Title: Differential carbohydrate utilization and organic acid production by honey bee symbionts

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Abstract:

The honey bee worker gut is host to a community of bacteria that primarily comprises 8-10 bacterial species. Collectively, these microbes break down and ferment saccharides present in the host's diet. The model of metabolism for these gut symbionts is rooted in previous analyses of genomes, metagenomes, and metatranscriptomes of this environment. Importantly, there is a correlation between the composition of the gut microbiome and weight gain in the honey bee, suggesting that bacterial production of organic acids might contribute to the observed phenomenon. Here we identify potential metabolic contributions of symbionts within the honey bee gut. We show significant variation in the metabolic capabilities of these microbes, highlighting the fact that although the microbiota appears simple and consistent based on 16S rRNA gene profiling, strains are highly variable in their ability to use specific carbohydrates and produce organic acids. Finally, we confirm that the honey bee core microbes, especially a clade of γ-proteobacteria (i.e. *Gilliamella*), are highly active *in vivo*, expressing key enzymatic genes critical for utilizing plant-derived molecules and producing organic acids. These results suggest that *Gilliamella*, and other core taxa, may contribute significantly to weight gain in the honey bee, specifically through the production of organic acids.

33 **Introduction:**

34 Insects are some of the most abundant and diverse species on earth, inhabiting
35 a plethora of unique environments, ranging from the arid sands of the hottest desert to
36 frigid temperatures of glaciers (1). In order to thrive in such unusual environments, many
37 species of insects have evolved specialized symbioses with microbes capable of
38 synthesizing essential nutrients (*e.g.,* amino acids and vitamins) or degrading complex
39 macromolecules (2). The world's most important agricultural pollinator, the honey bee
40 (*Apis mellifera*), is no exception, as it relies on microbes to aid in the digestion of its
41 plant-derived diet of honey and bee bread (3). After consumption, food ingested by the
42 bee is metabolized by the microbes that inhabit the honey bee gut, which include a small
43 number of dominant taxa that constitute bacterial species considered to be "core" to the
44 honey bee gut microbiota, including the proteobacteria *Snodograssella alvi*
45 (Betaproteobacteria), *Parasaccharibacter apium* (Alphaproteobacteria), *Frischella*
46 *perrara* and *Gilliamella apicola* (Gammaproteobacteria), two *Lactobacillus* species
47 (Firmicutes), a *Bifidobacterium* species (Actinobacteria), and a rarer *Bacteroidetes* (4).
48 Three of these phyla, the Gammaproteobacteria, Firmicutes, and Bifidobacteria,
49 dominate predicted functional capacity of the honey bee gut microbiome, based on a
50 metatranscriptomic survey on the honey bee gut (5). The current proposed model of
51 bacterial community metabolism in the bee gut, based on annotated gene presence and
52 expression, suggests that these core microbes participate in the breakdown and
53 fermentation of host dietary macromolecules into an array of alcohols, gases, short chain
54 fatty acids (SCFAs), and other organic acids, some of which likely serve to sustain the
55 host (5-10). Complementing this work, some studies have demonstrated, *in vitro*, the
56 ability of some strains to ferment a variety of substrates (5, 9, 11-14) and produce
57 organic acids (i.e. lactate and acetate)(11, 14). In addition to these observations, a
58 separate study also recently demonstrated that gnotobiotic, mono-colonized bees

possessed guts containing significant depletion in plant derivatives (i.e. flavonoids, ω-hydroxy acids, and phenolamides), likely originating from pollen consumed by the host, and an enrichment in fermentative products in the form of organic acids and SCFAs, compared to bees with a depleted microbiota (15). The production of these fatty acids may contribute to honey bee health. Indeed, a recent study identified a correlation between host weight gain and organic acids in the digestive tract of bees (3). Bees supplemented with gut bacteria showed an increase in the variety and amount of organic acids present within the host digestive system, as well as a significant increase in host weight gain and the expression of host genes related to insect growth and nutrient homeostasis, compared to controls. Collectively, these results link the honey bee symbionts to the production of organic acids and then to host growth and nutrient acquisition. It has been proposed that honey bee core bacteria likely convert carbohydrates in the bee diet to a variety of organic acids, which can either act as a signaling molecules eliciting a host response, or act as building blocks for host derived anabolism.

In light of these recent findings, we sought to determine the metabolic contribution of the Gammaproteobacteria, Firmicutes, and Bifidobacteria, within the bee gut, as these phyla dominate the metatranscriptomic profile of the digestive tract of the honey bee. Although there are well-defined species within these groupings, there is still a large diversity of honey bee strains found within individual bees and colonies (16). The strain diversity within each of the honey bee core clades is reflected in differences in genomic content (7, 16), suggesting that significant functional diversity may exist within each of the core 8-10 clades associated with the honey bee. In this study, we address fundamental questions regarding metabolic characteristics of the honey bee gut microbiota, such as: 1) What is the pattern of anaerobic utilization of environmentally relevant carbon sources across dominant symbionts, and what are the direct byproducts

85 produced, per taxa, *in vitro*? 2) Are there functional differences in the patterns of
86 utilization between isolates of the same species? 3) What contribution does an individual
87 taxon make to the utilization and the subsequent fermentation of environmentally
88 relevant substrate throughout the digestive tract of the honey bee? Through utilizing a
89 combination of molecular, biochemical, and physiological approaches, both *in vitro* and
90 *in vivo*, we perform an in-depth metabolic analysis on 17 isolates from the honey bee
91 gut. Our results suggest that overall, bee-associated microbes are primed to metabolize
92 certain components of the honey bee diet, but that significant variation exists within
93 clades, suggesting that each member contributes differentially to honey bee metabolism.
94
95 **Materials and Methods:**
96 *Bee Sampling and Bacterial Culture*
97 Worker honey bees were collected from colonies located at an apiary in
98 Bloomington, IN and transported to lab in sterile vials on ice. Hindgut dissections were
99 performed using aseptic technique and sterile equipment. Whole digestive tracts of 2-3
100 bees were homogenized in 1X phosphate buffered saline (PBS) with a sterile disposable
101 pestle and a dilution series was plated onto Brain-Heart Infusion (BHI), de Man, Rogosa
102 and Sharpe (MRS), and Luria-Bertani (LB) agar (BD Difco™). All agar plates were
103 incubated under anaerobic conditions and supplemented with CO_2 using the GasPak EZ
104 system (BD, New Jersey) at 37°C for 4 – 5 days. Isolated colonies were subcultured on
105 agar before genotyping (below).
106
107 *16S rRNA genotyping of bacterial isolates*
108 For each isolate, genomic DNA was extracted using the QIAGEN DNeasy Blood &
109 Tissue kit with one modification: the inclusion of a bead-beating step before lysates were
110 loaded onto the Qiagen column. DNA was quantified using a spectrophotometer (BioTek

