Social communication between microbes colonizing the social honey bee *Apis mellifera*

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Abstract

The European honey bee (*Apis mellifera*) is a charismatic species that plays a critical role in the pollination of agriculturally important crops and native flora. One emerging field of research is that of the host-associated honey bee microbiome: a group of bacterial phylotypes consistently found within the honey bee, which may play critical roles such as protection from pathogens and nutrient acquisition. In other model systems, host-associated microbial communities are known to participate in a form of bacterial communication known as quorum sensing. This type of communication allows bacteria to sense their environment and respond with changes in gene expression, controlling a number of factors including virulence, biofilm formation, and cell motility. Here, we have investigated the production of a specific quorum sensing molecule by honey bee microbes *in vivo* and *in vitro*. We specifically focused on the inter-species signaling molecule, autoinducer-2 (AI-2). We identified the production of AI-2 by both the entire community (using honey bee gut homogenates) and by cultured isolates, using a *Vibrio harveyi* biosensor. By comparing newly emerged and adult bees, we showed this signal is likely coming from the core microbial community. Finally, using honey bee specific bacterial isolates, we identified changes in biofilm production when isolates are exposed to increased levels of exogenous AI-2. Altogether, these data provide multiple lines of evidence for the presence of quorum sensing inside the honey bee host. The effect of AI-2 on biofilm formation by honey bee specific bacteria identifies one potential avenue for quorum sensing to affect host health.

Author summary

Microbial communities associate with every animal on the planet and can have dramatic effects on the health of their host. The honey bee is one such animal, home to a characteristic community of bacteria, which may provide various benefits. Here, we show that these microbes are producing quorum sensing molecules which could support interactions between bacterial members and facilitate host colonization.

Introduction

Host-associated microbial communities (the microbiota) can have dramatic effects on the health, fecundity, and longevity of many insect hosts. For example, germ free *Drosophila* are unable to survive the larval stage when in low nutrient environments and their survival can be restored with the addition of just one bacterial strain (1, 2). Additionally, alterations in the microbiome can affect traits as from mating behavior (3) to protection from pathogens (4, 5). These examples point to the critical and varied roles of the microbiota in insects. Insects are the most numerically and taxonomically abundant animal group on the planet and play important roles in disease ecology (6), herbivory (7), pollination (8), and other ecosystem processes (9, 10). It is therefore vital that we understand how insect associated microbes may shape insect health and subsequently or directly, impact their ecological roles.

The honey bee gut microflora is described as a consistent group of bacterial clades, dominated by Gamma-proteobacteria, Firmicutes, and Actinobacteria (11–14). Basic characterization of these microbial groups has led to speculations about their role in honey bee health and whether they are

45 responsible for provisioning nutrients (15) or assisting in the breakdown of plant-derived
46 carbohydrates (16, 17), as is the case for other insect-associated microbes. Additionally, honey
47 bees are more susceptible to pathogens after their microbiome is disrupted by antibiotics,
48 supporting a protective role of the microbiota (5). Although we are just starting to understand the
49 functions of these microbial species, we do know that they interact with each other *in vivo* (18)
50 and *in vitro* (19). For example, *Gilliamella, Snodgrassella,* and *Lactobacillus* strains together form
51 a biofilm on host tissue in the ileum of honey bees (18). When grown in co-culture, lactic acid
52 bacteria found in the honey bee promote each other's growth, suggesting a mutualistic or
53 syntrophic interaction (19). However, little is currently known about *how* the honey bee associated
54 microbes interact with each other and how these interactions impact the host.

55 It is important to emphasize that bacterial species do not exist in isolation; although studied in
56 monoculture in the laboratory, their natural ecology includes other microbial organisms. One way
57 bacteria communicate in their natural ecology is through a process referred to as "quorum sensing".
58 Quorum sensing is broadly phylogenetically conserved, found in a variety of bacterial classes (20).
59 This density-dependent communication allows bacteria to sense their environment and make
60 population scale behavioral changes in response. Bacteria participating in quorum sensing produce
61 signaling molecules, termed autoinducers, and the concentration of these molecules correlates with
62 bacterial density. When a threshold is reached, autoinducers often elicit changes in gene expression
63 which affect many processes including biofilm formation, symbiosis, motility, virulence and a
64 number of others (21, 22). For example, the bobtail squid forms an intimate, mutualistic
65 relationship with *Vibrio fisheri*, which produces bioluminescence at high densities, providing
66 beneficial camouflage for the host (28). In contrast, quorum sensing from *Sodalis praecaptivus* in
67 grain weevils suppresses virulence factors after establishment, allowing for persistent infection

(29). Additionally, *Vibrio cholerae* uses quorum sensing to mediate transmission by decreasing biofilm formation to increase dissemination (20, 23). These examples show the complexity of quorum sensing signals inside hosts and highlight the importance of understanding interactions in a host-specific manner.

Autoinducer-2 (AI-2) is a quorum sensing communication molecule that has received a lot of attention because of its effects on both gram-positive and negative bacteria. This molecule is predicted to be produced by 50% of all sequenced bacteria and detected by many more, controlling a broad array of behaviors including virulence, motility, nutrient acquisition, and biofilm formation (22). Because many bacteria produce, sense, and respond to AI-2 signals, quorum sensing via this molecule can result in community level effects. One example is the assembly of multispecies biofilms. In human oral cavities, *Streptococcus gordonii* and *Porphyromonas gingivalis* grow together to form a symbiotic, multispecies biofilm through the production and sensing of this interspecies signaling molecule. In the absence of AI-2, no biofilm is produced; however, biofilm production is restored if either species is able to produce AI-2 (24–26). Also, AI-2 production by gut microbiota may help to mitigate microbial community changes during antibiotic treatment. Mice with a microbiota that produced increased levels of AI-2 maintained a greater diversity of their bacterial gut microbiome after exposure to antibiotics (27). Taken together, these and other studies show that quorum sensing can cause changes in microbial gene expression, bacterial behavior, or community structure, which have resulting effects on host health.

