Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling

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Social bees harbor a simple and specialized microbiota that is spatially organized into different gut compartments. Recent results on the potential involvement of bee gut communities in pathogen protection and nutritional function have drawn attention to the impact of the microbiota on bee health. However, the contributions of gut microbiota to host physiology have yet to be investigated. Here we show that the gut microbiota promotes weight gain of both whole body and the gut in individual honey bees. This effect is likely mediated by changes in host vitellogenin, insulin signaling, and gustatory response. We found that microbial metabolism markedly reduces gut pH and redox potential through the production of short-chain fatty acids and that the bacteria adjacent to the gut wall form an oxygen gradient within the intestine. The short-chain fatty acid profile contributed by dominant gut species was confirmed in vitro. Furthermore, metabolomic analyses revealed that the gut community has striking impacts on the metabolic profiles of the gut compartments and the hemolymph, suggesting that gut bacteria degrade plant polymers from pollen and that the resulting metabolites contribute to host nutrition. Our results demonstrate how microbial metabolism affects bee growth, hormonal signaling, behavior, and gut physicochemical conditions. These findings indicate that the bee gut microbiota has basic roles similar to those found in some other animals and thus provides a model in studies of host–microbe interactions.

honeybee | gut microbiota | short-chain fatty acids | insulin | metabolomics

Honey bees (*Apis mellifera*) provide a critical link in global food production as pollinators of agricultural crops (1), and their economic value is over $15 billion annually in the United States alone (2). Honey bee populations have undergone elevated colony mortality during the last decade in the United States, Canada, and Europe (3). A potential role of gut microbial communities in the health of honey bees has recently become more widely appreciated (4). Perturbation of the gut microbiota leads to higher mortality within hives and greater susceptibility to a bacterial pathogen, suggesting a crucial role of normal microbiota in bee health (5). Honey bees are associated with specific intestinal microbiota that is simpler than the microbiota found in mammals but shares some features, including host specificity and social transmission (6) and a shared evolutionary history of bacterial and host lineages (7, 8). The bee gut is dominated by eight core bacterial species that are spatially organized within specific gut regions. Few bacteria colonize the crop and midgut; the hindgut (ileum and rectum) harbors the greatest abundance of bacteria. The ileum, a narrow tube with six longitudinal folds, is dominated by two Gram-negative bacterial species: *Snodgrassella alvi*, a nonsugar fermenter forms a layer directly on the gut wall, together with *Gilliamella apicola*, a sugar fermenter that resides more toward the center of the lumen (9). The distal rectum is dominated by the Gram-positive *Lactobacillus* spp (10).

In both insects and mammals, the gut microbiota can possess a large repertoire of metabolic capabilities and can contribute substantively to dietary carbohydrate digestion within the intestinal ecosystem (11). Short-chain fatty acids (SCFAs), namely acetate, propionate, and butyrate, produced by the gut microbiota as main fermentation products of dietary fiber accumulate in the human colon in concentrations up to 80–130 mM (12) and serve as a major energy source for intestinal epithelial cells (13) or as the main respiratory substrate of the host (14). Moreover, microbial metabolites can have a profound effect on gut physiology; for example, their effects on oxygen concentration, pH, and redox potential can be essential for host health (15). As neuroactive compounds, SCFAs produced by the gut microbiota can affect neural and immune pathways of the host and thereby influence brain function and behavior (16).

In the case of honey bees, genome-based investigations showed that *G. apicola* strains potentially digest complex carbohydrates (i.e., pectin from pollen cell wall) that are otherwise indigestible by the host (17). A recent study documents that *G. apicola* strains can also use several sugars that are harmful to bees (18). However, how the bee gut microbiota affects host physiology and the gut microenvironment has not yet been described. Hence, we compared germ-free (GF) honey bees to those with a conventional gut community (CV) to identify how the gut microbiota affects weight gain, expression of genes underlying hormonal pathways, gut physicochemical conditions, and metabolite pools in the gut and hemolymph.