instruments) and used in PCR using Phusion® High Fidelity PCR mix with HF buffer (New England BioLabs INC.), with 27F (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3') and 1492R (5'–ACG-GCT-ACC-TTG-TTA-CGA-CTT-3') primers. A reaction consisted of 0.4 mM primers, 1x Phusion mix, PCR grade water, and 1 µL of sample DNA, under the following conditions: 5 min at 98°C; 35 cycles-10 sec at 98°C, 30 sec at 55°C, 30 sec 72°C; Extension-10 min at 72°C. PCR products were visualized using gel electrophoresis and all isolates with PCR products of the correct size were used for sequencing (Beckman Coulter Genomics). Resulting ab1 files were concatenated and exported to Fasta. To align, trim, and classify sequences, Mothur v.1.33.3 software was used with the following commands (align.seqs, trim.seqs, and classify.seqs), using a custom reference database (Greengeen + honey bee specific dataset) (Newton and Roeselers, 2011) for classification. To demonstrate the evolutionary relationship between isolates, a 16S rRNA gene phylogeny was constructed, using the concatenated 16S rRNA gene sequence used for classification. The tree was rooted with *Aquifex aeolicus* (Accession: AJ309733.1) and to serve as representatives of the four dominant bacterial families within the honey bee (*Gilliamella apicola* - Accession: NR_118433.1 (Gilliamella), *Lactobacillus* sp. wkB8 - Accession: NZ_CP009531 - Locus Tag: LACWKB8_RS00480 (Firm-5), Uncultured *Lactobacillus* sp. - Accession: HM113352.1 (Firm-4), and *Bifidobacterium asteroides* strain Hma3 - Accession: EF187236.1 (Bifido) were used. Sequences were uploaded to SINA 1.2.11 aligner (ARB SILVA), and aligned using the default settings, after which the FASTA formatted alignment was converted to MEGA format (MEGA v6.06) and a maximum likelihood tree (GTR + G + I) was constructed using 1000 bootstrap replicates. Divergence estimates were performed in BLASTClust (Alva et al, 2016).

Carbohydrate Utilization & Byproduct Production of Isolates

137 To prepare cell suspensions to assay substrate utilization and product formation,
138 all isolates classified as actinobacteria or bacilli were grown on MRS agar, while gamma-
139 proteobacteria isolates were grown on BHI; these media correspond to the medium
140 under which the microbes were isolated. Broth cultures were grown anaerobically at
141 30°C for 6 days, after which cultures were pelleted via centrifugation and the supernatant
142 removed. The pellet was washed with salt solution (137.0 mM NaCl, 2.7 mM KCl, pH
143 6.0) to remove remaining media. Bacilli and actinobacteria, grown in MRS, were washed
144 twice to decrease the transfer of nutrients present in the spent MRS media. Isolates
145 were normalized for growth based on OD, across taxonomic classes, such that isolates
146 with more robust growth were diluted in a larger amount of salt solution.
147 To test the ability of isolates to utilize carbon substrates, we used the MT2
148 MicroPlate™ assay. In the assay, each well in a 96-well plate contained an aliquot of a
149 TTC dye. To each well, 150 µL of cell suspension from each isolate was added, with or
150 without 15 µL of a filter-sterilized carbon source (either 1.1 M of D-(+)-glucose (SIGMA
151 G8270), D-(-)-fructose (ACROS Organics 57-48-7), D-sucrose (Fischer Scintific BP220-
152 1), D-(+)-mannose (SIGMA M6020), L-rhamnose (SIGMA W373011), D-(+)-galactose
153 (SIGMA G0625), D-(+)-xylose (SIGMA X1500); or 13.1% D-(+)-cellobiose (SIGMA
154 22150), or 2.1% pectin (SIGMA – Pectin from apple – SIGMA 76282) solutions).
155 Negative controls received 15 µL of salt solution. To verify that substrate solutions were
156 void of microbes and that cross-contamination between wells was not occurring, an
157 additional negative control was filled with 15 µL of a sole carbon source with 150 µL of
158 salt solution. Each experimental condition and control was analyzed in triplicate within a
159 single plate. 96 well plates were covered with an optical adhesive film, and incubated
160 anaerobically at 30°C for 3 days. To measure reduction of TTC (and utilization of a
161 substrate), absorbance readings at 590 nm were taken, in triplicate, for each well.
162 Significant utilization was determined by performing a t-Test comparing 590 nm readings

163 between experimental (cell suspension + 15 µL substrate) and negative control (cell
164 suspension + 15 µL of salt solution) wells. All statistics were performed in IBM SPSS
165 Statistics Version 24 on datasets tested for a normal distribution. Biological triplicates for
166 each condition or control were then combined in a screw-top cryovial and stored at -80°C
167 until high performance liquid chromatography (HPLC) analysis (below).
168 To determine the soluble fermentation products, pooled samples were thawed,
169 vortexed, and then centrifuged at 13,000 RPM for 5 minutes to pellet bacterial debris.
170 After centrifugation, samples were filtered through a 0.45 µm HAWP membrane
171 (Millipore Corp., Bedford, Mass.) and acetate, lactate, formate, and succinate were
172 quantified by using a Shimadzu HPLC (Kyoto, Japan) as previously described (17).
173 *Assaying the expression of enzymatic genes*
174 For each dissection, the crop, midgut, and hindgut were aseptically incised from
175 the full digestive tract, and placed in a 2.0 mL Lysing matrix E tube with 500 uL of
176 Trizol® reagent. Samples were homogenized using a FastPrep®-24 instrument, twice, at
177 6.0 m/s for 40 seconds. RNA were extracted from samples using the standard protocol
178 provided by the manufacturer. After the final washing, RNA was resuspended in RNA
179 Storage Solution (Bioline) and stored at -80°C for downstream analysis. For each gut
180 chamber, each biological replicate consisted of three separate individuals. All analyses
181 were performed in triplicate with the same biological samples.
182 Primer sets specific for a particular enzymatic gene and group of honey bee
183 microbes (Gilliamella, Firm-5, and Bifido), were designed for qRT-PCR analyses
184 (Supplementary Table 1, Supplementary Figure 2). Selected primer sets fulfilled the
185 following requirements: 1) they amplified a product of the expected size, 2) the resulting
186 sequenced product had a top BLAST hit(s) to the intended bacterial taxa and the gene of
187 interest, or for the reference sequence used to design the primer set and 3) they showed
188 specificity for the bacterial group of interest in laboratory experiments (Supplemental

189 Table 1, Supplemental Figures 2). RNA extracted from individual gut chambers
190 (foregut, midgut, and hindgut) were used as a template for qRT-PCR reactions. Prior to
191 running reactions, all RNA samples were treated with RNase-free DNase I (Ambion) as
192 prescribed by the manufacture. RT-qPCR was performed using SensiMix ™ SYBER R
193 Hi-ROX One-Step kit and protocol. As a reference locus in these analyses, we used the
194 16S rRNA gene, using a universal bacterial primer set (S-*-Univ-05150a0S-19/S-D-Bact-
195 0787-b-A-20 (18)). Reactions were run on an Applied Biosystems StepOne RT-PCR
196 system: Initial denaturation-5 min at 98°C; 40 cycles -15 sec at 98°C, 1 min at 60°C, 30
197 sec 72°C; Extension-10 min at 72°C. A standard curve was generated for each primer
198 set separately, to account for primer efficiencies in our analyses. All statistics were
199 performed using IBM SPSS Statistics Version 24.
200 To quantify the expression of metabolic genes from honey bee specific clades of
201 bacteria, across gut chambers of the honey bee, Ct values acquired from the
202 quantification analyses of individual genes were initially normalized to the mean
203 amplification of the reference locus (16S rRNA gene) across gut sections. The
204 advantage of normalizing the data to 16S RNA gene is that it demonstrates the presence
205 of genes, relative to a proxy for the whole bacterial community present throughout the
206 digestive tract of the honey bee. Next, for each metabolic gene prescribed to a taxa of
207 interest, we standardized the data to the gut section with the lowest observed
208 amplification (i.e the crop) for calculating the relative expression (RNA). To determine
209 the relative ratio of expression of metabolic loci attributed to a particular taxa, across gut
210 sections, normalized datasets were standardized to the gut section and locus with the
211 lowest level of expression. To determine variation in the relative abundance, relative
212 expression, or relative ratio of expression of loci across gut sections, we performed an
213 ANOVA or Kruskal-Wallis test on the standardized data acquired for each individual
214 gene analyzed, which was dependent on results obtained from normality test of

individual gene datasets. Results with statistical significance ($p \leq 0.05$) were subjected to pairwise comparisons using one-way ANOVA/post hoc or Mann-Whitney test.