In this study, we present the first analysis of quorum sensing by honey bee associated bacteria. We identified genes encoding the AI-2 producing enzyme (LuxS) in the genomes of both honey bee and bumble bee associated microbes and we predict that this gene is functional by identifying the important catalytic residues. We also provide evidence for the production of the AI-2 molecule

within the honey bee gut using a *Vibrio harveyi* biosensor and hypothesize that the hindgut of the honey bee may be a focal chamber for interspecies quorum sensing. We compared newly emerged bees with mature adult worker bees to show that the level of *luxS* expression by one honey bee gut community member (*Gilliamella*) increases as the bacterial community matures. We then used cultured representatives from the major groups associated with the honey bee and identified two clades (*Gilliamella* (Gamma-1), and *Bifidobacteria*) that produce AI-2 when cultured *in vitro,* as expected based on the genomic analysis of LuxS in these genera. Finally, we show that AI-2 can modify an important density-dependent behavior for honey bee host colonization: the formation of a biofilm.

Methods

Bioinformatics analysis of luxS in existing genomic datasets

The *luxS* loci were identified based on functional annotations in previously published metagenomic scaffolds (Engel et al. 2012; JGI IMG/M project ID 2498). Scaffolds containing these loci were downloaded from the JGI IMG/M and manually annotated and curated using the Artemis genome browser software (30). Percent identities (amino acid) between LuxS homologs found in the scaffolds were elucidated using National Center for Biotechnology Information's (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST) suite. We found additional homologs of LuxS in honey bee specific *Gilliamella* and *Bifidobacterium* species through sequence homology with the type strains (accessions NZ_CP007445.1 and NC_018720.1 respectively) using NCBI's BLAST. All amino acid sequences were downloaded from the NCBI and aligned using MUSCLE with default parameters (31). The alignment was converted to relaxed

113 phylip format and RAxML was used to generate the phylogeny (raxmlHPC-SSE3 -m
114 PROTCATBLOSUM62) (32).

115 ***Isolation and culture of bacteria from honeybee guts***

116 Forager honey bees, identified by the presence of provisions in their pollen baskets, were collected
117 via aspirator from colonies maintained at Indiana University – Bloomington. Entire digestive tracts
118 were removed by dissection (the crop, midgut, and hindgut), whole guts were homogenized in
119 sterile PBS, and a dilution series was plated on Brain-Heart Infusion (BHI) agar. Plates were grown
120 anaerobically (via GasPak) at 37°C for 2 days. Isolated colonies were subcultured on BHI to obtain
121 and maintain a pure culture. After 2 days of growth, DNA was extracted using the DNeasy Blood
122 and Tissue kit (QIAGEN), the 16S rRNA was amplified with 27F/1492R primers and sequenced
123 using the 27F primer. Sequences were trimmed for quality and cover > 400bp. Percent identities
124 to known representative strains of honey bee gut microbes determined using NCBI's BLAST.

125 ***Phylogenetic analysis***

126 From these isolated and taxonomically characterized bacterial cultures, we chose a representative
127 sample that phylogenetically clade with known honey bee specific phylotypes (following (12)).
128 An alignment was generated using 16S rRNA gene sequences and the SINA aligner, which takes
129 into account the 16S rRNA structure. This alignment was used as input to R (version 3.3.3) to
130 create a phylogenetic tree using maximum likelihood with the *ape* and *phangorn* packages.
131 Bootstrap values were generated from 1000 replications.

132 ***Detection of AI-2 via Vibrio harvei reporter***

The assay to detect AI-2 production was performed as previously described using a *Vibrio harveyi* reporter strain (34). The *Vibrio harveyi* TL26 reporter strain (*ΔluxN ΔluxS ΔcqsS*; (35)) was used in combination with a positive control: *V. harveyi* BB120 (Wild type). *Vibrio* cultures were grown in autoinducer bioassay (AB) medium aerobically at 30°C overnight. Entire digestive tracts were removed from foragers by dissection and were homogenized in sterile PBS either by section or the entire tract. Honey bee bacterial isolates were grown anaerobically in BHI broth at 37°C for two days. For use in the assay, an overnight culture of TL26 strain was diluted to 1:1000 and 1:5000. Isolates and gut homogenates were tested in triplicate by adding the undiluted, cell free supernatant of each isolate to the 1:5000 dilution of TL26. The positive control contained the cell-free supernatant of BB120 and the 1:5000 dilution of TL26. Cell free supernatant were obtained by centrifuging one mL of culture for five minutes at 14K g. Negative controls contained sterile BHI media and the 1:1000 dilution of TL26. Cell controls contained sterile H_2O and the 1:1000 dilution of TL26. The plate was incubated at 30°C shaking aerobically for 8 hours. After incubation, luminescence and OD600 were measured on a Synergy H1 plate reader (BioTek Systems). Final luminescence values of the isolates are normalized to the final OD600 of the isolate.

