Early gut colonizers shape parasite susceptibility and microbiota composition in honey bee workers

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Microbial symbionts living within animal guts are largely composed of resident bacterial species, forming communities that often provide benefits to the host. Gut microbiomes of adult honey bees (*Apis mellifera*) include core residents such as the betaproteobacterium *Snodgrassella alvi*, alongside transient parasites such as the protozoan *Lotmaria passim*. To test how these species affect microbiome composition and host physiology, we administered *S. alvi* and/or *L. passim* inocula to newly emerged worker bees from four genetic backgrounds (G_H) and reared them in normal (within hives) or stressed (protein-deficient, asocial) conditions. Microbiota acquired by normal bees were abundant but quantitatively differed across treatments, indicating treatment-associated dysbiosis. Pretreatment with *S. alvi* made normal bees more susceptible to *L. passim* and altered developmental and detoxification gene expression. Stressed bees were more susceptible to *L. passim* and were depauperate in core microbiota, yet supplementation with *S. alvi* did not alter this susceptibility. Microbiomes were generally more variable by G_H in stressed bees, which also showed opposing and comparatively reduced modulation of gene expression responses to treatments compared with normal bees. These data provide experimental support for a link between altered gut microbiota and increased parasite and pathogen prevalence, as observed from honey bee colony collapse disorder.

cytochrome P450 | dysbiosis | microbial interaction | parasitism | vitellogenin

Animal guts contain diverse microbial communities that are often dominated by suites of bacteria but can also include archaea, viruses, protozoans, and fungi (1). Consistently present, or core, bacterial species are often beneficial to their host and are thought to be integral to animal evolution by influencing host growth, development, health, and behavior (2–7). Disrupted proportions among core bacterial communities can alter microbiome homeostasis, causing "dysbiosis" (1, 8, 9) that may alter host interactions. For example, shifts in bacterial population sizes in the mammalian gut have been linked to inflammatory gut disorders, diabetes, and obesity (8). Thus, causes of and consequences from dysbiosis are key to understanding animal health and disease. Whereas the diverse gut communities of mammals contain functional redundancy that may buffer shifts in composition (7, 10), insects usually have much lower microbiota diversity (4, 6, 9, 11) and consequently may be more affected by dysbiosis.

European honey bees, *Apis mellifera*, are globally significant to agriculture primarily due to their pollination services and honey production. As social insects, they form colonies with tens of thousands of mostly nonreproductive female workers that progressively perform a variety of tasks required for colony maintenance and growth as they age. This division of labor is centrally regulated by the expression of *vitellogenin* (*Vg*), a gene under strong positive selection in workers (12, 13) encoding a glycolipoprotein that affects development, lifespan, and immunity (14–18) and serves as a key biomarker for bee health and fitness (13, 19–21).

Gut bacteria acquired by honey bees as larvae are purged during pupation, and new communities are established following adult emergence through contact with nestmates and/or nest surfaces (4, 22–25). Eight bacterial symbionts typify the core adult honey bee gut microbiota, including two abundant Proteobacteria, *Snodgrassella alvi* (Betaproteobacteria) and *Gilliamella apicola* (Gammaproteobacteria), as well as two clusters of Firmicutes within the genus *Lactobacillus* (26–29). Functional data from some *Lactobacillus* species (30, 31) and from the alphaproteobacterium *Parasaccharibacter apium* (32, 33) support beneficial roles to larval survival and parasite resistance. However, data from a strain of *Frischella perrara* (Gammaproteobacteria) is associated with melanization in the bee gut epithelium (34), a response generally associated with host defense against potential pathogens. Roles for *S. alvi* and *G. apicola* are not clearly demonstrated, although nutritional and defensive roles have been hypothesized on the basis of genomic sequence data (35). Studies of bumble bee gut microbiota, which similarly include *S. alvi* and *G. apicola*, suggest a protective role against trypanosomatid protozoan parasites (36, 37). Related trypanosomatids can be common in honey bee guts (e.g., refs. 28 and 38–43) and have been linked to colony mortalities (39, 40, 44). Other potentially harmful microbes also occur transiently in bee guts and likely interact with other members of the community (45).

