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INVERTEBRATE MICROBIOLOGY

Established Cotton Stainer Gut Bacterial Mutualists Evade Regulation by Host Antimicrobial Peptides

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ABSTRACT Symbioses with microorganisms are ubiquitous in nature and confer important ecological traits to animal hosts but also require control mechanisms to ensure homeostasis of the symbiotic interactions. In addition to protecting hosts against pathogens, animal immune systems recognize, respond to, and regulate mutualists. The gut bacterial symbionts of the cotton stainer bug, *Dysdercus fasciatus*, elicit an immune response characterized by the upregulation of c-type lysozyme and the antimicrobial peptide pyrrhocoricin in bugs with their native gut microbiota compared to that in dysbiotic insects. In this study, we investigated the impact of the elicited antimicrobial immune response on the established cotton stainer gut bacterial symbiont populations. To this end, we used RNA interference (RNAi) to knock down immunity-related genes hypothesized to regulate the symbionts, and we subsequently measured the effect of this silencing on host fitness and on the abundance of the major gut bacterial symbionts. Despite successful downregulation of target genes by both ingestion and injection of double-stranded RNA (dsRNA), the silencing of immunity-related genes had no effect on either host fitness or the qualitative and quantitative composition of established gut bacterial symbionts, indicating that the host immune responses are not actively involved in the regulation of the nutritional and defensive gut bacterial mutualists. These results suggest that close associations of bacterial symbionts with their hosts can result in the evolution of mechanisms ensuring that symbionts remain insensitive to host immunological responses, which may be important for the evolutionary stability of animal-microbe symbiotic associations.

IMPORTANCE Animal immune systems are central for the protection of hosts against enemies by preventing or eliminating successful infections. However, in the presence of beneficial bacterial mutualists, the immune system must strike a balance of not killing the beneficial symbionts while at the same time preventing enemy attacks. Here, using the cotton stainer bug, we reveal that its long-term associated bacterial symbionts are insensitive to the host's immune effectors, suggesting adaptation to the host's defenses, thereby strengthening the stability of the symbiotic relationship. The ability of the symbionts to elicit host immune responses but remain insensitive themselves may be a mechanism by which the symbionts prime hosts to fight future pathogenic infections.

KEYWORDS AMP, gut microbiota, Hemiptera, Heteroptera, immune system, insect, regulation, symbiosis, vitamin, RNAi

Insects are the most diverse and successful group of animals on earth (1, 2). This can be attributed not only to their morphological and behavioral adaptations but also to ecological interactions with other organisms, including microorganisms, which confer insect hosts with novel traits, allowing them to expand into diverse ecological niches (2, 3). Insect-associated mutualistic bacteria can be essential for host nutrition and digestion, defense against natural enemies, detoxification of harmful compounds, adaptation to challenging environments, and host behavior manipulation (3–10). Thus,

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mutualistic bacteria are integral to the functional ecology of their insect hosts, and concordantly, hosts have evolved mechanisms ensuring reliable and efficient acquisition, maintenance, and transmission of the beneficial bacterial partners (11, 12).

While mutualistic bacteria confer their hosts with novel capabilities, their regulation is essential to avoid uncontrolled proliferation, which can be costly to the host (13). Accordingly, insect hosts have evolved mechanical, nutritional, chemical, and immunological mechanisms to ensure maintenance of mutualistic bacterial populations necessary for their needs. In some symbiotic relationships, insect hosts such as aphids and *Sitophilus oryzae* beetles have evolved specialized cells called bacteriocytes that physically confine and restrict the growth of their bacterial symbionts (14, 15). In other mutualistic relationships, hosts are known to restrict their extracellular symbionts in specialized structures such as midgut crypts, antennal reservoirs, or larval symbiont bearing organs, as reported, e.g., for stinkbugs, beewolves, and *Lagria* beetles, respectively (16–18).

In addition to confinement and restriction in specialized cells or structures, the availability and amount of essential nutrients required by the microbial partner play important roles in the establishment or proliferation of symbionts (19, 20). For instance, the population densities of *Spiroplasma poulsonii*, the *Drosophila melanogaster* endosymbiont, and *Buchnera aphidicola*, the pea aphid endosymbiont, correlate positively with the host lipid and nitrogen levels, respectively (19–21). This suggests that diet and host nutritional status have an impact on symbiont proliferation. This is supported by simulation experiments by Mitri et al. (22), who demonstrated that nutrient limitation on microbial colonization surfaces can drive the structure and functioning of microbial assemblages. In addition, intra- and interspecific antagonistic or cooperative interactions among cocolonizing symbionts can directly influence their composition and abundance in the host (23). The dominant gut bacterial symbionts of the honey bee, *Snodgrassella alvi* and *Gilliamella apicola*, for example, have complementary metabolic capabilities essential for joint resource utilization and cross-feeding interactions, which subsequently affect their abundance and ability to jointly colonize the host (23–25).

Insects are also known to possess an elaborate innate immune system that not only defends them against pathogens, but also has the ability to recognize and regulate bacterial mutualists (14, 15, 26–28). For instance, the *Burkholderia* symbiont of the bean bug *Riptortus pedestris*, which is confined to specialized midgut crypts, is highly susceptible to the insect's humoral immune responses (27, 29). A strong immunological response characterized by the upregulation of c-type lysozyme, pyrrhocoricin-like, and rip-thanatin antimicrobial peptides in the midgut efficiently controls *Burkholderia* symbiont populations in the crypts (27, 30). Likewise, *Sitophilus* sp. weevils' ColA antimicrobial peptide is important not only for containing the *Sitophilus* primary endosymbiont within the bacteriocyte but also for regulating symbiont growth by inhibiting cell division (14, 31, 32). While our knowledge of the interactions between the insects' immune system and beneficial microbes has increased considerably in the past decades, a general understanding of the molecular mechanisms underlying the maintenance of a mutualistic microbiota while at the same time ensuring an efficient defense against antagonists remains lacking.

The African cotton stainer bug, *Dysdercus fasciatus* (Hemiptera: Pyrrhocoridae), possesses a simple and stable core bacterial community in the midgut, which is composed of *Hungatella* sp., *Klebsiella* sp., *Coriobacterium glomerans*, *Gordonibacter* sp., and *Rickettsiales* bacteria (33, 34). These gut symbionts supplement the host with B vitamins that are limiting in their seed-based diet, and they were recently shown to provide protection against a trypanosomatid parasite, *Leptomonas pyrrhocoris* (33, 35, 36). Due to their functional importance, the symbionts are maintained in host populations through both vertical and horizontal transmission routes (37, 38), which are also exploited by the *L. pyrrhocoris* parasite for its own transmission within *D. fasciatus* populations (38). Dysbiotic insects (deprived of core gut bacteria and parasites) can be generated by interrupting the symbiont and parasite transmission routes (33, 37, 38), allowing investigation of the gut bacterial symbionts' contribution to host fitness and

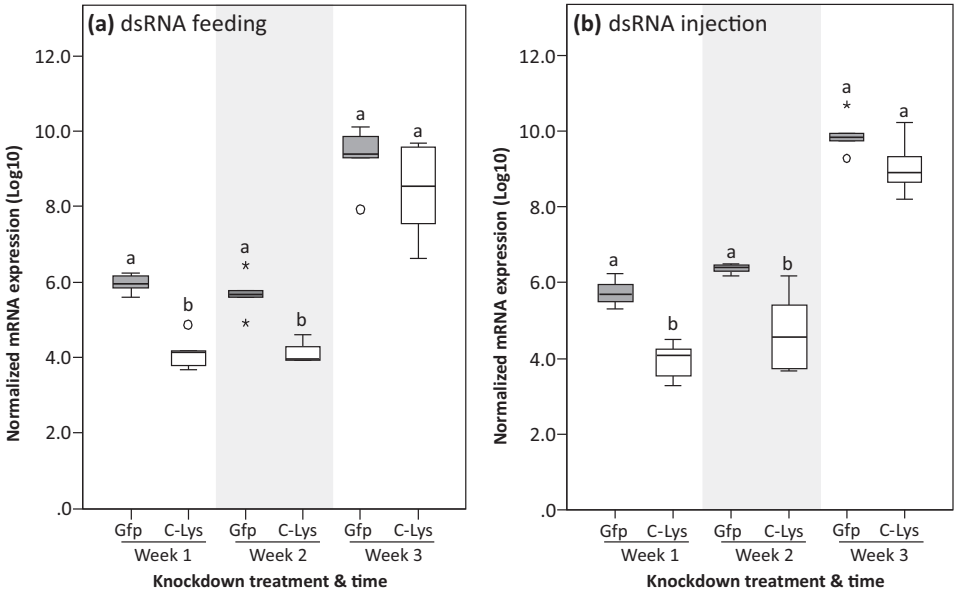

FIG 1 Efficiency of RNAi-mediated knockdown of *D. fasciatus*' c-type lysozyme after dsRNA feeding (a) and dsRNA injection (b). Dark boxes represent control knockdown individuals exposed to dsRNA for the green fluorescent protein (Gfp), white boxes show expression levels in bugs treated with dsRNA targeting c-type lysozyme (C-Lys). Significant differences between treatments and controls are represented by different lowercase letters above boxes (Mann-Whitney U tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the ranges, circles represent outliers, and stars represent extreme values.

physiology as well as host-symbiont-parasite interactions. Comparative transcriptomics of cotton stainer insects with native gut bacterial communities and dysbiotic insects revealed a differential expression of genes of the insect's innate immunity pathways, i.e., Imd, Toll, JAK/STAT, and phenoloxidase pathways (39). In particular, c-type lysozyme and the antimicrobial peptide (AMP) pyrrhocoricin showed significantly higher expression levels in insects with native bacteria, while the expression levels of the AMPs hemipterin and defensin were upregulated in dysbiotic insects (39).

