Report

Current Biology

Gut Microbiota Modifies Olfactory-Guided Microbial Preferences and Foraging Decisions in *Drosophila*

Highlights

- *Drosophila* display microbe-seeking behaviors as both larvae and adults
- These microbial preferences are shaped by host-microbe association
- Foraging decisions involve balancing cues from both microbes and nutrients

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In Brief

Wong et al. find a role of the gut microbiota in fly foraging, showing that fly preferences for foods with beneficial bacteria are shaped by the gut microbiota composition and history of host-microbe association. These bacteria also affect fly nutrient preferences, and flies trade off acquiring microbes against balancing nutrition in foraging.

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Gut Microbiota Modifies Olfactory-Guided Microbial Preferences and Foraging Decisions in *Drosophila*

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SUMMARY

The gut microbiota affects a wide spectrum of host physiological traits, including development [1–5], germline [6], immunity [7–9], nutrition [4, 10, 11], and longevity [12, 13]. Association with microbes also influences fitness-related behaviors such as mating [14] and social interactions [15, 16]. Although the gut microbiota is evidently important for host wellbeing, how hosts become associated with particular assemblages of microbes from the environment remains unclear. Here, we present evidence that the gut microbiota can modify microbial and nutritional preferences of *Drosophila melanogaster*. By experimentally manipulating the gut microbiota of flies subjected to behavioral and chemosensory assays, we found that fly-microbe attractions are shaped by the identity of the host microbiota. Conventional flies exhibit preference for their associated *Lactobacillus*, a behavior also present in axenic flies as adults and marginally as larvae. By contrast, fly preference for *Acetobacter* is primed by early-life exposure and can override the innate preference. These microbial preferences are largely olfactory guided and have profound impact on host foraging, as flies continuously trade off between acquiring beneficial microbes and balancing nutrients from food. Our study shows a role of animal microbiota in shaping host fitness-related behavior through their chemosensory responses, opening a research theme on the interrelationships between the microbiota, host sensory perception, and behavior.

RESULTS

Flies' Microbial Preference Depends on Their Microbiota

Based on previous studies [17, 18] and our 16S pyrosequencing survey, the fly egg surface inherited a high abundance of *Acetobacters* and *Lactobacillus*, especially *Acetobacter pomorum* (*AP*) and *Lactobacillus plantarum* (*LP*), which made up 75%–95% reads in our samples (Figure 1A; Table S1). These bacteria have been known to persist throughout developmental stages [2, 3, 19]. At the population level, *Lactobacillus* abundance is generally higher in eggs and at early development but declines, whereas *Acetobacter* abundance increases, as flies age [18, 19]. At the individual level, the relative abundances of *Lactobacillus* and *Acetobacter* vary greatly among flies [20] and are easily perturbed by environmental conditions such as diet and population density [18, 21]. Here, we report a similar trend (Figure 1A; Figures S1A and S1B). Flies deprived of food overnight showed a 9-fold reduction in *Acetobacter* and *Lactobacillus* colony forming unit (CFU) (Figure S1C), pointing to a possibility that *Drosophila* need to replenish their gut microbiota [22]. Based on this assumption, we investigated the chemosensory and behavioral responses of *Drosophila* toward cues associated with beneficial bacteria present in food and tested whether the gut microbiota plays a role in shaping these responses.

We monitored flies' behavioral responses to microbes using a foraging assay (Figure S2A; see STAR Methods). Food-deprived flies were allowed to forage in an arena containing seven patches of yeast-sucrose diet that was either unseeded or seeded with one of six bacteria species isolated from fly guts or bodies: *AP*, *Acetobacter tropicalis* (*AT*), *Lactobacillus casei* (*LC*), *Lactobacillus pantheris* (*LPa*), *LP*, and *Staphylococcus saprophyticus* (*S*). Foraging assays were conducted 1 hr after the bacteria were added to the diets to avoid substantial food modifications by microbial activities. We tested flies raised from conventional, axenic, or monoassociated eggs with the dominant *LP* or *AP*. Conventional flies showed a strong preference for foods seeded with both *Acetobacter* strains tested, *AP* and *AT*, as well as *LP* relative to unseeded food (Figure 1B1; Data S1A). A slight but significant preference was also observed for *LC*, a strain detected at low abundances in fly eggs and adults (0.15%–1.2% and 0.25%–3.9% of pyrosequencing reads, respectively; Figure 1A; Table S1). Flies showed no preference for food seeded with *LPa* or *S*, a strain isolated from fly whole bodies. Both *LPa* and *S* were undetected in our pyrosequencing dataset.

