Microbiology and Infectious Disease

Hosts Manipulate Lifestyle Switch and Pathogenicity Heterogeneity of Opportunistic Pathogens in the Single-cell Resolution

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Abstract

Host-microbe interactions are virtually bidirectional, but how the host affects their microbiome is poorly understood. Here, we report that the host is a critical modulator to regulate the lifestyle switch and pathogenicity heterogeneity of the opportunistic pathogens *Serratia marcescens* utilizing the *Drosophila* and bacterium model system. First, we find that *Drosophila* larvae efficiently outcompete *S. marcescens* and typically drive a bacterial switch from pathogenicity to commensalism toward the fly. Furthermore, *Drosophila* larvae reshape the transcriptomic and metabolic profiles of *S. marcescens* characterized by a lifestyle switch. More important, the host alters pathogenicity and heterogeneity of *S. marcescens* in the single-cell resolution. Finally, we find that larvae-derived AMPs are required to recapitulate the response of *S. marcescens* to larvae. Altogether, our findings provide an insight into the pivotal roles of the host in harnessing the life history and heterogeneity of symbiotic bacterial cells, advancing knowledge of the reciprocal relationships between the host and pathogen.

eLife assessment

The **fundamental** findings of this work substantially advance our understanding of the impact of the host on its gut microbes. The authors provided **compelling** evidence at single-cell resolution that the host can drive heterogeneity in the populations of gut microbes with significant consequences for the host physiology.

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Introduction

All metazoans ranging from insects to humans harbor a plethora of microbes referred to as the microbiome. The microbiome plays a pivotal role in host physiology and pathophysiology, with some species conferring benefits to the host and others causing damage (Delannoy-Bruno et al., 2021; Lynch and Hsiao, 2019; Morais et al., 2021). Over the past decades, tremendous effort, including ours (Jia et al., 2021; Liu et al., 2022; Liu et al., 2017), has been devoted to understanding the impact of microbial strains or more complex communities on their hosts. In fact, interactions between the host and microbiome are mutually bidirectional (Backhed et al., 2005; Jiang et al., 2019), conferring benefits to the host and microbial sides. Nevertheless, the knowledge of the effect of the host on the resident microbial community is still in its infancy. Studies reveal that hosts play a crucial role in shaping the assembly and composition of their unique microbiome (Muegge et al., 2011; Olm et al., 2022; Valeri and Endres, 2021). However, environmental fluctuations frequently happen at much shorter time scales that preclude bacterial adaptation by genetic mutation or species displacement. To cope with these situations, microbial communities have developed sophisticated transcriptional reprogramming to globally regulate gene expression (Avraham et al., 2015; Becattini et al., 2021; Prescott and Decho, 2020). Pathobionts routinely sense and specialize on host-derived substrates, execute context-dependent transitions from harmful to commensal states and generate the host-association continuum (Barak-Gavish et al., 2023; Proctor et al., 2023; Somvanshi et al., 2012). In this regard, the molecular mechanism by which the host restrictively controls gene transcription and metabolism of their microbiome remains much undefined.

Microbial populations frequently aggregate in several dozen micrometres and stochastically undergo specific differentiation into subpopulations with strikingly distinct properties, employing a strategy known as bet-hedging to survive rapid changes in the environment (Eldar and Elowitz, 2010; Raj and van Oudenaarden, 2008). As such, bacteria manifest heterogeneous transcriptional profiles, leading to phenotypic heterogeneity among individual bacterial cells (Dar et al., 2021; Gasch et al., 2017). Traditionally, gene expression of bacterial cells has been investigated in bulk or on a population level by mixing and averaging mRNA simultaneously from sorts of cells. Single-cell transcriptomics is revolutionizing the analysis of phenotypic cell- to-cell variation in eukaryotes, enabling us to explore the heterogeneity heretofore hidden within population behavior (Klein et al., 2015; Mancio-Silva et al., 2022; Perez et al., 2022). Recently, droplet-based high-throughput bacterial single-cell RNA sequencing methods have been developed and applied to study antibiotic-associated heterogeneous cellular states (Ma et al., 2023; Xu et al., 2023). We took advantage of this approach to tackle the challenge that the host induces transcriptionally distinct subpopulations of target bacteria during the interaction, contributing to a full appreciation of the phenotypic heterogeneity within microbiome.

Drosophila has a relatively simple bacterial community of commensal and opportunistic pathogens, typically predominated by 5 to 20 bacterial species. All *Drosophila* microbes are facultative bacteria that alternate between free-living and host-associated lifestyles. *Serratia marcescens* is an opportunistic pathogen and generates a pink pigment prodigiosin that is characteristically oscillatory to metabolic activities, making it a visible bioindicator of *S. marcescens* metabolism. In this regard, *Drosophila* has provided a promising model to study symbiosis, dysbiosis (Chandler et al., 2011; Kim et al., 2020).

To investigate whether and/or how the host modulates bacterial lifestyles, we used a reductionist approach in which germ-free (GF) *Drosophila* was re-associated with *S. marcescens* (Kuraishi et al., 2011). We found that *Drosophila* larvae sufficiently outcompeted *S. marcescens*, and resulted in shifts in the transcriptomic and metabolomic profile in bulk and single-cell resolution, providing a robust paradigm to further study the host-microbe interaction with the *Drosophila* model.

Results

Drosophila larvae alter surface topography and population size of microbes

All *Drosophila* microbes are facultative bacteria that alternate between free-living and host-associated lifestyles. Free-living bacteria initially form small microcolonies extending from the substratum, continue to expand, coalesce, and eventually generate a surface slick (a biofilm-like cell mat) that is visible to the naked eye (Koo and Yamada, 2016). Fascinatingly, the topography of surface slick was typically altered by fly colonization. The surface slick associated with strong wild-type flies was corn-like yellow, and the mature layer of the bacterial community was completely broken (**Figure 1A**). The destruction process was further exacerbated by overcrowded larvae that liquefied the upper food layer, leading to a yellow aqueous layer (**Figure 1B**). However, it was grey and partially segmented by weak flies (**Figure 1C**), and even pale and intact associated with infertile flies (**Figure 1D**). These results indicate that *Drosophila* plays a critical role in altering the topography of bacterial community on *Drosophila* media (Deines et al., 2020). Our data showed that the cultivable bacterial loads associated with weak or infertile flies were dramatically higher than those of strong stocks (**Figure 1E**), indicating that the host diminishes the bacterial load of the shared niche.

Given that *Drosophila* larvae and adults coexist in the bottles, we sought to determine which were directly responsible for these alterations. Similarly, bacteria loads were substantially higher in the medium associated with males and virgin females (without progenies) than in the control (**Figure 1F**), suggesting that larvae mainly cause a decline in bacterial population size. Consistently, the 50-day-aged flies that deposited few eggs mimicked weak flies with grey surface slick and a decline in bacterial loads. Moreover, transplanted larvae directly caused a dose-dependent decline in the overall bacterial loads in the habitat (**Figure 1G**). These findings demonstrate that *Drosophila* larvae play a critical role in outcompeting their symbionts in the shared habitat.

Drosophila larvae antagonize *S. marcescens* in the niche

To experimentally characterize the impact of the host on the symbiont, we applied a reductionist approach in which *Drosophila* mono-associated with *S. marcescens* was generated as depicted in **Figure 2A**. Crawling larvae (~96 h post oviposition) were concurrently transferred to fly food vials with 10^7 CFU bacterial load. We found that *S. marcescens* alone formed a pink surface slick in the medium over time (**Figure 2B**, top). The color intensity of surface slicks was initially accumulated, peaked at 24 h timepoint post-inoculation, but gradually faded thereafter. We quantified the optical density of prodigiosin inside surface slicks with the spectrophotometer as described (Kalivoda et al., 2010; Pan et al., 2020). The time-course amount of prodigiosin was congruent with the color density visible to the naked eye above (**Figure 2C**). Compared to *S. marcescens* alone, the color intensity of surface slicks was significantly dampened by larval transplantations (**Figure 2B**, bottom). As expected, the amount of prodigiosin of *S. marcescens* in co-culture substantially declined compared with that of *S. marcescens* alone. Next, we examined bacterial titers in the food at regular intervals. Our result showed that single *S. marcescens* grew more rapidly than coexisting *S. marcescens*, and the population of single *S. marcescens* reached the plateau value within 1 d (**Figure 2D**), indicating that the niche possesses a finite carrying capacity of bacteria. However, the population of coexisting *S. marcescens* reached the plateau value within 2 d. Moreover, the number of CFUs was predominantly suppressed by larva transplantation at the initial phases of colonization (within 72 h post-inoculation) compared to that of *S. marcescens* alone (**Figure 2D**), consistent with the result that larvae thwarted the number of total mixed bacteria in the diet (**Figure 2B**). Noteworthily, the growth of single *S. marcescens* was comparable (at 96 h post-inoculation) or even lower (at 120 h post-inoculation)

Legend

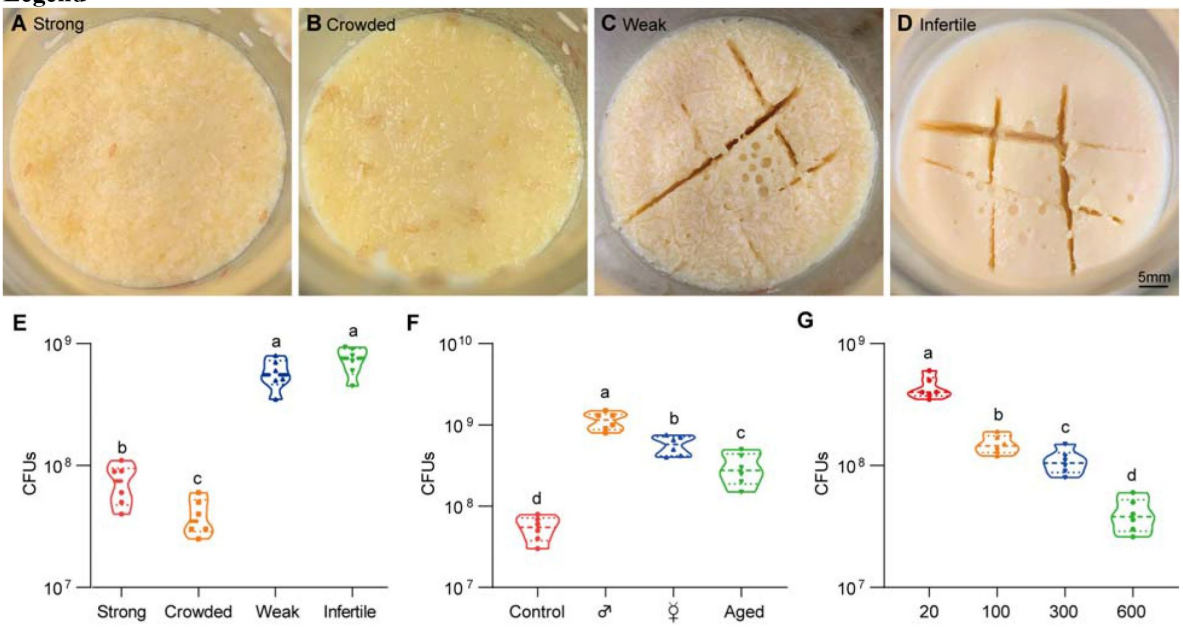

Figure 1.

Drosophila larvae shape topographies and carrying capacities of bacterial community. (**A**-**D**) Representative images of sticky "biofilm-like" formation on the surface of the sugar-corn- yeast medium whereby *Drosophila* flies with differential robustness were raised. The topographies of surface slick are differentially deconstructed and segmented by flies with different robust flies. (**E**) Bacterial loads of the diet associated with strong, crowed, weak and infertile flies, respectively. Strong flies (wild-type fly CS), weak flies (*yw; Sp/CyO;MKRS/TM6B*), infertile flies ($dfmr1^{50M}$ null mutant). n = 6 for each. (**F**) Bacterial loads of the diet associated with control, male, virgin and aged flies, respectively. Each bottle contained 100 flies with a 1:1 ratio of male: female in control and aged groups, and 50-day-aged flies were used. n = 6 for each. (**G**) Bacterial loads of the diet associated with *Drosophila* larvae in a dosage- dependent manner. n = 6 for each. Means ± SEMs. All variables have different letters, and they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

than corresponding *S. marcescens* in co-culture, likely that single *S. marcescens* rapidly exhausted their nutritional resources and underwent ecological suicide (Ratzke et al., 2018). These results suggest that the larva acts as a potential competitor that efficiently prevents the overgrowth of *S. marcescens* in the habitat before 72 h post-inoculation. To verify it, different numbers of larvae were added to vials inoculated with *S. marcescens*. Indeed, the more were larvae, the less the colour intensity of the surface slick (**Figure 2**-figure supplement 1A, B). In addition, the population size of *S. marcescens* in food was in restricted control by larvae in a dosage-dependent manner (**Figure 2**-figure supplement 1C). Altogether, the cumulating results suggested that *Drosophila* larva has a competitive advantage in the habitat, and acts as a critical regulator of *S. marcescens*.

Drosophila larvae modulate the pathogen-to-commensal transition of *S. marcescens*

S. marcescens is a *Drosophila* pathobiont with the potential to switch between commensalism and pathogenicity toward the host. To this end, we sought to examine the *S. marcescens* lifestyle switch from pathogenicity to commensalism by assessing the respective survival of flies on the fly medium that had been processed by single or coexisting *S. marcescens*. Our data showed that flies challenged with *S. marcescens* alone manifested higher mortality than flies with *S. marcescens* in co-culture (**Figure 2E**), suggesting that larva antagonizes the pathogenicity of *S. marcescens*. In addition, the expression of virulent factors was thwarted by larvae (**Figure 2F**). However, *S. marcescens* more efficiently sustained optimal larval development upon nutrient scarcity than both axenicity and commensal *Lactobacillus plantarum* (Matos et al., 2017; Storelli et al., 2011) (**Figure 2**-figure supplement 1D), indicating that larvae-associated *S. marcescens* could impart fitness benefits to their hosts. To rule out the possibility that phenotypic alterations could stem from genomic mutations, we examined the prodigiosin yield and CFUs of re- culturing *S. marcescens* that had coexisted with larvae. Our results showed that neither prodigiosin yield nor CFUs of re-culturing *S. marcescens* differed from the original strain (**Figure 2**-figure supplement 3A-C), suggesting that a phenotypic switch was driven primarily by transcriptional reprogramming. The cell wall of Gram-negative bacteria is composed of a single layer of peptidoglycan surrounded by a membranous structure, which may function as a virulence factor (Chu et al., 2016; Pan et al., 2022). Indeed, transmission electron microscopy confirmed a significant reduction in the width of the cell wall of *S. marcescens* in co-culture compared to that of *S. marcescens* alone (**Figure 2G**). Additionally, we observed that the expression of genes related to cell wall biosynthesis was dampened by larva transplantation (**Figure 2H**). Taken together, these results suggest that *Drosophila* larvae modulate *S. marcescens* lifestyle from pathogenicity to commensalism toward the host.

Presumably, surface slicks can be destroyed by mechanical force from larva crawling and burrowing (Dufrene and Persat, 2020). To tackle this issue, we agitated fly food to imitate larva locomotion (**Figure 2**-figure supplement 2A). Indeed, agitation decreased colour intensity, prodigiosin yield and population size in fly food (**Figure 2**-figure supplement 2B-D), implying that mechanical force accounts for the negative regulation of *S. marcescens*. However, larval transplantation resulted in a much more robust decline in colour intensity of surface slicks, prodigiosin yield and population size than agitation alone. Of note, the surface of the slick with agitation appeared lighter than that of larvae, possibly due to a stratification of prodigiosin following agitation. In addition, the expression of prodigiosin synthesis genes was thwarted by the agitation (**Figure 2**-figure supplement 2E). These results indicated that larva-derived biofactors and/or synergism of force and biofactors primarily confer the inhibition of *S. marcescens* (see the later results).

