Genomic changes underlying host specialization in the bee gut symbiont *Lactobacillus* Firm5

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Abstract

Bacteria that engage in longstanding associations with particular hosts are expected to evolve host-specific adaptations that limit their capacity to thrive in other environments. Consistent with this, many gut symbionts seem to have a limited host range, based on community profiling and phylogenomics. However, few studies have experimentally investigated host specialization of gut symbionts and underlying mechanisms have largely remained elusive. Here, we studied host specialization of a dominant gut symbiont of social bees, *Lactobacillus* Firm5. We show that Firm5 strains isolated from honey bees and bumble bees separate into deep-branching host-specific phylogenetic lineages. Despite their divergent evolution, colonization experiments show that bumble bee strains are capable of colonizing the honey bee gut. However, they were less successful than honey bee strains, and competition with honey bee strains completely abolished their colonization. In contrast honey bee strains of divergent phylogenetic lineages were able to coexist within individual bees. This suggests that both host selection and interbacterial competition play important roles for host specialization. Using comparative genomics of 27 Firm5 isolates, we found that the genomes of honey bee strains harbor more carbohydrate-related functions than bumble bee strains, possibly providing a competitive advantage in the honey bee gut. Remarkably, most of the genes encoding carbohydrate-related functions were not conserved among the honey bee strains, which suggests that honey bees can support a metabolically more diverse community of Firm5 strains than bumble bees. These findings advance our understanding of genomic changes underlying host specialization.

47 **Introduction**

48 Symbiotic relationships between bacteria and eukaryotes are pervasive and range
49 from loose associations to obligate interdependencies (McFall-Ngai *et al.* 2013; Kostic
50 *et al.* 2013). The evolution of a host-associated lifestyle is typically accompanied by
51 the loss of generalist characteristics, limiting a symbionts' capacity to compete and
52 survive in other environments. This in turn results in host specialization (Bobay &
53 Ochman 2017; Sriswasdi *et al.* 2017). In particular, bacteria with longstanding
54 associations are often host-specific and undergo marked genomic changes (Toft &
55 Andersson 2010). Among the most extreme examples are primary endosymbionts of
56 plant-sap feeding insects. These obligate mutualists reside within host cells, are
57 vertically inherited through the germ-line, and have experienced extreme genome
58 reduction due to population bottlenecks and genetic drift (McCutcheon & Moran
59 2012).

60

61 Based on phylogenetic analyses, host specialization has also been inferred for many
62 gut symbionts, as bacterial lineages are frequently found to be exclusively associated
63 with particular hosts (Ley *et al.* 2008; Oh *et al.* 2010; Ochman *et al.* 2010; Eren *et al.*
64 2015; Moeller *et al.* 2016; Kwong *et al.* 2017). This is remarkable considering that gut
65 symbionts can be horizontally transmitted and are exposed at least at some point to
66 the environment outside the host, which in principle provides opportunities for host
67 switching.

68

69

70 This leads to the question whether the observed host association is determined by
71 differences in the symbiont's fundamental niches (the set of environmental and
72 nutritional requirements that allow growth in specific hosts), or by constrains due to
73 biological interactions (e.g. cross-feeding, competition, dispersion), i.e their realized
74 (observed) niche is a subset of their fundamental niche (Macarthur & Levins 2015;
75 Hutchinson 1957).
76
77 In the case of the gut symbiont *Lactobacillus reuteri*, strains isolated from mice are
78 capable of colonizing the mouse gut, whereas those from humans, pigs, or chickens
79 are not, suggesting that host association in this case has resulted in the restriction of
80 the fundamental niche (Oh *et al.* 2010; Frese *et al.* 2011). In contrast, in another study
81 it was shown that bacterial communities from diverse habitats can colonize and
82 persist in the mouse gut (despite the fact that these species naturally do not occur in
83 the mouse gut), suggesting that a species' realized niche is frequently more restricted
84 than its fundamental niche (Seedorf *et al.* 2014). A notable difference between the
85 two studies is that host specificity of *L. reuteri* was tested in mice that were free of
86 *Lactobacilli*, but otherwise harbored a conventionalized microbiota, whereas in the
87 second study, most experiments were carried out in microbiota-free mice, supporting
88 the notion that competition can limit the realized niche of gut symbionts (Seedorf *et*
89 *al.* 2014).
90
91 Given that both experimental and phylogenetic evidence is required to determine
92 host specialization, our understanding of host specialization is limited for most gut
93 symbionts. Moreover, little is known about the underlying mechanisms and the

genomic changes accompanying host-specific evolution of gut symbionts. Selective forces acting on gut symbionts may differ between hosts due to varying degrees of population bottlenecks during transmission, or due to differences in dietary preferences, gut structure or host physiology, resulting in distinct evolutionary patterns.

A good model to study host specialization of bacterial inhabitants is the gut microbiota of corbiculate bees (Kwong & Moran 2015). Most species of honey bees, bumble bees, and stingless bees share a specialized core gut microbiota that is composed of five phylotypes (strains sharing ≥97% 16S rRNA sequence identity as estimated from amplicon sequencing studies): the gammaproteobacterium *Gilliamella*, the betaproteobacterium *Snodgrassella alvi*, two Lactobacilliales (Firm5 and Firm4), and a Bifidobacterium (Cox-Foster *et al.* 2007; Moran *et al.* 2012; Corby-Harris *et al.* 2014). These phylotypes are likely to have been acquired in a last common ancestor of the corbiculate bees, as they are widely distributed among contemporary species of honey bees, bumble bees, and stingless bees (Kwong *et al.* 2017). Moreover, there is evidence for host specialization and coevolution, because strains isolated from the three groups of corbiculate bees separate into divergent sublineages for most phylotypes (Koch *et al.* 2013; Kwong *et al.* 2014; Ellegaard *et al.* 2015; Zheng *et al.* 2016; Kwong *et al.* 2017; Steele *et al.* 2017).

The best-studied member of the bee gut microbiota with respect to host specialization is *S. alvi* (Kwong & Moran 2015). Reciprocal mono-colonization experiments of microbiota-depleted bees showed that *S. alvi* isolates from honey bees

118 (*Apis mellifera*) colonize the gut of bumble bees (*Bombus impatiens*) poorly, and vice
119 versa, suggesting that the host-specific evolution of these isolates has led to
120 specialization (Kwong *et al.* 2014). Based on the comparison of three *S. alvi* genomes,
121 it was suggested that bumble bee isolates tend to have smaller genomes and contain
122 larger amounts of mobile elements than honey bee isolates. Genomic differences were
123 also identified among isolates from different host groups (honey bees and bumble
124 bees) for the phylotype *Gilliamella*: honey bee isolates encoded more carbohydrate-
125 related functions than bumble bee isolates (Kwong *et al.* 2014). However, recent
126 genome sequencing of a larger number of *Gilliamella* strains revealed that some
127 isolates from honey bees have genomes as small as those from bumble bees,
128 suggesting that a large metabolic repertoire is not strictly needed for colonization of
129 the honey bee gut (Zheng *et al.* 2016; Ludvigsen *et al.* 2017; Steele *et al.* 2017).
130
131 For the other phylotypes of the bee gut microbiota, little is known about the link
132 between phylogeny, host range, and genome features. One of the most widely
133 distributed and abundant phylotypes of the bee gut microbiota is *Lactobacillus* Firm5.
134 In the gut of honey bees (*Apis mellifera*), four deep-branching sublineages have been
135 identified for this phylotype (Ellegaard et al. 2015), with pairwise average nucleotide
136 identity (ANI) values well below 90% across sublineages (Ellegaard & Engel 2019),
137 despite their relatively conserved 16S rRNA sequences (≥ 96.5% identity).
138 Overall, strains of different sublineages were found to vary up to 40% in gene content
139 (Ellegaard et al. 2015), suggesting that they may have adapted to distinct metabolic
140 or spatial niches within the honey bee gut, and leading to the proposition of different
141 species names (Olofsson *et al.* 2014). Interestingly, Firm5 strains isolated from other

142 corbiculate bees seem to belong to different sublineages than the honey bee isolates,
143 as indicated by single gene phylogenies (Kwong *et al.* 2017). Moreover, a divergent
144 Firm5 strain from bumble bees has been isolated and described as a new species,
145 *Lactobacillus bombicola* (Praet *et al.* 2015). Given that Firm5 strains can be cultured,
146 and that the honey bee is amenable to experimental colonization, this phylotype
147 represents an excellent opportunity to study evolutionary trajectories of host
148 adaptation and the consequences for the fundamental and realized niche of this gut
149 symbiont.
150
151 Here, we used experimental colonization, genome sequencing, and comparative
152 genomics to address host specialization in the Firm5 phylotype. First, we show that
153 isolates from honey bees and bumble bees belong to distinct sublineages, suggesting
154 longstanding host-specific associations. Second, we provide experimental evidence
155 that both host selection and interbacterial competition contribute to host
156 specialization. Third, our comparative genome analysis reveals marked differences in
157 carbohydrate utilization capacities between honey bee and bumble bee isolates,
158 suggesting that adaptation to the honey bee gut has resulted in larger metabolic
159 flexibility than adaptation to the bumble bee gut.

160 # Results

161 **Bumble bee and honey bee isolates belong to separate sublineages of the Firm5**
162 **phylotype.**

163 We sequenced the genomes of 15 new isolates of the Firm5 phylotype. Five isolates
164 were obtained from the honey bee *Apis mellifera* and ten isolates from three different
165 bumble bee species (five from *Bombus pascuorum*, four from *Bombus bohemicus*, and
166 one from *Bombus terrestris*). All bees were collected in Western Switzerland (**Table**
167 **S1**). We also included 12 previously sequenced isolates (one from a bumble bee, the
168 others from honey bees) to be more comprehensive in our analyses. All 27 isolates
169 shared >95% sequence identity across the full-length 16S rRNA gene (**Table S2**), with
170 isolates from conspecific hosts having higher 16S rRNA sequence identities in most
171 cases. The draft genomes of the 15 newly sequenced isolates consisted of 11-24
172 contigs with total lengths of 1.63-2.11 Mb, which is in the range of the previously
173 sequenced Firm5 strains (**Table S1**). While the genomes of the bumble bee isolates
174 tended to be smaller (1.63-1.70 Mb) than those of the honey bee isolates (1.68-2.15
175 Mb) (Kruskal-Wallis: chi=14.8, d.f.=1, p-value = 0.0001186), genome synteny was
176 largely conserved across the entire Firm5 phylotype (**Figure S1 and S2**).

177

178 To assess the evolutionary relationship between the 27 sequenced Firm5 strains, we
179 inferred a genome-wide phylogeny (including 15 close and three more distant
180 outgroup strains, see methods) (**Figure 1**), and calculated pairwise average
181 nucleotide identities (ANI) (**Figure S3, Table S3**). These analyses showed that the
182 Firm5 strains fall into six monophyletic sublineages with >96% ANI for within-

183 lineage divergence in all cases except "Firm5-4", for which pairwise ANI values were
184 as low as 91%. All ANI values were <86% between sublineages, indicating the
185 presence of a discontinuity zone between 86 and 91% (Jain et al. 2018), and
186 suggesting that the sublineages correspond to distinct species. Four of these six
187 sublineages consisted of only honey bee isolates and corresponded to the previously
188 identified Firm5 sublineages (Ellegaard *et al.* 2015). The two other sublineages
189 consisted of only bumble bee isolates and formed a monophyletic clade within Firm5
190 (**Figure 1**). One sublineage comprised isolates from three different bumble bee
191 species (*B. lapidarus, B. terrestris, B. pascuorum*) including the previous isolate
192 described as species *L. bombicola* (Praet *et al.* 2015). The other sublineage comprised
193 exclusively isolates from *B. bohemicus*. Based on its deep divergence from the other
194 sublineages (ANI <80%, **Figure S3**), this second sublineage of bumble bee isolates is
195 likely to represent a novel species.
196
197 Out of the 27 Firm5 isolates included in the current study, five isolates (ESL0262,
198 ESL0234, ESL0236, ESL0245, ESL0247) from three different sublineages were
199 identical or almost identical to other isolates (ANI >99.99%, **Table S3**). In all cases,
200 the nearly identical isolates were obtained from the same individual. Hence, they
201 were excluded from all subsequent analyses to avoid biases due to repeated sampling
202 of the same genotype.
203 In summary, our phylogenetic analysis of the Firm5 phylotype revealed a pattern
204 suggesting host specialization, because strains of each of the six deep-branching
205 sublineages were exclusively associated with either honey bees or bumble bees.
206