Results:

The honey bee core microbiota utilizes carbohydrates from the host diet

To determine the ability of gut isolates to utilize environmentally relevant substrates, we isolated 17 honey bee associated bacteria, spanning three transcriptionally dominant clades: *Gilliamella*, *Lactobacillus* Firm-5, and *Bifidobacterium* (Figure 1, Supplemental Figure 1). Based on the construction of a 16S rRNA gene phylogeny, including data from the 17 cultured isolates and 145 sequences previously obtained from bee-associated microbiota, some of the microbial cultures used herein are closely related to core honey bee bacteria while others fall outside of the core clades (Supplemental Figure 1). When comparing the diversity within the clades (based on 16S rRNA genes), all isolates are unique at 98% and 99% divergence, and some are unique at 97% divergence (Table 1), indicating that these microbes represent different bacterial strains and species.

To test the ability of gut bacteria to utilize sole carbon sources relevant to the honey bee diet, we used Biolog MT2™ plates, containing an aliquot of an indicator tetrazolium chloride dye (TTC), that when combined with a sole carbon source, along with an aliquot of culture, detects substrate utilization via a concomitant color change due to reduction of the dye. Each isolate was examined for the sole utilization of 9 carbon sources (glucose, fructose, sucrose, xylose, pectin, cellobiose, mannose, rhamnose, and galactose) in pure culture under anaerobic conditions. Honey, which provides the host with simple carbohydrates (e.g. glucose, fructose, and sucrose) and trace amounts of secondary sugars (*e.g*. mannose, rhamnose, and galactose) (19-21). Matured pollen (beebread), provides the host with essential nutrients (i.e. amino acids,

241 minerals, lipids), and is also rich in complex plant-derived substrates (e.g. cellobiose,
242 xylose, and pectin)(22, 23). Therefore, the compounds selected for analysis have
243 previously been identified, through biochemical assays, as molecules that constitute part
244 of the honey bee diet.

245 When examining the individual and collective data for the utilization of glucose,
246 fructose, and sucrose, nearly all isolates were able to utilize at least one or more of
247 these substrate(s), with the exception of 4 taxa: *Staphylococcaceae* EBHJ3 and
248 *Lactobacillales* MAH G07-3 within the Firmicutes and *Enterobacteriaceae* LB Tet D09
249 and LB Tet C11 (Figure 2, Supplemental Figure 2). At the phylum-level, at least 3
250 isolates within each phylum were capable of utilizing glucose, fructose, and sucrose,
251 with varying degrees of consistency (Figure 2A, Supplemental Figure 2A). Collectively,
252 when comparing isolates representative of taxa specific to the honey bee gut microbiota
253 (i.e. *Gilliamella*, *Lactobacillus* Firm-5, and *Bifidobacterium*), the general trend for
254 substrate utilization suggested that the honey bee bacteria were primed to utilize these
255 substrates (Figure 2). Interestingly, however, we did observe differences among strains
256 within clades in their ability to use different compounds. For example, although all
257 *Bifidobacteria* and *Lactobacillus* Firm-4 strains used glucose, fructose, and sucrose, the
258 *Lactobacillus* Firm-5 strain MAH H05-3 did not metabolize either monosaccharaide to a
259 significant extent. Similarly, *Gilliamella* strains differentially processed sucrose; although
260 both strains tested could utilize glucose and fructose, only Gamma-1 LB D06 could
261 utilize sucrose, although to a modest extent (Figure 2).

262

263 *Acetate, lactate, succinate, and formate are the primary fermentation products produced*
264 *by honey bee gut microbiota*

265 To determine the soluble fermentation products produced by each individual
266 isolate, post anaerobic metabolism, we analyzed the contents within each experimental

267 and control well of the MT2™ plates described in the previous section using HPLC. For
268 this analysis, we focused on the products in pure cultures with glucose, fructose, and
269 sucrose. We chose to use simple sugars abundant in honey, rather than complex
270 polymeric carbon sources, because complex carbon sources are degraded to simple
271 sugars and funneled into the same catabolic pathways. With the exception of pectin, all
272 other saccharides analyzed for utilization are mono- or di- saccharides and are only a
273 few enzymatic reactions away from glycolysis or the pentose phosphate and Entner-
274 Doudoroff pathways. While the ratio of fermentation products is expected to change
275 during growth on different substrates, it is less likely that different products would be
276 produced from different substrates. We used known standards (*e.g.,* acetate, lactate,
277 formate, succinate) and identified peaks at the appropriate retention times within each
278 biological sample.

279 HPLC analysis revealed a variety of organic acids produced by honey bee
280 associated microbes, under anaerobic conditions. When comparing the supernatant of
281 experimental and control wells of isolates, almost all isolates that were positive for
282 utilizing a substrate exhibited the presence of fermentative products (as derived from
283 retention times identical to known substrate standards, *i.e.* lactate, acetate, succinate,
284 and formate; Figure 2). We expected to observe ethanol, acetoin, propanoate, 2,3-
285 butanediol, based on the metabolic pathways identified in the metatranscriptomic data
286 but did not identify other significant peaks beyond the four described above, even though
287 the instrument is capable of detecting these compounds.

288 When comparing the general pattern of products produced across taxa, bacteria
289 classified within the *Bifidobacterium* clade unanimously produced lactate and acetate,
290 regardless of substrate. However, production of succinate and formate by this same
291 clade was variable both across the phylogeny and across substrates (Figure 2). The
292 Firm-5 and *Gilliamella* isolates produced these same organic acids, but with an even

