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Microbiomes associated with European foul brood disease, and idiopathic brood disease syndrome in honey bees.

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Abstract

Critical for pollination, commercial honey bees (*Apis mellifera*) are faced with a variety of pathogenic and environmental stressors that often result in colony or crop loss. One highly prevalent pathogen is the larval disease European foulbrood (EFB) attributed to the bacterium *Mellisococcus plutonius*. However, many disease phenotypes classified morphologically as EFB do not contain *M. plutonius* based on microscopy and molecular markers, and are designated as idiopathic. Here we improve understanding of the larval disease microbiome with the end goal of developing diagnoses and management strategies for EFB and EFB-like disease. We used next generation sequencing and qPCR to detail the microbiomes of phenotypically healthy and diseased larvae from six different apiaries in the state of Illinois, USA. A state apiary inspector diagnosed disease at all six apiaries as EFB according to a gestalt morphological phenotype. At five of the six apiaries, *Mellisococcus plutonius* dominated the larval disease microbiome, showing associations with three different firmicutes dependent on apiary. At the sixth site, diseased larvae were uniquely described as “melty, sunken and deflated”, and the resulting microbiota indicates a novel disease state that corresponds to idiopathic brood disease syndrome. Our results contribute to brood disease diagnosis, a growing problem for beekeeping and agriculture worldwide.

Introduction

Honey bees (*Apis mellifera*) are valuable pollinators of agriculture and ecosystems worldwide [1–3]. Recent colony loss has necessitated a review of pollination services and their sustainability including microbiome associations of health and disease [4,5]. Colony loss is described as multifactorial, often involving combinations of environmental stress and disease agents [3,6,7]. Strongly associated with colony decline, bacterial diseases of honey bee larvae have become more prevalent worldwide [8–10]. Despite this growing threat, the patterns and processes underlying honey bee larval disease remain poorly understood and poorly diagnosed [11], with more than half of reported cases in the United States attributed to an unidentified cause.

One primary function of the eukaryotic microbiome is protection from pathogens, and changes to the normal gut microbiota can range from mildly anticommensal to pathogenic [12]. Many non-communicable and chronic disease states are associated with microbiome variation, highlighting the importance of microbiome integrity or taxonomic membership in disease susceptibility. A variety of factors may weaken the core microbiota of adult honey bees rendering the host organism susceptible to disease [5,13]. The microbiome associated with reproductive queens reveals prevalent core gut bacterial species shared with larvae including *Bombella apis* and *Apilactobacillus kunkeei*, previously referred to as *Parasaccharibacter apium and Lactobacillus kunkeei*. Much of the queen and worker mouthparts and anterior alimentary tract of queens, classify as these two oxygen tolerant species [14]. Both are associated with decreased abundance of honey bee-specific disease, and likely represent protection from many aerobic opportunists including bacteria, microsporidia, and fungi, omnipresent throughout the hive environment [5,15–18]. The

influence of the microbiome on host fitness highlights the role of commensals in mediating susceptibility to disease [12,13,19,20].

Two major bacteria cause larval disease in honey bees: American Foulbrood (AFB) caused by *Paenibacillus larvae* and European Foulbrood (EFB), attributed to *Melissococcus plutonius* [21,22]. While AFB disease is overt, highly virulent, and caused by a singular bacterial species, the causative factor of EFB and EFB-like symptomology is less apparent. Historically, EFB has been considered an opportunistic disease, affecting stressed hives. Many other bacteria are found in association with EFB symptoms, suggesting a non-commuicable disease state associated with opportunistic microbes typically found in the hive environment, and EFB is anecdotally associated with particular crops, seasons or environmental conditions [23–26]. Although considered the primary cause of EFB disease, *M. plutonius* often goes undetected when EFB-like symptoms are present. Larval disease that is similar to EFB, without the presence of *M. plutonius* was first described as parasitic mite syndrome, but was renamed Idiopathic Brood Disease Syndrome (IBDS, vanEngelsdorp et al. 2013) because the disease phenotypes occur in the absence of Varroa infestation. IBDS is diagnosed by the presence of brood at different ages that appear molten (melted) on the bottom of cells or a collection of symptoms similar to, but not matching EFB, AFB or sac brood, a virus known to afflict larvae.

In this study we analyzed microbial abundance and diversity of larvae classified by a State Apiary Inspector as EFB disease according to morphology. We used high-throughput sequencing (HTS) analysis of the V3-V4 region of the 16S rRNA gene to explore both healthy and disease symptomology within the same apiary. To discover secondary bacteria that may contribute to pathology, we sampled healthy larval phenotypes from both healthy and diseased colonies to distinguish saprophytes from potential causes of, or contributors to disease. We sequenced young, middle, and old aged larvae (3rd, 4th and 5th instars) to characterize shifts in disease progression associated with age and development. Overtly diseased larvae were sequenced to explore potentially saprophytic bacteria associated with the final stages of disease.

Methods

Sampling

In Summer and Fall of 2016 we sampled larval honey bees from six apiaries in the state of Illinois USA during outbreaks of larval disease. An Illinois State Apiary inspector diagnosed the colonies as healthy or diseased with European Foul brood (EFB). Disease phenotypes were sampled to represent the progression of the disease from incipient through the final stages of disease progression. Thus our samples are comprised of healthy and diseased phenotypes and three distinct larval sizes that loosely correspond to three developmental larval stages; 3rd, 4th and 5th instar. Due to simple ergonomic constraints on bacterial growth, we hypothesized that younger larvae in a diseased hive may possess an incipient disease state and that healthy larval phenotypes would contain non-disease microbiomes. Further if larvae were socially distant from the disease phenotype they would show an increasingly

healthy (non-disease) microbiome, but that healthy phenotypes would likely possess some degree of diseased microbiome as they became more proximate to diseased individuals. We therefore sampled from each yard one phenotypically healthy hive and one diseased hive. Then within the diseased hive we sampled larvae with both healthy and diseased phenotypes. Briefly, the healthy hives show healthy (non-disease) phenotypes, and the diseased hives present a variety of disease phenotypes attributed to EFB or EFB-like disease (Table 1 and S1).