207 **Bumble bee strains can colonize microbiota-depleted honey bees, but are**
208 **outcompeted by honey bee strains.**
209 To test whether the fundamental niche of the bumble bee strains extends to the honey
210 bee (*A. mellifera*) we experimentally tested the ability of bumble bee strains to
211 colonize the honey bee gut. In the absence of competitors (i.e. when microbiota-
212 depleted bees were mono-colonized), we found that all bumble bee strains were able
213 to colonize the honey bee hindgut (**Figure 2A**). The number of recovered bacterial
214 cells at day 5 post colonization (10^5 – >10^8 CFUs per gut) was substantially higher
215 than in the inoculum (**Figure S5A**) indicating active growth of the bumble bee strains
216 in the honey bee gut. The fact that they can successfully colonize and grow means that
217 the fundamental niche of the bumble bee strains also includes the honey bee gut.
218 However, the percentage of successfully colonized bees was lower for bumble bee
219 strains (10-80%) than for honey bee strains (80-100%) (chi=14.1, d.f.=1, p = 0.0002,
220 and colonization efficiency was slightly lower compared to mono-colonizations with
221 honey bee strains, which reached 10^7–10^9 CFUs per gut (**Figure 2A**) (F=38.1, d.f=1, p
222 = 0.0008).
223
224 To test if the colonization success depends on the number of cells in the inoculum, we
225 colonized microbiota-depleted honey bees with different inocula of the bumble bee
226 Firm5 strain ESL0228 (**Figure S5B**). We chose this particular strain for follow-up
227 experiments, because it had an intermediate colonization success, yet resulted in
228 relatively high bacterial loads compared to the other bumble bee strains. We found a
229 statistically significant difference in the colonization success (two-sided test of equal
230 proportions; chi-squared = 10.946, df=3, p = 0.0120) and the colonization levels (one-

231 way ANOVA, d.f.=3, F=6.646, p = 0.0011) across treatments. With the lowest
232 inoculum, no colonization was obtained at day 5 post colonization (n=10), while with
233 the highest inoculum, all bees were colonized, yielding between 10^6 – >10^8 CFUs per
234 gut as in the previous experiment (**Figure 2B**). The relatively high number of bacteria
235 that was needed to achieve a robust colonization suggests that stronger host selection
236 is at play on bumble bee than on honey bee Firm5 strains for gut colonization.
237
238 To test for the effect of competitive exclusion between strains, we co-colonized
239 microbiota-depleted bees with the bumble bee Firm5 strain ESL0228 and a mix of
240 four honey bee strains (ESL0183, ESL0184, ESL0185, and ESL0186), each from one
241 of the four divergent sublineages. We kept the number of bacteria in the inoculum
242 constant for the honey bee strains (1:1:1:1), but provided the bumble bee strain at
243 ratios of 1:1, 10:1, or 100:1 relative to the honey bee strains (**Figure S5B**). All bees in
244 the experiment (n=30, n=10 per treatment) were successfully colonized by the Firm5
245 phylotype and the total numbers of CFUs per gut were in the same range as for the
246 mono-colonizations (10^8 – 10^9 CFUs). We used amplicon sequencing of a short
247 fragment of a conserved housekeeping gene to determine the relative abundance of
248 the five Firm5 strains tested in the community (see Methods). This analysis revealed
249 that overall all four honey bee strains successfully colonized and coexisted in the gut,
250 except for strain ESL0184 which was absent from a few samples (**Figure 2C**). In
251 contrast, the bumble bee strain ESL0228 was detected in only a few bees and at very
252 low relative abundance (<0.1%), even when inoculated with a ratio of 100:1.
253

254 Collectively, these experiments show that bumble bee strains of the Firm5 phylotype
255 are capable of colonizing the honey bee gut, but consistent colonization can only be
256 achieved with a relatively high inoculum and when honey bee strains are absent.
257 Therefore, we conclude that both host selection and interbacterial competition
258 contribute to the restriction of the realized niche of bumble bee strains.
259
260 **Firm5 strains harbor a large gene pool of phylotype-specific functions of which**
261 **few are conserved.**
262 In order to identify genomic characteristics that may contribute to host specialization
263 among Firm5 strains, we carried out a detailed comparative genome analysis. We first
264 determined the distribution of the entire pan genome across the analyzed Firm5
265 strains. We included 15 divergent outgroup strains in this analysis (i.e. strains not
266 belonging to the Firm5 phylotype, see **Figure 1** and methods) to identify Firm5-
267 specific gene families that could play a role in adaptation to the bee gut environment.
268 In total, 8,248 gene families were identified across the 37 genomes, of which 2,131
269 gene families were only represented by Firm5 strains ("Firm5-specific"). Of those,
270 571 and 1,222 gene families were only represented by bumble bee and honey bee
271 strains, respectively, and 338 gene families were represented by members of both
272 hosts (**Figure 3A**).
273
274 Despite this relatively large gene pool of Firm5-specific functions, few gene families
275 were core gene families (defined as gene families shared by all members of a group)
276 (**Figure 3A**). Among the 19 Firm5-specific core gene families, we found an ABC
277 transporter system for branched chain amino acids and two putative adhesin genes

278 (DUF4097). Amino acid transporter genes were also present among the 20 honey
279 bee-specific core gene families, whereas the 8 bumble bee-specific core gene families
280 were annotated as either hypothetical proteins or transcriptional regulators,
281 providing few clues about their functional roles for host adaptation (**Dataset S1**).
282 Altogether, this analysis revealed very few phylotype- or host-specific gene functions
283 as potential candidates for general determinants of host adaptation across the
284 analyzed Firm5 strains.
285
286 **Firm5-specific gene content is restricted to sublineages**
287 As strains from the same host can belong to divergent sublineages, it is possible that
288 sublineage-specific gene functions are involved in host specialization, e.g. by
289 adaptation to different metabolic niches within the gut. Such genes could also explain
290 the ability of the four honey bee sublineages of Firm5 to coexist within bees
291 (Ellegaard & Engel 2019).
292
293 Indeed, we found that a relatively large fraction of the Firm5-specific gene families
294 only contained members of a single sublineage (840 of 1,222 for honey bee strains,
295 532 of 571 for bumble bee strains). However, as for the previous analysis, sublineage-
296 specific core gene families represented a minor fraction (**Figure 3B**). In the honey
297 bee sublineage Firm5-2 (*L. helsingborgensis*) 53 gene families were present in all
298 three strains (i.e. 34% of the sublineage-specific gene content), including several
299 sugar transporter genes and a genomic island for the breakdown of
300 rhamnogalacturonan, a major polysaccharide of pectin (**Dataset S1**). This genomic
301 island was also found in a recent metagenomic study to correlate in abundance with

302 core genes of this sublineage (Ellegaard & Engel 2019), suggesting that
303 rhamnogalacturonan utilization is a conserved function of strains belonging to Firm5-
304 2 (*L. helsingborgensis*). For the other three honey bee sublineages, only 9-20 gene
305 families (0.3%-1.1%) represented sublineage-specific core gene content (**Figure 3B**).
306 In sublineage Firm5-3 (*L. melliventris*), functions for rhamnose utilization were
307 present in all four strains, whereas the annotations of the gene families in the other
308 two sublineages provided little functional insights (**Dataset S1**). The same was the
309 case for the two sublineage-specific core gene families found in bumble bees (9 and
310 59 gene families, **Figure 3B**), most of which were annotated as hypothetical proteins
311 (**Dataset S1**). Overall, these results indicate a high degree of gene content variability
312 among strains from the same host, also within sub-lineages.
313
314 **Honey bee strains harbor a larger diversity of carbohydrate-related functions**
315 **than bumble bee strains.**
316 The high degree of gene content plasticity within the Firm5 phylotype prompted us
317 to look at the functional composition of the entire Firm5-specific gene pool. This
318 analysis revealed marked differences between honey bee and bumble bee strains
319 with respect to carbohydrate-related functions. While 'Carbohydrate transport and
320 metabolism' (COG category 'G') was by far the most dominant COG category among
321 the gene families specific to the honey bee strains (184 gene families, 50% of those
322 with COG annotation), this category was nearly absent among the gene families
323 specific to the bumble bee strains (4 gene families, 10% of those with COG annotation)
324 (**Figure 3C**). In fact, most gene families specific to bumble bee strains had no COG
325 annotation at all. Analysis of the sublineage-specific gene content revealed a similar

326 pattern. For three of the four honey bee sublineages, COG category 'G' was the most
327 abundant COG category, while for the two bumble bee sublineages this category was
328 much less prominent (**Figure 3D**).
329
330 This pattern was further explored by quantifying the carbohydrate-related functions
331 within individual genomes. Notably, both the relative and total number of genes
332 assigned to COG category 'G' was higher for most honey bee strains compared to
333 bumble bee strains or outgroup strains (**Figure 4A, Figure S6)** (chi-squared=29.647,
334 d.f.=2, $p = 3.7*10^{-7}$). However, the Firm5-1 sublineage represented an exception to
335 this pattern. All three strains of this sublineage encoded fewer COG category 'G' genes
336 than other honey bee strains. A large proportion of the genes assigned to COG
337 category 'G' encoded phosphotransferase systems (PTSs), i.e. transporters involved
338 in sugar utilization. Correspondingly, these gene families showed a similar
339 distribution as the COG category 'G' genes across the Firm5 strains, with most honey
340 bee strains harboring a much larger number of PTS genes than bumble bee strains
341 (**Figure 4B)** (chi-squared = 31.057, d.f. = 2, $p = 1.8*10^{-7}$).
342
343 Within genomes, PTS transport systems are often co-localized with glycoside
344 hydrolases (GHs), which mediate the cleavage of sugar residues from polysaccharides
345 or other glycosylated compounds. To assess if bee gut bacteria harbor a specific
346 arsenal of these sugar-cleaving enzymes, we identified all GH genes in the analyzed
347 genomes. As for COG category 'G' and PTS transporters, we found a larger number of
348 GH genes for honey bee strains compared to bumble bee strains (**Figure 4C, Dataset**
349 **S3**)(chi-squared=11.458, d.f.=2, p = 0.0033). Some honey bee strains harbored twice

350 as many GH genes than bumble bee strains. However, there was remarkable variation
351 in the number of GH genes among the honey bee strains, both within and across
352 sublineages. Specifically, all strains of sublineages Firm5-3 and Firm5-4 harbored a
353 relatively high number of GH genes, while strains of sublineage Firm5-1 varied
354 substantially in the number of GH genes, and those of sublineage Firm5-2 were
355 consistently low.
356 The identified GH genes belonged to 79 different gene families (**Figure 4D, Dataset**
357 **S3**), of which 43 were specific to the Firm5 phylotype. Most of these (67%) were only
358 detected among honey bee strains, 19% were shared, and only 14% were specific to
359 bumble bee strains. Moreover, honey bee strains also shared more GH gene families
360 with the outgroup strains than bumble bee strains (18 vs 1 gene families).
361 While the substrate specificity of GH gene families cannot be unambiguously inferred
362 from sequence data, many of the Firm5-specific GH gene families included
363 glucosidases, fucosidases, mannosidases, xylosidases, and arabinofuranosidases (e.g.
364 GH29, GH38, GH39, GH43, and GH51), as based on the CAZY (Carbohydrate-Active
365 enZYmes) database classification (**Figure 4E).** A similarity search against the publicly
366 available non-redundant database NCBI nr (NCBI Resource Coordinators 2018)
367 revealed that many of the Firm5-specific GH families, especially those exclusively
368 present among the honey bee strains, have best hits to other taxonomic groups than
369 lactobacilliales (**Figure S7**). While these gene families may have been acquired by
370 horizontal gene transfer or secondarily lost in other lactobacillus, their limited
371 distribution among lactobacilliales suggests specific functions in the bee gut
372 environment.
373

374 Overall, the analysis of the carbohydrate-related gene content shows that Firm-5
375 strains from honey bees harbor a larger diversity of PTS transporters and glycoside
376 hydrolases than bumble bee strains. However, differences in the type and abundance
377 of these functions between strains and sublineages suggest that honey bee strains
378 have diversified in their ability to utilize different sugar resources.
379

380 **Firm5 strains from bumble bees harbor class II bacteriocins, Firm5 strains**
381 **from honey bees do not**

382 Most gene families specific to the bumble bee strains were annotated as hypothetical
383 proteins (**Figure 3C and D**), providing no insights about the possible genetic basis of
384 adaptation to the bumble bee gut environment. However, we found several short
385 open reading frames encoding putative class II bacteriocins. Bacteriocins are small
386 peptide toxins that act against closely related bacterial strains (Cotter *et al.* 2003). In
387 the case of class II bacteriocins, an ABC-like transporter usually facilitates toxin
388 secretion, and a dedicated immunity protein provides self-protection. Except for
389 strain ESL0228, all bumble bee strains harbored at least one class II bacteriocin gene
390 with homology to lactococcin 972, described to inhibit septum formation (Martínez
391 *et al.* 2000). Consistent with the genetic organization of lactococcin loci in other
392 species (Letzel *et al.* 2014), putative immunity proteins and ABC transporter genes
393 were encoded downstream of the bacteriocin gene **(Figure 5)**. We identified four
394 distinct genomic regions with this genetic organization. All four regions exhibited a
395 high degree of genomic plasticity, with many non-conserved open reading frames
396 close by (**Figure 5 and Figure S8**). Each bacteriocin locus was specific to one of the
397 two bumble bee sublineages and only present in a subset of the analyzed strains. In

398 sublineage Firm5-5, one region encoded two adjacent bacteriocin loci, and in several
399 instances one of the immunity protein or toxin genes was pseudogenized **(Figure 5)**.
400 Strikingly, none of these genomic regions were present in the analyzed honey bee
401 strains, suggesting that this genetic feature is specific to bumble bee strains. However,
402 we found homologs of genes for helveticin-J in honey bee strains, another protein
403 with known bactericidal activity against related bacteria. This gene family was
404 conserved in all strains of Firm5 as well as in some of the outgroup strains **(Figure**
405 **S9**).
406 In summary, while the two bumble bee sublineages of Firm5 harbored a large pool of
407 host-specific gene families, bacteriocins were the only conserved genes with
408 annotated functions, and thus the only identified candidates to play a role for niche
409 specialization in the present state of our knowledge.