293 larger degree of variability within the clades (Figure 2). Altogether, the data for the 17
294 isolates supports the hypothesis that honey bee gut microbiota are primed to consume
295 and ferment the components of the honey bee diet, but significant variation exists within
296 strains such that no uniform profile could be ascribed to a clade or species group. For
297 example, even if isolates of the same species used a substrate, they did not produce the
298 same organic acids; formate was detected from some strains of *Bifidobacterium* under
299 glucose but not under fructose, while all produced formate when given sucrose (Figure
300 2).
301
302 *Microbes utilize more complex carbohydrates and sugars toxic to the honey bee*
303 We extended our analysis to plant derivatives of pollen (cellobiose, xylose, and
304 pectin), as well as secondary sugars commonly found in honey (mannose, rhamnose,
305 and galactose) and known to be toxic to the host in high quantities. For the plant derived
306 carbohydrates, we observed the same trend noted for the utilization of simple sugars,
307 with at least 2 isolates within each major taxon capable of utilizing cellobiose, xylose,
308 and pectin. Interestingly, all honey bee specific bacteria tested could utilize pectin while
309 more distantly related bacteria (*Staphilococcaceae, Lactobacillales, Enterobacteriaceae*)
310 could not (Figure 3). However, we found variation in the ability of strains within honey
311 bee associated clades in their use of other complex carbohydrates; xylose was only
312 utilized by half of the *Bifidobacterium* strains tested and only 1 out of 4 *Lactobacillus*
313 strains. In contrast, we observed no difference between honey bee specific and more
314 distantly related bacteria in their ability to utilize sugars toxic to the bee (Figure 3). Again,
315 however, we observed variation in the ability of strains to utilize these toxic sugars with
316 only some core members able to use all of these toxic sugars and some core members
317 unable to use any (*Bifidobacteriales* LB C04 and *Lactobacillus* Firm-4 MaH G10-2,
318 Figure 3). Overall, these results suggesting the honey bee associated bacteria utilize

319 mannose, rhamnose, and galactose, secondary sugars common in the honey bee diet,
320 but toxic to the host.
321
322 *The honey bee gut compartments are associated with altered gene expression by the*
323 *microbiota*
324 We determined that isolates from the honey bee gut microbiota ferment sugars
325 common to the honey bee diet into organic acids *in vitro* (Figure 2). Recent work by
326 Zheng et al. (2017) demonstrated *in vivo*, the presence of a variety of organic acids
327 across distinct gut chambers of the honey bee digestive tract. Based on the
328 observations described above, we hypothesized that symbionts of the honey bee gut are
329 likely contributing to presence of organic acids *in vivo*, and since each gut chamber
330 houses a specific compilation of symbionts (24, 25), perhaps functional differences exist
331 in the metabolic activity of symbionts across individual gut chambers. To address this
332 hypothesis, we quantified the expression of genes that encode for fermentative enzymes
333 throughout the three gut chambers of the honey bee digestive tract (i.e. crop, midgut,
334 and hindgut (anterior to posterior)). We designed taxon-specific primers for two genes:
335 acetate kinase (*ackA*) and L-lactate dehydrogenase (*ldh*), encoding for enzymes
336 responsible for the production of acetate and lactate, respectively. In addition, we also
337 designed primers for clade specific enzymes (that is, the metabolic function was only
338 identified in one of the three phyla involved in cleaving glycosidic bonds within plant-
339 derived compounds (*e.g.* cellulase and cellodextrinase for *Bifidobacteria*, pectate lyase
340 for *Gilliamella*, and glucosidase for the *Lactobacillus* Firm-5 clade; Supplementary Table
341 1)). We used qRT-PCR on RNA extracted from each gut section to quantify the
342 expression of these loci. For the *Gilliamella* and *Lactobacillus* Firm-5 clades, the midgut
343 and hindgut chambers were found to be the most active gut sections (*Gilliamella* (df = 2;
344 x^2 = 7.05; p = 0.029), Firm-5 (df = 2; F = 3.95; p = 0.040); Figure 4A and C). In contrast,

345 while the activity of the *Bifidobacterium* clade increases across gut sections from
346 posterior to anterior, the trend is not significant ((df = 2; 2 = 4.36; p = 0.113); Figure 4B).
347 To obtain a better understanding of genes responsible for the trends seen in the
348 *Gilliamella* and *Lactobacillus* Firm-5 clades (which both resulted in statistically significant
349 contrasts across the digestive tract), we performed a one-way ANOVA/Tukey-HSD to
350 analyze the differences in variation of relative gene expression across gut sections. For
351 *Gilliamella*, the analysis of ackA and ldh expression indicated that expression levels
352 significantly increased across gut sections in the following order: crop<midgut<hindgut
353 (Figure 4D). Pectate lyase was detected at equal levels of expression in the midgut and
354 hindgut, but no amplification was detected in the crop (Figure 4D). In contrast to
355 *Gilliamella*, we saw no pattern to the expression of ldh or ackA by *Lactobacillus* Firm-5
356 across the gut sections (Figure 4E). Importantly, we were not able to detect β-
357 glucosidase and cellulase gene expression from Firm-5 and *Bifidobacterium* clades, in
358 any gut section, suggesting that they are not active in the honey bee gut.
359
360 **Discussion:**
361
362 The honey bee gut microbiota is a deceptively simple community – although
363 dominated by 8-10 species (26) and transcriptionally dominated by only 3 phyla (5), the
364 strain diversity found within the gut is high (16). Here we show that strains within three
365 transcriptionally dominant clades associated with the bee are functionally distinct and
366 that there is indeed, no strict metabolic rule that describes each clade. Although overall,
367 the microbes seem primed to utilize carbohydrates in the honey bee diet, expressing
368 enzymes capable of cleaving glycosidic bonds and fermenting these substrates, strains
369 within each clade of core microbes investigated here differ in their ability to use different
370 simple sugars, sugars toxic to the bee, or complex carbohydrates found in the bee diet

371 (Figures 2, 3). They also differ in their production of organic acids, the production of
372 which are suspected to contribute to honey bee metabolism. The results presented in
373 this study reveal that bacterial taxa associated with the honey bee gut are differentially
374 present and metabolically active throughout the three major gut chambers, with
375 particular enrichment in the hindgut. Of the three taxa assayed for gene expression, the
376 *Gilliamella* clade of bacteria was the most active, showing exceptional expression of its
377 acetate kinase gene in the mid- and hindgut, a result supported by previous
378 metatranscriptomic analyses.

379 The utilization results presented in this study are supported by previous
380 observations of gross pH changes when honey bee isolates are cultured (9, 11-14) and
381 previous measures of acetic and lactic acid production from a few pure cultures (27, 28).
382 Although our results are well supported by predicted models based on the genomic
383 evidence, we did not detect the production of specific fermentative waste products
384 predicted (e.g. ethanol, acetoin, propanoate, 2,3-butanediol)(5). The lack of detection of
385 these products in our analysis could be due to the following: 1) a complete lack of
386 production under the *in vitro* conditions provided 2) or production below the detectable
387 range of the UV and refractive index detectors used.

388 In addition to analyzing the utilization of the substrates mentioned above, we also
389 examined the ability of isolates to utilize pectin, which is a heteropolysaccharide,
390 commonly found in the primary cell wall of terrestrial plants, such as pollen grains. In our
391 analysis, we identified multiple isolates within the *Gilliamella, Lactobacillus* Firm-5, and
392 *Bifidobacterium* clades able to utilize pectin (Figure 3). Importantly all tested core
393 microbiome strains utilized pectin in our assays – it was the only consistently utilized
394 carbohydrate tested – and more distantly related organisms (e.g. *Staphylococcaceae,*
395 *Lactobacillales, Enterobacteriaceae*) were unable to use it. Previous work has shown
396 that *Gilliamella* isolates are capable of degrading pectin, possessing a pectate lyase