Table 1
Locations and dates associated with larval sampling.

Illinois Site ID	Date Sampled	Latitude	Longitude	Sample size
1. Spring Valley	June 22, 2016	39.880494	-90.975457	47
2. Butler	July 8, 2016	39.183442	-98.543459	48
3. Hardin	Sept. 6, 2016	39.166328	-90.618965	50
4. Kampsville	Sept. 15, 2016	39.29642	-90.611419	48
5. Wright's Corner	Oct. 15, 2016	39.117567	-88.902383	32
6. Hull	Oct. 8, 2016	39.755862	-91.205011	48

DNA Extraction

Larvae were frozen at -80C until DNA extraction. Immediately after removal from the freezer, individual larvae or larval material were surface sterilized with 95% EtOH and immediately placed into a 2ml bead beating tube containing ~ 100ul of 0.1mm silica beads and 300ul of 1X TE buffer (10 mM Tris-HCl, 1 mM EDTA) and immediately frozen on dry ice. Prior to DNA extraction, each sample was bead beaten for a total of 2 min in 30 sec intervals. To each sample, 100 µl lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5% Triton X-100, 80 mg/ml lysozyme, pH 8.0) was added and the samples were incubated at 37°C for 30 min. Total genomic DNA was further extracted using a Fermentas GeneJet Genomic DNA Purification Kit (#K0722) following the protocol for gram-positive bacteria.

PCR and MiSeq

The V3–V4 region of the 16S rRNA gene was amplified using PCR primers (341F 5'-CCTACGGGNGGCWGCAG-3'; 805R 5'-GACTACHVGGGTATCTAATCC-3'). Amplification was performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 94°C for 3 min, followed by 28 cycles of 94°C for 30 s, 53°C for 40 s and 72°C for 1 min, with a final elongation step at 72°C for 5 min. PCR products were confirmed using a 2% agarose gel. PCR products were then used to prepare DNA libraries following Illumina MiSeq DNA library preparation protocol. Sequencing was performed at the University of Arizona Genetics Core (UAGC) on a MiSeq following the manufacturer's guidelines. All sequence data were deposited in GenBank under Sequence Read Archive number PRJNA810064.

MiSeq sequence analysis

Sequences were processed using MOTHUR v.1.43 [28]. Forward and reverse reads were joined using the make.contigs command. After the reads were joined the first and last five bases pairs were removed using the SED command in UNIX. Sequences were then screened, using the screen.seqs command, to remove any sequences containing ambiguous bases. Unique sequences were generated using the unique.seqs command. A count file containing group information was generated using the count.seqs command. Sequences were aligned to Silva SSUREF database v102 [29] using the align.seqs command. Sequences not overlapping in the same region and columns not containing data were removed using the filter.seqs command. Sequences were pre-clustered using the pre.culster command. Chimeras were removed using UCHIME [30] and any sequences that were not of known bacterial origin were removed using the remove.seqs command. All remaining sequences were classified using the classify.seqs command. All sequences with only one or two (single/doubletons) associated reads were removed using the AWK command in UNIX. A distance matrix was constructed for the aligned sequences using the dist.seqs command. Sequences were classified with the RDP Naive Bayesian Classifier [31] using a manually constructed training set containing sequences sourced from the greengenes 16S rRNA database [32], the RDP version 9 training set, and all full length honeybee-associated gut microbiota listed in NCBI. Operational taxonomic units (OTUs) were generated using the cluster command. Representative sequences for each OTU were generated using the get.oturep command. Alpha diversity was determined using the rarefaction.single command while sample coverage and the Inverse Simpson diversity estimates were generated using the summary.single command.

Bacterial Quantification

Plasmid vectors were created using Invitrogen's pCRTM2.1 TOPOTM cloning vectors per the manufacture's specifications. Ligated vectors were then transformed into DH5α™ cells per the manufacture's specifications. Successfully, transformed colonies subsequently grown overnight in broth. Plasmid DNA was purified using a Thermo Scientific GeneJET Plasmid Miniprep Kit (#K0503). To determine 16S copy number for each sample we first calculated the mass of a single plasmid containing our insert using the Applied Biosystems equation. An Implen nanophotometer P300 was used to assess DNA concentration of the purified plasmid solution and subsequent 10-fold serial dilutions were made. The dilutions were then used as standards for all subsequent qPCR quantifications. Total copy number was determined by first calculating the 'raw' copy number (n_{raw}) in 1µl of DNA based on the Cq value and the standard curve using the formula n_{raw} = logarithmic trendline function. To determine the total number of copies present in each extraction the n_{raw} value was normalized according to elution volume and any subsequent dilution(s).

Statistical analysis

To incorporate community size in the analysis, we multiplied the proportional abundance of OTUs returned by amplicon sequencing by the total bacterial 16S rRNA gene copies determined with qPCR for each individual tissue type. All core bacterial genomes contain four 16S rRNA gene copies except *L.*

kunkeei (5), *B. asteroides* (2) and *Bo. apis* (1). OTUs representing non-core diversity were summed, corrected for community size and mean 16S rRNA gene copy number (4.2) [33].