410 # Discussion

411 In this study, we combined honey bee colonization experiments with comparative
412 genomics to investigate host specialization of Lactobacillus Firm5, a dominant gut
413 symbiont of social bees. Our results show that strains isolated from honey bees and
414 bumble bees belong to separate, highly divergent sublineages of the Firm5 phylotype,
415 which parallels phylogenetic analysis of other bee gut symbionts (Kwong *et al.* 2014;
416 Zheng *et al.* 2016; Kwong *et al.* 2017; Steele *et al.* 2017).
417
418 Interestingly, all tested Firm5 strains from bumble bees were able to colonize the gut
419 of microbiota-depleted honey bees, indicating that the divergent evolution of Firm5
420 strains from different bee species has not resulted in strict host specialization.
421 However, the percentage of successfully colonized bees as well as the number of
422 bacterial cells per gut were both lower for bumble bee strains compared to honey bee
423 strains. Only by increasing the number of bacterial cells in the inoculum by 100-fold
424 were we able to achieve reliable colonization, which suggests strong negative
425 selection of bumble bee strains during passage through the honey bee gut, possibly
426 due to the lack of host-specific adaptation.
427 However, we currently do not know whether, inversely, bumble bee strains would
428 perform better, and honey bee strains worse, in microbiota-depleted bumble bees,
429 which would provide further evidence for host-specific adaptation. Nevertheless, the
430 fact that strains from both hosts can colonize the honey bee gut extends the
431 fundamental niche of bumble bee strains to other bee genera and shows a partial
432 fundamental niche overlap amongst Firm5 strains. This is in agreement with a

433 previous study showing that selected bacteria from diverse environments, including
434 zebrafish or termite gut, can establish in the gut of germ-free mice (Seedorf *et al.*
435 2014). Moreover, the gut symbionts *S. alvi* (social bee gut) and *L. reuteri* (vertebrate
436 gut) – for which host specialization has been experimentally demonstrated – are both
437 able to colonize non-native hosts, although at much lower levels than native hosts
438 (Frese *et al.* 2013, Kwong *et al.* 2014).
439
440 Niche overlap can result in either niche partitioning, a differential utilization of
441 resources by the organisms involved, or competitive exclusion, where the best
442 competitor drives the other to extinction in the community. (Macarthur & Levins
443 2015). Although the genomic differences in carbohydrate utilization suggests at least
444 partial niche partitioning between strains that would allow coexistence in the honey
445 bee gut, the bumble bee strain did not establish in any of the tested honey bees when
446 co-inoculated with honey bee strains, even when inoculated with up to 100x more
447 bacterial cells than the four honey bee strains. This clearly shows that the tested
448 bumble bee strain is competitively excluded by the honey bee strains in the honey
449 bee gut. Similar results were obtained for *S. alvi*, when a non-native strain was
450 challenged with a native competitor for gut colonization (Kwong *et al.* 2014).
451
452 Honey bees live in large colonies and engage in frequent social interactions. This
453 results in constant exposure to bacteria from nestmates, thereby providing few
454 opportunities for bacteria from non-native hosts to establish in the gut of young
455 worker bees during community assembly. However, even when given the ecological
456 opportunity for gut colonization (as in our colonization experiments), bumble bee

457 strains seem not be able to reliably colonize the gut. Hence we conclude that the
458 competitive disadvantage relative to honey bee strains as well as suboptimal host
459 adaptation both contribute to the exclusion of bumble bee Firm5 strains from the
460 honey bee gut in natural populations.
461
462 The bacteria-mediated exclusion of the bumble bee Firm5 strains from the honey bee
463 gut could arise via direct antagonistic interactions between bacteria (e.g. via bacterial
464 toxins), or from resource competition. We identified a number of genes encoding
465 bacteriocins, which are known to mediate interbacterial killing (Kommineni *et al.*
466 2015). These genes were either shared by strains isolated from both hosts, or they
467 were specific to the bumble bee strains. However, it is notable that the strain selected
468 for the competition experiments, ESL0228, was the only bumble bee strain lacking
469 bacteriocin gene homologs. We can thus not exclude at this point that bumble bee
470 strains carrying bacteriocin genes would be more competitive in the honey bee gut.
471 Vice versa, we did not identify any toxin genes specific to the honey bee strains, which
472 could mediate possible antagonistic effects towards bumble bee strains and hence
473 hinder their colonization in the gut.
474
475 Biofilm formation at the host epithelium has been shown to be a crucial factor for the
476 colonization success and competitiveness of the murine gut symbiont *L. reuteri* (Frese
477 *et al.* 2013; Duar *et al.* 2017). The honey bee gut symbiont *S. alvi* also colonizes the
478 epithelial surface and forms biofilm-like structures, making it conceivable that
479 competition for adherence is also a critical factor for colonization in the bee gut
480 (Kwong & Moran 2016). However, bacteria of the Firm5 phylotype do not seem to

481 attach to the host epithelium, as shown by fluorescence in situ hybridization
482 experiments, but rather colonize the gut lumen in the rectum (Martinson *et al.* 2012),
483 where competition for space seems less likely to be a predominant limiting factor.
484 Moreover, our genomic analysis did not identify genes involved in host interaction or
485 adherence to be specific to strains from one of the two host groups.
486
487 Instead our genomic analysis showed that the strains differ in terms of the quantity
488 and diversity of metabolic functions. Although we found relatively few honey bee-
489 specific core gene families, the genomes of honey bee strains consistently harbored a
490 larger arsenal of genes related to carbohydrate metabolism and transport compared
491 to bumble bee and outgroup strains. This suggests that honey bee strains have a
492 greater capacity to utilize diet-derived carbohydrates, which may give them a growth
493 advantage over bumble bee strains in the bee gut. A similar trend has also been
494 observed for strains of the gut symbiont *G. apicola* (Kwong *et al.* 2014).
495
496 The predominant energy metabolism of the Firm5 phylotype is predicted to be
497 fermentation of dietary carbohydrates, which is not surprising given that the diet of
498 social bees (pollen and nectar) is rich in simple sugars, polysaccharides (pectin,
499 hemicellulose and cellulose), and other glycosylated compounds (e.g. flavonoids)
500 (Engel *et al.* 2012; Ellegaard *et al.* 2015; Kešnerová *et al.* 2017). However,
501 bumble bees and honey bees have a similar dietary regime, as both eat nectar and
502 pollen. Hence, the reason for why bumble bee strains harbor significantly fewer
503 carbohydrate-related functions is currently unclear. Interestingly, almost none of the
504 carbohydrate-related gene families specific to honey bee strains of the Firm5

505 phylotype were conserved across the analyzed genomes, suggesting that the genetic
506 basis of host adaptation in regard of carbohydrate metabolism differs between
507 strains. Moreover, although a large proportion of the carbohydrate-related gene
508 content was specifically associated with one of the four sublineages of honey bee
509 Firm5 strains, only a few of these functions were conserved within sublineages (e.g
510 rhamnogalacturonan and rhamnose utilization in Firm5-2 and Firm5-3,
511 respectively), and the number of carbohydrate-related functions varied markedly
512 among strains of some sublineages. Taken together, these results suggest that
513 metabolic functions are also more frequently gained and lost in honey bee strains
514 compared to bumble bee strains.
515 This could possibly be related to known differences in the life cycle of bumble bees
516 and honey bees. Honey bees maintain perennial colonies of large population sizes (ca.
517 20-50,000), while bumble bees build smaller colonies (typically < 500 individuals)
518 from a single overwintering queen every year. This represents a population
519 bottleneck for the bacterial community in the gut of bumble bees, since only bacteria
520 colonizing the queen are expected to be transferred to colony members in the
521 following season. Likewise, smaller colonies would reduce the effective population
522 size, unless migration across colonies is frequent. If so, genetic drift would lead to
523 gene loss and decrease the selective pressure imposed by related bacteria. It would
524 also slow the acquisition of novel gene functions allowing bacteria to utilize diverse
525 carbohydrates. Strikingly, in the host-specialized vertebrate gut symbiont *L. reuteri*,
526 it was also speculated that genomic differences in genome size and pan genome
527 diversity may be due to differences in population bottlenecks across hosts (Frese *et*
528 *al.* 20131).

529

530 In conclusion, our study advances the understanding of host specialization of gut
531 symbionts. While previous studies on *L. reuteri* have shown that host interaction, and
532 specifically colonization of the gut surface, determine host specificity, we provide
533 evidence for metabolic flexibility that may facilitate adaptation to the host diet and
534 hence the competitive exclusion of non-adapted strains. As specific dietary
535 preferences are common among animals, similar processes may also be a determining
536 factor of host specialization among other gut symbionts.

537 # Materials and methods

538 **Bee sampling, bacterial culturing and DNA isolation.**

539 Bumble bees were collected from flowers in different locations in Western
540 Switzerland as indicated in **Table S1**. Honey bees were sampled from two healthy
541 looking colonies in the same region located at the University of Lausanne. Within 6h
542 after sampling, bees were immobilized on ice and the entire gut was dissected with
543 sterile scissors and forceps. Each gut tissue was individually placed into a screw cap
544 tube containing 1ml 1x PBS and glass beads (0.75-1mm, SIGMA) and homogenized
545 with a bead-beater (FastPrep-24 5G, MP Biomedicals) for 30s at speed 6.0. Serial
546 dilutions of the gut homogenates were plated on MRS agar and incubated at 34°C in
547 an anaerobic chamber (Coy laboratories, MI, USA) containing a gas mix of 8% H_2, 20%
548 CO_2 and 72% N_2. After 3-5 days of incubation, single colonies were picked, restreaked
549 on fresh MRS agar and incubated for another 2-3 days. Then, a small fraction of each
550 restreaked bacterial colony was resuspended in lysis buffer (10 mM Tris-HCl, 1 mM
551 EDTA, 0.1% Triton, pH 8, 2 mg/ml lysozyme and 1mg/ml proteinase K) and incubated
552 in a thermocycler (10 min 37°C, 20 min 55°C, 10 min 95°C). Subsequently, a standard
553 PCR with universal bacterial primers (5'-AGR GTT YGA TYM TGG CTC AG-3', 5'-CCG
554 TCA ATT CMT TTR AGT TT-3') was performed on 1 µl of the bacterial lysate and the
555 resulting PCR products were sent for Sanger sequencing. Sequencing reads were
556 inspected with Geneious v6 (Biomatters Limited) and compared to the NCBI nr
557 database (NCBI Resource Coordinators 2018) using BLASTN. Isolates identified to
558 have high similarity (i.e. >95% sequence identity) to honey bee strains of the Firm-5
559 phylotype were stocked in MRS broth containing 25% glycerol at -80°C. Genomic DNA

560 was isolated from fresh bacterial cultures of the strains of interest using the GenElute
561 Bacterial Genomic DNA Kit (SIGMA) according to manufacturers instructions. Bumble
562 bees were genotyped based on the COI gene by performing a PCR on DNA extracted
563 from the carcass with primers LepF1 and LepR1 (Hebert *et al.* 2004), sending the
564 PCR product for Sanger sequencing, and searching the resulting sequence read by
565 BLASTN against the NCBI nr database.
566
567 **Genome sequencing, assembly and annotation.**
568 Genome sequencing libraries were prepared with the TruSeq DNA kit and sequenced
569 on the MiSeq platform (Illumina) using the paired-end 2x250-bp protocol at the
570 Genomic Technology facility (GTF) of the University of Lausanne. The preliminary
571 genome sequence analysis was carried out in the framework of the student course
572 'Sequence-a-genome (SAGE)' at the University of Lausanne in 2016-2017. In short,
573 the resulting sequence reads were quality-trimmed with trimmomatic v0.33 (Bolger
574 *et al.* 2014) to remove adapter sequences and low quality reads using the following
575 parameters: ILLUMINACLIP:TruSeq3-PE.fa:3:25:6 LEADING:9 TRAILING:9
576 SLIDINGWINDOW:4:15 MINLEN:60. The quality-trimmed reads were assembled
577 with SPAdes v.3.7.1 (Bankevich *et al.* 2012), using the "--careful" flag and multiple k-
578 mer sizes (-k 21,33,55,77,99,127). Small contigs (less than 500 bp) and contigs with
579 low kmer coverage (less than 5) were removed from the assemblies, resulting in 11-
580 24 contigs per assembly. The contigs of each assembly were re-ordered according to
581 the complete genome of the honey bee strain ESL0183 using MAUVE v2.4 (Rissman
582 *et al.* 2009). The origin of replication was set to the first base of the *dnaA* gene, which
583 coincided with the sign change of the GC skew. The ordered assemblies were checked