Here, we hypothesized that the antimicrobial effectors overexpressed in *D. fasciatus* in the presence of native gut microbial symbionts may be involved in the regulation of the cotton stainer's gut bacterial community. To test this hypothesis, we established an efficient RNA interference (RNAi)-mediated gene knockdown procedure, which we used to silence the expression of key immunity-related genes of the Toll and Imd pathways. We subsequently measured the effect of silencing on insect fitness correlates (developmental time, weight, and survival rates) and quantified the abundance of the core bacterial community to determine the interaction between the host immunity-related genes and the essential nutritional and defensive gut bacterial symbionts.

RESULTS

Optimal dsRNA delivery method in cotton stainers. To determine the optimal method for delivering double-stranded RNA (dsRNA) to achieve significant knockdown of *D. fasciatus*' genes, we exposed bugs to dsRNA for the c-type lysozyme gene by either feeding or injecting, and we subsequently compared the knockdown efficiencies and the durations of silencing for both methods for 3 weeks. Our results show that both feeding and injecting efficiently delivered c-type lysozyme dsRNA molecules, resulting in a significant knockdown of up to 2 orders of magnitude in the first and second week after dsRNA exposure (Fig. 1a and b) (Mann-Whitney U tests, $P < 0.05$). Although the expression levels for both methods remained lower in the knockdown treatments than in the controls throughout the third week, the differences were no longer significant (Fig. 1a and b) (Mann-Whitney U tests, $P > 0.05$).

RNAi-mediated knockdown of immunity-related genes in *D. fasciatus*. To study the role of the immune system in the regulation of established gut bacterial symbionts,

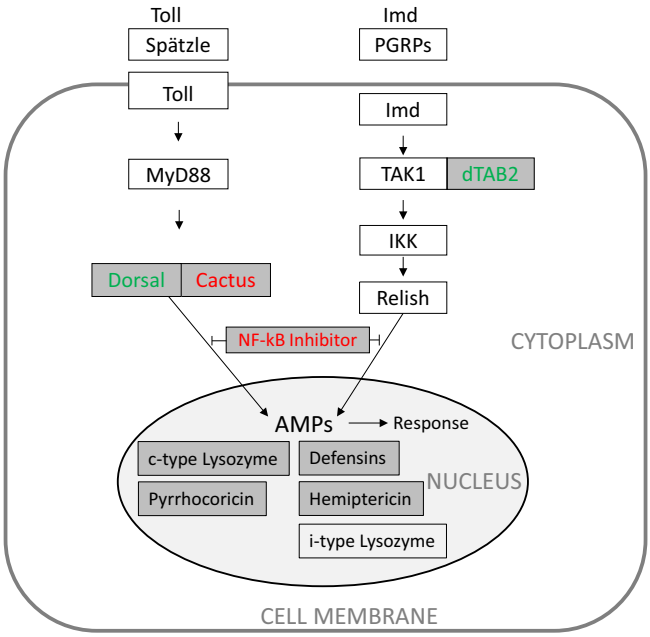

FIG 2 Schematic diagram of the insect Toll and Imd pathways and silenced genes in this study. Gray boxes represent all genes targeted for knockdown, genes in red inhibit transcription of effector genes, while genes in green enhance transcription of effector genes. Figure modified from reference 39.

we silenced the expression of candidate immunity-related genes by RNA interference (RNAi) in late 2nd instar *D. fasciatus* nymphs, a stage where the core gut bacterial community is already mostly established (34). By feeding the respective dsRNA to the bugs, we silenced genes encoding the immune effectors c-type lysozyme, pyrrhocoricin, two forms of defensin (defensin 1 and defensin 2), and hemipthericin (Fig. 2, black in gray boxes). We also targeted genes upstream in the Toll and Imd pathways, respectively, encoding Dorsal and Tab (Fig. 2, green) that enhance the expression of effector genes, as well as Cactus and NF-κB inhibitor (Fig. 2, red) that inhibit the expression of effector genes (40).

Quantitative PCRs 1 week after RNAi treatment revealed that the expression levels of the target genes in the knockdown treatments were lower than those of control individuals fed dsRNA of the green fluorescent protein (GFP) gene by at least 1 order of magnitude, except for NF-κB inhibitor and hemipthericin. The transcript levels of c-type lysozyme and pyrrhocoricin, which were previously found to be significantly overexpressed in the presence of *D. fasciatus*' native gut bacterial symbionts (39), were significantly reduced by 2 and 1 orders of magnitude, respectively, after knockdown (Fig. 3e and f). Wilcoxon signed-rank tests revealed that our knockdown strategy significantly decreased the expression levels of all targeted genes except for hemipthericin (Fig. 3) (Wilcoxon signed-rank test, $P < 0.05$). Although there were lower transcript levels of hemipthericin in the knockdown treatments than in the controls, this difference was not significant (Fig. 3g) (Wilcoxon signed-rank test, $P = 0.496$).

Impact of immunity-related gene knockdown on insect fitness. Once we established that the target genes had been successfully knocked down, we sought to evaluate the effect of knockdown on insect fitness correlates, i.e., developmental time (time between knockdown and adult emergence), weight upon adult emergence, and survival rate until adulthood (survivorship from the time of RNAi treatment until emergence). Across the seven treatments, developmental time and insect weight upon adult emergence were not significantly different (Fig. 4a and b) (Friedman test: developmental time, χ^2 [6] = 5.282, $P = 0.508$; weight at emergence, χ^2 [6] = 8.816, $P = 0.184$). Similarly, there were no differences in survival rates between each of the six knockdown treatments and the control treatment (Fig. 5) (Cox mixed-effects model, $P > 0.05$).

