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Microbial Communities in Different Developmental Stages of the Oriental Fruit Fly, *Bactrocera dorsalis*, Are Associated with Differentially Expressed Peptidoglycan Recognition Protein-Encoding Genes

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ABSTRACT The insect microbiota can change dramatically to enable adaptation of the host in different developmental stages and environments; however, little is known about how the host maintains its microbiota to achieve such adaptations. In this study, 16S rRNA sequencing revealed that the microorganisms in larvae and adults of the Oriental fruit fly, *Bactrocera dorsalis*, are primarily Gram-negative bacteria but that the major components in pupae are Gram-positive bacteria. Using suppression subtractive hybridization (SSH) and transcriptome analysis, we screened two specifically expressed genes encoding peptidoglycan recognition proteins (PGRP-LB and PGRP-SB1) and analyzed their relationship to *B. dorsalis* microbial communities. Knockdown of the PGRP-LB gene in larvae and adults led to increased ratios of Gram-positive bacteria; knockdown of the PGRP-SB1 gene in pupae led to increased ratios of Gram-negative bacteria. Our results suggest that maintenance of the microbiota in different developmental stages of *B. dorsalis* may be associated with the PGRP-LB and PGRP-SB1 genes.

IMPORTANCE Microorganisms are ubiquitous in insects and have widespread impacts on multiple aspects of insect biology. However, the microorganisms present in insects can change dramatically in different developmental stages, and it is critical to maintain the appropriate microorganisms in specific host developmental stages. Therefore, analysis of the factors associated with the microbiota in specific development stages of the host is needed. In this study, we applied suppression subtractive hybridization (SSH) combined with transcriptome analysis to investigate whether the microbiota in development stages of the Oriental fruit fly, *Bactrocera dorsalis*, is associated with expression of PGRP genes. We found that two different PGRP genes were specifically expressed during development and that these genes may be associated with changes in microbial communities in different developmental stages of *B. dorsalis*.

KEYWORDS *Bactrocera dorsalis*, association, developmental stages, immune-related genes, microbiota

Insects are colonized by a remarkable array of symbionts (1, 2). Certain microbial taxa can promote insect fitness by contributing to nutrition, especially by providing essential amino acids or B vitamins (3–7). Furthermore, symbionts can protect their insect hosts against pathogens, parasitoids, and other parasites by synthesizing specific toxins (8–10) or by modifying the insect immune system (11, 12). Other functions, such as production of detoxifying toxins (13–15) and determinants of insect communication

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(16), have also been reported. With regard to *Bactrocera dorsalis*, we have found that *Gluconobacter* bacteria are beneficial for larval development (17); in addition, *Citrobacter* can enhance the insecticide resistance of adults (13), and many *Actinobacteria* members have been identified in pupae (18). Overall, changes in the microbiota may enable adaptation of the host in different developmental stages and environments.

Symbionts are ubiquitous in insects and have pervasive impacts on multiple aspects of insect biology in different environments and developmental stages (19–21). Thus, the host must maintain the proper number of symbiotic microorganisms needed to remain healthy while combatting microbial infection (22) or reducing the abundance of other symbionts to conserve resources (23). In general, the identity and diversity of insect symbionts strongly depend on the environment, lifestyle, and life history of the host, and these factors are likely to have a major impact on the evolution of symbioses. Insects that have either a valuable but immobile resource (nest or food) or immobile developmental stages (eggs or pupae) are more likely to evolve defensive relationships with symbionts (24, 25). Some holometabolous insects that undergo four gradual stages of metamorphosis (egg, larva, pupa, and adult) also display significant differences in their microbial communities. For example, the fractions of mosquito-associated microflora significantly differ during the insect's life cycle (26), and in honeybees, the microbiota varies with age, caste, and season (27).

Peptidoglycan (PGN) recognition proteins (PGRPs, including PGRP-LB and PGRP-SB) are mediators of innate immunity in fruit flies (28). PGRP-LB cleaves *meso*-diaminopimelic acid-type (DAP-type) peptidoglycan to reduce immune deficiency (IMD) pathway activity, which should promote the growth of DAP-type bacteria (most of which are Gram-negative bacteria) (29, 30), and knockdown of the gene increases the load of Gram-negative bacteria (31). Moreover, PGRP-LB is expressed in specific life stages (32). PGRP-SB1 exerts bactericidal activity against DAP-type peptidoglycan, which should restrict the load of DAP-type bacteria, and it is preferentially expressed in pupae (33, 34). As PGRPs have multiple functions in the host immune response, including lysine-type and DAP-type peptidoglycan recognition (35), they may also affect the microbial community in some insects.

In our previous study, we found dramatic differences in microbial communities in *B. dorsalis* larvae, pupae, and adults (18). Thus, we explored three issues in the current study. First, we examined the characteristics (Gram positive or Gram negative) of the microorganisms in *B. dorsalis* larvae, pupae, and adults. Second, *B. dorsalis* larvae, pupae, and adults were infected with Gram-positive or Gram-negative bacteria to induce immune responses, and differentially expressed PGRP genes were identified using suppression subtractive hybridization (SSH) and transcriptome analysis. Third, we investigated whether the differentially expressed PGRP genes are associated with microbial communities in different developmental stages of *B. dorsalis*.

RESULTS

Microbial communities in different developmental stages of *B. dorsalis*. Shannon rarefaction curves based on 16S rRNA sequencing results tended toward saturation (see Fig. S1 in the supplemental material), indicating that the bacterial libraries produced from our samples well represented the microbial communities present in *B. dorsalis*. Analysis of the samples confirmed *Acetobacteraceae* to be the major microorganism component of *B. dorsalis* larvae. In pupae, *Micrococcaceae*, *Intrasporangiaceae*, *Dermabacteraceae*, *Staphylococcaceae*, and *Brevibacteriaceae* were the major microbiota members. *Enterobacteriaceae* was the major family in adults (Fig. 1a). By classifying all microorganisms as Gram positive or Gram negative, we found significantly different ratios of these bacteria across different developmental stages (Fig. 1b and Data Set S1). We also used the LEfSe method to identify bacteria that were likely to explain most of the differences between samples. Notable bacteria in larvae were *Acetobacteraceae* and *Lactobacillaceae*; in contrast, *Enterobacteriaceae* was a marker of adults and *Micrococcaceae*, *Intrasporangiaceae*, *Dermabacteraceae*, *Staphylococcaceae*, and *Brevibacteriaceae* were markers of pupae (linear discriminant analysis [LDA] scores $>$ 4.5) (Fig. 1c).