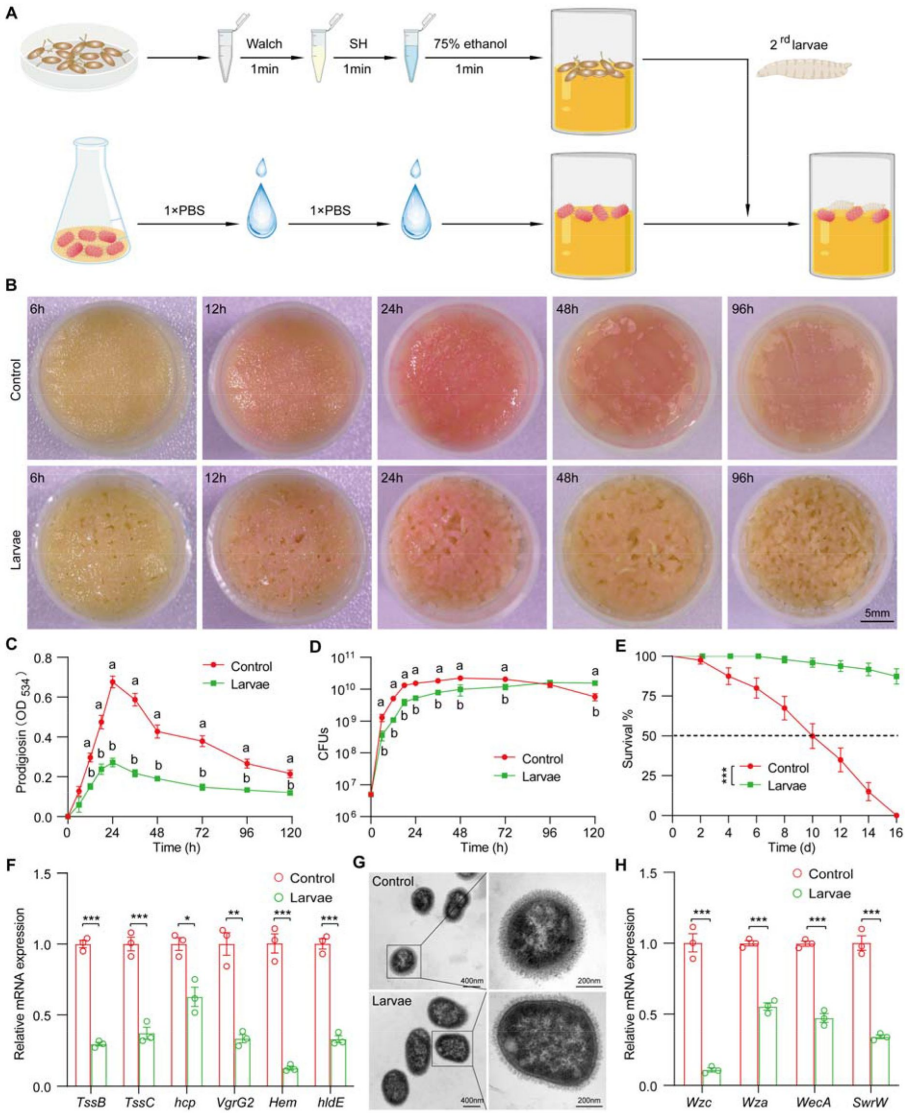

Figure 2.

Drosophila larvae outcompete *S. marcescens* in the diet. (**A**) A diagram of a reductionist approach to investigate the role of *Drosophila* in regulating the physiology and behavior of *S. marcescens*. Top: Germ-free *Drosophila* larvae were generated by successive sterilization of fresh eggs with sanitizer Walch, sodium hypochloride (SH), ethanol, and PBS containing 0.01% TritonX-100 (PBST). Bottom: *S. marcescens* was cultured in a liquid medium and re-inoculated to fly cornmeal food after washing with PBS buffer. In the meantime, GF crawling larvae were transferred to the fly medium in the shared vials with *S. marcescens*. (**B**) Representative images of surface slick inoculated with *S. marcescens* alone and with *S. marcescens* over time. (**C**) The prodigiosin production of *S. marcescens* alone and in coculture at different time points. Prodigiosin production was assessed with the spectrometer at OD_{534}. n = 6 for each. (**D**) Bacterial loads of *S. marcescens* alone and in coculture in the time course. n = 6 for each. (**E**) The survival rate of adult flies challenged with *S. marcescens* alone and in coculture. The bacterial suspension (20 µl, OD_{595} = 1) was supplemented to a vial with autoclaved fly food. Single and coculturing *S. marcescens* were obtained after 24-h incubation as described in **Figure 2A**, and the percentage of living female flies treated with *S. marcescens* alone and in coculture was calculated to monitor lifespan. n = 180 for each. The statistical analyses were performed using Log-rank test. ***$p < 0.001$. (**F**) RT-qPCR analysis of the expression levels of virulence- related genes of with *S. marcescens* alone and in coculture. n = 3 for each. (**G**) Transmission electron microscopy of *S. marcescens* alone and in coculture. Scale bars: 400 nm (left panel) or 200 nm (right panel). (**H**) RT-qPCR analysis of the expression levels of extracellular polysaccharide production-related genes in the control and larvae groups. n = 3 for each. Means ± SEMs. The statistical analyses were performed using two-tailed unpaired Student's t test in (F) and (H). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. All variables have different letters, and they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

Drosophila enforces bacterial global transcriptional and metabolic adaptation to the host

In order to understand the molecular basis for the bacterial lifestyle switch from pathogenicity to commensalism in response to *Drosophila* larvae, bulk RNA-seq analysis was applied to bacterial cells 24 h after inoculation. We devised an approach to efficiently collect bacteria from the agar fly food as described in Methods. As shown in **Figure 3A**, Principal Component Analysis (PCA) showed a larvae-dependent separation to *S. marcescens* alone on the first principal component of variance. Compared to single *S. marcescens*, larvae-associated *S. marcescens* exhibited significant upregulation of 360 genes and downregulation of 439 genes (**Figure 3B** and Supplementary table 2), respectively. Functional annotations of the differentially expressed genes (DEGs) were assigned using the Kyoto Encyclopedia of Genes and Genomes (KEGG). KEGG pathway enrichment analysis highlighted that the most differentially upregulated DEGs of coexisting *S. marcescens* were related to bacterial proliferation and growth, including ribosome, translation factors, transcription, DNA replication proteins, and energy metabolism (**Figure 3D**), implying that larval transplantations favored maintenance of *S. marcescens* in the diet. By contrast, the most differentially downregulated DEGs of single *S. marcescens* were related to bacterial pathogenicity, including transporters, excretion, quorum sensing, and exosome (**Figure 3C**). To validate our findings in bulk RNA-seq analysis, quantitative PCR was used to examine the expression of predicted genes involved in bacterial proliferation and pathogenicity. Indeed, most predicted genes associated with pathogenicity were downregulated in single *S. marcescens* (**Figure 3E**), while genes associated with bacterial proliferation were upregulated in response to larva colonization (**Figure 3F**). Consistent with the previous result that this phenotypic switch was driven by transcriptional changes, the expression of virulent and growth genes was recovered after re-culturing (**Figure 3**-figure supplement 3D, E). Overall, *Drosophila* forms a symbiosis with the bacterial community by harnessing bacterial global transcription.

Next, we investigated whether larvae could further elicit changes in the metabolism of *S. marcescens* using untargeted metabolomics. The data showed that 91 metabolites were identified and their concentrations were quantified (Supplementary table 3). Metabolomic profiles discriminated larva-associated *S. marcescens* from *S. marcescens* alone (**Figure 4A**), indicating that the host reshapes the global metabolic profile of bacterial cells. We found that larvae-associated *S. marcescens* displayed significant upregulation of 22 metabolites and downregulation of 69 metabolites (**Figure 4B**). Unsupervised hierarchical clustering of the metabolome revealed distinct clusters of metabolites in *S. marcescens* alone versus co-culture (**Figure 4C**). Moreover, we detected a significant decrease in amino acid metabolism, phosphotransferase system, and ABC transporters in *S. marcescens* in co-culture compared to *S. marcescens* alone (**Figure 4D**). Consistent with previous studies (Defoirdt, 2019; Wen et al., 2022), these results suggested that the host suppresses differentiation of *S. marcescens* into the population with pathogenicity. By contrast, the most differentially upregulated metabolites of *S. marcescens* in co-culture were related to the biosynthesis of fatty acids and unsaturated fatty acids, and the pentose phosphate pathway (**Figure 3D**). Taken together, these results suggest that the host affects the global metabolic profile of symbiotic cells and drives the switch of the bacterial lifestyle from pathogenicity to commensalism.

The co-expression network analysis of transcriptome and metabolome revealed that 42 differentially expressed metabolites were found to be related to the pathogen-to-commensal switch, including ribosome, transcription, DNA replication, energy metabolism, ABC transporters, phosphotransferase system, quorum sensing and exosome (**Figure 4**-figure supplement 4A). Serotonin, sorbitol and lactobionic acid were related to the pathogen-to- commensal switch (p value<0.05). To validate these results, we perturbed the *S. marcescens* extracellular environment by adding predicted metabolites to the fly food. Indeed, we found that serotonin, sorbitol and lactobionic acid efficiently reduced the prodigiosin yield and CFUs (**Figure 4**-figure supplement

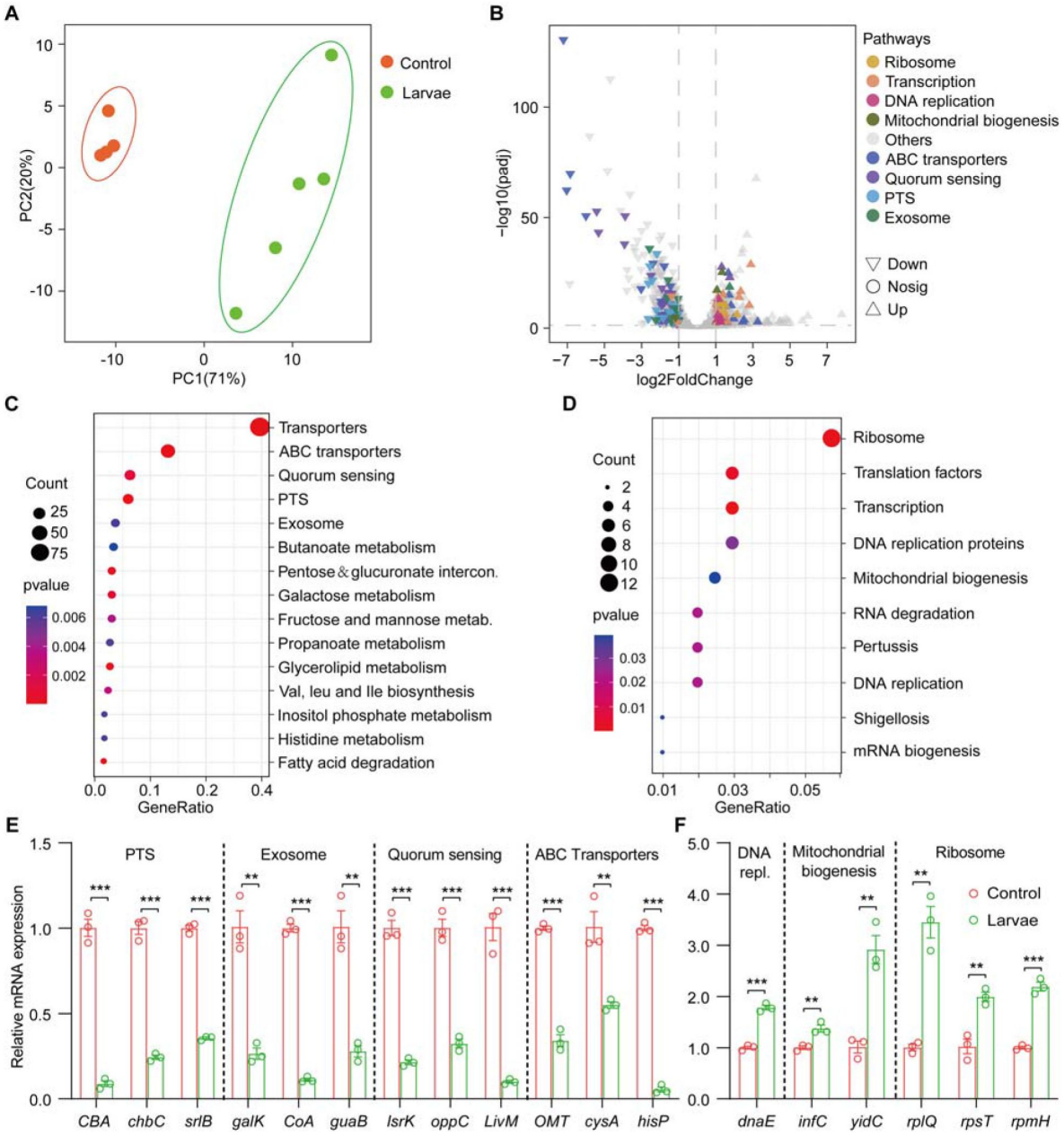

Figure 3.

Drosophila larvae adjust bacterial global transcriptional adaptation to the host. (**A**) Principal coordinate analysis (PCA) of unweighted, jack-knifed UniFrac distances of the transcriptional profile of *S. marcescens* alone and with larvae. PC1, principal coordinate 1; PC2, principal coordinate 2. Scattered dots in different colors represent samples from different experimental groups. n = 4-5. (**B**) Volcano plot comparing gene expression profiles of *S. marcescens* alone and with larvae after 24 h of incubation. X-axis represents the log2- transformed value of gene expression change folds between larvae and control groups. Y-axis represents the logarithmic transformation value of gene expression levels in *S. marcescens*. Genes belonging to different pathways are represented by different colored shapes as indicated. ▽depicts genes significantly upregulated in *S. marcescens* with larvae compared to *S. marcescens* alone (log2 fold change < 1; adjusted $p < 0.01$), and △ depicts genes significantly downregulated in *S. marcescens* with larvae (log2 fold change < 1; adjusted $p < 0.01$) compared to *S. marcescens* alone. ○ depicts genes without significant alteration compared to *S. marcescens* alone. (**C**, **D**) Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the significantly downregulated and upregulated genes in *S. marcescens* with larvae compared to *S. marcescens* alone. (**E**, **F**) RT-qPCR analysis of the expression levels of downregulated and upregulated genes in the control and larvae groups. n = 3 for each. Means ± SEMs. The statistical analyses were performed using two-tailed unpaired Student's t test. $^{**}p < 0.01$; $^{***}p < 0.001$.

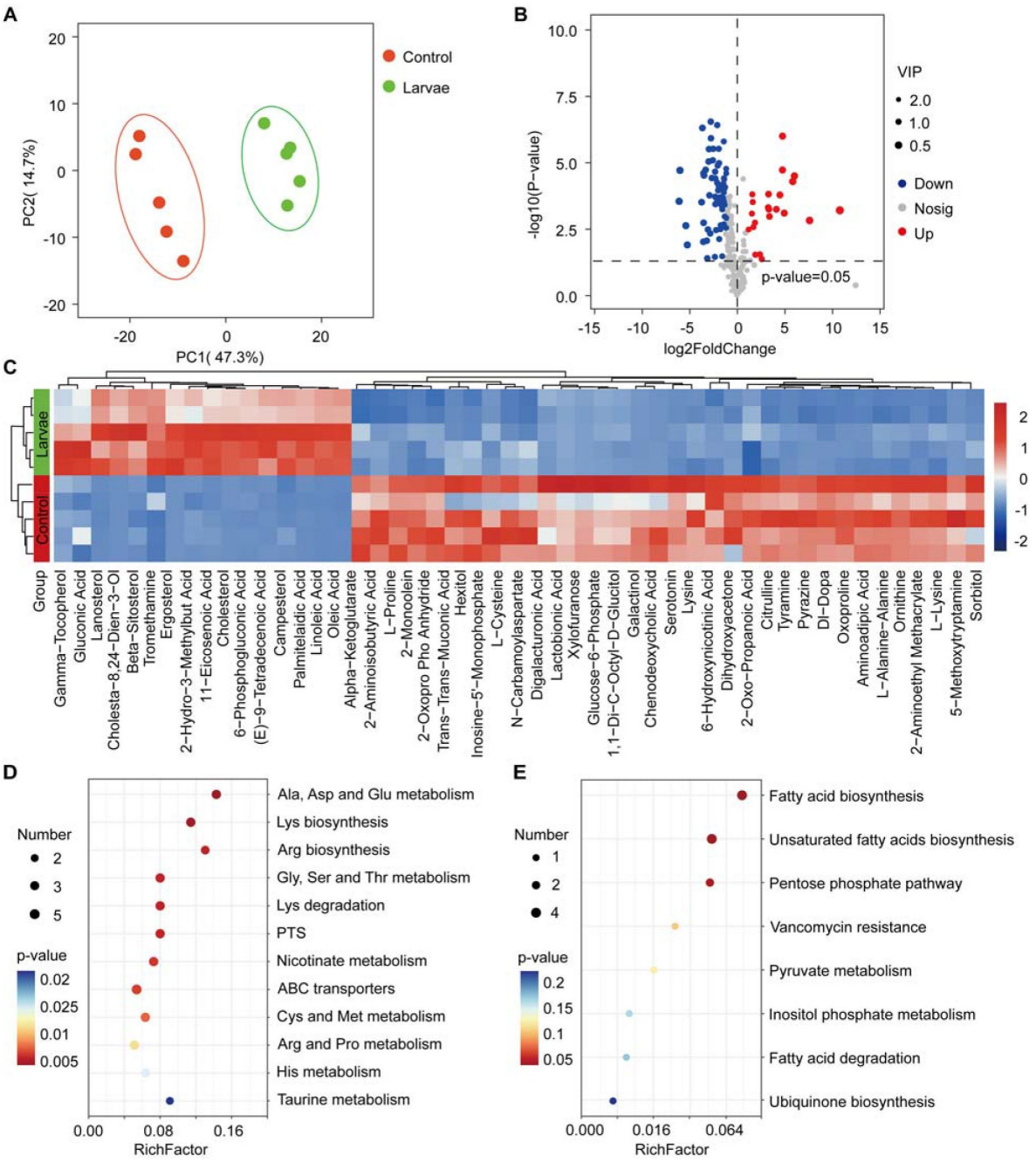

Figure 4.