Advancing animal health management requires understanding the interplay among stressors, microbiomes, and host genetics. For example, ongoing problems with honey bee health (46–49) are attributed to multiple stress factors including parasites and pathogens, pesticides, suboptimal nutrition, and stress associated with commercial management. Genetic diversity of bee stocks can influence their adaptability to stressors (e.g., refs. 50–53); these effects may involve key metabolic and detoxification genes, such as cytochrome P450s (54–56). Their gut microbiota also likely mediate stress responses, and bacterial strains cultivated from honey bees could be harnessed as probiotic mitigators of stress (30–33, 57).

Significance

Using a social insect model, we tested how supplementing young adult bees with a resident microbiota species affects host physiology and microbiome composition. This supplementation had significant consequences for host development and detoxification responses, parasite susceptibility, and microbiome community structure. Our results show that early perturbation of the microbiota composition can have sustained consequences for hosts. Additionally, this work provides a cautionary tale to the arbitrary use of probiotics in animal health management and highlights the importance of experimental research addressing factors that shape animal microbiome communities.

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Here, we used honey bees as a comparatively simple animal model to address the roles of microbial species in gut microbiome homeostasis and in host physiology. We explored how early colonization with a core bacterial symbiont, *S. alvi*, and a common parasite, *Lotmaria passim*, affected microbiome composition and host gene expression. We administered *S. alvi* and/or *L. passim* to newly emerged worker bees from four genetic backgrounds. We tested the prediction that gut colonization by *S. alvi* renders the host less susceptible to parasitism by *L. passim* during "normal" (hive-reared) and "stressed" (laboratory-reared, asocial, protein-deficient) conditions. Further, we tested for impacts of these treatments on host physiology by quantifying expression of *vitellogenin* and three cytochrome P450 (CYP450) genes, which are known central mediators of development and detoxification capabilities (14–18, 54–56).

Results

Newly emerged adults were inoculated with *S. alvi*, *L. passim*, both, or neither, and were then reared in either a normal hive environment ("normal") or in a nutritionally and socially stressful and largely sterile laboratory environment ("stressed"). At 6 d post-emergence, bees were harvested to examine effects on microbiota and expression of key bee genes used as indicators of stress responses and health.

Impacts on the Bacterial Microbiome. Bees with normal rearing developed high loads of *S. alvi* (Fig. 1A) whether or not they received experimental *S. alvi* inocula, as expected via natural social acquisition. Highest loads occurred in bees treated with both *S. alvi* + *L. passim* (S+L), larger than untreated controls (C) and *L. passim*-only (L) bees (one-way ANOVA, $P < 0.0001$ for both) and larger than direct comparison with bees treated with *S. alvi*

only (S) (Mann–Whitney U test, $P = 0.0094$; C, 17.71 ± 0.24 SEM; L, 17.31 ± 0.43 SEM; S, 18.34 ± 0.18 SEM; and S+L, 18.92 ± 0.18 SEM). Loads of *S. alvi* did not vary by host genotype (G_H) (Fig. S1A; one-way and two-way ANOVA, $P > 0.05$).

Under stressed conditions, bees experimentally inoculated with *S. alvi* developed distinctly larger mean *S. alvi* loads (Fig. 1B) than were found in bees not experimentally inoculated (S, 24.11 ± 0.22 SEM and S+L, 23.92 ± 0.2 SEM vs. C, 7.62 ± 1.55 SEM and L, 7.57 ± 1.27 SEM; one-way ANOVA, $P < 0.001$ for both). The G_H-associated variation in these experimentally administered *S. alvi* loads was significant (Fig. S1B; one-way ANOVA; S, $P = 0.0176$ and S+L, $P = 0.0003$) and was independent of *L. passim* challenge (two-way ANOVA, $P > 0.05$ for all comparisons). Whereas most (72%) stressed bees not experimentally inoculated with *S. alvi* had low (below the mean, $n = 35/96$) or no ($n = 34/96$) *S. alvi*, the remainder (28%) had levels that resembled those in experimentally inoculated bees (C, $n = 15$ and L, $n = 12$). These incidental *S. alvi* populations were likely acquired at emergence from brood cell caps contaminated with *S. alvi*. Such acquisition did not vary by G_H (one-way ANOVA, $P > 0.05$ for both C and L).

