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1 **Title:**

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

3

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21

22 **Short running head**

23 Study of a diet-microbiota-parasite interplay

24 **Abstract**

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

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

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

47

48 **Impact Statement**

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

52

53 **Keywords**

54 Bumblebee; Microbiota; Parasite; Phenolamides; Pollen diets

55

56 1. Introduction

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

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

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

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

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

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

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

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

137 **2. Materials and Methods**

138 *2.1. Bioassays*

139 Bumble bee males were sampled in microcolonies (i.e., small queenless colonies) from
140 an experiment described in Gekière et al. (2022). Briefly, five *Bombus terrestris* L. colonies
141 were ordered from Biobest *bvba* (Westerlo, Belgium) and fed on willow pollen (*Salix* sp.;
142 Rosids: Salicaceae) and Biogluc® (fructose 37.5%, glucose 34.5%, sucrose 25%, maltose 2%,
143 oligosaccharides 1%, preservatives potassium sorbate (E202) 0.15% and citric acid (E330)
144 0.06%) (Wäckers et al., 2017) *ad libitum* in laboratory conditions (26 ± 1 °C; $60 \pm 10\%$ relative
145 humidity, red light). Ninety microcolonies (i.e., plastic boxes $10 \times 16 \times 16$ cm) of five workers
146 were implemented from these five foundress colonies (i.e., 18 microcolonies per colony). These
147 microcolonies were allocated to one of six treatments (i.e., three microcolonies per colony per
148 treatment = 15 microcolonies per treatment). Microcolonies were fed on either (i) willow
149 pollen, (ii) sunflower pollen or (iii) willow pollen supplemented with sunflower pollen
150 phenolamides for 35 days (see Appendix 1 for phenolamide extraction and Table S1 for diet
151 preparation). Workers in microcolonies were either (i) uninfected or (ii) initially infected with
152 the gut parasite *Crithidia* sp. (see Appendix 2 for *Crithidia* sp. inoculation). In these queenless
153 microcolonies, one or several workers increased their dominance and laid haploid eggs that
154 brought forth male individuals ~25 days after microcolony establishment. At the end of the
155 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

181 μL of phenol:chloroform:isoamyl alcohol (49.5:49.5:1.0) solution (Sigma-Aldrich, St. Louis,
182 MO, USA). Phases were separated by centrifugation (20,800 g, 10 min, 4 °C) and the upper
183 aqueous phase was transferred to a new 2-mL Eppendorf tube. DNA precipitation was
184 conducted on ice for 15 min after the addition of 70 μL 5 M NaCl and 1 mL ice-cold 70%
185 ethanol. DNA was collected by centrifugation and the pellets were washed twice with 150 μL
186 ice-cold 70% ethanol (centrifugation in-between: 20,800 g, 1 min, 4 °C). DNA pellets were left
187 to dry under a laminar airflow cabinet and then solubilised overnight in 50 μL TE buffer. To
188 remove RNA materials, 2.5 μL RNase (2 mg/L) were added to the nucleic acid solutions which
189 were incubated at 37 °C for 60 min. The purity of the DNA samples was evaluated using 0.7%
190 (w/v) agarose gels stained in ethidium bromide and by spectrophotometric measurements at
191 280, 260, and 234 nm (Nanodrop). The concentration of the DNA samples was measured using
192 a Quantus Fluorometer (Promega) and diluted up to 20 ng/ μL with TE buffer (Appendix 3).
193 DNA samples were kept at -20 °C until sequencing. No blank negative sample was sent to
194 sequencing. Yet, because we did not find any aberrant or uncommon bacterial taxa in our
195 results, we are confident that contamination did not occur.

