Gut microbiota structure differs between honey bees in winter and summer

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Abstract

Honey bees harbor a relatively simple and highly conserved gut microbiota. Yet, little is known about the dynamics of the bee gut microbiota across seasons, especially in winter when adult worker bees live much longer and are critical for colony survival. We quantified the major community members of the bee gut microbiota in a single colony over two years and monitored 14 additional colonies during summer and winter. Our data shows that the total abundance of the gut microbiota in nectar/honey-feeding foragers was significantly lower compared to pollen-feeding nurses and winter bees. Strikingly, long-lived winter bees had the highest bacterial loads, with a characteristic shift towards a predominance of Lactobacillus Firm-5 and Bartonella apis. By colonizing microbiota-depleted bees with a community reconstituted from bacterial isolates, we provide experimental evidence that diet is a major contributor to the observed differences in bacterial loads between winter bees, nurses, and foragers. Overall, our study reveals that the gut microbiota of winter bees is remarkably different compared to foragers and nurses. Considering the importance of winter bees for colony survival, future work should focus on the role of the gut microbiota in health and disease in winter bees.

39 # Introduction

40 The European honey bee, *Apis mellifera,* is an important pollinator species for natural
41 ecosystems and agricultural production [1]. Its health status is threatened by numerous
42 factors including habitat loss, pesticide exposure, and high parasite and pathogen loads
43 [2–4]. Accumulating evidence point that the gut microbiota of adult honey bees plays a
44 critical role for bee health [5]. It converts dietary compounds [6, 7] and produces short
45 chain fatty acids [8] in the gut, enhances sucrose responsiveness of the host [8], and
46 stimulates the immune system [9, 10]. Moreover, disruption of the gut microbiota
47 composition by antibiotic treatment, pesticide exposure, or dietary manipulations was
48 associated with increased pathogen loads resulting in increased mortality [11–14].

49 A striking feature of the honey bee gut microbiota is its low taxonomic complexity. In
50 female worker bees, the community is dominated by seven to ten phylotypes (i.e. clusters
51 of strains sharing ≥97% sequence identity in the 16S rRNA gene), which typically make up
52 >95% of the bacterial cells in the gut [5, 15–18]. These phylotypes have been consistently
53 detected in honey bees, regardless of geographic location, life stage, or sampling season
54 [16, 19, 20], and are acquired horizontally through contact with nest mates and hive
55 components [21]. They include five core phylotypes (*Gilliamella, Snodgrassella alvi,*
56 *Lactobacillus* Firm-4 and Firm-5, and *Bifidobacterium*), which are present in all bees, and
57 two non-core phylotypes, i.e. *Frischella perrara* and *Bartonella apis,* which are highly
58 prevalent in colonies, but not in every bee [22]. On the other hand, phylotypes such as
59 Alpha 2.1, Alpha 2.2, or *Lactobacillus kunkeii* account for a relatively small proportion of
60 the gut community [23].

While these phylotypes are consistently present in adult worker bees, their abundance may change across bees influencing their impact on the host. For example, the type and amount of nutrients (i.e. pollen and nectar) available during the foraging season may impact the metabolic activity of the gut microbiota and therefore modulate its composition and function [24]. Likewise, distinct dietary habits or variation in life span of worker bees during summer and winter may influence gut microbiota composition. From spring to autumn, young worker bees (nurses) stay inside the hive to take care of larvae, and feed on nutrient-rich pollen, whereas older worker bees become foragers that feed on nectar and honey to fuel their energy-expensive flights [25]. In late autumn, newly emerged adult worker bees become winter bees (also called 'diutinus') that have an extended lifespan (~6 months) and that ensure the colony survival during the cold winter season in the absence of brood [26]. These bees form a tight cluster for thermoregulation inside the hive, feed strictly on food stores (pollen beebread and honey) and retain their feces all winter [27], which is likely to impact the ecology of their gut microbiota.

A number of studies have looked at the gut microbiota composition of different worker bee types or throughout seasons, with the overall conclusion that the community composition is relatively stable [19, 28–31]. However, previous studies were mostly based on comparative analyses of relative community member abundance using 16S rRNA amplicon sequencing. Such analysis cannot provide insights about the extent or directionality of changes in taxa abundance, especially if microbial loads vary substantially between samples [32]. In fact, a change in the total abundance of the microbiota could by itself be an important characteristic of different bee types, season, or environmental exposure. A striking example is the experimental exposure of bees to antibiotics which

did not result in a strong shift in the relative composition, but in an overall reduction of bacterial load, rendering bees more susceptible to pathogen invasion [11].

In addition to the limitations of current studies using relative abundance data, almost nothing is known about the gut microbiota of winter bees as compared to foragers or nurses. This is surprising, as winter bees are critical for colony health and survival during the cold season of the year, when resources are limited and most colony losses occur [33, 34].

Characterizing the gut microbiota structure of winter bees and identifying the factors that shape the gut communities may help us to understand the physiological adaptations that are necessary to survive the cold season of the year in temperate regions. In this study, we monitored the gut microbiota of 566 individual worker bees from a single hive over two years using a qPCR-based approach. We confirmed our initial findings by analyzing the microbiota of nurses, foragers, and winter bees from 14 hives in two subsequent years, and performed experiments with gnotobiotic bees to test the influence of diet on gut microbiota loads.

Materials and Methods

Sampling of honey bees

For monitoring the gut microbiota of worker bees over the period of two years, ~24 bees were collected monthly from a single hive located at the apiary of the University of Lausanne, Switzerland. During the foraging season, we sampled foragers returning to the hive entrance with pollen on their legs, while during the cold winter months, we sampled “winter bees” on top of the frames from inside the hive. Each sampling time point took

107 place at the middle of each month (+/- 3 days) between April 2015 and April 2017.
108 Samples from July 2017 were not included in the analysis due to a manipulation error
109 during DNA extraction.

110 To compare the gut microbiota of three different worker bee types (nurses, foragers, and
111 winter bees) across colonies, we sampled bees from 14 hives. Eleven hives were located
112 on the Dorigny campus of the University of Lausanne and three hives were located in the
113 village of Yens, about 17 km away. Foragers and nurses were sampled in July 2017 and
114 August 2018, and winter bees were sampled in January 2018 and January 2019. "Nurses"
115 and winter bees were collected inside the hive in the center of the colony. Foragers were
116 collected outside the hive at the same time point as nurses. The bees sampled in July
117 2017 and January 2018 were used for gut content visualization and correlation of gut
118 weight and 16S rRNA copy numbers. The guts of the bees sampled in August 2018 and
119 January 2019 were pooled (20 guts per bee type per hive) and used to monitor the
120 abundance of individual community members.

121 To dissect the guts, bees were anesthetized with CO_2 for 10 s, and the guts including crop,
122 mid- and hindgut and Malpighian tubules were carefully removed using sterile forceps.
123 For the monthly sampling of the single bee hive, each gut sample was placed in a drop of
124 PBS, scored for the scab phenotype [35], and placed in a bead beating tube containing
125 approx. 150 mg of glass beads (0.75-1 mm in diameter, Carl Roth) and 750 µL of CTAB
126 lysis buffer (0.2 M Tris-HCl, pH 8; 1.4 M NaCl; 0.02 M EDTA, pH 8; 2% CTAB, w/v, dissolved
127 at 56°C overnight; 0.25% β-mercaptoethanol, v/v). For the sampling of the 14 colonies in
128 August 2018 and January 2019, the dissected guts were pooled into a single Falcon tube

129 (14 hives x 3 bee types = 42 pooled samples). Tubes were flash frozen in liquid nitrogen
130 and stored at -80°C until DNA extraction.