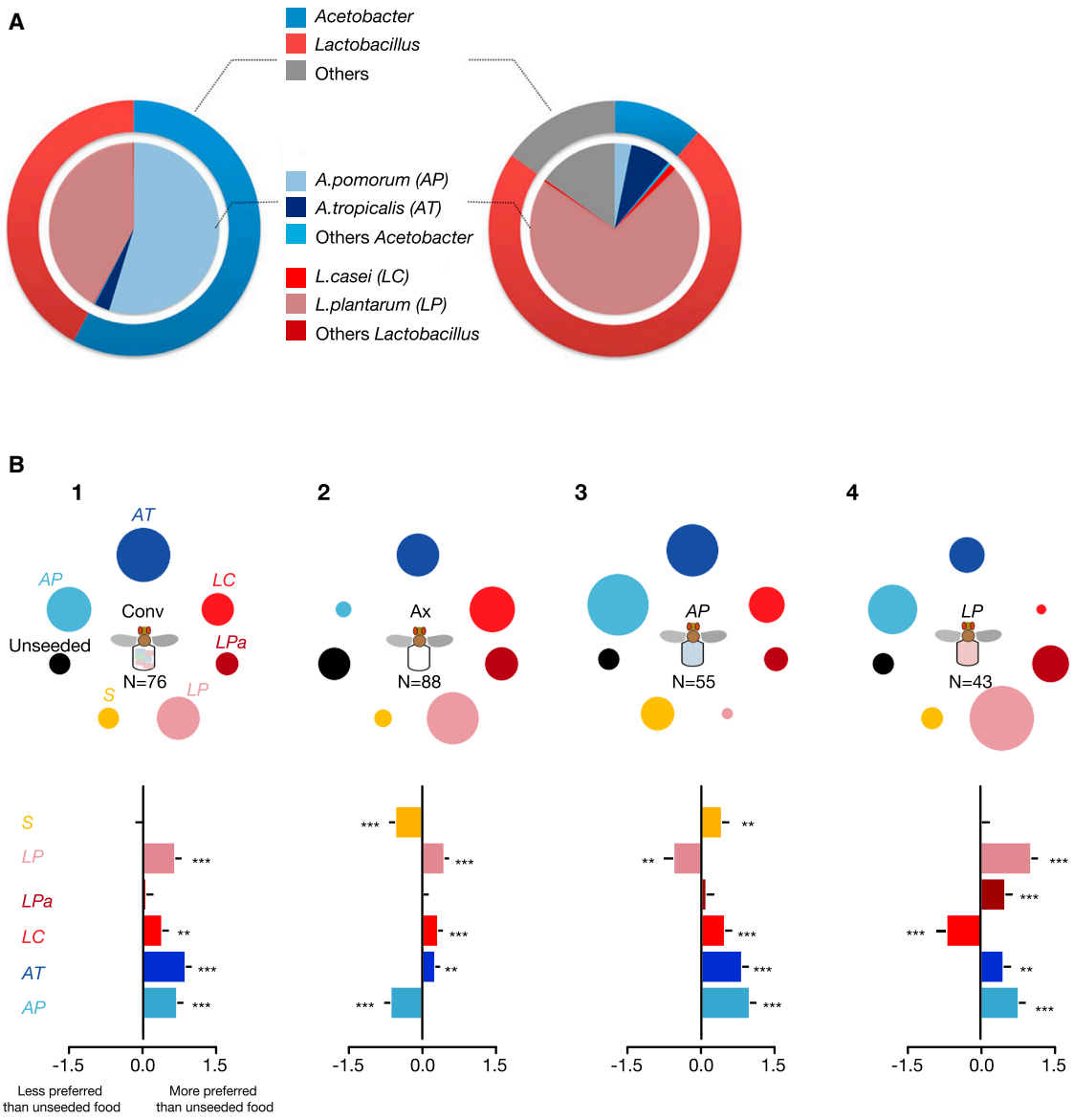

Figure 1. Microbiota Affects Adult *Drosophila* Microbial Preference

(A) 16S rRNA gene pyrosequencing of *Drosophila eggs*. Duplicate samples of eggs are shown. See also Figure S1and Table S1.

(B) Foraging of adult *Drosophila* on food patches unseeded or seeded with six bacteria isolated from flies: *Acetobacter pomorum* (*AP*), *Acetobacter tropicalis* (*AT*), *Lactobacillus casei* (*LC*), *Lactobacillus pantheris* (*LPa*), *Lactobacillus plantarum* (*LP*), and *Staphylococcus saprophyticus* (*S*). Multiple fly microbiota lines (conventional [Conv], axenic [Ax], and *AP* or *LP* monocolonized) were tested; n indicates the number of flies tested. Circle sizes are proportional to the total observations of flies. Bars indicate multinomial generalized linear model estimates of the log odds of a fly selecting a given inoculated food over the unseeded food (see Data S1A for model coefficients). Error bars indicate the SE. Statistical significance inferred from confidence intervals (CIs) is indicated by asterisks (*, 95% CI or $p \leq 0.05$; **, 99% CI or $p \leq 0.01$; ***, 99.9%CI or $p \leq 0.001$). See also Figure S2A and Data S1A.

Microbial preferences were dramatically altered in axenic flies. The preference for *AT* was diminished and the preference for *AP* abolished. Nonetheless, axenic flies retained preference for the two *Lactobacillus* strains (*LC* and *LP*) but displayed a higher chance of foraging on the unseeded food (Figure 1B2; Data S1A).

Flies raised in monoassociation with *AP* and *LP* showed increased preferences for foods seeded with the corresponding bacteria (Figures 1B3 and 1B4; Data S1A). Interestingly, *AP* monoassociation overrode fly preference for *LP*, although *AP* flies showed preferences toward *AT*, *LC*, and *S*. Conversely, *LP* monoassociation did not show the same antagonistic effects on flies' preference for *Acetobacter* strains. Together, our results demonstrate that flies' microbial preferences are influenced by their microbiota identity.

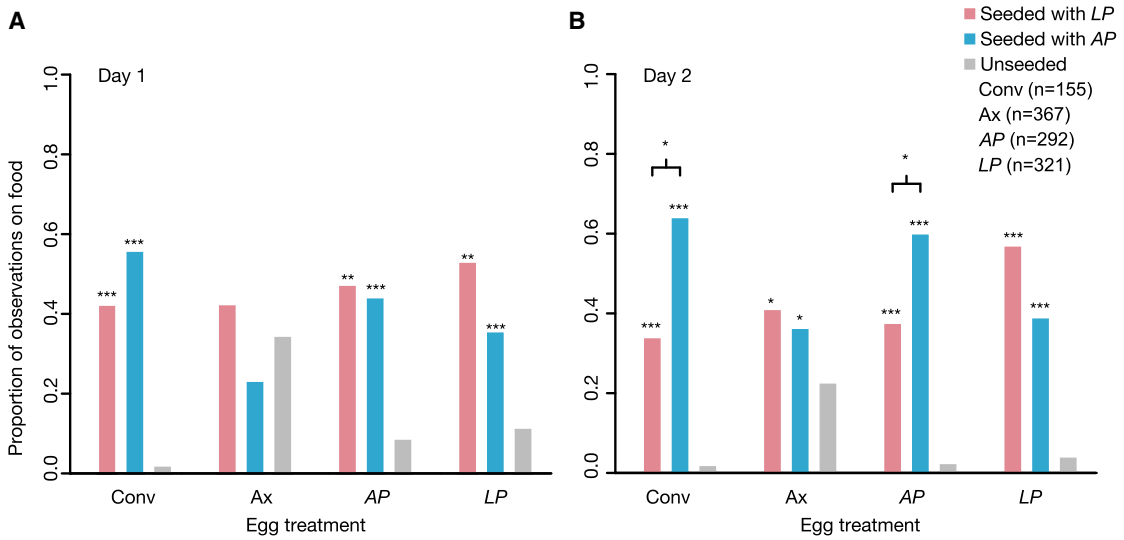

Figure 2. Early Microbial Exposure Influences *Drosophila* Larvae Microbial Preference