Detection of Gilliamella apicola LuxS Expression using quantitative RT-PCR analysis

Whole gut sections of newly emerged honey bees as well as adult bees (aged 3-12 days) were collected from established, healthy hives located at Wellesley College in Wellesley, Massachusetts. RNA was extracted using TRIzol reagent and quantitative RT-PCR analyses were performed using SensiFAST™ SYBER Hi-ROX One-Step (Bioline) with primers specific to Gamma-1 LuxS gene (Forward: TTGTATGCCAACACTGTCCTTT, Reverse: TGGCGCGATGATCTTAATTT) and the host actin gene (Forward: ATAGCCAAAACCATGGCAAC, Reverse: TAAAAACCAGTTCGGCAACC, (36)) using the

Applied Bioscience StepOnePlus qRT-PCR machine (Life Technologies). Specificity was determined by Sanger sequencing of the amplified product and using NCBI's BLAST to identify. The expression levels were normalized to the host actin gene using the ΔCt Method.

Amplicon sequencing of the bacterial community in newly emerged and adult bees

Using the same samples above, RNA was extracted from the homogenates using TRIzol reagent (Ambion). RNA was DNase treated (DNA I, New England Biolabs) and cDNA was synthesized using the SuperScript III First-Strand synthesis system (Invitrogen). cDNA from each sample was amplified via PCR using Earth Microbiome barcoded primers 515F and 806R (37). Earth Microbiome amplification protocols were followed, except for the polymerase used (HF Phusion, New England Biolabs) and amplicons were cleaned with a PCR cleanup kit (Qiagen). Picogreen protocol was used to quantify DNA concentration for each pool sample. Samples were then normalized and pooled collectively for sequencing. Sequencing was performed on an Illumina Miseq, using 250 PE cycles. Sequences are available to reviewers upon request and are currently being deposited at the DDBJ.

Sequence Analysis

All sequence processing was performed using the Mothur microbial ecology suite (38). Reads from each sample were combined into contiguous sequences and screened for quality (maxambig 0, maxlength 300). Sequences were then aligned with the Silva reference database (silva.bacteria.fasta), preclustered, and examined for chimeras via the uchime function. After removal of chimeric sequences, sequences were taxonomically classified using a honey bee specific training set as a reference (39) and binned into operational taxonomic units (OTUs) based

upon 97% sequence identity. The data set was also subsampled to the smallest sample size of 1230 sequences, in order to normalize across samples.

Biofilm production response to AI-2

To determine if autoinducers (AI) have an effect on biofilm formation we modified a common biofilm assay (40). Using the same isolates used for the AI-2 assays, cultures in early exponential phase were added to a 24-well culture plate (Falcon). For the exogenous autoinducer treatment, a final concentration of 10 μM of AI-2 was added (OMM Scientific). Sterile media was used for the negative control. After a 24-hour aerobic incubation at 37°C, crystal violet was added to stain the biofilm. The biofilm was then disrupted with acetic acid and the amount of stain was quantified using absorbance at 600 nm on a Synergy H1 plate reader (BioTek Systems).

Results

LuxS gene found in honey bee specific bacterial genomes

LuxS is required for the enzymatic synthesis of the AI-2 signaling molecule. This metalloenzyme, S-Ribosylhomocysteinase, cleaves thioether bonds in S-ribosylhomocysteine resulting in a homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) which is the precursor for AI-2. DPD can then spontaneously cyclize to form a furone known as AI-2. These reactions are catalyzed by a divalent metal ion, Fe^{2+}, and the activity requires three conserved residues (His-54, His-58, and Cys-126) (21, 41–43).

Using functional annotation of metagenomics scaffolds, the *luxS* gene was identified in the genomes of two honey bee specific microbes (*Gilliamella apicola* and *Bifidobacterium sp.*) (Figure

198 1A). To determine how conserved this locus was within these bacterial species, we performed a
199 search for homologs, using NCBI's BLAST, and were able to identify a *luxS* homolog in 91
200 *Bifidobacterium* species, including both honey bee and bumble bee associated strains
201 (*Bifidobacterium asteroides* and *bombi)*. Similarly, we found *luxS* within 41 *Orbus*-related species,
202 colonizing both honey bees and bumble bees (including *Frischella perrara, Gilliamella apicola,*
203 and *Schmidhempelia bombi,* Figure 1. Finally, alignment of the LuxS protein sequences identified
204 the conserved domains and residues known to be important for LuxS activity (Figure 1C). Highly
205 conserved regions included the catalytic active residue (the cysteine at position 87, arrowhead in
206 Figure 1C) and known metal co-factor binding sites (Asterisks in Figure 1C). This analysis
207 suggested that these LuxS proteins might be both conserved within two important honey bee gut
208 symbiont groups (the *Bifidobacteria* and *gamma-proteobacteria*), and potentially functional.

209 ***Detection of AI-2 production honey bee microbiota***

210 One approach to determine whether LuxS is functional in the honey bee gut symbionts is to identify
211 the production of autoinducer-2 (AI-2). Towards that end, we used a biological reporter assay,
212 where a strain of *Vibrio harveyi* (TL26), which is incapable of producing autoinducers and
213 responds to exogenous AI-2 only, was cultured in the presence of honey bee gut extracts. When
214 TL26 senses AI-2, it responds with the production of light, which we detected using a
215 spectrophotometer (see methods for more detail). We were able to detect significant AI-2
216 production in entire digestive tracts of honey bee workers as well as gut sections (fore, mid, and
217 hindgut) (Figure 2). These data suggest that AI-2 is being produced within honey bee gut digestive
218 tract.