A second core gut bacterium, *G. apicola*, was naturally acquired in the hive by normal bees (99% overall; Fig. 1A). Whereas *G. apicola* loads in bees from the *S. alvi*-only treatment did not differ from those in control bees, a significant buildup occurred in bees that received *L. passim* alone or following *S. alvi* inoculation (one-way ANOVA, $P < 0.001$; C, 14.09 ± 0.482 SEM; L, 17.54 ± 0.697 SEM; S, 16.16 ± 0.477 SEM; and S+L, 20.56 ± 0.558). These *G. apicola* loads did not vary by G_H (Fig. S1A, one-way ANOVA, $P > 0.05$ for all). *G. apicola* also occurred in stressed bees, but at a lower rate compared with normal bees (Fig. 1B; 80% overall, Fisher's exact test $P < 0.0001$). Stressed bees treated with *S. alvi* + *L. passim* developed the largest *G. apicola* loads (S+L, 16.53 ± 1.52 SEM vs.
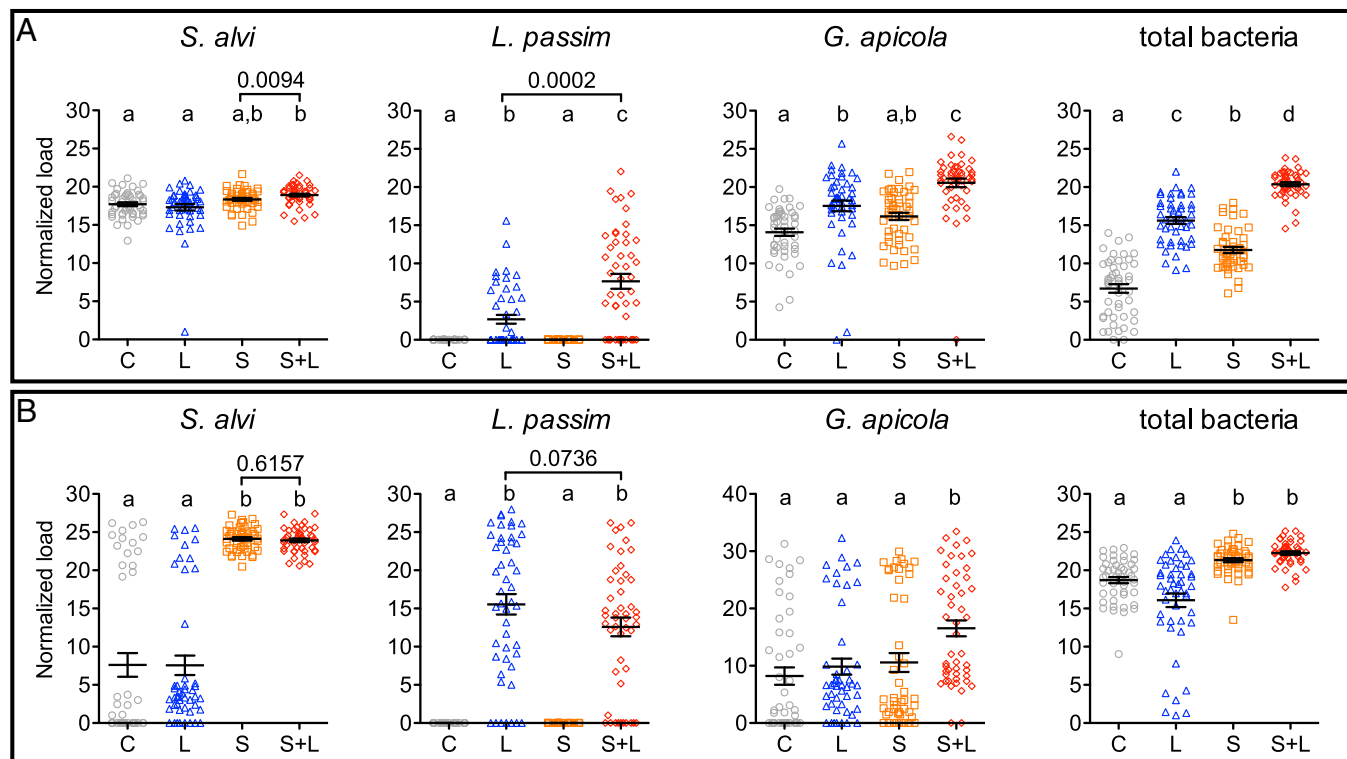

Fig. 1. Microbial loads in bees during normal (A) and stressed (B) rearing. Means ± SEM are shown by the black bars and whiskers. Treatments ($n = 48$ each): sugar water control (C, gray circles); *L. passim* only (L, blue triangles); *S. alvi* only (S, orange squares); and *S. alvi* and *L. passim* (S+L, red diamonds). Different letters above treatment groups indicate significantly different loads (one-way ANOVA with Dunn's multiple comparison tests). *P* values above brackets are from planned comparisons using two-tailed Mann–Whitney U tests. Data presented by each of four colonies in Fig. S1.