196 *2.3. 16S rRNA amplicon sequencing and processing*

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

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

215 *2.4. Statistical analyses*

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

231 explain as much variation as possible with the first (random) term (i.e., colony) before the other
232 factors (i.e., diet, parasite and their interaction) were evaluated (Bakker, 2022). Also, because
233 we focussed on comparing the sunflower and supplemented diets against the willow diet (and
234 not against each other), we used a custom contrast formula in the `adonis2` function. This was
235 the only way to run custom contrasts in a `perMANOVA` since the `pairwiseAdonis` function
236 (Martinez Arbizu, 2020) does not have custom contrasts implemented yet. Homogeneity of
237 variances among treatments was tested using the `betadisper` function (i.e., multivariate version
238 of Levene's test for homogeneity of variances; `vegan` package; Oksanen et al., 2019).
239 Differences in bacterial community composition were visualised through a NMDS plot of Bray-
240 Curtis dissimilarities (`plot_ordination` function, `phyloseq` package; McMurdie and Holmes,
241 2013). As a follow-up to analysing differences at the community level, we also assessed
242 whether there were differences in the relative abundance of different ASVs among treatments
243 (diet*parasite) using the `DESeq` function (`DESeq2` package; Love et al., 2014) and volcano
244 plots (i.e., plotting $-\text{Log}$ Bonferroni-adjusted p-values on the y-axis and Log_2 fold-change
245 values on the x-axis; `EnhancedVolcano` function, `EnhancedVolcano` package; Blighe et al.,
246 2021). Only volcano plots with significant results are shown. All data were analysed in R
247 version 4.1.3 (R Core Team, 2020).

248 **3. Results**

249 *3.1. Bacterial community composition*

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

256 139 amplicon sequence variants (ASVs). After curation, 42 ASVs were retained, of which 35
257 could be assigned up to genus level. The remaining ASVs were assigned up to family level (n
258 = 6) or order level (n = 1).

259 All the ASVs belonged either to the phylum Pseudomonadota (~55%; 23 ASVs;
260 synonym Proteobacteria), Bacillota (~31%; 13 ASVs; synonym Firmicutes), Actinomycetota
261 (~12%; 5 ASVs; synonym Actinobacteria) or Bacteroidota (~2%; 1 ASV; synonym
262 Bacteroidetes) (Figure 1). In the phylum Pseudomonadota, the ASVs were assigned to the
263 genera *Gilliamella* (~30%; 7 ASVs), *Snodgrassella* (~9%; 2 ASVs), *Acidocella* (~4%; 1 ASV),
264 *Asaia* (~4%; 1 ASV), *Achromobacter* (~4%; 1 ASV), *Arsenophonus* (~4%; 1 ASV), *Serratia*
265 (~4%; 1 ASV), *Pseudomonas* (~4%; 1 ASV) and *Stenotrophomonas* (~4%; 1 ASV), or to the
266 families Enterobacteriaceae (~22%; 5 ASVs) and Yersiniaceae (~4%; 1 ASV), or to the order
267 Rhizobiales (~4%; 1 ASV). In the phylum Bacillota, the ASVs were assigned to the genera
268 *Lactobacillus* (~31%; 4 ASVs), *Apilactobacillus* (~23%; 3 ASVs), *Staphylococcus* (~15%; 2
269 ASVs), *Bacillus* (~8%; 1 ASV), *Bombilactobacillus* (~8%; 1 ASV), *Pediococcus* (~8%; 1 ASV)
270 and *Lactococcus* (~8%; 1 ASV). In the phylum Actinomycetota, the ASVs were assigned to the
271 genera *Bifidobacterium* (40%; 2 ASVs), *Cutibacterium* (40%; 2 ASVs) and *Bombiscardovia*
272 (20%; 1 ASV). In the phylum Bacteroidota, the only ASV was assigned to the genus *Apibacter*.

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

278 3.2. Diversity across treatments

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

281 bacterial taxa in indoor-reared bumble bee males. The Shannon index differed among diets
282 ($F_{2,27} = 4.36$, $p = 0.027$) but custom contrasts showed that males that emerged in microcolonies
283 fed on the sunflower or supplemented diets did not differ from males that emerged in
284 microcolonies fed on the willow diet (Figure 3A). There was no significant effect of the parasite
285 infections ($F_{1,28} = 0.27$, $p = 0.610$) (Figure 3B) or the diet*parasite interaction ($F_{2,24} = 0.09$, $p =$
286 0.913) on the Shannon index. In addition, we found no impact of the diet ($F_{2,27} = 1.28$, $p =$
287 0.298) on the Gini-Simpson index (Figure 3C). Parasite infections ($F_{1,28} = 0.003$, $p = 0.951$)
288 (Figure 3D) or the diet*parasite interaction ($F_{2,24} = 0.07$, $p = 0.933$) had no impact on the Gini-
289 Simpson index.

