custom contrasts showed that bacterial communities differed between males that emerged in microcolonies fed on the willow or sunflower diet ($F_{1,18} = 3.043$, $p = 0.006$) and between males that emerged in uninfected or infected microcolonies ($F_{1,28} = 2.204$, $p = 0.028$; Figure 4). Conversely, bacterial communities did not differ between males that emerged in microcolonies fed on the willow or supplemented diet ($F_{1,18} = 0.400$, $p = 0.939$), and the diet*parasite interactions were not significant (all $p > 0.3$).

In our analysis, we identified specific gut bacterial taxa whose abundance was significantly influenced by diverse pollen diets, parasite infections, and their interactive effects. Employing DESeq2 and assessing log2 fold differences, we pinpointed bacterial ASVs exhibiting distinct abundance patterns within the gut samples of bumble bee males. The genus *Bifidobacterium* was more abundant in males that emerged in uninfected microcolonies fed on the sunflower diet in comparison to males that emerged in uninfected microcolonies fed on the willow diet (Figure 5A). Likewise, the taxa *Apilactobacillus*, *Bifidobacterium*,

306 *Bombilactobacillus* and Enterobacteriaceae were more abundant in males that emerged in
307 infected microcolonies fed on the sunflower diet in comparison to males that emerged in
308 infected microcolonies fed on the willow diet (Figure 5B). Parasite infections also had a direct
309 effect in microcolonies fed on the sunflower diet, wherein infected males had a reduced
310 abundance of *Bifidobacterium* but an increased abundance of *Lactobacillus* and
311 Enterobacteriaceae when compared to uninfected males (Figure 5C). No difference was found
312 between males fed on the supplemented diet and males fed on the willow diet, regardless of the
313 infection. Likewise, no difference was found in males that emerged in infected or uninfected
314 microcolonies fed on the willow or supplemented diet.

315 **4. Discussion**

316 *4.1. Core and non-core bacterial community*

317 The gut of bumble bees is typically dominated by seven bacterial taxa (Table 1) that
318 comprise > 90% of the bacteria in all individuals. These taxa are distributed into four families,
319 namely Bifidobacteriaceae, Neisseriaceae, Orbaceae and Lactobacillaceae (Kwong et al. 2017;
320 Hammer et al., 2021). Species representing each of these taxa were detected in the present
321 study, with the exception of *Schmidhempelia bombi*. Mockler et al. (2018) proposed that this
322 phylotype could be mistaken with the closely related bacterium *Gilliamella* using short-read
323 datasets. However, a recent study that sequenced the V3-V4 region of the 16S rRNA gene (i.e.,
324 the same region as used here) distinctly identified *Schmidhempelia* and *Gilliamella* (Choi et al.,
325 2023). It is also likely that the high abundance of other core phylotypes (e.g., *Snodgrassella*
326 *alvi*) hindered the detection of *Schmidhempelia bombi*, as discussed in Meeus et al. (2015) and
327 Choi et al. (2023). The Gram-negative Pseudomonadota *Snodgrassella* and *Gilliamella* were
328 found in all samples, mostly at high abundances, which is in accordance with previous studies
329 (Meeus et al. 2015; Kwong et al., 2017; Mockler et al., 2018). Interestingly, we observed the
330 core bacterium *Bombiscardovia* at high abundances in most samples, whereas this core genus

was found at low abundances or missing in the bumble bee guts in previous studies (Meeus et al., 2015; Parmentier et al., 2016; Mockler et al., 2018; Praet et al., 2018). Finally, we observed the core bacterium *Bifidobacterium* as well as the core lactic acid bacteria *Bombilactobacillus* and *Lactobacillus*, although at relatively low abundances in most samples.

Additionally, several non-core bacteria were detected in the gut samples of our bumble bee males, but always at very low abundances. Some reported bacteria were previously found in the gut of bumble bees such as *Asaia*, *Apibacter*, *Apilactobacillus*, *Bacillus*, Hyphomicrobiales, *Lactococcus*, *Pediococcus*, *Pseudomonas*, *Serratia* and *Staphylococcus* ( Kwong and Moran, 2016; Kwong et al., 2017; Mockler et al., 2018; Parmentier, Meeus, et al., 2018; Praet et al., 2018; Hammer et al., 2021; Amiri et al., 2023; Zhang et al., 2023). By contrast, to our knowledge, the present study is the first to report the genera *Achromobacter*, *Acidocella*, *Arsenophonus*, *Cutibacterium* and *Stenotrophomonas* in the gut of bumble bees. However, these bacteria have already been described in other bee species and other bumble bee organs. For instance, *Arsenophonus* was found in the fat bodies of bumble bees (Parmentier, Billiet, et al., 2018). *Cutibacterium* and *Stenotrophomonas* were found in the gut of carpenter bees (Graystock et al., 2017; Nguyen and Rehan, 2022). *Achromobacter* was found in the gut of honey bees (Cai et al., 2022) and *Acidocella* was recently detected in the gut of the invasive giant resin bee (Tuerlings et al., 2023). Among these non-core bacteria, some are associated with bee diseases such as *Achromobacter* and *Arsenophonus* that were correlated with European foulbrood (Erler et al., 2018) and *Apicystis bombi* infection (Parmentier, Billiet, et al., 2018) respectively. In addition, we reported the presence of *Serratia* which is considered an opportunistic pathogen of insects (Raymann et al., 2018).

