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Frequent Replenishment Sustains the Beneficial Microbiome of *Drosophila melanogaster*

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ABSTRACT   We report that establishment and maintenance of the *Drosophila melanogaster* microbiome depend on ingestion of bacteria. Frequent transfer of flies to sterile food prevented establishment of the microbiome in newly emerged flies and reduced the predominant members, *Acetobacter* and *Lactobacillus* spp., by 10- to 1,000-fold in older flies. Flies with a normal microbiome were less susceptible than germfree flies to infection by *Serratia marcescens* and *Pseudomonas aeruginosa*. Augmentation of the normal microbiome with higher populations of *Lactobacillus plantarum*, a *Drosophila* commensal and probiotic used in humans, further protected the fly from infection. Replenishment represents an unexplored strategy by which animals can sustain a gut microbial community. Moreover, the population behavior and health benefits of *L. plantarum* resemble features of certain probiotic bacteria administered to humans. As such, *L. plantarum* in the fly gut may serve as a simple model for dissecting the population dynamics and mode of action of probiotics in animal hosts.

IMPORTANCE   Previous studies have defined the composition of the *Drosophila melanogaster* microbiome in laboratory and wild-caught flies. Our study advances current knowledge in this field by demonstrating that *Drosophila* must consume bacteria to establish and maintain its microbiome. This finding suggests that the dominant *Drosophila* symbionts remain associated with their host because of repeated reintroduction rather than internal growth. Furthermore, our study shows that one member of the microbiome, *Lactobacillus plantarum*, protects the fly from intestinal pathogens. These results suggest that, although not always present, the microbiota can promote salubrious effects for the host. In sum, our work provides a previously unexplored mechanism of microbiome maintenance and an *in vivo* model system for investigating the mechanisms of action of probiotic bacteria.

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Recent innovations in metagenomics and microbial ecology have sparked an explosion of research on the human microbiome (1, 2). Resident microbes aid in digestion of complex substrates (3, 4) and protect the gut epithelium from damage by pathogenic bacteria (5, 6). Dysbiosis of the human gut microbiome is associated with many chronic diseases, such as diabetes (7, 8), obesity (9–11), colon cancer (12), and depression (13). Although details of the relationship between the microbiome and disease are still emerging, a surge of interest in manipulating the composition of gut communities has generated a need for model systems in which to learn the principles that govern the dynamics and function of microbial communities.

Due to its short rearing time and cultivable microbiota, *Drosophila melanogaster* presents an ideal model to study dynamics of the microbiota throughout the life span of its host. In spite of these advantages, little is known about the dynamics of the *Drosophila* microbiota. Previous studies using culture-independent techniques have shown that representations of members of the microbiome differ between younger and older adults (14). Another study found that total bacterial populations increased over the lifetime of the fly (15). However, *Drosophila* strategies for microbiome maintenance have been overlooked. This process has been well characterized in invertebrates, such as aphids, which maintain an obligate bacterial endosymbiont (16), and in the bobtail squid, which is colonized daily by its symbiont, *Vibrio fischeri* (17). In humans, it is thought that microbial growth within the intestine is sufficient to maintain many commensal species (18). Another strategy may involve repeated reintroduction of bacteria into the host from an environmental source. Since *Drosophila* feeds on decomposing foods and ingests polymicrobial mixtures of bacteria, we hypothesized that external sources may serve as a means to repopulate its microbiome.

RESULTS

Microbiome composition and establishment in *Drosophila*. Tag pyrosequencing of 16S rRNA genes revealed that *Lactobacillus* and
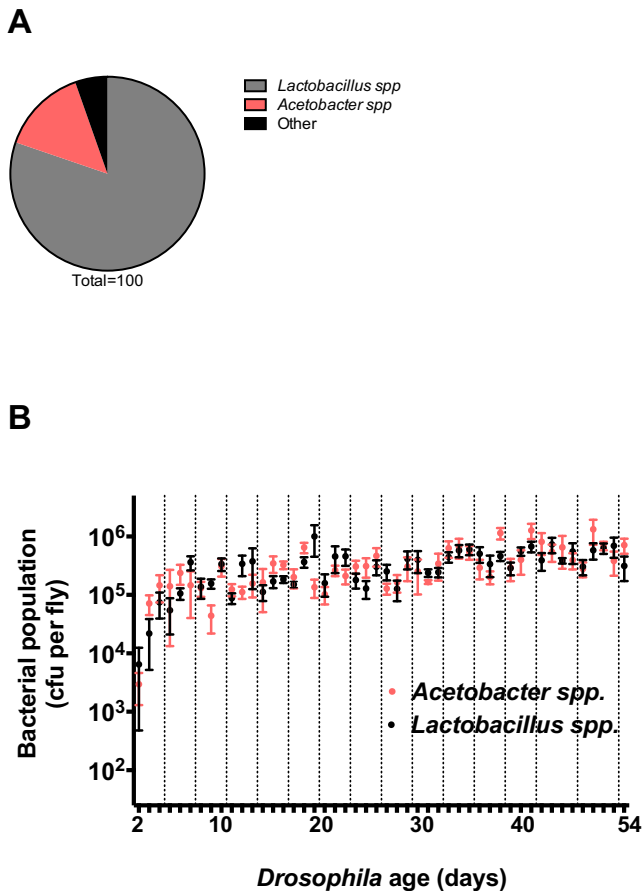
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A