Proportion of newly emerged larvae observed on food patches unseeded or seeded with *AP* or *LP* on day 1 (A) and day 2 (B); n indicates the total number of observations of larvae on food. Data were analyzed using a multinomial generalized linear mixed model (GLMM) with a random effect accounting for repeated measures of the same cohorts of larvae. Asterisks indicate a significantly greater number of observations on a given food relative to the unseeded food based on credible intervals (*, 95% CI; **, 99% CI; ***, 99.9% CI). See also Figure S2B and Data S1B.

Flies' Microbial Preferences Depend on Early-Life Exposure

The preferences of adult *Drosophila* toward their associated bacteria may be innate or exposure dependent, for instance the result of associative learning between food stimuli and microbial cues. To disentangle between these possible mechanisms, we developed a larval food choice assay (Figure S2B; see STAR Methods). Newly emerged larvae from conventional, axenic, and *AP*- or *LP*-inoculated eggs were given the choices of yeast-sucrose medium either unseeded or seeded with *AP* or *LP*. Larvae from conventional and inoculated eggs promptly sought out media seeded with *AP* or *LP* from day 1 (Figure 2A; Data S1B). In contrast, larvae from axenic eggs evenly spread among all media on day 1. On day 2, larvae from axenic eggs began to show preferences for *AP* and *LP*, although the magnitude of preference was smaller compared to larvae associated with *AP* and *LP*, and a notable proportion of the larvae remained in unseeded medium (Figure 2B; Data S1B). Larvae from conventional and *AP*-inoculated eggs showed the strongest preference for *AP* medium on day 2. Larvae from *LP*-inoculated eggs preferred *LP* and *AP* medium comparably, on both days (Figures 2A and 2B; Data S1B). Together, our results suggest that early-life microbial exposure influences host microbial preference.

Fly Attraction toward Beneficial Microbes Is Guided by Olfaction

In the previous assays, fly adults and larvae were allowed to migrate freely in and out of the food patches. Hence, it remained unclear whether the host microbial preferences were mediated via close contact with the microbes (e.g., taste), via volatile cues over a distance (i.e., olfaction), or both. To test for the contribution of close contact, we used a proboscis extension response (PER) assay in which conventional flies were presented single bacterial suspensions (*AP* or *LP*) across a series of doses onto the tarsi (Figure S2C; see STAR Methods). Responses were measured at different recovery times (i.e., 0, 20, 40, and 60 min). We used bacteria re-suspended in water along with a water-only negative control and a sugar solution as positive control. Flies showed a non-linear, positive dose-dependent PER to *LP* (Figure 3A; Data S1C) with a maximum response to CFU of 1.17×10^9 per ml. However, PER to *AP* did not differ from the water-only control (Figure 3A, lower panel), suggesting that direct sampling or other close contact mechanisms are unlikely to contribute to fly attraction toward *Acetobacter.*

We then tested the contribution of olfaction by setting up a two-choice trap assay, in which flies were given a choice between an unseeded medium and a *AP*- or *LP*-seeded medium (Figure S2D; see STAR Methods). Conventional flies were strongly attracted to the *LP*-seeded medium and, to a lesser but significant extent, the *AP*-seeded medium (Figure 3B; Data S1D), suggesting that attraction to *LP* and *AP* cues are mediated by olfaction. We further tested olfactory-guided microbial preference in axenic flies. Parallel to our adult fly foraging data, axenic flies preferred the *LP*-seeded medium, but not the *AP*-seeded medium, over the unseeded medium (Figure 3B; Data S1D). Together, our results suggest that olfaction plays an important role in *Drosophila* microbial preferences, but that other chemosensory mechanisms (such as taste) can also be at play for different microbes.

Foraging Decisions Involve Balancing Cues from Both Microbes and Nutrients

Animals sense and respond to a changing nutritional environment by adjusting their food choices and consumption patterns [23]. Studies using semi-defined diets have shown that insects can behaviorally balance their food intake to specific