C, 8.20 ± 1.52 SEM; L, 9.85 ± 1.40 SEM; and S, 10.57 ± 1.65 SEM; one-way ANOVA, $P < 0.0001$), although this was highly influenced by G_H (Fig. S1B; one-way ANOVA, $P = 0.0210$).

Assessment of total bacterial numbers using universal bacterial primers showed that each microbial treatment quantitatively altered the honey bee microbiota in bees reared normally (Fig. 1A; C, 6.72 ± 0.563 SEM; L, 15.61 ± 0.438 SEM; S, 11.76 ± 0.392 SEM; and S+L, 20.37 ± 0.261 SEM; one-way ANOVA, $P < 0.01$ to < 0.001). Highest total bacterial loads occurred in *S. alvi* + *L. passim*-treated bees, which corroborated results observed in this treatment for *S. alvi* loads and *G. apicola* loads. In contrast, total bacterial microbiota loads from stressed bees (Fig. 1B) showed variation that corresponded to the presence/absence of *S. alvi* inoculation (S, 21.33 ± 0.263 SEM and S+L, 22.26 ± 0.226 SEM vs. C, 18.71 ± 0.399 SEM and L, 16.08 ± 0.888 SEM; one-way ANOVA, $P < 0.001$ for all comparisons). In addition, G_H influenced the total bacterial loads in normal bees treated with *S. alvi* + *L. passim* (Fig. S1A; one-way ANOVA, $P = 0.0332$) and in stressed bees treated with *L. passim* only and *S. alvi* + *L. passim* (Fig. S1B; one-way ANOVA, $P = 0.0053$ and $P = 0.0056$).

Impacts on *L. passim*. Only bees experimentally inoculated with *L. passim* had detectable levels of this parasite, whether reared normally (Fig. 1A) or under stress (Fig. 1B). Stressed bees were more susceptible to *L. passim* infection than bees reared under normal conditions (Fig. 2), with overall infection rates of 82.3% vs. 54.2% ($n = 96$ each; Fisher's exact test of independence, $P < 0.0001$). Infection rates were lower in *L. passim*-only-treated bees reared under normal vs. stressed conditions (116% lower; Fisher's exact test, $P < 0.0001$), whereas the rate difference in *S. alvi*- pretreated bees was not significant. Unexpectedly, normally reared bees pretreated with *S. alvi* had higher *L. passim* loads (Fig. 1A; 185% higher; Mann–Whitney U test, $P = 0.0002$) and infection rates (Fig. 2; 74% higher; Fisher's exact test, $P = 0.0074$) in a G_H-dependent manner (Fig. S1A; one-way ANOVA, $P = 0.0178$).

Pretreatment with *S. alvi* did not affect susceptibility to *L. passim* in stressed bees ($P > 0.05$ for loads, rates, and G_H). However, bees from one colony (058) had a significant 44% reduction in *L. passim* loads when they were pretreated with *S. alvi* (Fig. S1B; two-way ANOVA, $P < 0.01$), supporting a G_H-dependent dynamic with *S. alvi* during stress. In addition, *L. passim* loads varied by G_H when bees were not pretreated with *S. alvi* (one-way ANOVA, $P = 0.0005$), whereas *S. alvi* pretreatment abrogated this association ($P > 0.05$).
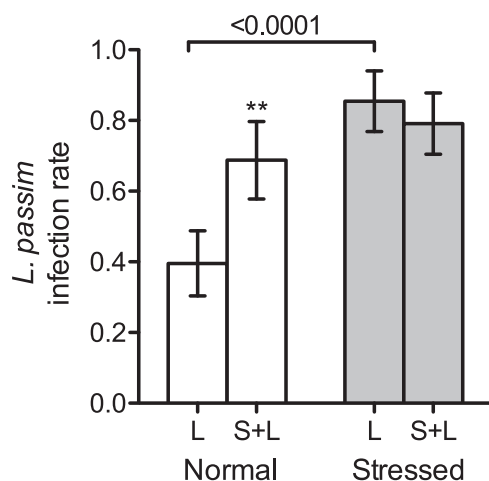