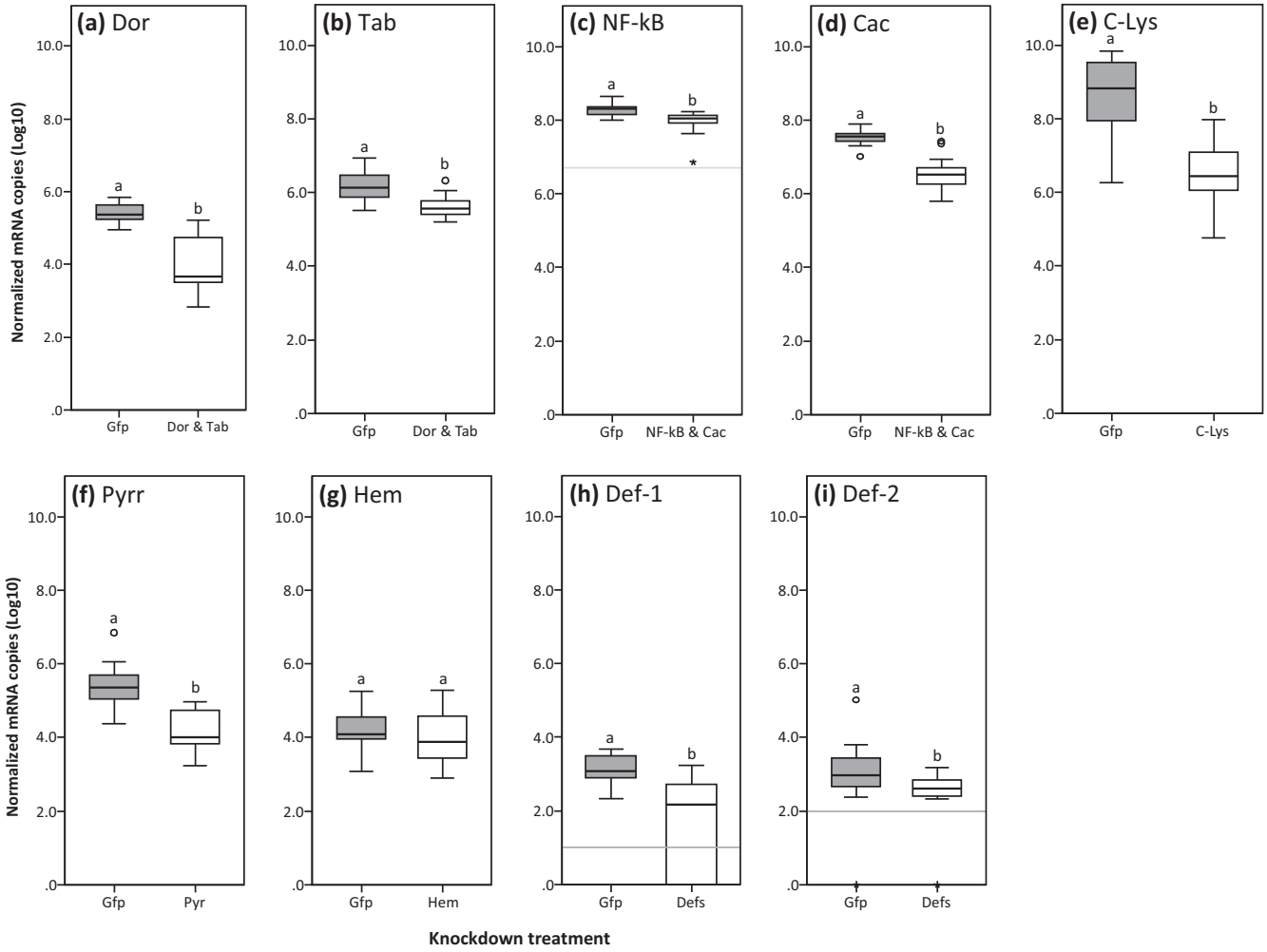

FIG 3 Expression of target immunity-related genes in *D. fasciatus* nymphs 1 week after knockdown. RNAi-mediated knockdown resulted in significant decreases in the expression of Dorsal (Dor) (a), Tak1 binding protein (Tab) (b) NF-kappa B inhibitor (NF-κB) (c), Cactus (Cac) (d), c-type lysozyme (C-Lys) (e), pyrrhocoricin (Pyr) (f), and defensin 1 (h) and defensin 2 (i) (Defs) in comparison to those in control individuals fed with dsRNA targeting the GFP gene, while hemipterecin (Hem) (g) showed an insignificant decrease in transcript levels after knockdown. Significant differences are represented by different lowercase letters above boxes (Wilcoxon signed-rank tests). Detection threshold is 0 if not indicated by the gray horizontal line (negative control in the qPCR). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the ranges, circles represent outliers, and stars represent extreme values.

Impact of immunity-related gene knockdown on the composition of the *D. fasciatus* gut bacterial community. Establishment of the core members of the gut bacterial community of firebugs (*Hungatella* sp., *C. glomerans*, *Gordonibacter* sp., and *Klebsiella* sp.) occurs in the 2nd instar stage (34). To determine if *D. fasciatus*' immune system is actively involved in the regulation of the already established gut bacterial symbionts, we knocked down the expression of target genes in the late 2nd instar nymphs and quantified by quantitative PCR (qPCR) the 16S rRNA copy numbers of the core gut bacterial symbionts 1 week after knockdown as well as after emergence as adults. After 1 week of RNAi knockdown, normalized 16S rRNA copy numbers of *C. glomerans* and *Hungatella* sp. in nymphs were statistically significantly different across treatments (Fig. 6a and c) (Friedman test: *C. glomerans*, χ^2 [6] = 16.286, $P = 0.012$; *Hungatella*, χ^2 [6] = 14.971, $P = 0.02$). However, Dunn-Bonferroni *post hoc* tests did not reveal any significant differences between the control and any of the six knockdown treatments. Instead, significant differences were observed between c-type lysozyme (C-Lys) and Dorsal and Tak1 binding protein (Dor&Tab) knockdown treatments ($P < 0.05$) as well as between C-Lys and defensin 1 and defensin 2 (Defs) knockdown treatments ($P < 0.05$) for *C. glomerans* (Fig. 6a) and between pyrrhocoricin (Pyr) and

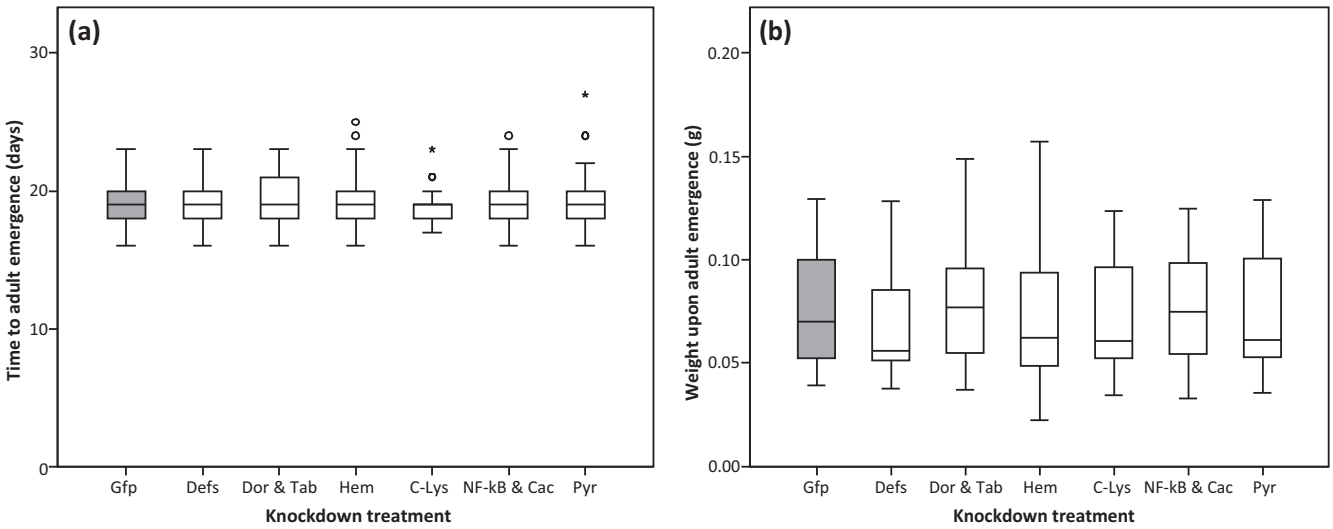

FIG 4 *D. fasciatus* developmental times (a) and weights upon adult emergence (b) after RNAi-mediated knockdown of immunity-related genes. Times between gene knockdown and adult emergence (a) and weights after adult emergence (b) were not significantly different across treatments (Friedman tests with Dunn-Bonferroni *post hoc* tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the ranges, circles represent outliers, and stars represent extreme values. Gfp, green fluorescent protein; Defs, defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, hemipticin; C-Lys, c-type lysozyme; NF-κB, NF-kappa B inhibitor; Cac, Cactus; Pyr, pyrrhocoricin.

Dor&Tab knockdown treatments for *Hungatella* sp. ($P = 0.05$) (Fig. 6c). Although significant, the symbiont abundance in the treatments was decreased by less than 1 order of magnitude. Normalized 16S rRNA copy numbers of *Gordonibacter* sp. and *Klebsiella* sp. were not significantly different across the seven treatments (Fig. 6b and d) (Friedman test: *Gordonibacter*, χ^2 [6] = 4.041, $P = 0.671$; *Klebsiella*, χ^2 [6] = 12.143, $P = 0.059$).

Similarly, adults that emerged from the dsRNA-treated nymphs showed minor changes in the bacterial community, with only *Hungatella* sp. showing statistically

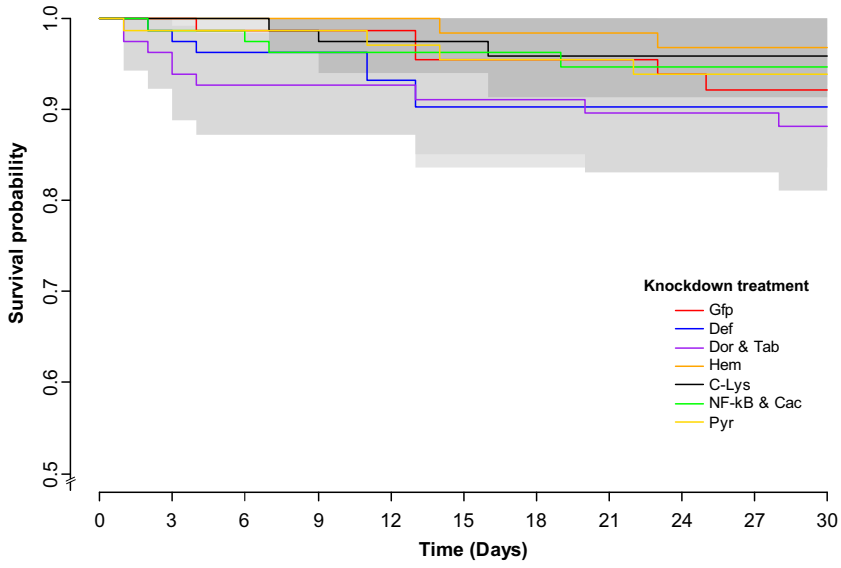

FIG 5 *D. fasciatus* survival rates after RNAi-mediated knockdown of target immunity-related genes. Survival rates of insects in each immunity-related gene knockdown treatment were not significantly different from those of the control treatment ($P > 0.05$, Cox mixed-effects model). Gray blocks represent 95% confidence levels. Gfp, green fluorescent protein; Defs, defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, hemipticin; C-Lys, c-type lysozyme; NF-κB, NF-kappa B inhibitor; Cac, Cactus; Pyr, pyrrhocoricin.