131

132 **Experimental colonization of honey bees**

133 Microbiota-depleted bees were generated and colonized as described in Kešnerová et al.
134 [6]. The treatment group of bees fed on pollen and sugar water was the same as in this
135 previous study. The other treatment group (bees fed on sugar water only) was carried out
136 in parallel with the same batch of bees. Bees were fed *ad libitum* with sterilized bee
137 pollen and sterilized sugar water (50% sucrose, w/v) (SW+P treatment), or with only
138 sterilized sugar water (SW treatment). Bees were sampled 10 days after colonization and
139 the guts were dissected as described above.

140

141 **DNA extraction from honey bee gut tissue**

142 A previously established CTAB/phenol-based extraction protocol [6] was used to extract
143 DNA/RNA from individual guts. At the end of the protocol, the precipitated dried pellet
144 was resuspended in 200 µl and split into two samples à 100 µl. One sample was
145 processed with the Nucleospin PCR Clean-up kit (Macherey-Nagel, Germany) according to
146 the manufacturer's instructions and the resulting DNA was used for qPCR. For the pooled
147 samples containing 20 guts, 2 ml of glass beads and 15 ml of CTAB lysis buffer were added
148 to each Falcon tube. Samples were then homogenized in a Fast-Prep24^{TM}5G homogenizer
149 at 6 m/s for 40 s, briefly centrifuged, and an aliquot of 750 µl corresponding to the
150 sample volume of one bee gut was transferred to a new 2 ml bead beating tube with glass

151 beads and homogenized again. All further steps in DNA extractions were performed as
152 previously described [6].

153

154 **Quantitative PCR (qPCR) to determine absolute abundance of community members**

155 We used the same qPCR methods and data analysis as described in Kešnerová et al. [6]. In
156 short, to determine the absolute copy number (i.e. abundance) of each target in each
157 sample, we performed standard curves on serial dilutions of plasmids containing the
158 targeted sequence. The number of plasmid copies in these dilutions ranged from 10^1–10^7
159 copies per µl. The plasmid copy number was calculated from the molecular weight of the
160 plasmid and the DNA concentration of the purified plasmid measured with NanoDrop.
161 The slope and intercept of standard curves were calculated based on the Cq values
162 (quantification cycle [36]) obtained from the dilutions with 10^2 - 10^7 copies. For most
163 primer pairs, no amplification was obtained for the highest dilution, or the amplification
164 reached a similar Cq as the negative water control. As the *limit of detection* (LOD) of a
165 given primer pair, we consider the detectable Cq value of the highest plasmid dilution.
166 The target was considered absent in the sample when the Cq value was higher than the
167 LOD Cq. The *Efficiency of primers (E)* was estimated from the slope according to the
168 equation: $E = 10^{(-1/slope)}$ [37]. Primer characteristics and their performance are summarized
169 in **Table S1**.

170 All samples for which the Cq value of actin was >24 were excluded from the analysis, as
171 the extracted DNA was considered to be not of sufficient quality. For each DNA sample
172 that passed the initial quality check, we determined the number of bacterial cells per gut
173 as follows: We first calculated the 'raw' copy number (*n*) of each target in 1 µl of DNA

174 sample from the Cq value and the standard curve using the formula n = $E^{(intercept - Cq)}$ [38].
175 Then, we normalized bacterial 16S rRNA gene copies to the median number of actin gene
176 copies in a given dataset and corrected this number considering the total volume of the
177 samples (200µl) to obtain normalized copy numbers per gut (*Normn*): $Normn =$
178 $n_{target}/n_{actin} \times Median(n_{(actin)}) \times 200.$ Normalization with the actin gene was carried out to
179 reduce the effect of gut size variation and extraction efficiency. To infer the number of
180 microbial cells from the normalized 16S rRNA gene copy number we additionally divided
181 it by the number of 16S rRNA loci present in the genome of the given species (as listed in
182 **Table S1**).
183

184 **Statistics and diversity analyses of the gut microbiota composition**

185 All statistical analyses were performed using R (version 3.5.1). We measured alpha
186 diversity based on the absolute quantification of the seven dominant gut microbiota
187 phylotypes using effective number of species [39] that is calculated by taking the
188 exponent of Shannon's diversity index ("diversity function", from "Vegan" package in R
189 [40].
190 We performed a principal components analysis (PCA) with the *prcomp* function of the R
191 package "stats" to determine the similarity of the bacterial communities between
192 foragers and winter bees across the monthly sampling over two years using absolute
193 abundance measures of the seven gut microbiota phylotypes.
194 We tested the effect of bee type on bacterial loads, diversity indices and wet gut weight
195 using Student's t-test (in case of two group comparisons) or general linear models (in case
196 of three group comparisons). Since the residuals obtained for certain models showed
197 heteroscedasticity, we used a permutation approach to test the significance of the effects

198 as described before [41]. Briefly, we randomized the values of the response variable
199 10’000 times and computed the F-values/t-values for the tested effect for each
200 randomized dataset. The p-values corresponding to the effects were calculated by the
201 proportion of 10'000 F-values that were equal or higher than the observed one. Pairwise
202 comparisons between different factors were performed by Tukey’s HSD using
203 “multcomp“ package [42] using *glht* function on the model. P-values were adjusted using
204 the Bonferroni method.

205

206 # Results

207 ***Bacterial loads and community composition differ between foraging and winter season***
208 ***in a honey bee colony monitored over two years.***

209 To characterize the gut microbiota of worker bees across seasons, we tracked the total
210 abundance of five core (*Gilliamella*, *S. alvi, Bifidobacterium*, *Lactobacillus* Firm4, and
211 *Lactobacillus* Firm5) and two non-core members (*F. perrara* and *B. apis*; **Table S1**) in adult
212 worker bees from a single hive over two years. Our analyses included 566 individual bee
213 samples.

214 The core members *Gilliamella*, *S. alvi*, *Lactobacillus* Firm-5 and *Bifidobacterium* were
215 present in all analyzed bees, and the core member *Lactobacillus* Firm-4 was detectable in
216 98.4% of all bees (**Supplementary Figure S1A**). Notably, the two designated non-core
217 members *B. apis and F. perrara* were also present at relatively high prevalence with
218 94.7% and 73.1%, respectively (**Supplementary Figure S1B** & **C**). Consistent with our
219 previous results, *F. perrara* prevalence strongly correlated with the presence of the scab
220 phenotype (**Supplementary Figure S2**), a local melanization response that is induced by
221 *F. perrara* upon colonization [35].

222 The absolute abundance of the monitored phylotypes varied little among the bees
223 sampled at the same time point, with the exception of the non-core members *F. perrara*
224 and *B. apis* (**Supplementary Figure S3**). However, there were clear differences in bacterial
225 abundances between months (Permutation ANOVA P=1e-4) for all microbiota members.
226 In particular, we observed remarkable differences in the bacterial loads between bees
227 sampled during the foraging and the winter season in both years. This became evident
228 from the abundance of individual phylotypes and from the total bacterial load as based

229 on the summed abundances of all seven phylotypes (**Supplementary Figure S3, Figure**
230 **1A**). Specifically, we found a 10- to 100-fold increase in the levels of the core members
231 Firm-4, Firm-5, and *Bifidobacterium*, as well as the non-core members *B. apis* when
232 comparing across all winter bees relative to foragers (**Figure 1B**, Permutation T-Test P=1e-
233 4). We also observed a small increase of *S. alvi* levels in winter bees (**Figure 1B**,
234 Permutation T-Test P=6e-4), but no difference in the levels of *Gilliamella* (**Figure 1B**,
235 Permutation T-Test P=0.7). *F. perrara* was the only member of the community which
236 displayed the opposite trend, i.e. lower abundance in winter bees (**Figure 1B**,
237 Permutation T-Test P=1e-4). The overall bacterial load was about 10x larger in winter
238 bees than in foragers as based on both the summed abundances of all seven phylotypes
239 (**Figure 1B**, Permutation T-Test P=1e-4) as well as the number of universal 16S rRNA gene
240 copies, which was determined for a subset of the samples (**Figure 1C**, Permutation T-Test,
241 P=1e-4).