We analyzed microbiome size and taxonomic structure using bactiquant qPCR and Illumina high throughput 16S rRNA gene sequencing. To allow the use of parametric multivariate analyses [34], we converted bacterial relative abundance to ratios among all OTUs [35] using the software CoDaPack's centered log-ratio (CLR) transformation [36]. Principle component analysis (PCA and PCoA) bi-plots were generated to first assess the variance in the data. Microbial community structure was compared with PermANOVA and MANOVA using site, age and disease phenotype as factors. Bacterial copy number comparisons were made using one-way ANOVA (Tukey HSD post-hoc) and two-sample t-tests. W e performed correlations examining log transformed bacterial abundance with each major bacterial taxon. All analyses were conducted in either JMP_ v11 (JMP_ 1989–2007) and/or SAS_ v9.4 (SAS Institute Inc. 2013)

Results

Microbial community analysis

Next generation sequencing returned 11,736,227 quality trimmed reads (400 bp) for the 273 libraries (Table S2). A total of 1646 OTUs were resolved at 97% similarity. The top 19 OTUs represent 95.6% of the total sequences (respectively), and were used for all downstream statistical analyses.

Alpha diversity, depicted as Shannon index and observed OTUs differed significantly by site, age and phenotype (Table S3). Healthy phenotypes contained more bacterial diversity than diseased phenotypes according to both metrics ($t = -7.3$, $p < 0.0001$). Young larvae were more diverse than older larvae ($F = 31.6$, $p < 0.0001$), and the idiopathic disease state more diverse than the EFB disease state ($F = 7.9$, $p < 0.0001$).

Clustering according to PCA, PCoA and CCA reveal significantly different groupings according to site, age and phenotype (Fig. S1). Based on PermANOVA, larval microbiomes differed by site; $F = 5.8$; R-sq: 0.10; $p < 0.001$, age; $F = 13.5$; R-sq = 0.16; $p < 0.001$, and phenotype; $F = 43.4$; R-sq = 0.16, $p < 0.001$. Site 6 clustered independently from sites 1–5. Sites 1–5 were defined by *M. plutonius* dominance, but Site 6 contained a low abundance of *M. plutonius* regardless of age or disease phenotype, and contained a different assemblage of bacterial OTUs (Fig. S2). Thus, we performed statistical analysis on the microbiomes of sites 1–5 separately from site 6.

In general, bacterial density increased with larval size and disease progression (Table S4). Total bacterial abundance was positively correlated with the abundance of *M. plutonius* (R-sq = 0.49, $F = 44.9$, $p < 0.0001$). There was a significant difference in total bacterial abundance between healthy larval instars by age (Fig. 1, $p < 0.008$), between healthy larval instars and those with incipient signs of disease ($p < 0.005$), and between incipient and advanced disease ($p < 0.0001$). All developing larval stages classified as healthy and sampled from a spotty brood pattern in a hive that also contained disease phenotypes

revealed significantly more *M. plutonius* than did larvae from hives with no phenotypic indication of disease (Fig. S2).

MANOVA analyses revealed many differences in community structure between sites and stages of disease progression (Table S4 and S5). *Bo apis* and *L. kunkeei* were abundant in healthy larval phenotypes (Fig. 2). For sites 1–5 with EFB disease, *Bo. apis* decreased with disease progression and various Firmicutes were significantly associated with advanced disease states by site (Fig. 2A-E, $p < 0.001$). At sites 1–5, *M. plutonius* abundance and occurrence differed significantly between healthy hives and diseased hives, and, *M. plutonius* (EFB) was the dominant bacterium (Fig. 3). *M. plutonius* occurred with significantly different microbial taxa and structures by sampled apiary suggesting multiple hypotheses for disease progression based on microbiota variation (Fig. 3, Table S5). For example, *Enterococcus faecalis* was more abundant at sites 1, 3 and 4, *F. fructosus* was more abundant at sites 1 and 4, while *Bo. apis* was more abundant at sites 1, 4 and 5). As part of our experimental design, sequencing microbial succession by larval age also resulted in multiple hypotheses for the role of the microbiota in disease progression (Fig. 3, Table S5).

We analyzed the apiary associated with idiopathic brood disease syndrome (IBDS) separately (Table S6). At this site, only samples classified as advanced disease showed increased microbiome size (Table S4). Three bacteria associated with the worker ileum (*F. perrara, G. apicola* and *S. alvi*) were significantly more abundant in larvae with IBDS, as was *Serratia marcescens* (Table 1). Additionally, *S. alvi* abundance was positively correlated with *S. marcescens* abundance (Fig. 4). *Bombella apis*, and the group of six OTUs (Bacteroidetes, Ralstonia, Caulobacterales, Bradyrhizobium, Burkholderiales and Cyanobacteria) were all more abundant in healthy larval phenotypes sampled from site 6.

Discussion

High throughput sequencing has been the foundation of microbiome science, but must be interpreted carefully [38]. Here we applied this method to distinguish microbiomes associated with health and disease in honey bee larvae. Most serendipitously, we discovered and characterized two major disease related larval microbiomes, one caused by *M. plutonius* (European Foul Brood, EFB), the other caused by an unknown factor (Idiopathic Brood Disease Syndrome, IBDS). Below we discuss the general nature of the results, healthy larval microbiomes, EFB associated microbiomes, and a novel disease microbiome associated with the symptomology of IBDS.