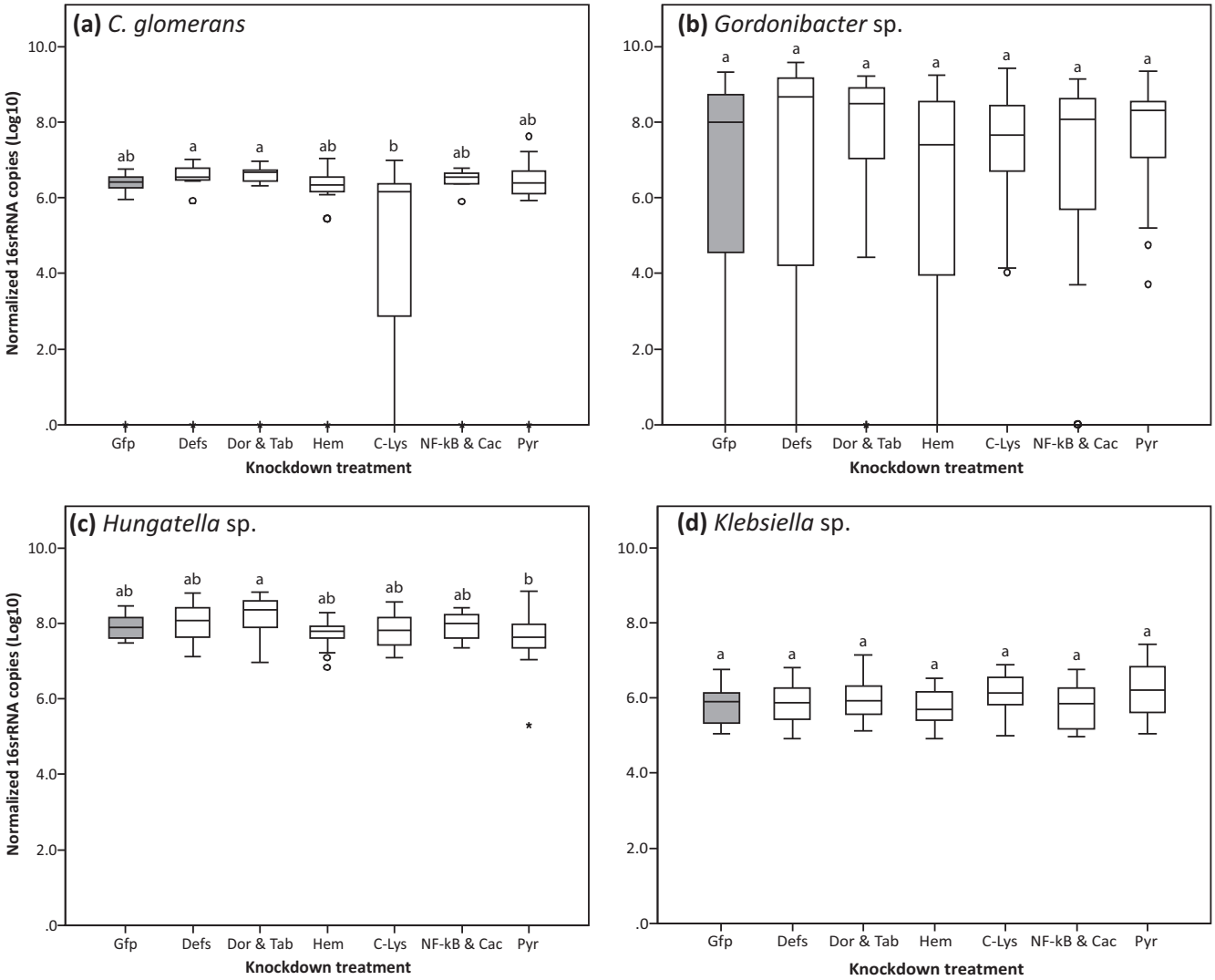

FIG 6 Abundances of the core bacterial taxa in *D. fasciatus* nymphs 1 week after knockdown of key immunity-related genes. Significant differences of 16S rRNA copies of *C. glomerans* (a), *Gordonibacter* sp. (b), *Hungatella* sp. (c), and *Klebsiella* sp. (d) as revealed by qPCR are indicated by different lowercase letters above the boxes (Friedman tests with Dunn-Bonferroni *post hoc* tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the ranges, circles represent outliers, and stars represent extreme values. Gfp, green fluorescent protein; Defs, defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, hemipterricin; C-Lys, c-type lysozyme; NF-κB, NF-kappa B inhibitor; Cac, Cactus; Pyr, pyrrhocoricin.

significant differences across treatments (Fig. 7c) (Friedman test: χ^2 [6] = 16.071, P = 0.013). Dunn-Bonferroni *post hoc* tests revealed statistically significant differences between the GFP control treatment and both the Pyr and Defs knockdown treatments ($P < 0.05$). *Hungatella* abundance in these treatments was decreased by approximately 1 order of magnitude compared to that in the GFP controls. The abundances of *C. glomerans*, *Gordonibacter*, and *Klebsiella* did not differ significantly across treatments (Fig. 7a, b, and d) (Friedman test: *C. glomerans*, χ^2 [6] = 12.429, P = 0.053; *Gordonibacter*, χ^2 [6] = 7.745, P = 0.257; *Klebsiella*, χ^2 [6] = 1.469, P = 0.962).

DISCUSSION

In addition to defending hosts against pathogenic infections, animal immune systems play an important role in the molecular cross talk of hosts and their beneficial microbes in many animal-bacterial symbioses (14, 28). Here, we studied the interaction of the pyrrhocorid bug *D. fasciatus* with its nutritional and defensive gut bacterial symbionts (*Coriobacterium glomerans*, *Gordonibacter* sp., *Hungatella* sp., and *Klebsiella* sp.) via the host immune system. Using a target gene knockdown approach, we report

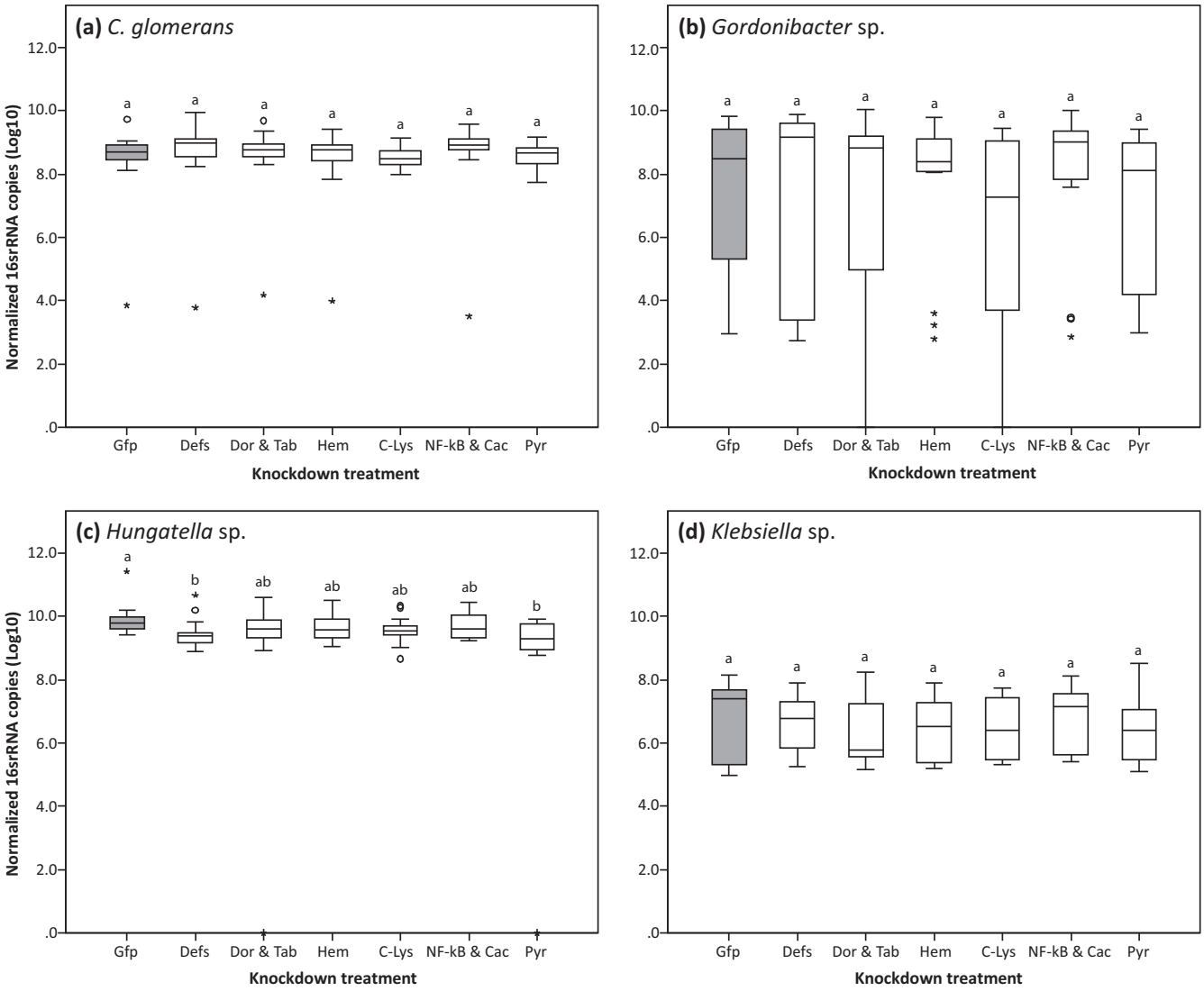

FIG 7 Abundances of the core bacterial taxa in adult *D. fasciatus* that emerged from nymphs treated with dsRNA silencing target immunity-related genes. Significant differences of 16S rRNA copies of *C. glomerans* (a), *Gordonibacter* sp. (b), *Hungatella* sp. (c), and *Klebsiella* sp. (d) as revealed by qPCR are indicated by the different lowercase letters above the boxes (Friedman tests with Dunn-Bonferroni *post hoc* tests). Boxes comprise 25th to 75th percentiles, lines in boxes represent medians, whiskers denote the ranges, circles represent outliers, and stars represent extreme values. Gfp, green fluorescent protein; Defs, defensins; Dor, Dorsal; Tab, Tak1 binding protein; Hem, hemipterican; C-Lys, c-type lysozyme; NF-κB, NF-kappa B inhibitor; Cac, Cactus; Pyr, pyrrhocoricin.

