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Origin and Effect of Alpha 2.2 *Acetobacteraceae* in Honey Bee Larvae and Description of *Parasaccharibacter apium* gen. nov., sp. nov.

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The honey bee hive environment contains a rich microbial community that differs according to niche. *Acetobacteraceae* Alpha 2.2 (Alpha 2.2) bacteria are present in the food stores, the forager crop, and larvae but at negligible levels in the nurse and forager midgut and hindgut. We first sought to determine the source of Alpha 2.2 in young larvae by assaying the diversity of microbes in nurse crops, hypopharyngeal glands (HGs), and royal jelly (RJ). Amplicon-based pyrosequencing showed that Alpha 2.2 bacteria occupy each of these environments along with a variety of other bacteria, including *Lactobacillus kunkeei*. RJ and the crop contained fewer bacteria than the HGs, suggesting that these tissues are rather selective environments. Phylogenetic analyses showed that honey bee-derived Alpha 2.2 bacteria are specific to bees that "nurse" the hive's developing brood with HG secretions and are distinct from the *Saccharibacter*-type bacteria found in bees that provision their young differently, such as with a pollen ball coated in crop-derived contents. *Acetobacteraceae* can form symbiotic relationships with insects, so we next tested whether Alpha 2.2 increased larval fitness. We cultured 44 Alpha 2.2 strains from young larvae that grouped into nine distinct clades. Three isolates from these nine clades flourished in royal jelly, and one isolate increased larval survival *in vitro*. We conclude that Alpha 2.2 bacteria are not gut bacteria but are prolific in the crop-HG-RJ-larva niche, passed to the developing brood through nurse worker feeding behavior. We propose the name *Parasaccharibacter apium* for this bacterial symbiont of bees in the genus *Apis*.

Honey bees (*Apis mellifera*) are highly eusocial insects that live together as a colony unit or "superorganism" (1). Queens lay almost all of the eggs in the hive (the exception being haploid eggs laid by workers), and the facultatively sterile female workers support the queen's developing brood through a series of nurse behaviors. Nurse workers are young (<~2 weeks old) in-hive bees that have not yet transitioned to foraging. They nourish larvae and newly emerged adults—their full or half-sisters—with a lipid and protein-rich substance called royal jelly (RJ) that is secreted from the nurse hypopharyngeal glands (HGs). These paired exocrine glands occupy much of the nurse's head volume apart from the brain. Young first- and second-instar larvae are fed a diet of RJ only, which has antiseptic, antifungal, and antitumorigenic qualities (2). Older worker larvae (third through fifth instars) are fed a mixture of RJ, pollen, and sugar regurgitated from the nurse crop (i.e., the honey stomach or social stomach). Larvae have an incomplete (closed) gut until they reach the pupal phase and are continuously fed by the nurse workers in the hive. These larvae therefore retain both undigested material as well as any fecal material until they make the final defecation that signals that their gut development is complete. At this point, the cell containing the developing individual, now a pupa, is capped by the workers in the hive, and the bee does not receive any more food until emergence. Pupae do not contain any bacteria and are reinoculated after they emerge (3). There are therefore three main nutritional phases of honey bee preadult development: the period when larvae receive only royal jelly (first and second instars), the period when they receive a mixture of royal jelly, crop contents, and regurgitated pollen (third, fourth, and fifth instars), and the pupal stage when they receive no nourishment and must rely on the energetic stores gained during the larval instars until they emerge as adults.

The honey bee microbiome has been studied for the last half of the 20th century (4) but has received renewed attention in the past 5 years owing to the historically high colony losses experienced of late (5) and the emergence of high-throughput sequencing methods for studying microbial communities. The adult honey bee midgut and hindgut have been the most extensively studied tissues and harbor a core microbiota of approximately seven bacterial phylotypes that are consistently present at very high levels in adults collected across time and space (3, 6–9). The hive is a remarkably dynamic environment, however, and recent studies show that the food stores, the larvae, and the crop are not dominated by these same core microbiota (10, 11). Instead, it appears that some of the major microbial players in nongut hive niches are those able to tolerate the sugary and acidic environments of the crop, bee bread, larval guts, and royal jelly, such as *Acetobacteraceae* Alpha 2.2 (Alpha 2.2) (10, 12). *Acetobacteraceae* are symbionts of a wide variety of insects, providing nutrition to insects on limited sugar-rich diets (13), benefiting development and the formation of tissues (14, 15), and modulating immunity (16). They are commonly found in the insect gut (13) but have also been isolated from salivary glands and reproductive tissues (14). *Acetobacteraceae* Alpha 2.2 bacteria are closely related to *Saccharibacter* sp. bacteria isolated from honey bee guts based on 16S rRNA sequence (7), and the genome of a *Saccharibacter* sp. from the honey

bee gut was recently sequenced and characterized along with 15 other *Acetobacteraceae* members by Chouaia et al. (17). Despite the ability of *Acetobacteraceae* to colonize insect guts, Alpha 2.2 bacteria are largely absent from the midgut and hindgut (8–11). However, culture-based assays of the microbial communities in honey bee larvae show that early larval instars, which receive only RJ for nutrition, are comprised predominantly of Alpha 2.2 bacteria and *Lactobacillus kunkeei* (12). The existing literature (4, 10–12) therefore suggests that the nongut hive environment (including the social stomach or crop) is a diverse but understudied aspect of honey bee hive microbial ecology.

Honey bees used for commercial pollination experience long periods of nutritional stress, and recent surveys of beekeepers rank starvation as a major cause of colony loss (18, 19). Nutrition at the larval stage is a particularly underappreciated aspect of colony health. It is possible that larval nutrition is a combination of not only the proteins, carbohydrates, lipids, and micronutrients that nurses supply but also the microbes they pass to larvae during bouts of nursing activity. We addressed this overarching question by first asking what bacteria are present in the nurse HGs, nurse crops, and RJ and then by asking how one of these bacteria impacts larval survival. We used high-throughput sequencing to determine the relative abundance of Alpha 2.2 bacteria and diversity of other bacteria in the RJ, nurse HGs, and nurse crops. Given the antiseptic qualities of RJ (2) and previous observations that young larvae contain only Alpha 2.2 and *L. kunkeei* (12), we hypothesized that the RJ collected from 1st- and 2nd-instar larvae would contain more *Acetobacteraceae* Alpha 2.2 bacteria than the crop and HGs and also that RJ would contain a less diverse microbiota than the HGs. Using a phylogenetic approach, we tested whether Alpha 2.2 is specific to bees that provision their young with HG-derived secretions (i.e., RJ in honey bees) compared to bee species that do not. We then tested whether Alpha 2.2 is a critical component of larval nutrition by testing whether the Alpha 2.2 found in larvae survives in the RJ passed from nurses to larvae and whether it confers a fitness benefit to its larval host. We find that Alpha 2.2 is in all of the tissues and substrates key to the nurse worker feeding behavior, that it is specific to bees that feed their larvae with HG-derived secretions, and that Alpha 2.2 increases larval survival.

MATERIALS AND METHODS

Isolation, culturing, and characterization of *Acetobacteraceae* Alpha 2.2. In June of 2013, 20 first-instar larvae were collected from three different hives housed at the Carl Hayden Bee Research Center (CHBRC) in Tucson, AZ, USA. The three hives were headed by *A. mellifera ligustica* queens less than 1 year of age, and the hives were of equal size and strength (10 frames total, with approximately 6 frames of adult bees, 1.5 frames of brood, and 2 frames of food). Second-instar larvae were collected directly from the hive into physiological saline, gently vortexed, and then placed into 75% ethanol, where they were gently vortexed again. After these surface washings, the 20 larvae were transferred into 250 µl of physiological saline and were macerated with a sterilized pestle. Fifty microliters of this solution of crushed larvae was then plated onto five plates of Sabouraud dextrose agar (SDA) and incubated at 34°C under low-oxygen (5% CO_2) conditions for 48 h according to the methods of Vojvodic et al. (12). After 48 h, individual colonies were picked and placed into 1 ml of Sabouraud dextrose broth (SDB), where they grew under identical conditions for 48 h or until the broth appeared cloudy. Two hundred microliters of each culture was plated onto new SDA and grown under identical conditions; 200 µl of each culture was used for long-term storage (by adding sterile glycerol to a final concentration of 12% and freezing samples at −80°C), and 200 µl was used for 16S rRNA gene sequencing.

DNA was isolated from each bacterial isolate growing in 200 µl of SDB using a Fermentas GeneJET DNA purification kit according to the manufacturer's protocol for Gram-positive bacteria so as not to exclude any non-*Acetobacteraceae* taxa. The isolated DNA was then subjected to a PCR using the universal bacterial primers 27F (5′-AGAGTTTGATCCTGGCT CAG-3′) and 338R (5′-TGCTGCCTCCCGTAGGAGT-3′) that amplify 311 bp of the V1/V2 variable region of the 16S rRNA gene. Cycling conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 50°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 2 min. Strains A29, B8, and C6 were subjected to PCR using the bacterial primers 27F (above) and 1522R (5′-AAGGAGGTGATCCAGCCGCA-3′) to obtain a longer section (1,495 bp) of the 16S rRNA gene sequence. Cycling conditions were as follows: 95°C for 9 min, followed by 15 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension step at 60°C for 10 min. Ten microliters of the resulting PCR products was cleaned using ExoSAP-IT (USB) according to the manufacturer's protocol, and the products from each isolate were sequenced in one direction using the 27F primer. To assess whether the isolates belonged to the *Acetobacteraceae* Alpha 2.2 group, the sequences were compared to published sequences from honey bees (7) as well as species of *Gluconobacter*, *Acetobacter*, *Commensalibacter intestini* strain A911, and *Saccharibacter floricola* strain S-877. A total of 275 positions were included in the final data set and were aligned using Muscle (20).