Healthy larval microbiomes

While many of the OTUs returned by Illumina sequencing have been confirmed by culturing and cloning, some of the unverified OTUs may represent a form of 'kitome' contamination [38], and require validation by alternate methods including cloning and shotgun sequencing. Six OTUs were uniformly abundant and highly intercorrelated, a phenomenon most apparent in younger healthy larvae with smaller microbiomes (Tables S2 and S4). These OTUs were; Bacteroidetes, *Ralstonia*, Caulobacterales, Bradyrhizobium,

Burkholderiales and Cyanobacteria (Fig. S1). This pattern indicates either amplified contamination or a highly structured core larval microbiota. Some of the same OTUs were detected on the mouths of queens, with greatest abundance in the proximal queen digestive tract, also associated with smaller microbiomes (Anderson et al. 2018). Low BactQuant yields from first and second instar larvae (not shown here) further suggests that PCR/sequencing artifacts may bias amplicon results particularly in low abundance environments. For this reason, we discuss the development of disease-associated microbiomes focusing on bacteria that have been cultured or cloned within the honey bee system [39,40,49,41–48].

We sequenced larval microbiomes from colonies and larvae with no symptoms of disease, but these healthy colonies came from apiaries that also contained colonies diagnosed with EFB disease. We found that the healthy larval microbiome (Fig. 2) does not resemble the worker hindgut microbiome as previously hypothesized [40], but seemingly shares more similarity with the queen microbiome [14,50]. Workers drifting from one colony to another is suspected to be the major mode of EFB transmission, such that healthy hives during an apiary outbreak of EFB may harbor a substantial load of *M. plutonius*. Sequences matching *M. plutonius*, the causal agent of EFB, were found in 95% of healthy larvae from healthy hives, at an average abundance of 0.06 (0-0.92) across the 71 healthy larvae (Fig. 2). We found that the size of the microbiome increased with larval development (Fig. 1), and the microbiomes of healthy larvae varied by both location and developmental stage. Microbial succession throughout larval development suggests a role for the larval microbiome in disease prevention and/or the development of immunity [18,51]. The healthy larval microbiome is often dominated by *Bo. apis* or *L. kunkeei*, both prevalent core gut bacteria of reproductive queens [14,50,52]. Over 95% of the queen and worker mouthparts and anterior alimentary tract of queens, classify as these two oxygen tolerant species (Anderson et al. 2018). Both are associated with decreased abundance of honey bee-specific disease, and likely provide protection from many aerobic opportunists including bacteria, microsporidia, and fungi, omnipresent throughout the hive environment [6, 16–18,53].

European Foul Brood Microbiomes

Larvae afflicted with EFB turn from pearly white to a yellowish/brownish tinge, become deflated, and sometimes translucent [54]. Symptomology varies considerably and in many cases, the expression of EFB disease phenotype is attributed to secondary or “helper” bacteria, but this hypothesis has been difficult to verify due to variation in methods across studies, and historical taxonomic treatment [54,55]. It is unknown if the helper bacteria are saprophytic, or if the cause of EFB disease can be polymicrobial [56]. Many bacteria species in the honey bee worker microbiota form metabolic partnerships with other species and this is likely true for many disease pathologies [57]. With the progression of EFB disease, species considered co-infective or saprophytic may affect virulence, and accelerate larval pathology. Based on a literature search, potential helper species associated with EFB disease include *Paenibacillus alvei, Enterococcus faecalis, Brevibacillus laterosporus and Achromobacter eurydice* [54,55,58]. We confirm *E. faecalis* in abundance, but our high throughput sequencing method did not return *Paenibacillus* or *Brevibacillus* with any frequency or prevalence in EFB diseased larvae (Fig. 3, Table S2). Considered

somewhat omnipresent by past investigations, the putative secondary invader *Paenibacillus alvei* was also detected at very low prevalence and abundance in diseased larvae, and larvae in general, indicating that the universal 16S rRNA gene primers amplified *Paenibacillus alvei*, but that it is not associated with EFB disease progression. In contrast, *Enterococcus faecalis* was prevalent and abundant in larvae with overt EFB symptoms, occurring with abundance in a site-specific manner, at two of five EFB locations (Fig. 3, Table S2).

From EFB diseased colonies, we sampled diseased larvae with both incipient and advanced symptoms. We found that *Melissococcus plutonius* (EFB) occurred with significantly different microbial taxa and community structures by sampled apiary suggesting multiple hypotheses based on microbiota variation and disease progression. We report seven abundant bacteria that increased significantly with *M. plutonius* in diseased larvae; *Enterococcus faecalis*, *Lactobacillus* Firm5, *Fructobacillus fructosus*, *G. apicola, Brenneria quercina, S. alvi, Bifidobacterium* and Enterobacteriaceae. In agreement with culture based results [11], gram positive bacteria, primarily *L. kunkeei, F. fructosus* and *L. firm5* became more abundant with advanced disease in a site-specific manner (Fig. 3). In agreement with our findings, it was recently deduced that *A. euyridice*, implicated in past research, was most likely *Lactobacillus kunkeei* [55], an abundant species in many larval guts (Figs. 2 and 3), both healthy and diseased [40].

As part of our experimental design, sequencing microbial succession by larval age also resulted in multiple hypotheses for the role of the microbiota in EFB disease progression. All developing larval stages classified phenotypically as healthy and sampled from a spotty brood pattern in an EFB diseased hive have significantly different microbiomes containing significantly more *M. plutonius* than larvae from hives with no sign of disease (Fig. 3, Table S5). That every larvae in a diseased hive may have significantly elevated levels of *M. plutonius*, suggests that transmission is rampant within infected hives, distributed by the nursing activity and mouthparts. This may indicate that every larvae in a diseased hive is exposed to a virulent dose of *M. plutonius*, but some fight it off more effectively than others perhaps due to a protective microbiome structure or host genotype-specific immune response or both.