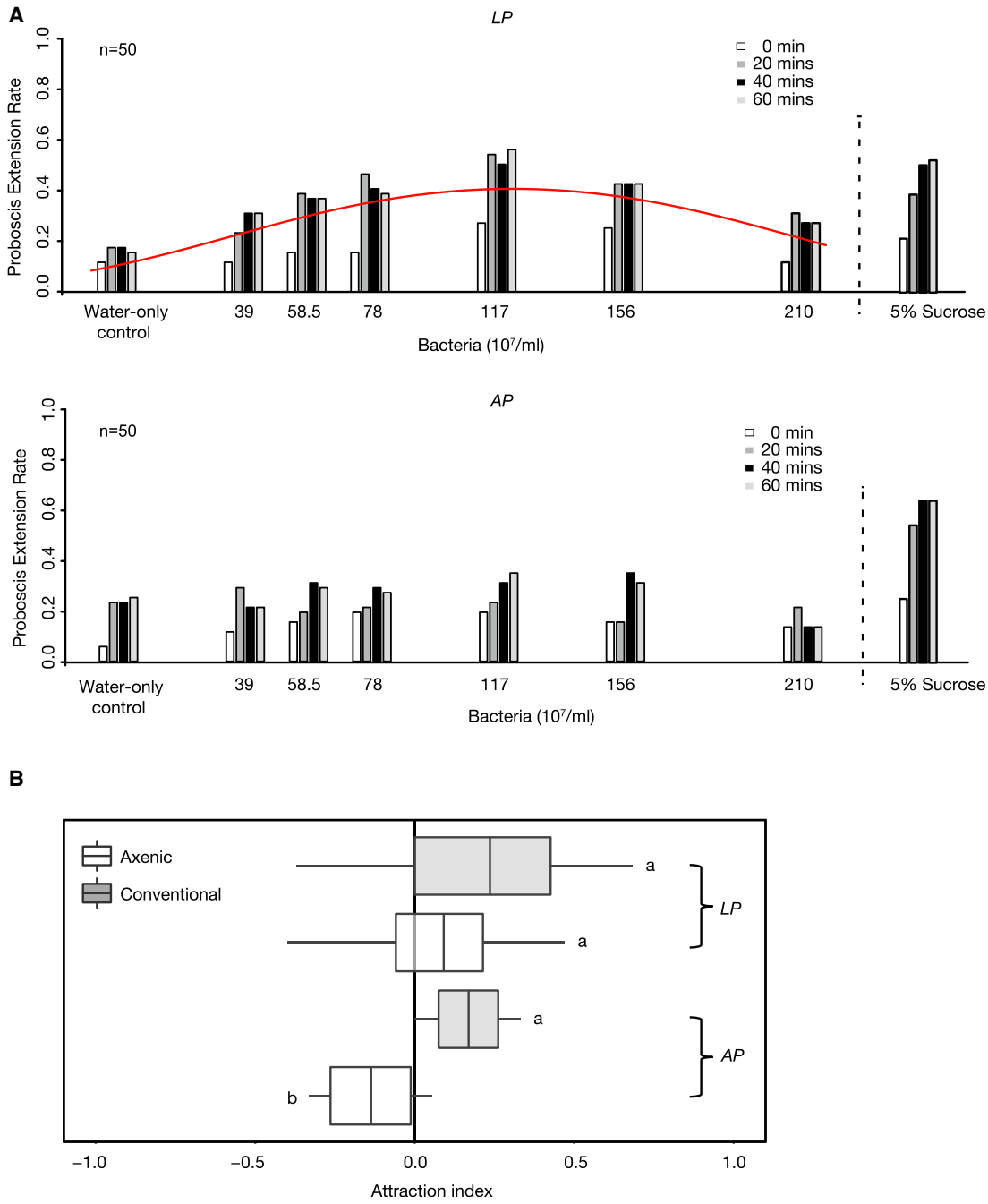

Figure 3. *Drosophila* Chemosensory Responses toward Beneficial Bacteria

(A) Proboscis extension response (PER) of adult *Drosophila* toward substrates as a function of dosage of *LP* (top) and *AP* (bottom) and test time (bar colors); n indicates the number of flies tested. Data were analyzed using binomial generalized linear mixed models (GLMMs), with a random effect accounting for repeated-measurements of individual flies. The red curve indicates the GLMM estimated probability of PER as a function of concentration of bacterial cells in the suspension. See also Figure S2C and Data S1C.

(B) Attraction index (AI) indicating olfactory preference of conventional and axenic flies toward medium seeded with *LP* and *AP*. AI was calculated as follows: number of flies in *LP*- or *AP*-seeded trap minus number of flies in unseeded trap divided by the total number of flies placed in the foraging arena. Statistical significance was assigned by a two-way type II ANOVA. Different letters indicate significant differences (Student-Newman-Keuls [SNK] post hoc test). See also Figure S2D and Data S1D.

protein-to-carbohydrate ratios (P:C) that maximize correlates of evolutionary fitness (e.g., lifetime egg production [24, 25] and resistance to infection [26–28]). Given the importance of the gut microbiota to host nutrition and our observed differing fly responses toward bacterial cues on food, we questioned how these microbes affect fly foraging decisions.