584 by re-mapping the quality-trimmed reads (**Figure S2**). Except for a few prophage
585 regions that showed increased read coverage, no inconsistencies in terms of read
586 coverage or GC skew were revealed suggesting that the overall order of the contigs
587 was correct. The median read coverage of the sequenced genomes ranged between
588 135x-223x (**Figure S2**). The genomes were annotated using the 'Integrated Microbial
589 Genomes and Microbiomes' (IMG/mer) system (Markowitz *et al.* 2014).
590
591 **Inference of a genome-wide phylogeny.**
592 Gene families, i.e. groups of homologous genes, were determined using OrthoMCL (Li
593 *et al.* 2003) between all publicly available and newly sequenced genomes of the Firm5
594 phylotype as well as a set of outgroup genomes of other lactobacilli strains. The
595 outgroup strains were selected based on their phylogenetic relatedness with the
596 Firm5 phylotype using a previously published phylogeny of the entire genus
597 *Lactobacillus* (Zheng *et al.* 2015). Based on this analysis, we included the genomes of
598 15 closely related outgroup strains that belong to the same *Lactobacillus* clade as
599 Firm5 ('delbrueckii group') and three more distantly related strains for rooting the
600 phylogeny. All-against-all BLASTP searches were conducted with the proteomes of
601 the selected genomes, and hits with an e-value of $\leq 10^{-5}$ and a relative alignment length
602 of >50% of the query and the hit protein lengths were kept for OrthoMCL analysis. All
603 steps of the OrthoMCL pipeline were executed as recommended in the manual and
604 the mcl program was run with the parameters '--abc -I 1.5'.
605
606 The core genome phylogeny was inferred from 408 single copy orthologs extracted
607 from the OrthoMCL output (i.e. gene families having exactly one representative in

608 every genome in the analysis). The protein sequences of each of these core gene
609 families were aligned with mafft (Katoh *et al.* 2017). Alignment columns represented
610 by less than 50% of all sequences were removed and then the alignments were
611 concatenated. Core genome phylogenies were inferred on the concatenated trimmed
612 alignments using RAxML (Stamatakis 2014) with the PROTCATWAG model and 100
613 bootstrap replicates.
614
615 **Comparison of genome structure, genome divergence, and gene content.**
616 To compare and visualize whole genomes we used the R-package genoPlotR (Guy *et*
617 *al.* 2010). BLASTN comparison files were generated with DoubleACT (www. hpa-
618 bioinfotools.org.uk) using a bit score cutoff of 100. To estimate sequence divergence
619 between genomes, we calculated pairwise average nucleotide identity (ANI) with
620 OrthoANI (Lee *et al.* 2016) using the exectutable 'OAT_cmd.jar' with the parameter '-
621 method ani'.
622
623 For analyzing the distribution of gene families across Firm5 sublineages and closely
624 related outgroup strains, we carried out a second OrthoMCL analysis, in which we
625 excluded the three distantly related outgroup strains. To remove redundancy in our
626 database, we also excluded the genomes of five Firm5 isolates that were identical, or
627 almost identical, to other Firm5 strains in the analysis, based on ANI values of
628 >99.99%. This resulted in a total 37 genomes (22 Firm5 genomes and 15 outgroup
629 genomes) that were included in the analysis. BLASTP and OrthoMCL were run with
630 the same parameters as before. Gene family subsets of interest (e.g. families specific
631 to honey bee, bumble bee or outgroup strains) were extracted from the OrthoMCL

632 output file using custom-made Perl scripts. COGs (Cluster of Orthologous Groups)
633 were retrieved from IMG/mer genome annotations (Markowitz *et al.* 2014).
634
635 For the detection and visualization of the genomic regions encoding bacteriocin
636 genes, Bagel3 (van Heel *et al.* 2'13) and MultiGeneBlast (Medema *et al.* 2013)
637 were used. For the MultiGeneBlast analysis, bacteriocins-encoding genomic regions
638 of strains ESL0233 and ESL0247 served as query sequences for searching a custom-
639 made database composed of all non-redundant Firm5 genomes.
640
641 **Identification of glycoside hydrolase gene families.**
642 Glycoside hydrolase gene families were identified in all analyzed genomes (excluding
643 the redundant Firm5 strains and the three distant outgroup strains) using the
644 command-line version of dbCAN (Database for automated Carbohydrate-active
645 enzyme Annotation) (Yin *et al.* 2012)
646 . In short, we searched each genome against dbCAN using hmmscan implemented in
647 HMMER v3 (Eddy 2009). The output was processed with the parser script 'hmmscan-
648 parser.sh', and genes with hits to Hidden Markov Models of glycoside hydrolase
649 families were extracted (for alignments > 80aa an e-value cut-off of < 10^{-5} was used,
650 otherwise an e-value cut-off of <10^{-3} was used, the covered fraction of the HMM had
651 to be > 0.3).
652
653 For determining the taxonomic distribution of related genes, we searched one
654 homolog of each glycoside hydrolase gene family against the NCBI *nr* database (NCBI
655 Resource Coordinators 2018) using BLASTP. The taxonomy of the first 50 BLASTP

656 hits (e-value <10^{-5}) was extracted at the family level using the Perl script 'Tax_trace.pl'
657 and the database files nodes.dmp and names.dmp. The latter two files contain the
658 NCBI taxonomy nodes and names.
659
660 **Bee colonization experiments.**
661 Newly emerged, microbiota-depleted bees were generated as described in (Emery *et*
662 *al.* 2017) and colonized within 24-36h after pupal eclosion. To this end, bacterial
663 strains were grown on MRS agar containing 2% fructose and 0.2% L-cysteine-HCl
664 from glycerol stocks for two days in an anaerobic chamber at 34°C. Then, 1-10
665 colonies were inoculated into 5ml of carbohydrate-free MRS supplemented with 4%
666 fructose, 4% glucose and 1% L-cysteine-HCl and incubated for another 16-18h
667 without shaking. Bacteria were spun down and resuspended in 1xPBS/sugar water
668 (1:1). The optical density (600nm) was adjusted according to the experimental
669 condition (OD=0.0001, 0.001, 0.01, or 0.1) and 5 µl of the final bacterial suspension
670 was fed to each newly emerged bee. Before feeding, the bees were starved for 2-3h.
671 After colonization, bees were given 1 ml of sterilized polyfloral pollen and sugar water
672 ad libitum. Bees were co-housed in groups of 20-40 bees. For the competition
673 experiment, each of the four honey bee strains was adjusted to an optical density
674 (600nm) of 0.001. The bumble bee strain ESL0228 was adjusted to an optical density
675 (600nm) of either 0.001, 0.01, or 0.1. Then equal volumes of the five strains were
676 mixed together and fed to newly emerged bees as described before. As negative
677 control, bees were fed with 5 µl of 1xPBS/sugar water. Dilutions of the bacterial
678 inocula were plated on MRS agar containing 2% fructose and 0.2% L-cysteine-HCl and

679 incubated as described before to determine how many CFUs correspond to a given
680 optical density (see Figure S5).
681 Ten bees per condition were dissected on day 5 after colonization. The hindgut was
682 separated from the midgut with a sterile scalpel and tweezers, and added to 1 ml or
683 500 ul of 1x PBS (depending on the experiment). The tissues were homogenized by
684 bead-beating as described before, dilutions plated on MRS agar containing 2%
685 fructose and 0.2% L-cysteine-HCl and the number of CFUs counted two to three days
686 after incubation. For the negative control, bacterial colonies were detected for only
687 one out of 30 bees with a relatively low abundance (10^3 CFUs per gut). Moreover, the
688 colonies looked different from the colonies of the Firm5 strains and were identified
689 as being *E. coli* and *Staphylococcus aureus* by 16S rRNA gene sequencing.
690 The relative abundance of the five strains in the competition experiment was
691 analyzed using amplicon sequencing of a 199-bp fragment of a conserved
692 housekeeping gene (COG0266). To this end, a two-step PCR protocol was established.
693 In the first PCR, the 199-bp fragment of COG0266 was amplified from crude cell
694 lysates of gut homogenates with primers 1133 (5' -
695 CGTACGTAGACGGCCAGTATGCCNGAAATGCCRGARGTTGA – 3') and 1134 (5' -
696 GACTGACTGCCTATGACGACTAARCGATAYTTRCCYTCCATRCG) (3' – 95°C; 25x: 30'' -
697 95°C, 30'' – 64°C, 30'' – 72°C; 5' – 72°C). After removing primers with exonuclease and
698 shrimp alkaline phosphatase, barcoded Illumina adapters were added in the second
699 PCR. The resulting PCR products were pooled at equal volumes, gel purified (MinElute
700 Gel Extraction Kit, Qiagen) and loaded on an Illumina MiniSeq instrument in mid-
701 output mode. Reads were demultiplexed and filtered on quality using trimmomatic
702 (LEADING:28 TRAILING:28 SLIDINGWINDOW:4:15 MINLEN:90) (Bolger *et al.* 2014).

703 Then, each forward and reverse read pair was assembled using PEAR (-m 290 -n 284
704 -j 4 -q 26 -v 10 -b 33) (Zhang *et al.* 2014). The resulting contigs were assigned to the
705 five strains based on base positions with discriminatory SNP variants with the help
706 of a custom-made Perl script.
707
708 **Statistical Analysis.**
709 Colonization success (as proportion of bees in trial successfully colonized above
710 detection limit) was compared in all experiments with a two-sided test of equal
711 proportions across groups, and again pairwise between all strains/treatments.
712 Differences in colonization efficiency (as the number of CFUs per gut in those bees
713 that were successfully colonized), were tested with a nested analysis of variance
714 where strains are nested within host groups, or with a one-way analysis of variance
715 for the initial inoculum experiment. Normality was tested with a Shapiro-Wilk test
716 and an inspection of the QQ-plots of both raw data and residuals. P-values in post-hoc
717 tests were adjusted with the Benjamin-Hochberg correction (Benjamini & Hochberg
718 1995) for multiple comparisons, and were considered significant below 0.05. All tests
719 were performed in R.
720 Genome length and differences in functional gene number were tested with a Kruskal-
721 Wallis test and a Games-Howell post-hoc test, for COG 'G' and PTS categories, and with
722 a Dunn's post-hoc test for genome length and GH.
723

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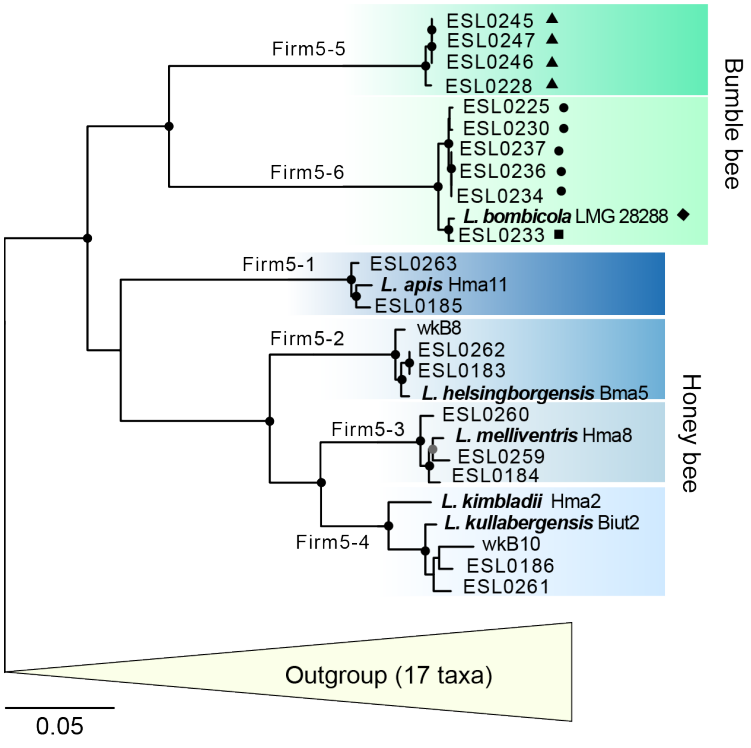
Data accessibility