397 gene capable of cleaving de-esterified pectin (7). In addition to *Gilliamella*, we also
398 report utilization of pectin and/or its derivatives by isolates within the Firm-4/-5 and
399 *Bifidobacterium* clades, observations supported by a recent publication that observed
400 growth of several core gut isolates in minimal media supplemented with pollen extract
401 (15). These results suggest that multiple constitutes of the honey bee microbiota may be
402 capable breaking-down and utilizing plant material abundant in the host's diet, an idea
403 that has previously been hypothesized (10). Indeed, the genome of *Bifidobacterium*
404 *asteroides* has an annotations for glycosidases and a pectinesterase, enzymes capable
405 of degrading pollen (6). Contrary to *Bifidobacterium*, genomes from bacteria in the
406 *Lactobacillus* Firm-5 clade currently have no annotated pectinases or glycosidases (8),
407 but these and many of the genomes of honey bee specific bacteria possess a plethora of
408 hypothetical genes of unknown function that might aid in the degradation and
409 metabolism of pectin (9). Importantly, although the pectin used in our *in vitro* assays was
410 of high quality, it is not a completely uniform nor pure substance and therefore it is
411 possible that trace amounts of other compounds might have been present and
412 metabolized by the honey bee specific microbes. Further experiments need to be
413 conducted to confirm the pectinase activity of the isolates analyzed. It should also be
414 noted, that the conditions provided in the utilization assay likely do not support the
415 growth of all 17 isolates as the salt solution lacks several elements required for growth.
416 However, although the assay does not rely on growth, it is possible that the utilization
417 patterns observed for individual isolates could potentially alter under alternative
418 conditions conducive for growth.

419 Recent important work has identified a link between the presence of canonical
420 gut symbionts and weight gain in honey bees; organic acids, which are presumed to be
421 bacterially derived, are thought to contribute to the observed influence symbionts to host
422 health (28). The fact that these microbes live within the bee gut and are consistently

423 associated with honey bees has led to the assumption that microbes isolated from the
424 bee are mutualists in this context – that they benefit the bee in some way. However,
425 supplementation of the bee diet with one of these core members (*Snodgrasella)* results
426 in an increased susceptibility to parasites (29) and another (*Frischella*) results in scab
427 formation in the intestine (7). The converse can also be true; non-core members of the
428 bee can provide a benefit as well. *Lactobacillus kunkeii*, an environmentally acquired
429 microbe, protects from pathogens (30) and although *Gilliamella* strains have been found
430 to use sugars that are toxic to the bee (31), here we show that both non-core bacteria
431 and core microbes can utilize these sugars.

432 The difference in the enzymatic activity pattern between members of the honey
433 bee microbiota suggests they might occupy different niches in the honey bee food,
434 digestive tract, or built environment. The idea that niche partitioning might exist in the
435 gut of the honey bee is physically plausible, as the alimentary system in the honey bee is
436 subdivided into three distinct chambers. Indeed, our analysis of gene expression (qRT-
437 PCR) suggests that expression of different enzymes can differ across gut sections,
438 genes, and taxa. It should be noted, however, that we could not detect the expression of
439 genes responsible for the breakdown of complex plant-derived molecules (i.e.
440 glucosidase, cellodextrinase, and cellulase). These results indicate that these genes are
441 either completely inactive, or are expressed at levels below detection. In either scenario,
442 the data suggest that perhaps complex carbohydrates are either present at minimal
443 levels in honey bee gut, or that the taxa analyzed in this study thrive on other resources
444 abundant in the environment (i.e. glucose, fructose, sucrose, and/or other plant
445 derivatives) by expressing other enzymatic loci capable of contributing to catabolism.

446 Our work suggests a dominant profile for *Gillamella in vivo.* Of the three analyzed
447 taxa, the *Gilliamella* clade was the most active in our analysis, with the *Bifidobacterium*
448 and *Lactobacillus* Firm-5 clades being roughly equal; this result fits well with previously

449 published transcriptomic data, where the gamma-proteobacteria dominated the
450 transcriptional profile of the gut (5). When assaying the relative expression of certain
451 genes (i.e. ackA and ldh) for the *Gilliamella* clade (Figure 4A, D), we saw highest levels
452 of expression in the hindgut, suggesting that *Gilliamella* may function differently in each
453 gut chamber or may be particularly active in the hindgut. Additionally, within each gut
454 section, *Gilliamella* consistently expresses more ackA than ldh, which might suggest a
455 preference for this taxon to produce acetate, a byproduct produced *in vitro* by *Gilliamella*
456 isolates under nearly all tested conditions (Figure 2). It is intriguing that *Gilliamella*
457 dominates transcriptomic analyses of the honey bee and related species are found in
458 bumble bees (8). *Gilliamella* and other proteobacterial species in the honey bee require
459 direct social contact between bees beyond mere trophallaxis (i.e. fecal exposure) to
460 achieve transmission (32). The fact that the honey bee workers acquire this important
461 symbiont through their social interactions, and that *Gilliamella* dominates the functional
462 profile of the honey bee worker gut suggests it to be an important, if not critical, part of
463 the honey bee core microbiota.
464 Recent work elegantly demonstrated through metabolomics that honey bees
465 primarily colonized by individual bacterial strains of taxa core to the gut microbiota show
466 depletion in plant derived substrates and an enrichment in fermentation product present
467 in the digestive system, compared to bees with a depleted gut microbiota (33). These
468 results are promising, as they demonstrate substrate utilization by individual core taxa
469 and enrichment of metabolites within the gut *in vivo.* However, our work here suggests
470 that the choice of strain used in colonization experiments may substantially influence
471 results; not all *Gilliamella apicola* strains produce the same organic acids under the
472 same conditions. It is perhaps the case that the diversity of strains found within the
473 honey bee gut complement each other, providing a range of organic acids under most
474 conditions and diets. This hypothesis awaits testing. Nevertheless, it is obvious that

individual taxa found in the core microbiome of bees might not accurately reflect the metabolic dynamics occurring in the gut of a honey bee with a natural microbiota, where microbe-microbe and microbe-host interactions likely govern niche partitioning and microbiome function.

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Conflicts of interest statement:

The authors declare no conflict of interest.

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595

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Tables:

Table 1. Cluster analysis of the 16S rRNA gene sequences of 17 cultured honey bee gut isolates used for metabolic analysis. A FASTA file with near full-length 16S rRNA sequences for each isolate was uploaded to BLASTClust (Alva et al., 2016). Standard settings were used for the analysis, using 97% or 98% identity thresholds. The total number of isolates analyzed for each class of bacteria is provided, along with the number of phylotype clusters at 97% and 98% ID.