After emergence, the gut microbiota of bumble bees is not acquired through proctodeal nor oral trophallaxis transmission routes, but is rather driven by a faecal-oral route through contaminated resources and nesting materials (Hammer et al., 2021). It is therefore assumed

that emerged workers and males from the same colony harbour identical gut symbiont communities since they are in contact with the same resources and nesting materials (Li et al., 2021), but see Kapheim et al. (2015) and Krams et al. (2022). To our knowledge, there is no study that directly compared the gut microbiota of bumble bee males and workers from indoor-reared colonies, and hence potential discrepancies between sexes cannot be ruled out.

*4.2. Effects of sunflower pollen and its phenolamides on the gut bacterial community*

Even though bumble bees harbour a consistent microbiota across the globe, variation in their gut bacterial composition could occur due to environmental factors, including their diet. Here, bumble bee males that emerged in microcolonies fed on sunflower pollen harboured a gut microbiota distinct from bumble bee males that emerged in microcolonies fed on willow pollen, with some bacterial phylotypes showing higher relative abundances in bumble bees fed on sunflower pollen. Although, the alpha diversity of their communities did not differ. A difference in beta diversity but not in alpha diversity means that bacterial communities differed between the two diets, but these discrepancies were not due to within-diet dissimilarity in diversity. Differential analyses further underlined an increase in *Apilactobacillus*, *Bombilactobacillus*, *Bifidobacterium* and Enterobacteriaceae in bumble bee males fed on sunflower pollen, especially in infected microcolonies. These results are in line with Billiet et al. (2015) who observed that *Lactobacillus bombi* (now *Bombilactobacillus bombi*; Zheng et al., 2020) was differentially abundant in bumble bees fed on distinct pollen diets. Also, *Lactobacillus kunkeei* (now *Apilactobacillus kunkeei*; Zheng et al., 2020) was found to grow well in fructose-rich environment – and not in glucose-rich environment (Maeno et al., 2016) – but these carbohydrate concentrations seem rather equivalent between willow and sunflower pollen (Taha, 2015; Radev, 2022).

The diet-mediated modifications in gut bacterial communities observed in the present research are supported by several earlier studies (Maes et al., 2016; Castelli et al., 2020) and

may be explained by three non-mutually exclusive reasons. Firstly, willow and sunflower pollen differ in their nutritional profiles (e.g., protein, lipid and carbohydrate content; Vaudo et al., 2020), which could shape the gut communities by favouring some bacterial species that are usually found at low abundances in the bumble bee gut (Ricigliano et al., 2022). Secondly, sunflower pollen harbours a spiny exine which could physically scrape the gut bacterial biofilm, as shown for trypanosomatid cells (Figueroa et al., 2023). Thirdly, although pollen-borne bacteria do not include core microbiota of corbiculate bees, they are ingested by bees in large numbers (Steffan et al., 2019) which may affect the host microbiome composition (Ambika Manirajan et al., 2016; Billiet et al., 2017; Graystock et al., 2017; Dharampal et al., 2020). Overall, although sunflower pollen induced variation in the gut bacterial community of bumble bees, such changes did not induce any severe dysbiosis in the gut of bumble bees. The absence of difference in alpha diversity between the two pollen diets further emphasised that sunflower pollen did not induce any severe dysbiosis.

Sunflower extracts (Fatrcová-Šramková et al., 2016) and especially its phenolamides (Kyselka et al., 2018) have antimicrobial properties *in vitro*. Furthermore, phenolamides were also shown to upregulate genes facilitating excretion – and hypothetically gut symbiont flushing – in insects (Chahine and O'Donnell, 2011). Contrary to our expectations, feeding bumble bee males with a phenolamide-enriched diet did not alter their gut microbial composition. This absence of difference in bacterial communities between bumble bees fed on the supplemented or the control diet is surprising. The assumption was that introducing new nutritional elements in the diet, especially one with demonstrated biological effects (Chahine and O'Donnell, 2011; Fatrcová-Šramková et al., 2016; Kyselka et al., 2018), would perturb the overall microbial community. Honey bee gut symbionts from the genus *Lactobacillus* were perfectly able to metabolise pollen phenolamides (Kešnerová et al., 2017), but we did not observe any increase in *Lactobacillus* in bumble bees fed on the phenolamide-enriched diet. However, providing

honey bees with various phytochemicals modified the gut symbiont communities only in the short term (i.e., three days), and the communities tended to return to the baseline after six days (Geldert et al., 2020). It is hence possible that these short-term effects were missed in the present study. We postulate that the core bumble bee gut symbionts were not negatively impacted by phenolamides, or that potential slight modifications in the bacterial communities did not persist over time. It is reasonable to assume so given the widespread occurrence of phenolamides in plant pollen (Roumani et al., 2021), which means core gut symbionts of bumble bees have perfectly co-evolved with these phytochemicals.