A microbiome of Idiopathic Brood Disease Syndrome

Idiopathic brood disease syndrome (IBDS) is associated with substantial colony loss and the cause is unknown according to present molecular tests and microscopy [27]. Larval phenotype has been unreliable as a diagnostic tool in the field and IBDS is typically confused with EFB. At the Hull apiary, larval disease was described by the apiary inspector as “melty, sunken and deflated”, descriptors distinguishing it from the EFB-only sites in this study (Table S1). At the Hull apiary, we found that the microbiome associated with IBDS-melty larval disease shared little to no resemblance with any of the EFB associated microbiomes (Figs. S1 and S2). At the Hull apiary, *M. plutonius* abundance and occurrence was exceedingly low, and did not differ between diseased vs. healthy phenotypes (Table S6). The IBDS disease microbiome showed significant increases in *S. alvi, G. apicola*, and *F. perrara*; all enteric bacteria specialized to inhabit the adult worker pylorus/ileum, and *Serratia marcesens*, a demonstrated pathogen of adult and larval honey bees [13,59]. In the five apiaries typified as *M. plutonius*-dominant EFB disease,

these same four hindgut bacteria occurred stochastically across treatments, and were represented by very low (incidental) abundance and prevalence (Tables S2 and S5). The IBDS phenotype also showed a significant decrease in *Bo. apis* compared to the healthy larval phenotype from the same hive (Table S6). Moreover, *Snodgassella alvi* and *Serratia marcesens* from the guts of IBDS larvae were strongly correlated following a log transformation of bacterial abundance suggesting synergistic co-existence (Fig. 4, Adj Rsq = 0.59, F = 31.1, $p < 0.0001$).

While the effects of *S. marcescens* in honey bees are becoming evident [10,13,60,61], this bacterium is a known secondary invader following viral and other infections, and can be abundant in Varroa mites and adult bees sampled from stressful overwintering conditions [61]. Many honey bee associated bacteria have been detected in parasites; the small hive beetle and Varroa mite including known pathogens *S. marcescens* and *E. faecalis*, and group living bacteria native to the honey bee worker ileum [62,63]. In a recent paper, *S. marcescens* is proposed as a widespread opportunistic pathogen of adult honey bees that may be often go undetected, but is highly virulent when the host is compromised [13]. Because symptoms of *Serratia* infection are not definitive, this bacterium is considered under-reported as a cause of bee losses. Given the high prevalence of *S. marcescens* in IBDS disease, it may serve as a bacterial marker of opportunistic disease.

According to the Apiary inspectors notes (Table S1), the IBDS diseased hive from Illinois was treated with Tetra Bee (oxytetracycline) a couple weeks prior to sampling but the disease state did not exhibit strong response. Also, both healthy and disease microbiomes from this location showed a near complete lack of gram positive bacteria, found somewhat uniformly across the five EFB apiaries (Fig. 3, Table S2). It appears that antibiotic application selectively diminished the gram positive species. In contrast, gram negative species known to carry antibiotic resistance genes were the only bacteria found blooming in the larvae including species core to the worker or queen ileum; *P. apium, S. alvi, G. apicola*, and *F. perrarra* (Table 2). The antibiotic resistance genes for tetracycline are largely found in the gram negative species [64] which may in part explain the microbiome composition of IBDS larvae in this study. This suggests a scenario wherein the putative causative agents or secondary invaders are resistant to oxytetracycline and the overtreatment with antibiotics interferes with the normal microbiome function of primarily gram positive bacteria, making the host more susceptible to *Serratia* infection. The mortality rate of bees infected with *S. marcescens* was previously shown to be much higher following exposure to the antibiotic tetracycline [65].

Table 2
Microbiome changes associated with IBDS (Hull apiary) in honey bee larvae.

Bacterial species	Change	P value
Parasaccharibacter apium	Decrease	< 0.0001
Frishella perrara	Increase	< 0.0001
Gilliamella apicola	Increase	< 0.001
Snodgrassella alvi	Increase	< 0.0001
Serrtia marcescens	Increase	< 0.0001
*Wilcoxon tests comparing absolute abundance in healthy vs. diseased larvae		

A suite of genes associated with *Serratia maercescens* strain *sicaria* (SS1) isolated from honey bees suggests a range of adaptation to the honey bee system not seen in other *Serratia* strains [61]. Interestingly, this strain is adapted for survival in the honey bee system (SS1), but does not produce genes involved in iron regulation or siderophores, which may explain the significant association with *S. alvi* as detected in the Hull apiary microbiome survey (Fig. 4). Iron can be scarce in host associated gut environments, but *S. alvi* possesses multiple systems for iron uptake and synthesizes siderophores [66]. Other bacteria in the community can “cheat” and take up siderophores they did not produce. Thus a potential metabolic explanation for co-infection of *S. marcescens* with *S. alvi* includes siderophore production and iron acquisition genes present in *S. alvi* but absent in *Serratia marcescens* and the ability of *S. alvi* to use bacterial waste products for growth. Bacteria lacking siderophore production are less virulent [67], but within a local community, the presence of siderophores can benefit all bacteria, and in this case may lead to virulence. Moreover, *Serratia marcescens* strain SSI has lost motility (flagellar genes), a trait typically seen in obligate endosymbionts and intracellullar pathogens because flagella production is costly. If transmission of SS1 is provided by the social hive context (social grooming or parasite transmission) flagella may be unnecessary.