242 Considering that the monitored phylotypes comprise the majority of the detected species
243 in the honey bee gut microbiota, we analyzed the diversity of microbial communities
244 based on our dataset neglecting the presence of other (likely very low-abundant) non-
245 core members. In both years, the communities of winter bees were largely dominated by
246 the phylotypes Firm-5 and *B. apis.* In contrast, forager bees seem to have more even
247 community compositions (**Figure 1D**). The dominance of Firm-5 and *B. apis* in winter bees
248 was reflected by the lower effective number of species (calculated from Shannon's
249 diversity index) in winter bees compared to foragers (**Figure 1E**, Permutation T-Test,
250 P=1e-4). Moreover, PCA revealed a clear separation between foragers and winter bees
251 (**Figure 1F,** MANOVA Wilks =0.6 $F_{(7, 392)}$=39.4, P<2.2e-4) along the principal component 1

252 (PC1). This separation was driven by Firm-4, Firm-5, *B. apis* and *Bifidobacterium*, the four
253 phylotypes with the largest differences in abundance between the two types of bees
254 (**Figure 1B**).

255 Taken together, these results suggest that the gut microbiota of winter bees and foragers
256 markedly differ from each other in the monitored hive, both in terms of the absolute
257 bacterial abundance and the relative community composition, with a few phylotypes
258 dominating the community in winter bees.

259

260 ***Consistent difference in bacterial loads and community composition between foragers,***
261 ***nurses, and winter bees across colonies***

262 The observed differences in bacterial loads between foragers and winter bees prompted
263 us to check for similar patterns across 14 different hives in two subsequent years. In
264 addition to foragers and winter bees, we also monitored the bacterial loads in nurses,
265 because this would help us understand whether the differences between foragers and
266 winter bees are linked to different seasons or to behavioral or dietary differences.

267 Strikingly, we found that the pooled gut samples from the fourteen different hives
268 showed a similar pattern as the individual samples of the hive monitored over two years.
269 Winter bees had higher bacterial loads than forager bees based on the summed
270 abundances of the seven monitored phylotypes (**Figure 2A,** Permutation ANOVA followed
271 by Tukey P=3.782-10) and the overall bacterial quantification with the universal 16S rRNA
272 primers (**Figure 2B,** Permutation ANOVA followed by Tukey, P=5.1e-9). As for the
273 previously monitored hive, *Bifidobacterium*, *Lactobacillus* Firm4, *Lactobacillus* Firm5, and
274 *B. apis* had increased levels (Permutation ANOVA followed by Tukey, P=1e-4), while *F.*

275 *perrara* had lower levels in winter bees compared to foragers (**Figure 2A,** Permutation
276 ANOVA followed by Tukey P=3.36e10-3). The only two phylotypes which showed
277 abundance patterns inconsistent with the results from the previous hive were *S. alvi* and
278 *Gilliamella*. *S. alvi* did not experience any differences in abundance (**Figure 2A,**
279 Permuation ANOVA P=5.52e-1), while *Gilliamella* had increased levels in winter bees as
280 compared to foragers (**Figure 2A,** Permutation ANOVA followed by Tukey P=4.33e-08).
281 Interestingly, we also found that nurse bees had increased bacterial loads compared to
282 foragers (**Figure 2A,** P=1.33e-5; and **Figure 2B**). The levels of *Gilliamella* and Firm4 in
283 nurse bees were as high as in winter bees, while the levels of *Bifidobacterium,*
284 *Lactobacillus* Firm5*, and B. apis* were at intermediate levels between foragers and winter
285 bees (see **Supplementary Table S2** for statistics).
286 The differences of the gut microbiota of foragers, nurses, and winter bees were also
287 evident from the analysis of the relative abundances at the community level. *B. apis* and
288 *Lactobacillus* Firm5 were both more dominant in winter bees as compared to foragers,
289 while in foragers the phylotypes *F. perrara* and *S. alvi* accounted for a larger proportion of
290 the community (**Figure 2C,** see **Supplementary Table S2** for statistics). In the PCA,
291 foragers and winter bees clustered separately along PC1, explaining 57.3% of the
292 variation in the data (**Figure 2D**). The communities of most nurse samples were less
293 dominated by *B. apis* than those of winter bees (**Figure 2C**). However, they still clustered
294 more closely with the winter bees than the foragers (**Fig. 2D,** MANOVA Wilks =0.1,
295 $F_{(14,66)=}$7.9, P=1.7e-9).
296 In summary, our analysis across 14 hives suggests that the differences in bacterial loads
297 and community structure between winter bees, foragers, and nurses is a general pattern,

298 and that the microbiota of winter bees resembles more closely the one of nurses in terms
299 of absolute bacterial abundances.
300
301 ***Pollen diet increases gut community size in gnotobiotic bees***
302 One of the drivers of the observed differences in bacterial load and community structure
303 between winter bees, foragers, and nurses could be diet. Dietary differences between the
304 three types of bees were clearly visible from the dissected guts (**Figure 3A-C**). The
305 rectums of winter bees and nurses appeared yellow indicating the presence of pollen,
306 while the rectums of forager bees appeared translucent indicating the absence of pollen.
307 Moreover, the wet weight of the guts was significantly different between the three types
308 of bees (ANOVA $F_{(2,68)}$ = 24.13, P= 1.205e-8), with foragers having on average two times
309 lighter guts than nurses (Tukey P=1.14e-6) and winter bees (Tukey P=6.81e-8) (**Figure 3D**).
310 When plotting normalized 16S rRNA gene copy numbers as a function of gut weight, we
311 found that gut weight positively correlated with total microbiota abundances across the
312 three bee types (**Figure 3E**).

313 In order to demonstrate that pollen diet is directly associated with increased bacterial
314 loads in honey bees, we experimentally colonized newly emerged bees with a community
315 of 11 bacterial strains representing the seven major bacterial phylotypes of the bee gut
316 microbiota [6]. The colonized bees were kept in the laboratory for ten days and fed *ad*
317 *libitum* either sterile sugar water and pollen (SW+P treatment), or sugar water only (SW
318 treatment). We found a significant difference in the gut weights between the two
319 treatments (**Figure 3F**, Welch's T-Test *t*=9.433, P=1.452e-11). While the gut weights of the
320 bees of the SW treatment were comparable to those of forager bees, the gut weights of

321 the bees of the SW+P treatment were markedly higher, exceeding even those of winter
322 bees (**Figure 3D** & **3F**). Strikingly, we observed a positive correlation between gut weight
323 and microbiota abundance for both the colonized bees in the laboratory (**Figure 3G**) and
324 the worker bees sampled from the hive (**Figure 3E**). Moreover, differences in bacterial
325 loads of individual community members between the two experimental treatments
326 mirrored, to a large extent, the difference found between winter bees, nurses, and
327 foragers: most phylotypes were more abundant in bees fed on pollen as compared to
328 bees fed sugar water only (**Figure 3H**, see **Supplementary Table S2** for statistics). Two
329 exceptions were *B. apis* and *F. perrara.* While *B. apis* had similar levels between the two
330 experimental treatments **(Figure 3H)**, its abundance was higher in winter bees and nurses
331 as compared to foragers **(Figure 2A)**. Interestingly, *B. apis* was able to colonize only 75%
332 of all bees when pollen was absent. The dependence on pollen for gut colonization was
333 even more pronounced for *F. perrara.* Less than 50% of the experimentally colonized bees
334 of the SW treatment had detectable levels of *F. perrara,* and the loads in bees that were
335 colonized were relatively low. In contrast, bees of the SW+P treatment were all colonized
336 and had relatively high and consistent loads *of F. perrara* **(Figure 3H)***.*

337 Taken together, these results show that a pollen diet leads to an increase in gut weight
338 and overall bacterial load providing a plausible explanation for some of the differences in
339 the loads observed between foragers, nurses, and winter bees.