290 We also explored the variance in gut bacterial taxa composition (i.e., beta diversity)
291 influenced by diverse pollen diets, parasite infections, and their combined interplay in indoor-
292 reared bumble bee males. A permutation test with custom contrasts showed that bacterial
293 communities differed between males that emerged in microcolonies fed on the willow or
294 sunflower diet ($F_{1,18} = 3.043$, $p = 0.006$) and between males that emerged in uninfected or
295 infected microcolonies ($F_{1,28} = 2.204$, $p = 0.028$; Figure 4). Conversely, bacterial communities
296 did not differ between males that emerged in microcolonies fed on the willow or supplemented
297 diet ($F_{1,18} = 0.400$, $p = 0.939$), and the diet*parasite interactions were not significant (all $p >$
298 0.3).

299 In our analysis, we identified specific gut bacterial taxa whose abundance was
300 significantly influenced by diverse pollen diets, parasite infections, and their interactive effects.
301 Employing DESeq2 and assessing log₂ fold differences, we pinpointed bacterial ASVs
302 exhibiting distinct abundance patterns within the gut samples of bumble bee males. The genus
303 *Bifidobacterium* was more abundant in males that emerged in uninfected microcolonies fed on
304 the sunflower diet in comparison to males that emerged in uninfected microcolonies fed on the
305 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

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

335 Additionally, several non-core bacteria were detected in the gut samples of our bumble
336 bee males, but always at very low abundances. Some reported bacteria were previously found
337 in the gut of bumble bees such as *Asaia*, *Apibacter*, *Apilactobacillus*, *Bacillus*,
338 Hyphomicrobiales, *Lactococcus*, *Pediococcus*, *Pseudomonas*, *Serratia* and *Staphylococcus* (
339 Kwong and Moran, 2016; Kwong et al., 2017; Mockler et al., 2018; Parmentier, Meeus, et al.,
340 2018; Praet et al., 2018; Hammer et al., 2021; Amiri et al., 2023; Zhang et al., 2023). By
341 contrast, to our knowledge, the present study is the first to report the genera *Achromobacter*,
342 *Acidocella*, *Arsenophonus*, *Cutibacterium* and *Stenotrophomonas* in the gut of bumble bees.
343 However, these bacteria have already been described in other bee species and other bumble bee
344 organs. For instance, *Arsenophonus* was found in the fat bodies of bumble bees (Parmentier,
345 Billiet, et al., 2018). *Cutibacterium* and *Stenotrophomonas* were found in the gut of carpenter
346 bees (Graystock et al., 2017; Nguyen and Rehan, 2022). *Achromobacter* was found in the gut
347 of honey bees (Cai et al., 2022) and *Acidocella* was recently detected in the gut of the invasive
348 giant resin bee (Tuerlings et al., 2023). Among these non-core bacteria, some are associated
349 with bee diseases such as *Achromobacter* and *Arsenophonus* that were correlated with
350 European foulbrood (Erler et al., 2018) and *Apicystis bombi* infection (Parmentier, Billiet, et
351 al., 2018) respectively. In addition, we reported the presence of *Serratia* which is considered
352 an opportunistic pathogen of insects (Raymann et al., 2018).

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

356 that emerged workers and males from the same colony harbour identical gut symbiont
357 communities since they are in contact with the same resources and nesting materials (Li et al.,
358 2021), but see Kapheim et al. (2015) and Krams et al. (2022). To our knowledge, there is no
359 study that directly compared the gut microbiota of bumble bee males and workers from indoor-
360 reared colonies, and hence potential discrepancies between sexes cannot be ruled out.

361 4.2. *Effects of sunflower pollen and its phenolamides on the gut bacterial community*