Although not quantified by this survey, detailed photographs suggests high mite loads associated with the Hull apiary, suggestive of parasitic mite syndrome (PMS), another IBDS disease state described as a complex of symptoms associated with *Varroa* mite infestation, viruses, or a combination of both. Presently, the only verified viral infection of honey bee larvae is sacbrood [68]. We did not test for sacbrood virus because it is reliably distinguished by larval morphology, as larvae sit up in the middle of their cells with their heads raised, then become fluid filled sacs as the disease progresses. Future investigations of IBDS in honey bees should apply an approach that allows multiplexed detection of established and novel virus [69].

Declarations

Data availability

Next gene sequencing libraries were deposited in GenBank under Sequence Read Archive (SRA) accession PRJNA810064.

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Contributions

K.E.A. designed the experiment, analyzed the data, and wrote the paper. P.M. and A.S.F. performed the experiment, analyzed the data, and approved the final manuscript. B.M.M. and D.C.C. analyzed data, and approved the final manuscript.

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Ethics declarations

Competing interests

The authors declare no competing interests.

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Figures

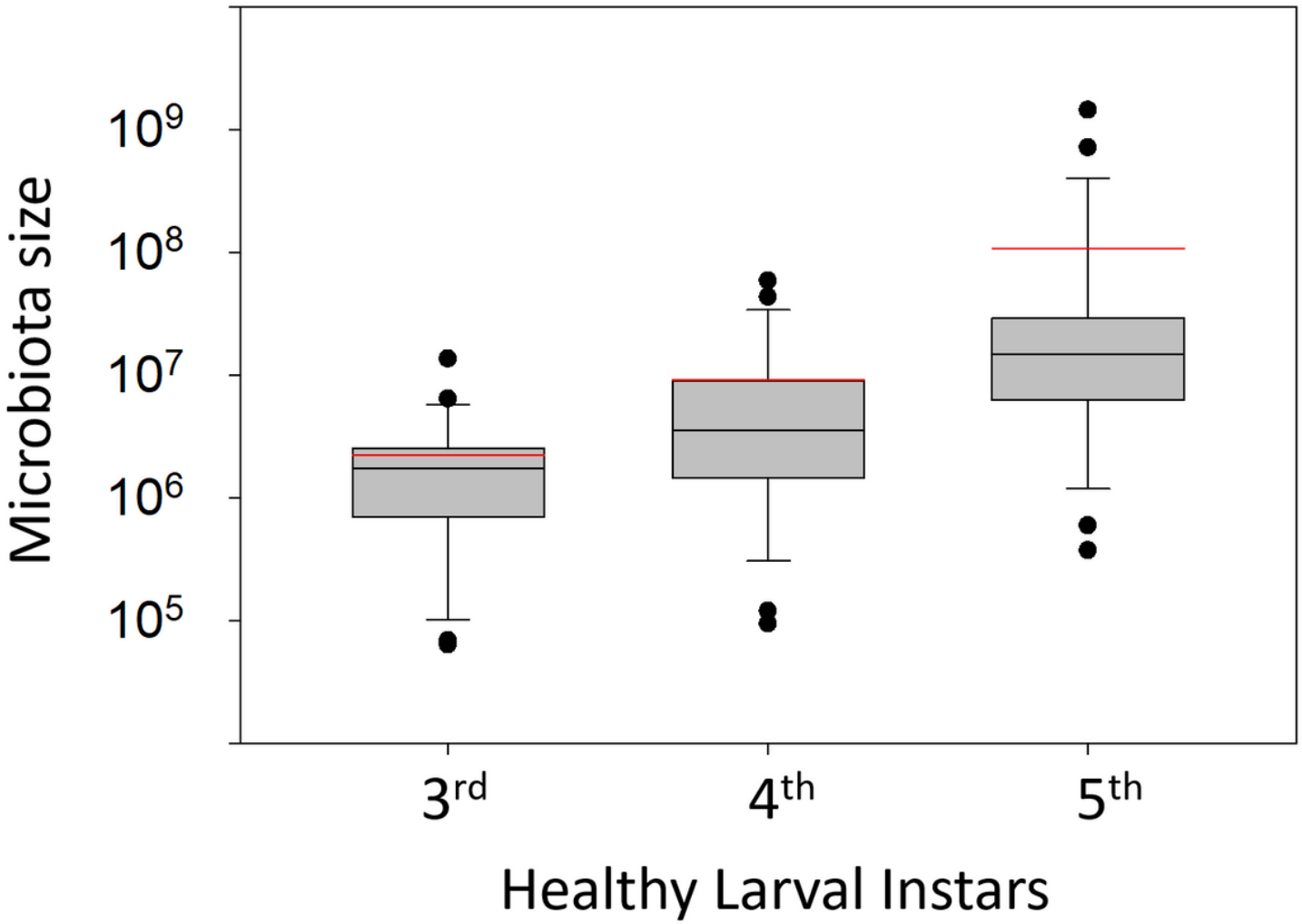

Figure 1

Microbiota size of healthy larval instars as determined by qPCR. Box plots contain 75% of the variation, whiskers 90%, and the dots are outliers. The red horizontal line is the mean, black the median.