340 ## Discussion

341 In contrast to 16S rRNA amplicon sequencing, qPCR provides information about the
342 absolute abundances of bacteria and therefore allows determining whether individual
343 community members increase, decrease, or remain the same in terms of bacterial cell
344 number across samples. For example, in our study, the relative abundance of *S. alvi* went
345 down in winter bees as compared to foragers and nurses (**Figure 2C**). However, this effect
346 was not due to a decrease of the total number of *S. alvi* cells, but rather an increase of
347 other community members as identified by qPCR. In fact, the total abundance of *S. alvi*
348 remained the same in foragers, nurses, and winter bees **(Figure 2A)**. Such quantitative
349 microbiome profiling approaches can reveal important associations between bacterial
350 symbionts and the host, as previously demonstrated for the human microbiota [32] or the
351 microbiota of caterpillars [43]. We argue that absolute abundances should be routinely
352 assessed when analyzing microbial communities, as the absolute number of cell of a
353 bacterium - not its relative abundance – may determine its impact on ecosystem
354 processes.

355 Noteworthy, qPCR is a targeted approach, i.e. we can only quantify members for which
356 specific primer sets have been designed. This is a limitation for microbial communities
357 that are highly diverse. However, in the current study, we took advantage of the fact that
358 the honey bee gut microbiota consists of a relatively simple community typically
359 dominated by the seven analyzed phylotypes [5, 22, 23, 44–46]. Hence, while our
360 approach did not cover some of the non-core members that are sporadically present in
361 the bee gut, we are confident that the majority of the diversity of the bee gut microbiota
362 has been quantified. This is supported by the fact that the community profiles obtained

363 from the qPCR data looked very similar to those obtained in previous studies based on
364 16S rRNA gene amplicon sequencing [21, 28, 47–49].

365

366 Almost all members of the gut microbiota had increased levels in winter bees when
367 compared to foragers, and many also showed increased levels when compared to nurses.
368 The most pronounced differences were found for *Lactobacillus* Firm-5 and *B. apis,*
369 resulting in a remarkable shift of the community towards these two phylotypes.
370 Strikingly, this shift was observed across fourteen different colonies and in three different
371 years. Hence, the "reconfiguration" of the microbiota in winter bees seems to be a
372 conserved feature in colonies in Western Switzerland. However, regional differences in
373 floral diversity [24] or climate may influence these changes. Therefore, additional surveys
374 of winter bees in other geographic regions are needed to test for the conservation of this
375 pattern. Interestingly, a recent study carried out in Germany on the effects of winter
376 supplementation feeds also revealed that the relative abundance of *Lactobacillus* Firm-5
377 (and in one treatment group also the relative abundance of *B. apis*) markedly increased in
378 winter bees compared to foragers [19], resulting in a community shift. The results of this
379 study were also consistent with our findings in that the levels of *F. perrara* were
380 significantly lower in winter bees as compared to forager bees.

381

382 What drives the changes in bacterial loads and community composition in winter bees? A
383 possible explanation could be dietary differences between the analyzed bee types. It is
384 well known that foragers mainly feed on nectar and honey, while nurses and winter bees
385 also consume pollen [50]. These dietary differences were also evident from the three bee

386 types sampled in our study based on consistent changes in appearance and weight of the
387 gut (**Figure 3A-C**). Strikingly, our bee colonization experiment with a defined bacterial
388 community showed that the presence of pollen in the diet substantially increased gut
389 weight and bacterial loads to levels comparable to those in winter bees, while the levels
390 in bees fed on sugar water were more similar to those of foragers (**Figure 3F-H**).
391 Therefore, we conclude that diet is an important factor that can explain many of the
392 differences observed between worker bee types. Seasonal changes in gut microbiota
393 composition in wild rodent populations [51] and humans [52, 53] have also been found to
394 coincide with dietary shifts, which is in agreement with the general notion that dietary
395 preferences is the main driver of community differences across a wide range of animals
396 [54–56].

397 In the case of honey bees, the larger amount of food in the gut is likely to increase the
398 carrying capacity for the gut microbiota. In addition, pollen is a more nutrient-rich diet
399 than nectar, honey, or sucrose offering a larger diversity of different metabolic niches for
400 gut bacteria. Both factors are likely to contribute to the increased bacterial loads in bees
401 fed on pollen as compared to those fed on sugar water only. The role of pollen as a main
402 driver for the observed differences is also supported by the fact that the bacteria with the
403 largest increase in total abundance (*Lactobacillus*, *Bifidobacteria*, *B. apis*) are located in
404 the rectum, which is the last part of the hindgut where pollen accumulates until bees
405 defecate. In line with these, a previous report showed that the abundances of total
406 bacteria, as well as certain individual phylotypes (*Lactobacillus* Firm-5, *Bifidobacterium*)
407 increase in rectum upon pollen consumption [57]. However, this increase was dependent
408 on the age factor and it was not significant when sterile pollen (similar to our pollen

treatment) was used instead of stored pollen [57]. In contrast to our study the experimental bees were not inoculated by bacteria [57], which may greatly impact community growth and dynamics. Overall, despite certain experimental differences, our results seem overall to be consistent with the data that has been published before.

However, not all changes observed in winter bees could be recapitulated in our colonization experiment. For example, the differences observed *in B. apis* levels between foragers, nurses, and winter bees were not observed in the experimental bees that were fed with or without pollen. Another example is *F. perrara*. While this bacterium was less abundant in winter bees than in foragers and nurses, the colonization success of *F. perrara* was largely dependent on the presence of pollen in the experimental bees. This suggests that other factors may contribute to community differences found in winter bees as compared to foragers or nurses. First, winter bees have an extended lifespan with an average life expectancy of ~6 months as compared to ~4 weeks in the case of summer bees (i.e. nurses and foragers) [58]. In contrast, the bees of the colonization experiment were age-matched and sampled ten days after emergence. It is well known that the physicochemical state of the gut changes as animals age, modulating their gut microbiota [59–61]. Second, winter bees feed on pollen that has been stored in the hive for several weeks to months. It has previously been shown that the consumption of an aged pollen-diet affects the gut microbiota composition of nurses, causing dysbiosis and higher mortality [12]. Third, winter bees retain their feces in the gut for extended periods of time, which likely affects the physico-chemical conditions and the availability of nutrients in the gut. Forth, the body temperature of bees is at ~21°C during winter and at ~35°C

432 during summer [62]. This may influence bacterial growth rates. Indeed, in a recent
433 metagenomic study, it was shown that gut bacteria have lower average population
434 replication in old winter bees as compared to young nurse bees, which is indicative of
435 decreased replication rates [18]. Fifth, winter bees show reduced expression of immune
436 genes [63–66], and have an altered protein metabolism [67].