### *4.3. Effects of* Crithidia *infection on the gut bacterial community*

In their host, gut parasites must compete with the gut symbionts for their spatial and nutritional niches. Studies that assessed the *Bombus*-*Crithidia*-microbiota interaction are not scarce, yet originally they highlighted the importance of the microbiota to face infection, leaving aside the consequences of infection on the microbiota itself (Koch and Schmid-Hempel, 2011, 2012; Näpflin and Schmid-Hempel, 2016; Palmer-Young et al., 2018; Praet et al., 2018; Barribeau et al., 2022). Studies then looked at correlations between bacterial diversity or specific bacterial strain abundance and infection intensity (Cariveau et al., 2014; Koch et al., 2012; Mockler et al., 2018; Näpflin and Schmid-Hempel, 2018; Palmer-Young et al., 2019). Here, the presence of ileum-dwelling *Crithidia* sp. cells in bumble bee males did not impact the alpha diversity but modified the relative composition of the gut bacterial community. Differential analyses showed that *Bifidobacterium* was less abundant while *Lactobacillus* and Enterobacteriaceae were more abundant in infected bumble bee males compared to uninfected ones in the sunflower diet. Our results are in line with a previous study showing that *Crithidia*-infected bumble bees harboured distinct gut microbiota when compared to uninfected ones (Felden et al., 2021). However, the higher relative abundance of *Lactobacillus* and Enterobacteriaceae in infected individuals is surprising given that these bacteria were shown to

be negatively associated with *Crithidia* sp. infection (Mockler et al., 2018; Palmer-Young et al., 2018). In addition, *Bifidobacterium* was shown to be marginally correlated with *Crithidia* sp., which contrasts with our findings (Fernandez De Landa et al., 2023). The opposite trends in *Bifidobacterium* and *Lactobacillus* abundances is also intriguing given that these taxa were shown to be positively associated in the bumble bee gut (Meeus et al., 2015). In the present experiment, it could be that bacterial community changes are a consequence of infection that would ultimately enable to better deal with the parasite, but the hypothesis of such an adaptative response remains completely unexplored.

Studies of the *Bombus*-*Crithidia*-microbiota interaction have yielded contradictory results. Our results did not corroborate those of Mockler et al. (2018) who found that *Crithidia* sp. infection was inversely correlated with microbial diversity. In addition, some studies reported no effect of *Crithidia* sp. infection on the gut microbiota of bumble bees (Näpflin and Schmid-Hempel, 2018; Straw et al., 2023). Such discrepancies are likely multifactorial, namely because of divergences in bumble bee species (*B. impatiens* vs. *B. terrestris*), bacterial strains and parasite strains. Besides, it is important to highlight that our study is the first to address the *Bombus*-*Crithidia*-microbiota interaction in (i) bumble bee males and (ii) in individuals that were simultaneously inoculated with their microbiota and the parasite (i.e., by eating contaminated food in the microcolony right after emergence). Previous studies always used bumble bee workers that were inoculated with *Crithidia* sp. cells after their microbiota was established. Elucidating whether *Crithidia* sp. infection has different impacts on the microbiota depending on the sex of the host and the time of inoculation warrants further investigations.

### 4.4. *Interactive effects of sunflower pollen and* Crithidia *infection on the gut bacterial community*

Our study is the first to report an interactive effect between pollen diet and parasite infections on the gut microbial communities of bumble bees. Although we did not observe any

456 interactive effects when considering the alpha and beta diversity of the bacterial communities,
457 differential analyses showed infection-dependant changes in bumble bees fed on sunflower
458 pollen. Indeed, only one taxon (i.e., *Bifidobacterium*) was more abundant in sunflower-fed
459 bumble bees that were uninfected, while four taxa (i.e., *Apilactobacillus*, *Bombilactobacillus*,
460 *Bifidobacterium* and Enterobacteriaceae) were more abundant when sunflower-fed bumble bees
461 were infected. Likewise, differential analyses showed diet-dependant changes in infected
462 bumble bees. Indeed, infection only led to differential abundances in some taxa (i.e.,
463 *Lactobacillus*, *Bifidobacterium* and Enterobacteriaceae) in bumble bees fed on sunflower
464 pollen. Why sunflower pollen amplified the effects of infection – and *vice-versa* – on the
465 abundances of some gut bacterial taxa remains completely enigmatic. It could be that both
466 factors induced slight disruptions in the gut bacterial communities, subsequently facilitating the
467 effects of the other factor.