219 ***Gilliamella strains express luxS in mature adult bees***

Because our assay above included honey bee tissue, we sought to confirm that the AI-2 signal we observed was coming from the bacterial community members. We therefore marked, age matched, and collected newly emerged bees (< 1 day old) and mature adult worker bees (> 3 days old). After extracting RNA from these samples, we used qRT-PCR and a *Gilliamella*-specific *luxS* primer set to quantify the expression of the *luxS* gene relative to host actin. In addition, we also characterized the microbial community associated with these same samples to confirm the establishment of *Gilliamella* in the mature adult worker bees. Previous work suggests that newly emerged bees lack the characteristic gut microbiome found in adult worker bees (18) and we confirmed that our newly emerged bee samples also lacked the core community and instead were dominated by unclassified OTUs (Figure 3). In contrast, mature adult bees were colonized by the characteristic core community (Figure 3). Additionally, *luxS* expression by *Gilliamella* was not detected in newly emerged bees while we identified low, but consistent expression in mature adult bees (relative expression compared to host actin was 4.40E-05 +/- 1.35E-05 SE; Figure 3).

Gilliamella* and *Bifidobacteria* species *produce AI-2 in vitro

Our results using gut extracts suggested that the honey bee gut community members may be producing AI-2 *in vivo*. To support our qRT-PCR results and the bioinformatics analysis of the *luxS* locus, we cultured *Gilliamella* and *Bifidobacteria* species from the honey bee gut and subjected their supernatants to the *Vibrio harveyi* AI-2 reporter assay. Representative isolates from the prominent clades found in the honey bee were chosen for the assay based on their phylogenetic placement (based on 16S rRNA gene sequence; Figure 4A). Each chosen isolate formed a highly supported clade (100% confidence) with known, characterized honey bee microbiome members (Figure 4A). In addition, 16S rRNA sequences from the cultured isolates were 92% - 100% identical to known honey bee associated microbes (Figure 4B). After normalizing to the optical

density of the cultures, we were able to detect AI-2 production by strains from each of these genera (as inferred from the luminescence produced by TL26) (Figure 4C). Compared to the negative controls, we observed statistically significant luminescence by TL26 in the presence of supernatants from *Bifidobacteria* and *Gilliamella* species (Figure 4C).

Biofilm production is modulated by honey bee bacterial isolates in response to autoinducers

Previous work had identified the presence of a microbial biofilm in the honey bee digestive tract. Because biofilm production is known to be regulated by quorum sensing, we sought to identify a relevant and functional link between the production of AI-2 in the honey bee and host colonization. With the same representative isolates used above, we cultured these microbes with or without exogenously added AI-2. We identified a statistically significant increase in biofilm production with added AI-2 for all our four isolates from the *Gilliamella* genus (Figure 5). However, there were no significant changes in the biofilm production of the *Bifidobacteria* isolates in response to the addition of AI-2 (Figure 5).

Discussion

Bacterial infections of eukaryotic hosts are established and maintained using a variety of bacterial behaviors such as biofilm formation, motility, and virulence. These behaviors are often modulated and controlled in a density dependent fashion using signaling molecules (44). Here we present multiple lines of evidence to support bacterial autoinducer-2 based quorum sensing in the honey bee microbiota. We identified an open reading frame, annotated as encoding LuxS, the AI-2 producing enzyme, in honey bee specific isolates. We detected the production of AI-2 *in vivo* using whole bee gut extracts. Additionally, we showed that *luxS* expression by *Gilliamella* is only detected in adult bees that harbor the core bacterial community. Finally, we also demonstrated that

specific isolates, representative of the honey bee core microbiome, produce AI-2 and increase biofilm formation in response to AI-2. These data support our conclusion that honey bee associated bacteria produce AI-2 during colonization of the host.

The two genera we worked with here (*Bifidobacteria* and *Gilliamella*) have been implicated in honey bee health or nutrition. For example, lactic acid bacteria (*Bifidobacteria*) isolated from the honey bee crop have been shown to protect larvae from pathogens such as European Foulbrood, likely through the production of antimicrobial molecules (45, 46). Members from the *Gilliamella* likely contribute to degradation of plant carbohydrates as they degrade pectin *in vitro* (15). Therefore, our work identifies a potential mechanism by which functionally important members of the honey bee microbiota may communicate with each other during their host association and suggests that AI-2 may regulate density dependent behaviors, such as biofilm formation, in the honey bee microbiota. In fact, *Gilliamella* species are known to form a multispecies biofilms in the honey bee digestive tract (18). For example, the ileum is colonized by both Gamma- and Beta-proteobacteria and the rectum is dominated by Firmicutes and Gamma-proteobacteria (18). These stratified biofilms suggest that colonization dynamics or environmental gradients may play a role in the colonization of the host. While important for the establishment and persistence of the microbiota, biofilms may also facilitate the breakdown of plant materials in host digestion. Based on our data, we propose that the production of autoinducers may mediate the colonization of honey bee specific microbes, contributing to the stratified biofilm observed. While some *Bifidobacterium* isolates were able to form a biofilm, the production of which was unaffected by exogenous AI-2, all *Gilliamella* strains increased their biofilm production in the presence of added AI-2. We therefore hypothesize that species such as *Bifidobacterium* may colonize early and that production

287 of AI-2 by these early colonizers may allow other species to form biofilms at higher density. This
288 hypothesis awaits further testing.