454 amplicon sequencing of royal jelly (RJ), hypopharyngeal gland (HG), and nurse crop bacterial communities. Young 1st- and 2nd-instar larvae are fed a diet comprised exclusively of RJ (21). Culture-based assays by Vojvodic et al. (12) showed that 2nd-instar larvae contain mostly *Acetobacteraceae* Alpha 2.2 bacteria and *Lactobacillus kunkeei*, while other larval instars contain a combination of *Acetobacteraceae* Alpha 2.2, *L. kunkeei*, *Bacillus* sp., and *Lactobacillus* sp. Firm5. To complement the culturing described above and to establish the source of the Alpha 2.2 bacteria found in larvae, we determined the composition and diversity of bacteria in the RJ, the HGs, and nurse crops. Royal jelly and nurse bees were collected from six replicate hives housed at the CHBRC in Tucson, AZ, USA, in August 2013. All six hives were headed by *A. mellifera ligustica* queens, and the hives were of equal size and strength compared to each other and to the hives used in the earlier experiments. For each hive, a total of 250 µl of RJ was collected from multiple (≥20) cells containing 1st- and 2nd-instar larvae and diluted with 750 µl of sterile distilled water. One hundred microliters of this diluted sample was sampled and spun down, the supernatant was removed, and 180 µl of lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, pH 8.0, plus 20 mg/ml lysozyme added immediately before use) was then added. Fifteen nurses were collected from each of the same six hives that the royal jelly was harvested from and were discriminated from other hive bees based on their behavior (i.e., visiting a larval cell for >5 s). Nurse bees were flash frozen in liquid nitrogen upon collection and kept at −80°C until they were dissected. Each bee was decapitated, and the head was placed face up on a surface containing dental wax (Electron Microscopy Sciences) and steadied with a small portion of melted wax in the center that anchored it upon cooling. Insect pins were placed through the center of each of the eyes to further steady the specimen. Breakable double-edge razor blades (Electron Microscopy Sciences) and sterile, sharp, fine Vannas spring scissors (Fine Science Tools) were used to carefully cut the face from the rest of the head, starting from the base of the mandible, along the inner margins of each compound eye, and through the ocelli. The antennal lobes were severed from the antennae, and the face was then lifted from the rest of the head at the mandible. Sterile, distilled water was added, and the HGs were severed from the head at the base of the gland. Crops were dissected from these same nurse bees. The thorax and abdomen were placed ventral side up on the same wax surface and affixed using insect pins. The abdomen was cut from the rectum toward the thorax along the abdominal midline, exposing the digestive tract. The crop was dissected by cutting it at the top and bottom with sterile, sharp, fine Vannas spring scissors (Fine Science Tools). The crops and hypopharyngeal glands from 15 nurses per hive

TABLE 1 Library-specific bar codes used for the pyrosequencing of bacterial 16S rRNA genes from royal jelly, nurse hypopharyngeal glands, and nurse crops

Library bar code[a]	Sample type[b]	Colony no.	Data file no.[c]
ACGAGTGCGT	RJ	1	IIY86TY03
ACGCTCGACA	RJ	2	IIY86TY03
AGACGCACTC	RJ	3	IIY86TY03
AGCACTGTAG	RJ	4	IIY86TY03
ATCAGACACG	RJ	5	IIY86TY03
ATATCGCGAG	RJ	6	IIY86TY03
CGTGTCTCTA	Nurse crop	1	IIY86TY03
CTCGCGTGTC	Nurse crop	2	IIY86TY03
TAGTATCAGC	Nurse crop	3	IIY86TY03
ACGAGTGCGT	Nurse crop	4	IIY86TY04
ACGCTCGACA	Nurse crop	5	IIY86TY04
AGACGCACTC	Nurse crop	6	IIY86TY04
AGCACTGTAG	HG	1	IIY86TY04
ATCAGACACG	HG	2	IIY86TY04
ATATCGCGAG	HG	3	IIY86TY04
CGTGTCTCTA	HG	4	IIY86TY04
CTCGCGTGTC	HG	5	IIY86TY04
TAGTATCAGC	HG	6	IIY86TY04

[a] Library-specific 454 sequencing bar code.

[b] RJ, royal jelly; HG, hypopharyngeal gland.

[c] File name containing data archived in the NCBI under study PRJNA252625 (accession number SRP043168). All files have the extension sff.

were dissected directly into 180 μl of lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 1.2% Triton X-100, pH 8.0, plus 20 mg/ml lysozyme added immediately before use), pooled by hive, and homogenized with a sterile pestle. DNA extraction followed using a GeneJET Genomic DNA purification kit (Fermentas) according to the manufacturer's protocol for Gram-positive bacteria. The extracted DNA was subjected to 16S rRNA PCR amplification using universal primers (27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 338R, 5′-TGCTGCCTCCCGTAGGAGT-3′) to confirm the presence of bacterial DNA. Cycling conditions were as follows: 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 50°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 2 min.

For pyrosequencing, the V1/V2 region of the 16S rRNA gene of the samples was PCR amplified using universal 16S rRNA primers fitted with 454 FLX Titanium adapter sequences (27F, 5′-CCATCTCATCCCTGCG TGTCTCCGACTCAGNNNNNNNNNNNNagagtttgatcctggctcag-3′; 338R, 5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGtgctgcctcccgtaggagt-3′; uppercase letters denote the adapter sequences, Ns indicate library-specific bar codes, and lowercase letters indicate universal 16S rRNA primers) (Table 1). Amplicons were sequenced using Roche 454 GS FLX Titanium sequencing at the University of Arizona Genomics Core Facility (http://uagc.arl.arizona.edu/).

The 18 sequence libraries containing RJ-, crop-, and HG-derived sequences from the six replicate colonies were concatenated and analyzed using Mothur, version 1.26.0 (22). Sequences in the sff files were quality filtered using the trim.flows command, and all sequences of <220 bp with more than two base mismatches to the 27F primer sequence or one mismatch to the 10-bp pyrotag after trimming were eliminated using the trim.seqs command. Pyrotags were removed, and the sequences were aligned to the Silva SSURef database (version 102) using the align.seqs command. Sequences that did not align to the 27F primer position were eliminated using the screen.seqs command. Chimeras were removed using UCHIME (23) in addition to any sequences that were mitochondrial, chloroplast, archaeal, eukaryotic, or of unknown origin. Sequences that differed by one base pair were clustered together using the pre.culster command. A distance matrix was constructed for the aligned sequences using the dist.seqs command and the default parameters. Sequences were then grouped into operational taxonomic units (OTUs) based on 97%

sequence similarity. Representative sequences from each OTU with the smallest maximum distance to the other sequences in that OTU were obtained through the get.oturep command. The taxonomy of each representative sequence was determined using the Ribosomal Database Project (RDP) naive Bayesian classifier (24) with a manually constructed training set that contained sequences from the Greengenes 16S rRNA database (version gg_13_5_99 accessed May 2013), the RDP version 9 training set, and all full-length honey bee-associated gut microbiota listed in NCBI trimmed to the V1/V2 region of the 16S rRNA gene. We then linked each representative sequence to sequences published in the NCBI nucleotide database. Each representative sequence was used to query the NCBI nucleotide database for the best hit using an E value cutoff of 1×10^{-10} and 97% sequence similarity. Any remaining sequences that were of chloroplast or mitochondrial origin, that were classified with less than 80% confidence at the phylum level, or that contained fewer than two sequences in at least two libraries (9) were also removed. A Venn diagram representing the number of OTUs shared or not shared among the three sample types (RJ, HGs, and crop) was constructed using the venn command, and the number of sequences belonging to these OTUs from each sample type was calculated. Collector curves were obtained using the collect.single command to determine whether the Chao or inverse Simpson diversity index was sensitive to the library size. The Chao index was overly sensitive to library size and was not further analyzed; however, the inverse Simpson diversity index was not. The library coverage and inverse Simpson diversity index were calculated by subsampling each library equally 1,000 times and averaging the estimates using the summary.single command. An analysis of variance (ANOVA) was used to test whether sample type (i.e., crop, HGs, or RJ) significantly influenced (i) the proportions of Alpha 2.2 and *L. kunkeei* sequences, (ii) the diversity of bacterial taxa (i.e., inverse Simpson diversity index), and (iii) the number of 97% OTUs found in each library. A *post hoc* Tukey-Kramer honestly significant difference (HSD) test was used to compare means while correcting for multiple comparisons.

We investigated whether Alpha 2.2 bacteria formed a clade specific to bee taxa that perform the nurse behavior and secretion of brood food by investigating the relationships among the following: isolates obtained from 2nd-instar larvae (strains A29, B8, and C6); sequences obtained from honey bee RJ, crops, and HGs via pyrosequencing; Alpha 2.2 bacteria from *Apis dorsata* (7), which feed RJ to their larvae (25); *Acetobacteraceae* from native pollinators that do not nurse their young; *Acetobacteraceae* from floral sources; and published Alpha 2.2 (7, 26–28), Alpha 2.1 (7), *Saccharibacter floricola* (GenBank accession number NR_024819.1), *Neokomagataea thailandica* (GenBank AB513363.1), *Gluconobacter* sp., and *Acetobacter* sp. sequences. Representative sequences were chosen from each of the five *Acetobacteraceae* Alpha 2.2 OTUs obtained from RJ, crops, and HGs that contained >500 sequences in the 454 data set. The 16S rRNA gene sequences from Alpha 2.2 bacteria isolated from the guts of wild bees in the genera *Megachile* and *Osmia* and from wildflowers in the genera *Carduus*, *Hellenium*, and *Opuntia* are a subset of forthcoming (unpublished) studies. A sterile technique was employed when these flower and wild bee samples were collected, and 16S rRNA gene sequences were amplified using the primer pair Gray28F (5′-GAGTTTGATCNTGGCTCAG-3′) and Gray519R (5′-GTNTTACNGCG GCKGCTG-3′). The resulting amplicons were sequenced on a Roche GS FLX 454 sequencer using Titanium reagents. The sequences were aligned as described above, and the alignment was filtered using the filter.seqs command in Mothur, version 1.26.0 (22). A total of 163 nucleotide positions were included in the final data set.