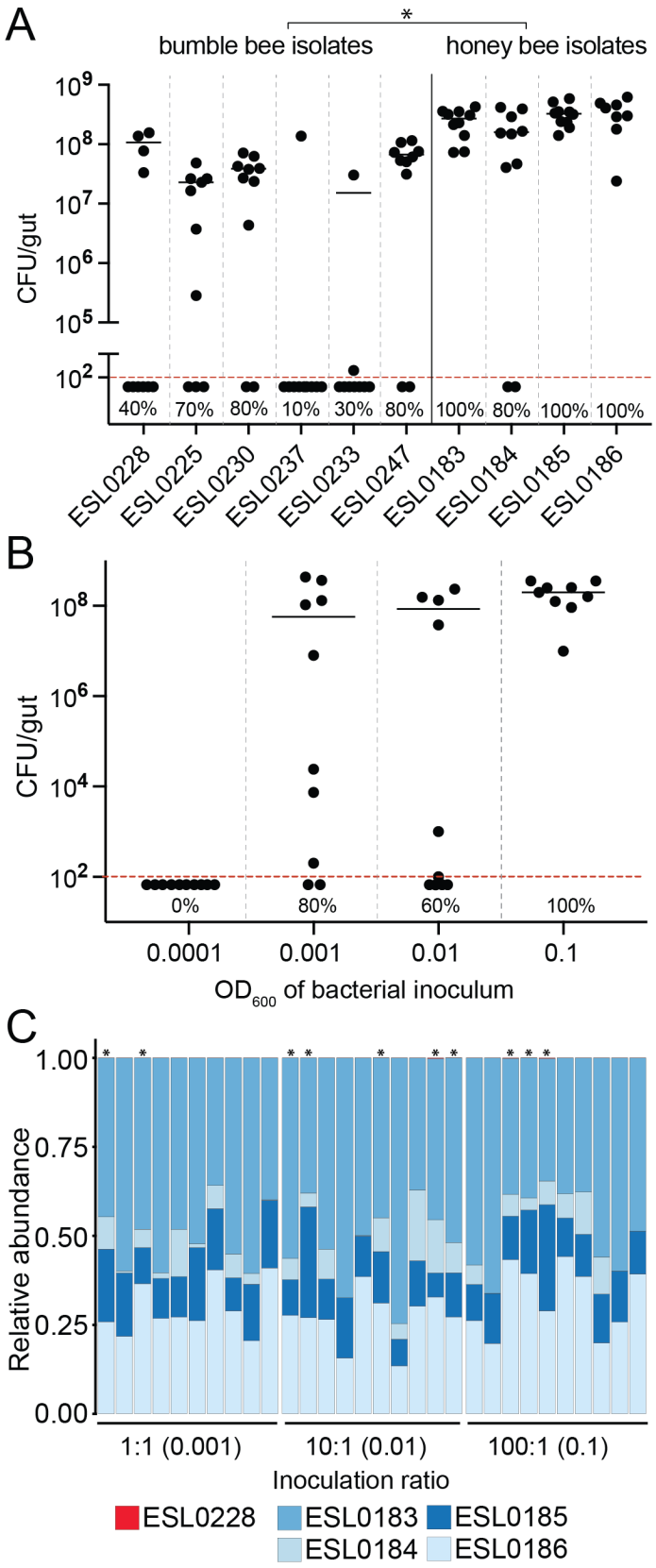
Genome sequences and short read datasets are available under NCBI Bioproject accession PRJNA392822. Annotations of the Firm5 strains used for this study can be found in IMG/mer. Data analyses including custom scripts and intermediate output files are available on Zenodo Data analyses including custom scripts and intermediate output files are available on Zenodo: https://doi.org/10.5281/zenodo.1010076.

745 # Figures



746

747 **Figure 1. Core genome phylogeny of *Lactobacillus* Firm5.** The tree was inferred
748 using maximum likelihood on the concatenated protein alignments of 408 single-copy
749 core genes (i.e. present in all Firm5 strains and in the outgroup strains). The collapsed
750 outgroup consisted of 17 strains that were used to root the tree (see **Figure S4** for
751 the complete tree). The two lineages of bumble bee strains and the four lineages of
752 honey bee strains are shown in green and blue color shades, respectively. Black and
753 grey circles indicate bootstrap support values of 100 and ≥80, respectively, out of 100
754 replicates. The strain designation of each isolate is given and the species names of the
755 type strains are indicated. The length of the bar indicates 0.05 amino acid
756 substitutions/site. Shapes behind strain name of bumble bee isolate indicate the bee
757 species that the strain was isolated from. Triangle, *Bombus bohemicus*; circle, *Bombus*
758 *pascuorum;* rhombus, *Bombus lapidarius;* square, *Bombus terrestris.*


A
*
bumble bee isolates
honey bee isolates
CFU/gut
40%
70%
80%
10%
30%
80%
100%
80%
100%
100%
ESL0228
ESL0225
ESL0230
ESL0237
ESL0233
ESL0247
ESL0183
ESL0184
ESL0185
ESL0186
B
CFU/gut
0%
80%
60%
100%
0.0001
0.001
0.01
0.1
OD600 of bacterial inoculum
C
Relative abundance
1:1 (0.001)
10:1 (0.01)
100:1 (0.1)
Inoculation ratio
ESL0228
ESL0183
ESL0185
ESL0184
ESL0186

759

760 **Figure 2. Colonization of microbiota-depleted honey bees with Firm5 strains**
761 **from bumble bees and honey bees. (A)** Mono-colonizations of microbiota-depleted
762 honey bees (n=10 per treatment) with six bumble bee strains and four honey bee
763 strains. Each bee was inoculated with 5 μL of an optical density of 0.001. The dashed
764 red line indicates the detection threshold. Data points below the detection limit show
765 bees that had no detectable colonization levels. The percentage of successfully
766 colonized bees is shown. Horizontal lines indicate median. The asterisk above the plot
767 indicates that there is a significant difference between isolates of different host
768 groups in both colonization success (two-sided test of equal proportions, chi=14.1,
769 d.f.=1, p = 0.0002) and colonization efficiency (nested analysis of variance, strains
770 nested within host groups, F=38.1, d.f=1, p = 0.0008). **(B)** Mono-colonizations of
771 microbiota-depleted honey bees with increasing inocula of the bumble bee strain
772 ESL0228. Colony forming units (CFUs) per gut were determined at day 5 post
773 colonization. The graph has the same layout as in panel A. The number of successfully
774 colonized bees and the colonization levels were significantly different across
775 treatments according to a two-sided test of equal proportions (chi-squared = 10.946,
776 df=3, p = 0.0120) and a one way ANOVA (d.f.=3, F=6.646, p = 0.0011). (**C**) Community
777 profiles of microbiota-depleted bees colonized with a community consisting of the
778 bumble bee strain ESL0228 and four honey bee strains (ESL0183, ESL0184, ESL0185,
779 and ESL0186). Three different inoculation ratios of bumble bee strain versus honey
780 bee strains were used. The optical density of the bumble bee strain in the inoculum is
781 given in brackets. Due to the absence or the very low abundance of ESL0228, the red
782 fraction of the graph is not visible. Asterisks indicate samples for which at least a few
783 reads of strain ELS0228 were detected.

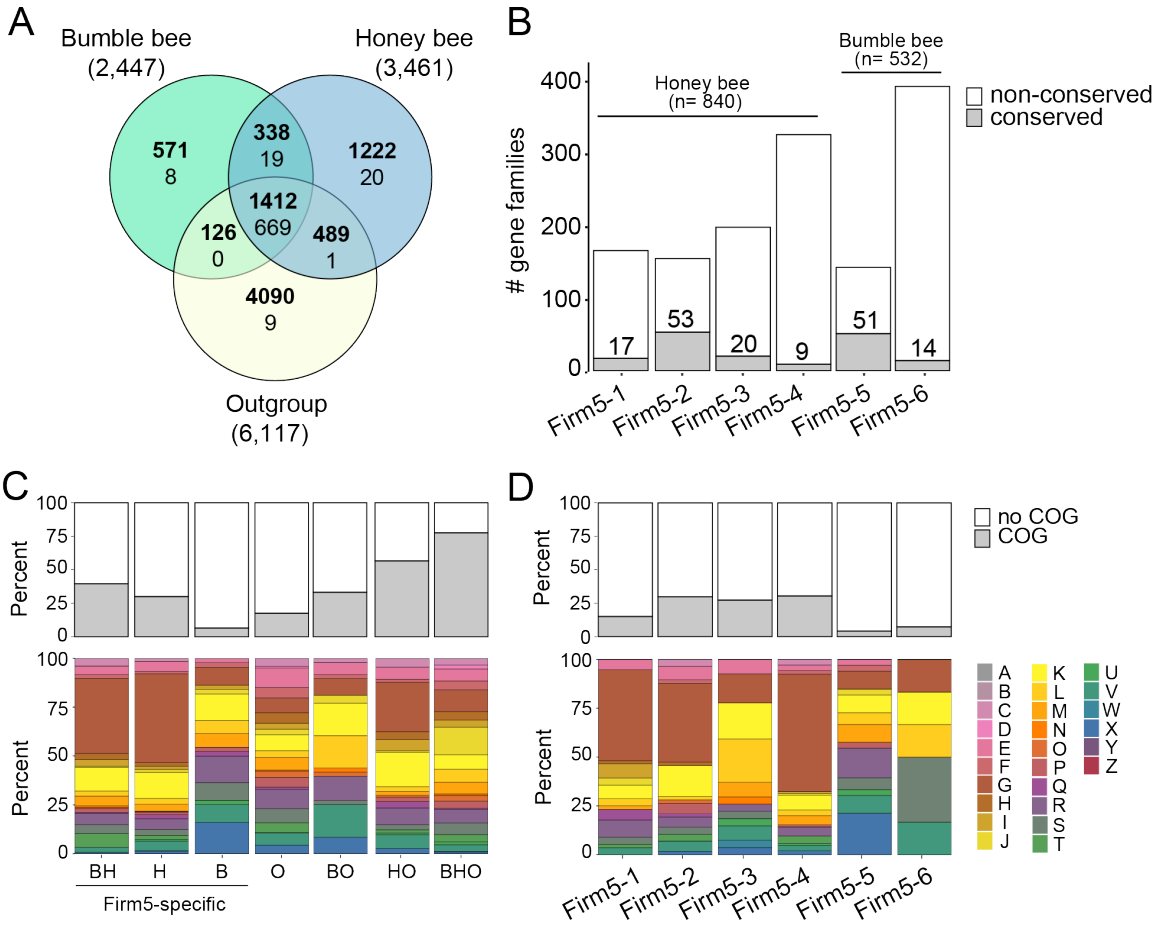


Figure 3. Pan genome analysis of Firm5 strains from honey bees and bumble bees and comparison to outgroup strains (i.e. closely related lactobacilli). (A) Venn diagram showing gene family distribution into the three major groups: Firm5 strains from bumble bees, Firm5 strains from honey bees, and outgroup strains. Numbers in bold indicate the total number of gene families (i.e. present in at least one genome of a given group). Numbers in regular font indicate core genome gene families (i.e. present in all genomes of a given group). **(B)** Number of Firm5-specific gene families exclusively present in strains of one sublineage. The fraction of the gene families belonging to the core genome (present in every genome of a given sublineage) and the accessory genome (present in at least one genome of a given sublineage) gene families is indicated by grey and white color, respectively.

796 Numbers above the graph indicate total number of lineage-specific gene families for
797 each host group. **(C)** Upper plot shows number of gene families with COG
798 annotation, and lower plot shows COG category distribution of the annotated gene
799 families for each subset of the Venn diagram in panel A. B, specific to bumble bee
800 strains; H, specific to honey bee strains; O, specific to outgroup strains; BH, shared
801 between honey bee and bumble bee strains; BO, shared between bumble bee and
802 outgroup strains; HO, shared between honey bee and outgroup strains; BHO, shared
803 between all three groups. **(D)** Same as in panel C, but for the sublineage-specific
804 gene families shown in panel B. Complete lists of all gene families and their
805 annotations can be found in **Datasets S1 and S2**. The dominant COG category 'G' is
806 shown in dark red and corresponds to 'Carbohydrate transport and metabolism'.
807 Other COG category abbreviations are given in **Dataset S2**.