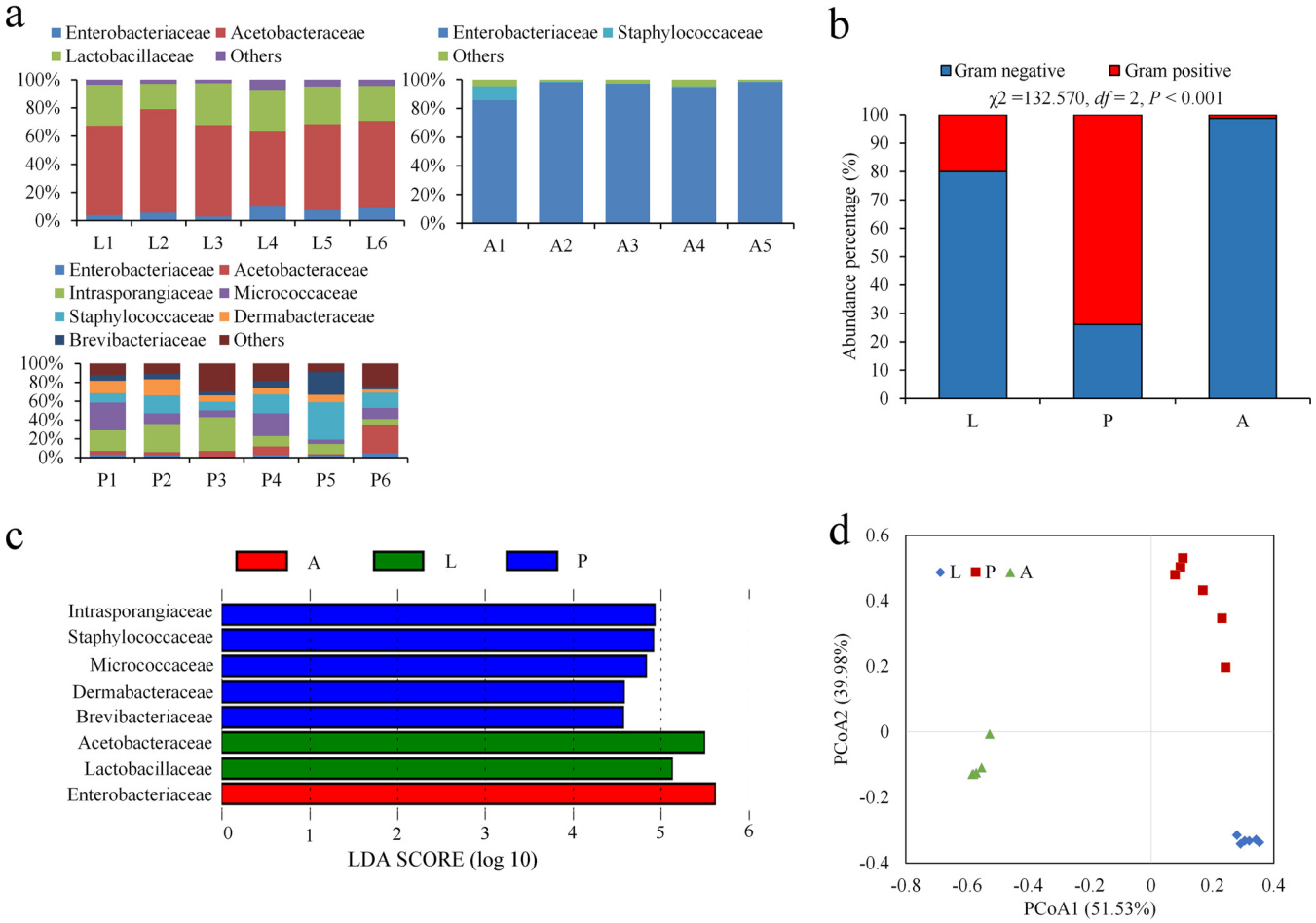

FIG 1 Comparison of the microbiotal structure in different developmental stages of *B. dorsalis*. (a) Percentages of bacterial phyla in different samples. Only phyla with a relative abundance greater than 2% are shown. (b) Percentages of Gram-positive and Gram-negative bacterial phyla in different samples. (c) LEfSe analysis based on OTU abundance in different development stages. (d) PCoA plot based on the microbiotal structure in different developmental stages. L, larva; P, pupa; A, adult.

Principal-coordinate analysis (PcoA) also demonstrated a separation of the samples from different developmental stages (Fig. 1d), and permutational multivariate analysis of variance (PERMANOVA) results indicated significant differences in the microbial communities of *B. dorsalis* across developmental stages ($P = 0.001$).

SSH results. Maintenance of microbial communities in different development stages may be associated with immune-related genes, and immune responses may be induced when larvae, pupae, or adults are infected with bacteria that produce different types of peptidoglycan (larvae and adults were infected with *Staphylococcus aureus* [Gram positive bacterium]; pupae were infected with *Escherichia coli* [Gram-negative bacterium]). The results of SSH analysis showed 54 (including 12 immune-related genes) and 66 (including 15 immune-related genes) differentially expressed genes for adults and larvae infected by *S. aureus*, respectively; 122 genes (including 17 immune-related genes) were obtained for pupae infected with *E. coli*. Among these immune-related genes, the gene for one peptidoglycan recognition protein (PGRP-LB) (GenBank accession number XM_019992218) was identified in both *S. aureus*-infected larvae and adults, whereas the gene for PGRP-SB1 (GenBank accession number XM_011211796) was identified in *E. coli*-infected pupae. Additionally, 15 other immune-related genes were identified in larvae, pupae, or adults (Data Set S2).

Screening of differentially expressed immune-related genes during developmental stages by transcriptome analysis. To validate the SSH results, the transcriptomes of larvae, pupae, and adults were investigated. More than 91% of the clean reads

TABLE 1 Differentially expressed PGRPs in adults and pupae[a]

Gene ID	A RPKM	P RPKM	$\log_2$ ratio[b]	Annotated function
Unigene0024927	44.27	0.00	15.43	PGRP-SC2-like isoform X1
Unigene0032727	34.98	0.24	7.22	PGRP-LB
Unigene0030318	15.98	2.98	2.42	PGRP-LA
Unigene0023841	15.42	5.93	1.38	PGRP-SA
Unigene0007218	7.92	3.17	1.32	PGRP-LC-like
Unigene0033183	29.23	12.27	1.25	PGRP-LC-like isoform X2
Unigene0026579	1.26	6.90	−2.45	PGRP-SB1

[a]The *P* values for reads per kilobase per million (RPKM) between adults (A) and pupae (P) were all <0.01. ID, identifier.
[b]Ratio of RPKM for adults to RPKM for pupae.

were successfully mapped to the reference transcriptomes of the six samples (Table S1), and correlation analysis of gene expression levels revealed agreement between 0.9649 and 0.994 for replicate treatments (Fig. S2). The numbers of differentially expressed genes generated for larvae versus pupae and pupae versus adults were 19,400 (3,409 downregulated and 15,991 upregulated) and 21,616 (16,721 downregulated and 4,895 upregulated), respectively (Fig. S3). Among the differentially expressed immune-related genes, those for 7 PGRPs were differentially expressed between adults and pupae (Table 1) and 8 between larvae and pupae (Table 2). The relative RNA expression level of the PGRP-LB gene was significantly higher in larvae and adults than in pupae, and PGRP-SB1 gene expression was significantly higher in pupae than in larvae and adults (one-way analysis of variance [ANOVA], $F_{3,8} = 126.229$ and $P < 0.01$) (Fig. 2a). The same results were obtained by normalization with the RpL32 gene (data not shown). Overall, these two genes are correlated with the shift in microbial community in different developmental stages.