Phylogeny construction. With the exception of the phylogeny created to compare the 44 Alpha 2.2 isolates to published sequences, all phylogenies were created using the neighbor-joining (29) and maximum-likelihood (30) methods in MEGA (31), and a bootstrap test (32) with 1,000 replicates was employed to test the reliability of the resulting phylogeny. The phylogeny used to compare the 44 Alpha 2.2 isolates derived from 1st- and 2nd-instar larvae was created using only the neighbor-joining (29)

method and a bootstrap test (32) with 1,000 replicates. In all cases, the rate variation among sites was modeled with a gamma distribution and 1st plus 2nd plus 3rd plus noncoding positions were included. Ambiguous positions were removed.

Culturable Alpha 2.2 and *Saccharibacter* bacteria in the nurse midgut. Previous culture-based and culture-independent work demonstrates that Alpha 2.2 and *Saccharibacter* sp. bacteria are rare in the honey bee gut (8–11). We sought to confirm this in nurse bees collected as part of the current study and given culturing conditions that enrich for Alpha 2.2 bacterial growth. Ten nurse bees were collected from each of two colonies identical to the colonies used in the previous pyrosequencing studies. Their midguts were dissected into 500 μl of sterile physiological saline and pooled by colony. The tissue was macerated, and 100 μl of each solution was plated onto SDA medium in triplicate as described above. The bacterial plates were then incubated at 34°C under low-oxygen (5% CO_2) conditions for 48 h. These conditions are favorable for the growth of *Saccharibacter* sp. and for *Acetobacteraceae* Alpha 2.2 bacteria as well as other bacteria tolerant of high sugar and slightly acidic conditions (10, 12). For each bee colony, 48 bacterial colonies were then randomly picked equally from each of the three plates into 20 μl of sterile distilled water. These colony picks were then directly subjected to a PCR as described above using the universal bacterial primers 27F and 338R to amplify 311 bp of the V1/V2 variable region of the 16S rRNA gene. These PCR products were sequenced, the sequences were aligned to the Silva SSURef database (version 102) using the align.seqs command, and uninformative sites were removed using the filter.seqs command in Mothur, version 1.26.0 (22). Chimeras were identified using UCHIME (23) and were removed, yielding high-quality DNA sequences that were further classified. The taxonomy of each sequence was determined using the RDP Naive Bayesian Classifier (24) as described above, and the proportion of sequences belonging to each genus or species was calculated.

Tests for growth inhibition in the presence of RJ. Previous work by Vojvodic et al. (12) showed that Alpha 2.2 bacteria isolated from early-instar larvae grow in the presence of RJ. To confirm this phenotype on the isolates from the present experiment, we repeated the experiments described by Vojvodic et al. (12) using the three Alpha 2.2 isolates and *Escherichia coli* strain DH5α that were used in the *in vitro* rearing experiments. Two hundred microliters of the three Alpha 2.2 strains were inoculated separately onto SDA, and 200 μl of *E. coli* was inoculated onto Luria broth (LB) solid medium. The inoculum was spread using sterile glass beads. After the inoculum had soaked into the medium, a sterile filter paper dipped in fresh RJ from hives in the CHBRC apiary was placed onto the inoculum. The Alpha 2.2 bacteria on SDA plates were incubated for 48 h at 34°C in 5% CO_2, and the *E. coli* bacteria on LB plates were incubated for 24 h at 34°C under atmospheric conditions (i.e., no added CO_2). After incubation, we recorded whether a zone of inhibition was present (or not) or whether growth was enhanced around the RJ. The size of the zone of inhibition was not measured.

***In vitro* rearing of *A. mellifera* larvae with or without Alpha 2.2 supplementation.** To test the hypothesis that Alpha 2.2 bacteria provide a fitness benefit to honey bee larvae, we determined whether larvae supplemented with Alpha 2.2 bacteria lived longer than larvae supplemented with either no bacteria or bacteria not known to associate with or cause disease in honey bees. Three Alpha 2.2 isolates were randomly selected from three of the nine major groups of Alpha 2.2 bacteria that were cultured from first-instar larvae. Survival of the larvae and pupae was measured in response to these three Alpha 2.2 isolates in addition to two negative controls: no bacteria or *E. coli* strain DH5α, which is not present in honey bee hives and not normally encountered by honey bee larvae. Honey bee queens from three colonies were caged over empty comb for a period of 2 days. Based on previous experience, we expected the queen to begin ovipositing after several hours of being caged. Three days after the queen was released, the frame where the queen was caged was removed from the hive, and the second-instar larvae (approximately 108 h after oviposition ±12 h) on the frame were utilized for *in vitro* rearing in the

presence of Alpha 2.2 bacteria or either of the negative controls. Larvae were visited by nurse workers in the hive that contained their own resident microbiota for a period of approximately 1 day prior to the start of the experiment.

In two separate trials, 48 second-instar larvae were assayed for each of the five experimental treatments, yielding a total of 480 larvae tested (2 trials times 5 treatments times 48 larvae). Second-instar larvae from the three source colonies were sampled equally and randomly for each of the treatments. Following the method of Huang (33), the diets were comprised of the following: 50 ml of sterile distilled water, 6 g of D-glucose (6%), 6 g of D-fructose, 1 g of yeast extract, and 50 g of fresh commercially available RJ (Stakich, Inc., MI, USA). The commercially available RJ was not sterilized because it is too viscous to be filter sterilized, and the antiseptic qualities of RJ that are conferred by the major royal jelly proteins are removed when the RJ is heated (34). However, because the RJ was frozen before use and because Alpha 2.2 bacteria do not survive −20°C temperatures (unpublished data), we reasoned that the RJ was free of Alpha 2.2 when the diet was prepared. However, the presence of microbes that can survive such temperatures could not be discounted. The negative control that did not contain any bacteria was comprised only of the above ingredients (glucose, fructose, yeast extract, and RJ). For the four treatments that contained bacteria (three Alpha 2.2 treatments and one *E. coli*), bacteria were grown to approximately the log phase in either SDB (Alpha 2.2) or LB (*E. coli*) medium at either 34°C in 5% CO_2 (Alpha 2.2) or 34°C under atmospheric conditions (*E. coli*). The number of CFU was standardized for each of the bacterial treatments to approximately 300 CFU/100 μl of broth. For the bacterium-supplemented diets, the above rearing diet was prepared omitting the RJ. For each bacterial type, 100 μl of each bacterial culture was spun down, and all of the liquid medium was removed before 50 g of fresh RJ was added to the spun-down cells. This mixture of bacteria and RJ was then added to the remainder of the *in vitro* diet and was used for the subsequent *in vitro* assays.

The growth of Alpha 2.2 strain C6 relative to the growth of *E. coli* in the *in vitro* rearing diet was tested. To first ensure that these bacteria were indeed viable prior to being added to the *in vitro* rearing diet and as a point of reference, 100 μl of Alpha 2.2 strain C6 and *E. coli* in liquid growth medium was plated onto SDA and LB, respectively. These plates were incubated either overnight at 37°C under atmospheric conditions (*E. coli*) or at 34°C in 5% CO_2 (Alpha 2.2) for 48 h. The growth of Alpha 2.2 bacteria and *E. coli* in the larval diet was next determined. The diet was prepared as indicated above for the *E. coli* and Alpha 2.2 strain C6 treatments and incubated at 34°C overnight without larvae present. A total of 100 μl (1/1,000) of the prepared diet was then plated onto SDA (Alpha 2.2 strain C6) or LB agar (*E. coli*). The numbers of CFU were then determined after the plates were incubated either overnight at 37°C under atmospheric conditions (*E. coli*) or at 34°C in 5% CO_2 (Alpha 2.2) for 48 h.

Larvae were assayed in a sterile 48-well cell culture plate, yielding a total of 5 plates of 48 larvae per trial. For the first 3 days of rearing, 100 μl of the diet containing the bacterial treatment (or the negative control) was provided to each larva. Standard diet containing no bacteria was provided to all larvae for all treatment groups from then on until they reached pupation (as evidenced by the final larval defecation that signals the completion of gut development). All of the remaining diet not ingested by the larvae was replaced with new diet each day. Larvae were maintained at 34°C and 95% relative humidity, and mortality was recorded daily. Spinning-phase larvae that were ready to pupate were moved to the cells of a dry 48-well cell culture plate that were each lined with autoclaved laboratory tissue. These larvae were allowed to pupate, and mortality was checked daily. In cells where the larva or pupa died, the dead insect was removed and the cell was cleaned with a Q-tip soaked in 70% ethanol. Mortality through the larval stages and also through the pupal phase was recorded for two separate trials.