Results and Discussion

Gut Microbiota Promotes Host Body and Gut Weight Gain. To observe effects of the microbiota on growth of individual hosts, we performed serial measurements of whole-body wet weight in the presence and absence of the gut microbiota. GF and CV bees were obtained from pupae that were removed from hives, allowed to emerge in sterile laboratory conditions, and then fed either

Significance

Honey bees are globally important plant pollinators. Guts of adult workers contain specialized bacteria not found outside bees. Experimental results show that gut bacteria increase weight gain in young adult bees, affect expression of genes governing insulin and vitellogenin levels, and increase sucrose sensitivity. Gut bacteria also shape the physicochemical conditions within the gut, lowering pH and oxygen levels. Peripheral resident bacteria consume oxygen, thus maintaining anoxia, as required for microbial activity. Additionally, gut bacteria produce short-chain fatty acids, with acetate and propionate as the major metabolites, as in guts of human and other animals. This study demonstrates how bacteria in the honey bee gut affect host weight gain and improves our understanding of how gut symbionts influence host health.

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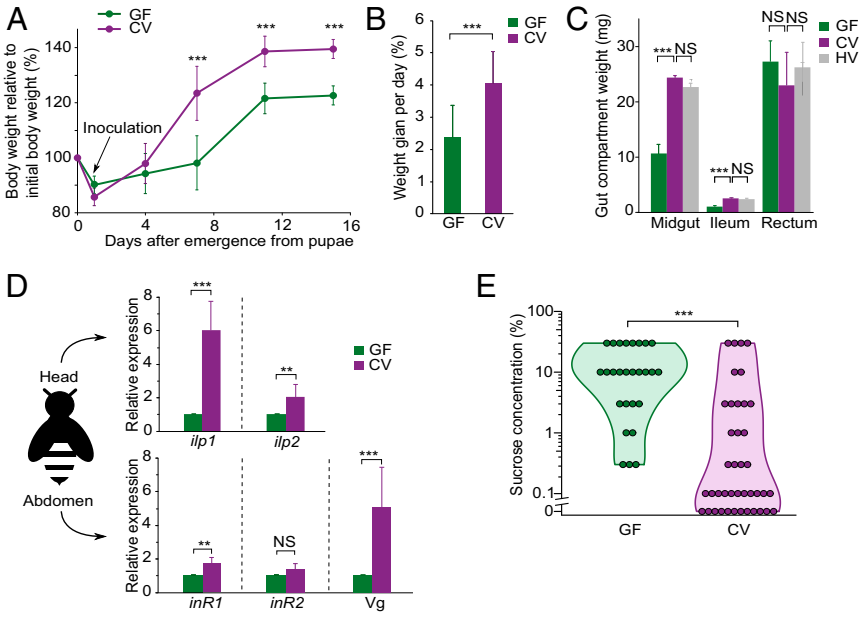

Fig. 1. Gut microbiota increases honey bee whole-body weight, gut weight, hormonal signaling, and sucrose sensitivity. (*A*) Whole-body wet weight growth curves of GF ($n = 45$) and CV bees ($n = 49$) originated from four different hives (the colony of origin was not statistically significant). (*B*) Daily weight gains of GF and CV bees after feeding with sterilized food or food supplemented with hindgut samples of hive nurse bees (day 1 to day 15). (*C*) Weights of different gut compartments of GF, CV, and hive nurse bees ($n = 25$). Bars indicate mean values of 10–15 pooled guts of bees from different hives (GF, $n = 4$; CV, $n = 4$) and hive bees from different hives ($n = 3$). (*D*) Differential expression of *ilp*, *inR*, and Vg genes in the head or abdomen of GF and CV bees originating from different hives. $n = 3$. (*E*) Distribution of sucrose-response thresholds of GF ($n = 27$) and CV ($n = 41$) bees shown as a violin plot. The colony of origin was not statistically significance. Each circle indicates a bee response to the provided concentration of sucrose. In *A–D*, $^{**}P < 0.01$, $^{***}P < 0.001$ (Mann–Whitney *u* test for the indicated comparisons); error bars indicate SD. In *E*, $^{***}P < 0.001$ (χ^2 test). NS, not significant.