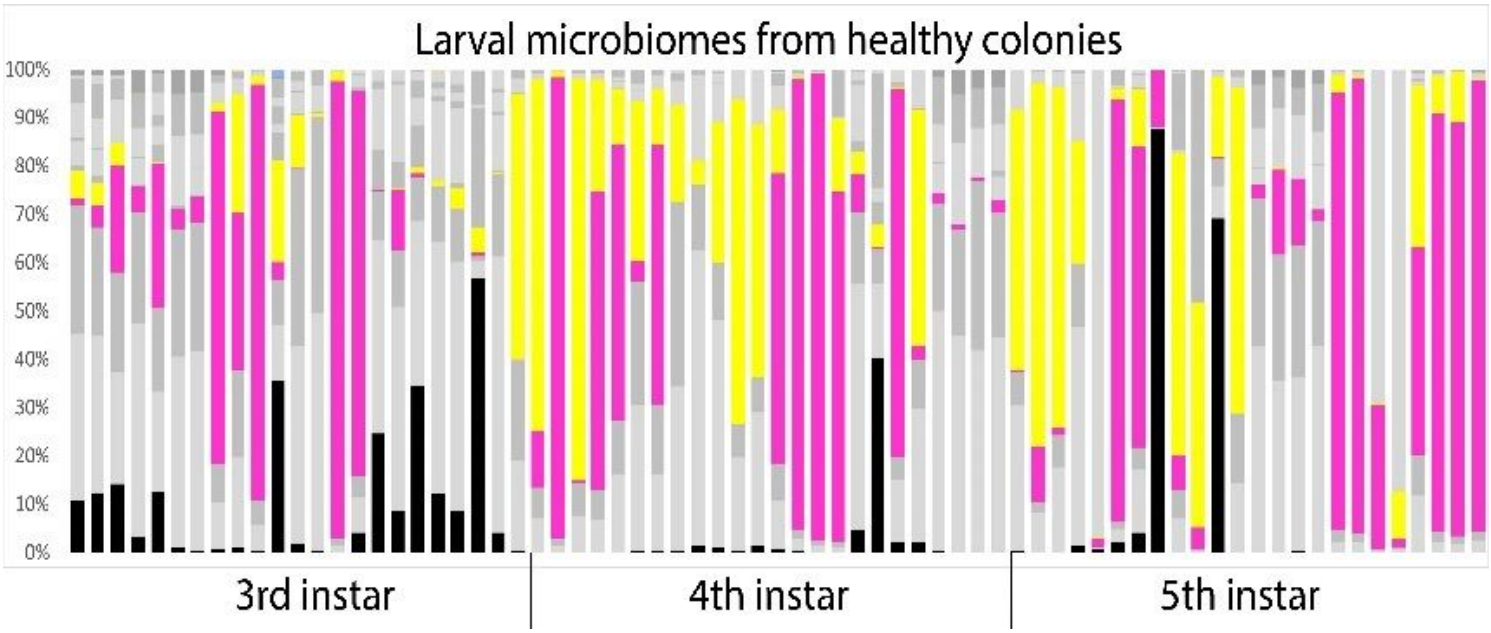

Figure 2

Larval microbiomes associated with healthy phenotypes. Results from five apiaries (sites) sampling phenotypically healthy larvae from healthy hives, Yellow is *L. kunkeei*, pink is *P. apium*, and black is *M. plutonius*.