Mortality data were analyzed using a logistic regression where death (or survival) at the end of the larval stages and pupal phase was the dependent variable and where treatment was the independent variable. We

opted against analyses such as the Cox proportional hazards model. Larval development is a short and rapid process. Hours separate instars, and substantial morphological changes occur during the ~24-h period that larvae are in the 2nd larval instar (see Fig. S1 in the supplemental material). The drastic outward differences in larval morphology within this 24-h period might reflect internal differences in the insect that might affect the larva's interaction with bacteria, such as the immune response or gut morphology. Because it was impossible to reliably control for the exact age that the larvae were inoculated (beyond the fact that they were late into the second instar) and because substantial morphological differences accompany relatively minor differences in chronological time, we reasoned that asking whether the treatment had an effect on whether the individual survived was adequate. Survival through the pupal phase was determined only for those individuals that survived the larval stage, and so n is <48 for each of these treatments. Sample sizes for the pupal-phase measurements are indicated in Fig. S6 in the supplemental material. Each trial was analyzed independently. Odds ratios were calculated to determine whether there were significant differences in mortality between each of the three Alpha 2.2 strain treatments and either of the *E. coli* or no-bacteria controls. A Dunn-Šidák correction (35) was applied to evaluate the *P* values of each odds ratio, controlling for experimental error in each of the six planned comparisons.

Phylogenetic analysis and general characteristics of the proposed novel taxon. Nearly full-length 16S rRNA gene sequences (1,495 bp, encompassing the V1 to V8 variable regions) from strains A29, B8, and C6 were compared to full-length *Acetobacteraceae* 16S rRNA gene sequences from crops, food stores, hindguts (i.e., not including the crop), larvae (10, 12), and other closely related cultured sequences (see Fig. 8). The sequences were aligned to the Silva SSURef database (version 102) using the align.seqs command in Mothur, version 1.26.0 (25). A phylogeny was created from the alignment using the methods described above.

A pure culture containing one strain of the *Acetobacteraceae* Alpha 2.2 phylotype, strain A29, was chosen for closer inspection of its morphology and motility. Strain A29 was grown overnight in SDB at 34°C in low oxygen (5% CO_2) for 48 h. Twenty microliters of culture was placed onto a microscope slide with a coverslip, and the culture was observed at magnifications of both ×400 and ×1,000 with a Nikon Eclipse 80i compound light microscope. Photographs were taken with the Nikon DS-Qi1Mc camera and the NIS Elements Software (version 3.22.00).

Accession numbers. Sequences of the 16S rRNA gene for the 44 isolates from first-instar larvae are available from NCBI under accession numbers KM014124 to KM014167. Included are the full-length 16S rRNA gene sequences for strains A29, B8, and C6 under accession numbers KM014158 (strain A29), KM014144 (strain B8), and KM014167 (strain C6). Additional *Acetobacteraceae* sequences from native pollinators and floral sources were deposited to the NCBI Sequence Read Archive under study PRJNA252627 (accession number SRP043429). Pyrosequencing data were deposited in the NCBI Sequence Read Archive under study PRJNA252625 (accession number SRP043168). Table 1 contains the bar code sequences corresponding to each sample type from each colony. The 72 nurse midgut-associated bacteria cultured on SDA at 5% CO_2 were deposited in the NCBI nucleotide database under accession numbers KM365336 to KM365407. Bacterial cultures for Alpha 2.2 strains A29, B8, and C6 have been stored at the ATCC under accession numbers SD-6836, SD-6837, and SD-6838, respectively, and are available by request.

RESULTS

Nine groups of *Acetobacteraceae* Alpha 2.2 bacteria were isolated from 1st-instar honey bee larvae. Forty-four bacterial isolates were successfully cultured from first-instar larvae obtained from standard honey bee hives. All of these isolates grew well over a 48-h period in slightly acidic SDA medium and 5% CO_2. Phylogenetic analysis of the 16S rRNA gene sequences obtained from these isolates showed that all 44 belonged to *Acetobacteraceae* Al-

pha 2.2 (see Fig. S2 in the supplemental material). Sequence alignments of these 44 Alpha 2.2 isolates revealed nine major groups of Alpha 2.2 bacteria based on variability at the V1 hypervariable region of the 16S rRNA sequence (see Fig. S3).

Three strains (A29, B8, and C6) were randomly selected for further tests from groups A, B, and C (see Fig. S3 in the supplemental material) to represent a group of isolates that were 100% identical to each other at the V1 region of the 16S rRNA gene sequence. While there was 100% sequence similarity at this site for the sequences within the same group, representative sequences from groups A, B, and C differed: A29 and B8 were 0.6% different, A29 and C6 were 0.6% different, and strains B8 and C6 were 1.2% different at the V1 region.

Royal jelly-, crop-, and hypopharyngeal gland-associated microbial communities. Young 1st- and 2nd-instar larvae are fed a diet exclusively of RJ, which is comprised mostly of the proteinaceous secretions of the hypopharyngeal glands (21). Although RJ is antiseptic (2), culture-based assays suggest that these young larvae contain mostly Alpha 2.2 and *L. kunkeei* bacteria (12). A total of 498,556 sequences were recovered from the 18 libraries, and 452,415 of these reads were nonchimeric (see Table S1 in the supplemental material). Further culling of suspect sequences resulted in a total of 346,380 sequences across the 18 libraries (see Tables S1 and S2). For the libraries containing these remaining sequences, average library coverage was high (average Good's coverage of 97.8% ± 0.2% standard error [SE]), and so we tested the hypothesis that Alpha 2.2 and *L. kunkeei* bacteria are adapted to this RJ niche by looking at the concentration of these bacteria and the overall microbial diversity in the RJ compared to the crops and HGs. Both Alpha 2.2 and *L. kunkeei* were present in all of the crop, RJ, and HG libraries (Fig. 1; see also Table S2 in the supplemental material). Crops, HGs, and RJ contained equivalent proportions of Alpha 2.2 bacteria, but *L. kunkeei* was more prevalent in the RJ than in the HGs but not in the crops ($F_{2,15} = 5.57$, $P = 0.016$) (Fig. 2). There was significant variation among sample types for both taxon diversity as measured by the inverse Simpson diversity index (overall ANOVA, $F_{2,15} = 5.29$, $P = 0.0182$) (Fig. 3) and the number of 97% OTUs discovered (overall ANOVA, $F_{2,15} = 13.78$, $P = 0.0004$) (Fig. 3). The HG libraries had higher taxon diversity than the RJ but not than the crop (Fig. 3). The HG libraries had more bacterial 97% OTUs than both the RJ and the crop (Fig. 3). Despite these differences in diversity, the majority of bacterial sequences across all three libraries belonged to OTUs that were common to all three sample types. The 192 OTUs that were shared among all three sample types contained 90% of the sequences from the HGs, 98% from the crops, and 98% from the RJ (Fig. 4; see also Table S2 in the supplemental material). The OTUs that were not shared among the three libraries contained mostly rare sequences (Fig. 4 and Table S2).

The core gut microbiota of honey bees (i.e., Alpha 2.1 phylotype, *Lactobacillus* sp. Firm4, *Lactobacillus* sp. Firm5, *Frischella perrara* [Gamma2 phylotype], *Gilliamella apicola* [Gamma1 phylotype], *Snodgrassella alvi* [Beta phylotype], and a honey bee-associated *Bifidobacterium* sp.) has been identified in almost all of the honey bee tissues and in hive environments studied to date (6–11, 27, 28, 36). Many of these core gut microbes were present in each of the crop (55% ± 13%), hypopharyngeal gland (31% ± 9%), and RJ (4% ± 1%) libraries. The Alpha 2.1 group was not found in any of the sample type libraries (Fig. 1).

The three Alpha 2.2 isolates from young larvae were closely
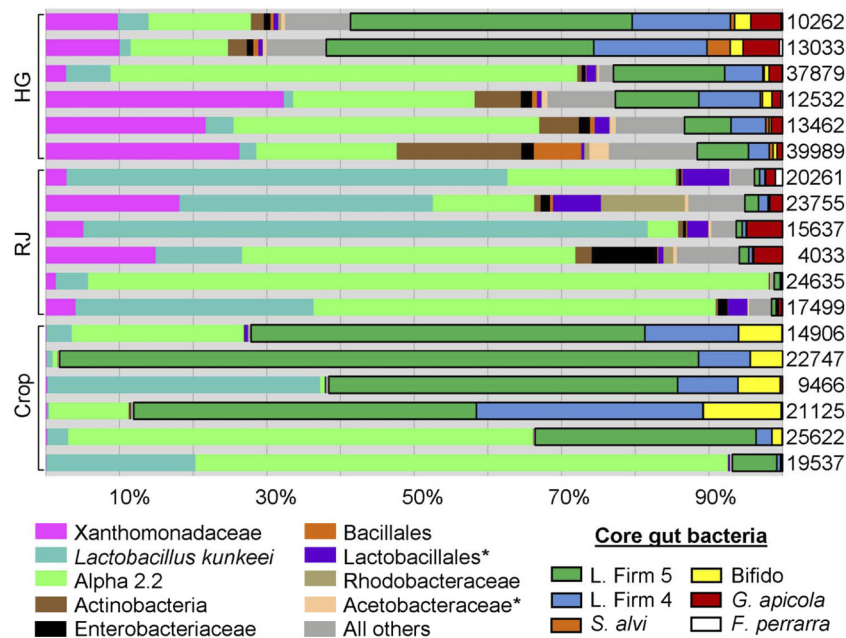
FIG 1 The distribution of bacterial taxa in royal jelly (RJ), crops, and hypopharyngeal glands (HGs) of nurse workers. The proportion of sequences belonging to each bacterial taxon was determined relative to the number of sequences in each individual sequencing library. The number of sequences from each library is given at the right of each group. Bacterial taxa boxed in black are members of the core gut microbiome. The *Lactobacillales* and *Acetobacteraceae* clades marked with asterisks represent the remaining sequences within these clades after *Lactobacillus kunkeei* and Alpha 2.2 bacteria were accounted for and indicated elsewhere in the graph. L. Firm5, *Lactobacillus* sp. Firm 5; L. Firm4, *Lactobacillus* sp. Firm 5; Bifido, *Bifidobacterium* sp.
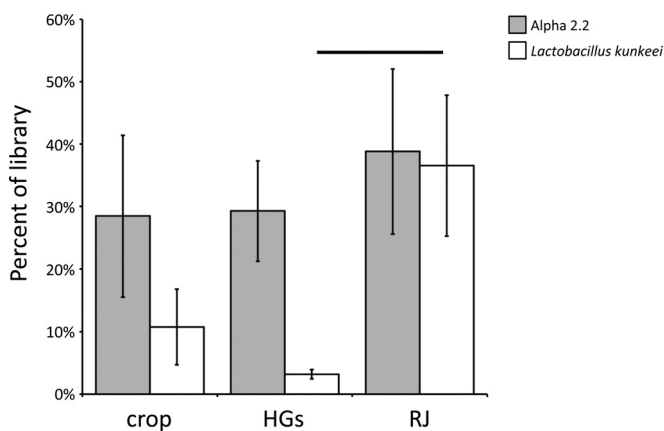
related to the most prevalent OTUs found in the nurse crops, RJ, and HGs and the Alpha 2.2 found the guts of *Apis mellifera* and *Apis dorsata* (Fig. 5). These gut-derived Alpha 2.2 sequences have also been found in other studies of honey bee hive food stores (10), corbicular pollen (11), and larvae (12) (Fig. 5). These Alpha 2.2 bacteria formed a clade separate from the *Saccharibacter* sp. bacteria found in bees that provision their young with pollen and floral samples (Fig. 5). The Alpha 2.2 and *Saccharibacter* sp. clades