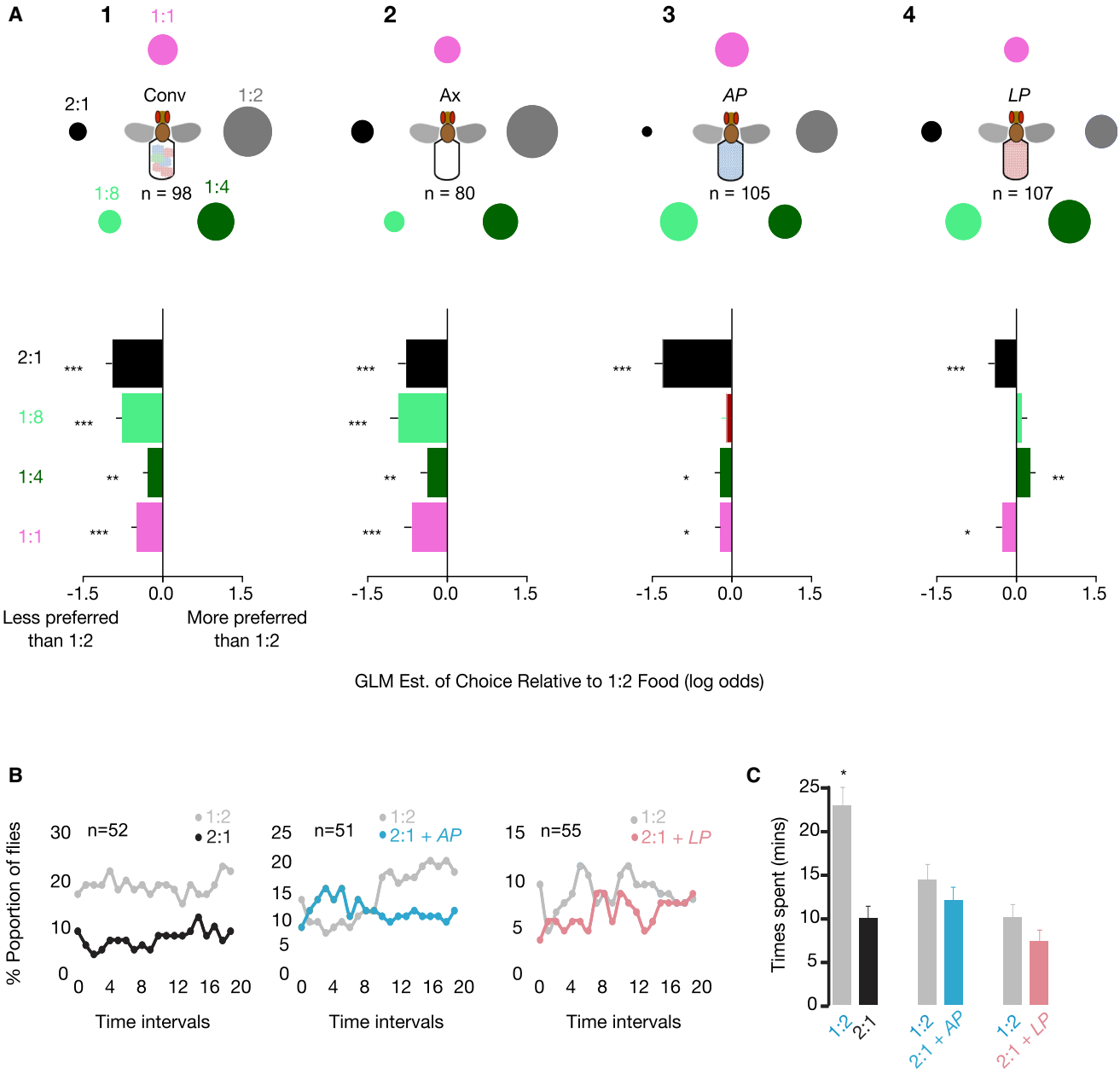

Figure 4. *Drosophila* Foraging Decisions Are Compounded by the Host and Food Microbiota

(A) Foraging of adult *Drosophila* on yeast-sucrose diets at five different protein-to-carbohydrate ratios (P:C). Multiple fly microbiota lines (conventional [Conv], axenic [Ax], and *AP* and *LP* monocolonized) were tested. n indicates the number of flies tested. Circle sizes are proportional to the total observations of flies on each food type. Bars indicate multinomial generalized linear model estimates of the log odds of a fly selecting a given P:C ratio relative to the 1:2 diet. Error bars indicate the SE. Statistical significance was inferred from CIs and is indicated by asterisks (*, 95% CI or $p \leq 0.05$; **, 99% CI or $p \leq 0.01$; ***, 99.9% CI or $p \leq 0.001$). See also Data S1E.

(B) Real-time foraging dynamics of flies toward P:C 1:2 and P:C 2:1 diets unseeded or seeded with *AP* or *LP*, indicated as the proportion of flies on each diet every 3 min for one hour. n indicates the number of flies tested.

(C) Average time spent per fly upon making a foraging decision on a given food during the 1 hr assay. Error bars indicate the SE. A paired Wilcoxon test (repeated measures) was used to test whether time spent per fly differed between the two foods in each of the three assay groups (1:2 versus 2:1, 1:2 versus 2:1+*AP*, 1:2 versus 2:1+*LP*). The asterisk indicates statistical significance, whereby p values < 0.016 are significant after Bonferroni correction for multiple comparisons. See also Data S1F.

We first tested whether the gut microbiota influences flies' nutritional preference. We offered adult *Drosophila* five unseeded yeast-sucrose diets with varying nutritional contents (P:C 2:1, 1:1, 1:2, 1:4, and 1:8) in the foraging assay (see STAR Methods). As expected (see [29]), conventional flies preferred foraging on the balanced diet (P:C 1:2) the most. Imbalanced

diets, either high in protein (P:C 2:1) or carbohydrate (P:C 1:8) (see [24, 29]), were least preferred (Figure 4A1; Data S1E). By manipulating the fly microbiota, we observed subtle yet notable changes on flies' nutritional preferences. Axenic flies retained the greatest preference for P:C 1:2 diet, although the relative preference strength diminished because a higher proportion foraged on the high protein diet (P:C 2:1) (Figure 4A2). In contrast, *AP* flies had a reduced tendency to choose the high-protein diet (P:C 2:1) (Figure 4A3), whereas *LP* flies shifted their greatest preference toward the diet higher in carbohydrate (P:C 1:4) (Figure 4A4). These results indicate that the microbiota can modify flies' nutritional preference (see also [30]).

An important ecological question arising from our findings is how flies prioritize and respond to nutritional and microbial signals in food. To address this, we examined the foraging pattern of conventional flies subjected to binary choices between the highly preferred P:C 1:2 diet and the least preferred P:C 2:1 diet that was unseeded or seeded with *LP* or *AP* (see STAR Methods). Without microbial supplementation, the majority of flies promptly foraged on the P:C 1:2 diet (Figure 4B), and the time spent on the P:C 1:2 diet was three times higher than on the P:C 2:1 diet (Figure 4C; Data S1F). The presence of *AP* or *LP* on the food dramatically altered flies' foraging pattern, promoting both the proportion of flies and their time spent on the high protein diet (P:C 2:1) when seeded, such that the average time spent between the diets was no longer different (Figures 4B and 4C; Data S1F). Our results suggest flies exhibit behavioral tradeoffs between acquiring beneficial microbes and balancing nutrients in foraging.

DISCUSSION

Our study provides new evidence that the gut microbiota can modify host chemosensory responses and behavior. Flies show preferences for beneficial bacteria, but these preferences vary depending on host-microbial history and identity. Members of the gut microbiota also affect flies' nutritional preference and can drive behavioral tradeoffs in foraging, as flies have to accommodate both microbial acquisition and nutritional balance. Our results corroborate recent proposals that the gut microbiota controls animal feeding preferences [30–33].