sterilized food or sterilized food inoculated with the gut content of conventional adult nurse bees. This procedure yields GF bees with $<10^5$ bacteria per gut consisting of an erratic mix of bacterial species versus CV bees with $>10^8$ bacteria per gut and consisting primarily of core bee gut species, as in naturally inoculated hive bees (19, 20). Although GF and CV bees showed similar survivorship in laboratory experiments, CV bees attained greater body weight (Fig. 1*A*) and achieved a weight gain 82% higher than that of GF bees (Fig. 1*B*). After 15 d, the wet weights of both midgut and ileum were also larger in CV bees than in GF bees and were similar to those of nurse bees collected from hives (Fig. 1*C*). In contrast, rectum weights were not significantly different and were more variable, probably because of variable levels of pollen and waste accumulation in this gut compartment (Fig. 1*C*). These results indicate that the presence of conventional gut microbiota is required for normal body and gut weight gain during the days

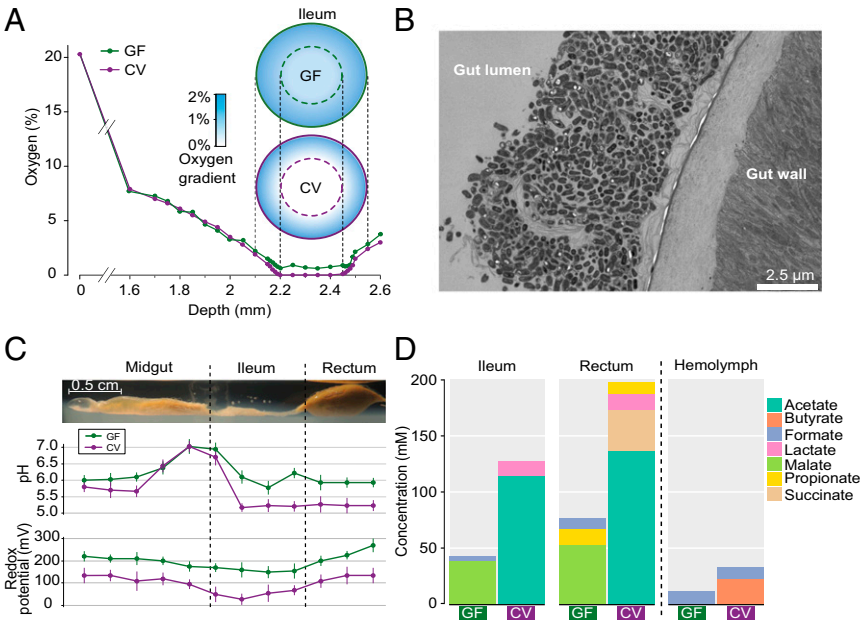

Fig. 2. Physicochemical conditions and SCFA profiles in the guts of GF and CV bees. (*A*) Radial profiles of oxygen concentration in the ileum of GF ($n = 3$) and CV ($n = 3$) honey bees from different hives. The central regions of CV ileums are always absolutely anoxic (0% oxygen). Deviation for each value is typically less than 0.2% oxygen. Depth refers to the distance between the electrode tip and the surface of the agarose. The schematic representation of the oxygen concentration gradient shows a microoxic periphery around the anoxic center in the CV bee ileum, whereas oxygen is present even in the center of the GF bee ileum. (*B*) Transmission electron micrographs of the CV ileum epithelium with a bacterial layer. (*C*) Microelectrode profiles of pH and redox potential along the GF and CV gut axis. The gut was embedded in a microchamber with agarose. Samples are originated from different hives, but colony of origin was not statistically significant. Error bars indicate SD ($n = 4$). (*D*) Concentrations of SCFA in the ileum, rectum, and hemolymph of GF and CV honey bees. Samples are originated from different hives; colony of origin was not statistically significant. A full list of concentration values is shown in Table S1.

following emergence from the pupal stage. A similar effect of gut microbiota has been documented in humans and mice (21, 22), and the commensal microbiota also influences the systemic development of *Drosophila* (23). Although the fitness effects of this weight gain were not measured directly at the colony level, several observations suggest that the greater increase in weight in the presence of microbiota is beneficial. As observed in previous studies, all bees gained weight during the first 10 d following emergence (24), but we found that the gain was almost halved for bees deprived of gut microbiota (Fig. 1*A*). Larger mass may assist in the initial tasks as nurses within the hive, whereas older bees (>20 d) are known to lose weight as they transition to foraging (24, 25).