that after establishment, *D. fasciatus*' gut bacterial symbionts were not affected by the insect's antimicrobial peptides, although some of them were overexpressed in the presence of the bacterial symbionts. Concordantly, the insect's developmental time, weight gain, and survival rate were not significantly affected by the knockdown of immunity-related genes.

The success of RNAi-mediated gene knockdown in insects is highly variable across species as well as genes or even life stages of the same insect (41). This is because of the difficulty associated with the delivery, uptake, processing, and trafficking of dsRNA molecules required to trigger RNAi and the variability in the transcript suppression period (41, 42). In our experiments, we tested the efficiency of the two traditional dsRNA delivery methods that are commonly used in insect gene function studies to knockdown the expression of important cotton stainer immunity-related genes. Our results show that dsRNA molecules delivered by both injecting and feeding were readily taken up by the cotton stainer and processed into small interfering RNAs (siRNAs) to initiate posttranscriptional gene silencing as witnessed by the significant

knockdown of the target genes (Fig. 1 and 3). In other hemipteran insect species, such as *Pyrrhocoris apterus* (Pyrrhocoridae), *Oncopeltus fasciatus* (Lygaeidae), and *Rhodnius prolixus* (Reduviidae), RNAi via injection has been used successfully to study genes associated with their growth and development (43, 44). Our results provide more evidence on the applicability of the injection method in the delivery of dsRNA for RNAi studies in heteropteran insects. Additionally, we show that feeding is an equally reliable technique for conducting successful RNAi-mediated silencing experiments, in terms of both the degree and the duration of knockdown (Fig. 1). Being a noninvasive and simple procedure compared to injection, feeding is more applicable in large-scale RNAi experiments. For example, in the honey bee (*Apis mellifera*), where both injection and feeding methods efficiently deliver dsRNA for gene knockdown (45, 46), feeding has been used successfully in the ecological application of RNAi in improving honeybee health and resistance against ecologically important viral infections (46).

Removal of the essential cotton stainer gut bacterial symbionts not only affects host fitness but also changes the expression pattern of the host immunity-related genes (39). Transcriptome and qPCR analyses showed a higher expression of c-type lysozyme and pyrrhocoricin in bugs harboring native gut bacteria than in dysbiotic bugs (39). This suggested that these immune effectors might be involved in the regulation of the beneficial gut bacterial symbionts. Contrary to this expectation, however, knockdown of these immunity-related genes did not have an effect on host insect fitness or on the abundance of the established core gut bacterial symbionts throughout the insect's development (Fig. 4 to 6), indicating that the symbionts are insensitive to the host immune effectors. The cotton stainer's gut bacterial symbionts are important in B vitamin supplementation and protection against *L. pyrrhocoris* infections (33, 35, 36). Similarities in insect fitness correlates (developmental time, survivorship, and weight at emergence) between the different knockdown treatments and the control treatment affirm that all seven treatments had access to the essential symbionts supplying B vitamins required for development. This is corroborated by the qualitative and quantitative consistency of the core gut bacterial symbionts across the seven treatments (Fig. 6 and 7). Thus, the symbionts may have adapted and become insensitive to the host immune responses that they trigger, stabilizing this nutritional and protective mutualism. The ability of the gut bacterial symbionts to elicit the cotton stainer's immune responses may be a mechanism by which they stimulate or prime the firebug immune system to fight pathogenic infections, thereby protecting the host (5, 47, 48). A similar effect has been observed in honey bees, where individuals with high levels of hymenoptaecin and apidaecin antimicrobial peptides as a result of harboring native gut bacterial symbionts are better protected upon infection with *Escherichia coli* (49).

The insensitivity of gut bacterial symbionts to AMPs as suggested by our results is contrary to results reported for other insects such as bean bugs (*Riptortus pedestris*) and *Sitophilus* grain weevils, where host antimicrobial peptides are actively involved in the regulation of established symbionts (14, 27, 29, 31). Nevertheless, our findings agree with other studies demonstrating resistance of symbionts to host AMPs. Similar to the cotton stainers, honey bees mount an innate immune response against their core gut bacterial symbionts (48, 49). In particular, honey bees harboring natural gut bacterial communities show a higher expression of hymenoptaecin and apidaecin antimicrobial peptides in the gut and hemolymph than bees with perturbed gut bacterial communities (49). Investigations into the function of these two antimicrobial peptides through *in vitro* experiments revealed that the major honey bee gut bacterial symbionts (*Snodgrassella alvi*, *Lactobacillus* Firm-5, *Bifidobacterium* sp., and *Gilliamella apicola*) are resistant to one or both AMPs (49), suggesting that the elicited AMPs are not directly involved in regulating the symbionts. Similarly, in the tsetse fly *Glossina morsitans morsitans*, the innate immunity system does not seem to be involved in the regulation of its *Wigglesworthia* and *Sodalis* endosymbionts, although these symbionts activate the tsetse fly's humoral and cellular immunity (47, 50). Furthermore, a strong immune response characterized by a higher expression of antimicrobial peptides as a result of *E. coli* and trypanosomatid challenge does not affect the titers of the two endosymbi-

onts (51). Therefore, our findings corroborate these studies, demonstrating that bacterial symbionts can be recognized by the host, resulting in the activation of the host immune responses to which the bacterial symbionts remain insensitive.

Pathogens successfully infect hosts either by evading immune system detection due to the lack of immune response elicitors or by avoiding killing through the suppression of host defense mechanisms (52). Likewise, long-term coevolution of beneficial bacterial symbionts with hosts may result in the adaptation of the symbionts to the hosts' immune system in a way that they are not recognized as foreign or they remain insensitive to the host immune responses (52, 53). The association of *D. fasciatus* and other pyrrhocorids with their characteristic symbiotic bacteria for approximately 80 million years (54) may have resulted in the evolution of resistance mechanisms by the symbionts aimed at evading the host antimicrobial immune responses, as suggested for other symbiotic bacteria (52, 53, 55). For instance, *Sodalis glossinidius*, a secondary endosymbiont of the tsetse fly, harbors a gene cluster whose expression products are responsible for the modification of the negative charge of lipid A, a component of the cell membrane lipopolysaccharides (53). This modification interferes with the ability of the tsetse fly's cationic AMPs to bind to the endosymbiont's cell membrane, resulting in resistance to these AMPs (53). Mutation of the regulatory systems for this gene cluster results in the susceptibility of *S. glossinidius* to the host's cationic AMPs and inability to colonize the host (53). Similarly, *Bacteroides thetaiotaomicron*, a human gut bacterium, harbors an enzyme responsible for the alteration of the negative charge on the lipopolysaccharides leading to decreased binding ability of AMPs responsible for membrane disruption (55). In *Riptortus pedestris*, the composition of the cell membrane lipopolysaccharides of its *Burkholderia* symbiont is essential not only for successful host colonization but also for symbiont titer regulation (28). As some of the cotton stainer gut bacteria are not easily cultivable, it is difficult at the moment to investigate whether any changes in their cell membrane composition are responsible for the ability of the gut bacterial symbionts to evade regulation by host AMPs.

Our findings do not, however, rule out other possible host mechanisms regulating the bacterial symbionts. Other than activating the expression of AMPs, the gut bacterial symbionts may also elicit other immune responses, such as the production of reactive oxygen species and cellular immune mechanisms, which could be important in symbiont regulation (40). In addition to providing a surface area for the adherence of gut bacterial symbionts, the peritrophic matrix of *D. fasciatus* provides the physicochemical conditions and nutrients that can influence symbiont growth dynamics (22, 34). For instance, the oxygen gradient present in the M3 midgut region of firebugs can act as a selection and regulation mechanism that influences microbial community growth dynamics within the midgut (34, 36). Additionally, cocolonization of the cotton stainers' peritrophic matrix by the different members of the gut bacterial community presents an opportunity for either antagonistic or synergistic interactions between symbionts that can greatly influence the composition and stability of the gut microbial community (25, 56).