Drosophila larvae affect the global metabolic profile of *S. marcescens*. (**A**) Principal coordinate analysis (PCA) of unweighted, jack-knifed UniFrac distances of metabolic profile of *S. marcescens* alone and with larvae. PC1, principal coordinate 1; PC2, principal coordinate 2. Scattered dots in different colors represent samples from different experimental groups. n = 5 for each. (**B**) Volcano plot comparing metabolic profiles of between control and larvae groups after 24 h of incubation. X-axis represents the log2-transformed value of gene expression change folds between larvae and control groups. Y-axis represents the logarithmic transformation value of gene expression levels in *S. marcescens*. Red dots depict genes significantly upregulated in *S. marcescens* with larvae compared to *S. marcescens* alone (log2 fold change < 1; adjusted $p < 0.01$), and blue dots depict genes significantly downregulated in *S. marcescens* with larvae (log2 fold change < 1; adjusted $p < 0.01$) compared to *S. marcescens* alone. Grey dots depict genes without significant alteration compared to *S. marcescens* alone. (**C**) The distinct clusters of metabolites in *S. marcescens* alone versus co-culture. (**D**, **E**) KEGG pathway analysis of the significantly downregulated and upregulated metabolites in *S. marcescens* with larvae compared to *S. marcescens* alone.

4B, C), highlighting the high quality of the prediction. Moreover, *S. marcescens* perturbed with sorbitol manifested less virulent to flies (**Figure 4**-figure supplement 4D) and the impaired transcription of virulence-related genes (**Figure 4**-figure supplement 4E), resembling the lifestyle of co-culturing *S. marcescens*. Collectively, these findings suggest that *Drosophila* enforces bacterial transcriptional and metabolic adaptation to the host.

Larvae modulate pathogenicity heterogeneity of *S. marcescens*

Isogenic microbial populations can generate transcriptional variation across individual cells, thereby prompting us to analyze bacterial populations at the single-cell resolution. We characterized the transcriptome of individual *S. marcescens* cells by implementing bacterial single-cell RNA sequencing on a platform available. To validate this approach, we first determined the capacity of this platform to distinguish bacterial populations of heat-shocked *S. marcescens* grown in a liquid medium as previously described (Dar et al., 2021). Indeed, the reults provide strong evidence that the bacterial single-cell RNA sequencing approach used was sufficient and reliable to capture transcriptional responses to heat shock (**Figure 5**-figure supplement 5).

Next, we speculated about the heterogeneous transcriptional response of *S. marcescens* challenged with larvae as well as mechanical force. The growth of *S. marcescens* was well understood in fly food conditions as described in **Figure 2**, so we collected *S. marcescens* at the late exponential phase when certain bacterial cells manifested their differentiation and pathogenicity. We captured 2800 cells in total and detected a median of 198 genes per cell for control, 333 for force, and 175 for larvae (**Figure 5A**). Strikingly, bacterial cells from each group tended to form three distinct clusters that correspond to the agitated, larva-associated and control cultures could be visualized by graph-based clustering of their gene expression profiles (**Figure 5B**). This result suggested that larval transplantation, as well as agitation, induce significant changes in gene expression patterns, consistent with the bulk RNA-seq result that larvae caused the shift in bacterial global transcription using Seurat (Kuchina et al., 2021). Next, we attempted to partition the total bacterial population of *S. marcescens* into subpopulations with diverse predicted functional capabilities by the algorithmic grouping of cell-expression profiles. As shown in **Figure 5C**, we further partitioned the three groups into 7 subclusters (Subcluster 0-6) representing subpopulations with distinct expression profiles, while the gene expression in each cluster was continuous. Namely, the sampled populations of *S. marcescens* alone and in coculture were respectively partitioned into three coexisting subgroups (Subcluster 0,1, and 2 for co-culturing *S. marcescens* and Subcluster 3, 4, and 6 for single *S. marcescens*), implicating that phenotypic diversity is a general feature of bacteria in clonal populations.

To understand the heterogeneity of gene expression patterns, we identified the DEGs associated with pathogenicity to characterize the transcriptional profile (Supplementary table 4), and ranked marker genes in each cluster (**Figure 5D**). We selected pathogenicity-related DEGs, including *livI* (ABC transporter), *oppA* (quorum sensing), *secY* (secretion system), and *fp* (virulence), and charted the feature expression of them in each cluster in low-dimensional space (**Figure 5E**, F and I, J). We found most genes involved in pathogenicity displayed a substantial decline upon larvae, which was consistent with the previous findings of reduced pathogenicity of coexisting *S. marcescens*. Moreover, we found that *livI* exhibited gradually higher expression down along Subcluster 3 to Subcluster 6 (**Figure 5G**), implicating the potentially hierarchical virulence-regulatory network in these three subclusters. Analogously, *oppA*, *secY* and *fp* exhibited a similar expression pattern in these three subclusters (**Figure 5H**, K and L). Given that increased pathogenicity along three subclusters. It was likely that Subcluster 6 could be developed from Subcluster 4, thereby inferring that Subcluster 6 could be identified as subpopulations of single *S. marcescens* in a bona fide pathogen. Intriguingly, many virulence- associated genes were highly expressed in Subclusters 5, implying that mechanical force could not attenuate the virulence of *S. marcescens*. We tested it by assessing the survival of flies challenged with processed fly medium from agitated *S. marcescens*. Indeed, flies treated with agitated *S. marcescens* displayed a significantly shorter lifetime than flies

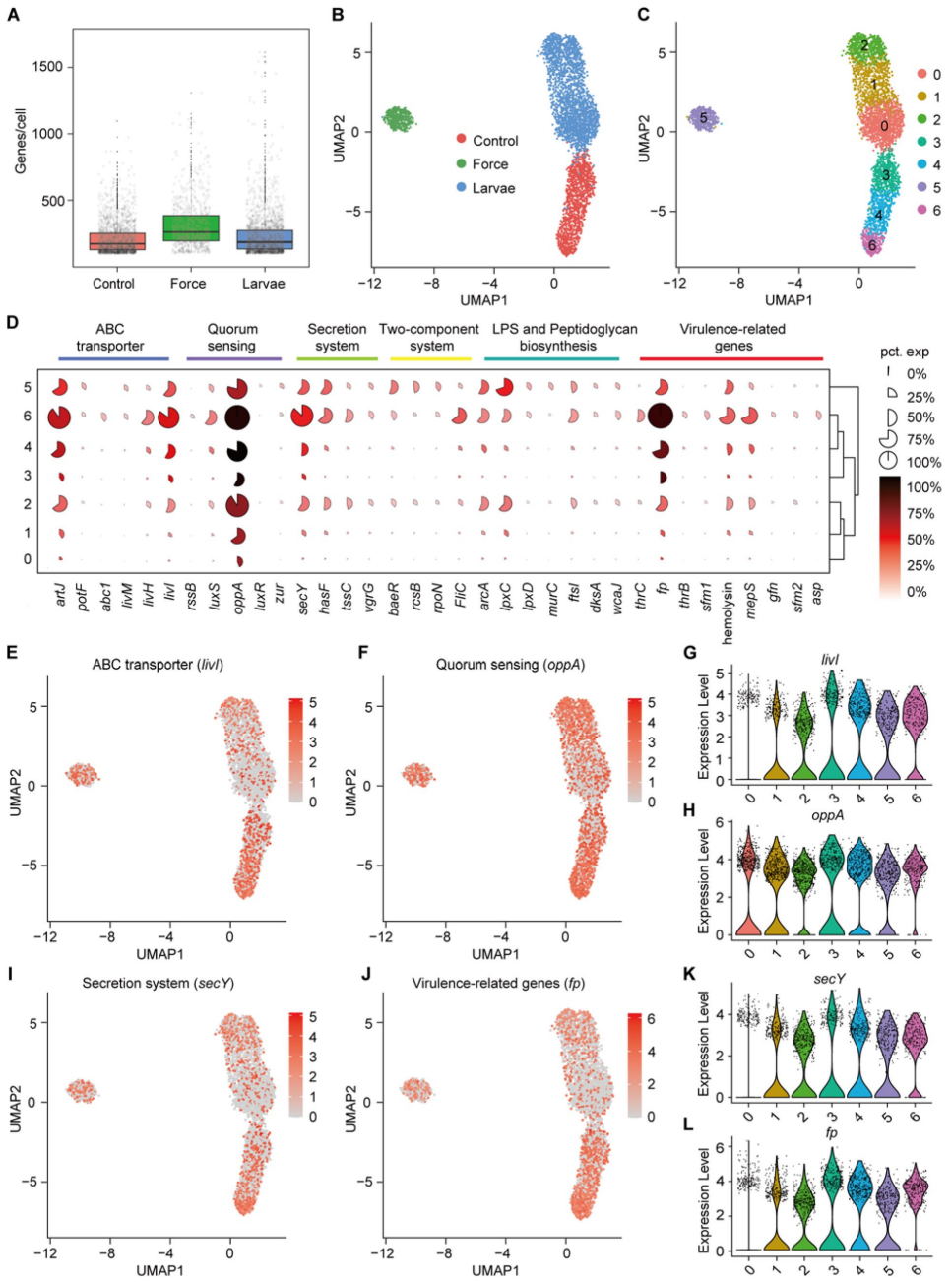

Figure 5.

Pathogenicity heterogeneity of *S. marcescens.* (**A**) mRNA gene counts per cell for *S. marcescens* alone, with force and with larvae. Each dot represents a bacterial cell of *S. marcescens*. (**B**) Joint UMAP two-dimensional analysis showing that are distinct clusters among *S. marcescens* alone, with force and with larvae. (**C**) The cell subpopulation among the control, force and larvae groups. There were three distinct subpopulations in the control and force groups.(**D**) Mean expression levels of genes involved in ABC transporter, Quorum sensing, Secretion system, Two-component system, LPS and Peptidoglycan biosynthesis and Virulence-related genes in different subclusters. The shape of each dot indicates the proportion of cells in the cluster, while the color indicates the average activity normalized from 0 % to 100 % across all clusters. (**E**, **F**) The expression of a representative gene of ABC transporter and Quorum sensing was highlighted on the UMAP. The red color bars represent the normalized expression of a gene across all cells analyzed. (**G**, **H**) Violin plots of *livI* and *oppA* gene in different subclusters. Each dot represents a single cell and the shapes represent the expression distribution. (**I**, **J**) The expression of a representative gene of Secretion system and Virulence-related genes was highlighted on the UMAP. The red color bars represent the normalized expression of a gene across all cells analyzed. (**K**, **L**) Violin plots of *secY* and *fp* gene in different subclusters. Each dot represents a single cell and the shapes represent the expression distribution.

with coculturing *S. marcescens* (**Figure 5**-figure supplement 5A). Taken together, our results demonstrated the presence of pathogenicity heterogeneity of *S. marcescens* at the single-microbe-level transcriptional landscape.

Larvae modulate growth heterogeneity of *S. marcescens*

We showed that coexisting *S. marcescens* were related to growth, so we continued to identify the DEGs associated with bacterial propagation to characterize the transcriptional profile (Supplementary table 4), and ranked marker genes in each cluster (**Figure 6A**). Among DEGs of ribosome between single *S. marcescens* and co-culturing *S. marcescens*, *rpsL* encodes the highly conserved rps12 protein of the ribosomal accuracy center, while, tryptophan, biosynthesized by *trpD* among DEGs of DNA replication, is an essential nutrient and serves as a building block for protein synthesis and DNA replication. As anticipated, most genes involved in growth showed an evident increase in larvae. Interestingly, *rpsL* and *trpD* similarly exhibited gradually higher expression up along Subcluster 0 to Subcluster 1 (**Figure 6B**), indicating the potentially hierarchical growth-regulatory network in these three subclusters. Taken together, our results demonstrated the presence of bacterial propagation heterogeneity of *S. marcescens* at the single-microbe-level transcriptional landscape.

Nitrogen is the fundamental element of nucleic acids and proteins. In the clusters corresponding to larvae (clusters 0, 1 and 2), we observe peak expression of genes involved in nitrogen metabolism, such as P-II family nitrogen regulator (*glnK*) and glutamate-ammonia ligase (glnA; **Figure 6**-figure supplement 6B, D). *glnK* plays a critical role in regulating the activity of glutamine synthetase (e.g. *glnA*), which promotes glutamine synthesis. Glutamine is the most abundant non-essential amino acid, and serves as nitrogen and carbon sources for cell growth and differentiation. Moreover, these three subclusters showed a significant increase in the activity of genes involved in urea transport and utilization, such as urea ABC transporter substrate-binding protein (*urtA*), allophanate hydrolase (*atzF*) and urea carboxylase (*uca*). *urtA* accounts for the transport of urea, and then *atzF* and *uca* synergize to convert urea to ammonia that can be used for glutamine biosynthesis. The regulatory schematic is shown in **Figure 6J**. Additionally, glutamine synthesis needs a large amount of energy, so we then turned to carbon metabolism. Indeed, we observed higher expression of phosphoglycerate kinase (*pgk*) and bifunctional acetaldehyde-CoA/alcohol dehydrogenase (*adhE*) that participate in glycosis in the subclusters 0, 1 and 2 (**Figure 6G** and **Figure 6**-figure supplement 6C). Moreover, we found that *pgk* and *adhE* exhibited gradually higher expression in these three subclusters relative to subclusters with single *S. marcescens* (**Figure 6I** and **Figure 6**-figure supplement 6E), suggesting that bacteria could establish a dedicated replicative niche to efficiently replicate. Altogether, these findings that the host globally results in transcriptional reprogramming of single-cell *S. marcescens*, facilitating the cooperation among individual cells.

Larvae-derived AMPs efficiently antagonize *S. marcescens*

Our results showed that the host keeps strict control over the pathobiont, we next asked whether one or multiple compound(s) secreted by the host could recapitulate this phenomenon. Indeed, the data showed that diets with intestinal excreta exhibited a modest but significant decline in color intensity of the slick, prodigiosin yield and population size compared to control (**Figure 7A-C**), in line with the result above (**Figure 2**-figure supplement 2C, D). Larval excreta contains short antimicrobial peptides (AMPs) that efficiently combat a variety of pathogens (Marra et al., 2021), prompting us to whether AMPs could recapitulate the response of *S. marcescens* to the presence of *Drosophila* larvae. To refine this concept, AMPs were supplemented to fly food vials with 10^7 CFU *S. marcescens* at the same time. We found that AMPs suppressed color intensity of the slick, prodigiosin yield and population size in a dosage-dependent manner, indicating that AMPs, to a lesser extent, played a role in reshaping *S. marcescens*. To verify it, we turned to a compound mutant strain lacking *Defensin*, *Cecropins* (4 genes), *Drosocin*, *Diptericins* (2 genes), *Attacins* (4 genes), *Metchnikowin*, and *Drosomycin*, referred to as "*ΔAMP*" (Hanson et al., 2019), allowing

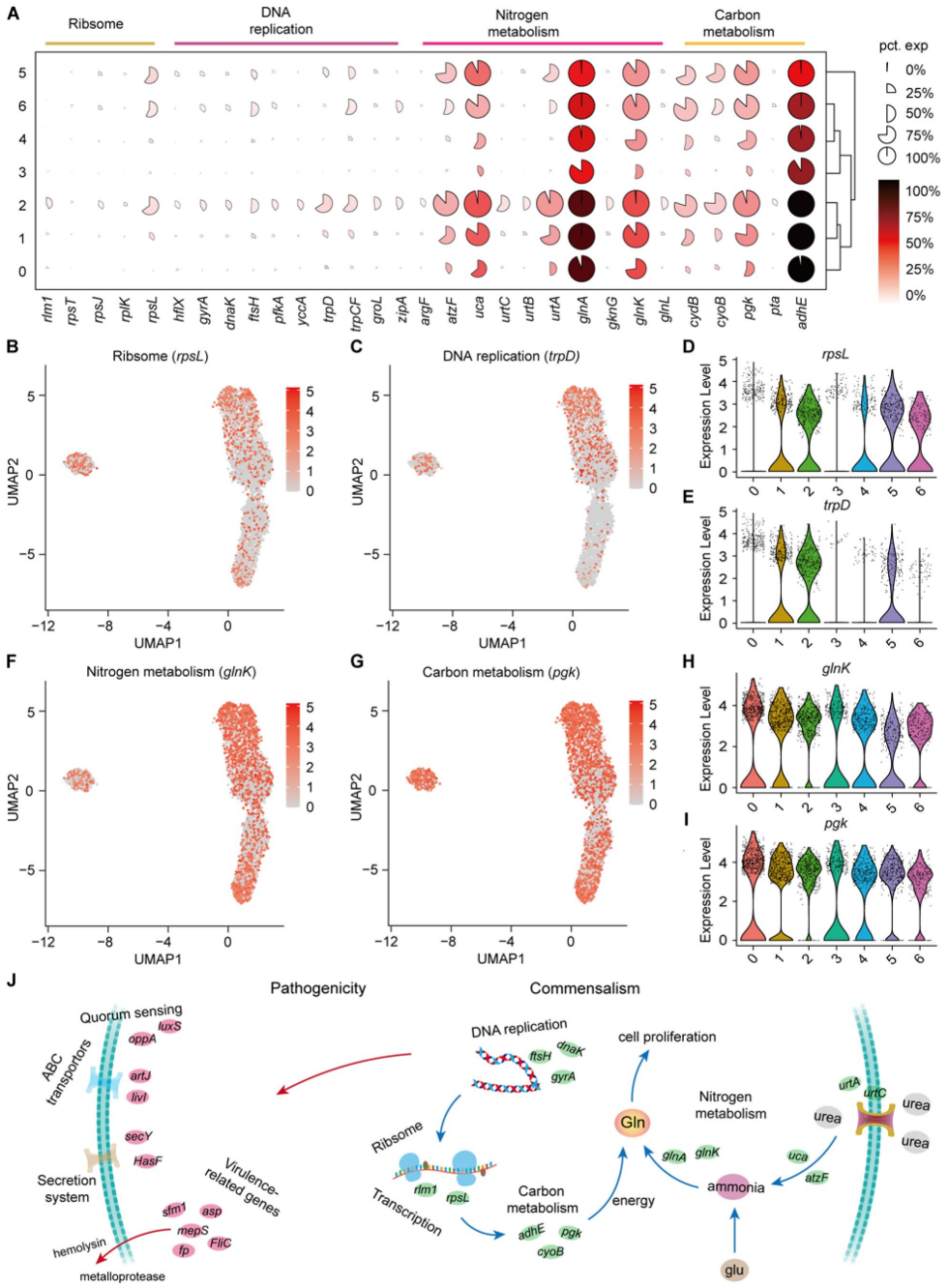

Figure 6.