289 The presence of *luxS* in *Frischella* as well as isolates from *Bombus* species suggests that social
290 behaviors in these microbes, such as the production of AI-2, may be conserved across bee
291 associated microbes, both pathogens and mutualists. The production of AI-2 and quorum sensing
292 *writ large* is not uniformly beneficial to a host, as virulence is another density dependent behavior
293 often regulated by quorum sensing. For example, in the cabbage white butterfly (CWB), quorum
294 sensing in pathogenic *Pseudomonas aeruginosa* contributes to virulence such that, when quorum
295 sensing pathways are disrupted, CWB larval survival rates are increased (49). Similarly in
296 mammals, *Pseudomonas aeruginosa* utilizes quorum sensing during chronic lung infections,
297 upregulating biofilm formation and adhesion (50, 51). Importantly, we identified a *luxS* in
298 *Frischella perrara*, a putative bee pathogen, and in this organism, AI-2 may be utilized to promote
299 pathogenicity within the honey bee host (52).

300 To our knowledge, this is the first time quorum sensing has been shown to occur in the honey bee
301 microbiota. Investigations such as this one can help to identify not only behaviors mediated by
302 quorum sensing but potential cross-talk and communication *between* microbial members in the
303 gut. For example, although we focused on a single quorum sensing molecule (AI-2), there are
304 likely many other molecules (such as AHLs and oligopeptides) produced *in vivo* by honey bee gut
305 microbes. To form a complete picture of microbial communication between community members,
306 additional quorum sensing molecules need be examined as well as their effects on gene regulation.
307 We know that the honey bee bacterial community is specific and consistent (in terms of the
308 presence of members), however the proportion of different bacteria within individual bees can vary
309 (16, 19). If these bacterial members are participating in intra-species communication and

mediating important behaviors, their relative proportions may impact other community members and potentially host health. Future work is needed to understand how these persistent infections are maintained and influenced by quorum sensing.

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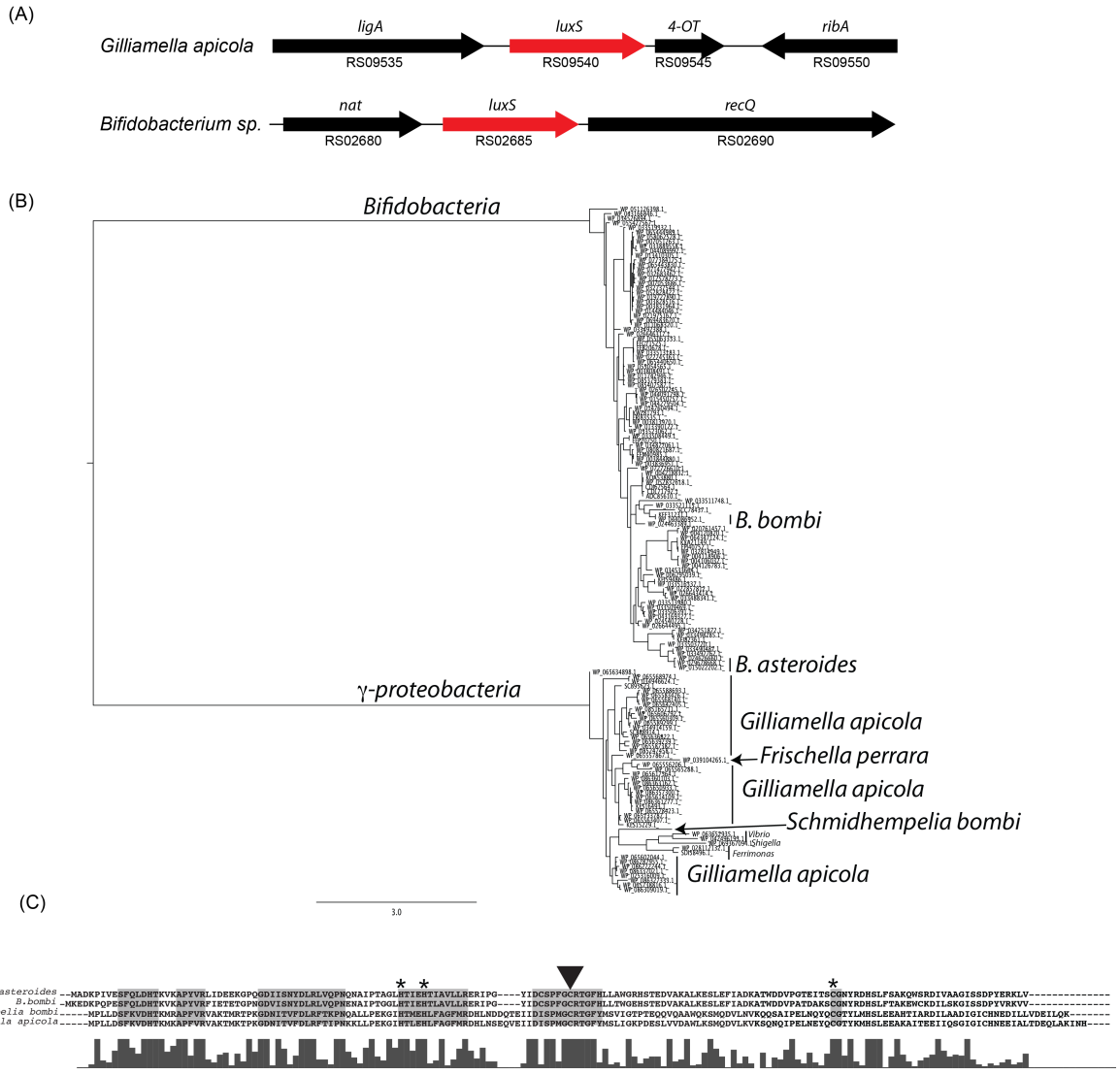


Figure 1. Honey bee associated microbes encode LuxS. (A) The *luxS* gene and its syntenic region is shown within the genomes of two honey bee specific isolates (*Gilliamella apicola* and *Bifidobacterium sp.)*. **(B)** A phylogeny generated based on aligned LuxS amino acid sequences from honey bee and bumble bee associated microbes. **(C)** LuxS homologs from honey bee specific isolates (*Gilliamella apicola and Bifidobacterium asteroids)* and bumble bee isolates (*Schmidhempelia bombi and Bifidobacterium bombi)*, were identified by functional gene annotation in an existing metagenomic dataset. Shaded areas represent highly conserved regions among these sequences as well as those of other published LuxS homologs (36, 38, 48). Asterisk = conserved iron binding sites; arrowhead = catalytic cysteine.