In conclusion, our results indicate that the cotton stainer AMPs do not regulate already established gut bacterial symbionts, as knockdown of the AMPs that are overexpressed in the presence of the symbionts (or any other AMPs) did not change the qualitative or quantitative composition of the gut bacterial community. We speculate that the ability of the gut bacterial symbionts to elicit host immune responses may be a mechanism of immune priming to enhance the host's protection against future pathogenic infections. The insensitivity of the symbionts to the host immune responses may be due to the evolution of resistance against the host AMPs during the long-term association of the symbionts with the host. However, colonization succession studies of firebug gut bacterial symbionts show that the core gut bacterial symbionts are qualitatively and quantitatively established in the 2nd instar (34). Since we performed our knockdown experiments in the late 2nd instar stage when the core symbionts had already established, we cannot rule out that the investigated AMPs are important in shaping the gut microbial community during host colonization and early development.

We therefore propose further investigation into the role of AMPs before and during symbiont establishment.

MATERIALS AND METHODS

Insect culture source. We used cotton stainer insect cultures that are currently maintained in the laboratory at the Johannes Gutenberg University Mainz. The insects were originally collected in Comoé National Park, Côte d'Ivoire, in 2001 and were previously maintained at the University of Würzburg, Germany, and at the Max Planck Institute for Chemical Ecology, Jena, Germany. The composition and the abundance of the cotton stainer's gut microbial community remained unaffected despite the long-term maintenance in the laboratory (54). All experiments in this study were conducted in Fitotron standard growth chambers (Weiss Technik, Leicestershire, UK) under controlled environmental conditions: temperature of 26°C, 60% humidity, and long light regimes of 16-h days and 8-h nights.

Double-stranded RNA preparation. To generate dsRNA for the target genes, we used *D. fasciatus* transcript sequences from a previous transcriptomic study (39). Candidate sequences for dsRNA generation were first checked for the possibility of off-target knockdown by blasting them against a local BLAST database generated using all *D. fasciatus* transcript sequences. Only the unique regions of these sequences that had no matching sequences in the database other than the intended target were selected for further processing. Using Primer-BLAST, we designed specific primers for these unique regions of the target sequences and used them for a diagnostic PCR. A 12.5-μl PCR mixture was set up containing 6.4 μl H_2O, 1.5 μl reaction buffer S, 1.5 μl deoxynucleoside triphosphate (dNTP) mix, 1 μl of each primer (10 μM), 0.1 μl of Peqlab DNA polymerase (Peqlab, Erlangen, Germany), and 1 μl of the cDNA template synthesized from *D. fasciatus* total RNA. The following PCR conditions were used: denaturation at 94°C for 30 s, annealing at 58°C for 40 s, extension at 72°C for 45 s, and a final extension at 72°C for 4 min. The PCR product was run on an agarose gel, purified with the innuPREP PCRpure kit (Analytik Jena, Jena, Germany), and sequenced with an ABI 3130 capillary sequencer (Applied Biosystems, CA, USA). Specificity of the primers was further confirmed by blasting the primer sequences and their respective PCR products against the local database. Once the primers' specificity was confirmed, T7 RNA polymerase promoter sequence was added to the primers' 5′ ends and then used to perform a second PCR. The PCR conditions stated above were used, but the annealing temperature was increased by 5°C after the first 5 cycles because of the additional T7 RNA polymerase promoter sequence. The PCR product with the T7 RNA polymerase promoter sequence was purified as stated above and used as a template for dsRNA synthesis using a MEGAscript RNAi kit (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacturer's protocol. Briefly, a 20-μl reaction mixture was set up containing ~0.1 to 0.2 μg of the template, 2 μl of each of the dNTPs (75 mM each), 2 μl of the T7 reaction buffer, 2 μl of the T7 enzyme mix, and nuclease-free water. The reaction components were mixed by gentle pipetting and then incubated at 37°C for 12 h for dsRNA synthesis. The transcribed dsRNA was mixed with 1 μl TURBO DNase and incubated at 37°C for 30 min to digest the DNA template. The DNA-free dsRNA was checked on a low-percentage agarose gel, and once it was confirmed to be of the expected size, it was purified using the ethanol precipitation protocol. Briefly, 30 μl of nuclease-free water and 5 μl of 3 M sodium acetate were added followed by 150 μl of absolute ethanol. The solution was mixed thoroughly by vortexing and incubated at −20°C for 1 h, after which it was centrifuged at 4°C and 15,000 rpm for 15 min. The pellet was washed once with 200 μl of 70% ethanol and air dried for 15 min before it was dissolved in 50 μl of nuclease-free water. RNA concentration was determined using the Varioskan LUX multifunction microplate reader (Thermo Scientific, MA, USA) before storage at −20°C until use.

Establishing the most efficient method for dsRNA delivery. To establish the optimal method for delivering dsRNA to knock down cotton stainer target genes, two *D. fasciatus* egg clutches (~35 eggs each) were collected from the main cultures, one for each of the two methods investigated, i.e., feeding and injecting. The eggs were maintained under the conditions specified above until hatching. The newly hatched nymphs were fed *ad libitum* with autoclaved water and a linden seed diet until they reached the late second instar, when they were divided into two groups, i.e., knockdown and control treatments. RNAi was performed by depriving the insects of water for 24 h and then feeding or injecting them with dsRNA of c-type lysozyme to test for the efficiency of feeding and injection methods of dsRNA delivery, respectively. One microliter of dsRNA with a concentration of ~2 $\mu g \cdot \mu l^{-1}$ was used per insect for both methods. For the feeding method, a droplet of the dsRNA was offered to the group of insects in a cage, while for the injection method, the insects were first anaesthetized with CO_2 for 1 min and then attached to a suction pump before dsRNA was injected into the first abdominal segment using the ES-blastocyst injection straight pipette with a spike needle (Biomedical Instruments, Zöllnitz, Germany) mounted to the CellTram vario manual microinjector (Eppendorf, Hamburg, Germany). For the controls, mock knockdown was performed using dsRNA targeting the transcript for green fluorescent protein (GFP). After dsRNA delivery, the insects were reared in their respective cages and fed *ad libitum* with autoclaved water and a linden seed diet. To evaluate the efficiency and duration of knockdown for each of the two dsRNA delivery methods, 3 to 6 bugs, depending on the number surviving after silencing, were sampled on day 7, day 14, and day 21 after the RNAi knockdown procedure for RNA extraction and subsequent qPCR.

Experimental set-up to investigate the role of immunity-related genes in symbiont regulation. Fifteen adult *D. fasciatus* mating pairs were collected from the main cultures and kept in small sterile box cages (14 cm by 8 cm by 6 cm) until they laid eggs. Egg clutches with >35 eggs were collected for this experiment and incubated in sterile petri dishes lined with moist filter papers at 26°C and 60% humidity until hatching. The newly hatched nymphs were fed *ad libitum* with autoclaved water and a linden tree seed diet until they reached the late 2nd instar (~7 days after hatching), when they were randomly divided into seven groups of equal sizes. RNAi targeting single genes or a combination of two genes/two

TABLE 1 qPCR primers used to quantify the expression of target gene transcripts

Primer name	Primer sequence (5′→3′)	*D. fasciatus* target gene[a]	Sequence ID(s)[b]
Cact-1F	GGCCTGATCTCTTCGCCTAC	Cactus	Dfas-16185
Cact-1R	AACAAAAAGGCAGTCGTCGC		
Nf_kappa_qPCR_1F	ACTCTTCCGGTCCTCTCGAA	NF-kappa B inhibitor	Dfas-48512 and Dfas-53732
Nf_kappa_qPCR_1R	AGCTTAACACGCTCGACCAA		
Dorsal_1F	CCGGCTCTTTAGCCAACATC	Dorsal	Dfas-36948
Dorsal_2R	ACAGTTGCCAAGGTTGAAACA		
Lyso_For_1	CTTTCCAACCCTGAATGCTC	C-type lysozyme	Dfas-30397
Lyso_Reverse	AGCACGGACTACGGACTGTT		
Hemi_1_qpc_for	TGAAGGCTCAGGGTAAC	Hemipticerin	Dfas-00011 and Dfas-46208
Hemip_2_rev	GTTTTCCTGTGCATCGCTGT		
Pyrrho qpc for	GCCAGAGCTTGAACAGGAA	Pyrrhocoricin	Dfas-00911 and Dfas-33105
Pyrrho qpc rev	TGTTGTATATCGGCCTTGGA		
Tab_qPCR_1F	AAAGGCCACCAGTTGTCAGG	Tak1 binding protein	Dfas-09234 and Dfas-30553
Tab_qPCR_1R	TGCAGCTAAACGGGCACTAA		
Def_for	CAACTTTCCAAACAAATCCACA	Defensin 1	Dfas-33854
Dfas_Def_1R	ACTGTCTTCTTGCAGCTCCC		
Defensin-1F	GGGTGTGAACCACTGGGATT	Defensin 2	Dfas-51099
Defensin-1R_Modified	TATGCGCCGCTATGGTC		

[a]Sequences of the genes can be retrieved from European Nucleotide Archive in the Sequence Read Archive database under accession number PRJEB6171 (39).
[b]ID, identifier.

isoforms of the defensin gene was performed as per the feeding protocol described above for all seven groups: Dor&Tab (Dorsal and Tak1 binding protein), NF-κB and Cac (NF-κB inhibitor and Cactus), Defs (defensin 1 and defensin 2), Hem (hemipticerin), Pyr (pyrrhocoricin), C-Lys (c-type lysozyme), and GFP (control). All bugs undergoing knockdown treatments were maintained on autoclaved water and linden seed diets in sterile cages until the end of the experiment. Survival rates, developmental time to adulthood, measured as the duration from silencing until 50% of the bugs in a replicate treatment had molted into adults, and weight at emergence, computed as the average weight of individuals within each replicate treatment, were recorded to determine the effect of knockdown on *D. fasciatus* fitness. 1 week post-RNAi, one insect was sampled from each of the 7 treatments for all 15 replicates and subjected to RNA extraction and qPCR to check for knockdown success and the stability of the core gut microbial community. Additionally, upon reaching adulthood, the M3 midgut region was dissected from one insect per replicate treatment to check for the effect of knockdown on the core gut bacteria upon completion of the developmental period of the insects.