B

FIG 1 Populations within the adult *Drosophila* microbiome. (A) One- to 2-day-old surface-sterilized adults were analyzed for bacterial community membership by 454 tag pyrosequencing. The proportions of the community represented by the two most abundant taxa, *Lactobacillus* and *Acetobacter*, which represent 94% of the community, are shown. (B) Two- to 54-day-old adult *Drosophila* were sampled daily (points at 43, 45, 50, and 51 days were omitted). Five adult females were sampled at most time points (with three samples on days 14, 21, 25, and 44 and four samples on days 16, 17, 19, 20, 26, 28, and 46). Values represent mean bacterial population per fly ± the standard error of the mean as estimated by culturing. The limit of detection is 25 CFU per fly. Dashed lines represent transfer of flies to sterile vials.

Acetobacter spp. comprise 94% of the microbiome in our lab colony of *Drosophila*, which is consistent with a previous description of the microbiome (Fig. 1A) (14). To distinguish *Acetobacter* and *Lactobacillus* spp. by colony morphology, *Drosophila* homogenates were cultured on semiselective and differential media; 4 tan colonies on *Acetobacter* (Ace) medium and 10 white colonies on de Man, Rogosa, and Sharpe (MRS) medium were identified as *Acetobacter* and *Lactobacillus* spp., respectively, by Sanger sequencing of the 16S rRNA gene from each colony. Subsequent analysis of 40 tan and white colonies has consistently discriminated the two genera based on media type and colony morphology (data not shown).

We sought to measure the dynamics of the dominant bacterial members of the *Drosophila* microbiome. *Acetobacter* and *Lactobacillus* spp. were assessed in newly emerged flies and the environment in which they emerged. Although they emerged from pupae into an environment laden with bacteria, 9 out of 10 adult flies did not contain detectable, culturable bacteria 1 h after emergence
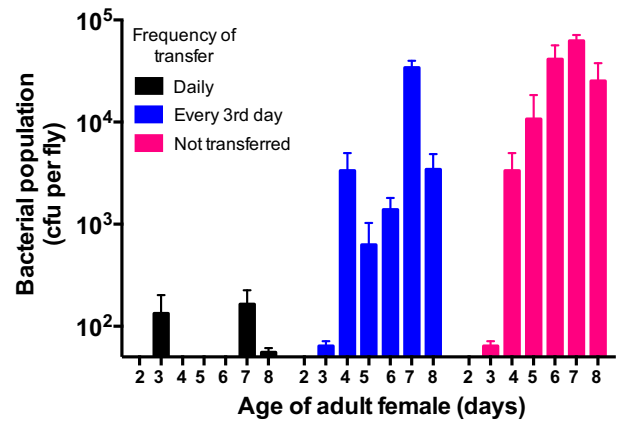

FIG 2 Establishment of the *Drosophila* microbiome requires frequent consumption of bacteria. Cultured populations of *Acetobacter* and *Lactobacillus* spp. in newly emerged *Drosophila* subjected to various transfer regimens were measured. (B) Following eclosion, flies were transferred to fresh food daily (black), every 3rd day (blue), or not at all (pink) for 7 days. A total of nine females obtained from three vials (three/vial) were sampled daily throughout the 7-day period. The height of each bar represents the mean bacterial population (*Lactobacillus* and *Acetobacter* spp.) in flies sampled that day ± the standard error of the mean.

from the pupal case as adults (see Fig. S1A and B in the supplemental material). This observation is consistent with a previous report (14). Within 24 h of eclosion, 6 out of 10 flies contained detectable bacteria (see Fig. S1B). Collectively, our results show that newly emerged flies harbor low microbial populations, suggesting that the members of the microbiome of adult *Drosophila* come from their environment.

The abundance of bacteria in the *Drosophila* microbiome increased over the lifetime of the fly, reflecting the amount of time that the flies spent on the same food source (Fig. 1B). Throughout the 54-day time course, flies were maintained on the same food source for 3 days and then transferred to fresh food. Populations of the bacterial members declined when flies were transferred to new food and peaked when *Drosophila* were maintained on the same food source for 2 to 3 days. We propose that upon transfer, flies seed the sterile food with members of the microbiome that grow to high abundance and repopulate the microbiome when consumed by the fly.