The PGRP-LB and PGRP-SB1 genes are associated with shifted microbial communities. Based on the results presented above, we hypothesize that the PGRP-LB gene may be associated with maintaining the larval and adult microbial communities, whereas the PGRP-SB1 gene may influence maintenance of the microbial community in pupae. Thus, we assessed changes in the microbiota after silencing the PGRP-LB gene in larvae and adults and the PGRP-SB1 gene in pupae via siRNA injection. We found that the levels of PGRP-LB and PGRP-SB1 gene transcripts were significantly reduced at 24 h after siRNA treatment and that the reduction in PGRP-LB and PGRP-SB1 gene expression reached 50% after 72 h (Fig. 2b).

16S rRNA high-throughput sequencing was also employed to investigate microbial communities in *B. dorsalis* with PGRP-LB and PGRP-SB1 gene knockdown. The Shannon rarefaction curves tended toward saturation (Fig. S4), suggesting good representation of the microbiota structure in each library. In larvae, analysis of control samples confirmed previous observations that the microbiota composition exhibited moderate complexity with *Acetobacteraceae* the major component; the major components in PGRP-LB gene knockdown samples were *Acetobacteraceae* and *Lactobacillaceae* (Fig. 3a). Moreover, the ratios of Gram-positive and Gram-negative bacteria between the RNA interference (RNAi) and control treatments were significantly different (Fig. 3b and

TABLE 2 Differentially expressed PGRPs in larvae and pupae[a]

Gene ID	L RPKM	P RPKM	$\log_2$ ratio[b]	Annotated function
Unigene0024927	31.12	0.00	14.93	PGRP-SC2-like isoform X1
Unigene0013730	158.58	0.04	11.97	PGRP-SC2
Unigene0032727	8.84	0.24	5.23	PGRP-LB
Unigene0031273	5.41	0.19	4.81	PGRP-SB1-like
Unigene0023841	16.62	5.93	1.49	PGRP-SA
Unigene0026579	1.26	5.49	−2.12	PGRP-SB1
Unigene0007218	1.14	3.17	−1.48	PGRP-LC-like
Unigene0030319	6.09	13.10	−1.11	PGRP-LA

[a]The *P* values for reads per kilobase per million between larvae (L) and pupae (P) were all <0.01.
[b]Ratio of RPKM for larvae to RPKM for pupae.

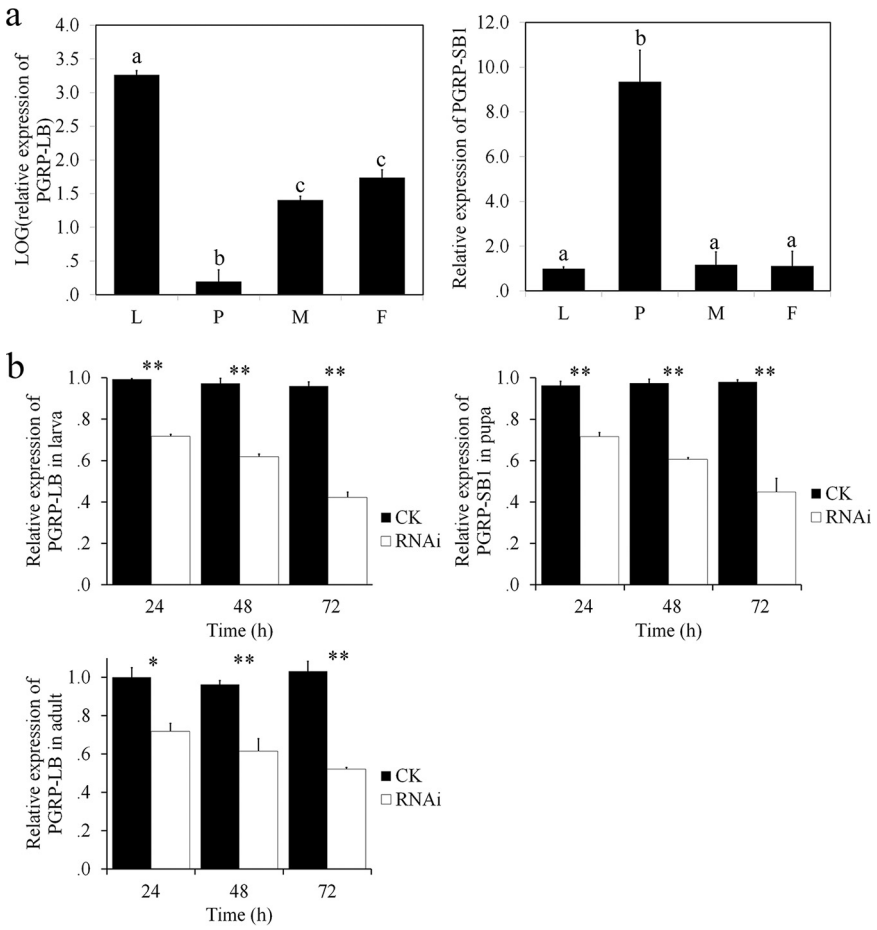

FIG 2 Relative expression of PGRP-LB and PGRP-SB1 genes. (a) Relative expression of PGRP-LB and PGRP-SB1 genes during different developmental stages. (b) Effects of RNAi interference of PGRP-LB and PGRP-SB1. The statistical comparison was based on one-way analysis of variance (different letters indicate significant difference) or the independent-sample *t* test (*, $P < 0.05$; **, $P < 0.01$). M, male; F, female; CK, control.

Data Set S3). Regarding the major taxa, significant differences were observed between the control and PGRP-LB gene knockdown samples (LDA scores > 4.5 [Fig. 3c]). PCoA also illustrated separation between the RNAi and control samples (Fig. 3d), and PERMANOVA indicated a significant difference for microbiotal structures between RNAi and control samples ($P = 0.011$). These results indicate that the PGRP-LB gene may be associated with the relative abundance of specific groups in the larval microbiota.

For adults, the major taxa in PGRP-LB gene knockdown samples were *Micrococcaceae*, *Brevibacteriaceae*, *Intrasporangiaceae*, *Enterobacteriaceae*, *Sphingobacteriaceae*, and *Brucellaceae*, whereas the major family in control samples was *Enterobacteriaceae* (Fig. 4a). The ratios of Gram-positive and Gram-negative bacteria between RNAi and control treatments were also significantly different (Fig. 4b and Data Set S3). Among the major microbiota components, significant differences were observed between the control and PGRP-LB gene knockdown samples (LDA scores > 4.5 [Fig. 4b]), and PCoA showed separation between RNAi and control samples (Fig. 4c). Moreover, there were significant differences for microbiotal structures between RNAi and control samples according to PERMANOVA ($P = 0.021$). Thus, there appears to be a correlation between the PGRP-LB gene and the adult *B. dorsalis* microbiotal structure.