468 *4.5. Diet-microbiota-parasite interplay*

469 In one of our previous studies, sunflower pollen and its phenolamides had various
470 effects on bumble bees at the microcolony (i.e., brood development) and individual (i.e., fat
471 body content) levels (Gekière et al., 2022). Here, we showed that the detrimental consequences
472 of sunflower pollen on bumble bees could be due to the alteration of the gut microbiota it
473 triggered, although sunflower pollen did not lead to a serious dysbiosis. By contrast, the
474 detrimental impacts of phenolamides found on bumble bees could not be due to any gut
475 microbiota alteration, since phenolamide-enriched pollen did not induce any change in the gut
476 microbial communities. With regards to *Crithidia* sp. infection, our previous research found an
477 increase in parasite load in bumble bees fed on phenolamide-enriched pollen. This increase in
478 infection was thus not mediated by any change in the gut microbial communities. Because in
479 our previous study the infection intensity did not change in bumble bees fed on sunflower pollen
480 when compared to bumble bees fed on willow pollen, we hereby demonstrated that the gut

481 microbiota alteration did not shape infection susceptibility. Finally, our previous research found
482 a reduction in fat body content in infected bumble bees, and the present study thus showcased
483 a potential association between a gut microbial alteration and changes in fat body content. Yet,
484 this hypothesis remains to be demonstrated properly.

485 *4.6. Conclusion and future directions*

486 In the present study, we showed that bumble bee males that emerged in microcolonies
487 fed on different diets and infected by a gut parasite or parasite-free harboured relatively
488 different gut bacterial communities. Although no treatment led to a severe dysbiosis (i.e., high
489 abundance of environmental and pathogenic bacteria), some bumble bee-associated core
490 bacteria were differentially abundant among treatments. The effects of diets on the abundance
491 of some core bacteria were infection-dependant – and *vice-versa*. It stresses that environmental
492 factors such as diet variability and parasite infections, as well as their interaction, could shape
493 the gut microbiota of bumble bees. Our findings highlight that the microbial communities found
494 in bumble bees are influenced by external conditions, and that the 'worldwide consistency' of
495 the gut microbiota of bumble bees should be considered cautiously. In addition, given the
496 commercial availability of several bumble bee species (Osterman et al., 2021), their quick and
497 undemanding rearing (Klinger et al., 2019) as well as simple and cultivable microbiota ( Praet
498 et al., 2018; Hammer et al., 2021), the present research confirms that bumble bees are suitable
499 models to study the effects of diets and parasites on gut symbiotic bacteria, as already suggested
500 for honey bees (Zheng et al., 2018). Commercial bumble bees could therefore be seriously
501 considered as biological surrogates to conduct gut microbiota studies (Douglas, 2019).
502

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**Conflict of interests**

None declared.

**Data Availability Statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethical Approval Statement**

No specific approval was required for this study.

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**CRediT authorship contribution statement**

**Antoine Gekière**: Conceptualisation, Methodology, Formal analysis, Investigation, Visualisation, Writing - Original Draft, Writing - Review & Editing. **Maryse Vanderplanck**: Conceptualisation, Methodology, Supervision, Writing - Review & Editing. **Amanda Hettiarachchi**: Methodology, Investigation, Writing - Review & Editing. **Irène Semay**: Methodology, Writing - Review & Editing. **Pascal Gerbaux**: Methodology, Resources, Writing - Review & Editing. **Denis Michez**: Funding acquisition, Supervision, Writing - Review & Editing. **Marie Joossens**: Methodology, Resources, Supervision, Writing - Review & Editing. **Peter Vandamme**: Methodology, Resources, Writing - Review & Editing.

858 **Supplementary data titles**

859

860 **Appendix 1.** Phenolamide extraction and diet preparation.

861

862 **Appendix 2.** Inoculation protocol.

863

864 **Appendix 3.** Male sampling details.

865

866 **Appendix 4.** Comparison between the number of ASVs identified through the SILVA SSU
867 r138 and RDP v18 databases after singleton removal.

868

869 **Table**

870

871 **Table 1.** Core bacteria described in the gut of *Bombus* spp. (Kwong et al. 2017; Hammer et al.