RNA extraction, cDNA synthesis, and qPCR. Total RNA was extracted from the whole insect for the nymphs or M3 gut region for the adults using the innuPREP RNA Mini isolation kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. Concentration and quality (optical density [OD] 260/280 nm and OD 260/230, respectively) of extracted RNA samples were determined using a Varioskan LUX multifunction microplate reader (Thermo Scientific, MA, USA). Reverse transcription-quantitative PCR (qRT-PCR) was performed using a Quantitect reverse transcription kit (Qiagen, Hilden, Germany) according to the manufacturer's guidelines. Briefly, genomic DNA was removed by setting up a 14-μl reaction mixture comprising ~0.2 μg of RNA template, 2 μl of gDNA wipeout buffer, and RNase-free water. DNA was digested by incubating the mixture at 42°C for 2 min, followed by the addition of 1 μl of the RT primer mix, 4 μl of the Quantiscript RT buffer, and 1 μl of the Quantiscript reverse transcriptase enzyme. This mixture was then incubated at 42°C for 30 min for cDNA synthesis, after which the enzyme was inactivated at 95°C for 3 min and the generated cDNA stored at −20°C until required for qPCR to measure gene expression or bacterial abundance.

To determine if RNAi-mediated knockdown of target genes was successful, qPCRs targeting immunity-related genes were set up with gene-specific primers (Table 1), which were designed and the specificity of determined as described above. For the quantification of core gut bacterial symbionts, qPCRs targeting the 16S rRNA genes were set up with specific primers for each of the symbionts as described previously (36). A qPCR was set up with a final reaction volume of 10 μl containing 0.5 μl of each primer (10 μM), 5 μl SYBR mix, 3 μl of qPCR H_2O, and 1 μl of either template or standard or negative control (H_2O). An additional qPCR targeting *D. fasciatus*' 18S rRNA was performed for normalizing the expression of knockdown genes and abundance of the core gut bacterial symbionts. qPCRs were conducted on the Rotor-Gene Q cycler (Qiagen, Hilden, Germany) with cycling conditions as described previously (36). Quantification of the copy number of the expressed immunity-related genes and bacterial 16S rRNA was determined using Rotor-Gene Q software as previously described (36). Gene expression levels and abundance of the core bacterial symbionts were normalized with host 18S rRNA copy numbers prior to data analyses.

Data analysis. All our experiments were performed by splitting up the same egg clutches and distributing the individuals equally across treatments, resulting in each replicate consisting of siblings. Therefore, our data met the requirements to be analyzed with paired test statistics, since individuals in each treatment were related. Accordingly, we used Wilcoxon signed-rank tests to assess differences in relative gene expression between the knockdown versus the control treatments. However, we used Mann-Whitney U tests to compare knockdown success when determining the efficient method to deliver

dsRNA for knocking down genes in *D. fasciatus* because of unequal sample size distribution for the injected treatments where the invasive procedure resulted in the death of some individuals. Friedman tests with Dunn-Bonferroni *post hoc* tests were used to compare differences in the times of development, weights of emerged individuals, and symbiont abundances across the different treatments. These tests were conducted using SPSS statistics 23.0 (IBM, NY, USA). Survival probabilities of insects were compared across the different treatments as described previously, by using Cox mixed-effects models as implemented in R 3.4.1 (36).

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REFERENCES

1. Grimaldi D, Engel MS. 2005. Evolution of the insects. Cambridge University Press, Cambridge, United Kingdom.
2. Schowalter T. 2011. Insect ecology: an ecosystem approach. Academic Press, Cambridge, MA.
3. Douglas AE. 2015. Multiorganismal insects: diversity and function of resident microorganisms. Annu Rev Entomol 60:17–34. https://doi.org/10.1146/annurev-ento-010814-020822.
4. 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.
5. Flórez 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.
6. Berasategui A, Salem H, Paetz C, Santoro M, Gershenzon J, Kaltenpoth M, Schmidt A. 2017. Gut microbiota of the pine weevil degrades conifer diterpenes and increases insect fitness. Mol Ecol 26:4099–4110. https://doi.org/10.1111/mec.14186.
7. Bosch TJM, Welte CU. 2017. Detoxifying symbionts in agriculturally important pest insects. Microb Biotechnol 10:531–540. https://doi.org/10.1111/1751-7915.12483.
8. Moran NA, Yun Y. 2015. Experimental replacement of an obligate insect symbiont. Proc Natl Acad Sci U S A 112:2093–2096. https://doi.org/10.1073/pnas.1420037112.
9. Engl T, Eberl N, Gorse C, Krüger T, Schmidt THP, Plarre R, Adler C, Kaltenpoth M. 2018. Ancient symbiosis confers desiccation resistance to stored grain pest beetles. Mol Ecol 27:2095–2108. https://doi.org/10.1111/mec.14418.
10. Engl T, Kaltenpoth M. 2018. Influence of microbial symbionts on insect pheromones. Nat Prod Rep 35:386–397. https://doi.org/10.1039/C7NP00068E.
11. Salem H, Florez L, Gerardo N, Kaltenpoth M. 2015. An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. Proc Biol Sci 282:20142957. https://doi.org/10.1098/rspb.2014.2957.
12. Onchuru TO, Martinez AJ, Ingham CS, Kaltenpoth M. 2018. Transmission of mutualistic bacteria in social and gregarious insects. Curr Opin Insect Sci 28:50–58. https://doi.org/10.1016/j.cois.2018.05.002.
13. Oliver KM, Moran NA, Hunter MS. 2006. Costs and benefits of a superinfection of facultative symbionts in aphids. Proc Biol Sci 273:1273–1280. https://doi.org/10.1098/rspb.2005.3436.
14. Login FH, Balmand S, Vallier A, Vincent-Monégat C, Vigneron A, Weiss-Gayet M, Rochat D, Heddi A. 2011. Antimicrobial peptides keep insect endosymbionts under control. Science 334:362–365. https://doi.org/10.1126/science.1209728.
15. 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.
16. Kaltenpoth M, Göttler 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.
17. Kikuchi Y, Hosokawa T, Fukatsu T. 2011. An ancient but promiscuous host-symbiont association between *Burkholderia* gut symbionts and their heteropteran hosts. ISME J 5:446–460. https://doi.org/10.1038/ismej.2010.150.
18. Flórez LV, Scherlach K, Gaube P, Ross C, Sitte E, Hermes C, Rodrigues A, Hertweck C, Kaltenpoth M. 2017. Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. Nat Commun 8:15172. https://doi.org/10.1038/ncomms15172.
19. Herren JK, Paredes JC, Schüpfer F, Arafah K, Bulet P, Lemaitre B. 2014. Insect endosymbiont proliferation is limited by lipid availability. Elife 3:e02964. https://doi.org/10.7554/eLife.02964.
20. Wilkinson TL, Koga R, Fukatsu T. 2007. Role of host nutrition in symbiont regulation: impact of dietary nitrogen on proliferation of obligate and facultative bacterial endosymbionts of the pea aphid *Acyrthosiphon pisum*. Appl Environ Microbiol 73:1362–1366. https://doi.org/10.1128/AEM.01211-06.
21. Paredes JC, Herren JK, Schüpfer F, Lemaitre B. 2016. The role of lipid competition for endosymbiont-mediated protection against parasitoid wasps in *Drosophila*. mBio 7:e01006-16. https://doi.org/10.1128/mBio.01006-16.
22. Mitri S, Clarke E, Foster KR. 2016. Resource limitation drives spatial organization in microbial groups. ISME J 10:1471–1482. https://doi.org/10.1038/ismej.2015.208.
23. Scherlach K, Hertweck C. 2018. Mediators of mutualistic microbe-microbe interactions. Nat Prod Rep 35:303–308. https://doi.org/10.1039/c7np00035a.
24. Kwong WK, Engel P, Koch H, Moran NA. 2014. Genomics and host specialization of honey bee and bumble bee gut symbionts. Proc Natl Acad Sci U S A 111:11509–11514. https://doi.org/10.1073/pnas.1405838111.
25. Kešnerová L, Mars RAT, Ellegaard KM, Troilo M, Sauer U, Engel P. 2017. Disentangling metabolic functions of bacteria in the honey bee gut. PLoS Biol 15:e2003467. https://doi.org/10.1371/journal.pbio.2003467.
26. Uvell H, Engström Y. 2007. A multilayered defense against infection: combinatorial control of insect immune genes. Trends Genet 23:342–349. https://doi.org/10.1016/j.tig.2007.05.003.
27. Park KE, Jang SH, Lee J, Lee SA, Kikuchi Y, Seo YS, Lee BL. 2018. The roles of antimicrobial peptide, rip-thanatin, in the midgut of *Riptortus pedestris*. Dev Comp Immunol 78:83–90. https://doi.org/10.1016/j.dci.2017.09.009.
28. Kim JK, Son DW, Kim CH, Cho JH, Marchetti R, Silipo A, Sturiale L, Park HY, Huh YR, Nakayama H, Fukatsu T, Molinaro A, Lee BL. 2015. Insect gut symbiont susceptibility to host antimicrobial peptides caused by alteration of the bacterial cell envelope. J Biol Chem 290:21042–21053. https://doi.org/10.1074/jbc.M115.651158.
29. Kim JK, Lee JB, Jang HA, Han YS, Fukatsu T, Lee BL. 2016. Understanding regulation of the host-mediated gut symbiont population and the symbiont-mediated host immunity in the *Riptortus-Burkholderia* symbi-