Growth heterogeneity of *S. marcescens*. (**A**) Mean expression levels of genes involved in Ribsome, DNA replication, Nitrogen metabolism and Carbon metabolism in different subclusters. The shape of each dot indicates the proportion of cells in the cluster, while the color indicates the average activity normalized from 0 % to 100 % across all clusters. (**B**, **C**) The expression of a representative gene of Ribsome and DNA replication was highlighted on the UMAP. The red color bars represent the normalized expression of a gene across all cells analyzed.

(**D**, **E**) Violin plots of *rpsL* and *trpD* genes in different subclusters. Each dot represents a single cell and the shapes represent the expression distribution. (**F**, **G**) The expression of two representative genes of Nitrogen metabolism and Carbon metabolism was highlighted on the UMAP. The red color bars represent the normalized expression of a gene across all cells analyzed.

(**H**, **I**) Violin plots of *glnK*, *glnA, pgk* and *adhE* genes in different subclusters. Each dot represents a single cell and the shapes represent the expression distribution. (**J**) Schematic of the pathogenicity and commensalism regulatory pathway.

direct investigation of their role in the lifestyle transition of the pathobiont. Indeed, *ΔAMP* recapitulated an increment in color intensity of surface slick, prodigiosin yield and population size compared to wild-type counterparts (**Figure 7D-F**). More important, the recapitulation in color intensity of slick, prodigiosin yield and population size was substantially attenuated and even abolished by the addition of AMPs, suggesting that much of the inhibition of the microbiome can be ascribed to AMPs. To rule out the potential role of other immune effectors, we turned to the IMD pathway mutant Rel^{E20} that is deficient in total immune effectors. Our result showed that the optical density and yield of prodigiosin in Rel^{E20} group did not significantly differ from the ones in *ΔAMP* group (**Figure 7**-figure supplement 7A, B). Moreover, the load of *S. marcescens* associated with Rel^{E20} mutant was comparable to that of *S. marcescens* associated with Delta AMP mutant (**Figure 7**-figure supplement 7C). Subsequently, quantitative PCR was employed to confirm the expression of altered genes as depicted above (**Figure 7G**, H). Together, our data demonstrated that larva-derived AMPs were a key factor that accounts for the restrictive control over diet microbes.

Discussion

The important role of the host in affecting microbial physiology and behavior is only beginning to be appreciated. In the current study, we found that *Drosophila* larvae act as a competitive regulator that prevents *S. marcescens* overgrowth and antagonizes its pathogenicity. *Drosophila* larvae reshaped the transcriptomic and metabolomic profile of *S. marcescens*, characterized by the lifestyle switch from pathogenicity to mutualism toward the fly. More importantly, we highlight that the host alters the single-cell transcriptomic atlas of *S. marcescens* and phenotypic heterogeneity in bacterial populations.

Drosophila adults are routinely attracted to oviposit their eggs on rotting fruits that possess both commensal and pathogenic microbes (Liu et al., 2017; Wong et al., 2017). Extensive attention has been dedicated to the essential roles of symbiotic bacteria in modulating the physiology of their animal partner, or to the molecular mechanisms underlying physiological benefits to their host. However, it's necessary to explore both sides of symbioses to obtain a more complete understanding of the host-bacteria interaction. *S. marcescens* encounters a cost associated with symbiosis, as the population size for it is diminished in the shared niche before 72 hours post inoculation by the larvae (**Figure 2D**). Our findings highlight that larvae alleviate intraspecies competition of *S. marcescens* through population size control at the initial stage. Taking into account these findings, the competition model is more plausible to the larvae- pathogen system where larvae efficiently prevent the overgrowth of the potential pathogenic bacterial community (Burns et al., 2017; Foster et al., 2017). Interspecies competion for nearly the same or similar nutrients and space occurs in the habitat, making *Drosophila* larvae a potential competitor for *S. marcescens*. Intriguingly, a previous study reported the contrasting result that *Drosophila* farms its commensal *L. plantarum*, and is required to optimize the extraction of dietary nutrients and sustain the growth of their symbionts upon chronic undernutrition (Storelli et al., 2018). To reconcile the two contradictions, another model of species cooperation is suggested. Consequently, larval transplantations favored long-term maintenance of *L. plantarum* on the diet, and benefits from symbiosis override the costs of the initial competition in the long run, conferring the beneficial effect of larval presence on bacterial maintenance. This paradox possibly reflects a strategy developed by *Drosophila* to preserve its own fitness. Potential pathogens differ from commensal bacteria, because they generate secondary metabolites threatening insect life at the static stage of growth (**Figure 2E**). If the larvae obtain benefits to promote their development and growth, they must exert strict control over them through antimicrobial peptides and reactive oxygen species (Sharp and Foster, 2022). We found that larvae-derived AMPs efficiently antagonized *S. marcescens* (**Figure 7A-F**), thereby recapitulating the response of *S. marcescens* to *Drosophila* larvae. Yet, the full complement of larva-derived factors required for bacterial controls as well as their mode of functions remains elusive, paving the way to seek the

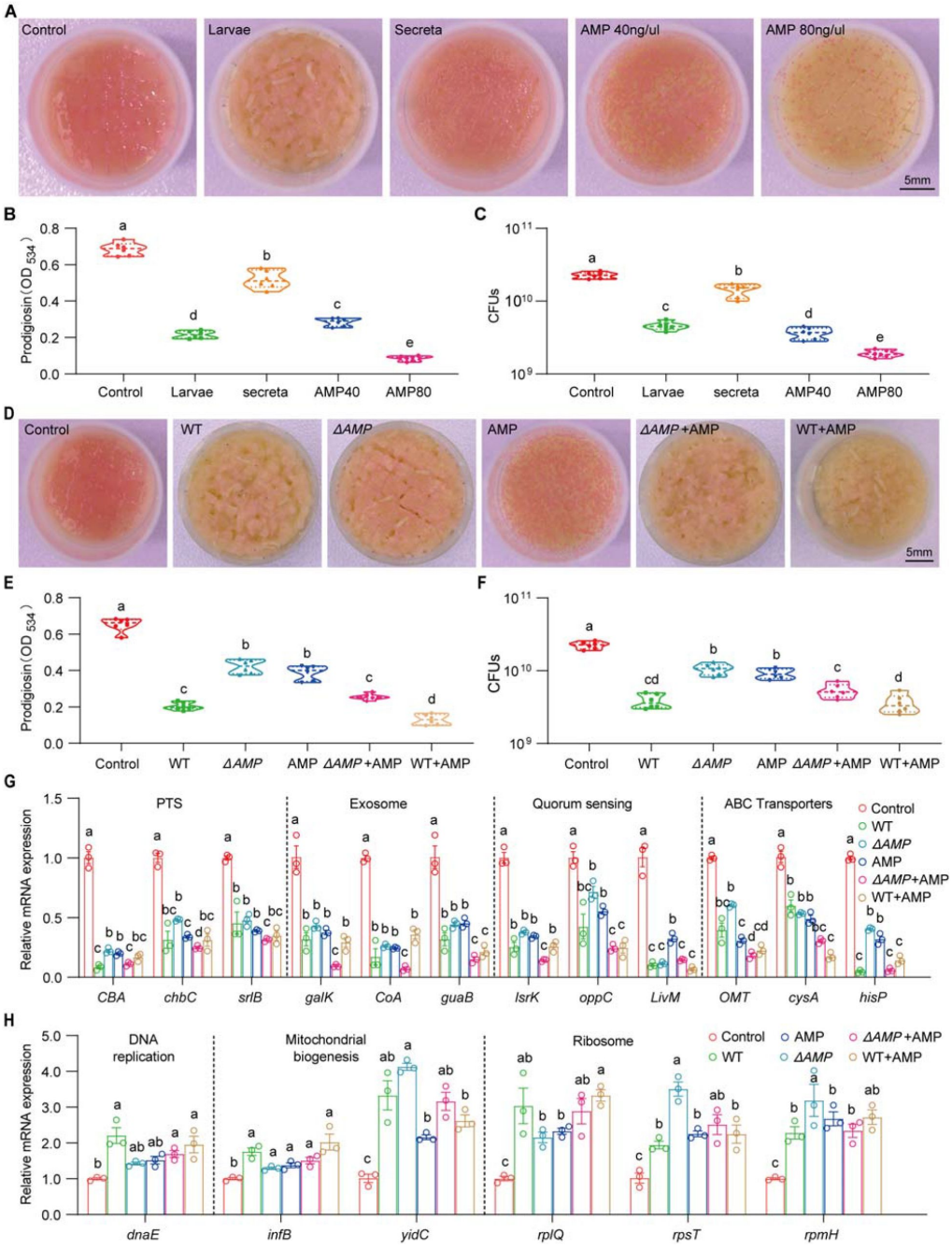

Figure 7.

Larvae-derived AMPs antagonize *S. marcescens.* (**A**) Representative images of surface slick with *S. marcescens* alone, with larvae, with secreta and with AMPs. (**B**) The prodigiosin production of *S. marcescens* alone, with larvae, with secreta and with AMPs. (**C**) Bacterial loads of *S. marcescens* alone, with larvae, with secreta and with AMPs. AMPs: cecropin A (40 μg/μl, 80 μg/μl). (**D**) Representative images of surface slick with *S. marcescens* alone, with wild-type larvae, with *ΔAMP14* larvae, with AMPs, with *ΔAMP14* larvae+AMPs and wild-type larvae+AMPs. (**E**) The prodigiosin production of *S marcescens* alone, with wild-type larvae, With *ΔAMP14* larvae, with AMPs, with *ΔAMP14* larvae+AMPs and wild-type larvae+AMPs. (**F**) Bacterial loads of with *S. marcescens* alone, with wild-type larvae, with *ΔAMP14* larvae, with AMPs, with *ΔAMP14* larvae+AMPs and wild-type larvae+AMPs. AMPs cecropin A = 40 μg/μl. (**G**, **H**) RT-qPCR analysis of the expression levels of downregulated and upregulated genes in the *S. marcescens* alone, with wild-type larvae, with *ΔAMP14* larvae, with AMPs, with *ΔAMP14* larvae+AMPs and wild-type larvae+AMPs. For B-C and E-F, n = 6 for each. Fo G-H, n = 3 for each. Means ± SEMs. All variables have different letters, and they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal- Wallis test followed by Dunn's multiple comparisons test.

evolutionary-conserved animal factors essential to maintain symbiosis. Taken together, our findings of the host on microbial communities would improve our understanding of the ecology of host-symbiont interactions.

Owing to their saprophagous foraging behavior, *Drosophila* has to cope with many potential pathogens in the environment (Black et al., 2018). We're appreciating the profound influence of the host on the resident microbial community, but the underlying molecular mechanisms by which the host is potentially involved in the perpetuation of host-bacteria symbiosis are still poorly understood. Our result showed that *S. marcescens* versatilely displayed transcriptional and metabolomic adaptations to *Drosophila* larvae (**Figure 3** and **Figure 4**), avoiding being competitors when they are associated with the host. Indeed, *S. marcescens* rapidly reached the plateau and exhausted its nutritional resources, and then generated secondary metabolites that could endanger insect life (**Figure 2E**). Consistently, our transcriptome data suggest the pathogen-to-commensal transition of *S. marcescens* by the presence of larvae, including ABC transporters, phosphotransferase system, quorum sensing and exosome. In gram- negative bacteria, these exporters transport lipids and polysaccharides from the cytoplasm to the periplasm, or certain substances that need to be extruded from the cell, including surface components of the bacterial cell (e.g., capsular polysaccharides, lipopolysaccharides, and teichoic acid), proteins involved in bacterial pathogenesis (e.g., hemolysis), hydrolytic enzymes, competence factors, toxins, bacteriocins, peptide antibiotics, and siderophores. In addition, quorum sensing, used by pathogens in disease and infection, regulates gene expression following population density through autoinducers, allowing bacteria populations to communicate and coordinate group behavior (Mukherjee and Bassler, 2019). The findings suggest that the microbiome responds to host physiology by altering gene expression and metabolism (Penterman et al., 2014). Consistently, a study observed that commensal bacteria calibrate their transcriptional and metabolic output to different systemic inflammatory responses (Fyhrquist et al., 2019). Taken together, these findings suggest that larvae elicit a set of expressed genes in their harmful symbionts, favoring the shift from a pathogenic to a commensal stage.

Most microbial communities consist of a genetically diverse assembly of different organisms, and the level of genetic diversity plays an important part in community properties and functions (Davies et al., 2022). However, genetically identical microbial cells can show different behaviors, including differences in growth speed, gene expression and metabolism, and biological diversity can arise at a lower level of biological units (Ackermann, 2015; Avraham et al., 2015). Fortunately, the recent development of methods to interrogate populations on the single-cell level has dramatically altered our understanding of cellular heterogeneity by providing much greater resolution of different cell types and cell states (Hare et al., 2021; Imdahl et al., 2020; McNulty et al., 2023). By applying this technique, we indeed observed that single cells differ from each other with respect to gene expression even when genetic and environmental differences between cells are reduced as much as possible in a shaking liquid medium (**Figure 5**-figure supplement 4). Recent development in single-cell techniques facilitates to reveal that distinct bacterial subpopulations contribute unique colonization and growth strategies to infection sites (Llorens-Rico et al., 2022). For example, the host can drive *Salmonella* phenotypic heterogeneity at the single-cell level throughout the course of infection, highlighting how variation in gene expression, and metabolic activity contribute to overall bacterial success (Tsai and Coombes, 2019). Consistently, we found that the host changes the transcriptomic atlas of *S. marcescens* individual cells, and attenuates phenotypic heterogeneity of virulent factors (**Figure 5D-L**). To date, transcriptional approaches-profiling the host, the pathogen, or both-have been employed to uncover substantial molecular details about the host and bacterial factors that underlie infection outcomes. During the interaction, the niche environment diversifies into 3D areas with varying degrees of growth conditions (Koo and Yamada, 2016). In a single population, both in vitro and in vivo, *S. typhimurium* has been shown to display significant cell-to-cell variation in attributes such as growth rate, expression of virulence factors, and sensitivity to antibiotics (Claudi et al.,

2014). With the development of this approach, it is possible to identify and modify the transcription of certain target bacterial cells with the expression of virulence factors in order to selectively treat human diseases in the gut microenvironment.

Utilizing the *Drosophila* model system, we revealed a natural ecological phenomenon whereby the host was a prerequisite for regulating the population size and lifecycle switch of indigenous bacteria. It's of importance to understand the ecological and evolutionary processes that shape host-associated microbial communities. Little is known about the effect of the host on the commensal bacteria, and it would be also interesting to investigate whether the host and commensal bacteria could synergize to antagonize the pathogenicity of potential pathogens. Future studies that evaluate the molecular mechanism underlying the effect of the host on microbial communities would improve our understanding of host-symbiont co-evolution in nature.