Fig. 2. Box plots of *L. passim* infection rates from bees with normal and stressed rearing. Bar height indicates the mean ($n = 48$) with SEM (error bars). **Significant difference between *L. passim*-challenged bees with *S. alvi* (S+L) vs. without (L); $P = 0.0074$, Fisher's exact test of independence.

Host Gene Expression Responses and Correlations with Microbial Loads. Treatment impacts on host physiology were measured by quantifying the expression of development mediators (*Vg* and *Cyp306A1*) and detoxification mediators (*Cyp4g11* and *Cyp6AS7*). Suppressed *Vg* expression occurred in bees treated with *S. alvi* + *L. passim* under normal conditions (Fig. 3A). Although these bees showed suppressed *Vg* expression overall, individuals that had higher *L. passim* loads had higher *Vg* expression ($P = 0.0474$, $r = 0.2877$) and had higher *Cyp306A1* expression ($P = 0.0480$, $r = 0.2870$) (Fig. 4A; Spearman correlation tests). In contrast, stressed bees had no significant treatment-dependent changes in *Vg* expression overall, but G_H-dependent induction was detected in the *L. passim*-only treatment (Fig. 3B). There were no significant correlations between *Vg* and microbial loads in stressed bees.

Overall, each CYP450 gene was significantly affected by microbial treatments, showing suppression under normal conditions (Fig. 3A) and induction under stressful conditions (Fig. 3B). Bees treated with *L. passim* only and reared in normal conditions suppressed all CYP450 genes, whereas those reared in stressed conditions induced all CYP450 genes, detectable overall and at G_H. Individual expression levels of *Cyp4g11* from bees treated with *L. passim* and reared in stressed conditions were positively correlated with total bacteria load (Fig. 4B; $P = 0.0352$, $r = 0.3047$).

Bees treated with *S. alvi* alone suppressed *Cyp6AS7* and *Cyp306A1* expression under normal conditions. There was no overall change in CYP450 expression in stressed bees treated with *S. alvi*, but G_H-dependent induction of *Cyp6AS7* was detectable (Fig. 3B) and larger *S. alvi* loads were positively correlated with *Cyp306A1* expression (Fig. 4B; $P = 0.0253$, $r = 0.3262$). Combined *S. alvi* + *L. passim* treatment reduced *Cyp6AS7* and *Cyp4g11* expression under normal conditions and induced *Cyp6AS7* when stressed.

Discussion

Dysbiosis from *S. alvi* Supplementation and from Stress Increases Susceptibility to Parasitism. Given that *S. alvi* is a core microbiota species in both honey bees and bumble bees (29, 35, 58) and that trypanosomatids are common bee parasites (e.g. refs. 28, 38–43, and 59–61), this study provides important insight into the functional roles of prominent microbes in key pollinator species. Experimental supplementation with *S. alvi* before challenge with *L. passim* led to measurable perturbation, or dysbiosis, of the core microbiota (i.e., *S. alvi*, *G. apicola*, and total bacteria) and, contrary to our hypothesis, increased susceptibility to parasitism by *L. passim* despite normal rearing conditions within colonies. Presumably, the microbiota acquired naturally in colonies without prior application of *S. alvi* result in a community that more effectively limits this parasite. These findings offer experimental support linking parasite susceptibility to dysbiosis of the core microbiota, a proposed "biomarker" of colony collapse disorder (CCD) (39). Metagenomic analysis of bee colonies diagnosed with CCD, an enigmatic rapid decline of honey bee colonies, showed "a strong and consistent pattern" of increased Gammaproteobacteria (i.e., *G. apicola* and/or *F. perrara*), Betaproteobacteria (i.e., *S. alvi*), and Firmicutes (i.e., *Lactobacillus* spp.) along with a decreased relative abundance of Alphaproteobacteria and Actinomycetes (i.e., *Bifidobacteria* spp.) compared with healthy bee colonies (39). This dysbiosis in CCD colonies was also associated with an overall increase in parasite and pathogen loads, including increased trypanosomatid abundance. Preliminary analysis of CCD bee colonies (28) noted a similar but nonsignificant dysbiosis with a "trend toward increased abundance" of *G. apicola* (i.e., "Gamma-1").