Olfaction is the central mechanism through which flies sense environmental cues and adjust their behaviors. Our study suggests that olfaction participates in host-microbe recognition, potentially facilitating processes such as replenishment of the gut microbiota [22] and symbiont dispersal and transmission [34, 35]. We further show that the effects of microbial exposure on host microbial preference begins at early life stages, signifying maternal microbiota deposition on eggs and offspring, observed in many animals [36], may promote symbiotic association. Together, our findings open new questions about the evolutionary processes that shape animal microbial recognition and foraging behaviors.

In flies, different olfactory receptors and pathways have been implicated in sensing and responding to microbes including pathogens [37], beneficial *Acetobacter*, and yeast [35]. Candidate metabolites that can attract flies through olfaction have recently been suggested [38], raising the question as to whether the gut microbiota can modify host responses to these microbial fermentation products. One possibility is that the gut bacteria produce metabolites that shape the gut-brain axis in flies, like in mammals [39]. The conservation of these microbial-recognition mechanisms and the significance of gut microbiota in other animals remain to be explored.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Larval food choice assay
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- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND SOFTWARE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, two tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.07.022.

AUTHOR CONTRIBUTIONS

A.C.-N.W., Q.-P.W., S.J.S., and F.P. designed the experiments. A.C.-N.W., Q.-P.W., J.M., M.L., and F.P. ran the experiments. A.C.-N.W., Q.-P.W., J.M., A.M.S., M.L., and F.P. analyzed the results. A.C.-N.W., J.M., A.M.S., M.L., G.G.N., S.J.S., and F.P. wrote the manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and Virus Strains		
Acetobacter pomorum (Canton S) isolated	This study	GenBank: MF191595
Acetobacter tropicalis (Canton S) isolated	This study	GenBank: MF191596
Lactobacillus casei (Canton S) isolated	This study	GenBank: MF191597
Lactobacillus plantarum (Canton S) isolated	This study	GenBank: MF191598
Lactobacillus pantheris (Canton S) isolated	This study	GenBank: MF191599
Staphylococcus saprophyticus (Canton S) isolated	This study	GenBank: MF191600
Chemicals, Peptides, and Recombinant Proteins		
Sucrose	MP Biomedicals	Cat# 02902978
Agar	MP Biomedicals	Cat# 02100262
Glycerol	Sigma Aldrich	Cat# G5516
MRS broth	Oxoid	Cat# CM0359
Brewer's yeast	MP Biomedicals	Cat# 02903312
Yeast Hydrolysate	MP Biomedicals	Cat# 02103304
PBS pH 7.4	Sigma Aldrich	Cat# P3813
D-(+)-Glucose	Sigma Aldrich	Cat# G8270
Phosphoric Acid	Sigma Aldrich	Cat# P5811
Propionic Acid	Sigma Aldrich	Cat# P1386
Platinum Taq DNA Polymerase	Thermo Scientific	Cat# 10966026
Taq DNA Polymerase PCR Buffer	Thermo Scientific	Cat# 18067017
dNTP mix	Thermo Scientific	Cat# 18427088
$MgCl_2$	Sigma Aldrich	Cat# M8266
LB Broth	Oxoid	Cat# CM1018
Sodium Hypochlorite (Bleach)	Peerless JAL	Cat# 9326157002270
Critical Commercial Assays		
DNeasy Blood & Tissue Kits	QIAGEN	Cat# 69506
Deposited Data		
All behavioral data from this study is available at the Dryad Digital Repository	This study	http://dx.doi.org/10.5061/dryad.2np32
16S rRNA amplicon pyrosequencing reads on the *Drosophila* microbiota composition	This study	GenBank: Bioproject PRJNA389291; ID: SAMN07194310–SAMN07194330; https://www.ncbi.nlm.nih.gov/sra
Experimental Models: Organisms/Strains		
D. melanogaster Canton-S strain	Bloomington Drosophila Stock Center	BDSC: 64349; FlyBase: FBst0064349
Oligonucleotides		
Primer 16S Forward: 27F 5′-AGAGTTTGATCMTGGCTCAG-3′	This study	N/A
Primer 16S pyrosequencing: Reverse: 1522R 5′-AAGGAGGT GATCCAGCCGCA- 3′	This study	N/A
Primer 454 pyrosequencing Forward: 28F 5′-TTTGATCNTG GCTCAG-3′	This study	N/A
Primer 454 pyrosequencing Reverse: 519R 5′-GTNTTACNGC GGCKGCTG-3′	This study	N/A
Software and Algorithms		
QIIME for the analysis of bacterial sequencing data	[40]	http://qiime.org/
R for statistical analysis and figures	[41]	http://www.r-project.org

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Pestle (autoclavable)	VWR	Cat# 47747-358
Petri Dish (55mm diameter)	BRAND	Cat# BR452010
Petri Dish (85mm diameter)	Thermo Scientific	Cat# R80085

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Adam C.N. Wong (cw442@cornell.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Drosophila stocks

Wolbachia-free Canton-S *Drosophila melanogaster* were maintained at room temperature under a 12 hr:12 hr light–dark cycle on yeast-sucrose food, comprising 100 g glucose l^{-1} (Sigma, St Louis, MO, USA), 100 g Brewer's yeast l^{-1} (MP Biomedicals, Santa Ana, CA, USA) and 13 g agar l^{-1} (MP Biomedicals) and preservatives (0.04% phosphoric acid, 0.42% propionic acid; Sigma). The age and sex of *Drosophila* used in the different experiments are as followed: pyrosequencing (4 days old females); isolation of cultured bacteria (mixed age and sexes); larval food choice assay (24-48 hr old larvae); adult foraging assay, proboscis extension response (PER) assay and two-choice trap assay (5-10 days old mated females).