872 2021). The relative abundance of these phylotypes may vary between species.

| Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|
| Actinomycetota (synonym Actinobacteria) | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | *Bombiscardovia*<br>*Bifidobacterium* |
| Pseudomonadota (synonym Proteobacteria) | Betaproteobacteria | Neisseriales | Neisseriaceae | *Snodgrassella* |
| | Gammaproteobacteria | Orbales | Orbaceae | *Gilliamella*<br>*Schmidhempelia* |
| Bacillota (synonym Firmicutes) | Bacilli | Lactobacillales | Lactobacillaceae | *Bombilactobacillus*<br>*Lactobacillus* |

873

874 **Figure legends**

875

876 **Figure 1.** Phylum-level bar plots with relative abundances of all the bacterial ASVs found in
877 gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on
878 different pollen diets (five samples per treatment). **Willow.** Microcolonies fed on *Salix* sp.
879 pollen. **Sunflower.** Microcolonies fed on *Helianthus annuus* pollen. **Supp.** Microcolonies fed
880 on *Salix* sp. pollen spiked with phenolamides from *Helianthus annuus* pollen. + **P.**
881 Microcolonies in which workers were originally inoculated with the gut parasite *Crithidia* sp.

882

883 **Figure 2.** Genus-level bar plots with relative abundances of the five most abundant ASVs found
884 in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed
885 on different pollen diets (five samples per treatment). **Willow.** Microcolonies fed on *Salix* sp.
886 pollen. **Sunflower.** Microcolonies fed on *Helianthus annuus* pollen. **Supp.** Microcolonies fed
887 on *Salix* sp. pollen spiked with phenolamides from *Helianthus annuus* pollen. + **P.**
888 Microcolonies in which workers were originally inoculated with the gut parasite *Crithidia* sp.

889

890 **Figure 3.** Alpha diversity of the bacterial communities found in gut samples of bumble bee
891 males that emerged in infected and uninfected microcolonies fed on different pollen diets (five
892 samples per treatment). **AB.** Shannon diversity index taking into consideration the number of
893 ASVs (i.e., richness) and their relative abundance (i.e., evenness). **CD.** Gini-Simpson index
894 indicating the probability that two randomly chosen ASVs are different. **AC.** Comparison
895 among pollen diets. **BD.** Comparison between infection status. **n.s.** Not significant. The
896 diet*parasite interaction was not significant for any alpha diversity metric.

897

898 **Figure 4.** Nonmetric multidimensional scaling (NMDS) ordination based on the Bray-Curtis
899 dissimilarity matrix of the bacterial communities found in gut samples of bumble bee males
900 that emerged in infected and uninfected microcolonies fed on different pollen diets (five
901 samples per treatment). **A.** Comparison among pollen diets. **B.** Comparison between infection
902 status. The diet*parasite interaction was not significant.
903
904 **Figure 5.** Volcano plots of significance (Wald test and Benjamini-Hochberg-adjusted p-value)
905 versus log2-fold change showing differentially abundant ASVs in gut samples of bumble bee
906 males that emerged in infected and uninfected microcolonies fed on different pollen diets (five
907 samples per treatment). **A.** Uninfected microcolonies fed either the sunflower or willow diet.
908 **B.** Infected microcolonies fed either the sunflower or willow diet. **C.** Microcolonies either
909 infected or uninfected by the parasite *Crithidia* sp. fed the sunflower diet. The vertical dashed
910 lines indicate $\log_2$ fold change thresholds of -1 and 1 (i.e., ASVs that are twice more or less
911 abundant). The horizontal dashed line indicates an adjusted p-value threshold of $10^{-5}$. This
912 figure only shows comparisons between treatments with significant results.
913

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915

**Table 1.** Core bacteria described in the gut of *Bombus* spp. (Kwong et al. 2017; Hammer et al. 2021). The relative abundance of these phylotypes may vary between species.

| Phylum | Class | Order | Family | Genus |
|---|---|---|---|---|
| Actinomycetota (synonym Actinobacteria) | Actinobacteria | Bifidobacteriales | Bifidobacteriaceae | *Bombiscardovia*<br>*Bifidobacterium* |
| Pseudomonadota (synonym Proteobacteria) | Betaproteobacteria | Neisseriales | Neisseriaceae | *Snodgrassella* |
| | Gammaproteobacteria | Orbales | Orbaceae | *Gilliamella*<br>*Schmidhempelia* |
| Bacillota (synonym Firmicutes) | Bacilli | Lactobacillales | Lactobacillaceae | *Bombilactobacillus*<br>*Lactobacillus* |