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

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

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

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

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

414 4.3. Effects of *Crithidia* infection on the gut bacterial community

415 In their host, gut parasites must compete with the gut symbionts for their spatial and
416 nutritional niches. Studies that assessed the *Bombus-Crithidia*-microbiota interaction are not
417 scarce, yet originally they highlighted the importance of the microbiota to face infection,
418 leaving aside the consequences of infection on the microbiota itself (Koch and Schmid-Hempel,
419 2011, 2012; Näpflin and Schmid-Hempel, 2016; Palmer-Young et al., 2018; Praet et al., 2018;
420 Barribeau et al., 2022). Studies then looked at correlations between bacterial diversity or
421 specific bacterial strain abundance and infection intensity (Cariveau et al., 2014; Koch et al.,
422 2012; Mockler et al., 2018; Näpflin and Schmid-Hempel, 2018; Palmer-Young et al., 2019).
423 Here, the presence of ileum-dwelling *Crithidia* sp. cells in bumble bee males did not impact the
424 alpha diversity but modified the relative composition of the gut bacterial community.
425 Differential analyses showed that *Bifidobacterium* was less abundant while *Lactobacillus* and
426 Enterobacteriaceae were more abundant in infected bumble bee males compared to uninfected
427 ones in the sunflower diet. Our results are in line with a previous study showing that *Crithidia*-
428 infected bumble bees harboured distinct gut microbiota when compared to uninfected ones
429 (Felden et al., 2021). However, the higher relative abundance of *Lactobacillus* and
430 Enterobacteriaceae in infected individuals is surprising given that these bacteria were shown to

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

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

452 4.4. *Interactive effects of sunflower pollen and Crithidia infection on the gut bacterial* 453 *community*

454 Our study is the first to report an interactive effect between pollen diet and parasite
455 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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512 **Conflict of interests**

513 None declared.

514 **Data Availability Statement**

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

517 **Ethical Approval Statement**

518 No specific approval was required for this study.

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848

849 **CRedit authorship contribution statement**

850 **Antoine Gekière:** Conceptualisation, Methodology, Formal analysis, Investigation,
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853 **Hettiarachchi:** Methodology, Investigation, Writing - Review & Editing. **Irène Semay:**
854 Methodology, Writing - Review & Editing. **Pascal Gerbaux:** Methodology, Resources,
855 Writing - Review & Editing. **Denis Michez:** Funding acquisition, Supervision, Writing -
856 Review & Editing. **Marie Joossens:** Methodology, Resources, Supervision, Writing - Review
857 & 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	<i>Bombiscardovia</i>
				<i>Bifidobacterium</i>
Pseudomonadota (synonym Proteobacteria)	Betaproteobacteria	Neisseriales	Neisseriaceae	<i>Snodgrassella</i>
	Gammaproteobacteria	Orbales	Orbaceae	<i>Gilliamella</i> <i>Schmidhempelia</i>
Bacillota (synonym Firmicutes)	Bacilli	Lactobacillales	Lactobacillaceae	<i>Bombilactobacillus</i> <i>Lactobacillus</i>

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 log₂ 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

914

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	<i>Bombiscardovia</i>
				<i>Bifidobacterium</i>
Pseudomonadota (synonym Proteobacteria)	Betaproteobacteria	Neisseriales	Neisseriaceae	<i>Snodgrassella</i>
	Gammaproteobacteria	Orbales	Orbaceae	<i>Gilliamella</i>
				<i>Schmidhempelia</i>
Bacillota (synonym Firmicutes)	Bacilli	Lactobacillales	Lactobacillaceae	<i>Bombilactobacillus</i>
				<i>Lactobacillus</i>