The major microbiota components in PGRP-SB1 gene knockdown pupae were *Enterobacteriaceae*, *Acetobacteraceae*, *Lactobacillaceae*, *Intrasporangiaceae*, *Pseudomonadaceae*, *Dermabacteraceae*, *Cyclobacteriaceae*, and *Sphingobacteriaceae*. However, the major taxon in control samples was *Intrasporangiaceae* (Fig. 5a). Ratios of Gram-positive

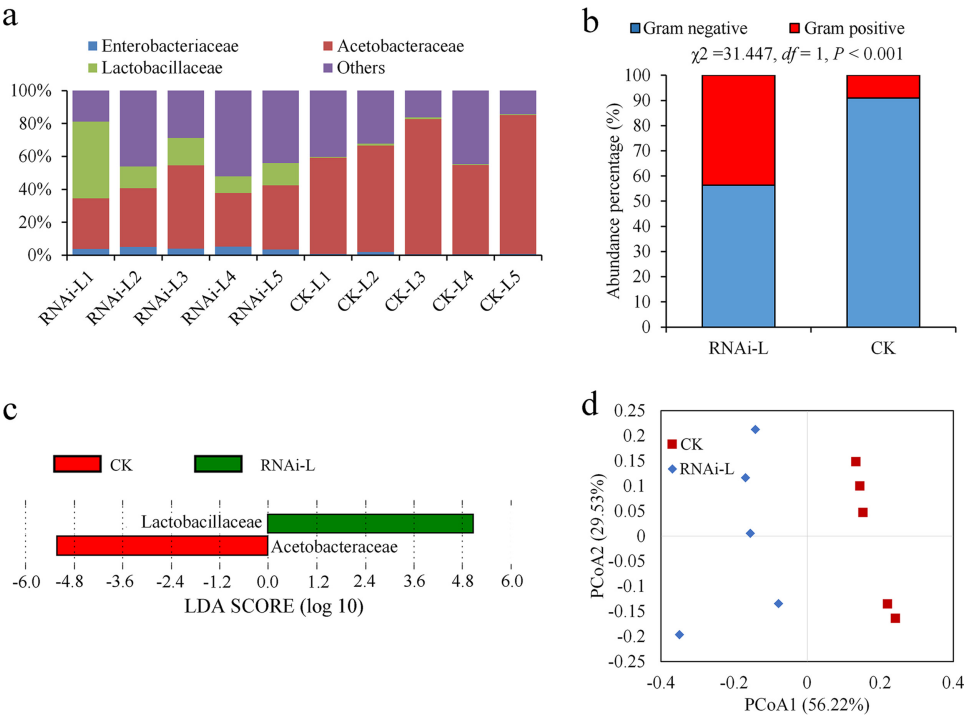

FIG 3 Comparison of the microbiotal structure between RNAi and control larva samples. (a) Percentage of bacterial phyla in the RNAi and control groups. Only phyla with a relative abundance greater than 2% are shown. (b) Percentages of Gram-positive and Gram-negative bacteria in RNAi and control larvae. (c) LEfSe analysis based on OTU abundance in RNAi and control larvae. (d) PCoA plot based on the microbiotal structure in different treatments.

and Gram-negative bacteria between RNAi and control treatments were also significantly different (Fig. 5b and Data Set S3). In addition, LEfSe analysis revealed significant differences regarding the abundance of *Enterobacteriaceae* and *Intrasporangiaceae* between control and RNAi samples (LDA scores $>$ 4.5 [Fig. 5c]), and PCoA also indicated separation between RNAi and control samples (Fig. 5d); PERMANOVA also showed significant differences in microbiotal structure between RNAi and control samples (P = 0.018). These results suggest a correlation between the PGRP-SB1 gene and the microbiotal structure in pupae.

DISCUSSION

Studies have indicated that diet and environment can greatly influence the structure of the microbiota (36–38), and differences in the *B. dorsalis* microbiota in different developmental stages support this conclusion. *B. dorsalis* may rely on multiple microbial species for fitness, and such differences provide a model for investigating and comparing the population dynamics of the microbiota.

In our research, we found dramatic changes in the microbiota during *B. dorsalis* development. In larvae and adults, the major components of the microbiota were Gram-negative bacteria, whereas the major taxa in pupae were Gram-positive bacteria. Some regulatory effectors may be associated with maintaining the proper microorganisms in different stages of *B. dorsalis*. For instance, previous research on antimicrobial peptides (AMPs) of the weevil *Sitophilus zeamais* indicates that the immunological status of the host might be important for regulating the microbiota (39). This mechanism has also been revealed to occur in tsetse flies (*Glossina* spp.) through downregulation of the PGRP-LB gene (40). In our study, we identified two PGRP genes by SSH and transcriptome analysis. By knocking down their expression, we found these genes to be associated with the observed changes in the microbiota of larvae, pupae, and adults. Similar results were reported for *Drosophila*, whereby PGRP-SB1 gene expression was

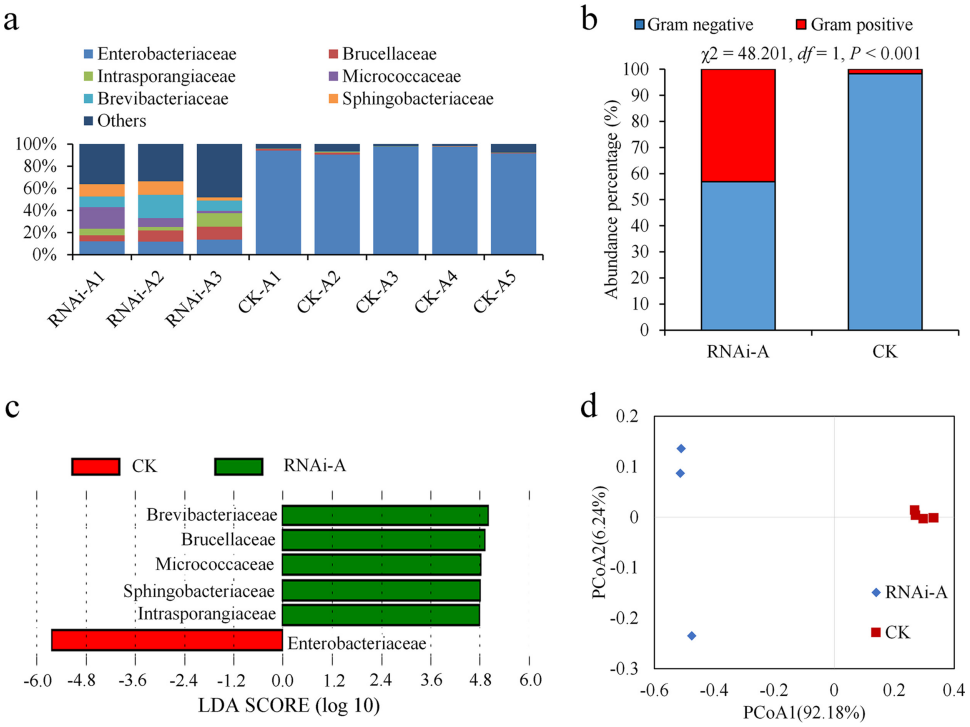

FIG 4 Comparison of the microbiotal structure between RNAi and control adult samples. (a) Percentages of bacterial phyla in the RNAi treatment and control groups. Only phyla with a relative abundance greater than 2% are shown. (b) Percentage of Gram-positive and Gram-negative bacteria in RNAi and control adults. (c) LEfSe analysis based on OTU abundance in RNAi and control adults. (d) PCoA plot based on the microbiotal structure in different treatments.

significantly increased after exposure to DAP-type peptidoglycan bacteria (most of which are Gram-negative bacteria) (34). In *B. dorsalis*, the PGRP-LB and PGRP-SB1 genes may be associated with altered microbiotal structure in different developmental stages.