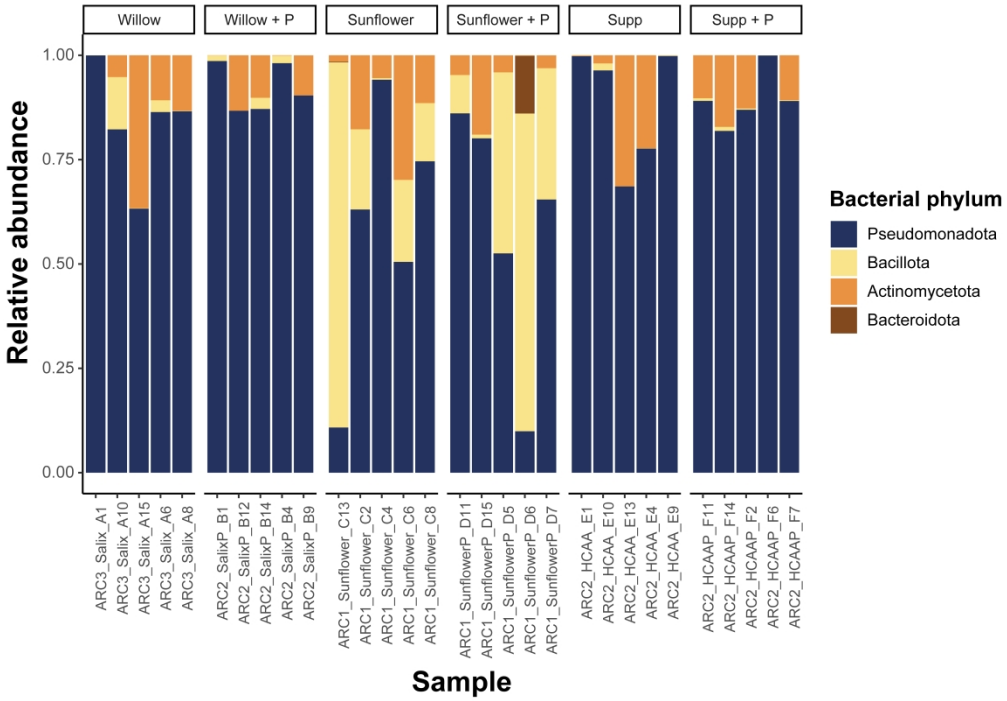


Figure 1. Phylum-level bar plots with relative abundances of all the bacterial ASVs found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). Willow. Microcolonies fed on Salix sp. pollen. Sunflower. Microcolonies fed on Helianthus annuus pollen. Supp. Microcolonies fed on Salix sp. pollen spiked with phenolamides from Helianthus annuus pollen. + P. Microcolonies in which workers were originally inoculated with the gut parasite Crithidia sp.

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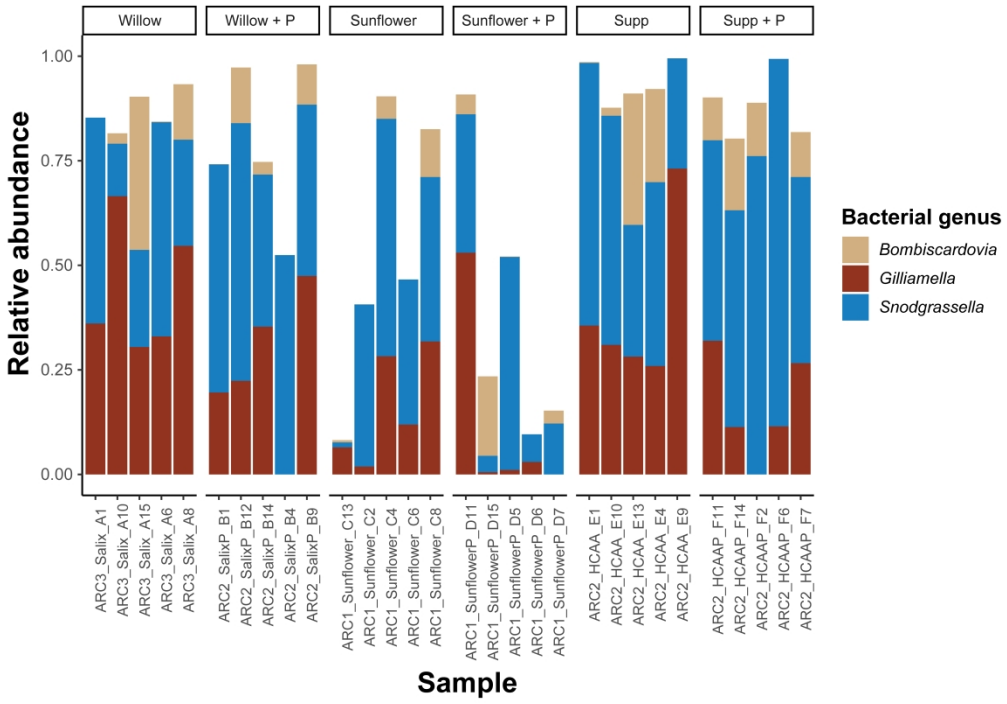