Fly-associated bacteria

Ten adult *Drosophila* whole bodies or guts were homogenized in 1ml PBS buffer, plated onto the de Man, Rogosa and Sharpe (MRS) medium (Oxoid, UK) and incubated for 72h at room temperature under aerobic or microaerophilic conditions. The resultant colonies of distinct morphology were subjected to end-point PCR using general 16S primers 27F-1522R (Key Resources Table), followed by Sanger sequencing (Macrogen, Korea). The PCR reactions contained 1 × Taq DNA polymerase buffer, 0.24 mM of each dNTP, 2 mM MgCl2, 0.32 μM primers, 1 μL template DNA and 0.25 U Platinum Taq in 25 μL. The cycling conditions were 5 min at 94°C, followed by one cycle of 1 min at 56°C, 72°C for 2 min and 25–30 cycles of 1 min at 94°C, 1 min at 56°C and 2 min at 72°C with a final incubation of 8 min at 72°C.

To prepare glycerol stocks and for generating gnotobiotic flies, *Drosophila*-associated bacteria were grown on the MRS medium at 30°C under aerobic (*Acetobacter*) or microaerophilic (*Lactobacillus*) conditions using two-position sap cap tubes. *Staphylococcus* was grown on the Luria-Bertani (LB) medium at room temperature aerobically.

METHOD DETAILS

Fly microbiota manipulation

Axenic (Ax) flies were derived from dechorionated eggs as described in [42]. Eggs deposited from conventional (Conv) mated females overnight were collected and rinsed 3 times in 0.6% sodium hypochlorite before inoculating onto autoclaved yeast-sucrose food in a biosafety cabinet. Flies mono-associated with *Acetobacter pomorum* (*AP*) or *Lactobacillus plantarum* (*LP*) were generated by adding 50 μL bacterial suspension at 10^6 cells mL^{-1} density onto each food vial (30 mm diameter) containing dechorionated eggs. The administered bacteria were clonal isolates originally from the *D. melanogaster* guts. The microbiota status of the adult flies was measured at day 4 post-eclosion. Five flies were homogenized with an autoclaved pestle (VMR) in 200 μL deionized water, serially diluted to 1 in 10000 and plated onto the MRS medium in Petri dishes (diameter: 85mm). The plates were incubated at 30°C under aerobic or microaerophilic conditions. The number of colony-forming unit (CFU) corresponding to *Acetobacter* and *Lactobacillus* were scored 48h after plating.

Pyrosequencing

DNA was extracted from *Drosophila* samples, comprising: duplicate of embryos (estimated 100-300 each), six individual food-deprived flies and twelve individual non food-deprived flies, alongside a reagent-only control using the QIAGEN DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA) following the manufacturer's instructions for Gram-positive bacteria as in [19]. Purified DNA samples were sent to a sequence service provider (Research and Testing Laboratory, Lubbock, TX), with amplicons prepared using protocols based on primer pair 28F and 519R incorporating domains for 454 sequencing and sample-specific identification tags (Key Resources Table, Table S2). Amplicon pyrosequencing was performed using the Roche 454 FLX instrument with standard Titanium chemistry. Pyrosequencing flowgram were analyzed as in [20] using QIIME 1.7.0 virtualbox with default parameters [40], except that the denoising cutoff was set to remove singletons. OTUs with fewer reads than in the reagent-only control were excluded.

Species identities of the OTUs were assigned by NCBI StandAlone BLAST (megablast program) using the 16S microbial database (December 2014) with supplementary manual curation. Principal component analysis (PCA) plots of the bacterial communities were created after read numbers were log-transformed.

Adult foraging assay

Adult foraging assays were conducted in arenas (350 × 260 × 150 mm) containing food patches made of 2 mL agar-based diets set in open Petri dishes (55mm diameter) as shown in Figure S1A [43]. All food patches were made of sucrose (S) and yeast hydrolysate (Y) (MP Biomedicals) in a total (Y+S) of 180 g l^{-1} at specific P:C ratios [24]. Macronutrient calculations were based on the yeast containing 45% protein and 24% carbohydrates (as glucose equivalents). To test for fly microbial preference, the food patches were made with the Y-S diet at P:C 1:2 seeded with single bacteria (isolated from fly guts: *Acetobacter pomorum [AP]*, *Acetobacter tropicalis [AT]*, *Lactobacillus casei [LC]*, *Lactobacillus plantarum [LP]*, *Lactobacillus pantheris [LPa]*; isolated from fly body surface: *Staphylococcus saprophyticus [S]*) by plating 100 μL bacterial suspension at 10^6 cells mL^{-1} density onto each dish; along with an unseeded food patch (no-bacteria control). To test for fly nutritional preference, food patches were made with five different diets (P:C 2:1, 1:1, 1:2, 1:4 and 1:8). To test whether flies prioritize nutritional or microbial signals, we used binary choices with one preferred diet (P:C 1:2) and one non preferred diet (P:C 2:1) that was either unseeded or seeded with *LP* or *AP*. Food patches were arranged in a randomized, circular array (not applicable to the binary choices assay). Flies were food-deprived (provided with water) for 18h before being placed individually into each cage, and fly foraging was sampled every 3min for 1h. Pilot tests were performed at different time food deprivation length (3h, 6h and 18h) and we observed that flies food-deprived for 18h were the most ready to forage (data not shown). All adult foraging experiments were conducted in biological replicates of single flies placed in the arenas, denoted as the n number shown in the figures (Figures 1B and 4A). Data were aggregated from assays performed over at least 3 separate days. Flies that did not forage at all throughout the assays were excluded from statistical analysis.

Larval food choice assay

Larval choice assays were conducted as shown in Figure S2B. Conv, Ax, *AP*- or *LP*-inoculated eggs were transferred to the center of a Petri dish (85mm diameter) containing 2% agarose. Three choices of yeast hydrolysate (5%; MP Biomedicals)-sucrose (5%) agar media differed in bacterial content (plated with *AP*, *LP* or no bacteria) were placed at equal distance to the eggs on the side of each dish. The translucent nature of the medium allowed efficient tracking of the larvae. Two observations (morning and afternoon) of the number of larvae on each medium were made on each of the two days (day 1 and 2). Experiments were performed in triplicate with ~20 Conv, Ax, *AP*- or *LP*-inoculated eggs each time, with the assays repeated four times. n refers to the total number of observations of larvae on each food type (Figure 2). Replicates with no larvae observed on media were excluded from statistical analysis.