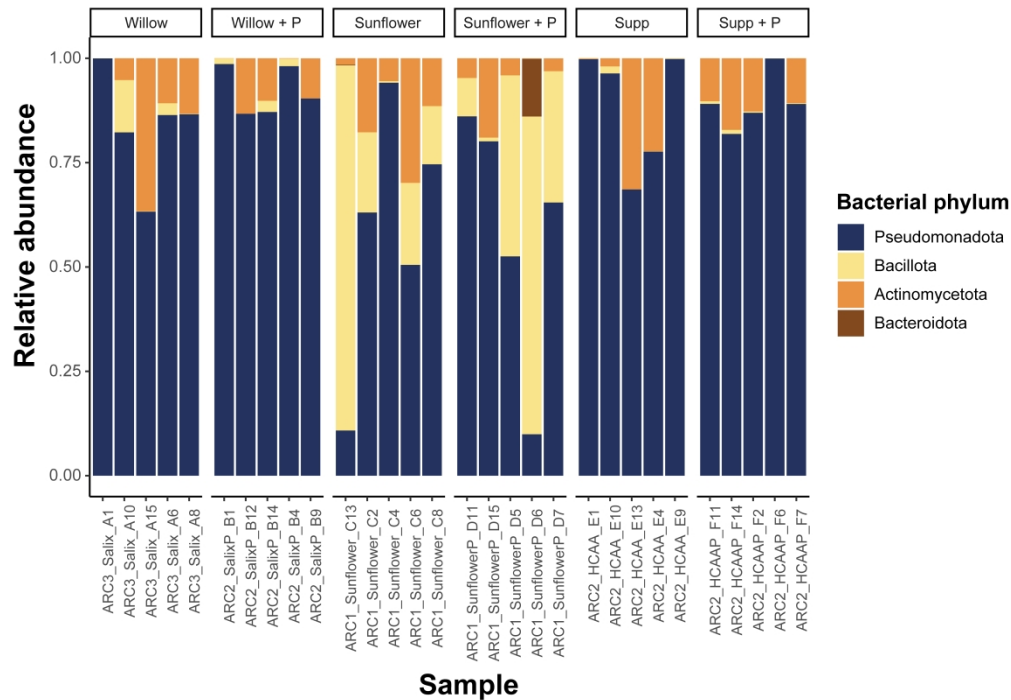


Figure 1. Phylum-level bar plots with relative abundances of all the bacterial ASVs found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). Willow. Microcolonies fed on *Salix* sp. pollen. Sunflower. Microcolonies 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.

212x150mm (700 x 700 DPI)