All microbial treatments we administered to bees reared normally in hives were associated with a disproportionally large buildup of bacteria. Microbiota buildup in response to *S. alvi* might indicate that this species facilitates colonization by other microbiota species, as proposed elsewhere (24, 62). Microbiota buildup in response to *L. passim* may be a protective measure against the parasite, because competitive exclusion of pathogens
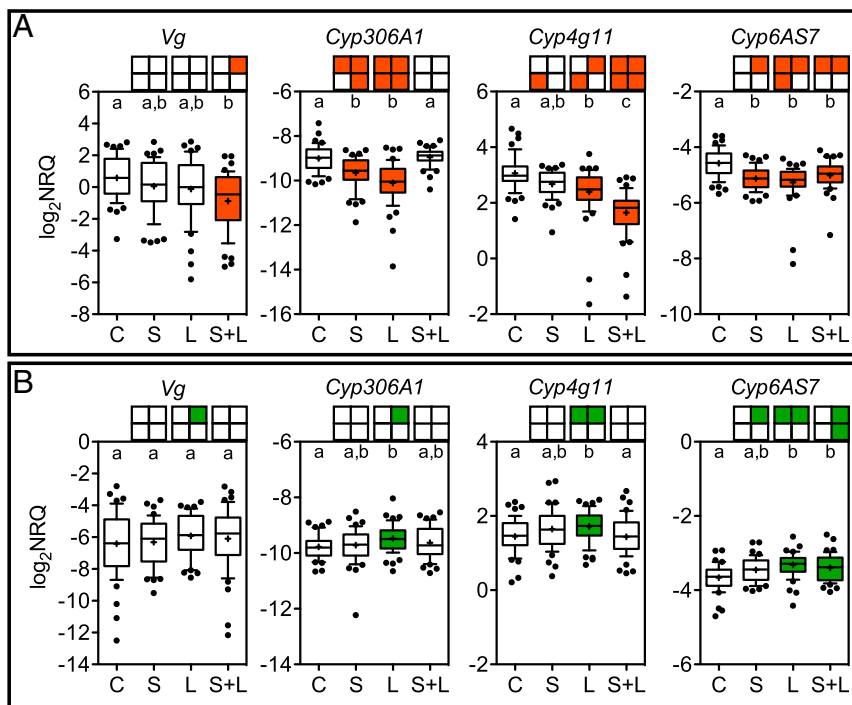
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Fig. 3. Gene expression in bees ($n = 48$ each) reared under normal (*A*) or stressed (*B*) conditions. Boxes above each plot summarize G_H data by quadrant: upper left, colony 58; upper right, colony 66; lower left, colony 68; and lower right, colony 106. Significant changes compared with control are shaded: green, induced and red, suppressed. Groups that do not share a letter are significantly different ($P < 0.05$; Kruskal–Wallis with Dunn's multiple comparison tests). Data are presented after reference gene normalization and primer efficiency correction. Whiskers span the 10th–90th percentile. Outliers are shown as dots. + indicates expression mean. Treatments: *S. alvi* (S), *L. passim* (L), both *S. alvi* and *L. passim* (S+L), and sugar water control (C).

is a repeatedly identified role of animal microbiota communities (3, 6). Alternatively, because *L. passim* was more abundant in bees with greatest total bacterial biomass, this may be indicative that *L. passim* benefits from the gut microbiota in some way, perhaps via provisioned nutrients.

Stress was another factor, in addition to dysbiosis, that affected host susceptibility to parasitism. Stressed bees reared with poor nutrition (no protein), asocial context, and lack of exposure to microbiota from the hive were much more susceptible to

Fig. 4. Significant correlations between microbial loads and host gene expression during normal (*A*) and stressed (*B*) rearing. Treatment groups are given above each graph. Best-fit lines are plotted and *P* values are from two-tailed Spearman nonparametric correlation tests. Data presented are normalized to internal references. One outlier was removed from this dataset (*); removal made the correlation less significant (shown).