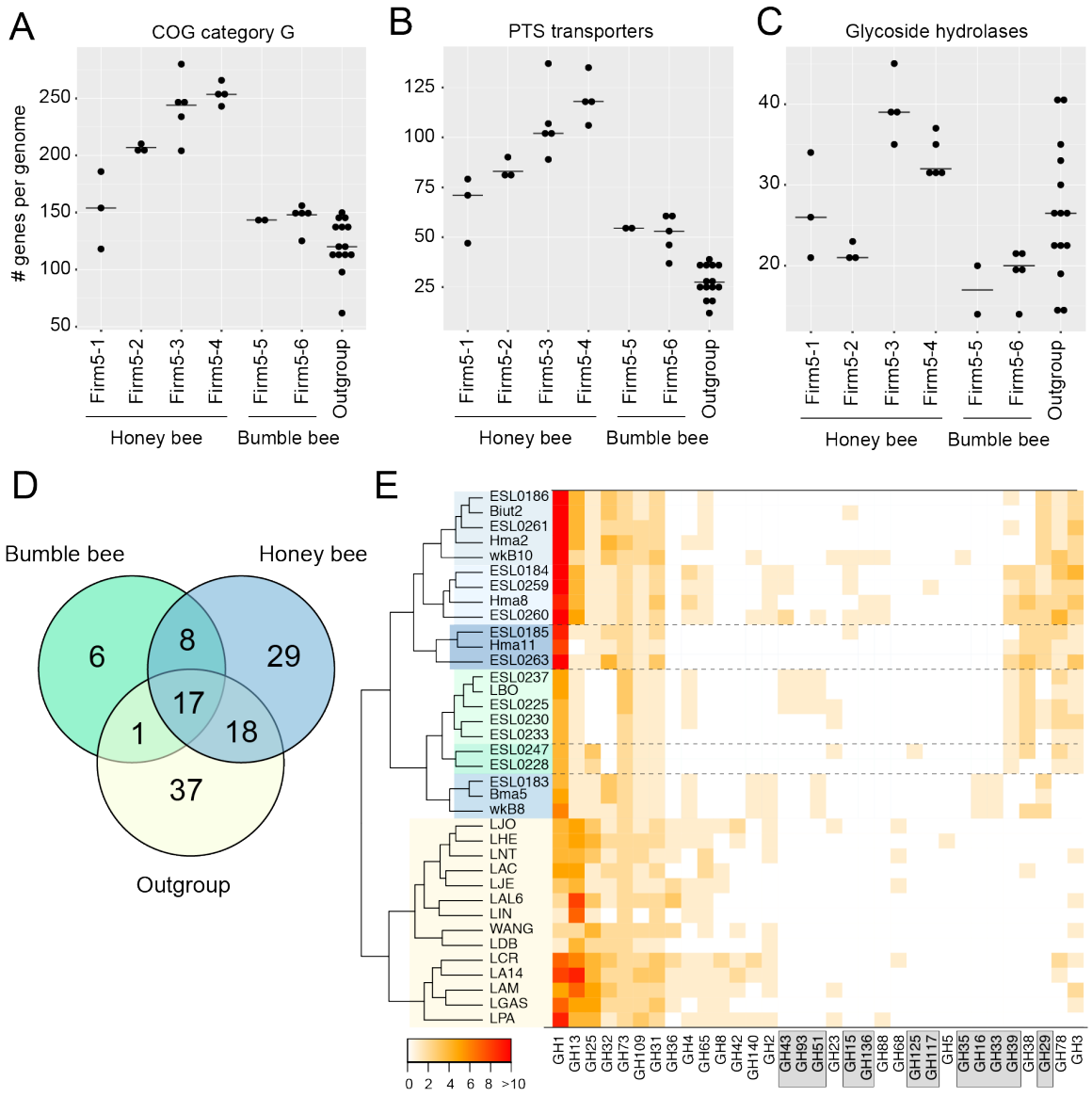


Figure 4. Distribution of carbohydrate-related gene families across strains of the Firm5 phylotype. (A) Total number of COG category 'G' gene families per genome per sublineage. **(B)** Total number of PTS (Phosphotransferase system) gene families per genome per sublineage. **(C)** Total number of glycoside hydrolase gene families per genome per sublineage. In all three panels, the genomes of the outgroup strains were included as a reference. **(D)** Venn diagram of glycoside hydrolase gene family distribution into the three major phylogenetic groups: Firm5 strains isolated from bumble bees, Firm5 strains isolated from honey bees, and outgroup strains. **(E)**

817 Heatmap showing the distribution of the identified glycoside hydrolase (GH) families
818 across the analyzed genomes. The dendrogram on the left shows a hierarchical
819 clustering based on glycoside hydrolase distribution. Strains are colored according to
820 the major groups (green, bumble bee strains; blue, honey bee strains; yellow,
821 outgroup) and sublineage (color tones). GH families specific to the Firm5 phylotype
822 are indicated by grey boxes.

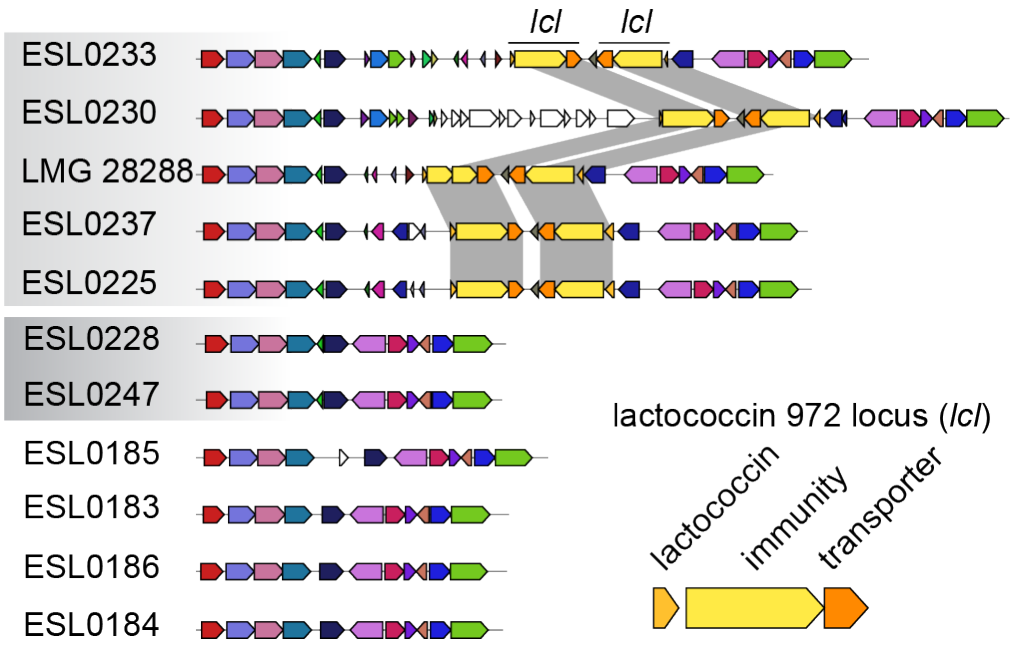


Figure 5. Genomic region encoding class II bacteriocins in Firm5 strains of bumble bee strains. Genomic regions encoding bacteriocin genes were identified and visualized with MultiGeneBlast v1.1.14 (Medema *et al.* 2013). Arrows present genes and same color indicates homology. A black line indicates the lactococcin 972 locus (*lcl*) and vertical grey blocks connect the homologous genes in other strains. An enlarged version of the three genes of the *lcl* locus with annotation is shown in the lower right. Grey shading over strain names indicates two sublineages of bumble bee strains; the four honey bees strains are representatives of the four sublineages. Other genomic regions encoding bacteriocins genes are given in Figure S8.

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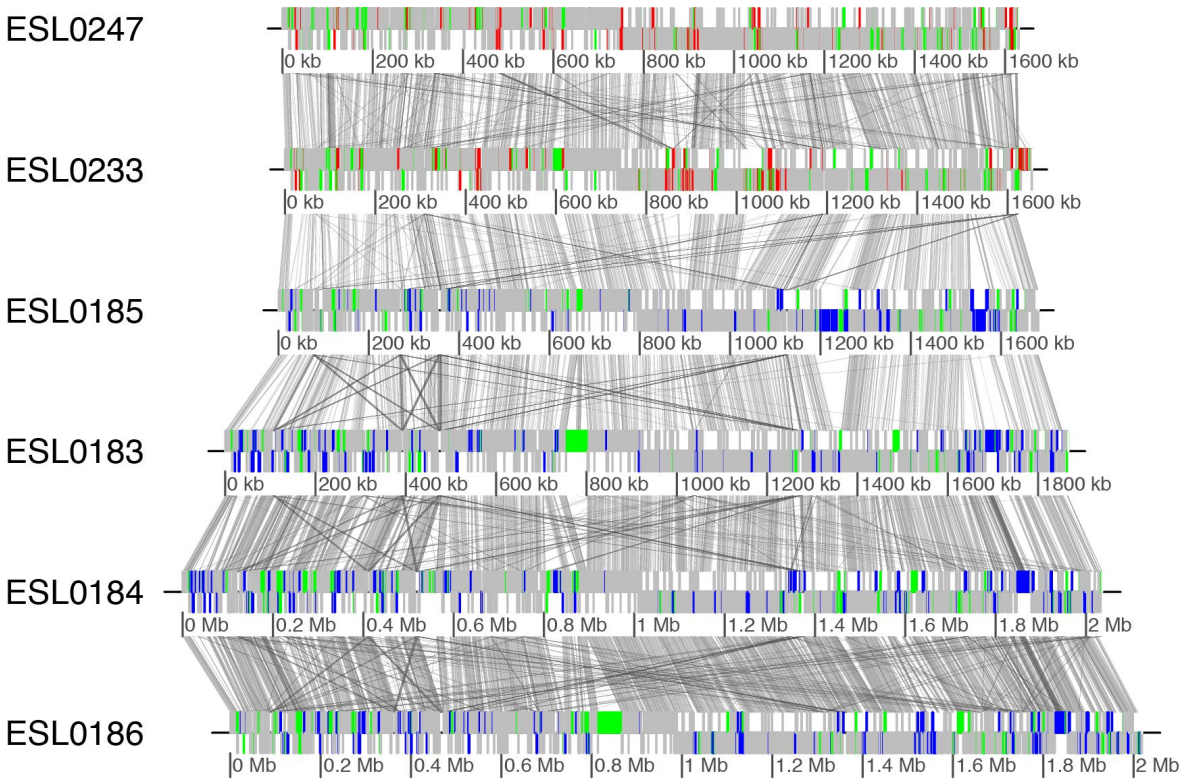
982

983 # Supplementary Figures

984



985

986 **Figure S1. Whole genome alignments of divergent Firm5 strains.** ESL0247 and
987 ESL0233 are bumble bee strains. ESL0183-186 are honey bee strains. Vertical grey
988 lines indicate blocks of nucleotide sequence similarity. Different color intensities
989 correspond to different degree of similarity based on BLASTN hits with a bit score of
990 at least 100. Genes in color correspond to Firm5-specific genes relative to the
991 outgroup (blue, genes specific to honey bee strains; red, genes specific to bumble bee
992 strains; green, genes shared between honey bee and bumble bee strains). Other genes
993 are shown in grey.