437

438 How the different factors influence the dynamics of specific gut microbiota members
439 should be the focus of future studies. In the case of *F. perrara* it is tempting to speculate
440 that the decrease in colonization levels in old winter bees, may be a consequence of the
441 specific immune response elicited by the host towards this bacterium [10], eliminating it
442 from the gut as the bee ages. Another interesting finding is that the levels of *S. alvi* barely
443 changed across worker bee type or the two diet treatments in the experiment. This
444 suggests that the colonization of *S. alvi* is not modulated by the dietary state, the
445 physicochemical conditions in the gut, or the abundance of other community members. A
446 possible explanation could be that the niche of *S. alvi* is dependent on the host rather
447 than the diet, because it selectively colonizes the epithelial lining of the ileum and was
448 suggested to rely on the presence of oxygen for growth released from the host
449 epithelium [8, 44, 68].

450

451 The two phylotypes that predominate in winter bees, *Lactobacillus* Firm5 and *B. apis*,
452 seem to profit from the specific gut conditions in winter bees. The primary metabolism of
453 *Lactobacillus* Firm5 is fermentation of sugars or sugar derivatives into organic acids [22,
454 69]. Genomic analysis revealed that these bacteria harbor a large diversity of glycoside

455 hydrolases and phosphotransferase transporters for the cleavage and transport of sugars.
456 Moreover, a recent metabolomics study has found that flavonoids, secondary plant
457 metabolites that are abundant in pollen, are prominent substrates of these bacteria. In
458 contrast to *Lactobacillus* Firm5, *B. apis* encodes a complete TCA cycle and harbors a
459 respiratory chain. Some strains of *B. apis* also possess genes for the degradation of
460 aromatic compounds. It is possible that metabolic interactions among these two
461 particular phylotypes or the extended gut-retention time of pollen (resulting in the
462 availability of additional nutrients) facilitates their increased abundance in winter bees.

463

464 Most of the recent colony losses occur during the winter months [33, 34]. Consequently,
465 winter bees are highly critical for colony survival and a better understanding of the
466 factors influencing their health status - including the gut microbiota - is needed. In
467 summary, our analysis revealed that the gut microbiota of winter bees undergo
468 characteristic shifts. These changes may have important consequences for the host. For
469 example, they may influence the physiology or metabolic state of the host by optimizing
470 the energy uptake [70], or increase colonization resistance against pathogens. Therefore,
471 future studies should specifically focus on the functional role of the gut microbiota in
472 winter bees, and colony health.

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Competing Interests

Authors declare to have no conflicts of interest.

Data accessibility

All scripts and datasets can be found on:

https://drive.switch.ch/index.php/s/GsX9TBGcNJ8WEuv

Supplementary Material

Supplementary Figures: Contains Figures S1-S3

Supplementary Table S1: Contains information on the primers that are used in this study.

Supplementary Table S2: Contains the results of the statistical tests used in this study.

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Figures and Figure Legends

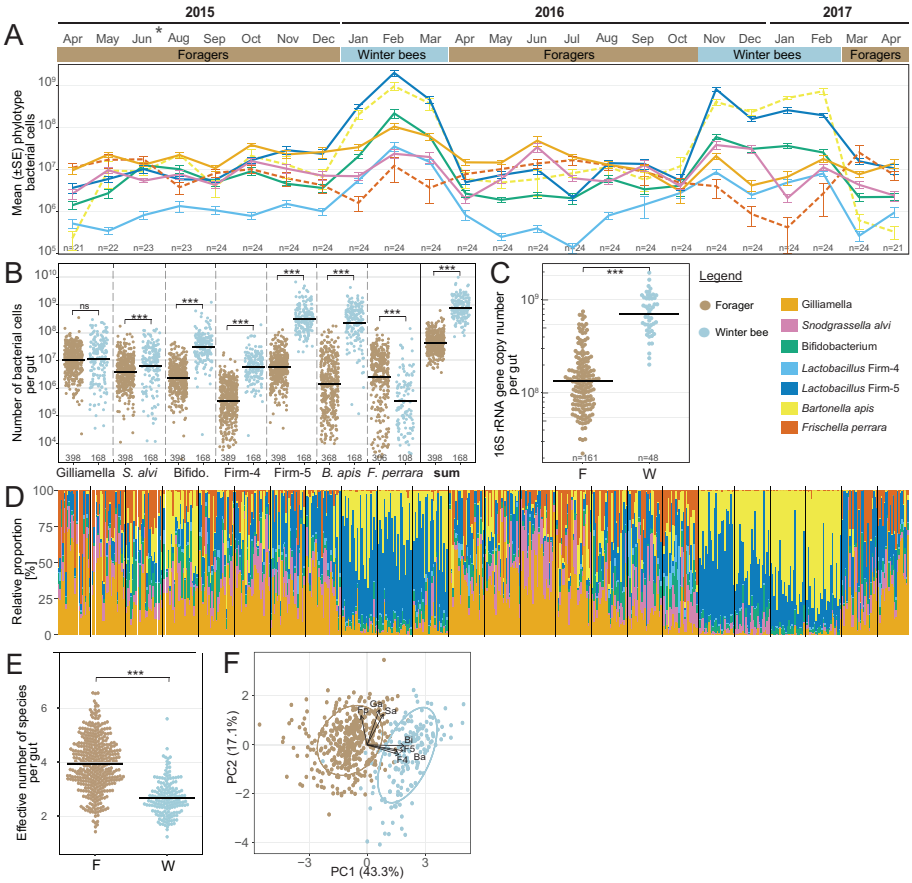
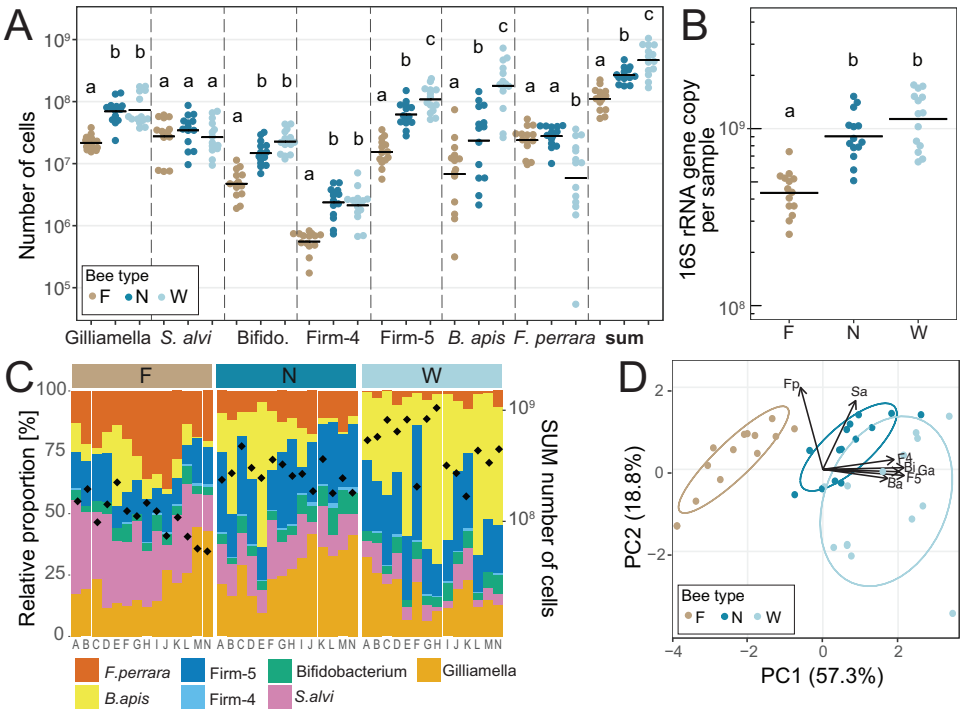