Bacterial Class	Total Number of isolates	Number of Phylotypes (%ID)	
		97	98
y-Proteobacteria	5	5	5
Bacilli	6	5	6
Actinobacteria	6	1	6

Figures:

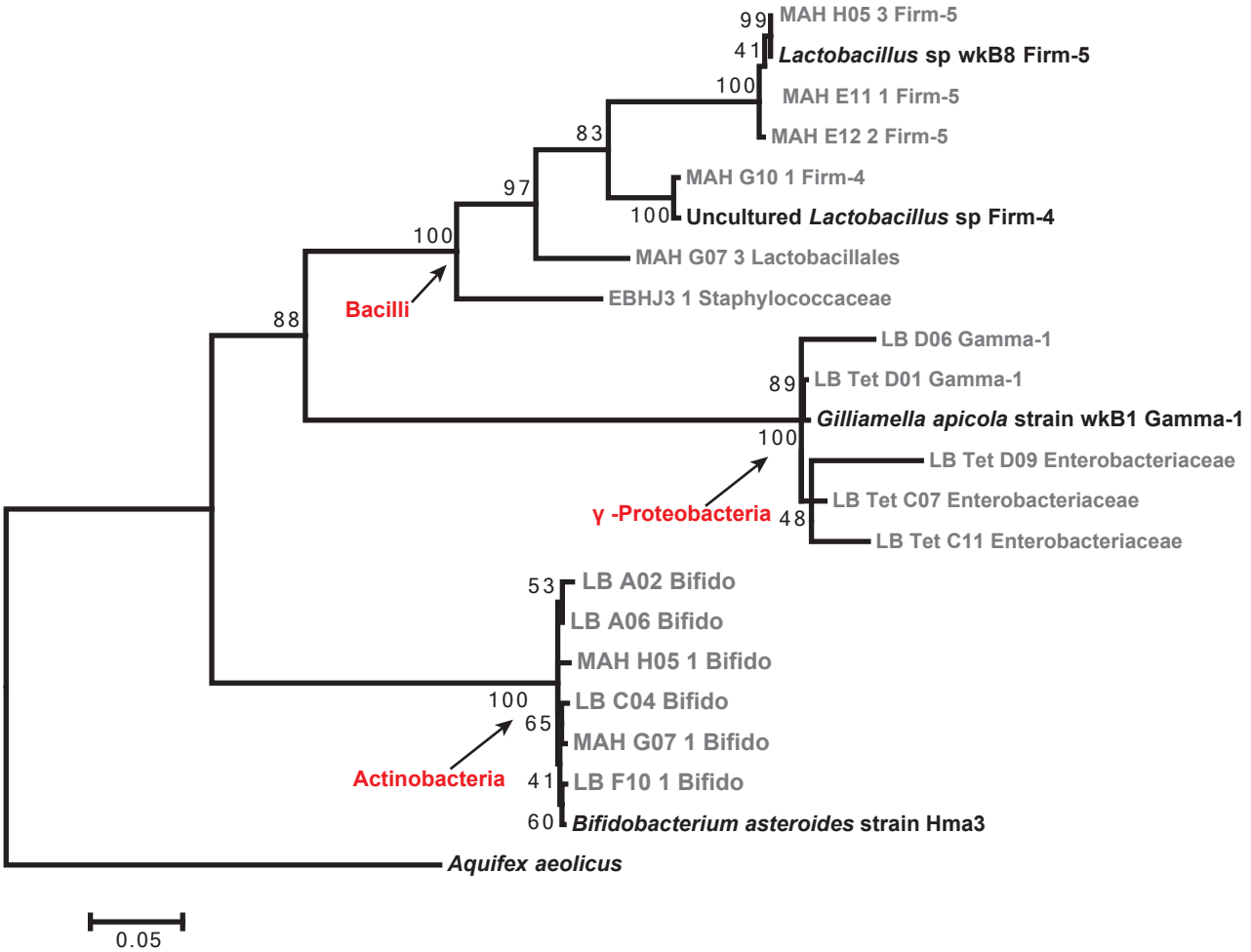
Figure 1. 16S rRNA gene phylogeny of gut isolates selected for metabolic analyses, in relationship to reference sequences of honey bee specific taxa. Sequences were aligned using SINA Aligner 1.2.11, the output file was used to construct a Maximum likelihood phylogeny, in MEGA 6.06 with GTR (G+I) and 1000 bootstraps. Bootstrap support is provided at nodes. Reference sequences indicated by black text. Cultured isolates indicated by grey text. Taxonomic class highlighted by arrow.

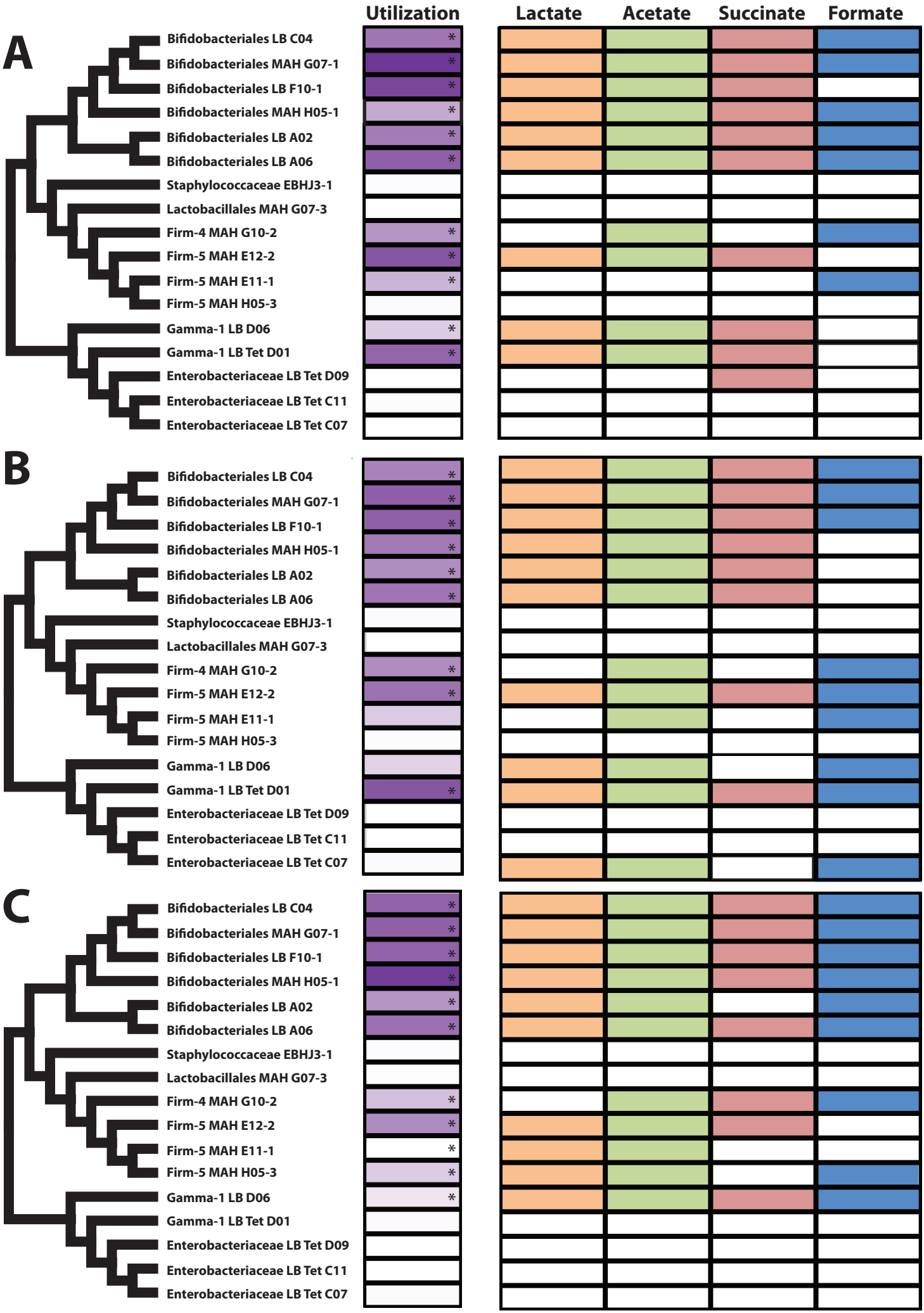
Figure 2. Phenotypic profile of substrate utilization by gut isolates. On the left of the figure are consensus phylogenetic trees derived from the 16S rRNA gene phylogeny. Utilization column is a heat map representation of OD590 measurements (background subtracted) from 1.4 (most purple) to zero (white). Asterisks indicate statistically significant results, compared to negative control, for glucose (A), fructose (B), or sucrose (C). Similarly, the detection of organic acids is denoted by boxes filled will the color that corresponds to a given compound detected: formate (blue), acetate (green), lactate (orange), and succinate (red), based on retention peaks on HPLC chromatograms.