Insulin/Insulin-Like Signaling and Sucrose Sensitivity. Weight gain in honey bees has been shown to be associated with insulin insulin/insulin-like signaling (IIS) (26). The IIS pathway plays a key role in insect growth, reproduction, and aging (27) and is a regulator of nutrient homeostasis and behavior in honey bees (28, 29). The honey bee genome contains genes encoding two insulin-like peptides (ILPs) and two putative insulin receptors (InRs) for these peptides (30). In addition, vitellogenin (Vg), an egg yolk protein, interacts with the IIS pathway to regulate bee nutritional status (31). The ILPs are preferentially expressed in the heads of worker bees, whereas InRs and Vg are more highly expressed in abdomens (32).

We examined the expression levels of the two ILP genes (*ilp1* and *ilp2*) in heads of 7-d-old bees and of two InR genes (*inR1* and *inR2*) and one Vg gene in the abdomens of the same bees. The *ilp1* and Vg genes were expressed 5.8 and 4.9 times higher in CV than in GF bees, respectively, and *ilp2* and *inR1* also increased expression in CV bees (Fig. 1*D*). Thus, CV bees have enhanced insulin production and responsiveness. The IIS pathway responds to diet, and *ilp1* expression is highest on a protein-rich diet; moreover, bees fed on this diet obtain more body weight (26). Our results suggest that the gut bacteria supply amino acids, which increase IIS gene expression and weight gain. Bees on high-protein diets exhibit harmful weight gain and short lifespans (26); however, the similar survival of CV and GF bees indicates that the weight gain of CV bees does not affect longevity. In contrast, the expression of *inR2* was not significantly altered, suggesting that it is unresponsive to nutrient manipulations as shown for *ilp2* (33, 34).

We also determined how the presence of microbiota impacts the overall transcriptome of gut epithelial cells. Of 10,189 genes detected in the RNA sequencing analysis, 221 host genes are significantly more highly expressed in CV bees (Dataset S1). Interestingly, the most significantly up-regulated gene belongs to the low-density lipoprotein receptor superfamily (Fig. S1 and Dataset