Fly food as a bacterial reservoir for the *Drosophila* microbiome. To test whether fly food itself supports the growth of microbiome members, we measured the population of bacteria on fly food over time. As flies remained on food, the bacterial population increased, peaking at 72 h (see Fig. S2 in the supplemental material). These data demonstrate that *Drosophila* inoculate their food with bacteria that then multiply on the food.

Establishment of the *Drosophila* microbiome. To assess the time scale of microbiota acquisition in *Drosophila*, we transferred newly emerged flies to fresh food daily, every 3rd day, or not at all for 7 days, evaluating bacterial populations daily. Flies that were not transferred harbored larger bacterial populations than those that were transferred (Fig. 2). The dynamics of these populations were similar in males and females, and bacterial population size corresponded to the time spent on the same food source (see Fig. S3 and S4 in the supplemental material). These results collectively support the idea that establishment of the *Drosophila* microbiome requires access to and consumption of exogenous bacteria.
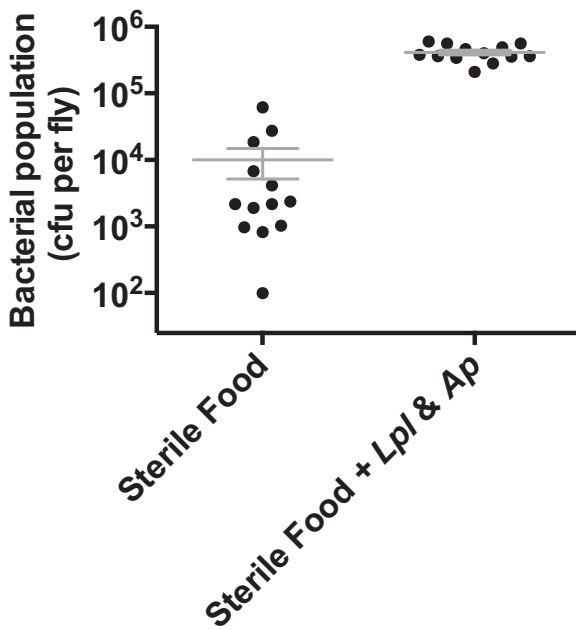
FIG 3 Maintenance of the *Drosophila* microbiome requires frequent consumption of bacteria. Cultured populations of *Acetobacter* and *Lactobacillus* spp. in conventionally reared flies introduced into environments with or without exogenous bacteria. Sixteen-day-old flies were transferred twice daily to sterile food or sterile food amended with *A. pasteurianus* and *L. plantarum*. Three to five female *Drosophila* were sampled every 3rd day for 9 days after transfer to either environment. Data points represent the mean total bacterial population (*Lactobacillus* and *Acetobacter* spp.) ± the standard error of the mean in flies sampled on days 3, 6, and 9. An unpaired two-tailed *t* test revealed significant differences ($P < 0.0001$) between bacterial populations under the two rearing regimens.

Maintenance of the *Drosophila* microbiome. We measured microbial populations in 16-day-old conventionally reared flies that were transferred twice daily to either sterilized food or food inoculated with *Lactobacillus plantarum* and *Acetobacter pasteurianus*. Flies feeding on the amended medium contained more bacteria than those feeding on sterilized food, although populations in the flies on sterilized food were not completely eliminated and varied in size (Fig. 3). These results indicate that bacterial populations in the *Drosophila* microbiome are influenced by fly access to exogenous bacteria. Without repeated consumption of bacteria, established microbiota populations decline and are much smaller than those achieved when *Drosophila* has access to an environmental reservoir of bacteria. Reduction of bacterial population size is detectable within 6 h of transfer to fresh food or under starvation conditions (see Fig. S5 in the supplemental material), although bacterial populations in starved flies were larger than those in flies transferred to sterile food. This is consistent with the observation that starvation reduces the rate of defecation by *Drosophila*, thereby slowing the loss of bacteria from the gut (19).

Impact of the community on fly fitness. To test whether the abundance of organisms in the *Drosophila* microbiome has functional consequences for host fitness, we fed *L. plantarum* to conventionally reared and germfree flies and then challenged them with *Serratia marcescens*, a *Drosophila* and nosocomial human pathogen, and assessed fly mortality. *L. plantarum*, a member of the native gut microbiome, protected flies from mortality induced by *S. marcescens* (Fig. 4A). Feeding *L. plantarum* reduced mortality more in germfree than in conventionally reared flies (Fig. 4B). Overall, the level of protection corresponded to the size of the *L. plantarum* population detected in the fly (Fig. 4C).