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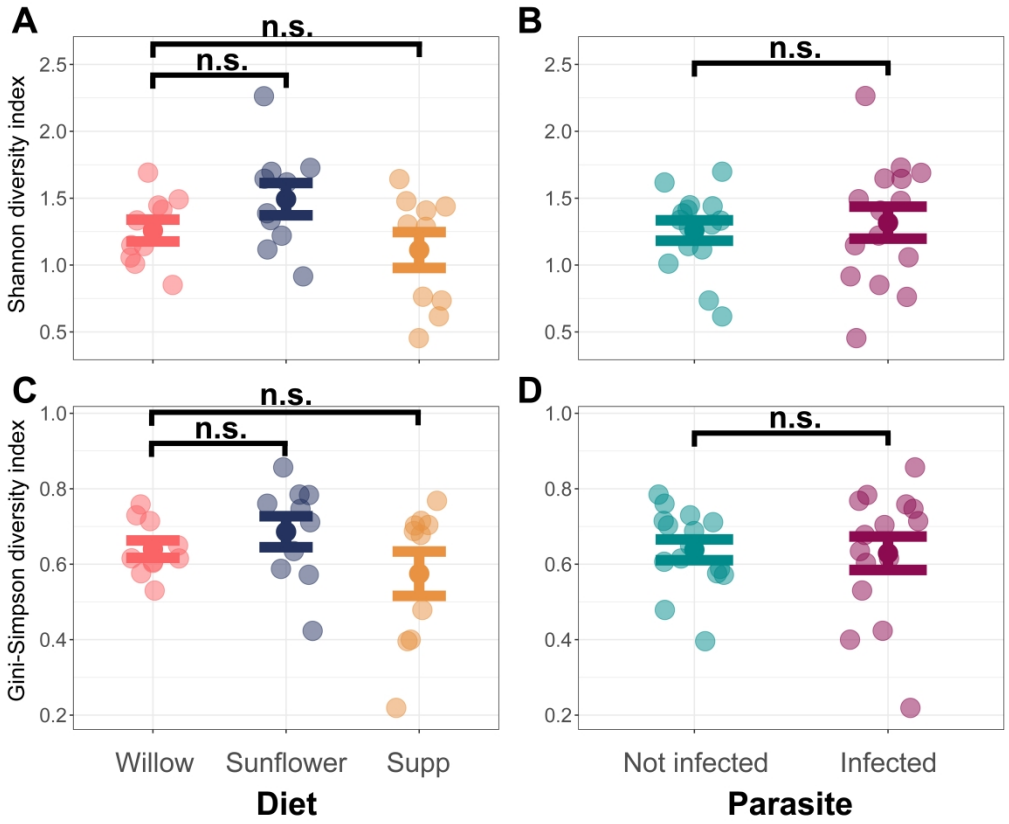


Figure 3. Alpha diversity of the bacterial communities found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). AB. Shannon diversity index taking into consideration the number of ASVs (i.e., richness) and their relative abundance (i.e., evenness). CD. Gini-Simpson index indicating the probability that two randomly chosen ASVs are different. AC. Comparison among pollen diets. BD. Comparison between infection status. n.s. Not significant. The diet*parasite interaction was not significant for any alpha diversity metric.

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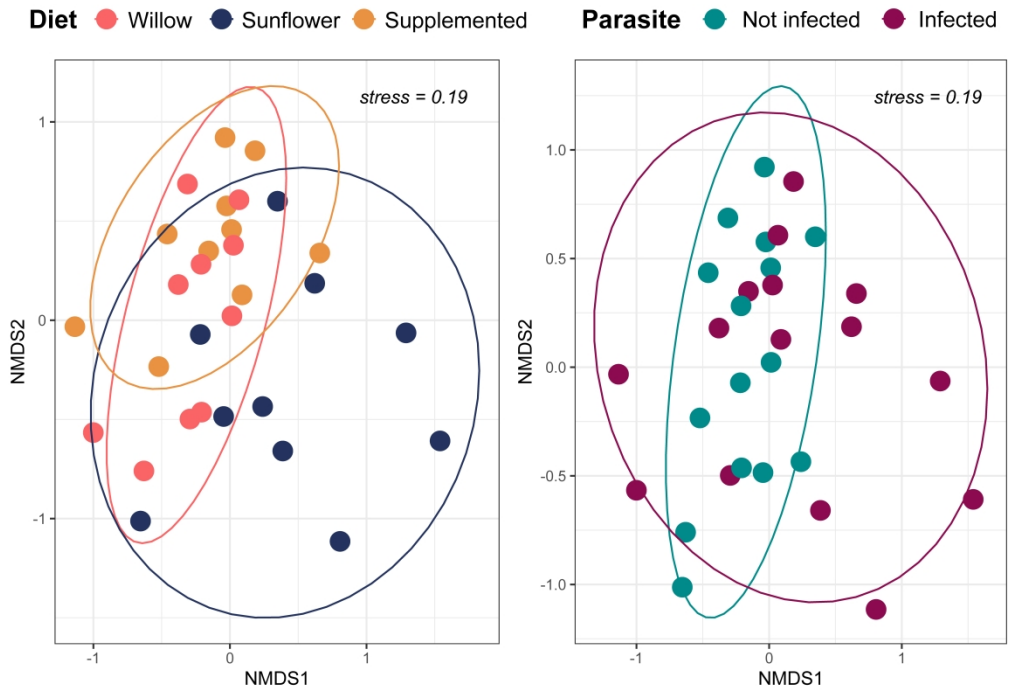