Materials and methods

Fly culture and stocks

Drosophila flies were routinely reared and kept at the condition of 25°C, 55%-65% humidity with a 12 h: 12 h light-dark circle unless otherwise noted. The Canton-S strain was used as the wild-type fly in this work. Delta *14 AMP* mutant and Rel^{E20} mutant was generated as described (Hanson et al., 2019) and kindly gifted by Dr. Zhai in Hunan Normal University. The *yw; Sp/CyO; MKRS/TM6B* and $dfmr1^{50M}$ null mutant were used as weak flies and infertile flies (Liu et al., 2012). The fly was raised on standard cornmeal-sugar-agar medium (1 liter): 105 g dextrose, 7.5 g agar, 26 g yeast, 50 g cornmeal, 0.25 g Sodium benzoate (Sigma Aldrich) dissolved in 8.5 ml 95% ethanol and 1.9 ml propionic acid (99%, Mallinckrodt Baker).

Generation of germ-free and gnotobiotic flies

Germ-free flies were generated as described (Jia et al., 2021). In brief, fresh embryos within 8 hours post egg-laying were collected from agar media with 1.5% grape juice, rinsed with ddH_2O, and transferred into 1.5 ml Eppendorf tubes. Diluted sanitizer Walch (1:30), 2.5% sodium hypochloride (Sigma-Aldrich), 75% ethanol, and sterile PBS containing 0.01% TritonX-100 were successively applied to bleach embryos. The germ-free embryos were cultivated in autoclaved fly food with 10% yeast. Axenia of germ-free larvae was normally tested by plating the larval homogenates on nutrient agar plates (peptone 10 g/L; beef extract powder 3 g/L; NaCl 5 g/L; agar 15 g/L). Gnotobiotic flies were generated by inoculating bacterial strains to germ-free flies.

Survival assay

Newly eclosed female flies were collected and transferred into vials (15 flies/vial), and each group contained 6 vials with a total of 180 flies in each group based on two replicates for each. Before bacterial treatment, vials were pre-inoculated with *S. marcescens* alone and *S. marcescens* with 40 larvae, and incubated at 25°C for 24 h for culture. The flies were then transferred to vials processed with *S. marcescens* alone and *S. marcescens* in-coculture every 3 days. The number of dead flies was counted each day, and the proportion of surviving flies was calculated at each time point of the experiment. Experiments were independently replicated twice.

Bacteria culture and bacterial load quantification

All the material to manipulate bacteria was sterilized before usage. The strains of *Serratia marcescens* with the Genbank accession number CP053378 and *Lactobacillus plantarum* with the Genbank accession number KY038178 were used. *S. marcescens* and *L. plantarum* were cultured in LB and MRS broth medium at 30°C, respectively. Bacteria cells were harvested by centrifugation

(5000 rpm, 3 min), washed twice in 1×PBS, and resuspended in 1×PBS to obtain 10^8 cells/ml (OD_{595} = 1). The bacterial suspension (20 l) was supplemented to a vial with μ autoclaved fly food, and crawling GF larvae were then added to vials to generate a host-microbe interaction model. For the heat-shock experiment, the culture was transferred to a 45°C incubator at 12 h timepoint after postinoculation, and kept for 15 min. Heat-shocked bacteria were used for bacterial single-cell RNA seq below.

Fly food in vials was agitated and homogenated with 5 ml ddH_2O. Bacterial load was assessed by plating tenfold serial dilutions of the food homogenates on LB agar plates and incubating the plates at 30°C for 16 h. Mixed bacteria (bacteria in the living environment of *Drosophila*) were quantified in the NA medium that supports the growth of *Drosophila* microbiota (Jia et al., 2021). The numbers of CFUs were counted, and expressed as the total number of living bacteria per vial.

Prodigiosin production assay

The determination of the prodigiosin yield of bacteria was carried out with acidified ethanol and absorbance measurement as previously described (Kalivoda et al., 2010; Pan et al., 2020). The relative concentration of prodigiosin produced by solid-grown cultures was quantified as follows. Samples were added to 1.2 ml acidified ethanol (4% 1 M HCl in ethanol) to extract prodigiosin from the culture for 10 min. Cell debris and impurities were removed by centrifugation at 13000 rpm for 5 min, and the supernatant was transferred to a cuvette for measurement of absorbance at 534 nm. Prodigiosin production of samples was calculated as the optical density at 534 nm. Experiments were independently replicated four times.

AMP and antibiotics treatment

Cecropin A produced by silkworms (Sigma-Aldrich) was used as the representive of AMPs. Cecropin A was added to food at 40 μg/μl and 80 μg/μl. Antibiotic food was prepared by adding 5 μg/μl Kanamycin (Sigma-Aldrich) and 10 μg/μl Ampicillin (Sigma-Aldrich). The prodigiosin concentration and bacterial loads of *S. marcescens* challenged with Cecropin A, Kanamycin and Ampicillin were examined as described above.

Transmission electron microscopy

Transmission electron microscopy (TEM) was conducted to observe the structures of the bacterial cells as previously depicted (Morgelin, 2017). Briefly, bacterial cells were collected after 24-h incubation and then fixed in 2.5% glutaraldehyde for 5 h. The cells were dehydrated in a gradient series of ethanol solutions from 30 to 100% by incubation. For TEM analysis, the samples were treated with acetone and embedded in epoxy resin. Thin sections (70 nm) were cut with a diamond knife mounted on a Leica UC-7 ultramicrotome and collected on carbon-coated Cu grids. TEM observation was performed with a JEOL JEM1400 TEM at 200 kV. Micrograph films were digitally acquired at high resolution with EMSIS Morada G3 (Winey et al., 2014).

Real time-PCR analysis

RT-qPCR assay was performed as described previously (Liu et al., 2022). In brief, to assess the expression levels of prodigiosin synthesis-related genes, extracellular polysaccharide production-related genes, and partial down-regulated and up-regulated genes, the cultures of different groups of *S. marcescens* were collected after 24 h incubation. The collected cells were then subjected to total RNA extraction using a Bacteria RNA Extraction Kit (Vazyme, China). After treating the total bacterial RNA with DNase I (Vazyme, China), the concentration and quantity of the total bacterial RNA were determined using a NanoDrop spectrophotometer (Thermo Scientific), and 0.6 μg of the total bacterial RNA was subjected to reverse transcription using the HiScript III All- in-one RT SuperMix Kit (Vazyme). The mixture was subjected to RT-qPCR analysis using the ChamQ Universal SYBR qPCR master mix kit (Vazyme) in a CFX96™ Real-Time System (BioRad, Hercules, CA, USA). RNA from three biological replicates were analyzed and four technical replicates were

performed. The relative expression values were calculated using the following formula: ΔCt= Ct (target gene) - Ct (reference gene), and the relative expression was equal to $2^{-\triangle \triangle Ct}$. The 16S rRNA protein-encoding gene was used as an internal control. The primers used for RT-qPCR analysis are listed in Supplementary table 1. Experiments were independently replicated three times.

Bacterial bulk RNA Sequencing

To analyze the effect of larvae on the transcriptome in *S. marcescens*, the cultures of different groups were collected after 24 h incubation. 2 ml ice-cold 1×PBS were added to the vial for 5 min to suspend bacteria cells, and then suspended bacteria cells were removed food remainder by centrifugation (900 rpm, 3 min), washed twice in 1×PBS. Bacteria cells were harvested by centrifugation (5000 rpm, 4 min), and washed twice in 1×PBS. The collected cells were then subjected to total RNA extraction using a Bacteria RNA Extraction Kit (Vazyme, China). The integrity, concentration and quantity of the bacterial RNA were determined using a NanoDrop spectrophotometer (Thermo Scientific) and a 1% agarose gel. 1 μg total RNA with RIN value above 7 was used for following strand-specific library construction using VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina (Vazyme, China). Library quality was evaluated using an Agilent 2100 Bioanalyzer. The sequences were sequenced and processed using Novaseq-PE150 by the company (Novogene, Beijing, China).

For annotation, the genome of *S. marcescens FY* was used as a reference. The significant differentially expressed genes were determined in different groups using the DESeq software (DESeq 2), with the standards of P-value ≤ 0.05, and fold change |log2Ratio|≥1.2. KEGG KAAS database was used to annotate the genes with significantly differential expression based on their functions using the BLAST.

Metabolomics analysis

Untargeted metabolomics was performed using a modified version of a previously reported protocol (Tsugawa et al., 2015). In brief, L-2-chlorophenylalanine (0.06 mg/ml) dissolved in methanol was taken as internal standard, the samples were porfomed to GC-MS analysis. QC samples were prepared by mixing aliquots of all samples to be a pooled sample. Samples were analyzed on an Agilent 7890B gas chromatography system coupled to an Agilent 5977A MSD system (Agilent Technologies Inc., CA, USA). The temperature of the MS quadrupole and ion source (electron impact) was set to 150°C and 230°C, respectively. The collision energy was 70 eV. Mass spectrometric data were acquired in a full-scan mode (m/z50-500). For data processing, MZmine 2 and MultiQuant software programs were used.

Bacterial single-cell RNA-Seq and analysis

Bacterial single-cell RNA sequencing was carried out on a commercially available platform (M20 Genomics Company) (Xu et al., 2023). In brief, half a million *S. marcescens* cells were collected by centrifuging at 4°C. The supernatant was removed and cell pellets were resuspended in 2 ml ice-cold 4% formaldehyde (Sigma, 47608) and incubated with shaking overnight at 4LJ. The fixative was removed by centrifuging at 4000 rpm for 5 min at 4°C and cells were washed twice with 1 mL PBS-TRI (1x PBS supplemented with 0.1% Tween-20 and 0.1 U/mL Murine RNase inhibitor (Vazyme, R301)). The supernatant was removed and cells were resuspended in 200 μl PBS-TRI (1x PBS supplemented with 0.1% Tween-20 and 0.2 U/mL Murine RNase inhibitor (Vazyme, R 301)). The cell suspensions were counted with a Moxi cell counter and diluted according to the manufacturer's instructions to obtain single-cell.

The bacterial single-cell RNA-Seq library was prepared according to the protocol of VITAPilote® kit (M20 Genomics, R20114124). In situ reverse transcription of bacteria was performed with random primers and the resulting cDNA fragment was added with adaptor. The droplet barcoding for a single bacterium was performed on VITACruiser® Single Cell Partitioning System (M20 Genomics,

Hangzhou, China). Bacteria, DNA extension reaction mix, and hydrogel barcoded beads were encapsulated using the VITACruiser. The aqueous phase containing cDNAs was purified with magnetic beads. The cDNAs were amplified by PCR, and purified with magnetic beads. All products were pooled to construct a standard sequencing library. Sequencing was done on a PE150 (Illumina), and raw reads were aligned against the *S. marcescens FY* genome and counted by StarSolo followed by secondary analysis in the Annotated Data Format. Sequencing data was further analyzed using Seurat (v.4.3.0.1 with default parameters except where indicated). Cells were filtered to retain only those with at least 100 genes. Genes were also screened to remove genes expressed only in fewer than five cells. Then, We first log-transformed the data using the NormalizeData function, and selected the 2000 most variable genes using FindVariableFeatures. Then, we z-scored these highly-variable genes using 'ScaleData'. Next, we performed linear dimensionality reduction using principal component analysis (PCA) down to 50 dimensions ('RunPCA'). Points in this embedding were used to construct UMAP plots and find neighbours for clustering. FindClusters was used to run the Louvain clustering algorithm and generate clusters. We confirmed that the clusters highlighted in the main text appeared consistently for a range of resolutions from 0.5 to 1.5. DEG was performed using the FindMarkers function with a log-fold change cutoff of 0.25.

Statistical analysis

Statistical analysis is performed using GraphPad Prism 9.0 and indicated inside each figure legend. The layout of all figures used Adobe Illustrator 2022. Experimental flies were tested at the same condition, and all data are collected from at least two independent experiments. D'Agostino-Pearson normality test was used to verify the normal distribution of data. If normally distributed, a two-tailed Student's t-test was used to compare two groups, and one-way ANOVA with two-stage step-up method of Benjamini, Krieger and Yekutieli followed by Tukey's multiple comparisons test was used for comparisons of multiple groups. If not normally distributed, a two-tailed Mann-Whitney U-test was performed to compare two groups of samples, while Kruskal-Wallis test followed by Dunn's multiple comparisons test was used for multiple comparisons among three or more groups.

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Author contributions

Z.W., H.C., E.T., Y.W., Y.W. and W.L. conceptualized the study, designed experiments, and interpreted all the data. Z.W. performed bacterial counting, survival assay, bull RNA-seq runs, TEM, metabolomics runs, bacterial single-cell RNA-seq and RT-PCR, with help from S.Z., Y.W., A.L. and Y.Z.; Y.W. contributed to bacterial single-cell RNA-seq. S.L. and S.Z. wrote codes and analyzed data of bull RNA-seq, metabolomics, and bacterial single-cell RNA-seq. Z.W., Y.Z., Y.W. and W.L. wrote the manuscript with input from all authors. Y.W., Y.W. and W.L. conceived and supervised the project.

Data availability

The authors declare that all data supporting the findings of this study are available within this article. The bulk RNA-seq data and single-cell RNA-seq date can be accessed at the GEO: GSE232120 and GSE232484. Metabolomics data can be found under MetaboLights: MTBLS7962. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Wei Liu (liuwei5@ahau.edu.cn).

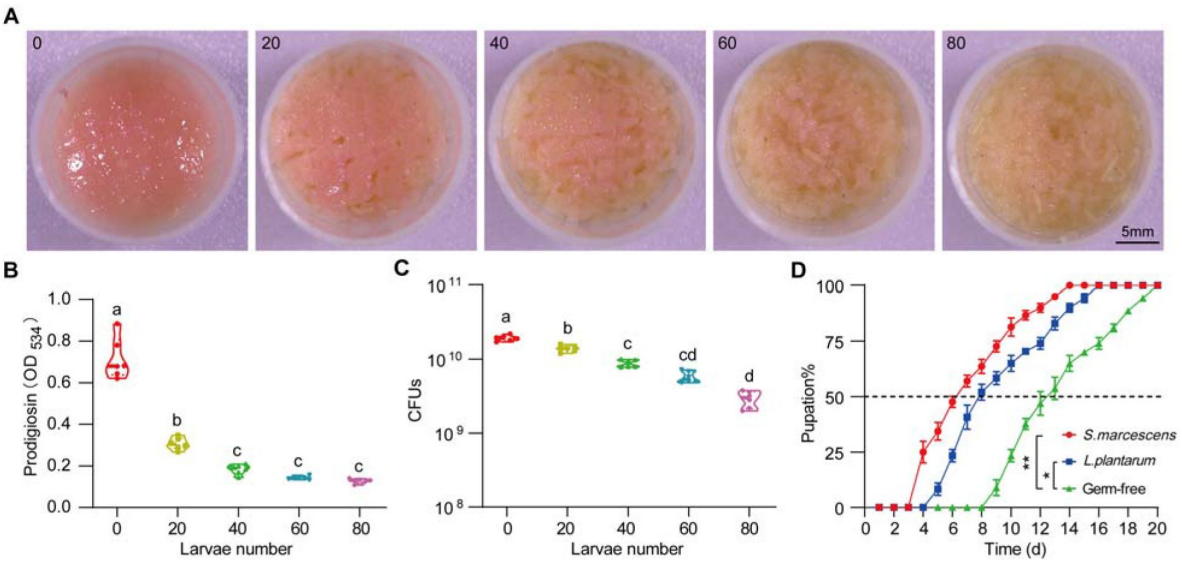

figure supplement 1.