Figure 1. Gut bacterial communities differ between foraging and winter season in a single colony monitored over two years. (A) Monthly changes in the absolute abundance of the seven phylotypes monitored over a period of two years depicted as mean values (±SE) of the analyzed bees. The number of bees per month is indicated. Asterisk indicates missing data for July 2015 due to extraction failure. **(B)** Number of cells per individual honey bee gut in foragers (F) and winter bees (W) for each bacterial phylotype and their sum. Mean values are shown as black horizontal lines. Only bees with detectable levels were plotted (number of bees is given at the bottom of the plot; for prevalence see **Supplementary Figure S1**). **(C)** Copy number of the 16S rRNA gene in gut samples of a subset of the analyzed months (Apr 2015, Aug 2015, Oct 2015, Jan 2016, Apr 2016, Jul

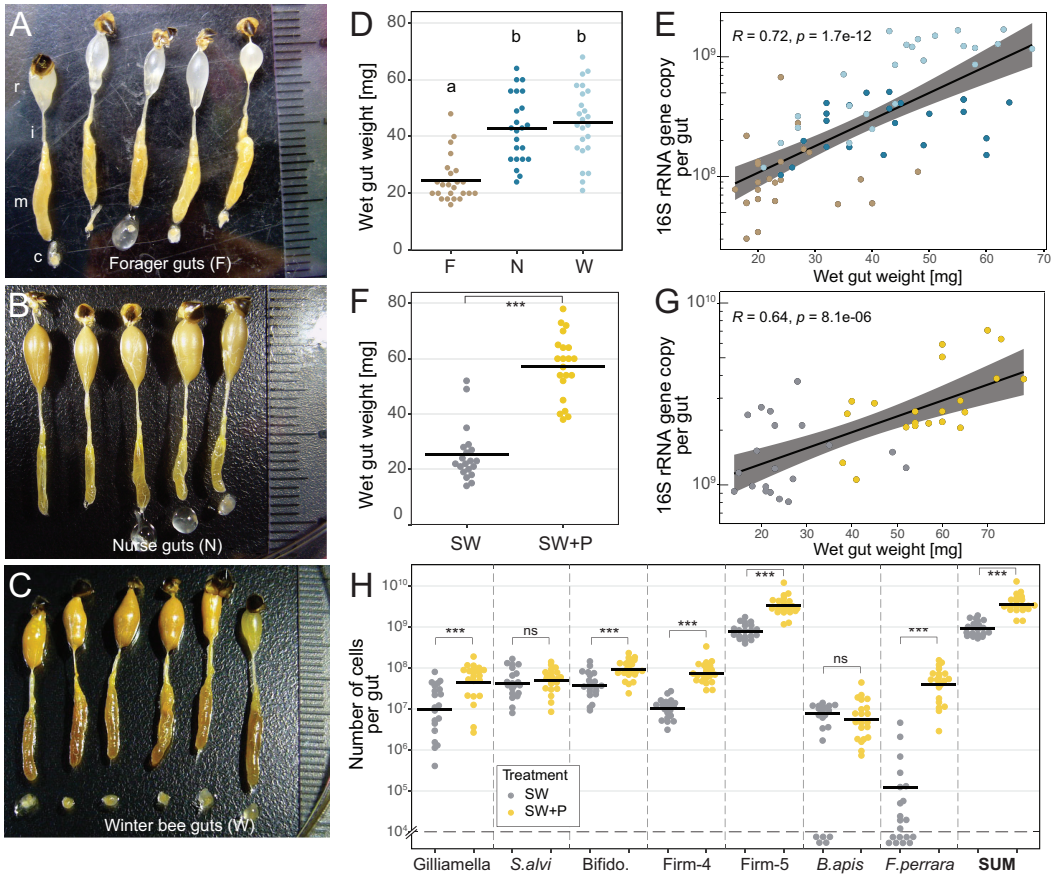
679 2016, Oct 2016, Jan 2017, Apr 2017). **(D)** Relative community composition of the gut
680 microbiota of bees sampled in each month. Community composition is based on the
681 abundances of the seven monitored phylotypes **(E)** Shannon's diversity index calculated
682 from cell numbers of different bacterial phylotypes in foragers (F) and winter bees (W).
683 **(F)** Projection of the abundances of monitored phylotypes into first and second principle
684 components in all analyzed bees, together with correlation vectors representing variables
685 driving the separation on both axes. Permutation T-Test was used for pairwise
686 comparisons. ns, non-significant; ***, P < 0.001.



687

688 **Figure 2. Gut community size and structure differ between foragers, nurses, and winter**
689 **bees across 14 colonies.** Data from 20 pooled guts sampled from foragers (F), nurses (N)
690 and winter bees (W) across multiple hives (n=14). **(A)** Normalized cell numbers (see
691 methods) of the seven monitored phylotypes and their summed abundances. (**B**) 16S
692 rRNA gene copy numbers per sample. (**C**) Relative community composition of the gut

693 microbiota based on the abundances of the seven monitored phylotypes (left y-axis).
694 Black diamonds indicate the sum of the total abundances of seven monitored phylotypes
695 (right y-axis). Capital letters below the stacked bars indicate the hive of origin. **(D)**
696 Projection of the abundances of monitored phylotypes into first and second principle
697 components in the pooled samples. Each dot represents a different hive. Correlation
698 vectors represent different variables driving the separation on both axes. Black lines show
699 mean values. Different letters indicate groups that are significantly different from each
700 other based on Tukey's post-hoc tests following Permutation ANOVA. See **Supplementary**
701 **Table S2** for full statistics results.



702

703 **Figure 3. Diet is a major factor shaping the gut communities of honey bees. (A-C)**
704 Dissected guts of foragers **(A)**, nurses **(B)**, and winter bees **(C)** consisting of the crop (c),

705 (missing in some samples), the midgut (m), the ileum (i), and the rectum (r) with attached
706 stinger and last abdominal tergite. **(D)** Wet gut weight of individual foragers (F), nurses
707 (N), and winter bees (W). **(E)** Spearman correlation between gut weight and bacterial
708 loads (assessed with universal bacterial 16S rRNA primers) across all bee types. The grey
709 area indicates the 95% confidence interval. **(F)** Wet gut weight of experimentally
710 colonized bees that were fed either sugar water (SW) or sugar water and pollen (SW+P).
711 **(G)** Spearman correlation between gut weight and bacterial loads (assessed with
712 universal bacterial 16S rRNA primers) across the two treatment groups of colonized bees.
713 **(H)** Total abundances of the seven monitored phylotypes in the two treatment groups of
714 colonized bees. Bees with loads below the detection limit of the qPCR method are shown
715 below the dashed line. Different letters indicate groups that are significantly different
716 from each other based on Tukey's post-hoc tests following Permutation ANOVA. Two-
717 group comparisons were done by Permutation T-Test. ns, non-significant; ***, $P < 0.001$.
718