Figure 4. Nonmetric multidimensional scaling (NMDS) ordination based on the Bray-Curtis dissimilarity matrix of the bacterial communities found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). A. Comparison among pollen diets. B. Comparison between infection status. The diet*parasite interaction was not significant.

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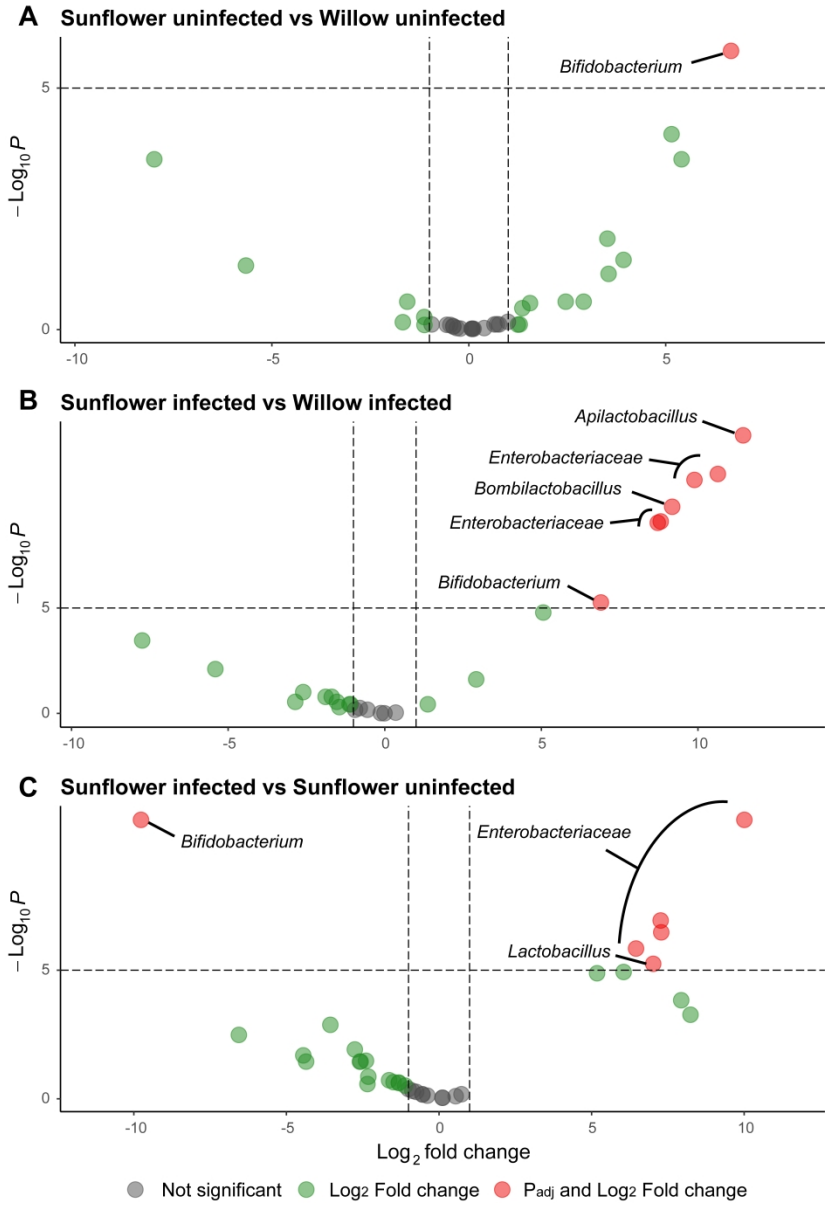


Figure 5. Volcano plots of significance (Wald test and Benjamini-Hochberg-adjusted p-value) versus log2-fold change showing differentially abundant ASVs in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). A. Uninfected microcolonies fed either the sunflower or willow diet. B. Infected microcolonies fed either the sunflower or willow diet. C. Microcolonies either infected or uninfected by the parasite Crithidia sp. fed the sunflower diet. The vertical dashed lines indicate log2 fold change thresholds of -1 and 1 (i.e., ASVs that are twice more or less abundant). The horizontal dashed line indicates an adjusted p-value threshold of 10-5. This figure only shows comparisons between treatments with significant results.

211x308mm (600 x 600 DPI)