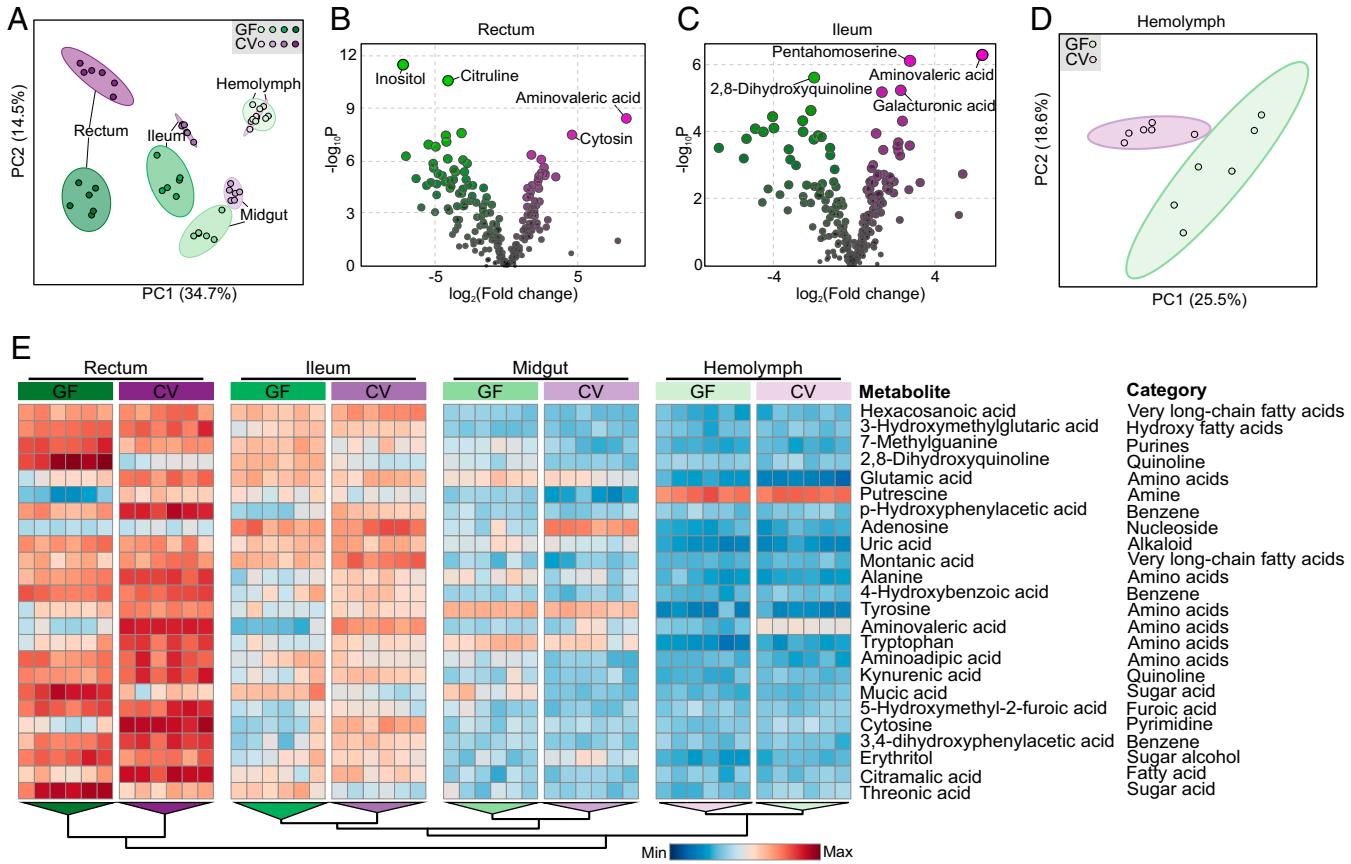

Fig. 3. Metabolomic analysis of different gut compartments of GF and CV bees. (*A*) Results of PCA based on 795 metabolites (Dataset S3) detected from three gut compartments and hemolymph of GF ($n = 6$) and CV ($n = 6$) bees showing different clustering groups (95% confidence regions) according to the presence of gut microbiota. (*B* and *C*) Differentially regulated metabolites in rectum (*B*) and ileum (*C*). The metabolites enriched in CV bees are shown in purple, and those enriched in GF bees are in green. The area and color of each circle are proportional to the significance ($-\log_{10} P$). Full lists of the values in all gut compartments and hemolymph are given in Dataset S2 *A*–*D*. (*D*) Results of PCA based on all metabolites detected in hemolymph of GF and CV bees (excluding gut compartments from the analysis). (*E*) Unsupervised hierarchical clustering heat map of the 25 metabolites that contribute most to the separation of hemolymph, midgut, ileum, and rectum. Each column represents one sample. Colors indicate the normalized relative concentration of each metabolite (minimum–maximum). The tree on the bottom illustrates a dendrogram of clustering (Ward's method).

S1), which encodes the Vg receptor in *Drosophila* (35). However, the Vg receptor is usually expressed in the ovary, suggesting that the transcripts originated from the residue of ovaries with the dissected guts. Nevertheless, such stimulation is consistent with the increase in Vg expression in the abdomen.

The IIS pathway regulates the behavior of worker honey bees, including division of labor and sucrose sensitivity (29, 36). Sucrose sensitivity is an indicator of energy status and satiety in honey bees (37). By measuring the proboscis extension response of both CV and GF bees (Movie S1), we found that the gut microbiota significantly elevates sucrose sensitivity: More CV bees responded to lower concentrations of sucrose (i.e., CV bees are "hungrier" than GF bees) (Fig. 1*E*). Our combined results provide evidence that the gut microbiota of honey bees stimulates IIS and Vg expression, which in turn affects bee satiety and ultimately promotes host weight gain. This observation is consistent with results showing that IIS promotes growth in other gut microbiota models such as *Drosophila* (23) and mammals (38).