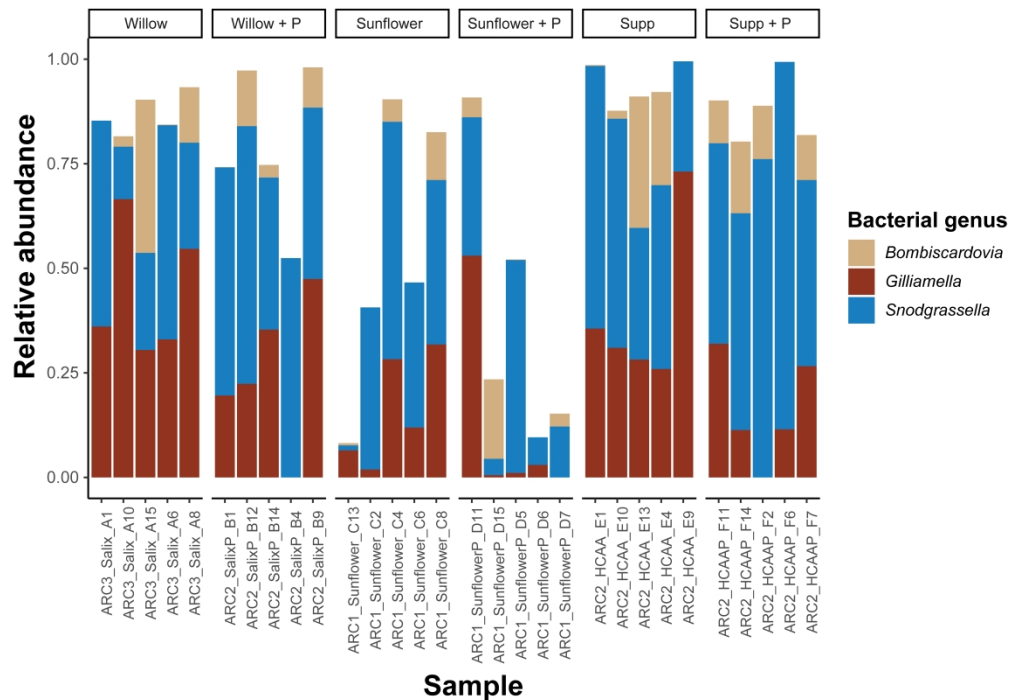


Figure 2. Genus-level bar plots with relative abundances of the five most abundant ASVs found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). Willow. Microcolonies fed on *Salix* sp. pollen. Sunflower. 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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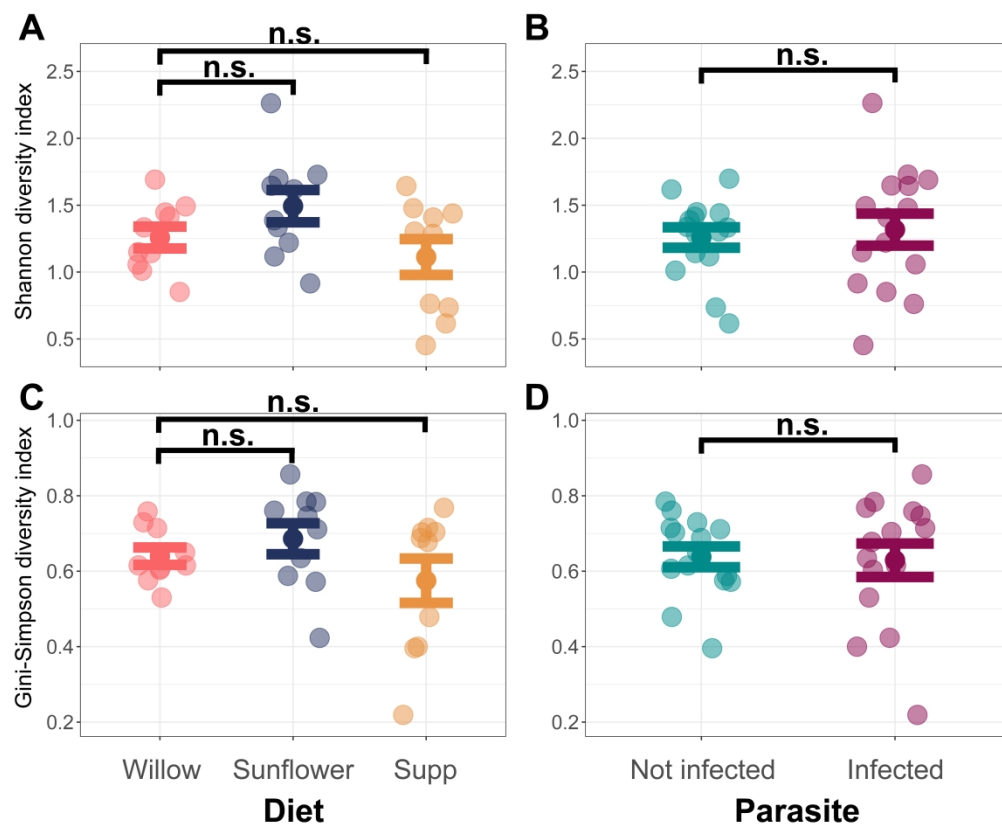


Figure 3. Alpha diversity of the bacterial communities found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). AB. Shannon diversity index taking into consideration the number of ASVs (i.e., richness) and 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.

283x234mm (600 x 600 DPI)

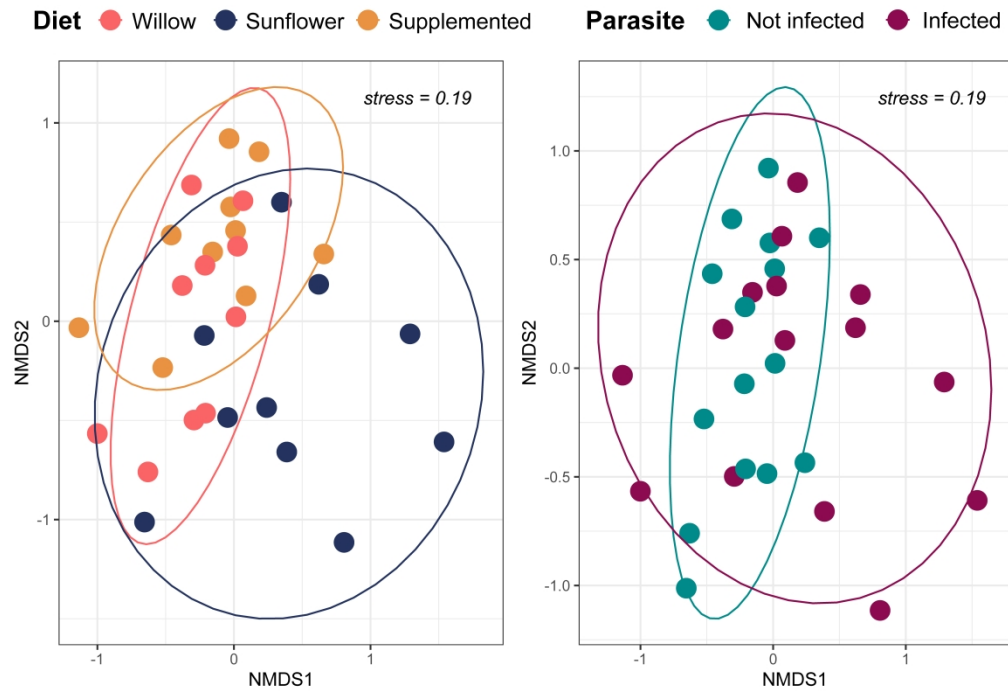


Figure 4. Nonmetric multidimensional scaling (NMDS) ordination based on the Bray-Curtis dissimilarity matrix of the bacterial communities found in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). A. Comparison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