FIG 2 The percentage of Alpha 2.2 and *Lactobacillus kunkeei* sequences in nurse worker crops, hypopharyngeal glands (HGs), and royal jelly (RJ). The average percentages of sequences in the sequence libraries from each sample type are shown for Alpha 2.2 and *L. kunkeei*. Alpha 2.2 bacteria were represented equally in all sample types. *L. kunkeei* was more prevalent in the RJ than in HGs (indicated with a line connecting the significant comparison). Although the levels of *L. kunkeei* bacteria appeared higher in the RJ than in the crops, the difference was not statistically significant.

were distinct from and basal to the Alpha 2.1 bacteria found in bee guts and other acetic acid bacteria. The Alpha 2.1 bacteria formed two separate clades, one related to *Gluconobacter* sp. and *Acetobacter* sp. and another related to *Commensalibacter* sp. found in *Drosophila melanogaster* guts.

Alpha 2.2 and *Saccharibacter* bacteria are rare or absent in the nurse midgut. Seventy-two high-quality nonchimeric sequences were recovered from the nurse midguts sampled across two colonies after the guts were cultured under conditions favoring the growth of *Acetobacteraceae*. Of these 72 sequences, two were classified as *Acetobacteraceae* Alpha 2.2 and were most similar to a sequence isolated from the guts of honey bees in Europe (GenBank accession number AJ971850.1) (28). The majority of the bacteria that did grow under these conditions were *Lactobacillus kunkeei* bacteria (58 sequences), while the remainder were *Fructobacillus* sp. (1), *Morganella* sp. (5), *Cronobacter* sp. (5), and *Enterobacter* sp. (2) (see Fig. S5 in the supplemental material).
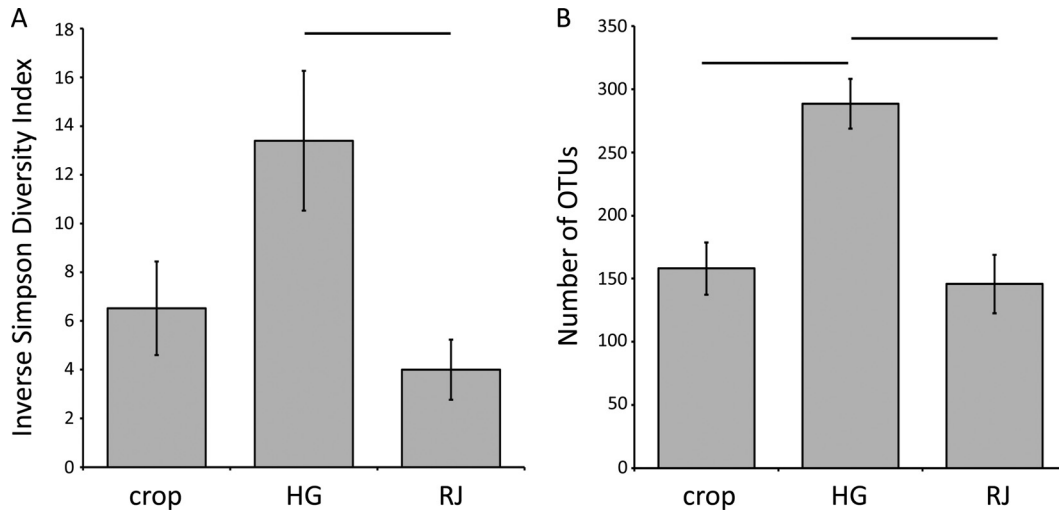
Acetobacteraceae Alpha 2.2 bacteria thrive in the presence of royal jelly (RJ). The three strains of *Acetobacteraceae* Alpha 2.2 isolated from 1st- and 2nd-instar larvae and used in the *in vitro* rearing experiments grew in the presence of RJ. Alpha 2.2 strains A29, B8, and C6 all grew in the presence of an RJ disk when plated on SDA (Fig. 6). In contrast, *E. coli* grown on LB agar in the presence of an RJ disk showed a clear zone of inhibition (Fig. 6). In all of the replicate plates, there was a zone of inhibition for the *E. coli* grown on LB agar and for growth around and on top of the RJ disk for all three strains of Alpha 2.2 bacteria grown on SDA. Additionally, Alpha 2.2 strain C6 grew by a factor of five, and *E. coli* yielded zero surviving CFU (Fig. 6) when each type of bacteria was added to the *in vitro* rearing diet containing RJ.

Alpha 2.2 bacteria increased the survivorship of larvae. Lar-

FIG 3 The diversity and number of bacterial OTUs in the hypopharyngeal glands (HG), royal jelly (RJ), and crop samples. (A) The mean inverse Simpson index ± SE is plotted for each sample type (HGs, RJ, or crop). (B) The mean number of 97% OTUs ± SE found in each sample type. *Post hoc* analyses yielding significant differences among sample types are indicated with a line.

val mortality varied significantly among treatments (for trial 1, $\chi^2_4 = 10.94$, $P = 0.0272$; for trial 2, $\chi^2_4 = 39.32$, $P < 0.0001$), but only Alpha 2.2 strain C6 impacted larval survival compared to the two controls in both trials (trial 1, C6/*E. coli* odds ratio of 4.53, $P = 0.0074$; C6/no-bacteria odds ratio of 4.53, $P = 0.0074$; trial 2, C6/*E. coli* odds ratio of 5.8, $P < 0.0001$; C6/no-bacteria odds ratio of 5.32, $P = 0.0001$) (Fig. 7). Strain C6 was beneficial compared to both *E. coli* and the negative control, suggesting that larvae that were fed strain C6 did not live longer simply because the bacteria were used as a food source or provided a hormetic benefit (37). In the second trial but not the first, Alpha 2.2 strain A29 also improved survival through the larval stages (trial 2, A29/*E. coli* odds ratio of 4.11, $P = 0.0009$; A29/no-bacteria odds ratio of 3.77, $P = 0.0018$) (Fig. 7). Larval survivorship decreased between the first

FIG 4 The number of taxa and sequences shared among hypopharyngeal glands (HG), royal jelly (RJ), and nurse crops (C). OTUs are defined based on 97% sequence similarity. The numbers of OTUs are indicated, and below them are the percentages of sequences of each sample type comprising the respective OTU group.

and second trials for both of the controls (Fig. 7). Pupal survivorship did not vary significantly among the five treatments in either trial (trial 1m $\chi^2_4 = 9.18$, $P = 0.0568$; trial 2, $\chi^2_4 = 8.54$, $P = 0.0735$) (see Fig. S6 in the supplemental material).

Phylogenic analysis and general properties of Alpha 2.2 bacteria. Phylogenetic analysis of the 16S rRNA gene sequence of all three *Acetobacteraceae* Alpha 2.2 isolates indicates that this phylotype represents a unique clade of *Acetobacteraceae* related to but distinct from the genera *Saccharibacter* and *Gluconobacter* (Fig. 5 and 8; see also Fig. S4 and S7 in the supplemental material). The 16S rRNA gene sequences for the most diverged of the isolates, strains A29 and B8, are both 4.5% diverged from the closest cultured relative, *Saccharibacter floricola* strain S-877 (GenBank accession number NR_024819.1). The 16S rRNA gene sequence for strain C6 was 4.4% diverged from *Saccharibacter floricola* strain S-877 (GenBank NR_024819.1). The three strains were ≥99.9% similar to each other based on sequence similarity over the nearly full (V1 to V8 regions) 16S rRNA gene sequence. Microscopic observations of Alpha 2.2 strain A29 indicated that it is a Gram-negative, nonmotile, rod (see Fig. S8 in the supplemental material). We propose the epithet *Parasaccharibacter apium* for the *Acetobacteraceae* Alpha 2.2 clade associated with the hive environment and social interactions of bees that provision brood with royal jelly.