Supplementary Material

Supplementary Figures

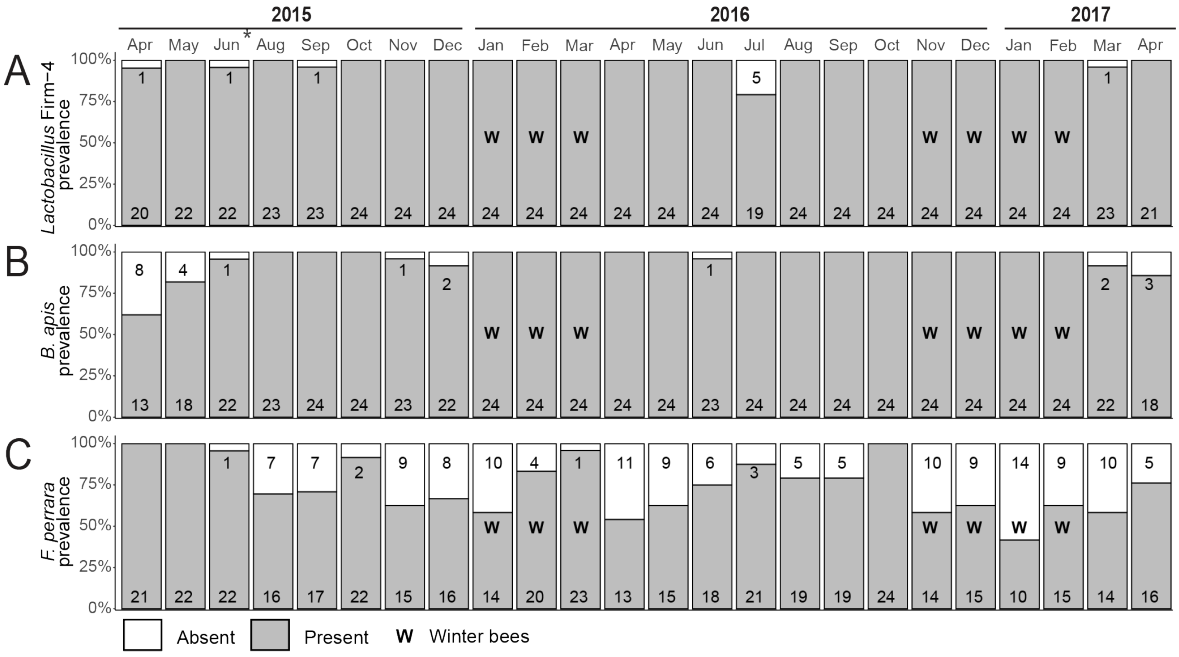


Figure S1. Prevalence of phylotypes that were not detect in every honey bee of the colony sampled over two years. Monthly prevalence (i.e. the proportion of bees in which the bacterial phylotype was detected in a given month) of **(A)** *Lactobacillus* Firm-4, **(B)** *B. apis* and **(C)** *F. perrara*. The targetted bacterium was considerred as absent when the qPCR signal was below the detection limit of the primer set (see Supplementary Table S1). The numbers in the upper and lower part of the bar plots indicate the number of bees in which the target is absent and present, respectively. Asterisk indicates missing data for July 2015 due to a sampling/extraction error.

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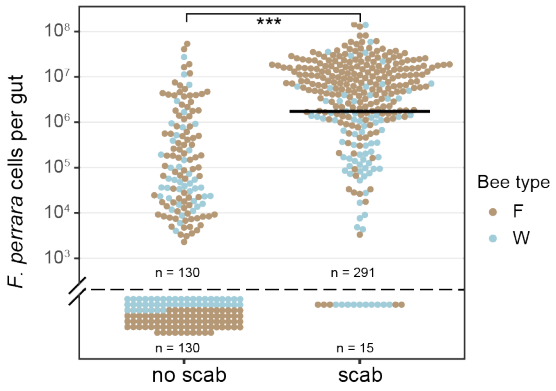

732

733 **Figure S2. *F. perrara* loads correlate with scab presence.** *F. perrara* normalized number

734 of cells (see methods) in guts of foragers (F) and winter bees (W) with or without scab

735 phenotype in the pylorus region. Data points under the black dashed line correspond to

736 samples with no detectable level of *F. perrara* as based on the detection limit of our qPCR

737 method. Statistics: permutation T-Test.

738

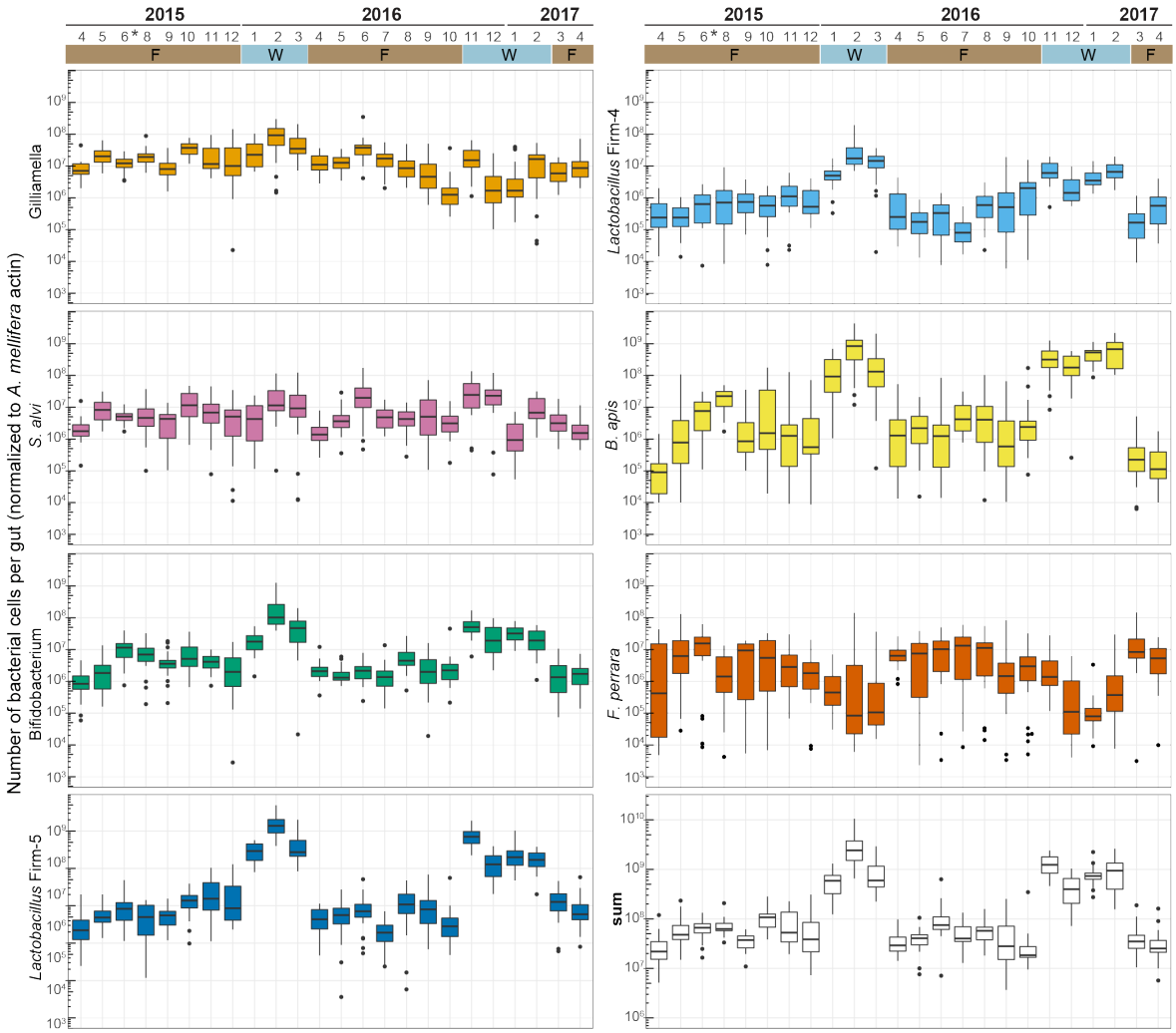

Fig. S3 Monthly trends in the abundance of honey bee-specific bacteria.

Absolute abundance of the seven monitored phylotypes in individual foragers and winter bees sampled over two years: Gilliamella, *S. alvi*, Bifidobacterium, Lactobacillus Firm-5, Lactobacillus Firm-4, *B. apis*, and *F. perrara*. The last plot shows the summed abundance of each bee. Only bees with detectable levels were considered. Bees with loads below the detection limit of the qPCR method are shown below the dashed line. Asterisk indicates missing data for July 2015 due to a sampling/extraction error.