In addition to increases in Gram-positive bacteria, the abundances of *Acetobacteraceae* and *Enterobacteriaceae* were decreased when the PGRP-LB gene was downregulated in larvae and adults. What led to this decrease in these families? One possible explanation may be that the growth of bacteria that are freed from host inhibition allows other taxa to occupy the niches that *Acetobacteraceae* and *Enterobacteriaceae* would have otherwise filled, as increased insect immune deficiency (IMD) activity due to reduced levels of PGRP-LB has been reported (39, 40). The IMD signaling pathway in response to bacterial cell wall peptidoglycan (PGN) fragments promotes production of AMPs that can regulate the microbiota (39, 40). Therefore, the abundance of *Acetobacteraceae* and *Enterobacteriaceae* may be reduced by AMPs in larvae and adults with PGRP-LB gene knockdown. In fact, we identified several AMPs (sarcotoxin II-3, attacin-A, attacin-C, and cecropin-1) (Data Set S2) in larvae and adults infected with *S. aureus*. Nonetheless, more evidence is needed to demonstrate whether expression of these AMPs is upregulated in larvae and adults with PGRP-LB gene knockdown. Moreover, it remains to be verified whether there is a negative correlation between the AMPs identified and the abundance of *Acetobacteraceae* and *Enterobacteriaceae* in larvae and adults.

In our study, we found dramatic changes compared with the natural status in the major components of the microbiota in larvae, pupae, and adults after PGRP genes were knocked down. For example, in PGRP-LB gene knockdown larvae, the major taxa (*Lactobacillaceae* and *Acetobacteraceae*) were not the same as those in pupae (*Micrococcaceae*, *Intrasporangiaceae*, *Dermabacteraceae*, *Staphylococcaceae*, and *Brevibacteriaceae*). Overall, a single mechanism may be not sufficient to explain the persistence of the microbiota in all insects. Indeed, some insect immune systems lack PGRPs and an

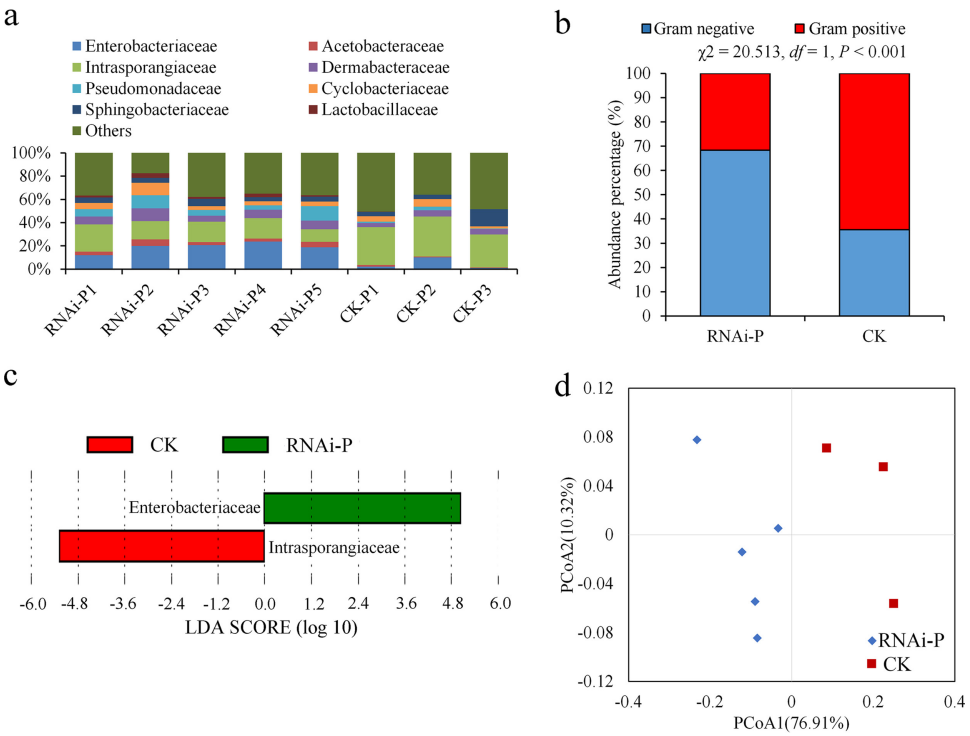

FIG 5 Comparison of the microbiotal structure between RNAi and control pupa samples. (a) Percentages of bacterial phyla in the RNAi treatment and control groups. Only taxonomic units with a relative abundance greater than 2% are shown. (b) Percentages of Gram-positive and Gram-negative bacteria between RNAi and control pupae. (c) LEfSe analysis based on OTU abundance in RNAi and control pupae. (d) PCoA plot based on the microbiotal structure in different treatments.

intact IMD pathway (41). Researchers have also hypothesized that other candidate immune effectors, including lysozyme and small cysteine-rich proteins, may contribute to the regulation of the microbiota in insect hosts (42, 43). Research in *B. dorsalis* has indicated that the dual-oxidase (BdDuox) gene can regulate intestinal bacterial community homeostasis (44). Thus, the immune effectors that regulate the microbial communities in *B. dorsalis* may vary. Regardless of the mechanism, our results support the hypothesis that the microbial communities in different developmental stages correlate with immune-related genes.

MATERIALS AND METHODS

Rearing and collection of *B. dorsalis*. *B. dorsalis* was collected from a carambola (*Averrhoa carambola*) orchard (23°06′53.09″N, 113°24′51.29″E) in Guangzhou, Guangdong Province, China, in April 2008 and reared as previously described (13). Briefly, the flies were reared under the following conditions: 25 ± 1°C, 16:8 h light:dark cycle, and 70 to 80% relative humidity (RH). The flies were reared for 72 generations.

Bacterial community characterization by Illumina Hiseq2500 sequencing. Seventeen samples of *B. dorsalis* (6 larvae, 6 pupae, and 5 adults) were collected. Each sample consisted of one individual. All samples were washed with 75% alcohol for 2 min and transferred to centrifuge tubes containing DNA extraction buffer (with lysozyme). Total DNA was extracted from the samples using a DNA extraction kit (Tiangen, Beijing, China) following the manufacturer's instructions. Approximately 465 bp of the bacterial 16S rRNA gene V3-V4 region was amplified and paired-end sequenced (2 × 250) using the Illumina platform according to standard protocols (13). To obtain high-quality clean reads, raw reads were filtered according to the following rules: (i) removal of reads containing more than 10% unknown nucleotides (N) and (ii) removal of reads containing less than 80% of bases with quality, i.e., a Q value of 20.