To assess the specificity of protection, conventionally reared flies were fed *Enterococcus faecalis* or *Bacillus subtilis* 3 days prior to challenge with *S. marcescens*.

Although *E. faecalis* was recovered from flies 3 days after feeding, *B. subtilis* was not. Neither *E. faecalis* nor *B. subtilis* reduced *S. marcescens*-induced mortality, demonstrating that protection by *L. plantarum* is specific and is not achieved with populations of all bacterial species (Fig. 5A). These data show that a prominent member of the *Drosophila* microbiome protects its host from intestinal infection.

Many members of the *Lactobacillus* genus besides *L. plantarum* are used as human probiotics—formulations of live bacteria ingested for their health benefits. Therefore, we investigated whether other probiotic strains could be studied in *Drosophila*. The commonly used human probiotic *Lactobacillus rhamnosus* GG protected conventionally reared flies from infection by *S. marcescens* and *Pseudomonas aeruginosa* PAO1, another nosocomial pathogen of humans (Fig. 6A and B). These results suggest that *Drosophila* may serve as a model system for studying human probiotics.

DISCUSSION

Our results show that *Drosophila* establishes and maintains its microbiome by frequently consuming bacteria, highlighting the contribution of external inputs, rather than internal maintenance, to sustaining the microbiome and revealing a novel facet of host-microbe interactions in this model system. We demonstrated that the lifetime abundance of the *Drosophila* microbiota was associated with the abundance of environmental microbes and that the microbiome was not established in newly emerged adults or maintained in flies with existing microbial populations when flies were deprived of exogenous bacteria. The influence of rearing regimen could extend to microbiome composition, as members may or may not be transferred to fresh food sources, perhaps revealing the cause of the variation in composition of the *Drosophila* microbiome among research labs (20). In light of these findings, we predict that both abundance and composition of *Drosophila* gut communities vary with differences in *Drosophila* husbandry techniques in various laboratory settings.

This work will advance several lines of inquiry about the *Drosophila* microbiome. First, the results raise questions about the mechanisms by which *Drosophila* and its symbionts come to be associated with one another. We speculate that innate or learned behaviors may enable the fly to replenish its microbiome. Recent work has highlighted an olfactory circuit used by *Drosophila* to avoid harmful microorganisms (21). Future work may identify an analogous circuit dedicated to detecting beneficial microorganisms. In this way, *Drosophila* would be attracted to and preferentially consume specific bacterial species, enabling it to maintain a beneficial microbiome. Though the population sizes of many host-associated microbial communities are dictated by host immunity and bacterial growth rates, the *Drosophila* system may represent an alternative mutualism strategy that we term "quotidian replenishment," which is intended to indicate the need for daily replenishment to obtain a consistent community in the animal. In this model, the symbiotic community in *Drosophila* is
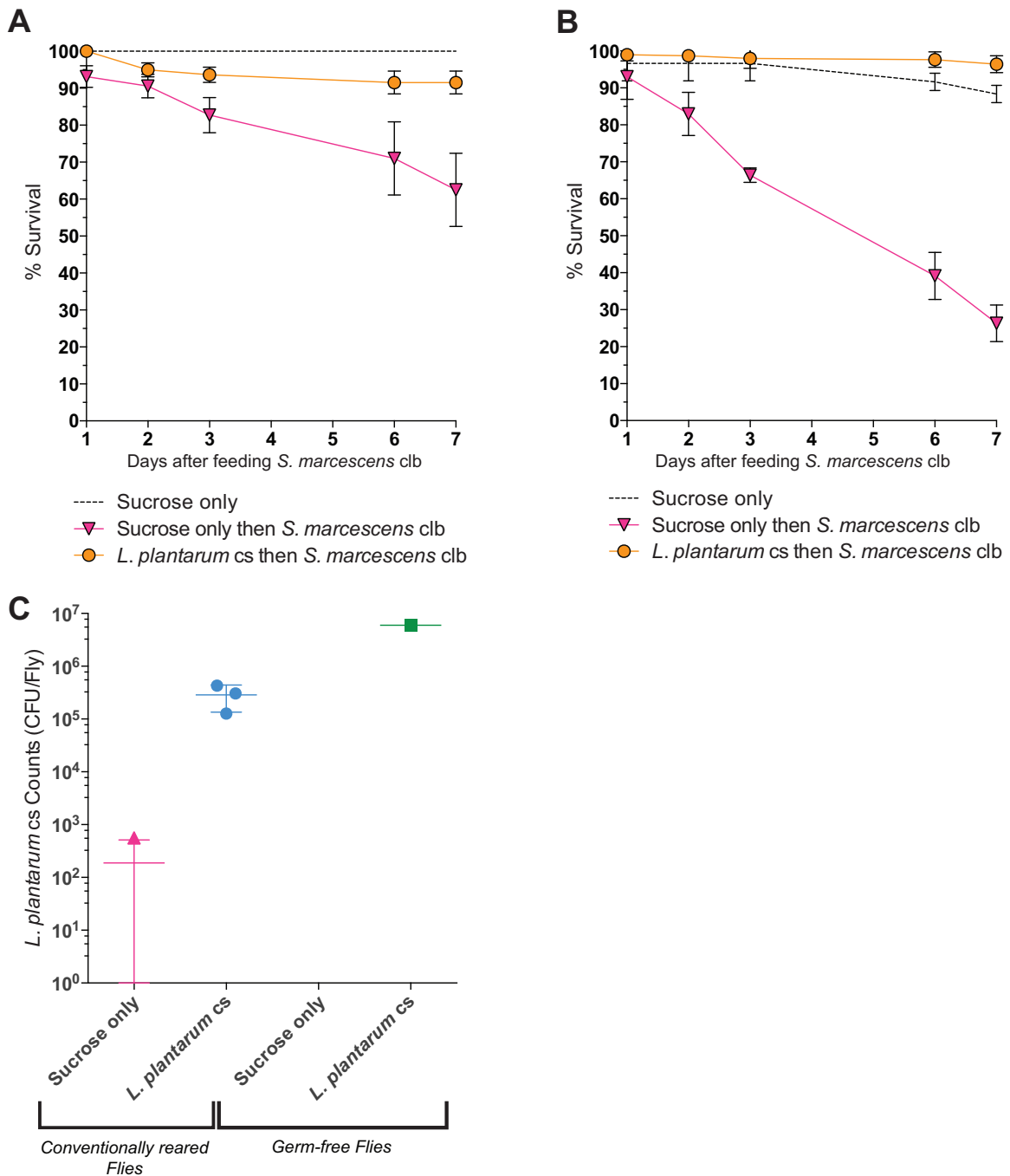
FIG 4 *Lactobacillus plantarum* protects flies from *Serratia marcescens* infection. (A and B) Conventionally reared (A) or germfree (B) flies were fed *L. plantarum* for 3 days prior to *S. marcescens* challenge. Flies were fed a sucrose suspension of *S. marcescens* for 2 days and then transferred to clean bottles with sterile sucrose solution. Fly mortality was recorded over time. *n* was 20 flies per treatment. Error bars represent the standard deviation of the mean for three replicate groups per treatment. (C) Cultured populations of *L. plantarum* in conventionally reared and germfree flies were measured 3 days after feeding. *L. plantarum* cs is an isolate from Canton-S *Drosophila* in our laboratory; *S. marcescens* clb is an isolate from cottonwood leaf beetle.