994

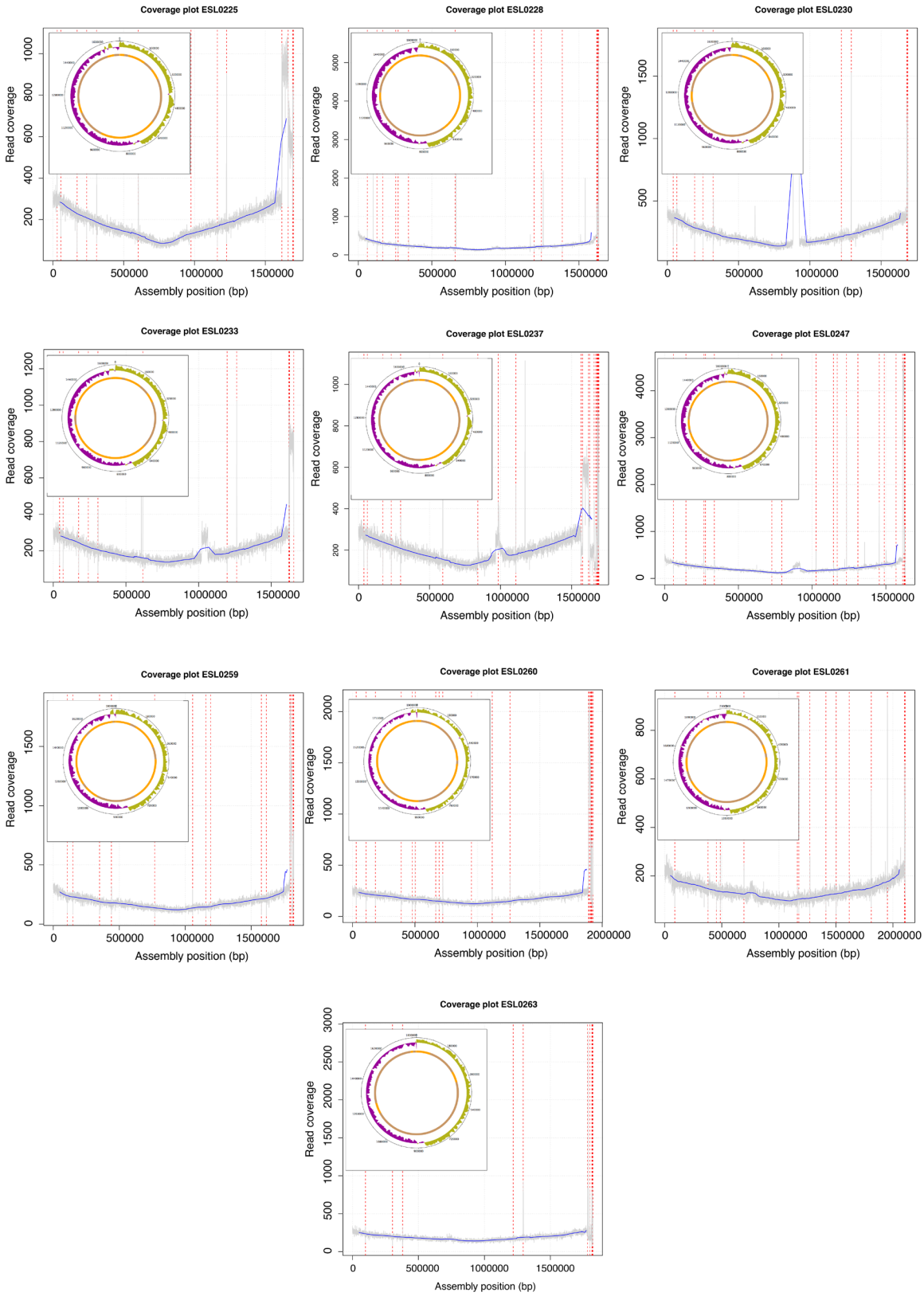
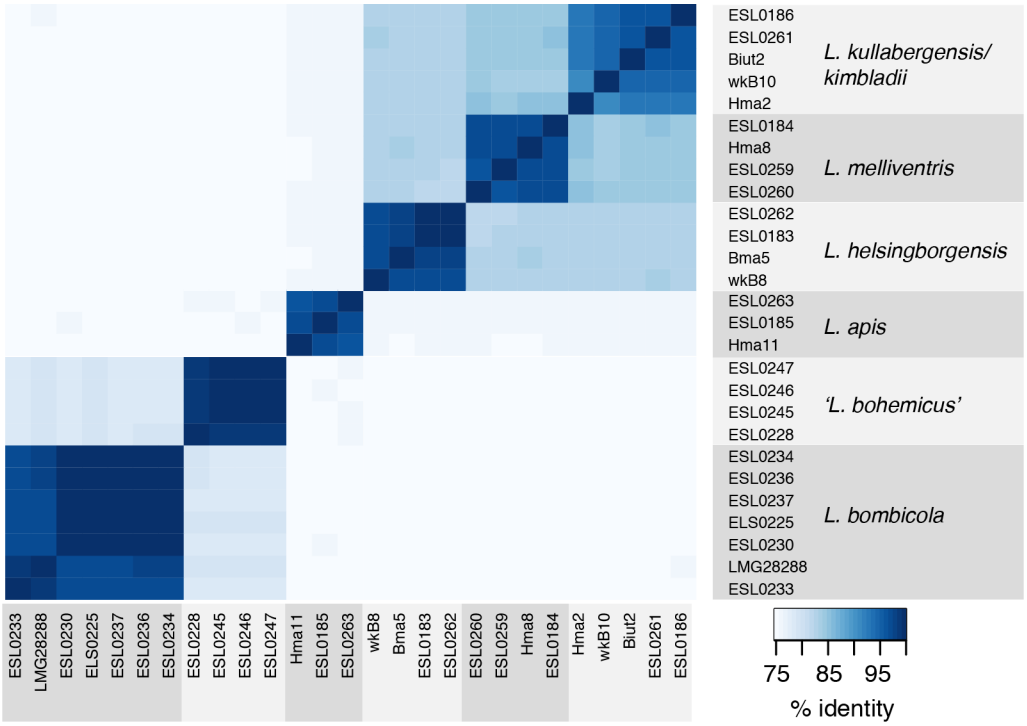


Figure S2. Read coverage and GC skew of the final genome assemblies. Assembly positions are shown on the x-axis for each sequenced strain. y-Axis shows Illumina

998 read coverage for a sliding window of 100 bp. Red dashed lines indicate contig breaks.
999 Contigs were ordered according to the fully sequenced reference strains ESL0183.
1000 Small contigs were left at the end of the assembly. Inset shows the circular form of the
1001 assembly with the GC skew indicated. We found a higher read coverage at the origin
1002 of replication, which is characteristic for replicating bacteria. Moreover, our
1003 assemblies showed the typical GC skew of bacterial genomes. Both characteristics
1004 indicate that the contigs of the assemblies were correctly assembled and ordered.
1005 Notably, regions of extremely high coverage correspond to prophages that apparently
1006 were amplified during culturing of some of the strains.

1007



1008 **Figure S3. Average nucleotide identity between the analyzed Firm5 genomes.**

1009 Intensity of heatmap indicates pairwise ANI. White areas correspond to genomes,

1010 which were too divergent for ANI calculation. The names of each strain included in

1011 the analysis are given next to the plot area (see also **Table S1**). Grey shading indicates

1012 the six different sublineages of Firm5. ANI values are given in **Table S3**.

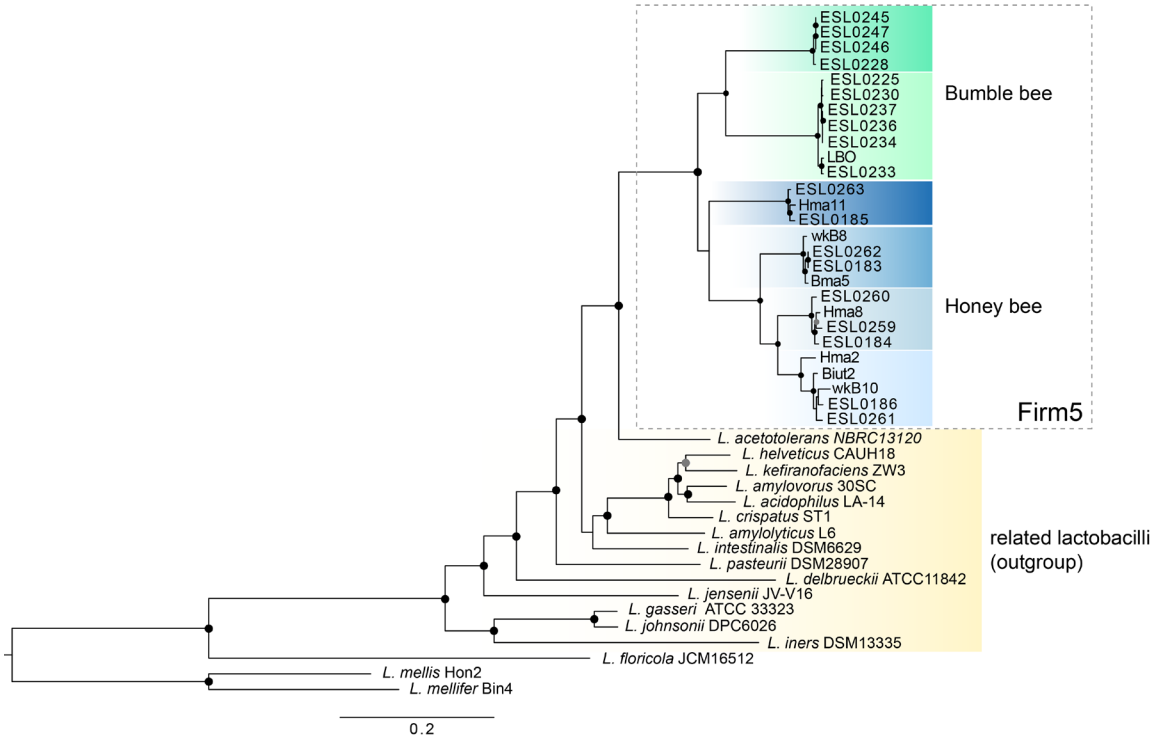
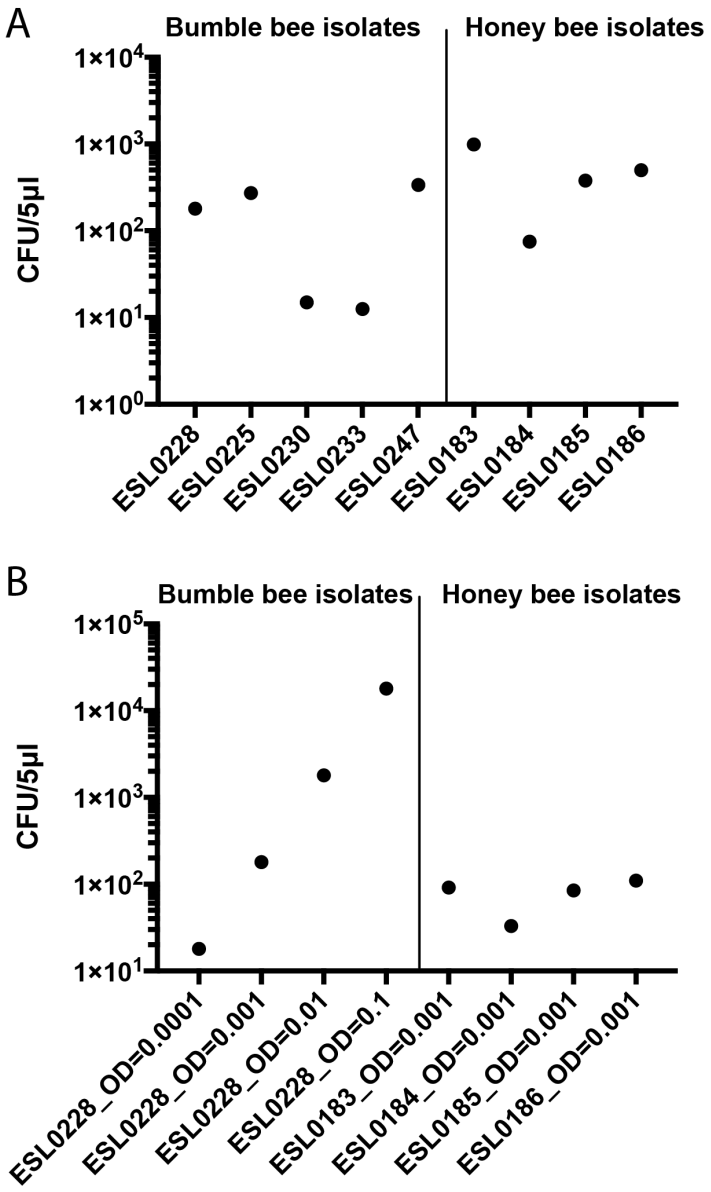


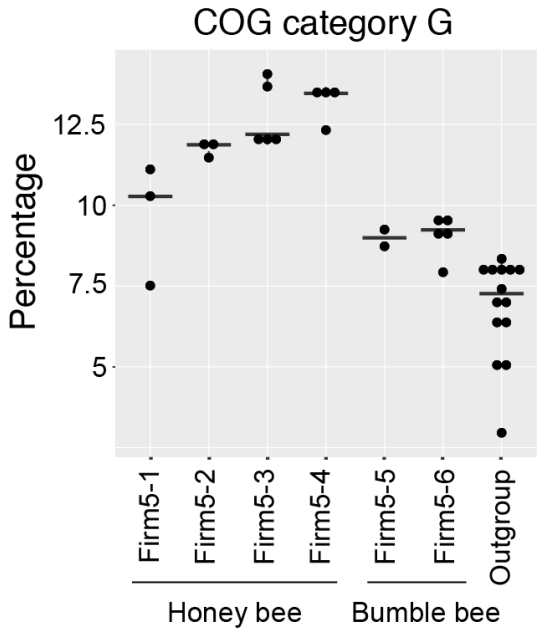
Figure S4. Complete core genome phylogeny of *Lactobacillus* Firm5. The tree was inferred using maximum likelihood on the concatenated protein alignments of 408 single-copy core gene families (i.e. present in all Firm5 strains and the outgroup strains). The two lineages of bumble bee strains and the four lineages of honey bee strains are shown in green and blue color shades, respectively. As outgroup, 15 representative strains of the *L. delbrueckii* group (to which Firm5 belongs to) were included in the analysis (shown in yellow) based on a previously published phylogeny of the entire genus *Lactobacillus* (Zheng *et al.* 2015). In addition, we included three more distantly related strains to root the tree. Noteworthy, these three distantly related lactobacilli were excluded for all subsequent comparative analysis of the Firm5 strains. Filled circles indicate 100 bootstrap support values. The strain designation of each isolate is given. The length of the bar indicates 0.05 amino acid substitutions/sites.