DISCUSSION

Culture-based and culture-independent studies show that *Acetobacteraceae* Alpha 2.2 bacteria are present in the hive environment (10), crops (11), and larvae (12) but are either not detected or incidental in the adult midgut and hindgut (8–11). Here, we tested whether Alpha 2.2 is a core hive rather than core gut microbe that is a critical component of larval nutrition, conferring a benefit to its honey bee larval host. Alpha 2.2 was readily cultured from 1st-instar honey bee larvae and thrived in the antimicrobial environment of royal jelly (RJ) (Fig. 6). High-throughput sequencing of the 16S rRNA gene sequence of RJ, hypopharyngeal glands (HGs), and nurse crops showed that Alpha 2.2 is abundant in each of these environments and cohabitates with a diverse array of
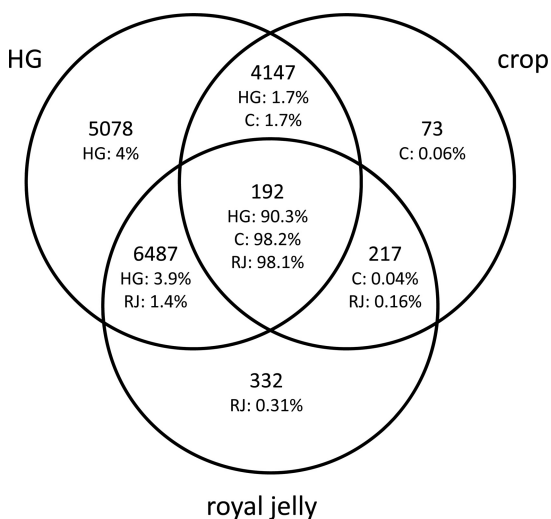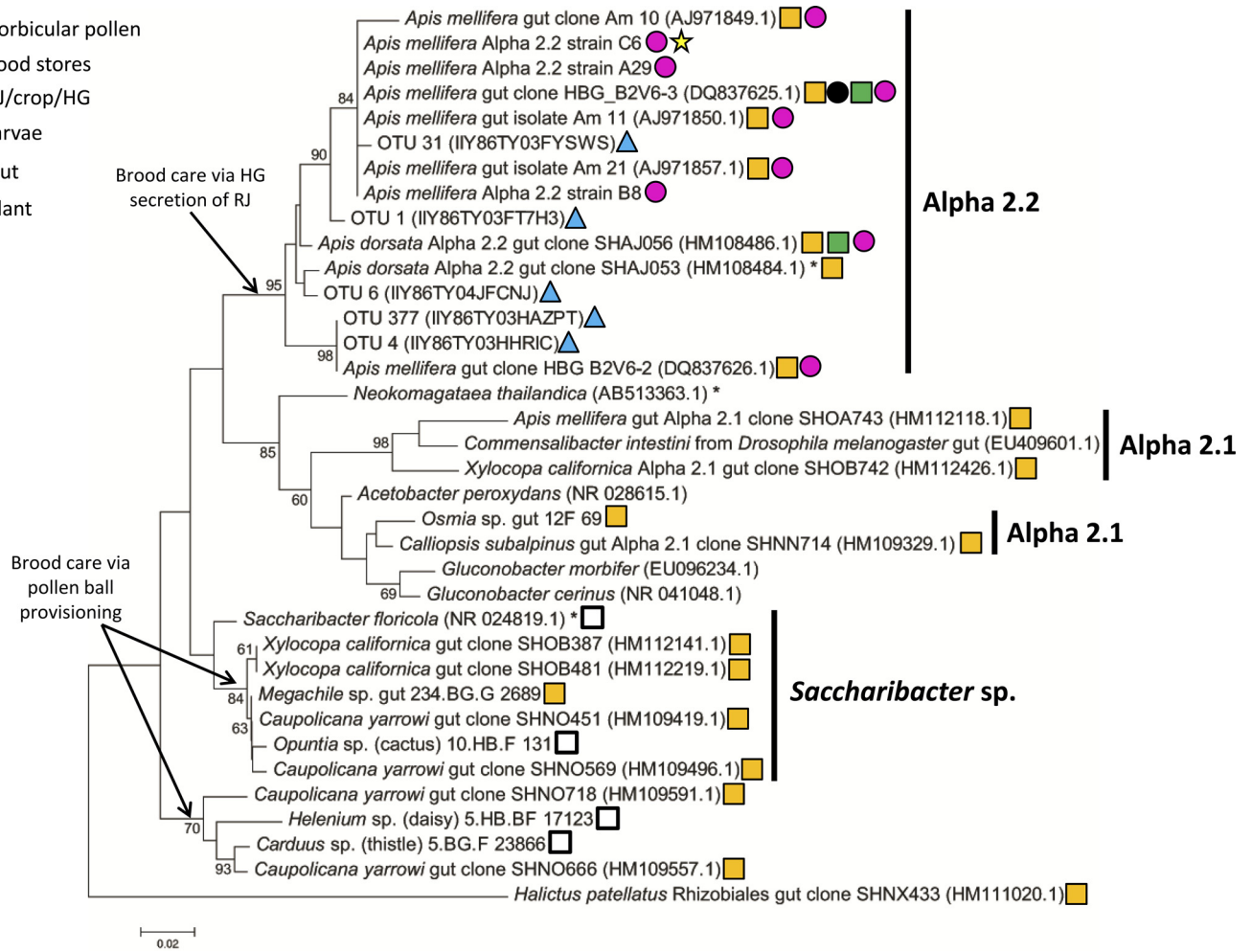
FIG 5 Neighbor-joining phylogeny of larval isolates relative to the Alpha 2.2 OTUs identified via high-throughput 16S rRNA gene sequencing. The V1 region of the 16S rRNA gene sequence was used to compare the representative sequences from the predominant Alpha 2.2 OTUs identified using high-throughput sequencing with the three isolates cultured from 1st-instar larvae that were used in the *in vitro* rearing experiments. Each Alpha 2.2 isolate is labeled with its OTU number (see Table S2 in the supplemental material) and its representative sequence title. Isolates found in larvae only are members of groups A, B, and C as described in the legend of Fig. S3 in the supplemental material. The yellow star represents the Alpha 2.2 strain (strain C6) that increased larval survival. Evolutionary distances were computed using the maximum composite likelihood method and are represented as the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Only bootstrap values of ≥60% are shown. Black asterisks (*) indicate reference sequences that are the best BLAST hits to OTUs associated with increased *Crithidia* infection in bumblebees (26). HG, hypopharyngeal gland; RJ, royal jelly.

other microorganisms (Fig. 1). It was not present at appreciable levels in the nurse midgut even under conditions that favor its growth (see Fig. S5 in the supplemental material). The Alpha 2.2 sequences isolated from bees that nurse their young with RJ formed a distinct clade separate from the *Saccharibacter*-type sequences found in species that provision their brood with pollen (Fig. 5 and 8), and one Alpha 2.2 strain increased the survivorship of larvae *in vitro* (Fig. 7). This combined evidence suggests that the Alpha 2.2 isolated from RJ, HGs, crops, and larvae is a core hive bacterium that (i) is readily cultured in the lab, (ii) is specific to bee taxa that feed their brood with RJ secreted from nurse HGs, and (ii) exerts a positive effect on honey bee larval survival.

Nine groups of Alpha 2.2 bacteria were recovered from 1st-instar honey bee larvae based on sequence variation at the V1 region of the 16S rRNA gene. Alpha 2.2 was easily cultured from larvae under low-oxygen environments and in a sugary, acidic medium (10, 12) which mirrors the conditions of the larval gut, RJ, and the crop. These isolates were either the same or closely related to the Alpha 2.2 bacteria that were numerous in nurse crops, nurse HGs, and the RJ surrounding young larvae. The sequences obtained from *A. mellifera* and *A. dorsata* guts as well as those obtained from honey bee hive food stores and corbicular pollen sequences formed a clade that was distinct from bacteria more closely related to *Saccharibacter floricola* and sequences obtained from bees that provision their young with pollen and do not perform the nursing behavior characteristic of bees that feed their brood with RJ. We suggest that because Alpha 2.2 is rarely found in the gut but is abundant in the royal jelly, the crop, and larvae, this microbe prefers these relatively antiseptic and extreme niches in the hive and follows the flow of nutrition between nurse workers and larvae in the hive.