Paired-end clean reads were merged as raw tags using FLASH (v 1.2.11) with a minimum overlap of 10 bp and mismatch error rates of 2%. The effective tags were clustered into operational taxonomic units (OTUs) of ≥97% similarity using the UPARSE pipeline. The tag sequence with highest abundance was selected as the representative sequence within each cluster. The representative sequences were classified into organisms by a naive Bayesian model using the RDP classifier (v 2.2) based on SILVA Database (https://www.arb-silva.de/). The Shannon index and all other alpha diversity indices were calculated in QIIME (v 1.9.1). OTU rarefaction and rank abundance curves were plotted in QIIME. Unweighted UniFrac

TABLE 3 Samples used to construct the SSH libraries

Sample name[a]	Bacterium	Insect stage	Sampling time after injection
SaA	*Staphylococcus aureus*	Adult	2 h, 4 h, 5 h, 6 h, 12 h, 30 h, 42 h, 48 h, 72 h
SaL	*Staphylococcus aureus*	Larva	2 h, 4 h, 5 h, 6 h, 12 h, 18 h, 24 h, 36 h, 42 h
EcP	*Escherichia coli*	Pupa	2 h, 4 h, 5 h, 6 h, 36 h, 54 h, 72 h

[a]SaA, *Staphylococcus aureus*-infected adult; SaL, *Staphylococcus aureus*-infected larva; EcP, *Escherichia coli*-infected pupa.

distance matrices generated by QIIME were used to calculate beta diversity and visualized by principal-coordinate analysis (PCoA).

The same methods were also used to analyze the microbiota community composition in larvae, pupae, and adults after RNAi treatment for 72 h. To exclude potential sequencing noise from the environment, OTUs with abundance below 0.02 in all samples were abandoned. OTUs were classified as Gram positive or Gram negative using *Bergey's Manual of Systematics of Archaea and Bacteria* (45).

Immune-related gene screening by SSH. Suppression subtractive hybridization (SSH) is a powerful technique that enables specific cloning of expressed sequence tags (ESTs) that are differentially expressed in different mRNA populations (46), and this method was used to identify immune-related genes that may be associated with microbial communities in *B. dorsalis*. Briefly, *S. aureus* (Gram positive) (10^8 CFU/ml) and *E. coli* (Gram negative) (10^8 CFU/ml) were prepared in insect saline solution (130 mM NaCl, 5 mM KCl, and 1 mM $CaCl_2$) to induce expression of immune-related genes. Three-day-old *B. dorsalis* adults were prepared and injected with 0.5 μl of *S. aureus* in the base of the middle leg and then placed in cages and reared as previously described (13). The samples consisted of 3 females and 3 males, with 3 replicate samples. For larvae, second-instar larvae were prepared and injected with 1 μl of *S. aureus* at the thoracolumbar junction. The treated larvae were placed in a box with an artificial diet, and samples of 6 larvae in 3 replicates were collected. For pupae, 2-day-old samples were prepared and injected with 0.2 μl of *E. coli* in the middle of the body and then placed in petri dishes. Six pupae per sample in 3 replicates were collected. The sampling information is listed in Table 3. Untreated flies (injected with saline solution) were used as controls (drivers).

Total RNA was isolated from the samples using TRIzol reagent (Invitrogen), and the RNA quantity was analyzed using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific Inc., USA). mRNA was purified from total RNA using an Oligotex dT30 mRNA kit (TaKaRa, Japan) according to the manufacturer's instructions. SSH was performed using the PCR-select cDNA SSH kit (Clontech, USA); the detailed protocol was previously reported (47). The resulting PCR products enriched for differentially expressed cDNAs were separately cloned into the TA cloning system (Invitrogen, USA) to construct forward and reverse libraries. These ligated products were transformed into competent cells of *E. coli* strain JM109. White transformants for both (forward and reverse) libraries were randomly selected and cultured in Luria-Bertani broth supplemented with 100 mg/ml of ampicillin, followed by plasmid isolation.

Plasmids of the 1128 bacterial clones of the resulting SSH library were isolated, and sequencing was performed using the T7/SP6 primers. The sequences of the clones were examined for vector contamination using VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/), and the vector and adaptor sequences were trimmed. Low-quality and short (<100-bp) sequences were excluded from the analysis. Computational annotation of ESTs was performed using Blast2GO version 3.3.5. Homology was assessed in the NCBI protein database by BLASTX searches using an E value set to 1.0e−1.

Transcriptome analysis for flies in different developmental stages. To verify the SSH results, transcriptomes for *B. dorsalis* larvae, pupae, and adults were sequenced and analyzed. Total RNA was isolated from the following developmental stages in a 1:1 female/male ratio: second-instar larvae, 2-day-old pupae, and newly emerged adults (within 5 days of eclosion). Two samples were collected for each developmental stage. The RNA extraction method was the same as that used for SSH, and transcriptome sequencing and analysis were performed according to a previously described protocol (48).

Expression validation by quantitative real-time PCR. cDNA was reverse transcribed from 1 μg of total RNA using a TIANscript reverse transcription kit (Tiangen). Relative mRNA expression levels were measured normalized to α-tubulin (48) and RpL32 (44). The primer information for the genes is listed in Table 4. PCR amplification was conducted using the Mx3000P spectrofluorometric thermal cycler (Stratagene) beginning with a 2-min incubation at 95°C, followed by 40 cycles of 95°C for 20 s, 55°C for

TABLE 4 Primer information for the genes in this study

Primer name	Primer sequence (5′–3′)[a]	Product size (bp)
16S rRNA	F, CCTACGGGNGGCWGCAG; R, RCCTACGGGNGGCWGCAG	465
PGRP-LB	F, AGCGAACTCTTTGGGAGACG; R, AAGAGATTACGACGTGGCCG	176
PGRP-SB1	F, ACCCAACTACAACCGCAACA; R, ACTCTTCCAATGGGGCCAAG	229
a-TUB	F, CGCATTCATGGTTGATAACG; R, GGGCACCAAGTTAGTCTGGA	184
RpL32	F, CCCGTCATATGCTGCCAACT; R, GCGCGCTCAACAATTTCCTT	148
PGRP-LB-SiRNA	Sense, GGGUUAUUCCUCUUAUCAAUG; antisense, UUGAUAAGAGGAAUAACCCAA	
PGRP-SB1-SiRNA	Sense, GCACCGAGACGGAGUGCAAGC; antisense, UUGCACUCCGUCUCGGUGCUG	

[a]F, forward; R, reverse.

30 s, and 72°C for 30 s. Relative gene expression data were analyzed using the threshold cycle ($2^{-\Delta\Delta CT}$) method.

siRNA synthesis and delivery by injection. Small interfering RNAs (siRNAs) targeting genes were synthesized using a chemical method with specific primers (Table 4). Needles were prepared with a puller at 60.8°C (PC-10; Narishige, Tokyo, Japan). Microinjection was performed using an Eppendorf micromanipulation system (microinjector for cell biology, FemtoJet 5247; Eppendorf, Hamburg, Germany) under conditions of Pi (injection pressure of microinjector) of 300 hPa and injection time of 0.3 s. Gene silencing experiments were performed by injecting 1 μl of a 2-μg/μl solution of siRNA into the ventral abdomen of larvae, pupae, or adults; 1 μl of elution buffer was injected into flies as a control.