maintained through frequent ingestion from an external reservoir of bacteria, as suggested by Storelli and colleagues (22).

Perhaps *Drosophila* rids its gut of most microbes to minimize the risk of acquiring and maintaining potential pathogens. *Drosophila* undoubtedly encounters both beneficial and pathogenic microbes in its natural environment, and if the fly provided an environment for a more permanent, actively growing microbial community, then it might also be more vulnerable to colonization by pathogens as well. Another possibility is that the microbiome may compete with its *Drosophila* host for nutrients in the gut. If so, we would predict that maintaining a bacterial population internally would present a cost to the host by reducing nutrients available to it. Further study of the relationship between the fly and its microbiome may reveal
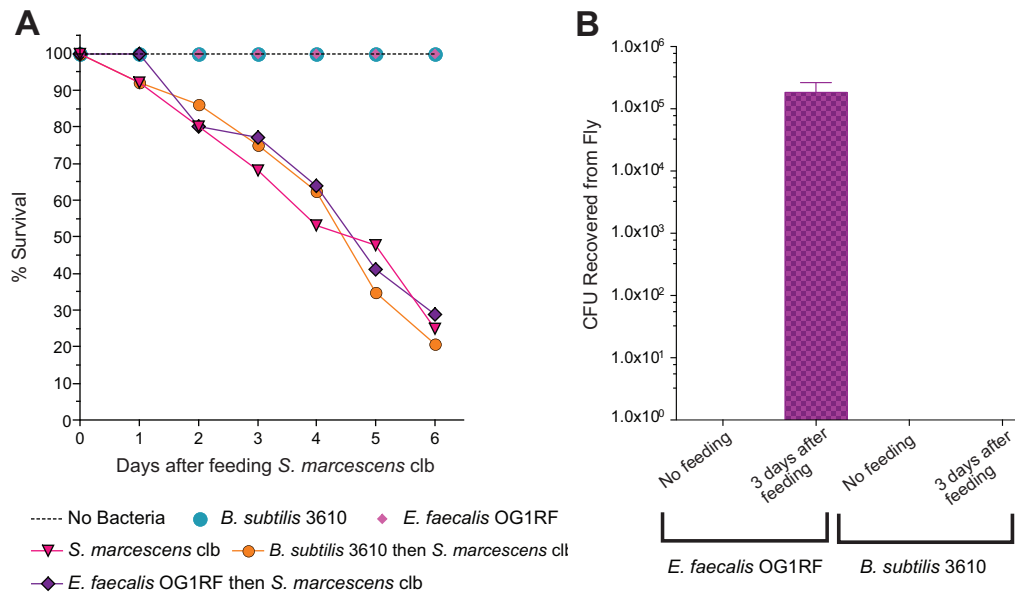
FIG 5 *Enterococcus faecalis* and *Bacillus subtilis* do not protect flies from *Serratia marcescens* infection. (A) Conventionally reared flies were fed *E. faecalis* or *B. subtilis* for 3 days prior to *S. marcescens* challenge. Flies were fed a sucrose solution containing *S. marcescens* for 2 days and then transferred to clean bottles with sterile sucrose solution. Fly mortality was recorded over time. *n* was 20 flies per treatment. (B) Conventionally reared flies were fed *E. faecalis*, *B. subtilis*, or sterile sucrose solution ("no feeding") for 3 days. Cultured populations of *E. faecalis* or *B. subtilis* were measured 3 days after feeding. *n* was 20 per treatment; data from one representative of three experiments are shown. Error bars represent the standard deviation of the mean of five flies.