Alpha 2.2 is one of a few examples of a bacterium that naturally
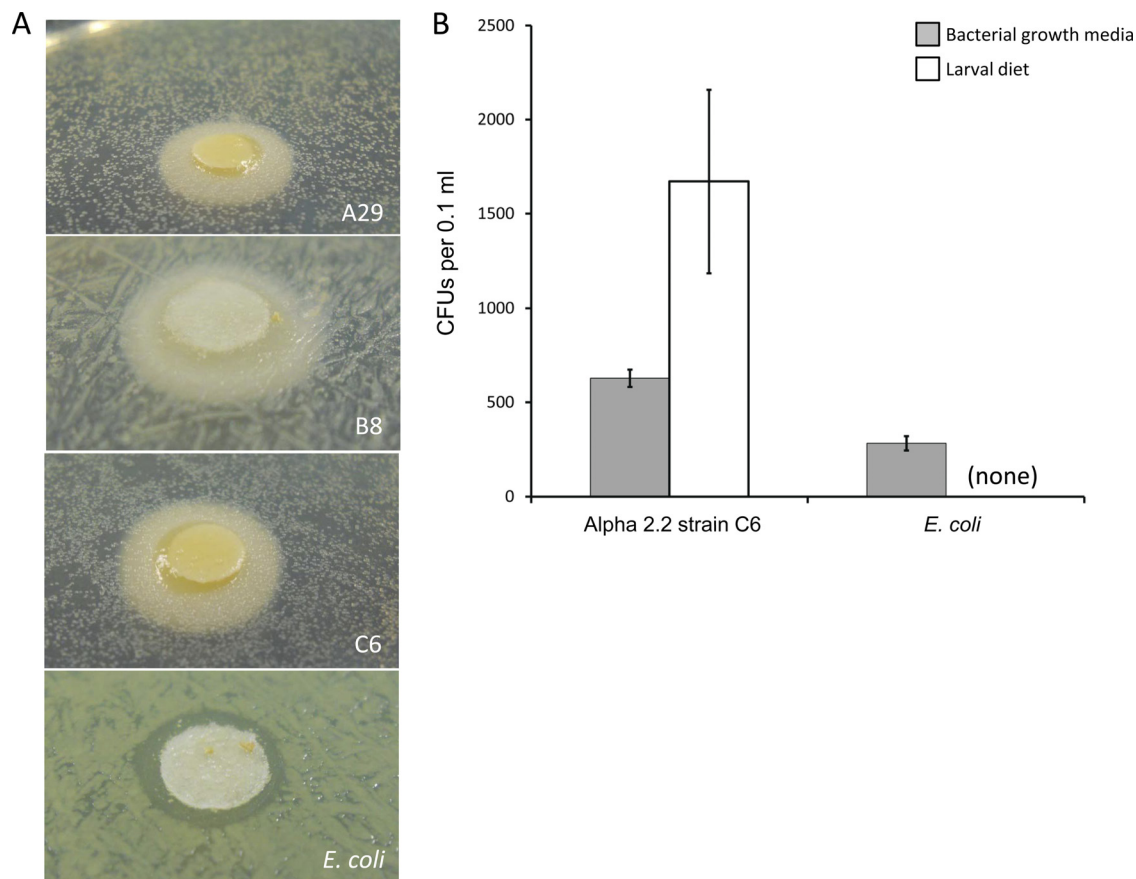
FIG 6 *Acetobacteraceae* Alpha 2.2 bacteria flourish in the presence of royal jelly compared to nonhive bacteria. (A) Three Alpha 2.2 strains and *E. coli* were plated onto SDA and LB medium, respectively. A royal jelly disk was added to the newly plated culture. (B) CFU of Alpha 2.2 strain C6 and *E. coli* per 100 μl of sample from culture (bacterial growth media) and from 100 ml of the larval diet incubated overnight at 34°C with 300 CFU of bacteria (larval diet). Error bars represent the standard error around the mean number of CFU for five replicate samples.

and stably occurs with honey bees that also increases honey bee health. Forsgren et al. (38) showed that individual and mixtures of lactic acid bacteria (LAB) can inhibit the growth of *Paenibacillus larvae* (American foulbrood) and that bee larvae fed a mixture of 11 LAB *in vitro* survive *P. larvae* infection better than larvae that are not fed LAB. However, while hindgut LAB (10, 11) showed almost total inhibition of *P. larvae*, the LAB found at high levels in both crops (11) and larvae (12) had limited effects on *P. larvae* growth, suggesting that the larvae did not survive the *P. larvae* infection due to the LAB that would realistically be found in the crop and larvae in nature. Similar questions arise when the effects of LAB on the larva's ability to resist European foulbrood are considered (39). Audisio and Benitez-Ahrendts showed that *Lactobacillus johnsonii*, a bacteria isolated from the honey bee intestinal tract, increases colony fitness (40), and further work showed that the metabolites produced by *L. johnsonii*—lactic acid, phenyllactic acid, and acetic acid—improved colony fitness (41). *L. johnsonii* is commonly found in mammalian intestines but has not been found with honey bees in any of the existing high-throughput sequencing studies to date. Lactic acid and acetic acid are the main metabolites produced by the lactic acid bacteria (*Lactobacillales*) and the acetic acid bacteria (*Acetobacteraceae*) commonly found in the guts and hives of honey bees, and so *Acetobacteraceae* Alpha 2.2 might increase larval fitness through the production of

such acids. Yet another possibility is that Alpha 2.2 induces an immune response against larval pathogens, similar to what was observed when *A. mellifera* larvae were supplemented with *Bifidobacteria* sp. and *Lactobacillus* sp. (42). This is possible if the *E. coli* used as a control does not induce the same immune response as the Alpha 2.2 and could be possible if a low level of infection persisted from the field to the lab. While the mechanism underlying the fitness benefit of Alpha 2.2 has yet to be determined, it does appear that certain lineages of Alpha 2.2 bacteria confer a survival benefit to their larval hosts.

Larval survivorship varied among the trials for larvae fed the three different strains of Alpha 2.2 *in vitro*. In both trials, strain C6 performed better than the two controls (*E. coli* and no bacteria). However, strain A29 showed a benefit only in the second trial, strain B8 showed marked differences between the trials, and the larvae fed the two control treatments performed better during the first trial than in the second. A variety of factors, such as differences in technique between trials or slight heat-related degradation of the royal jelly over time, could explain this variation. Proteins in royal jelly degrade when heated (43), and royal jelly contains fatty acids that may be sensitive to heat (2). Although we were careful not to subject the royal jelly used for *in vitro* rearing to more freeze-thaw cycles than necessary, it is possible that the use of fresh royal jelly each time as opposed to a bulk commercial
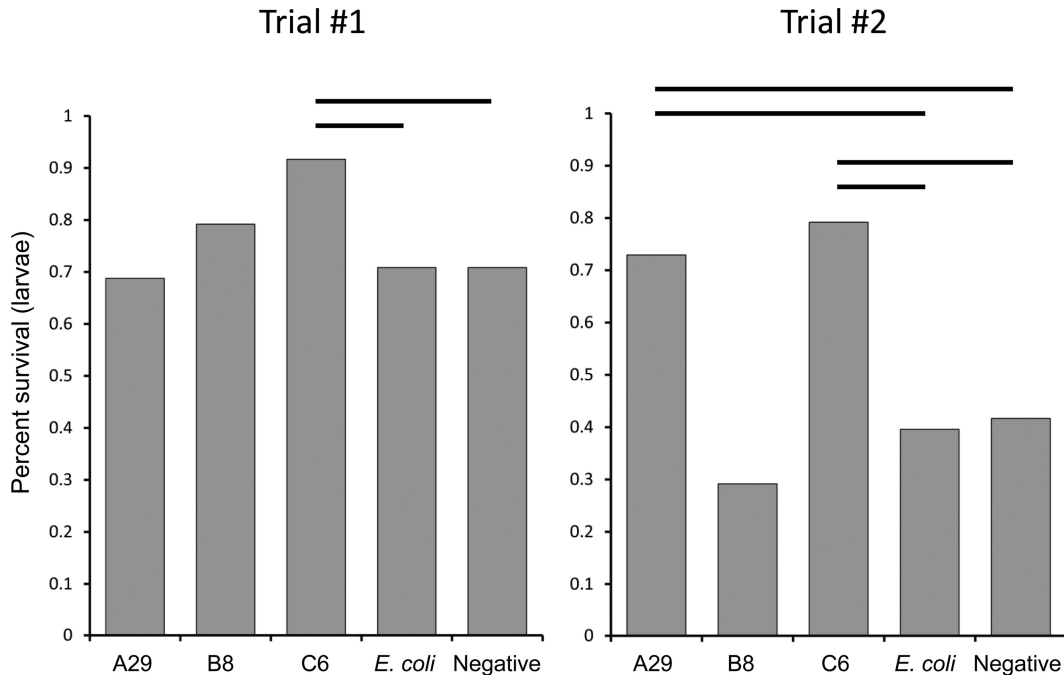
FIG 7 *Acetobacteraceae* Alpha 2.2 bacteria increase larval survival. The percentage of larvae surviving to the last larval instar when the larval diet was supplemented with Alpha 2.2 strain A29, B8, or C6, *E. coli*, or no bacteria (negative) is shown ($n = 48$ for each treatment per trial). Black lines indicate significantly different planned comparisons between the Alpha 2.2 treatments and the *E. coli* and negative-control treatments.

source would have resulted in higher repeatability among trials. Nonetheless, larvae fed the Alpha 2.2 strain C6 performed better than the controls in both trials, suggesting that the benefit of this strain is robust. Further, if we assume that the conditions were more stressful in the second trial based on the performance of larvae fed the controls, we note that the decline in survival of the larvae fed strain C6 was only approximately 10% between the two trials compared to the 30% difference observed for larvae fed either *E. coli* or no bacteria. Larvae fed strain A29 were also resistant to whatever factors caused the decreased survival between trials. The differences in the performance of the larvae on strain B8 is especially striking and unexplainable at this juncture but suggests that strain B8 is either more sensitive than the other strains to the factors causing the survival differences between trials or slightly pathogenic under certain conditions.

Recent work by Cariveau et al. ([26]) suggests that five Alpha 2.2 taxa (as defined by 97% sequence similarity) are positively correlated with levels of *Crithidia*, a protozoan gut parasite, in bumblebees. We cannot directly compare their data set with our data as different regions of the 16S rRNA gene were sequenced. However, we did investigate the relationships between the Alpha 2.2 bacteria from larvae, RJ, HGs, and crops identified in the present study with published full-length sequences that grouped closely with the taxa positively correlated with *Crithidia* incidence in Cariveau et al. ([26]) ([Fig. 5]). Based on this analysis, it appears that OTU 6 is closely related to an Alpha 2.2 isolate identified from *Apis dorsata* (GenBank accession number HM108484.1) and shares 98.7% sequence identity. However, 16S rRNA sequence similarity does not always translate directly to phenotypic similarity (see below). The biology of the Alpha 2.2-honey bee association suggests that the Alpha 2.2 from honey bees may not be as detrimental to the host as it is in bumblebees. Alpha 2.2 is very prevalent in the crop but is

virtually absent from the rest of the gut. Given that *Crithidia* attacks the midgut, it is unlikely that the two would directly interact for significant periods of time. Indeed, this may also be the case for bumblebees as the crop has never been studied in detail separate from the rest of the hindgut ([7], [26], [27]). Bumblebees and honey bees also have very different ecologies, and the niche and social mode of transmission that honey bee Alpha 2.2 bacteria use are largely absent in bumblebees. In particular, bumblebee HGs have a very different appearance and structure than honey bee HGs ([44]) and secrete digestive enzymes, not nutritive proteins such as RJ ([45], [46]). Taken together, the potentially negative effects of Alpha 2.2 that Cariveau et al. ([26]) observed may not occur in honey bees. However, more work must be done to determine whether similar associations between Alpha 2.2 and honey bee pathogens exist and whether the Alpha 2.2 found in honey bees is vectored to native pollinators.