1027

1028 **Figure S5. Number of bacterial cell in the inocula used to colonize microbiota-**
1029 **depleted honey bees with Firm5 strains. (A)** CFUs in the inocula used for the
1030 monocolonization experiments with individual strains. CFUs are given per 5µl, as
1031 each bee was inoculated with 5 µl of an OD600 of 0.0001. Despite the adjustment to
1032 the same OD600, the amount of live bacteria in each inoculum varied across strains.
1033 The inoculum of strain ESL0237 could not be assessed due to a handling mistake
1034 during dilution plating. **(B)** CFUs in the incocula used for the colonization experiment

1035 with the bumble bee strain ESL0228 (left part) and for the colonization experiment
1036 with the five-member community consisting of the bumble bee strain ES0228 (left
1037 panel, OD=0.001, 0.01, and 0.1) and the four different honey bee strains (right panel).



1038

1039 **Figure S6. Percentage of gene families annotated as COG category 'G' per**

1040 **genome per sublineage.** Same data as in Figure 4A, but expressed in relative

1041 numbers (percentage of all gene families per genome).

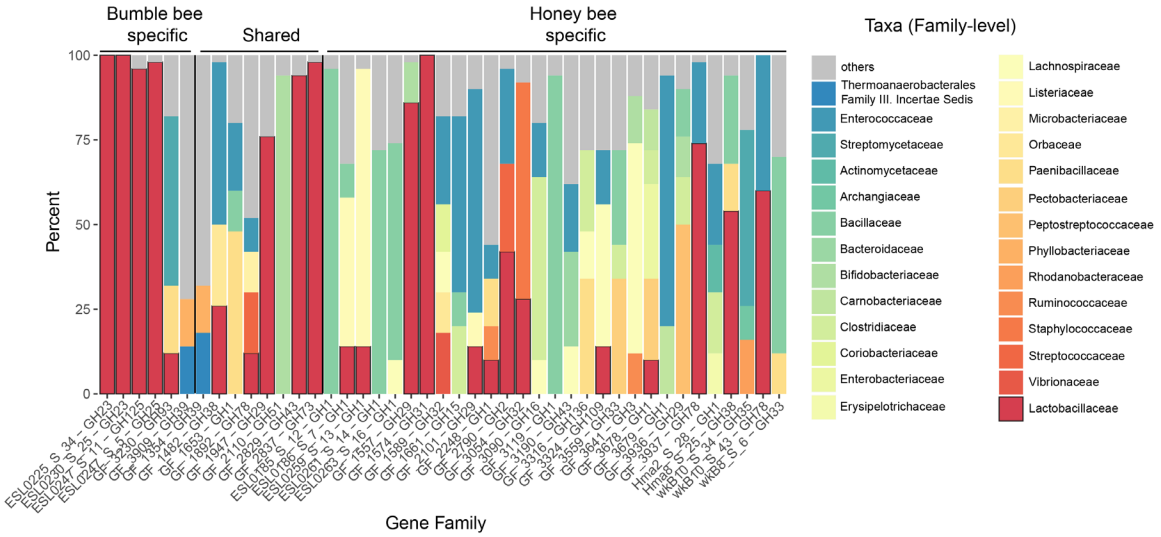
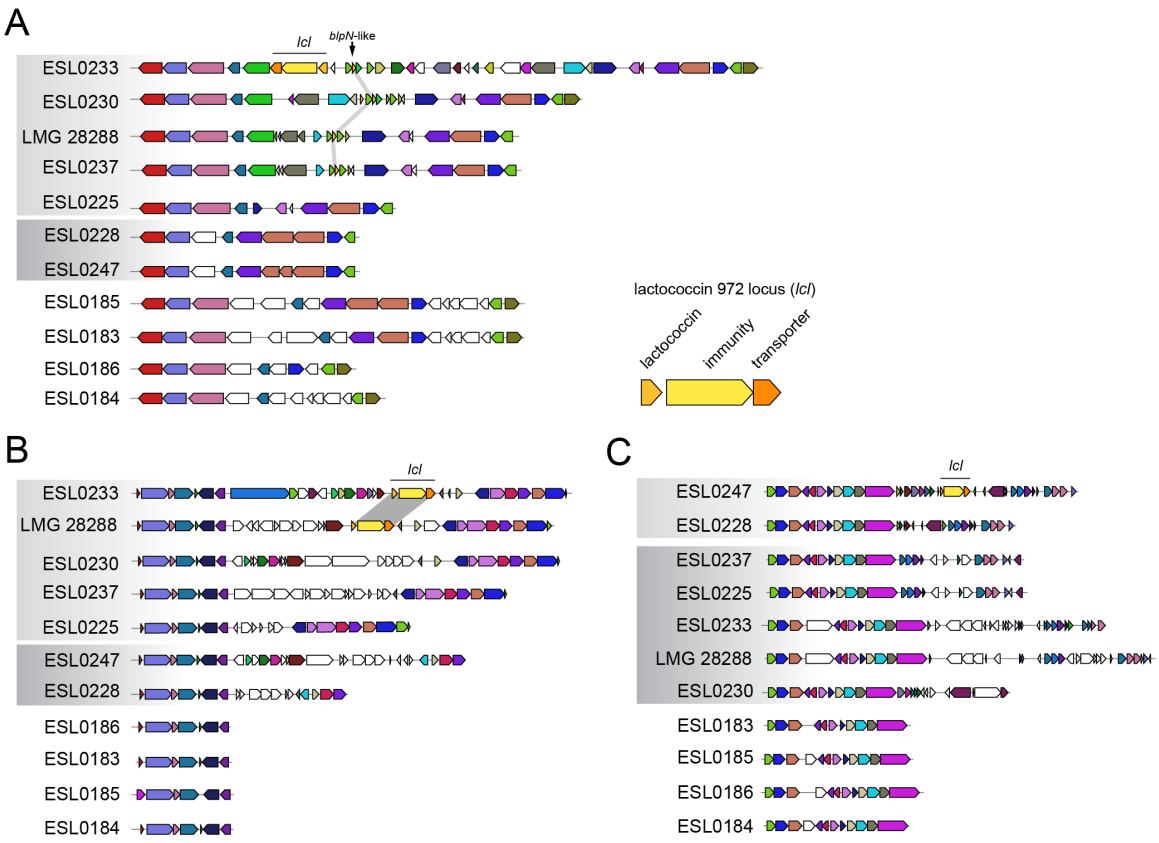


Figure S7. BlastP hit distribution of glycoside hydrolase (GH) gene families specific to Firm5. A representative protein sequence of each gene family was blasted against the NCBI nr database (NCBI Resource Coordinators 2018). The distribution of the taxonomic classification of the first 50 Blast hits is shown at the family level. The family of lactobacilliales is shown in red with black outlines. For each gene family, the gene family identifier and the glycoside hydrolase enzyme family (GHxx) are given.

1051



1052

1053 **Figure S8. Additional genomic regions encoding class II bacteriocins in Firm5**

1054 **strains of bumble bees.** Genomic regions encoding bacteriocin genes were

1055 identified and visualized with MultiGeneBlast v1.1.14 (Medema *et al.* 2013). Arrows

1056 represent genes, and same color indicates homology. A black line indicates the

1057 lactococcin 972 locus (*lcl*) and vertical grey blocks connect the homologous genes in

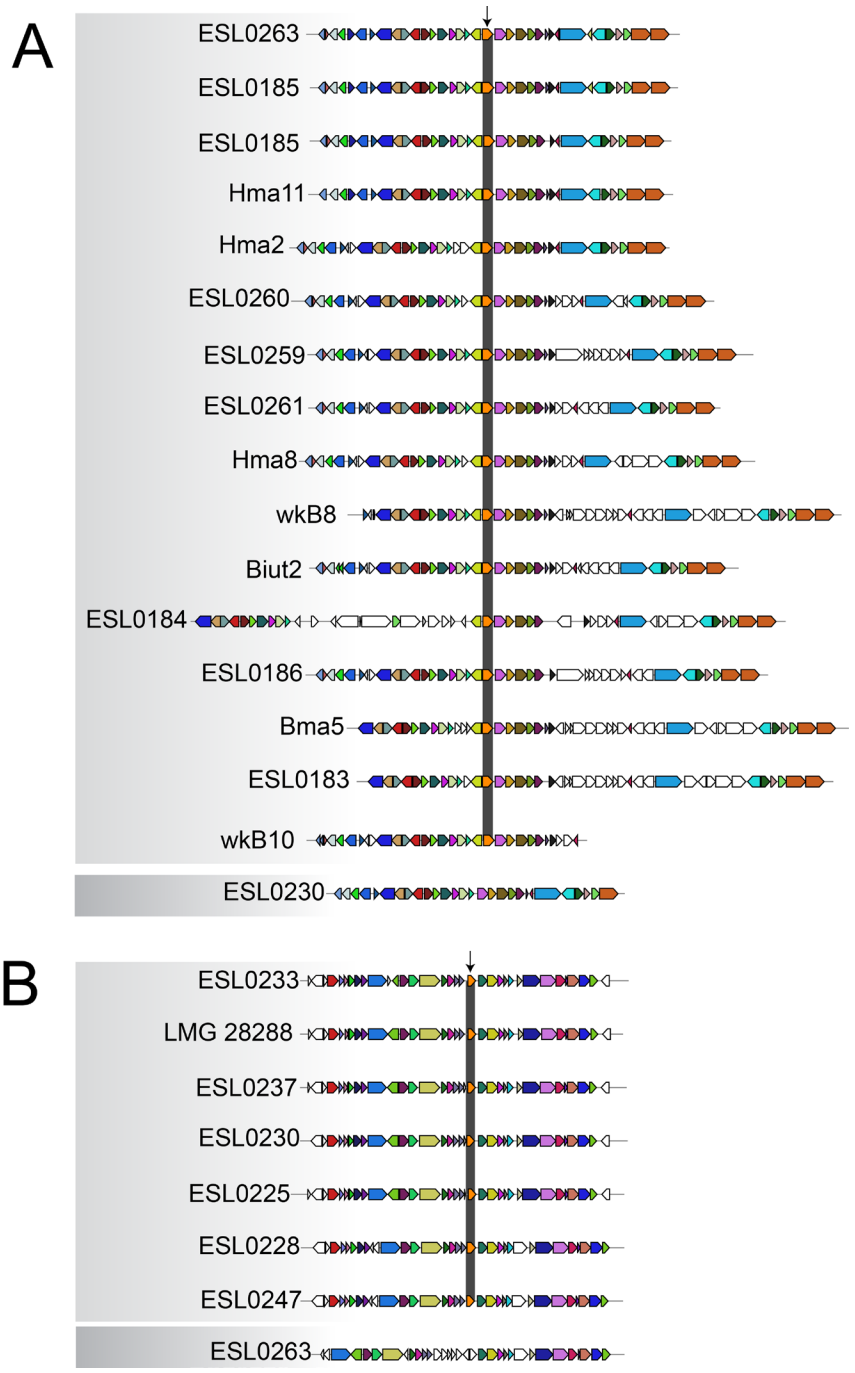
1058 other strains. An enlarged version of the three genes of the *lcl* locus with annotation

1059 is shown in the lower right of panel A. Grey shading over strain names indicates two

1060 sublineages of bumble bee strains; the four honey bees strains are representatives

1061 of the four sublineages.

1062


A
ESL0263
ESL0185
ESL0185
Hma11
Hma2
ESL0260
ESL0259
ESL0261
Hma8
wkB8
Biut2
ESL0184
ESL0186
Bma5
ESL0183
wkB10
ESL0230
B
ESL0233
LMG 28288
ESL0237
ESL0230
ESL0225
ESL0228
ESL0247
ESL0263

1063

1064 **Figure S9. Genomic regions encoding helviticin-J, a class III bacteriocin.** Genomic
1065 regions encoding bacteriocin genes were identified and visualized with
1066 MultiGeneBlast v1.1.14 (Medema *et al.* 2013). Arrows represent genes, and same
1067 color indicates homology. An arrow points at the helveticin-J gene homolog and
1068 vertical grey blocks connect the homologous genes in other strains. Strains with the
1069 two different types of grey shadings indicate strains from bumble bees and honey
1070 bees. **(A)** Genomic region encoding helveticin-J in honey bee strains, and **(B)** genomic
1071 region encoding helveticin-J in bumble bee strains.
1072

1073 # Supplementary Tables and Datasets (as separate files)

1074 **Table S1.** Strain list and genome features.

1075 **Table S2.** Pairwise 16S rRNA gene sequence identities.

1076 **Table S3.** ANI values.

1077 **Dataset S1.** List of gene families and their distribution according the three major

1078 groups: honey bee strains, bumble bee strains, outgroup strains.

1079 **Dataset S2.** List of sublineage-specific gene families and COG category

1080 abbreviations.

1081 **Dataset S3.** List of genes per genome with hits to the Carbohydrate-active enzyme

1082 (CAZY) database.