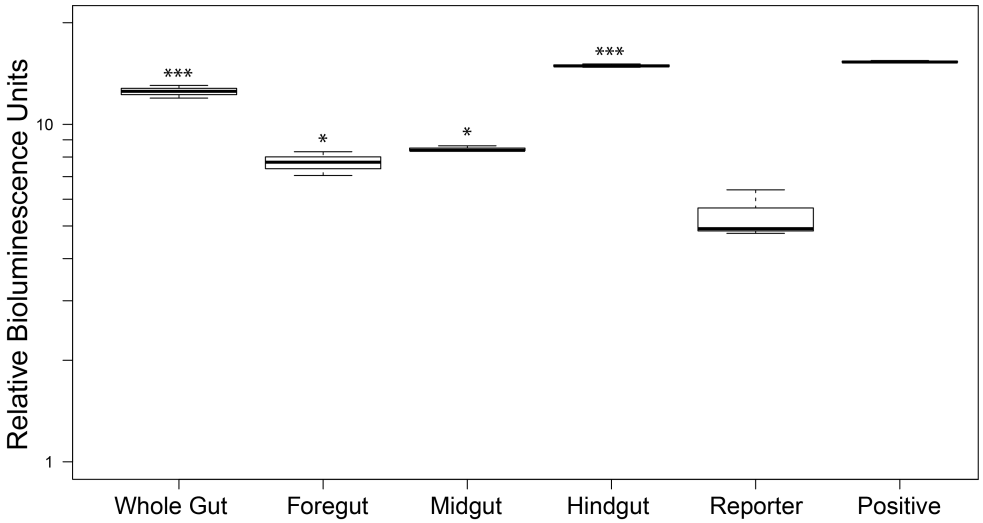


Figure 2. Detection of autoinducer-2 in the honey bee gut. After dissection, entire digestive tracts (Whole Gut) or gut sections (Foregut, Midgut, Hindgut) were homogenized and extracts were used in an autoinducer bioluminescence assay. The production of luminescence by *V. harveyi* TL26 is only observed in the presence of supernatants from the positive control (AI-2 producing *V. harveyi* strain BB120) or from extracts from the honey bee. Note log scale. Samples were compared to reporter alone with a t-test and significance designated by *** = 0.001, ** = 0.01, * = 0.05.