Physicochemical Conditions. The intestines of insects together with their symbiotic microbes can be viewed as minute ecosystems characterized by complex physicochemical conditions. We examined these conditions in the bee gut by using Clark-type, glass pH and Redox electrodes with a tip size of 50 μm to measure the oxygen status, pH, and redox potential in each gut compartment. All compartments of CV bee guts and the midgut and rectum of GF bees were entirely anoxic (0% oxygen) in their centers. In contrast, trace oxygen was detected in the center of the ileum of GF bees (Fig. 2*A*). For a more fine-grained view of how the microbiota affects oxygen levels within the ileum, we determined the radial oxygen profiles for the ileums of GF and CV bees. Oxygen concentration in the CV ileum the decreased rapidly from the gut wall inward, reaching 0% when the microsensor tip was only about 150–200 μm below the exterior surface of the gut wall. Thus, the ileums of CV bees contain a microoxic periphery around an anoxic center (Fig. 2*A*). By contrast, oxygen concentration was still 1% in the center of the GF ileum. Although gut epithelial cells can contribute to oxygen consumption, the different oxygen gradients of CV and GF ileums indicate that the major oxygen consumers must be resident bacteria in the peripheral region of the gut lumen.

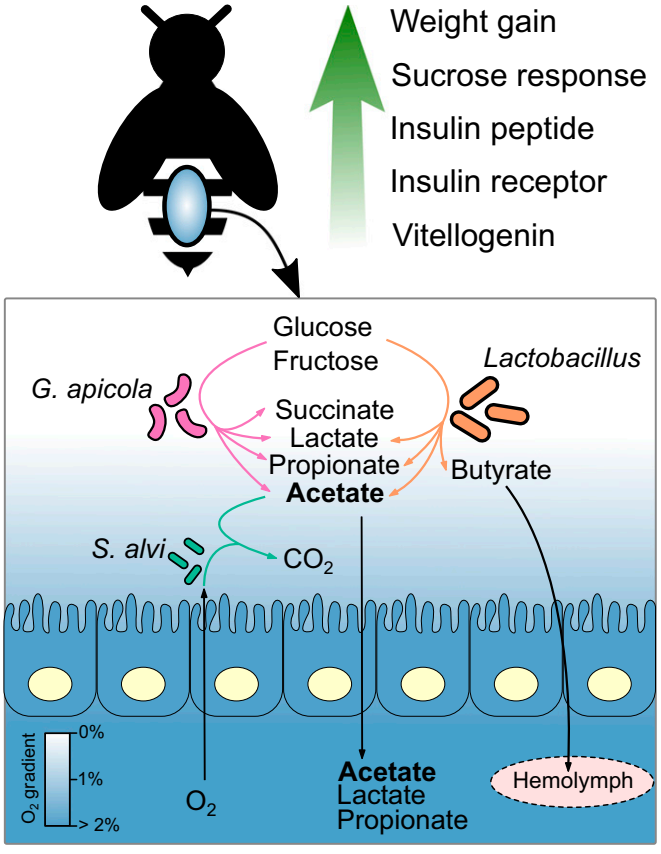

Fig. 4. Graphical summary of the main results. The conventional gut microbiota, including the major sugar fermenters *G. apicola* and *Lactobacillus* sp., produce SCFAs, which promote host growth. The gut wall-associated *S. alvi* uses acetate, the most abundant SCFA in the hindgut, to remove efficiently O_2 penetrating into the gut and to maintain a stable O_2 gradient. The presence of gut microbiota stimulated host weight gain, host sucrose sensitivity, and increased expression of genes related to hormonal signaling.

A favorable, anoxic environment is crucial for the anaerobic microorganisms in most animals' digestive tracts (39). Anoxia can be accomplished easily when the intestinal volume is large, and the ileums of honey bees possess a surface area:volume ratio of 6,700 m^2/m^3 that is much larger than that of human intestine or rumen (40). The inner wall of the honey bee ileum is associated with a bacterial layer formed by *S. alvi* (10), a non–sugar-fermenting *Betaproteobacteria* (Fig. 2*B*). The observed sharp drop in oxygen might result from *S. alvi*'s consumption of oxygen penetrating from outside or secreted from host tissue. This idea is supported by in vitro tests showing that *S. alvi* uses acetate to fuel its oxygen-consuming respiratory activity (Fig. S2*A*), as shown for gut wall-colonizing bacteria in human (41). The O_2 consumption rate of *S. alvi* wkB2 is $4.1 \pm 0.8 \times 10^{-5}$ pmol·min^{-1}·$cell^{-1}$ in buffer supplemented with acetate, which is higher than that of *Stenoxybacter acetivorans*, an O_2 consumer located in the peripheral region of termite hindguts, and that of *Citrobacter* sp. strain RFC-10 (Fig. S2*B*) (42). Not surprisingly, no O_2 was consumed in succinate- or glucose-supplemented buffer, because *S. alvi* does not use these as energy sources (Fig. S2*A*). *S. alvi* is an obligate aerobe (9), and genome-wide transposon insertion (Tn-Seq) screening has shown that the ntrX/ntrY two-component oxygen sensor is essential for host colonization (43). Considering the high abundance, peripheral localization in the gut, obligately aerophilic nature (9), and restriction to acetate as oxidizable energy sources, *S. alvi* must be responsible for the maintenance of anoxia in the gut, which is crucial for appropriate metabolism of other gut symbionts.