novel features of *Drosophila* mutualism and general principles of host-microbe relationships.

Our evidence indicates that *Drosophila* seeds its food with commensal organisms by depositing fecal matter on its food source on which the bacteria grow. In this way, *Drosophila* could be cultivating an inoculum with which to replenish the micro-
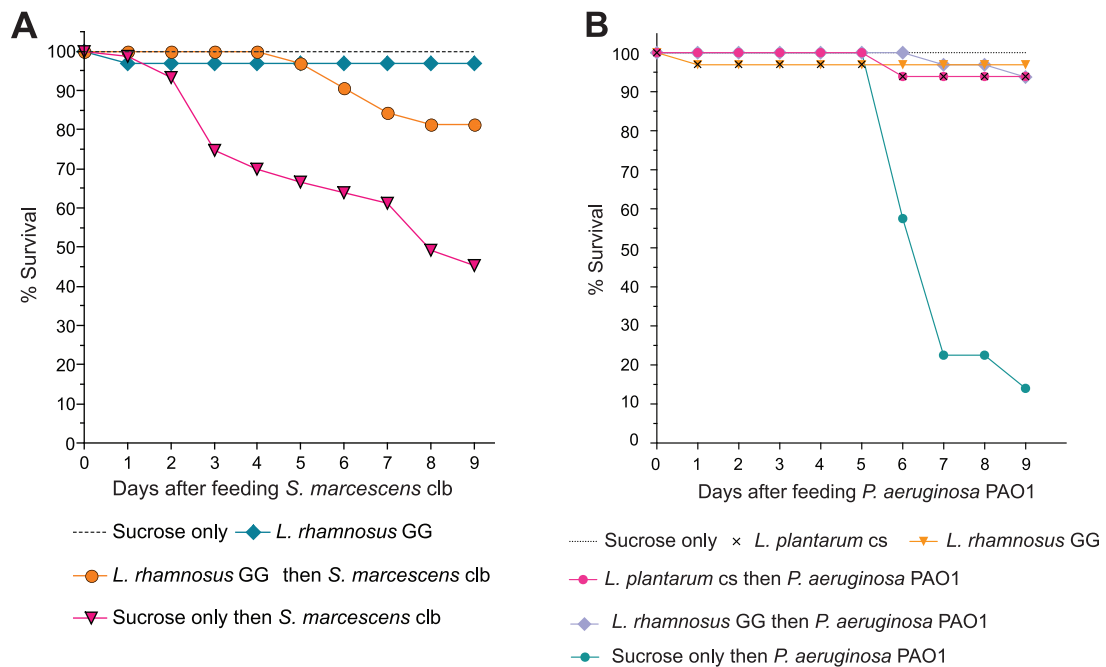

FIG 6 The probiotic fly model extends to other probiotics and pathogens. *Lactobacillus plantarum* and *L. rhamnosus* GG reduce fly death from *Serratia marcescens* and *Pseudomonas aeruginosa* when fed prior to infection. (A) Conventionally reared flies were fed *L. rhamnosus* GG for 3 days prior to *S. marcescens* challenge. Flies were fed a sucrose suspension of *S. marcescens* for 2 days and then transferred to clean bottles with sterile sucrose solution. Fly mortality was recorded over time. *n* was 20 per treatment; one representative experiment of three is shown. (B) Conventionally reared flies were fed *L. plantarum* or *L. rhamnosus* GG for 3 days prior to *P. aeruginosa* challenge. Flies were fed a sucrose suspension of *P. aeruginosa* for the duration of the assay. Fly mortality was recorded over time. *n* was 20 per treatment; data from one representative of three experiments are shown.