206x142mm (700 x 700 DPI)

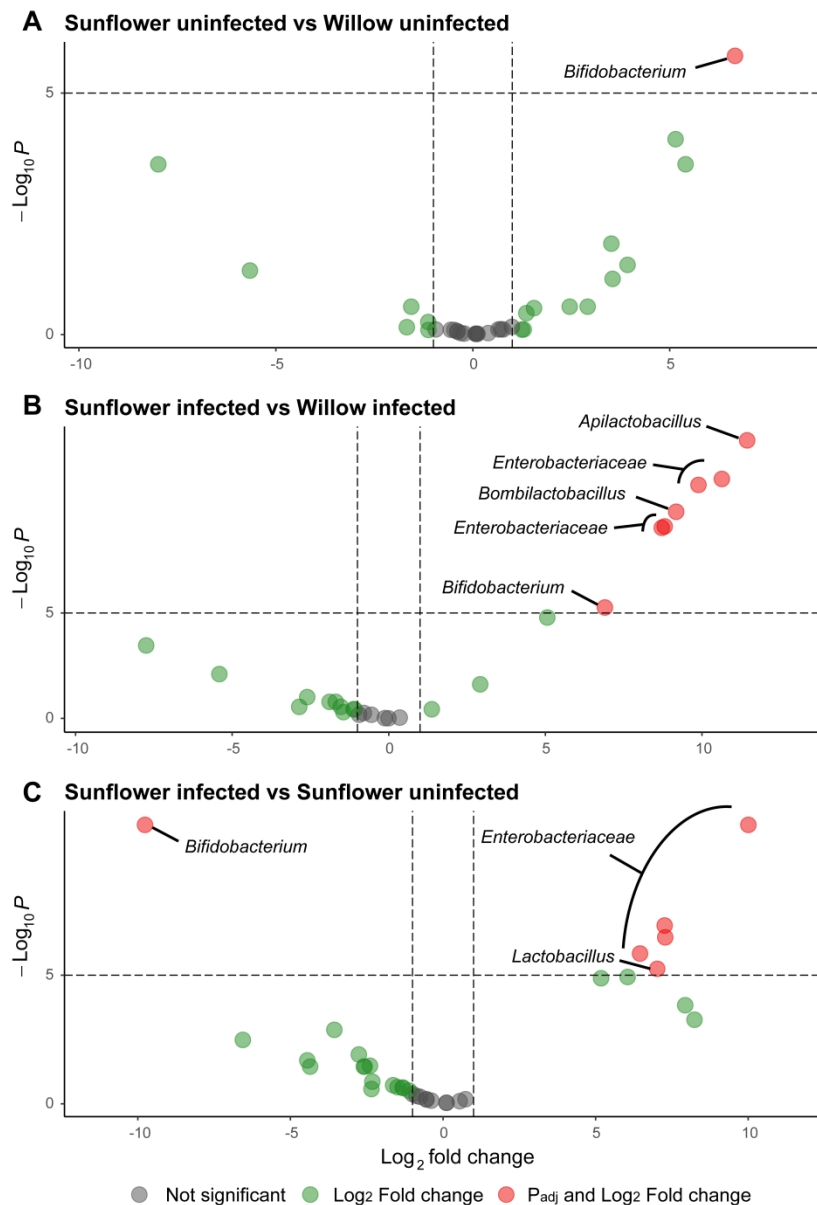


Figure 5. Volcano plots of significance (Wald test and Benjamini-Hochberg-adjusted p-value) versus log₂-fold change showing differentially abundant ASVs in gut samples of bumble bee males that emerged in infected and uninfected microcolonies fed on different pollen diets (five samples per treatment). 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)