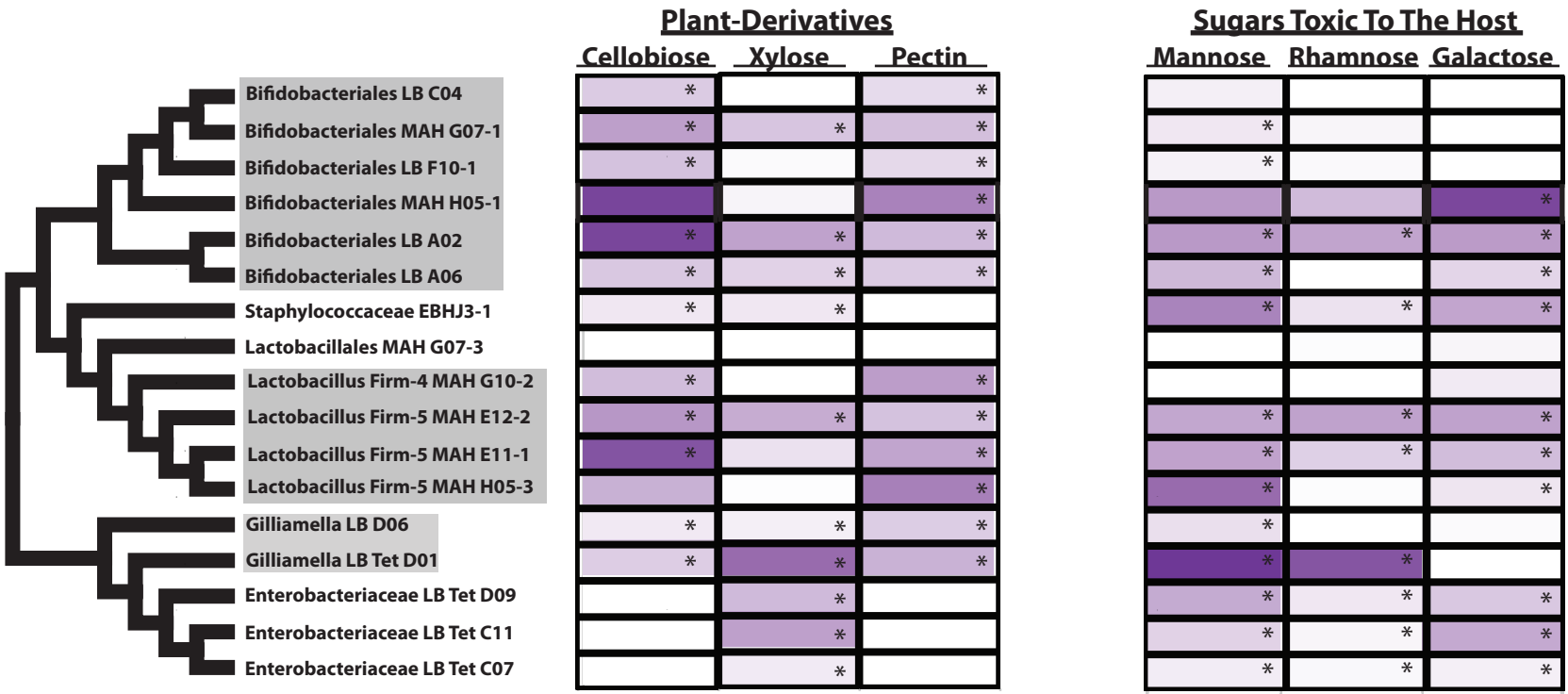
Figure 3. Phenotypic profile of substrate utilization by honey bee isolates. On the left are cladograms derived from the 16S rRNA gene phylogeny. Isolates highlighted in grey indicate taxa considered core to the honey bee gut, while all other isolates are representative of non-core members. The utilization of other plant-derived sugars (i.e. cellobiose, xylose, and pectin) and dietary saccharides toxic to the host (mannose, rhamnose, and galactose) is a heat map representation of OD590 measurements

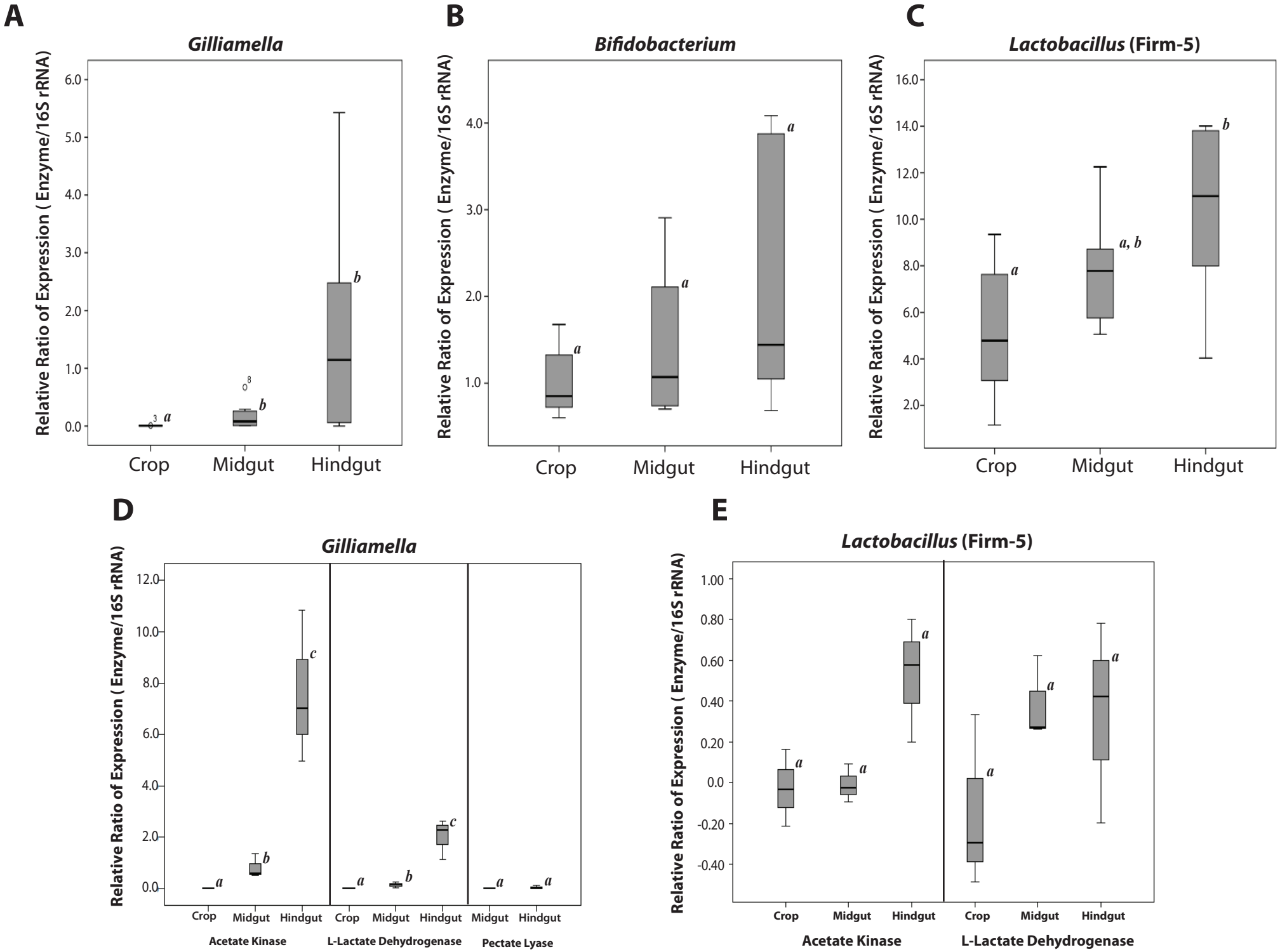
(background subtracted) from 1.4 (most purple) to zero (white). Asterisks indicate statistically significant results, compared to negative control.