The bee gut microbiota also causes both reduced pH and redox potential (Fig. 2*C*), reflecting bacterial metabolic activity. Axial profiles of pH showed greater acidity in CV bees than in GF bees in the center of each gut region, whereas the pH increases along the midgut and decreases toward the ileum and rectum in both (Fig. 2*C*). Especially in the ileum and rectum, where most bacteria localize, the pH values are lower in CV bees (around 5.2) than in GF bees (around 6.0), suggesting that the difference reflects microbial activity. In both types of guts, the redox potential is positive throughout the gut even though the gut is anoxic (Fig. 2*C*). In experiments on mammalian systems, colonic pH and redox status have important physiological effects on Ca^{2+} availability and on the composition of the gut community (44). A major cause of this reduced colonic pH is active fermentation resulting in significant increase of SCFAs (45).

Gut Metabolites. We investigated the composition of SCFAs in gut compartments of GF and CV bees. The metabolite pools from the different gut compartments differ strongly between GF and CV bees, demonstrating the role of the gut microbiota as the main producer of SCFA in vivo (Fig. 2*D*). In the CV ileum and rectum, the prevailing fermentation product is acetate (114–137 mM) (Table S1), which also is the most abundant SCFA observed in human intestine (46) and which has been shown to enhance gut epithelial barrier functions (47). By contrast, GF ileum and rectum show accumulation of malate, although in lower amounts; succinate was present only in CV rectum (Table S1). Thus, the gut

microbiota impacts host physiology. Because the redox potentials are always positive, we did not detect hydrogen production in the guts. In all compartments of the GF gut, the homogenates contain high concentrations of glucose and fructose. However, these sugars are much lower in the homogenates of CV ileums and rectum, indicating that they are consumed by the symbiotic bacteria. Hemolymph metabolite profiles also differ between CV and GF bees, suggesting that gut bacteria affect host metabolism. Although trace amounts of formate were detected in hemolymph of both CV and GF bees, butyrate (22.8 mM) was detected only in the CV hemolymph. In mammals, butyrate is an important fermentation product produced by the gut microbiota (48) and has been shown to serve as the main energy source for colonocytes (49). The absence of butyrate in the bee gut suggests that it is absorbed and used by the host.

The different gut fermentation profiles between GF and CV bees might be shaped by oxygen status, as shown in cockroaches (50). Indeed, in in vitro cultures of the two most abundant bee gut fermenters, *G. apicola* and *Lactobacillus* sp., fermentation products were strongly dependent on oxygen status (Tables S2 and S3), clarifying the differences in gut SCFA profiles (Fig. 2*D*). The major fermentation product of *G. apicola* under ambient air is malate, but with decrease of O_2, *G. apicola* shifts to producing more acetate and propionate (Table S2), as is consistent with the in situ results. Although *Lactobacillus* species are considered to be lactic acid producers (51), they produce substantial amounts of acetate when headspace contains 2% of O_2 (Table S3). The shift from lactate to acetate formation with the presence of O_2 has been documented in *Enterococcus* strain RfL6 from termite gut (52). Our results indicate that the resident gut bacteria contribute to the major SCFAs detected in the hindgut.