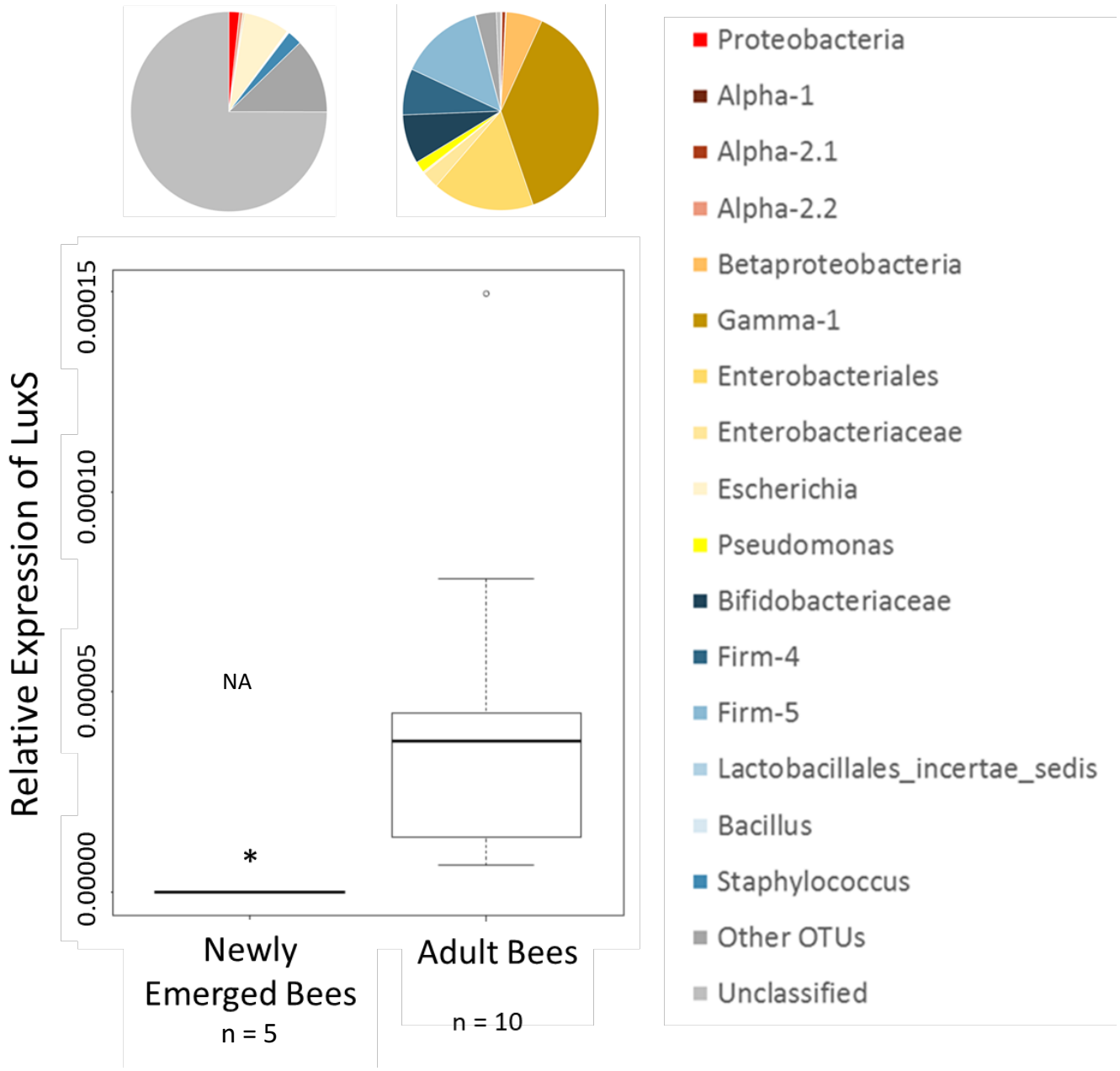
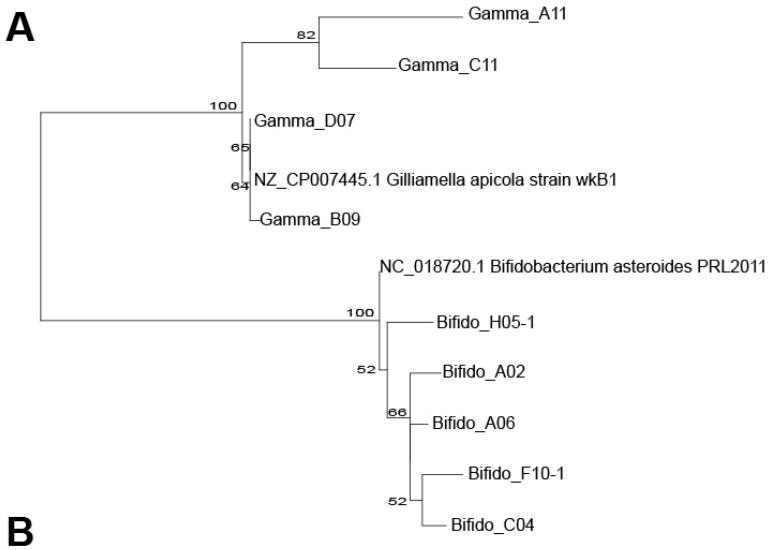


Figure 3. *luxS* gene expression increases and bacterial community composition changes as adult bees mature. Bacterial community composition, based on 16S rRNA, in newly emerged bees is dominated by unclassified bacterial taxa whereas adult bees have acquired the characteristic worker bee microbiome. Additionally, relative expression of *luxS* (qPCR) by *Gilliamella apicola* is detectable in mature adult bees while in newly emerged bees we observed no amplification of the transcript (NA = No Amplification).



B

Isolate		% ID	Accession
Bifido_A02	Bifidobacterium asteroides strain Hma3	96%	EF187236.1
Bifido_A06	Bifidobacterium asteroides strain Hma3	97%	EF187236.1
Bifido_C04	Bifidobacterium asteroides	96%	AB777204.1
Bifido_F10	Bifidobacterium asteroides strain Hma3	96%	EF187236.1
Bifido_H05	Bifidobacterium asteroides strain Bin7	97%	EF187234.1
Gamma_A11	Gilliamella apicola strain A11	100%	KT598279.1
Gamma_B09	Gilliamella apicola strain G5_4_4TCO2	99%	KF600252.1
Gamma_C11	Gilliamella apicola strain wkB1	92%	CP007445.1
Gamma_D07	Gilliamella apicola strain wkB1	99%	NR_121727.1