Drosophila larvae modulate *S. marcescens* lifestyle switch. (**A**) Representative images of surface slick inoculated with *S. marcescens* in coculture with different numbers of crawling larvae. (**B**) The prodigiosin production of *S. marcescens* in coculture with a series of crawling larvae. n = 6 for each. (**C**) Bacterial loads of *S. marcescens* in coculture with different numbers of crawling larvae. n = 6 for each. (**D**) *S. marcescens* promoted the development of GF larvae. Developmental timing of GF, *L. plantarum*-, and *S. marcescens*- associated *Drosophila* was assessed on the poor diet with 0.5% yeast. The cumulative percentage of the pupation emergence is shown over time. n = 60 for each. The statistical analyses were performed using two-tailed Mann-Whitney U-test in (D). *$p < 0.05$; **$p < 0.01$. Means ± SEMs. All variables have different letters, and they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

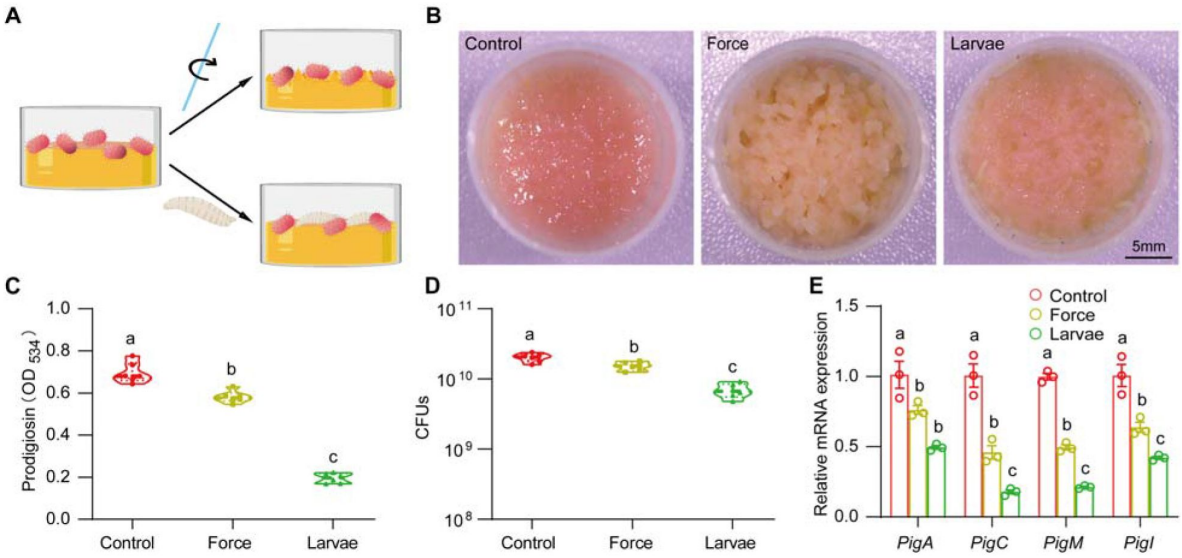

figure supplement 2.

Biological factors mainly determine *S. marcescens* lifestyle. (**A**) The schematic illustration of *S. marcescens* alone, with force and with larvae. Single and coculturing *S. marcescens* were generated as described in **Figure 2A**. In the meantime, *S. marcescens* with force were agitated using sterile glass sticks at the 2-h interval. (**B**) Representative images of surface slicks associated with *S. marcescens* alone, with force and with larvae. (**C**) The prodigiosin production of *S. marcescens* alone, with force and with larvae. n = 6 for each. (**D**) Bacterial loads of *S. marcescens* alone, with force and with larvae. n = 6 for each. (**E**) RT-qPCR analysis of the expression levels of the *pigA, pigC, pigM* and *pigI* genes of *S. marcescens* alone, with force and with larvae. n = 3 for each. Means ± SEMs. All variables have different letters, they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

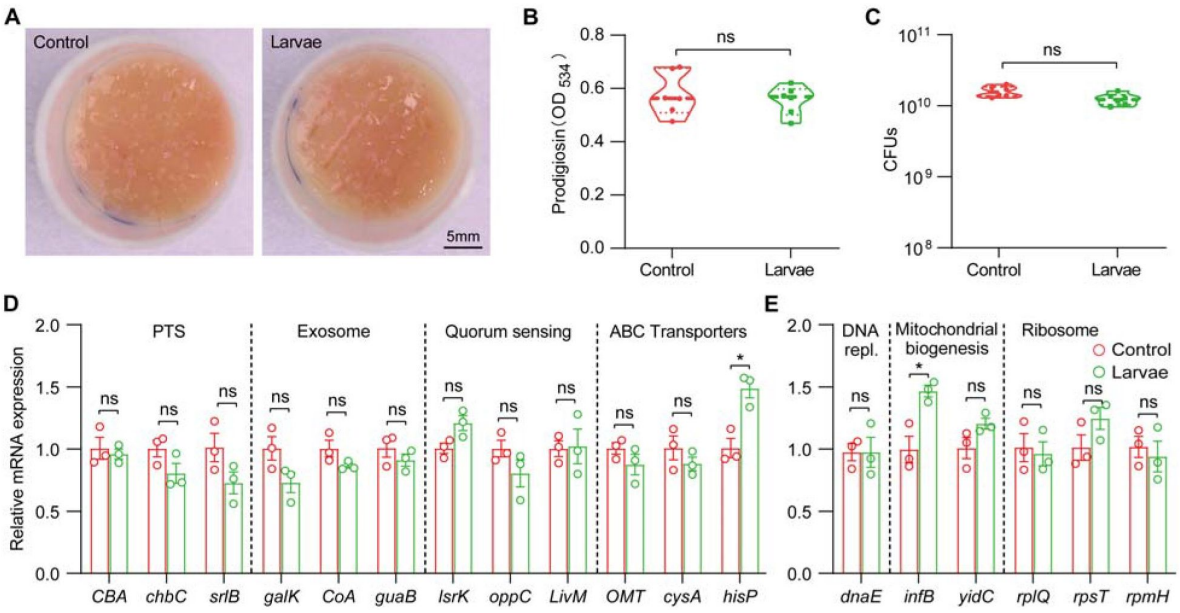

figure supplement 3.

The lifestyle switch of *S. marcescens* was driven by transcriptional alterations. (**A**) Representative images of surface slick with *S. marcescens* after reculturing 24 h from control and larvae groups isolated bacteria. (**B**) The prodigiosin production of *S. marcescens* after reculturing 24 h from control and larvae groups isolated bacteria. n = 6 for each. (**C**) Bacterial loads of *S. marcescens* after reculturing 24 h from control and larvae groups isolated bacteria. n = 6 for each. (**D, E**) RT-qPCR analysis of the expression levels of downregulated and upregulated genes in the *S. marcescens* after reculturing 24 h from control and larvae groups isolated bacteria. n = 3 for each. Means ± SEMs. The statistical analyses were performed using two-tailed unpaired Student's t test. *$p < 0.05$; ns, no significance.

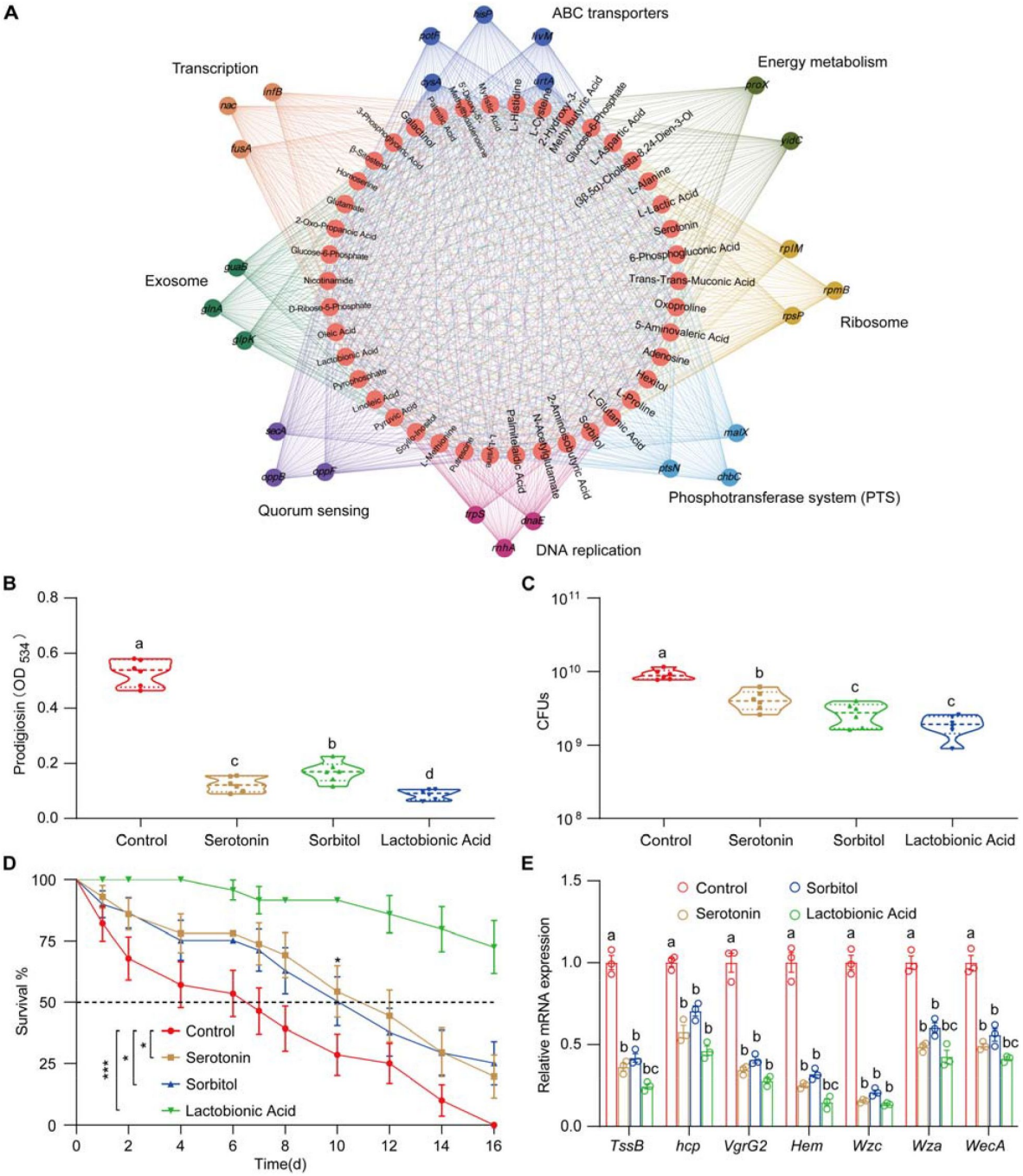

figure supplement 4.

Interaction network analysis of transcriptome and metabolome. (**A**) Interaction network analysis of transcriptome and metabolome of *S. marcescens* alone and with larvae. 42 differentially expressed metabolites (red circles) are related to differentially expressed genes involved in the ribosome (light brown), transcription (dark brown), DNA replication (light purple), energy metabolism (dark green), ABC transporters (dark blue), phosphotransferase system (light blue), quorum sensing (dark purple) and exosome (light blue). (**B**) The prodigiosin production of *S. marcescens* alone and with predicted metabolites. (**C**) Bacterial loads of *S. marcescens* alone and with predicted metabolites. n = 6 for each. (**D**) The survival rate of adult flies challenged with *S. marcescens* alone and with metabolites. n = 180 for each. The statistical analyses were performed using Log-rank test. *$p < 0.05$; ***$p < 0.001$. (**E**) RT-qPCR analysis of the expression levels of virulence-related genes of with *S. marcescens* alone and with metabolites. n = 3 for each. Means ± SEMs. All variables have different letters, and they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

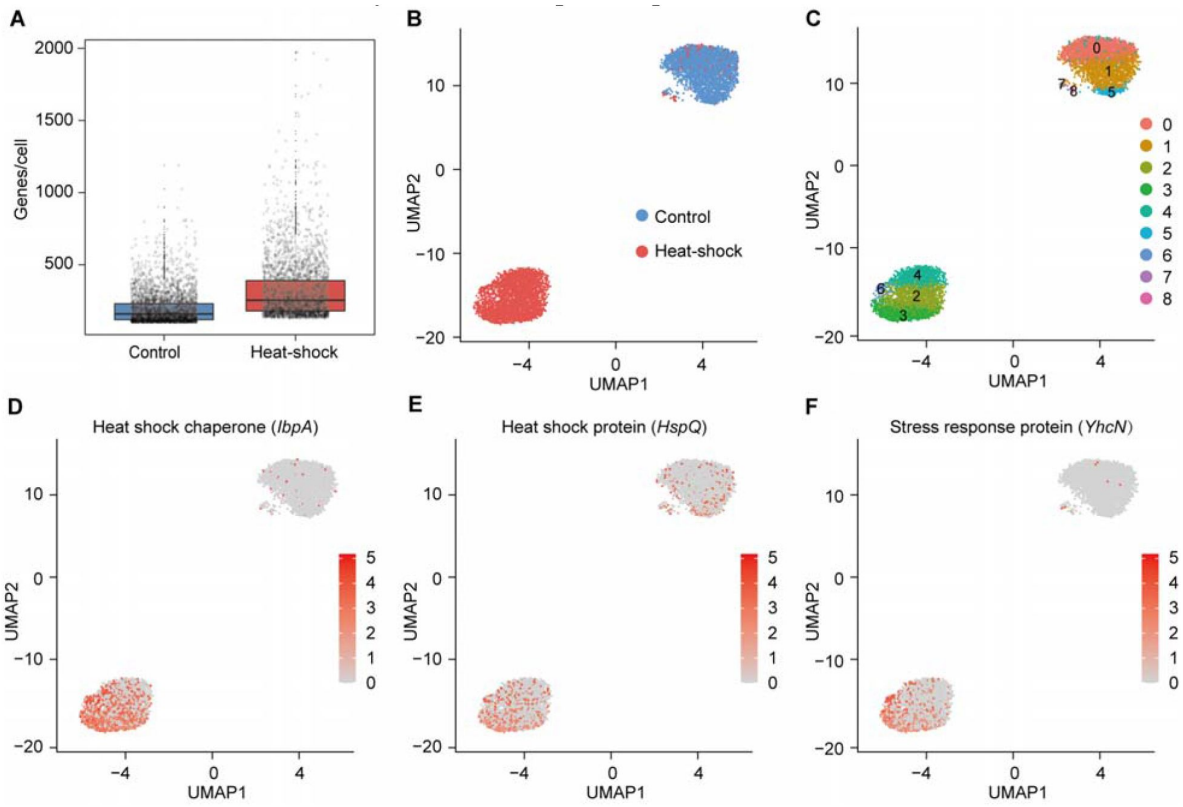

figure supplement 5.

Heat-induced phenotypic heterogeneity of *S. marcescens.* (**A**) mRNA gene counts per cell for the control and heat-shock groups. Each dot represents a bacterial cell of *S. marcescens.* (**B**) Joint UMAP two-dimensional analysis of *S. marcescens* showing that are distinct clusters between the control and heat-shock groups. (**C**) The cell subpopulation typing of the control and heat-shock groups. (**E**, **F**) The expression of two representative genes of heat shock and stress response was highlighted on the UMAP. The red color bars represent the normalized expression of a gene across all cells analyzed.

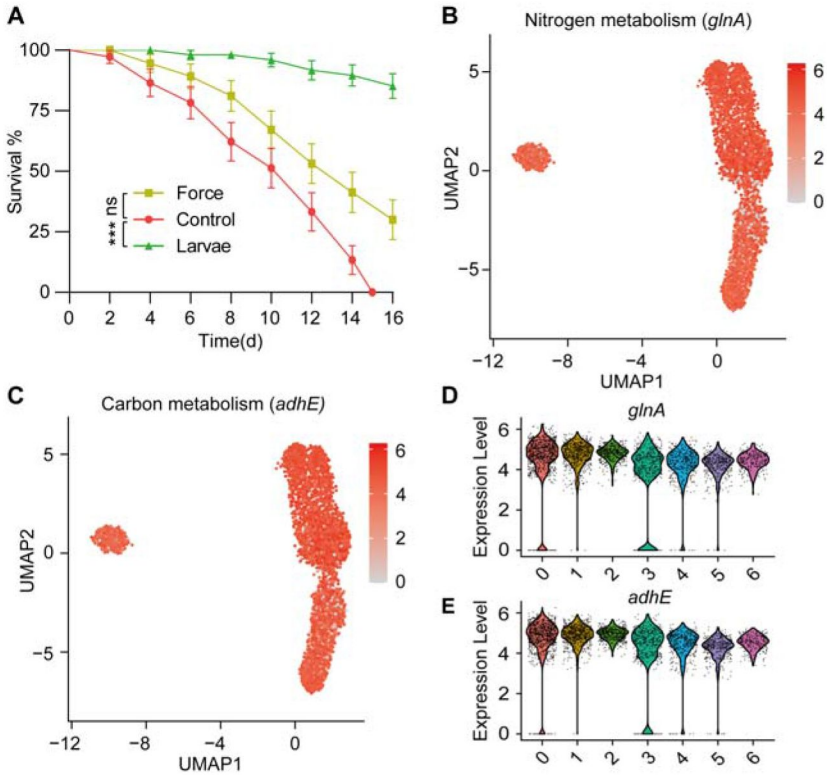

figure supplement 6.

Larvae affect the metabolism of *S. marcescens.* (**A**) The survival rate of adult flies challenged with *S. marcescens* alone, with agitation and in coculture. *S. marcescens* with alone, with agitation and in coculture were obtained after 24-h incubation as described in figure supplement 2A, and the percentage of living female flies was calculated to monitor lifespan. n = 180 for each. The statistical analyses were performed using Log-rank test. ***$p < 0.001$; ns, no significance. (**B**, **C**) The expression of two representative genes of Nitrogen metabolism and Carbon metabolism was highlighted on the UMAP. The red color bars represent the normalized expression of a gene across all cells analyzed. (**D**, **E**) Violin plots of *glnK*, *glnA*, *pgk* and *adhE* genes in different subclusters. Each dot represents a single cell and the shapes represent the expression distribution.