L. passim (infection rate and load). Young bees normally consume both protein from pollen and carbohydrates from honey during their early adult development. In addition, bees receive social cues (pheromones) in the hive from the queen, other adults, and larvae that affect worker behavior and physiology (63). Unlike normal conditions, the high susceptibility of stressed bees to *L. passim* was not altered when their guts were precolonized with *S. alvi*. The comparatively depauperate microbiota associated with higher *L. passim* susceptibility in stressed bees suggests that specific and stable proportions of core gut communities are likely important to successfully control this parasite.

These data provide experimental support for significant host genotype-dependent interactions between bacterial microbiota and eukaryotic parasites. Such dynamics are not unprecedented; experimental manipulation of microbiota has been shown to affect eukaryotic gut parasite population sizes in other insect models, such as populations of the apicomplexan parasite *Plasmodium* in mosquito hosts (64). In bumble bees, correlations between the size and composition of core gut microbiota and trypanosomatid populations have been observed (36, 37), as well as host genotype-dependent variation to trypanosomatid susceptibility (65, 66). Our present results unite the relevance of host microbiota composition, genetic background, and stress as important mediators of parasite susceptibility and insect health.

Gut Microbiome Changes Affect Host Developmental Pathways. Our analyses suggest an effect on host development from both *S. alvi* pretreatment and *L. passim* infection. Worker bees take on various social roles as they age, first within the hive then culminating in a final role as forager for food and other resources outside the hive. Transitioning to these different roles is associated in part with waning levels of vitellogenin, a central hormone of a developmental signaling network in bees (14, 16–18, 20). *Cyp306A1* encodes an essential regulator of another hormone in this network, ecdysteroid (55), which can control the production of vitellogenin. Although we found that *Vg* was suppressed overall in normally reared bees treated with both *S. alvi* and *L. passim*, individual analyses showed that bees with larger populations of *L. passim* had stronger *Vg* expression. The
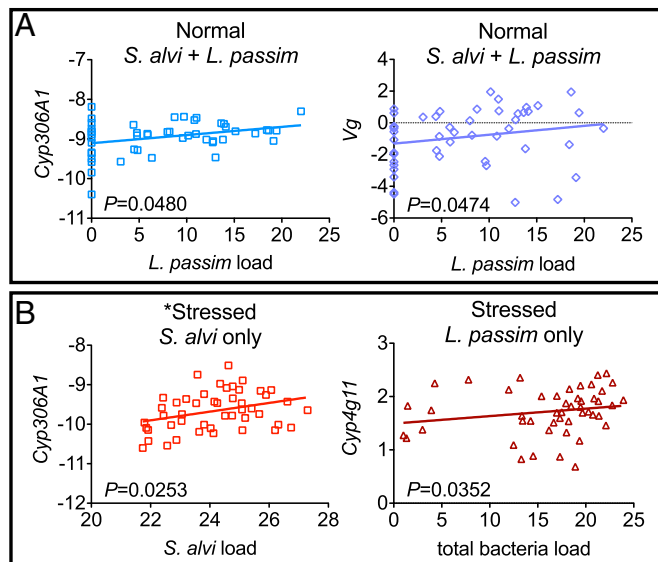
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same positive correlation was observed for *Cyp306A1* expression in these bees. Thus, dysbiosis made bees more susceptible to *L. passim*, and host developmental pathways were altered according to parasite load.

Elevated *Vg* expression corresponds with a slower onset to the forager stage, preferential foraging for pollen vs. nectar, and longer lifespan (14, 16–18, 20). As such, elevated *Vg* expression may increase *L. passim* transmission in the colony by extending potential contact time between infected and naïve individuals. In contrast, the reduced *Vg* expression we observed in normally reared worker bees inoculated with both *S. alvi* and *L. passim* corresponds with precocious development, preferential foraging for nectar, and shortened lifespan. Suppression of vitellogenin in bees parasitized by *Varroa destructor* mites (ectoparasites) and *Nosema ceranae* microsporidians (endoparasites) has been reported (19, 67). This phenomenon may be a defensive response to parasitism in social insects causing parasitized individuals to leave the colony sooner and die sooner, predictively mitigating the spread of parasites to other colony members. Our data support this theory and provide the additional observation that *L. passim* may reverse this response in highly infected individuals, enabling larger parasite population sizes and extended transmission potential to new hosts. We note that in the previously mentioned studies linking parasitism to reduced vitellogenin production bees had access to dietary protein. The absence of protein from the diet of stressed bees in our study likely compromised vitellogenin production and may explain the overall invariance in *Vg* from this treatment group.