748 **Supplementary Tables**

Target (gene name and accession no.)	Sequence (5' to 3')	Tm (°C)[a]	Amplicon size	# Gene loci per genome[b]	Standard curve			Reference
					Efficiency, R^2	Slope, intercept	LOD Cq[c] (# copies)	
Apis mellifera (*actin*, AB023025)	**F:** TGCCAACACTGTCCTTTCTG **R:** AGAATTGACCCACCAATCCA	58.4 56.4	156 bp	-	1.896 = 89.6 %, 1.0	-3.6, 37.579	33.726 (10)	[1]
Gilliamella (*16S rRNA*, JQ936674)	**F:** CTTTGTTGCCATCGGTTAGGCC **R:** CCGCTTGCTCTCGCGAGG	64.2 62.9	160 bp	4	1.86 = 86 %, 0.997	-3.709, 40.807	37.026 (10)	[2]
Frischella perrara (*16S rRNA*, JX8783306)	**F:** GGAAGTTATGTGTGGGATAAGC **R:** CTATTCTCAGGTTGAGCCCG	60.1 60.5	185 bp	4	1.946 = 94.6 %, 0.995	-3.457, 38.042	31.704 (100)	[3]
Snodgrassella alvi (*16S rRNA*, JQ746651)	**F:** CTTAGAGATAGGAGAGTGCCTT **R:** AACTTAATGATGGCAACTAATGACAA	60.1 60.1	132 bp	4	1.966 = 96.6 %, 0.999	-3.406, 38.236	31.256 (100)	[4], modified
Bartonella apis (*16S rRNA*, KP987885)	**F:** GTGGGAATCTACCTATTTCTACG **R:** AACGCGGGCTCATCTATCTC	60.9 60.5	103 bp	2	2.051 = 105.1 %, 0.997	-3.205, 36.771	30.752 (100)	[3]
Bifidobacterium (*16S rRNA*, AB437355)	**F:** ATGCAAGTCGAACGGGATCC **R:** CATCCCATRCCGGTAAACCC	60.5 60.5 – 62.5	174 bp	2	1.948 = 94.8 %, 0.997	-3.453, 38.983	34.906 (10)	[3]
Lactobacillus Firm-4 (*16S rRNA*, DQ837632)	**F:** AGTCGAGCGCGGGAAGTCA **R:** AGCCGTCTTTCAACCAGCACT	61.6 61.2	169 bp	4	1.912 = 91.2 %, 0.999	-3.554, 37.196	29.966 (100)	[3]
Lactobacillus Firm-5 (*16S rRNA*, JX099547)	**F:** GCAACCTGCCCTWTAGCTTG **R:** GCCCATCCTKTAGTGACAGC	60.5 60.5 – 62.5	118 bp	4	2.144 = 114.4 %, 0.998	-3.02, 36.306	29.94 (100)	[3]
Universal bacteria (*16S rRNA*)	**F:** AGGATTAGATACCCTGGTAGTCC **R:** YCGTACTCCCCAGGCGG	62.9 60 – 62	variable	-	1.946 = 94.6 %, 0.993	-3.457, 37.986	33.546 (10)	[3]

749

750 **Table S1. Primers used in this study and standard curve characteristics.** [a]Melting
751 temperatures were calculated with the online tool described in [5]. [b]The number of loci of
752 the target gene per genome of the corresponding organism was assessed based on JGI
753 database annotations. [c]*LOD* refers to the limit of detection of primers sets, expressed
754 here as the lowest number of plasmid copies reliably detected by qPCR when standard
755 curves were performed.

756

757 ## Supplementary References

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769

Supplementary Table S2

Statistics for data presented in Fig 1

Permutation ANOVA (Month Effect)	
Response Variable	**P-val**
Universal 16s	1.00E-04
Ba	1.00E-04
Bi	1.00E-04
F4	1.00E-04
F5	1.00E-04
Fp	1.00E-04
Ga	1.00E-04
Sa	1.00E-04

Permutation T-Test (F vs W)	
Response Variable	**P-val**
Sum	1.00E-04
Universal 16s	1.00E-04
Ba	1.00E-04
Bi	1.00E-04
F4	1.00E-04
F5	1.00E-04
Fp	1.00E-04
Ga	0.6572
Sa	6.00E-04

Regression for general trends During foraging season			
2015 foraging seaso	**slope**	**r^2**	**p-val**
Ba	0.02881199	0.0049618	0.3652
Bi	0.02773742	0.0153763	0.0943
F4	0.058836	0.070771	5.00E-04
F5	0.08169238	0.1790406	1.00E-04
Fp	-0.01085094	0.0006541	0.7533
Ga	0.01323004	0.0063768	0.2792
Sa	0.006376515	0.0008233	0.6935
2016 foraging season			
Ba	0.0412078	0.0085632	0.2464
Bi	0.0154952	0.0050785	0.3637
F4	0.0861126	0.0608465	0.0014
F5	0.007147429	0.0006722	0.7411
Fp	-0.08212353	0.0207844	0.1037
Ga	-0.1530585	0.2926288	1.00E-04
Sa	0.01242151	0.0018879	0.5773

Alpha-Diversity: Permutation T-Test on Shannon's Diversity Index	
F vs W	1.00E-04

Multivariate Analysis of Variance (MANOVA) Wilk's Lambda						
	Df	**Wilks**	**Approx F**	**Num Df**	**Den Df**	**P-Val**
Bee type	1	0.58162	57.342	7	558	< 2.2e-16
Residulas	564					

Statistics for data presented in Fig 2

Permutation One-Way ANOVA (F vs W vs N)	
Response Variable	**P-val**
Sum	1.00E-04
Universal 16S	1.00E-04
Ba	1.00E-04
Bi	1.00E-04
F4	1.00E-04
F5	1.00E-04
Fp	2.00E-04
Ga	1.00E-04
Sa	5.52E-01

Tukey's Post Hoc Test			
Response Variable	**P-Val N vsF**	**P-Val W vs F**	**P-Val W vs N**
Sum	1.33E-05	3.78E-10	0.00561
Universal 16S	1.66E-06	5.10E-09	0.224
Ba	0.039604	1.02E-07	0.000376
Bi	4.99E-07	2.67E-10	0.0602
F4	1.04E-07	5.42E-07	1.00E+00
F5	8.66E-09	1.08E-12	0.0118
Fp	1.00E+00	3.36E-03	0.00112
Ga	1.05E-07	4.33E-08	1.00E+00
Sa	1.00E+00	1.00E+00	0.957

Multivariate Analysis of Variance (MANOVA) Wilk's Lambda						
	Df	**Wilks**	**Approx F**	**Num Df**	**Den Df**	**P-Val**
Bee type	2	0.13955	7.9056	14	66	1.70E-09
Residulas	39					

Statistics for data presented in Fig 3

One-Way Anova (F vs N vs W) on gut weight			
	Df	**F-value**	**P-value**
Bee type	2	24,128	1.21E-08
residuals	66		
Tukey's Post Hoc Test			
	N vs F	**W vs F**	**W vs N**
P-value	1.14E-06	6.81E-08	1

Permutation T-Test Experimental Bees	
Response Variable	**P-val**
Universal 16s	3.00E-04
Ba	2.00E-02
Bi	2.00E-04
F4	1.00E-04
F5	1.00E-04
Fp	1.00E-04
Ga	2.00E-04
Sa	2.85E-01

Welch's T-test on gut weight of experimental bees	
t	9.43
Df	38.57
P-value	1.45E-11

Statistics for data Presented in Fig S2

Permutation T-Test onF. perrara abundance in the presence and absebce of scab	
P-Value	1.00E-04

Statistics for data Presented in Fig S2

Permutation T-Test onF. perrara abundance in the presence and absebce of scab	
P-Value	1.00E-04