biome. A similar system is the symbiosis strategy employed by the *Acromyrmex* leaf-cutting ant, which cultivates fungal mats that provide the insect with a rich food source (23). Like the leaf-cutter ant, *Drosophila* may digest its resident bacteria as a food source. Regardless of other similarities and differences between these biological systems, they both may represent examples of microbial farming by which animals cultivate beneficial microorganisms (24).

The discovery that both *Drosophila*-associated and human-administered probiotic strains protect *Drosophila* from infection provides a foundation for the use of the *Drosophila* system to study probiotic strains in a host that can be genetically altered and manipulated experimentally. Variation in human disease susceptibility and responses to treatment may be in part a consequence of variation between individuals' microbiomes. As such, managing the microbiome is an essential component of treatments intended to alter the host microbiome, including probiotics. Consumption of probiotics can alleviate symptoms of antibiotic-associated diarrhea (25), lactose intolerance (26), and childhood irritable bowel syndrome (27). However, the complexity of the human gut microbiome, the cost of clinical trials, and the limits of appropriate experimental procedures in human subjects have precluded elucidation of the *in vivo* mechanisms leading to these health benefits. Such understanding is needed to address the inconsistent performance of probiotics (28–30) and to direct their use in a targeted and precise manner.

The basis of probiotic action and failure will be advanced by studying a host model harboring a relatively simple microbial community, such as *Drosophila melanogaster*. The fly is a particularly attractive model in which to study probiotics because *L. plantarum*, a species formulated as a probiotic for humans, is a prominent symbiont of wild and laboratory-reared flies (14, 31–36). *Drosophila* and humans may share a mode of interaction with *Lactobacillus*: both hosts benefit from certain *Lactobacillus* strains, but the bacteria do not persist in either host, thereby necessitating quotidian replenishment in *Drosophila* (37). The lack of persistent colonization by probiotic lactobacilli has produced skepticism about their health-promoting effects on humans, but it may represent a common feature of animal-host symbioses. The *Drosophila* model provides a system that overcomes many of the experimental challenges of studying such interactions in the human gut, providing a path to understanding probiotics as well as diverse host-microbe interactions.

MATERIALS AND METHODS

Fly stocks and culture. *Drosophila melanogaster* Canton-S flies were reared at 25°C on medium containing 10% dextrose, 5% heat-killed yeast, 7% cornmeal, 0.6% propionic acid, and 0.6% agar. No microorganisms could be isolated from this sterile food. Flies raised in this manner are described as conventionally reared. A germfree Canton-S line was created by washing <12-h-old embryos successively in ethanol, 50% bleach, and ethanol followed by transfer of embryos to sterile medium in a sterile biosafety cabinet as described previously (34). Germfree status was checked by plating adult flies on MRS, Ace, and nutrient agar plates and sequencing the 16S rRNA gene (see Fig. S6A and B in the supplemental material). To maintain their sterility, germfree flies were transferred to autoclaved food within a sterilized biosafety cabinet.

Culture-independent identification of bacteria in *Drosophila*. DNA isolated from surface-sterilized flies (1 to 2 days old) was used to survey the *Drosophila* microbiome. Amplifications were performed in 25-μl reaction mixtures with Qiagen HotStar *Taq* master mix (Qiagen Inc., Va-

lencia, CA), 1 μl of each 5 μM primer, and 1 μl of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosystems, Carlsbad, CA) using the following conditions: 95°C for 5 min and then 35 cycles of 94°C for 30 s, 54°C for 40 s, and 72°C for 1 min, followed by one cycle of 72°C for 10 min and a 4°C hold. Amplification products were visualized with eGels (Life Technologies, Grand Island, NY). Products were then pooled at equimolar ratios, and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, NY) and selected by size using Agencourt AMPure XP (Beckman, Coulter, Indianapolis, IN) according to Roche 454 protocols (454 Life Sciences, Branford, CT). Size-selected pools were then quantified, and 150 ng of DNA was hybridized to Dynabeads M-270 (Life Technologies, Carlsbad, CA) to create single-stranded DNA according to Roche 454 protocols (454 Life Sciences). Single-stranded DNA was diluted and used in emulsion PCRs (emPCRs) which were performed, and the reaction mixtures were subsequently enriched. Sequencing followed the manufacturer's protocols (454 Life Sciences).