Statistical analysis. Differences in gene expression during different development stages were compared using one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons. Bacterial abundance comparisons were performed using the LEfSe method (49). For 16S rRNA sequencing results, unweighted UniFrac distance matrices were applied to calculate beta diversity and were visualized by principal-coordinate analysis. PERMANOVA (based on beta-diversity metrics) for the microbial community of *B. dorsalis* was implemented in PRIMER 7.0. The assignment of Gram-positive and Gram-negative bacteria identified in *B. dorsalis* was based on *Bergey's Manual of Systematics of Archaea and Bacteria* (45). Specifically, the classification information of annotated OTUs was searched in the manual individually, and references were added to the OTU tables (Data Sets S1 and S3) and other supplemental material. For OTUs with no classification information in the manual, detailed references with classifying information in Web of Science were searched. A chi-square test was then employed to compare the ratios of Gram-positive and Gram-negative bacteria across treatments.

Accession number(s). The sequencing data for paired-end clean reads have been uploaded to NCBI SRA under project number PRJNA523612. The sequencing data for microbiota community compositions for larvae, pupae, and adults have been uploaded to NCBI SRA under project number PRJNA523027. The sequencing data for transcriptome analysis of flies at different developmental stages have been uploaded to NCBI under project numbers PRJNA420604 and PRJNA528850.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00803-19.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.2 MB.

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REFERENCES

1. Frago E, Dicke M, Godfray H. 2012. Insect symbionts as hidden players in insect-plant interactions. Trends Ecol Evol 27:705–711. https://doi.org/10.1016/j.tree.2012.08.013.
2. Berasategui A, Shukla S, Salem H, Kaltenpoth M. 2016. Potential applications of insect symbionts in biotechnology. Appl Microbiol Biotechnol 100:1567–1577. https://doi.org/10.1007/s00253-015-7186-9.
3. Douglas AE, Minto LB, Wilkinson TL. 2001. Quantifying nutrient production by the microbial symbionts in an aphid. J Expe Biol 204:349–358.
4. Febvay G, Rahbe Y, Rynkiewicz M, Guillaud J, Bonnot G. 1999. Fate of dietary sucrose and neosynthesis of amino acids in the pea aphid, *Acyrthosiphon pisum*, reared on different diets. J Exp Biol 202:2639–2652.
5. Feldhaar H, Straka J, Krischke M, Berthold K, Stoll S, Mueller MJ, Gross R. 2007. Nutritional upgrading for omnivorous carpenter ants by the endosymbiont Blochmannia. BMC Biol 5:48. https://doi.org/10.1186/1741-7007-5-48.
6. McCutcheon JP, Moran NA. 2007. Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. Proc Natl Acad Sci U S A 104:19392–19397. https://doi.org/10.1073/pnas.0708855104.
7. Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp* APS. Nature 407:81–86. https://doi.org/10.1038/35024074.
8. Piel J. 2002. A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of Paederus beetles. Proc Natl Acad Sci U S A 99:14002–14007. https://doi.org/10.1073/pnas.222481399.
9. Kaltenpoth M, Gottler W, Herzner G, Strohm E. 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. Curr Biol 15:475–479. https://doi.org/10.1016/j.cub.2004.12.084.
10. Degnan PH, Moran NA. 2008. Diverse phage-encoded toxins in a protective insect endosymbiont. Appl Environ Microbiol 74:6782–6791. https://doi.org/10.1128/AEM.01285-08.
11. Cirimotich CM, Dong YM, Clayton AM, Sandiford SL, Souza-Neto JA, Mulenga M, Dimopoulos G. 2011. Natural microbe-mediated refractoriness to Plasmodium infection in *Anopheles gambiae*. Science 332:855–858. https://doi.org/10.1126/science.1201618.
12. Diaz-Albiter H, Sant'Anna MRV, Genta FA, Dillon RJ. 2012. Reactive oxygen species-mediated immunity against *Leishmania mexicana* and *Serratia marcescens* in the phlebotomine sand fly *Lutzomyia longipalpis*. J Biol Chem 287:23995–24003. https://doi.org/10.1074/jbc.M112.376095.
13. Cheng DF, Guo ZJ, Riegler M, Xi ZY, Liang GW, Xu YJ. 2017. Gut symbiont enhances insecticide resistance in a significant pest, the oriental fruit fly *Bactrocera dorsalis* (Hendel). Microbiome 5:13. https://doi.org/10.1186/s40168-017-0236-z.
14. Ha EM, Oh CT, Bae YS, Lee WJ. 2005. A direct role for dual oxidase in *Drosophila* gut immunity. Science 310:847–850. https://doi.org/10.1126/science.1117311.
15. Kikuchi Y, Hayatsu M, Hosokawa T, Nagayama A, Tago K, Fukatsu T. 2012. Symbiont-mediated insecticide resistance. Proc Natl Acad Sci U S A 109:8618–8622. https://doi.org/10.1073/pnas.1200231109.
16. Douglas AE, Dobson AJ. 2013. New synthesis: animal communication mediated by microbes: fact or fantasy? J Chem Ecol 39:1149. https://doi.org/10.1007/s10886-013-0343-7.
17. He MY, Jiang JJ, Cheng DF. 2017. The plant pathogen *Gluconobacter cerinus* strain CDF1 is beneficial to the fruit fly *Bactrocera dorsalis*. AMB Express 7:207. https://doi.org/10.1186/s13568-017-0514-y.