Figure 4. Expression of metabolic genes derived from honey bee specific taxa. Reverse transcriptase quantitative PCR (RT-qPCR) expression shown for each enzymatic genes, relative to the average 16S rRNA gene expression, across chambers of the honey bee gut. Standardized to data from the crop, the overall relative ratio of expression of enzymes for *Gilliamella* (A), *Bifidobacterium* (B), and *Lactobacillus* clades(C) were determined across gut chambers. (D-F) The relative expression data of individual genes, for *Gilliamella* (D), *Bifidobacterium* (E), and *Lactobacillus* (F), across the gut sections. For each dataset, relative expression data were compared across gut sections, using ANOVA or Kruskal-Wallis test in SPSS. For statistically significant results ($p \leq 0.05$), pairwise comparisons were conducted using one-way ANOVA/post hoc or Mann-Whitney test in SPSS, significant difference ($p \leq 0.05$) between gut sections are denoted with letters.


99
MAH H05 3 Firm-5
41
Lactobacillus sp wkB8 Firm-5
100
MAH E11 1 Firm-5
MAH E12 2 Firm-5
83
MAH G10 1 Firm-4
97
100
Uncultured Lactobacillus sp Firm-4
100
MAH G07 3 Lactobacillales
Bacilli
EBHJ3 1 Staphylococcaceae
88
LB D06 Gamma-1
89
LB Tet D01 Gamma-1
Gilliamella apicola strain wkB1 Gamma-1
100
LB Tet D09 Enterobacteriaceae
γ -Proteobacteria
48
LB Tet C07 Enterobacteriaceae
LB Tet C11 Enterobacteriaceae
53
LB A02 Bifido
LB A06 Bifido
MAH H05 1 Bifido
100
LB C04 Bifido
65
MAH G07 1 Bifido
Actinobacteria
41
LB F10 1 Bifido
60
Bifidobacterium asteroides strain Hma3
Aquifex aeolicus
0.05


Utilization
Lactate
Acetate
Succinate
Formate
A
Bifidobacteriales LB C04
Bifidobacteriales MAH G07-1
Bifidobacteriales LB F10-1
Bifidobacteriales MAH H05-1
Bifidobacteriales LB A02
Bifidobacteriales LB A06
Staphylococcaceae EBHJ3-1
Lactobacillales MAH G07-3
Firm-4 MAH G10-2
Firm-5 MAH E12-2
Firm-5 MAH E11-1
Firm-5 MAH H05-3
Gamma-1 LB D06
Gamma-1 LB Tet D01
Enterobacteriaceae LB Tet D09
Enterobacteriaceae LB Tet C11
Enterobacteriaceae LB Tet C07
B
Bifidobacteriales LB C04
Bifidobacteriales MAH G07-1
Bifidobacteriales LB F10-1
Bifidobacteriales MAH H05-1
Bifidobacteriales LB A02
Bifidobacteriales LB A06
Staphylococcaceae EBHJ3-1
Lactobacillales MAH G07-3
Firm-4 MAH G10-2
Firm-5 MAH E12-2
Firm-5 MAH E11-1
Firm-5 MAH H05-3
Gamma-1 LB D06
Gamma-1 LB Tet D01
Enterobacteriaceae LB Tet D09
Enterobacteriaceae LB Tet C11
Enterobacteriaceae LB Tet C07
C
Bifidobacteriales LB C04
Bifidobacteriales MAH G07-1
Bifidobacteriales LB F10-1
Bifidobacteriales MAH H05-1
Bifidobacteriales LB A02
Bifidobacteriales LB A06
Staphylococcaceae EBHJ3-1
Lactobacillales MAH G07-3
Firm-4 MAH G10-2
Firm-5 MAH E12-2
Firm-5 MAH E11-1
Firm-5 MAH H05-3
Gamma-1 LB D06
Gamma-1 LB Tet D01
Enterobacteriaceae LB Tet D09
Enterobacteriaceae LB Tet C11
Enterobacteriaceae LB Tet C07


Plant-Derivatives
Cellobiose
Xylose
Pectin
Sugars Toxic To The Host
Mannose
Rhamnose
Galactose
Bifidobacteriales LB C04
Bifidobacteriales MAH G07-1
Bifidobacteriales LB F10-1
Bifidobacteriales MAH H05-1
Bifidobacteriales LB A02
Bifidobacteriales LB A06
Staphylococcaceae EBHJ3-1
Lactobacillales MAH G07-3
Lactobacillus Firm-4 MAH G10-2
Lactobacillus Firm-5 MAH E12-2
Lactobacillus Firm-5 MAH E11-1
Lactobacillus Firm-5 MAH H05-3
Gilliamella LB D06
Gilliamella LB Tet D01
Enterobacteriaceae LB Tet D09
Enterobacteriaceae LB Tet C11
Enterobacteriaceae LB Tet C07


A
Gilliamella
Relative Ratio of Expression (Enzyme/16S rRNA)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
b
b
Crop
Midgut
Hindgut
B
Bifidobacterium
Relative Ratio of Expression (Enzyme/16S rRNA)
4.0
3.0
2.0
1.0
a
a
a
Crop
Midgut
Hindgut
C
Lactobacillus (Firm-5)
Relative Ratio of Expression (Enzyme/16S rRNA)
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
a
a, b
b
Crop
Midgut
Hindgut
D
Gilliamella
Relative Ratio of Expression (Enzyme/16S rRNA)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
a
b
c
a
b
c
a
a
Crop
Midgut
Hindgut
Crop
Midgut
Hindgut
Midgut
Hindgut
Acetate Kinase
L-Lactate Dehydrogenase
Pectate Lyase
E
Lactobacillus (Firm-5)
Relative Ratio of Expression (Enzyme/16S rRNA)
1.00
0.80
0.60
0.40
0.20
0.0
-0.20
-0.40
a
a
a
a
a
a
Crop
Midgut
Hindgut
Crop
Midgut
Hindgut
Acetate Kinase
L-Lactate Dehydrogenase