Metabolomics. For a more in-depth understanding of the effects of gut microbiota, we compared metabolite levels in separate gut compartments and hemolymph for CV and GF bees using untargeted metabolomics by GC–MS analysis. The principal component analysis (PCA) showed that the gut microbiota has a striking effect on the gut metabolome, especially in the rectum and ileum (Fig. 3 *A* and *E*), where most gut bacteria reside (10). By contrast, hemolymph samples were not clearly distinct between CV and GF bees. In the ileum and rectum, aminovaleric acid is the compound most elevated in CV bees in both the ileum (319-fold) and rectum (84-fold) (Fig. 3 *B* and *C* and Dataset S2, *A* and *B*). Aminovaleric acid is also a major product of microbial activity in the human gut (53). Another notable enrichment in the CV ileum is for galacturonic acid (Fig. 3*C*), the main constituent of pectin, indicating pectin degradation by gut bacteria and corroborating the presence of genes encoding enzymes that target pollen wall polysaccharides (17). Galacturonic acid was not enriched in the CV rectum, suggesting that it is degraded by the dense bacterial community. This result is consistent with the ability of *G. apicola* strains to use products of pectin degradation (18). In CV bees, adenosine was elevated within the midgut, despite the very low levels of bacteria colonizing this gut compartment (Fig. 3*E* and Dataset S2*C*). Potentially gut colonization triggers endogenous production of adenosine, which may reduce gut inflammation (54).

Although the impact on hemolymph is less than that on gut compartments, when we analyzed the hemolymph samples separately the metabolite profiles also show a clear effect of gut microbiota (Fig. 3*D*). Several amino acids are elevated in CV hemolymph (Dataset S2*D*), suggesting that they are absorbed by the host. Based on a previous mutational screen (43), biosynthetic pathways for amino acids are crucial for host colonization, suggesting that gut bacteria might contribute amino acids absorbed by the host. Alternatively, host pathways for vitamin and amino acid biosynthesis are among those significantly up-regulated in CV bees (Fig. S3), suggesting that the presence of gut microbiota stimulates host metabolism. The increased quantities of amino acids in the CV hemolymph might reflect the stimulation of the IIS pathway and subsequent weight gain, which has been linked to an amino acid-supplemented food in honey bees (26). Thus, gut symbionts may supply sufficient protein to affect hormonal signaling and to promote weight gain of the hosts.

Conclusions

Our results show that the gut microbiota of young adult honey bees promotes weight gain and that this effect is accompanied by the enhanced expression of genes affecting hormonal titers and by an increased sensitivity to sugar (Fig. 4). In addition, spatially organized microbial communities maintain an oxic–anoxic gradient in the gut with lowered pH and redox potential, effects similar to those demonstrated for the human gut microbiota (11). Our observations of the involvement of gut bacteria in the maintenance of gut physicochemical characteristics, SCFA production, and the digestion of complex dietary components (e.g., pectin) reveal further similarities between the gut microbiota of humans and honey bees and provide perspectives for future studies using the bee gut model for investigating host–microbe interactions. Further experimental work is needed to determine the extent to which similar cellular mechanisms underlie the observed similarities of gut microbiota in these different animal hosts.

Materials and Methods

Detailed protocols are available in *SI Materials and Methods*. GF and CV bees were obtained using the protocol described by Powell et al. (19). We obtained GF bees by removing pupae from brood frames of four different hives and allowing bees from each hive to emerge in a separate, sterile dish. CV bees were obtained by feeding newly emerged bees with homogenates of hindguts of nurse bees from their original hive. This inoculation method was chosen because it yields CV bees with robust gut communities similar in size and composition to those of normal bees sampled from hives (19, 20); however, we cannot entirely rule out effects of other components of the homogenized guts on the treatment bees. Bees were immobilized at 4 °C, and the whole-body wet weight was measured with an electric balance. The expression levels of genes encoding ILP, InR, and Vg were determined by quantitative PCR from RNA extracted from the heads or abdomens of GF and CV bees obtained as described above. Primers used in this study are listed in Table S4. The responsiveness of individual bees to sucrose was assessed by presenting sucrose solutions to the antennae. Examples of response or lack of response are shown in Movie S1. SCFA concentrations within different gut compartments and hemolymph were measured by HPLC. The gut O_2 concentrations, pH, and redox potential were measured using microsensors connected to a four-channel amplifier (Unisense). Metabolomic profiles were determined from extracted gut compartments at the University of California, Davis using GC-MS as described by Fiehn and Kind (55).

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