18. Zhao XF, Zhang XY, Chen ZS, Wang Z, Lu YY, Cheng DF. 2018. The divergence in bacterial components associated with *Bactrocera dorsalis* across developmental stages. Front Microbiol 9:114. https://doi.org/10.3389/fmicb.2018.00114.
19. Adams AS, Currie CR, Cardoza Y, Klepzig KD, Raffa KF. 2009. Effects of symbiotic bacteria and tree chemistry on the growth and reproduction of bark beetle fungal symbionts. Can J Res 39:1133–1147. https://doi.org/10.1139/X09-034.
20. Archie EA, Theis KR. 2011. Animal behaviour meets microbial ecology. Anim Behav 82:425–436. https://doi.org/10.1016/j.anbehav.2011.05.029.
21. Feldhaar H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36:533–543. https://doi.org/10.1111/j.1365-2311.2011.01318.x.
22. Ha EM, Lee KA, Seo YY, Kim SH, Lim JH, Oh BH, Kim J, Lee WJ. 2009. Coordination of multiple dual oxidase-regulatory pathways in responses to commensal and infectious microbes in *Drosophila* gut. Nat Immunol 10:949–957. https://doi.org/10.1038/ni.1765.
23. Kaltenpoth M. 2009. Actinobacteria as mutualists: general healthcare for insects? Trends Microbiol 17:529–535. https://doi.org/10.1016/j.tim.2009.09.006.
24. Florez LV, Biedermann PHW, Engl T, Kaltenpoth M. 2015. Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. Nat Prod Rep 32:904–936. https://doi.org/10.1039/C5NP00010F.
25. Kaltenpoth M, Engl T. 2014. Defensive microbial symbionts in Hymenoptera. Funct Ecol 28:315–327. https://doi.org/10.1111/1365-2435.12089.
26. Minard G, Mavingui P, Moro CV. 2013. Diversity and function of bacterial microbiota in the mosquito holobiont. Parasit Vectors 6:146. https://doi.org/10.1186/1756-3305-6-146.
27. Kwong WK, Moran NA. 2016. Gut microbial communities of social bees. Nat Rev Microbiol 14:374–384. https://doi.org/10.1038/nrmicro.2016.43.
28. Werner T, Liu G, Kang D, Ekengren S, Steiner H, Hultmark D. 2000. A family of peptidoglycan recognition proteins in the fruit fly *Drosophila melanogaster*. Proc Natl Acad Sci U S A 97:13772–13777. https://doi.org/10.1073/pnas.97.25.13772.
29. Charroux B, Capo F, Kurz CL, Peslier S, Chaduli D, Viallat-Lieutaud A, Royet J. 2018. Cytosolic and secreted peptidoglycan-degrading enzymes in *Drosophila* respectively control local and systemic immune responses to microbiota. Cell Host Microbe 23:215–228. https://doi.org/10.1016/j.chom.2017.12.007.
30. Bosco-Drayon V, Poidevin M, Boneca IG, Narbonne-Reveau K, Royet J, Charroux B. 2012. Peptidoglycan sensing by the receptor PGRP-LE in the *Drosophila* gut induces immune responses to infectious bacteria and tolerance to microbiota. Cell Host Microbe 12:153–165. https://doi.org/10.1016/j.chom.2012.06.002.
31. Dawadi B, Wang X, Xiao R, Muhammad A, Hou Y, Shi Z. 2018. PGRP-LB homolog acts as a negative modulator of immunity in maintaining the gut-microbe symbiosis of red palm weevil, *Rhynchophorus ferrugineus* Olivier. Dev Comp Immunol 86:65–77. https://doi.org/10.1016/j.dci.2018.04.021.
32. Wang J, Aksoy S. 2012. PGRP-LB is a maternally transmitted immune milk protein that influences symbiosis and parasitism in tsetse's offspring. Proc Natl Acad Sci U S A 109:10552–10557. https://doi.org/10.1073/pnas.1116431109.
33. Mellroth P, Steiner H. 2006. PGRP-SB1: an N-acetylmuramoyl L-alanine amidase with antibacterial activity. Biochem Biophys Res Commun 350:994–999. https://doi.org/10.1016/j.bbrc.2006.09.139.
34. Zaidman-Remy A, Poidevin M, Herve M, Welchman DP, Paredes JC, Fahlander C, Steiner H, Mengin-Lecreulx D, Lemaitre B. 2011. *Drosophila* immunity: analysis of PGRP-SB1 expression, enzymatic activity and function. PLoS One 6:e17231. https://doi.org/10.1371/journal.pone.0017231.
35. Kaneko T, Goldman WE, Mellroth P, Steiner H, Fukase K, Kusumoto S, Harley W, Fox A, Golenbock D, Silverman N. 2004. Monomeric and polymeric Gram-negative peptidoglycan but not purified LPS stimulate the *Drosophila* IMD pathway. Immunity 20:637–649. https://doi.org/10.1016/S1074-7613(04)00104-9.
36. Antwis RE, Haworth RL, Engelmoer DJP, Ogilvy V, Fidgett AL, Preziosi RF. 2014. Ex situ diet influences the bacterial community associated with the skin of red-eyed tree frogs (*Agalychnis callidryas*). PLoS One 9:e85563. https://doi.org/10.1371/journal.pone.0085563.
37. Egert M, Marhan S, Wagner B, Scheu S, Friedrich MW. 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris* L. (Oligochaeta: Lumbricidae). FEMS Microbiol Ecol 48:187–197. https://doi.org/10.1016/j.femsec.2004.01.007.
38. Rebollar EA, Hughey MC, Medina D, Harris RN, Ibanez R, Belden LK. 2016. Skin bacterial diversity of Panamanian frogs is associated with host susceptibility and presence of *Batrachochytrium dendrobatidis*. ISME J 10:1682–1695. https://doi.org/10.1038/ismej.2015.234.
39. Anselme C, Vallier A, Balmand S, Fauvarque MO, Heddi A. 2006. Host PGRP gene expression and bacterial release in endosymbiosis of the weevil *Sitophilus zeamais*. Appl Environ Microbiol 72:6766–6772. https://doi.org/10.1128/AEM.00942-06.
40. Wang JW, Wu YN, Yang GX, Aksoy S. 2009. Interactions between mutualist Wigglesworthia and tsetse peptidoglycan recognition protein (PGRP-LB) influence trypanosome transmission. Proc Natl Acad Sci U S A 106:12133–12138. https://doi.org/10.1073/pnas.0901226106.
41. Gerardo NM, Altincicek B, Anselme C, Atamian H, Barribeau SM, De Vos M, Duncan EJ, Evans JD, Gabaldon T, Ghanim M, Heddi A, Kaloshian I, Latorre A, Moya A, Nakabachi A, Parker BJ, Perez-Brocal V, Pignatelli M, Rahbe Y, Ramsey JS, Spragg CJ, Tamames J, Tamarit D, Tamborindeguy C, Vincent-Monegat C, Vilcinskas A. 2010. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. Genome Biol 11:R21. https://doi.org/10.1186/gb-2010-11-2-r21.
42. Nakabachi A, Shigenobu S, Sakazume N, Shiraki T, Hayashizaki Y, Carninci P, Ishikawa H, Kudo T, Fukatsu T. 2005. Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that harbors an endocellular mutualistic bacterium, *Buchnera*. Proc Natl Acad Sci U S A 102:5477–5482. https://doi.org/10.1073/pnas.0409034102.
43. Shigenobu S, Stern DL. 2012. Aphids evolved novel secreted proteins for symbiosis with bacterial endosymbiont. Proc Biol Sci 280:20121952. https://doi.org/10.1098/rspb.2012.1952.
44. Yao ZC, Wang AL, Li YS, Cai ZH, Lemaitre B, Zhang HY. 2016. The dual oxidase gene BdDuox regulates the intestinal bacterial community homeostasis of *Bactrocera dorsalis*. ISME J 10:1037–1050. https://doi.org/10.1038/ismej.2015.202.
45. Whitman WB, Rainey F, Kämpfer P, Trujillo M, Chun J, DeVos P, Hedlund B, Dedysh S (ed). 2012. Bergey's manual of systematics of archaea and bacteria. Wiley, New York, NY.
46. Diatchenko L, Lau YFC, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD. 1996. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 93:6025–6030. https://doi.org/10.1073/pnas.93.12.6025.
47. Shaw AK, Bhardwaj PK, Ghosh S, Azahar I, Adhikari S, Adhikari A, Sherpa AR, Saha SK, Hossain Z. 2017. Profiling of BABA-induced differentially expressed genes of Zea mays using suppression subtractive hybridization. RSC Adv 7:43849–43865. https://doi.org/10.1039/C7RA06220F.
48. Shen GM, Dou W, Niu JZ, Jiang HB, Yang WJ, Jia FX, Hu F, Cong L, Wang JJ. 2011. Transcriptome analysis of the oriental fruit fly (*Bactrocera dorsalis*). PLoS One 6:e29127. https://doi.org/10.1371/journal.pone.0029127.
49. Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RLV, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat Biotechnol 31:814–821. https://doi.org/10.1038/nbt.2676.