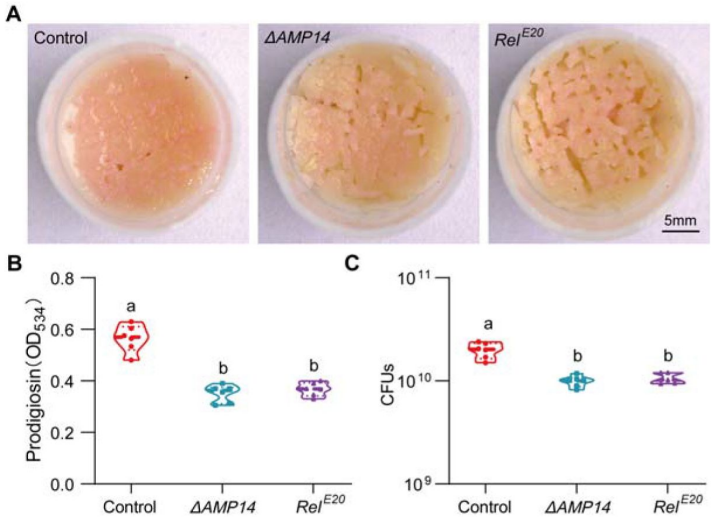

figure supplement 7.

AMPs play a major role in recapitulating the response of *S. marcescens* to larvae. (**A**) Representative images of surface slick with *S. marcescens* alone with *ΔAMP14* larvae and with Rel^{E20} larvae. (**B**) The prodigiosin production of *S. marcescens* alone, with *ΔAMP14* larvae and with Rel^{E20} larvae. n = 6 for each. (**C**) Bacterial loads of *S. marcescens* alone, with *ΔAMP14* larvae and with Rel^{E20} larvae. n = 6 for each. Means ± SEMs. All variables have differrent letters, they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). Kruskal-Wallis test followed by Dunn's multiple comparisons test.

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Reviewer #1 (Public Review):

Summary:

In this work, Wang and colleagues used Drosophila-Serratia as a host-microbe model to investigate the impact of the host on gut bacteria. The authors showed that Drosophila larvae reduce S. marcescens abundance in the food likely due to a combination of mechanical force and secretion of antimicrobial peptides. S. marcescens exposed to Drosophila larvae lost virulence to flies and could promote larval growth similar to typical Drosophila gut commensals. These phenotypic changes were reflected in the transcriptome and metabolome of bacteria, suggesting that the host could drive the switch from pathogenicity to commensalism in bacteria. Further, the authors used single-cell bacterial RNA-seq to demonstrate the heterogeneity in gut bacterial populations.

Strengths:

This is a valuable work that addresses an important question of the impact of the host on its gut microbes. The authors could convincingly demonstrate that gut bacteria are strongly affected by the host with important consequences for both interacting partners. Moreover, the authors used state-of-the-art bacterial single-cell RNA-seq to reveal heterogeneity in host-associated commensal populations.

Overall most parts of the study are solid and clear.

https://doi.org/10.7554/eLife.96789.2.sa2

Reviewer #3 (Public Review):

In this study, Wang and coworkers established a model of Drosophila-S. marcescens interactions and thoroughly examined host-microbe bidirectional interactions. They found that:

(1) Drosophila larvae directly impact microbial aggregation and density;
(2) Drosophila larvae affect microbial metabolism and cell wall morphology, as evidenced by reduced prodigiosin production and EPS production, respectively;
(3) Drosophila larvae attenuate microbial virulence;
(4) Drosophila larvae modulate the global transcription of microbes for adaptation to the host;
(5) Microbial single-cell RNA sequencing (scRNA-seq) analysis revealed heterogeneity in microbial pathogenicity and growth;
(6) AMPs are key factors controlling microbial virulence phenotypes.

Taken together, they concluded that host immune factors such as AMPs are directly involved in the pathogen-to-commensal transition by altering microbial transcription.

In general, in this revised version, I feel that the authors addressed all the points raised in the previous review process. Specifically, they demonstrated that sub-lethal doses of antibiotics such as kanamycin or ampicillin is sufficient to induce the virulence switch in S. marcescens. Furthermore, by testing IMD pathway mutant animals, they concluded that AMP plays a major role in the commensal-to-pathogen transition. In summary, I appreciate the authors' efforts, and I am satisfied with the revision.

https://doi.org/10.7554/eLife.96789.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***eLife assessment***
>
> *This valuable study examines the role of a host in conditions that shift pathogenicity of opportunistic microbes. The use of single-cell microbial transcriptomics and metabolomics to demonstrate the host's effects on pathogen dynamics is interesting and convincing. However, the connection to host antimicrobial peptides driving these effects is incomplete and would benefit from additional evidence and improved explanation in the text. This paper has the potential to be of broad interest to those working in host-microbe (microbiome and pathogen) interactions.*

We appreciate the editors for organizing our manuscript and providing eLife assessment. We went through each comment and carried out some necessary experiments. According to the comments, we here provide additional evidence that further supports our findings in this revised manuscript.

> ***Public Reviews:***
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> ***Reviewer #1 (Public Review):***
>
> *Summary:*
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> *In this work, Wang and colleagues used Drosophila-Serratia as a host-microbe model to investigate the impact of the host on gut bacteria. The authors showed that Drosophila larvae reduce S. marcescens abundance in the food likely due to a combination of mechanical force and secretion of antimicrobial peptides. S. marcescens exposed to Drosophila larvae lost virulence to flies and could promote larval growth similar to typical Drosophila gut commensals. These phenotypic changes were reflected in the transcriptome and metabolome of bacteria, suggesting that the host could drive the switch from pathogenicity to commensalism in bacteria. Further, the authors used single-cell bacterial RNA-seq to demonstrate the heterogeneity in gut bacterial populations.*
>
> *Strengths:*
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> *This is a valuable work that addresses an important question of the effect of the host on its gut microbes. The authors could convincingly demonstrate that gut bacteria are strongly affected by the host with important consequences for both interacting partners. Moreover, the authors used state-of-the-art bacterial single-cell RNA-seq to reveal heterogeneity in host-associated commensal populations.*
>
> *Weaknesses:*
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> *Some of the conclusions are not fully supported by the data.*
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> *Specifically, in lines 142-143, the authors claim that larva antagonizes the pathogenicity of S. marcescens based on the survival data. I do not fully agree with this statement. An alternative possibility could be that, since there are fewer S. marcescens in larvae-processed food, flies receive a lower pathogen load and consequently survive. Can the authors rule this out?*
>
> *Also, the authors propose that Drosophila larvae induce a transition from pathogenicity to commensalism in S. marcescens and provide nice phenotypic and transcriptomic data supporting this claim. However, is it driven only by transcriptional changes? Considering high mutation rates in bacteria, it is possible that S. marcescens during growth in the*

> *presence of larvae acquired mutations causing all the observed phenotypic and transcriptional changes. To test this possibility, the authors could check how long S. marcescens maintains the traits it acquires during growth with Drosophila. If these traits persist after reculturing isolated bacteria, it is very likely they are caused by genome alterations, if not - likely it is a phenotypic switch driven by transcriptional changes.*

We thank the reviewer for providing a feasible method to distinguish the shift in transcriptional profile from genomic mutations. According to this valuable suggestion, we checked phenotypic and transcriptional changes after re-culturing the bacterium that had coexisted with larvae. We found that all phenotypes can be recovered after re-culturing. The new data supported our previous result that a phenotypic switch was driven by transcriptional changes rather than genome mutations. We now add these results to the text with figure supplement 3 (line 147-151, 192-194). Please see the following text.

"To rule out the possibility that phenotypic alterations could stem from genomic mutations, we examined the prodigiosin yield and CFUs of re-culturing *S. marcescens* that had coexisted with larvae. Our results showed that neither prodigiosin yield nor CFUs of re-culturing *S. marcescens* differed from the original strain (Figure 2-figure supplement 3A-C), suggesting that a phenotypic switch was driven primarily by transcriptional reprogramming."
"Consistent with the previous result that this phenotypic switch was driven by transcriptional changes, the expression of virulent and growth genes was recovered after re-culturing (Figure 3-figure supplement 3D, E)."

For the first question, we admit the possibility that the high morality of flies could result from the acquirement of a higher pathogen load, because of an increase in the bacterial load of single *S. marcescens*. However, host pathogenesis is normally determined by the virulence of pathogens rather than the number of bacteria. For example, hosts constantly harbor astonishing commensals in their guts, but remain healthy. This evidence suggests that it was the property (virulence) of a pathogen that is more important to affect the health status of the hosts. Moreover, an increase in virulence of single *S. marcescens* was verified by real-time PCR (Fig. 2F) and TE (Fig. 2G). Taken together, we could draw a conclusion that the impaired survival of flies challenged with single *S. marcescens* mainly arose from an increase in the virulence of *S. marcescens*. Thanks for your understanding!

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
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> *While many studies have explored the impacts of pathogens on hosts, the effect of hosts on pathogens has received less attention. In this manuscript, Wang et al. utilize Drosophila melanogaster and an opportunistic pathogen, Serratia marcescens, to explore how the host impacts pathogenicity. Beginning with an observation that larval presence and density impacted microbial growth in fly vials (which they assess qualitatively as the amount of 'slick' and quantitatively as microbial load/CFUs), the authors focus on the impact of axenic/germ-free larvae on an opportunistic pathogen S. marcescens. Similar to their observations with general microbial load, they find that larvae reduce the presence of a pinkish slick of Sm, indicative of its secondary metabolite prodigiosin. The presence of larvae alters prodigiosin production, pathogen load, pathogen cellular morphology, and virulence, and this effect is through transcriptional and metabolic changes in the pathogen. Overall, they observe a loss of virulence factors/pathways and an increase in pathways contributing to growth. Given the important role the host plays in this lifestyle shift, the authors then examined host features that might influence these effects, focusing on the role of antimicrobial peptides (Amps). The authors combine the use of synthetic Amps and an Amp-deficient fly line and conclude much of the larval inhibitory effect is due to their production of AMPs.*

Strengths:

This is a very interesting question and the use of Drosophila-Serratia marcescens is a great model to explore these interactions and effects.

The authors have an interesting and compelling phenotype and are asking a unique question on the impact of the host on the pathogen. The use of microbial transcriptomics and metabolomics is a strength, especially in order to assess these impacts on the pathogen level and at the single-cell level to capture heterogeneity.

Weaknesses:

Overall, the writing style in the manuscript makes it difficult to fully understand and appreciate the data and its interpretation.

The data on the role of AMPs would benefit from strengthening. Some of the arguments in the text of that section are also counterintuitive. The authors show that △AMP larvae have a reduced impact on Sm as compared to wt larvae, but it seems less mild of an effect than that observed with wt excreta (assuming the same as secreta in Figures 7, should be corrected or harmonized). Higher doses of AMPs give a phenotype similar to wt larvae, but a lower dose (40 ng/ul) gives phenotypes more similar to controls. The authors argue that this data suggests AMPs are the factor responsible for much of the inhibition, but their data seems more to support that it's synergistic- you seem to still need larvae (or some not yet defined feature larvae make, although secreta/excreta was not sufficient) + AMPs to see similar effects as wt. Based on positioning and color scheme guessing that AMP 40ng/ul was used in Figures 7D-H, but could not find this detail in the text, methods, or figure legend and it should be indicated. This section does not seem to be well supported by the provided data, and this inconsistency greatly dampened this reviewer's enthusiasm for the paper.

We thank the reviewer's valuable comments and suggestions. We admitted that some photos of the pinkish slick (prodigiosin) are counterintuitive in Figure 7 as well as figure supplement 2B. Here comes the reason. Single *S. marcescens* produced prodigiosin that only stayed on the surface of fly agar medium. As we know, larvae can agitate food and form a stratification of prodigiosin, even making higher prodigiosin yield inside food lighter than the surface slick of prodigiosin. We mentioned it in the previous manuscript line 166-168. This is why some photos treated with excreta and a lower dose of AMP seemed more intense than those with WT larvae. However, we precisely quantified the prodigiosin yield inside food with the spectrophotometer, so we provided a prodigiosin yield following the photos of the slick. Therefore, we drew our conclusions mainly relying on the quantification of the prodigiosin yield. We actually used cecropin A for our experiments, so we added this information in the text. We hope that our replies can reignite your enthusiasm for our manuscript, and thanks for your great support!

Reviewer #3 (Public Review):

In this study, Wang and coworkers established a model of Drosophila-S. marcescens interactions and thoroughly examined host-microbe bidirectional interactions. They found that:

(1) Drosophila larvae directly impact microbial aggregation and density;

(2) Drosophila larvae affect microbial metabolism and cell wall morphology, as evidenced by reduced prodigiosin production and EPS production, respectively;

(3) Drosophila larvae attenuate microbial virulence;

(4) Drosophila larvae modulate the global transcription of microbes for adaptation to the host;

(5) Microbial single-cell RNA sequencing (scRNA-seq) analysis revealed heterogeneity in microbial pathogenicity and growth;

(6) AMPs are key factors controlling microbial virulence phenotypes.

Taken together, they concluded that host immune factors such as AMPs are directly involved in the pathogen-to-commensal transition by altering microbial transcription.

General comments:

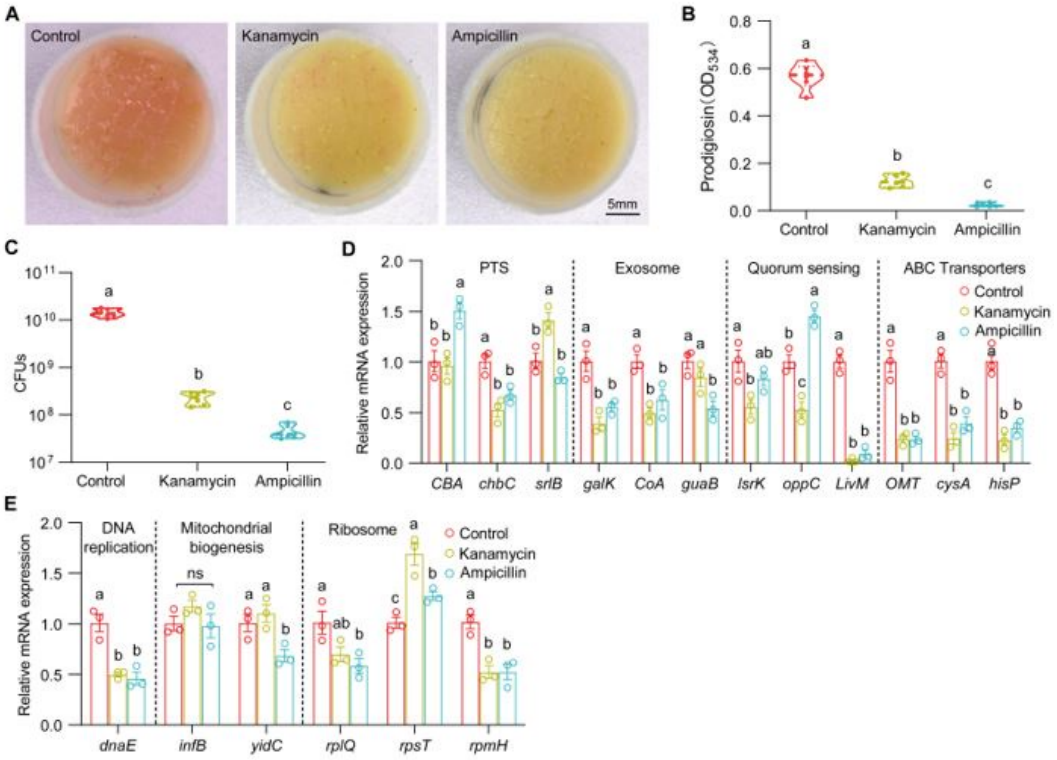
In general, this study is intriguing as it demonstrates that host immune effectors such as AMPs can serve as critical factors capable of modulating microbial transcription for host-microbe symbiosis. However, several important questions remain unanswered. One such question is: What is the mechanism by which AMPs modulate the pathogen-to-commensal transition? One hypothesis suggests that antimicrobial activity may influence microbial physiology, subsequently modulating transcription for the transition from pathogen to commensal. In this context, it is imperative to test various antibiotics with different modes of action (e.g., targeting the cell wall, transcription, or translation) at sub-lethal concentrations to determine whether sub-lethal doses of antimicrobial activity are sufficient to induce the pathogen-to-commensal transition.

Thank you for the important comments on our manuscript. We checked the effect of antibiotics (5 μg/μl kanamycin and 10 μg/μl ampicillin) on the virulence switch of *S. marcescens*. We found that the two antibiotics with the sub-lethal doses similarly resulted in a decrease in prodigiosin yield and virulence expression of *S. marcescens*. Intriguingly, the two antibiotics also resulted in a dramatic decline in the bacterial load and the expression of genes involved in cell growth. These results suggest that antibiotics reduced the virulence primarily through suppressing most activities of bacteria.