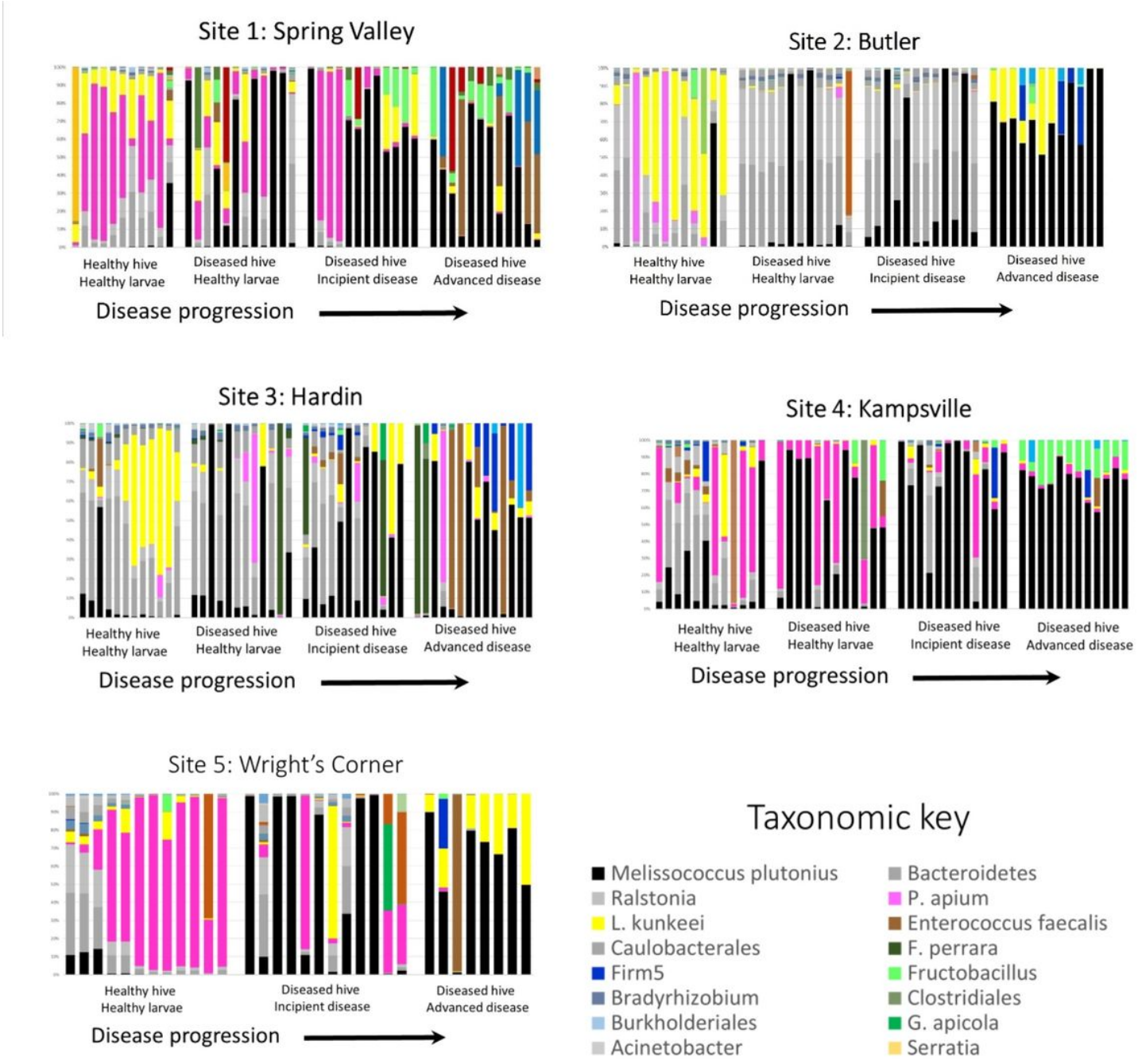

Figure 3

Larval microbiomes associated with the progression of EFB disease at each site molecularly diagnosed as EFB disease. Results from five apiaries (sites) sampling healthy hives, healthy larval phenotypes in diseased hives, and incipient and advanced EFB symptoms from the same hive.

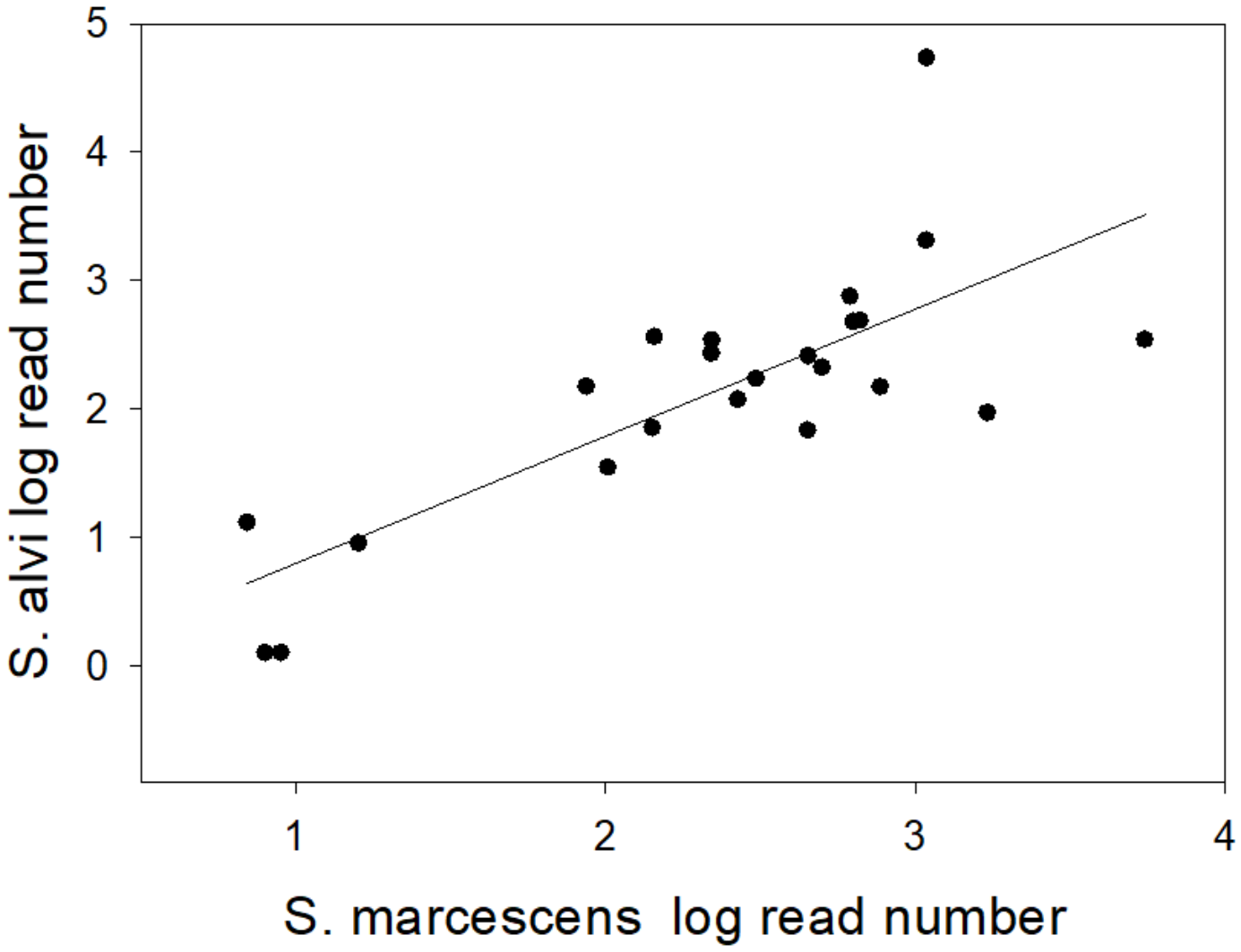

Figure 4

Correlation between *S. marcescens* and *S. alvi* in larvae with IBDS (Hull Apiary).

Supplementary Files

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