Proboscis extension response assay

Proboscis extension response (PER) assays were conducted as shown in Figure S1C. Flies were food deprived for 6h at room temperature, immobilized by chilling on ice and mounted on glass slides. While recovering from ice treatment, each fly was water-satiated before their tarsi were presented with suspensions of single bacteria at a series of doses, along with a 5% sucrose solution (positive control). Fly PER to each substance was recorded under a stereo microscope every 20min for 1h (i.e., four observations per fly). The flies were placed in a humidified chamber between each recording. Pilot tests were performed at different food deprivation length (3h, 6h and 18h) and we observed that flies food-deprived for 3h were less responsive in PER than 6h or 18h (data not shown), thus the flies were food-deprived for 6h for the experiment. Experiments were conducted in five replicates of ten flies, where n refers to the total number of flies (Figure 3A).

Two-choice trap assay

Two-choice trap assays were set up as shown in Figure S1D. Flies were food deprived for 3h before being transferred to the test cages (350 × 260 × 150 mm). Each cage contained two traps (transparent cups, 34mm diameter) of 1ml MRS medium diluted in water, one seeded with the bacterium (*AP* or *LP*) and the other unseeded. Experiments were conducted at the same time of the day (between noon – 2pm) at room temperature, 12h:12h light-dark cycle. The number of flies in the test traps and outside the traps (no choice) was scored after 24 and 48h. Pilot tests were performed at different food deprivation length (3h, 6h and 18h) and we observed no difference in the proportion of flies entering traps 24h or 48h after (data not shown). Thus, the shortest length (3h) was chosen for the experiment. Experiments were conducted in biological replicates of arenas with multiple flies (mean number of flies per arena = 19.4; median = 17; Figure 3B), where n refers to the number of arenas (see STAR Methods).

QUANTIFICATION AND STATISTICAL ANALYSIS

All analyses were performed in the statistical programming environment R [41]. Full statistical results are showed in Data S1. Descriptions of "n" in each assay can be found in the previous Method Details section.

Fly foraging data (inoculated food assay and P:C ratio assay) were analyzed with a multinomial generalized linear model (GLM; logit link), implemented with the 'multinom' function in the R package nnet [44]. The response variable was, the total counts of observations of each fly on each food, with the counts on the control food (unseeded in the inoculated foods assay and 1:2 P:C ratio in the

macronutrient assay) as the multinomial denominator; estimates are interpretable as a positive/negative estimate indicates higher/lower probability of observing a fly on a given food relative to the control food. The predictor in the GLM was a categorical variable describing the animal biotic treatment; Conv, *AP*, Ax and *LP*. Binary choice tests were analyzed with paired Wilcoxon tests to compare the amount of time spent by flies on diets varying in P:C ratios (1:2 or 2:1) and bacteria composition (unseeded or seeded with *LP* or *AP*) over 1 hr. Bonferroni corrections were applied for multiple comparisons.

Larval food choice data were assessed using multinomial generalized linear mixed-models (GLMMs; logit-link function), using the MCMCglmm function in the R package MCMCglmm [45]. The response was the counts of larvae on each food type at each observation, with the counts of larvae on the unseeded food as the multinomial denominator. The fixed-predictor in the GLMM was a categorical variable describing the egg treatment; Conv, *AP*, Ax and *LP*. Given that four observations were made from each larval cohort, a random-factor was fitted to the model denoting the cohort from which an observation was made.

PER assay data were assessed using binomial (logit-link) GLMMs fitted with the 'glmer' function in the R package lme4 [46], where the response was a binary outcome denoting whether the fly did (1) or did not (0) extend its proboscis when presented with a food. For each fly type (*AP* and *LP* treated), we first assessed the response of flies to biotic-treated food relative to water and sugar. The fixed predictor in the model was a three-level categorical variable denoting the type of food; water, sucrose or bacterial solution. Second, where a type of treated flies showed a significantly greater response to the inoculated food over water, we analyzed whether increasing the concentration of bacteria in the food affected probability of proboscis extension. Linear and quadratic effects of bacterial concentration on proboscis extension were explored. In both analyzes, a random-factor describing the identity of the fly from which each measurement was taken was also fitted. In addition, because treatments were performed every 20 min, a numeric fixed factor describing the time of the trial was also fitted (this variable was Z-transformed and centered to aid interpretation). Statistical significance of estimates for fly foraging data, larval food choice, and PER assay were based on 95% confidence/credible intervals (CI); where 95% CIs exclude zero estimates are considered statistically significant.

For the two-choice trap assay, we calculated the Attraction Index (AI) as shown in Figure 3B. We then used a two-way type II ANOVA to test for the effects of fly status (i.e., conventional or axenic), bacteria strain (i.e., *AP* or *LP*), and their interaction on AI while controlling for the effect of replicate and total number of individuals in a given trial [47]. Final sample sizes for the two-choice trap assay were: axenic *LP* (n = 4), and *AP* (n = 8), and conventional *LP* (n = 38), and *AP* (n = 25). Our data fitted the normality (Shapiro-Wilk test: $W = 0.976$, $p > 0.1$) and homoscedasticity assumptions (Bartlett's test: K-squared = 2.277, df = 3, $p > 0.516$). Differences between groups were assigned with the Student-Newman-Keuls (SNK) posthoc.

DATA AND SOFTWARE AVAILABILITY

The accession numbers for the 16S rRNA gene sequences obtained by Sanger sequencing reported in this paper are GenBank: MF191595–MF191600 and for the 454 pyrosequencing are SRA: SAMN07194310–SAMN07194330. Data were deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.2np32.