Culture-dependent identification of bacteria in *Drosophila*. Flies were washed with 10% bleach, 70% ethanol, and phosphate-buffered saline (PBS) in succession. Homogenates from surface-sterilized flies were cultured on de Man, Rogosa, and Sharpe (MRS) agar (Fisher Scientific, Hampton, NH), nutrient agar (Becton Dickinson, Franklin Lakes, NJ), and Ace agar (0.8% yeast extract, 1.5% peptone, 1% dextrose, 0.3% acetic acid, 0.5% ethanol, and 0.01% cycloheximide) at 28°C.

To identify *Lactobacillus* and *Acetobacter* spp. in the fly microbiome, colonies with distinct morphologies were selected from each medium type and placed in a 25-μl PCR mix containing HotStar HiFidelity DNA polymerase (Qiagen), water, MgSO₄, glycerol, and deoxynucleoside triphosphates (dNTPs) and amplified for 35 cycles in a thermocycler. 16S rRNA amplicons were cleaned using a QIAquick PCR purification kit (Qiagen), visualized by gel electrophoresis, gel purified using a QIAex II gel extraction kit (Qiagen), and sequenced using 8F and 1492R primers. Sequences were aligned using the Ribosomal Database Project. The resulting community profile was consistent with previous studies (14, 33).

Estimation of bacterial population size in *Drosophila*. Adult flies were collected within 24 h of emergence and placed in vials at a density of 30 flies per vial; these were designated 1-day-old flies. Flies were transferred to fresh food every 3rd day for the duration of the experiment. Homogenates of surface-sterilized flies were cultured on MRS, nutrient, and Ace agar using a Spiral Plating System Autoplater (Advanced Instruments Inc., Norwood, MA). Nutrient agar was used to monitor the growth of the culturable bacterial community. Plates were incubated at 28°C for 2 to 4 days, and bacterial CFU were estimated using a QCount automated colony counter (Advanced Instruments Inc., Norwood, MA) or manually.

Establishment and maintenance of the *Drosophila* microbiome. To assess establishment of the *Drosophila* microbiome, flies were collected within 24 h of eclosion, divided into three groups, and transferred to fresh fly food. One group remained on the fly food for the duration of the experiment, the second group was transferred every 3rd day, and the third group was transferred daily. Bacterial populations in three males and three females from each of three vials in each experimental group were sampled.

To assess maintenance of the *Drosophila* microbiome, 16-day-old flies were transferred twice daily either to fresh food or to fresh food amended with *Lactobacillus plantarum* and *Acetobacter pasteurianus*. *L. plantarum* was cultured for 24 h at 28°C, and *A. pasteurianus* was cultured in broth for 48 h at 28°C with shaking at 200 rpm. Five to nine females from each experimental treatment were sampled every 3 days for 9 days.

Probiotic feeding and pathogen infection assays. *L. plantarum* cs, an isolate from Canton-S *Drosophila* in our laboratory, and *Lactobacillus rhamnosus* GG were cultured overnight in MRS medium at 37°C. *Serratia marcescens* clb, an isolate from cottonwood leaf beetle; *Pseudomonas aeruginosa* PAO1; and *Bacillus subtilis* 3610 were cultured overnight in LB at 37°C with shaking at 225 rpm. *Enterococcus faecalis* OG1RF was cul-

tured in brain heart infusion medium (BHI) (VWR International) at 37°C with shaking at 225 rpm.

One-day-old flies were added to a glass bottle and fed a 5% sucrose suspension of either *S. marcescens* or *P. aeruginosa* on a sterile paper disc. In *S. marcescens* killing assays, flies were removed from the bacterium-sucrose suspension after 3 days and placed in new bottles with sterile sucrose solution. In *P. aeruginosa* killing assays, flies were reared on the bacterium-sucrose mixture for the duration of the experiment. Mortality was assessed daily.

To determine whether bacteria protected *Drosophila* from infection, 1-day-old flies were fed a sucrose solution inoculated with *B. subtilis*, *E. faecalis*, or *Lactobacillus* spp. for 3 days and then fed either *S. marcescens* or *P. aeruginosa*. When fed *S. marcescens*, the flies remained on the bacterial suspension for 2 days and then were transferred to bottles with sterile sucrose solution; when fed *P. aeruginosa*, the flies were transferred every 3 days.

Statistical analysis. All data analysis was performed using GraphPad Prism 6.0b software.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00860-13/-/DCSupplemental.

Figure S1, DOCX file, 0.1 MB.
Figure S2, DOCX file, 0.1 MB.
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Figure S4, DOCX file, 0.1 MB.
Figure S5, DOCX file, 0.1 MB.
Figure S6, DOCX file, 0.7 MB.

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