We found that larvae and AMPs at 40 μg/μl modestly resulted in a decrease in bacterial load and an increase in the relative level of genes involved in cellular proliferation, suggesting that AMPs could maintain the exponential phase of bacterial growth. This result is consistent that *Drosophila* larvae can support the long-term persistence of commensals in the shared habitat (DOI: 10.1016/j.cmet.2017.11.011). The inhibition could prevent bacteria from rapidly exhausting their nutritional resources, and consequently maintain symbiosis. It is likely that AMPs could maintain *S. marcescens* at the exponential phase of cell growth and prevent bacteria from rapidly exhausting their nutritional resources.

Author response image 1.

(A) Representative images of surface slick with *S. marcescens* alone, with kanamycin (5 μg/μl) and ampicillin (10 μg/μl). (B) The prodigiosin production of *S. marcescens* alone, with kanamycin (5 μg/μl) and ampicillin (10 μg/μl). n = 6 for each. (C) Bacterial loads of *S. marcescens* alone, with kanamycin (5 μg/μl) and ampicillin (10 μg/μl). n = 6 for each. (D, E) RT-qPCR analysis of the expression levels of downregulated and upregulated genes in the *S. marcescens* alone, with kanamycin (5 μg/μl) and ampicillin (10 μg/μl). n = 3 for each. Means ± SEMs. All variables have different letters, they are significantly different ($p < 0.05$). If two variables share a letter, they are not significantly different ($p > 0.05$). ns, no significance. Kruskal-Wallis test followed by Dunn's multiple comparisons test.



> ***Recommendations for the authors:***
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> ***Reviewer #1 (Recommendations For The Authors):***
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> *Here are some specific points that need to be addressed:*
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> *(1) Lack of statistical analysis for many figures. The authors should perform and report the statistical analysis for all figures where it is currently lacking, specifically, Figures 2C, D, E, F, H; Figures 3E, F; Figures 7G, H; Figure S2E, Figures S3D, E.*

Thanks for your valuable suggestions. We re-checked the manuscript and performed the statistical analysis for these figures.

> *(2) For graphs showing dots, it should be specified what exactly individual dots show and how many animals were used per replicate. Also, time points at which specific analysis was performed should be specified.*

We provided the important information in the legends in the revised manuscript.

> *(3) Figure 2. No letters illustrating statistical significance are shown, although this is claimed in the legend (line 848).*

We added statistical significance in the updated Figure 2.

> *(4) In Figure 7, the authors used AMPs of defined concentration, but it is not specified what exactly these AMPs are. Please provide the full composition of the AMP mix used.*

We used the antimicrobial peptide cecropin A produced by a silkworm. We added this information in the methods line 487-488 and Figure 7 legend.

> *(5) Figure S2B. To me, it looks like that medium with larvae is redder than after mechanical force. I find it hard to believe the quantification in panel C that the medium with larvae has 3 times less pigment as compared to the mechanical force.*

Larvae could only agitate the surface of food (~0.4 cm), but sticks completely agitated the food up to 3 cm. Thus, the layer of food with pink pigment with agitation seemed much deeper than with larvae, which was responsible for the counterintuitively. We explained it in the previous manuscript (line 166-168). “Of note, the surface of the slick with agitation appeared lighter than that of larvae, mainly due to a stratification of prodigiosin following agitation.”

> *(6) The authors need to proofread the manuscript as there are missing words, terms that need definition, and wrong terms. For example, L86 - naked eye?, L117 - what do the authors mean by co-culture?, L309 - not resist but rather combat, L347 - Species? or competition?, Figure 2A - 2nd?*

We have corrected these errors in the new manuscript. We added an "eye" in L86. Co-culture means “*S. marcescens* in co-culture”. Interspecies competition for nearly the same or similar nutrients and space occurs in the habitat.

> *(7) The authors should reorganize either the text or the figures' order in a way that the figures are described in a consecutive order (Figure 1A, B ... and not Figure 1D first and then 1A).*

Thanks for your valuable advice. We reorganize the order of the text.

> *(8) Do the authors have an idea which bacteria they quantified in Figures 1E to 1G? I didn't find the medium that was used for culturing. Also, in Figure 1F, Is the control group comprised of females or males?*

Mixed bacteria (bacteria in the living environment of *Drosophila*) were quantified in the NA medium that supports the growth of *Drosophila* microbiota (Jia Y, et al. Nat Commun. 2021) line 474-475. The control group comprised of both males and females with a 1:1 ratio. Similarly, the aged group contained 100 50-day-aged flies, male: female = 1:1. We provided details in Figure 1 legend line 849-850, 851-852.

> *(9) L118-129. it is not possible to make all these statements without any statistical analysis. To me, at 96h both treatments have the same CFUs, while the authors claim they are different.*

We added statistical analysis in the current version. In fact, single *S. marcescens* became collapsed after 72 h post inoculation, and the CFU number of single *S. marcescens* declined step by step. The bacterial load of *S. marcescens* in co-culture was comparable (at 96 h post-inoculation, $p>0.05$) or higher (at 120 h post-inoculation, $p<0.001$) than *S. marcescens* alone, possibly explained by the possibility that bacteria rapidly exhausted the nutritional resources and collapsed through population suicide. We rewrote this sentence line 125-129 in the updated manuscript.

> *(10) L136. term "symbionts" is not appropriate here.*

We change “symbionts” into “*S. marcescens*”.

> *(11) In Figure 1, the authors used flies of different fitness: weak, strong, and infertile. They should be specific and describe exactly what these terms mean, are these mutants*

> *or treatments that affect the fitness?*

We apologize for this missing information and add them in the method and legend. Strong flies (wild-type fly CS), weak flies (*yw; Sp/CyO; MKRS/TM6B*), infertile flies (*dfmr150M* null mutant) Figure 1 legend line 849-850.

> *(12) Figure S2. The title of this figure is misleading, please modify it. Mechanical force did affect S. marcescens but to a lesser degree as compared to larvae.*

Thank you for your suggestion. We admit that mechanical force affected *S. marcescens* but to a lesser degree as compared to larvae, so we changed the title to "Biological factors mainly determine *S. marcescens* lifestyle."

> ***Reviewer #2 (Recommendations For The Authors):***
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> *General improvement to writing and presentation (see below):*
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> *Describing confluent growth would make more sense than 'slick' and then using descriptions of broken, etc. "colour intensity of the surface slick".*

We used the slick to describe visible surface films of bacteria, which has been used in the previous study (DOI: 10.1038/s43705-023-00307-8). Slick is equal to confluent growth, but seems simple and easy than confluent growth. To make sense, we add this reference to the text.

We reorganized the text of Figure 1.

> *Suggest more specific language to describe observations. For example: Bacterial loading - S. marcescens growth (for example: the presence of dense fly populations reduced Sm growth).*

Thanks for the suggests. We replaced some of them.

> *Symbiont, microbiota, microbiome, etc were all used interchangeably throughout the manuscript, but I am not sure I would call Sm part of the indigenous microbiome. Suggest to ensure proper usage and then harmonize throughout the ms.*

We used microbes and microbiome to replace symbiont and microbiota, respectively.

> *Details missing from the message and Figure legends that would be helpful (including and especially Figure 7 - what AMP concentration?)*

Thanks for valuable comments. According to this comment, we provided concrete details in the Materials and methods and Figure 7 legend about AMPs, including the source and concentration of AMPs line 487-488, 954-955. Please see the response below.

> *L73: define 'these issues" maybe or lead better with the prior sentence, it is not evident as currently written.*

Change "to address these issues" to " To investigate whether and/or how the host modulates bacterial lifestyles," and merge two paragraphs.

> *L74: repetitive sentence with the above.*

Thanks for pointing out this detail. We deleted it.

> *L86: naked 'eye'.*

Added.

> *L87: what is meant by 'weak flies'?*

Genotypes were added in the updated manuscript. Weak fly stocks display weaker activity and generate fewer eggs than WT flies.

> *L96: bacterial load, not loading.*

Corrected.

> *L128: no evidence to support, could be reflective of increased numbers in dying/dead larvae that impact total numbers in the vial.*

The number of CFUs of *S. marcescens* alone was gradually decreased at 96 h post-inoculation. In addition, we observed pale biofilm on the surface of the medium at the late stage. The numbers of CFUs of *S. marcescens* alone at the later stages were reduced (compared to the peak load at 48 h post-inoculation), so it was deterred that bacteria could undergo ecological suicide. Ecological suicide of the bacterial population was similarly examined by recording the number of CFUs in the medium over time (Ratzke C, et al. Nat Ecol Evol. 2018.). Taken together, we draw a conclusion that bacteria possibly underwent ecological suicide.

> *L129: the prior sentence is in contradiction, reduced load only at early time points in the presence of larvae....*

Thanks for pointing out this detail. We added " before 72 h post-inoculation " in the sentence.

> *L134: data is only focused on S marcescens, so inferring to 'symbionts' broadly is outside study.*

We change “symbionts” into “*S. marcescens*”.

> *L139: sentence poorly written and confusing.*

We re-organized this sentence.

To this end, we sought to examine the *S. marcescens* lifestyle switch from pathogenicity to commensalism by assessing the respective survival of flies on the fly medium that had been processed by single or coexisting *S. marcescens*.

> *L189: evidence for long-term symbiosis is not well established in this paper, suggest editing this language throughout to more specifically reflect what the data supports and leave such interpretations to discussion points and future work.*

Thanks for your valuable advice. We deleted long-term and “thereby promoting the fitness of symbionts in the long maintenance.”.

> *L192; used metabolomics to assess the impacts of larvae on bacterial metabolism, as currently written does not make sense.*

We rewrote this sentence. “Next, we investigated whether larvae could further elicit changes in the metabolism of *S. marcescens* using untargeted metabolomics.”

> *L331: the use of monitored here is not correct/odd.*

We changed 'monitored' to 'reshaping’.

> *L340: While the authors initially see a cost to Sm in reduced load (CFUs) at 120 h populations associated with larvae become higher - there is also a cost to producing virulence factors, which their RNASeq and metabolomics data support - trade-offs between growth and virulence.*

Thanks for your suggestion. We added “before 72 hours post inoculation” to define the early stage of the bacterial growth in the sentence.

> ***Reviewer #3 (Recommendations For The Authors):***
>
> *(1) Figures 1 A-D: What defines weak and strong flies, and what criteria determine the robustness of flies? How was the experiment conducted? The manuscript lacks details on this matter.*

We thank you for your comments. We lack a criterium, but the robustness of flies comes from daily experience. Weak fly stocks display weak activity and generate fewer eggs than WT flies. Genotypes with different robustness were added in the legend in the updated manuscript

> *(2) The authors mentioned, "Noteworthily, the number of CFUs of S. marcescens alone was lower than S. marcescens in co-cultures at the late stage (at 96 h post inoculation), likely that bacteria rapidly exhausted their nutritional resources and underwent ecological suicide." How did they determine that the bacteria exhausted nutritional resources and underwent ecological suicide? One might speculate that larvae could have removed the bacteria simply by consuming them.*

Thanks for this comment. Virtually, there were no larvae inside the vials with single *S. marcescens*, so bacterial cells were not consumed. However, the numbers of CFUs of *S. marcescens* alone at the later stages were reduced (compared to the peak load at 48 h post-inoculation), so it was deterred that bacteria could undergo ecological suicide. Ecological suicide of the bacterial population was examined by recording the number of CFUs in the medium over time (Ratzke C, et al. Nat Ecol Evol. 2018.). A similar method was also applied to the number of CFUs of *S. marcescens*. Taken together, we draw a conclusion that bacteria possibly underwent ecological suicide.

> *(3) Figure 2E: The experimental details should be provided in the text. What was the CFU of the bacteria used in this survival experiment?*

We provided further experimental details in the legend line 869-870. The same amount of inocula was used in both single and coculturing *S. marcescens*.

> *(4) The experimental data in Figures 2G and 2H do not sufficiently prove the relationship between the width of the cell wall and virulence, as it lacks experimental validation.*

Previous studies (DOI: 10.1371/journal.ppat.1005946) reveal that glucosylating toxins on the surface are primary virulence determinants, so an increased surface-anchored polysaccharide and protein profile promotes the virulence of the pathogen. Alterations in cell

surface (the width of the cell wall) can be examined by TE. Moreover, TE was used to observe changes in the virulence of *S.* marcescens (DOI: 10.1093/nar/gkab1186). We think that the width of the cell wall could be used to reflect virulence in *S.* marcescens.

> *(5) While it's acknowledged that agitation decreases the color intensity of the bacteria, comparing mechanical agitation with larval crawling seems inappropriate, as the mechanical forces exerted by both methods are not of the same magnitude.*

Thanks for the suggestion. In fact, food was agitated more heavily by glass sticks than by larvae, because larvae merely agitated the surface of food (about 0.5 cm-depth). If the decrease in bacterial load and color was related to the magnitude of agitation, larvae would confer a less decrease (from the decrease in stick agitation) in bacterial load than the sticks. Consequently, it would further support our result that biofactors more importantly confer the inhibition of *S. marcescens* than force.

> *(6) Figure 4D: with this metabolome data, they mentioned, "host suppresses differentiation of S. marcescens into the population with pathogenicity." What evidence supports the claim that downregulation of amino acid metabolism, phosphotransferase system, and ABC transporter directly correlates with decreased pathogenicity?*

Thanks for the comment. Earlier studies showed that amino acid-derived quorum sensing molecules are closely related to bacterial pathogenicity (Defoirdt T. PLoS Pathog. 2019; Wen J, et al. Microbiol Spectr. 2022). Moreover, the phosphotransferase system and ABC transporter can transport and/or produce virulence factors. Therefore, we claimed that downregulation of amino acid metabolism, phosphotransferase system, and ABC transporter directly were related to decreased pathogenicity. To support this claim, we add some references in the updated manuscript line 662-664, 827-830.

> *(7) Serotonin: Does serotonin also reduce the virulence of S. marcescens?*

Our primary result showed that serotonin indeed could reduce the virulence of *S. marcescens* (figure supplement 4), because the survival rate of adult flies was increased and the expression levels of virulence-related genes of *S. marcescens* alone in the case of serotonin.

> *(8) Figures 6D, E, H, I: The expression of key genes should be verified using quantitative real-time polymerase chain reaction (qRT-PCR), as scRNA-seq expression levels might not accurately reflect the true expression levels.*

Bacterial single-cell RNA-seq can evaluate alterations in gene expression in the single-cell resolution. The expression of key genes screened by scRNA-seq was changed only in subpopulations, so the average expression of these genes would be comparable when mixed with a large population. We are afraid that qRT-PCR could be illegible to verify the expression of genes in subpopulations.

> *(9) Figure 7: The authors mentioned. "AMPs were supplemented to fly food". However, I could not find information regarding which AMPs and their respective concentrations (i.e., concentration of each AMP) were used in this study. This is a critical aspect of the research; therefore, details should be provided.*

Thanks for your important suggestions. We used the antimicrobial peptide cecropin A, which is produced by silkworms. We provided this information in the methods line 487-488. The concentrations of cecropin A were added in Figure 7 legend.

> *(10) Figure 7: Delta AMP + AMP exhibited a stronger effect on the bacteria compared to AMP alone, indicating that immune effectors other than AMP may be involved. Since the IMD pathway is necessary for most immune effectors, including AMP, it would be interesting to test IMD pathway mutant animals and compare them with Delta AMP. Delta AMP + AMP exhibited a stronger effect on the bacteria compared to AMP alone.*

We appreciate this important question. Indeed, Delta AMP + AMP exhibited a stronger effect on the bacteria compared to AMP alone. We admitted that immune effectors other than AMP may be involved. Alternatively, mechanical force, to a less extent, accounted for the stronger effect on the bacteria (Explained by larvae agitation in figure supplement 2). To rule out this possibility, we examined the effect of total immune effectors on the bacterial load and the prodigiosin yield of *S. marcescens* using the IMD pathway mutant (*RelE20* larvae). Our result showed that the optical density and yield of prodigiosin in Delta AMP group did not significantly differ from the ones in *RelE20* group. Moreover, the load of *S. marcescens* associated with Delta AMP mutant was comparable to that of *S. marcescens* associated with *RelE20* mutant. These results suggested that AMPs play a major role in recapitulating the response of _S. marcescens t_o larvae.

“To rule out the potential role of other immune effectors, we turned to the IMD pathway mutant *RelE20* that is deficient in total immune effectors. Our result showed that the optical density and yield of prodigiosin in *RelE20* group did not significantly differ from the ones in *DAMP* group (figure supplement 7A, B). Moreover, the load of *S. marcescens* associated with *RelE20* mutant was comparable to that of *S. marcescens* associated with Delta AMP mutant (figure supplement 7C).”

We now added these results in the text line 326-331.

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