S. alvi supplementation and its subsequent population size were positively correlated with *Cyp306A1* expression under stressed conditions. This suggests a potentially important role for *S. alvi* in developmental pathways when bees experience poor nutrition or have depauperate microbiota.

Microbiome Composition Affects Host Detoxification Responses. We determined that both normal and stressed bees treated with *S. alvi* and *L. passim* showed altered CYP450 gene expression. CYP450s comprise a large superfamily of metabolic enzymes with demonstrated or predicted roles in developmental hormone synthesis (i.e., *Cyp306A1* discussed in the prior section) and xenobiotic detoxification (i.e., *Cyp4g11* and *Cyp6AS7*) (54, 56). Because these genes can be highly expressed and locally specific in the adult insect gut (68), the proteins they encode are potentially significant players connecting gut microbial communities with the host. We found that *Cyp4g11* expression was altered only in bees treated with *L. passim* whereas *Cyp6AS7* was altered in bees from all microbial treatment groups. Stressed conditions dramatically reversed the regulation patterns of these genes.

As part of the CYP3 clade, *Cyp6AS7* is among the most diverse group of CYP450 genes in honey bees (54, 56), but no other experimental data are available for comparison. *Cyp4g11* is part of the CYP4 clan, which has the fewest genes in the honey bee CYP450 superfamily (54, 56). It is not known to be responsive to insecticides (56, 69) but is responsive to parasitization by *V. destructor* (70, 71) and is suppressed in bee guts from CCD colonies (72). It also has potential roles in sensory perception during foraging (73). Our data linking altered *Cyp4g11* expression to trypanosomatid infection provide a plausible explanation for modified foraging behavior observed in trypanosomatid-infected bumble bees (74).

Conclusion. In general, bees are capable of regulating a commonly encountered trypanosomatid gut parasite when provided with adequate nutrition and their normal bacterial microbiota, typically acquired in the hive. Bees dramatically lose their ability to control this parasite, however, when in a state of dysbiosis or when nutritionally and socially stressed. In our experiments, dysbiosis resulted from an early skew in the microbiota composition of young adult bees; application of antibiotics to colonies might also be expected to result in dysbiosis. These findings provide experimental support of past observations linking dysbiosis and high parasite loads in bee colony collapses. Using a core microbiota species from honey bees, *S. alvi*, we show that probiotic therapy is not always beneficial and has complicated consequences for parasite susceptibility, microbiota homeostasis, and host developmental and detoxification response pathways. This work does not refute the potentially beneficial nature of this core gut symbiont, but it does show that the succession and ratio of individual microbiota species is critical to the future structure and functioning of the microbiota as a whole. Because multiple strains of *S. alvi* and *L. passim* occur, it is possible that other strains will have different effects.

Methods

Adult worker bees were removed from four healthy (capped and uncapped brood with normal brood pattern, bee bread and honey stores, queen-right) colonies of *A. mellifera ligustica* US domestic hybrids upon emergence (*n* = 960) then separated into four treatment groups (*n* = 480 each): (*i*) 5 μL of 1:1 sugar water, (*ii*) *S. alvi* only (250,000 cfu, strain wkB2[T]), (*iii*) *L. passim* only (10,000 promastigotes, strain BRL), or (*iv*) *S. alvi* + *L. passim*, and color-coded accordingly. Bees were inoculated with sugar water (treatment *i*) or *S. alvi* (treatments *ii* and *iv*) within 8 h of emergence and with *L. passim* (treatments *iii* and *iv*) 24 h later. Half of the bees from each colony in each treatment group were returned to their parental colonies 12 h later ("normal rearing") and the remaining half were isolated according to treatment group within cages held at normal colony temperature (32 °C) within an incubator and maintained on sugar water/no protein diet ("stressed rearing"). Total RNA was extracted individually at 6 d postemergence and 10 μg was converted to first-strand cDNA for use as template to quantify microbes and honey bee genes with primers (Table S1) via quantitative PCR (qPCR) (24, 25, 75, 76). All qPCR data were normalized to empirically determined stable reference genes (Fig. S2) (77–80). Further details are available in *SI Methods*.

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