Despite the relatively high degree of similarity at the 16S rRNA gene, the isolates tested exhibited significant phenotypic diversity in how they affected larval survival. One main goal of high-throughput sequencing studies is to reveal the microbial diversity present within honey bees. This diversity is usually quantified based on the level of sequence variation at the 16S rRNA locus. Bacterial taxa uncovered by these sequencing experiments tend to be grouped into large functional categories (i.e., Firm4 or Alpha 2.2) based on 97% sequence similarity to define a genus or 99% sequence similarity to define a species. Our results suggest that the bacterial population associated with the hive environment contains an untapped pool of phenotypic diversity that we are only beginning to understand. The phylogenetic cutoffs that are commonly used in high-throughput studies to group taxa often do not align with phenotypic differences based on standard biochemical profiles or microscopy ([47], [48]). For example, Endo et al. ([49]) find
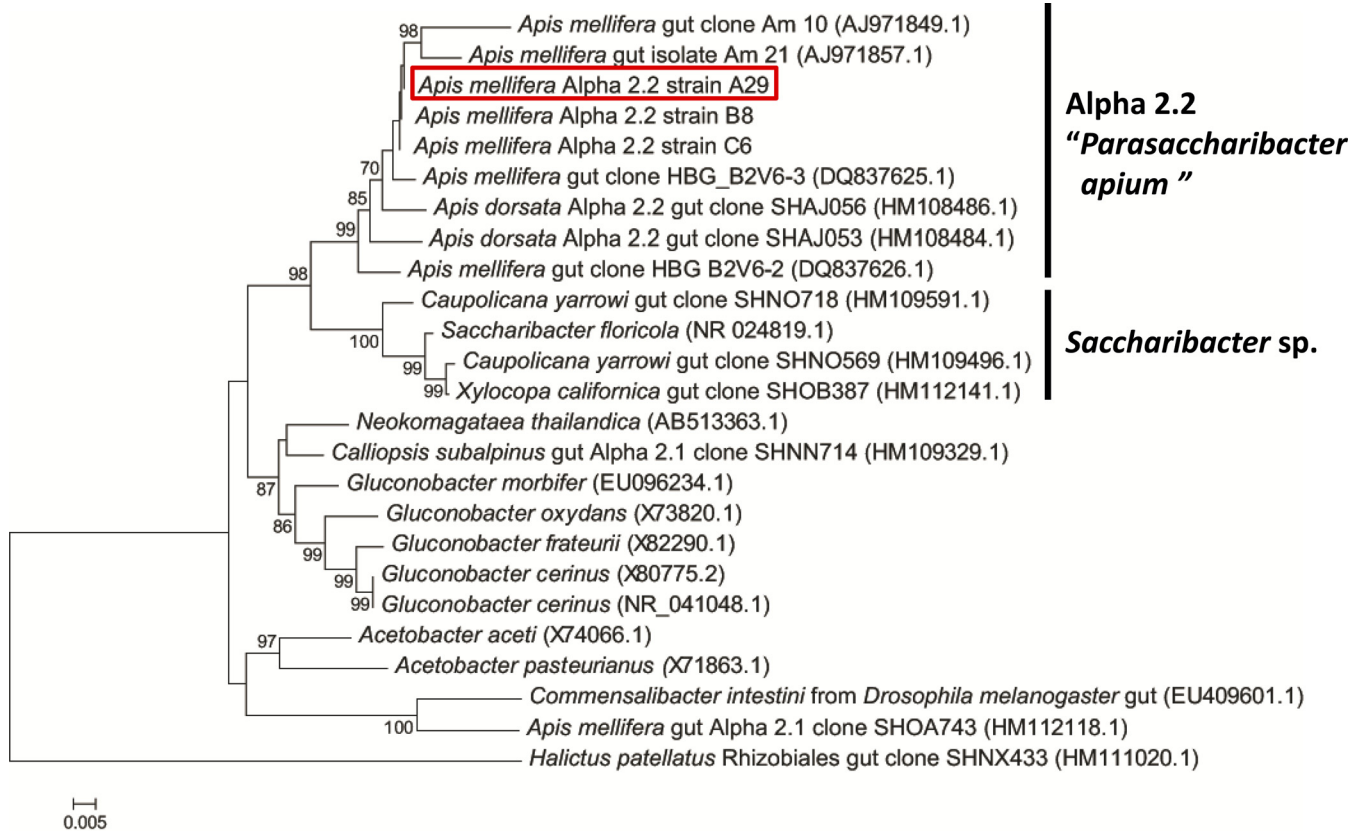
FIG 8 Taxonomy of Alpha 2.2 (*Parasaccharibacter apium*) isolates based on a longer 16S rRNA gene sequence. The regions of V1 through V8 of the 16S rRNA gene sequence were used to infer the taxonomy of the three Alpha 2.2 isolates cultured from 1st-instar larvae that were used in the *in vitro* rearing experiments. Larval isolates are members of groups A, B, and C as described in the legend of Fig. S3 in the supplemental material. A total of 1,306 bp was used to construct the original alignment. Evolutionary relationships were inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Only bootstrap values of ≥60% are shown. The type strain for the newly named *Parasaccharibacter apium*, Alpha 2.2 isolate A29, is boxed in red.

that *L. kunkeei* bacteria, which are widespread in the pollination environment, in the honey bee gut, and the honey bee colony (10–12), can have nearly identical 16S rRNA gene sequences yet very different biochemical profiles. This phenotypic diversity could have great implications for the study of bacterium-bee interactions and also the use of bacteria for improving honey bee health. Renewed efforts should be made to test the many hypotheses that have been generated using high-throughput sequencing methods.

Compared to forager crops (11), the crops of nurse workers had high levels of *Lactobacillus* sp. Firm5, similar levels of the Alpha 2.2 phylotype bacteria, and low levels of *L. kunkeei* although the levels of these taxa varied among individuals. Nurse crops had lower levels of *Enterobacteriaceae* than those of pollen forgers (11), suggesting that the pollination environment, not the hive, is the principle source of these bacteria for foragers. Additionally, *Lactobacillus* sp. Firm4 was in higher abundance in nurse crops than in forager crops (11). It is unclear where so many Firm4 bacteria originate from since the bee bread, honey, and corbicular pollen that could supply these bacteria to nurse workers through their diet of hive food stores have very low levels of Firm4 (10, 11). Hypopharyngeal glands and RJ also had appreciable levels of Firm4, suggesting that this bacterium may colonize adult digestive tracts through trophallaxis of RJ.

The lower taxonomic diversity and species richness in the RJ suggest that it is slightly antimicrobial, but it is certainly not devoid of microbes. Rather, there is an interesting array of microbial taxa that seem to thrive in the HG and RJ niches and could, in the same manner as Alpha 2.2, be passed as nurse workers care for young brood. Of great interest to the current experiment are Alpha 2.2 and *L. kunkeei*, the main bacteria found in early-instar larvae (12). Other bacteria such as the *Xanthomonadaceae* are also potentially important members of the hive environment transferred from nurses to larvae but not present in the crops at high levels. For example, OTU 167, a *Stenotrophomonas* sp., was 10 times more prevalent in the RJ and HGs than in the crops and is similar (≥97% sequence similarity) to two of the bacteria isolated by Evans and Armstrong (50) that repel the larval pathogen *Paenibacillus larvae*. *Rhodanobacter* sp. bacteria were also consistently present in the HG and RJ niches and largely absent from the crops. While some *Rhodanobacter* sp. bacteria contain complete denitrification pathways, others are able to break down components of insecticides (51) and produce β-galactosidase (52), which may hydrolyze the glycolipids and glycoproteins found in RJ. Such bacteria may confer benefits to honey bee larvae and provide candidates for further study.

Alpha 2.2 bacteria are passed to larvae as nurse workers feed the hive's developing brood and are specific to taxa that feed larvae with RJ. *Saccharibacter* sp. bacteria are closely related to the Alpha

2.2 bacteria found in honey bees but form a distinct clade comprised of bacteria found in bee species that use different means to provision their brood. Further, one strain of Alpha 2.2 is emerging as a mutualist, providing a survival benefit to larvae *in vitro*. The RJ-HG-crop niche is an underexplored aspect of the hive microbiota that is distinct from the core gut microbiota. Further work will determine whether overall hive health is impacted by microbes found in this niche and what mechanisms moderate host-bacteria interactions in honey bee larvae.

Taxonomy. *Parasaccharibacter apium* A29^T gen. nov., sp. nov. (ATCC SD-6836) (*Pa.ra.sac.cha.ri.bac'ter*. Gr. pref. para, close or similar to; N.L. n. *Saccharibacter* a bacterial genus; N.L. masc. n. *Parasaccharibacter*, a bacterial genus similar to *Saccharibacter*. *P. apium* sp. nov. (*a'pi.um*. L. gen. pl. n. *apium*, of bees; referring to the association with bees). This Gram-negative, nonmotile, rod-shaped bacterium flourishes under sugary and slightly acidic conditions. It is found in close association with bees of the genus *Apis* and is most likely transmitted via trophallaxis between honey bees, especially from nurse workers to larvae. The type strain is also known as *Acetobacteraceae* Alpha 2.2 strain A29.

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