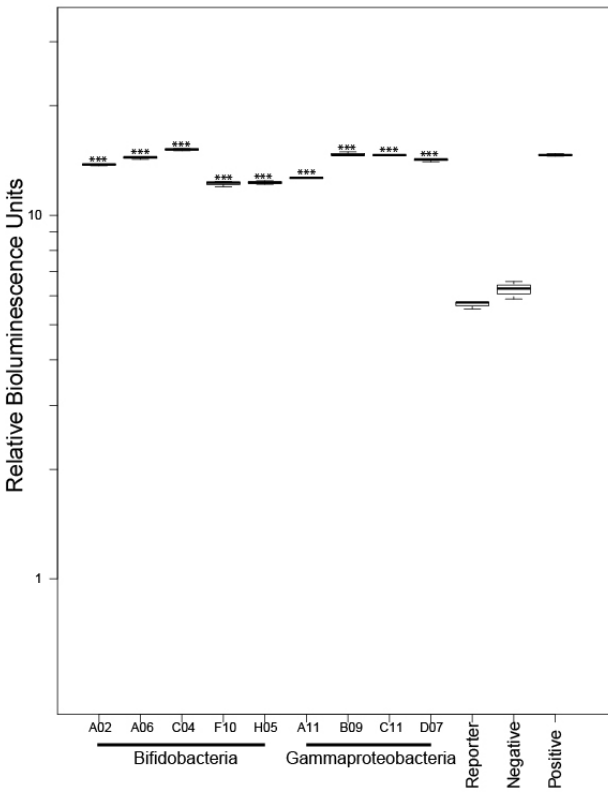


Figure 4. ***Gilliamella apicola*** **and** ***Bifidobacterium sp.*** **produce AI-2** ***in vitro*****. (A)** Phylogenetic tree of bacterial isolates utilized in this study and their evolutionary placement in the context of other honey bee gut microbes. 16S rRNA genes (> 400 bp) were used to construct this maximum likelihood phylogeny and bootstrap values are from 1000 iterations **(B)** 16S rRNA gene sequences from cultured isolates are 92-100% identical to known honey bee associated microbes. Percent identities of cultured isolates shown relative to accessions in the NCBI's nr database. **(C)** Detection of AI-2 in the overnight culture supernatants of *Gilliamella apicola* and *Bifidobacterium sp.* using *Vibrio harveyi* reporter strain luminescence. Controls (negative: sterile BHI; positive: *V. harveyi* BB120) (Note log scale). Samples compared to the Reporter only control with a t-test and significance designated by *** = < 0.001.

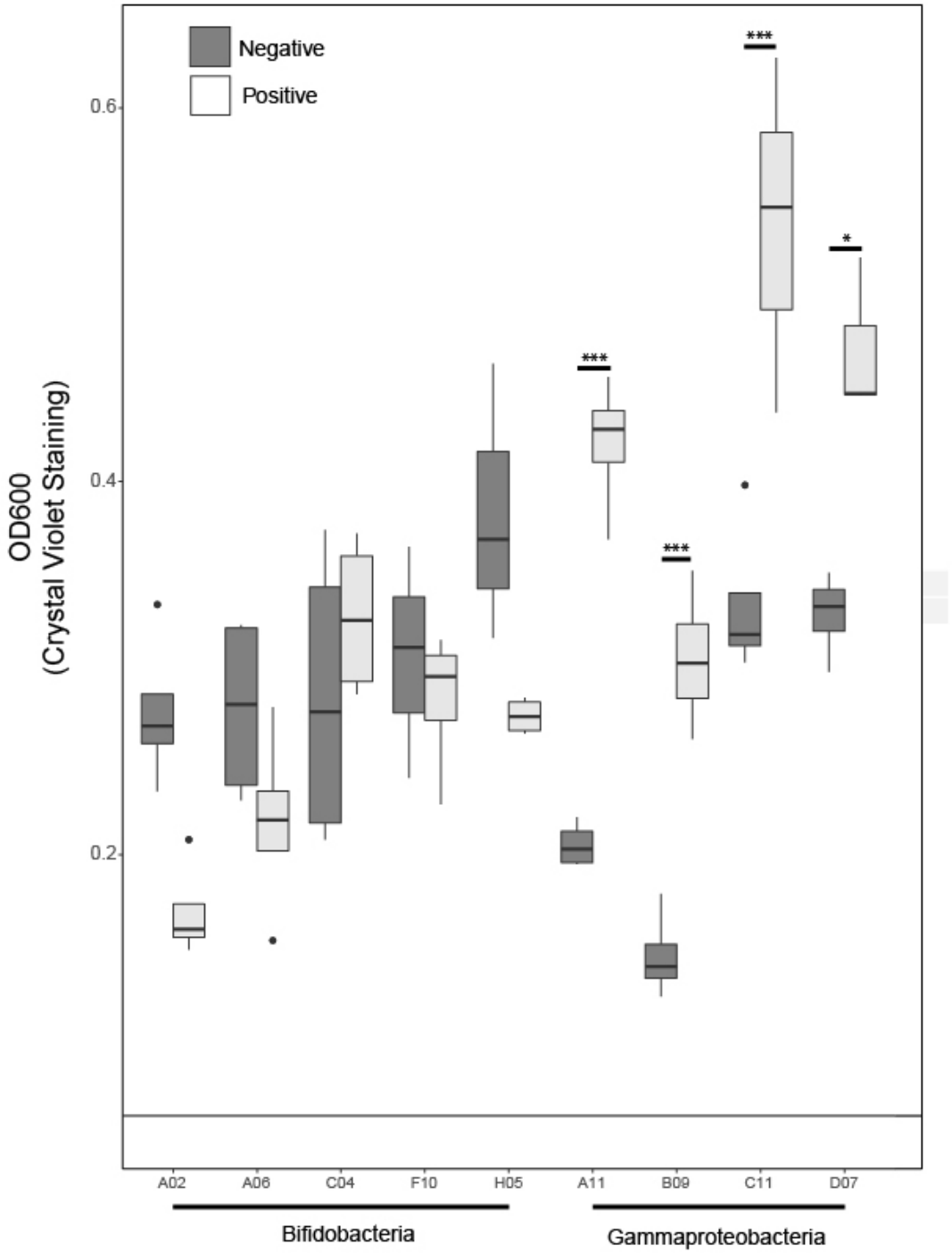


Figure 5. Quantification of crystal violet stained biofilms produced by honey bee gut microbiome members. Biofilm production on a chitin substrate by honey bee associated microbes was quantified using a standard crystal violet assay. Cultures were incubated either without (dark grey) or with (light grey) purified AI-2 added (see methods). Black line across the graph represents the average absorbance from sterile media controls across treatments. Comparisons were made between isolates treated with AI-2 and the same isolate without AI-2 added using t-tests. Significance designated by *** = < 0.001, * = p < 0.05.