osis system. Dev Comp Immunol 64:75–81. https://doi.org/10.1016/j.dci.2016.01.005.
30. Kim JK, Han SH, Kim CH, Jo YH, Futahashi R, Kikuchi Y, Fukatsu T, Lee BL. 2014. Molting-associated suppression of symbiont population and up-regulation of antimicrobial activity in the midgut symbiotic organ of the *Riptortus-Burkholderia* symbiosis. Dev Comp Immunol 43:10–14. https://doi.org/10.1016/j.dci.2013.10.010.
31. Maire J, Vincent-Monégat C, Masson F, Zaidman-Rémy A, Heddi A. 2018. An IMD-like pathway mediates both endosymbiont control and host immunity in the cereal weevil *Sitophilus* spp. Microbiome 6:6. https://doi.org/10.1186/s40168-017-0397-9.
32. Anselme C, Pérez-Brocal V, Vallier A, Vincent-Monegat C, Charif D, Latorre A, Moya A, Heddi A. 2008. Identification of the weevil immune genes and their expression in the bacteriome tissue. BMC Biol 6:43. https://doi.org/10.1186/1741-7007-6-43.
33. Salem H, Kreutzer E, Sudakaran S, Kaltenpoth M. 2013. Actinobacteria as essential symbionts in firebugs and cotton stainers (Hemiptera, Pyrrhocoridae). Environ Microbiol 15:1956–1968. https://doi.org/10.1111/1462-2920.12001.
34. Sudakaran S, Salem H, Kost C, Kaltenpoth M. 2012. Geographical and ecological stability of the symbiotic mid-gut microbiota in European firebugs, *Pyrrhocoris apterus* (Hemiptera, Pyrrhocoridae). Mol Ecol 21:6134–6151. https://doi.org/10.1111/mec.12027.
35. Salem H, Bauer E, Strauss AS, Vogel H, Marz M, Kaltenpoth M. 2014. Vitamin supplementation by gut symbionts ensures metabolic homeostasis in an insect host. Proc Biol Sci 281:20141838. https://doi.org/10.1098/rspb.2014.1838.
36. Onchuru TO, Martinez AJ, Kaltenpoth M. 2018. The cotton stainer's gut microbiota suppresses infection of a cotransmitted trypanosomatid parasite. Mol Ecol 27:3408–3419. https://doi.org/10.1111/mec.14788.
37. Kaltenpoth M, Winter SA, Kleinhammer A. 2009. Localization and transmission route of *Coriobacterium glomerans*, the endosymbiont of pyrrhocorid bugs. FEMS Microbiol Ecol 69:373–383. https://doi.org/10.1111/j.1574-6941.2009.00722.x.
38. Salem H, Onchuru TO, Bauer E, Kaltenpoth M. 2015. Symbiont transmission entails the risk of parasite infection. Biol Lett 11:20150840. https://doi.org/10.1098/rsbl.2015.0840.
39. Bauer E, Salem H, Marz M, Vogel H, Kaltenpoth M. 2014. Transcriptomic immune response of the cotton stainer *Dysdercus fasciatus* to experimental elimination of vitamin-supplementing intestinal symbionts. PLoS One 9:e114865. https://doi.org/10.1371/journal.pone.0114865.
40. Tzou P, De Gregorio E, Lemaitre B. 2002. How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions. Curr Opin Microbiol 5:102–110. https://doi.org/10.1016/S1369-5274(02)00294-1.
41. Scott JG, Michel K, Bartholomay LC, Siegfried BD, Hunter WB, Smagghe G, Zhu KY, Douglas AE. 2013. Towards the elements of successful insect RNAi. J Insect Physiol 59:1212–1221. https://doi.org/10.1016/j.jinsphys.2013.08.014.
42. Shukla JN, Kalsi M, Sethi A, Narva KE, Fishilevich E, Singh S, Mogilicherla K, Palli SR. 2016. Reduced stability and intracellular transport of dsRNA contribute to poor RNAi response in lepidopteran insects. RNA Biol 13:656–669. https://doi.org/10.1080/15476286.2016.1191728.
43. Konopova B, Smykal V, Jindra M. 2011. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. PLoS One 6:e28728. https://doi.org/10.1371/journal.pone.0028728.
44. Hughes CL, Kaufman TC. 2000. RNAi analysis of Deformed, proboscipedia and Sex combs reduced in the milkweed bug *Oncopeltus fasciatus*: novel roles for Hox genes in the hemipteran head. Development 127:3683–3694.
45. Amdam GV, Simões ZLP, Guidugli KR, Norberg K, Omholt SW. 2003. Disruption of vitellogenin gene function in adult honey bees by intra-abdominal injection of double-stranded RNA. BMC Biotechnol 3:1. https://doi.org/10.1186/1472-6750-3-1.
46. Hunter W, Ellis J, Vanengelsdorp D, Hayes J, Westervelt D, Glick E, Williams M, Sela I, Maori E, Pettis J, Cox-Foster D, Paldi N. 2010. Large-scale field application of RNAi technology reducing Israeli acute paralysis virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). PLoS Pathog 6:e1001160. https://doi.org/10.1371/journal.ppat.1001160.
47. Weiss BL, Maltz M, Aksoy S. 2012. Obligate symbionts activate immune system development in the tsetse fly. J Immunol 188:3395–3403. https://doi.org/10.4049/jimmunol.1103691.
48. Emery O, Schmidt K, Engel P. 2017. Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee (*Apis mellifera*). Mol Ecol 26:2576–2590. https://doi.org/10.1111/mec.14058.
49. Kwong WK, Mancenido AL, Moran NA. 2017. Immune system stimulation by the native gut microbiota of honey bees. R Soc Open Sci 4:170003. https://doi.org/10.1098/rsos.170003.
50. Weiss BL, Wang J, Aksoy S. 2011. Tsetse immune system maturation requires the presence of obligate symbionts in larvae. PLoS Biol 9:e1000619. https://doi.org/10.1371/journal.pbio.1000619.
51. Rio RV, Wu Y.-n, Filardo G, Aksoy S. 2006. Dynamics of multiple symbiont density regulation during host development: tsetse fly and its microbial flora. Proc Biol Sci 273:805–814. https://doi.org/10.1098/rspb.2005.3399.
52. Vallet-Gely I, Lemaitre B, Boccard F. 2008. Bacterial strategies to overcome insect defences. Nat Rev Microbiol 6:302–313. https://doi.org/10.1038/nrmicro1870.
53. Pontes MH, Smith KL, de Vooght L, van den Abbeele J, Dale C. 2011. Attenuation of the sensing capabilities of PhoQ in transition to obligate insect-bacterial association. PLoS Genet 7:e1002349. https://doi.org/10.1371/journal.pgen.1002349.
54. Sudakaran S, Retz F, Kikuchi Y, Kost C, Kaltenpoth M. 2015. Evolutionary transition in symbiotic syndromes enabled diversification of phytophagous insects on an imbalanced diet. ISME J 9:2587. https://doi.org/10.1038/ismej.2015.75.
55. Cullen TW, Schofield WB, Barry NA, Putnam EE, Rundell EA, Trent MS, Degnan PH, Booth CJ, Yu H, Goodman AL. 2015. Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation. Science 347:170–175. https://doi.org/10.1126/science.1260580.
56. Speare L, Cecere AG, Guckes KR, Smith S, Wollenberg MS, Mandel MJ, Miyashiro T, Septer AN. 2018. Bacterial symbionts use a type VI secretion system to eliminate competitors in their natural host. Proc Natl Acad Sci U S A 115:e8528–e8537. https://doi.org/10.1073/pnas.1808302115.