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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 envi- ronment 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. Con- ventional 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 *Aceto- bacters* 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 de- clines, 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 *Lacto- bacillus* colony forming unit (CFU) (Figure S1C), pointing to a possibility that *Drosophila* need to replenish their gut micro- biota [22]. Based on this assumption, we investigated the che- mosensory 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 prefer- ence 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 pref- erence for food seeded with *LPa* or *S*, a strain isolated from fly whole bodies. Both *LPa* and *S* were undetected in our pyrose- quencing dataset.

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.

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 micro- bial 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 magni- tude 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* (Fig- ure 3A; Data S1C) with a maximum response to CFU of 1.17 3 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 re- sults 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 environ- ment by adjusting their food choices and consumption pat- terns [23]. Studies using semi-defined diets have shown that insects can behaviorally balance their food intake to specific 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.

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) (Fig- ure 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 supplemen- tation, 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 prefer- ences 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 sug- gests 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, signi- fying maternal microbiota deposition on eggs and offspring, observed in many animals [36], may promote symbiotic associ- ation. 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-recogni- tion mechanisms and the significance of gut microbiota in other animals remain to be explored.

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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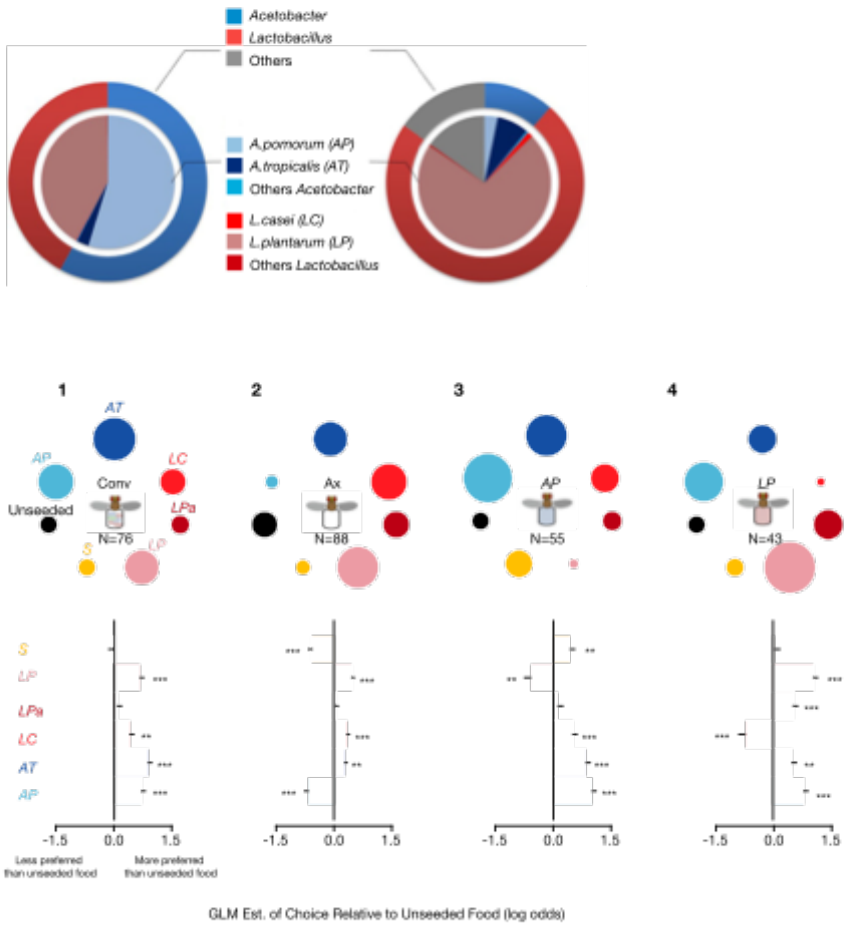

Figure 1. Microbiota Affects Adult Drosophila Microbial Preference

(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 % 0.05; **, 99% CI or p % 0.01; ***, 99.9%CI or p % 0.001). See also Figure S2A and Data S1A.

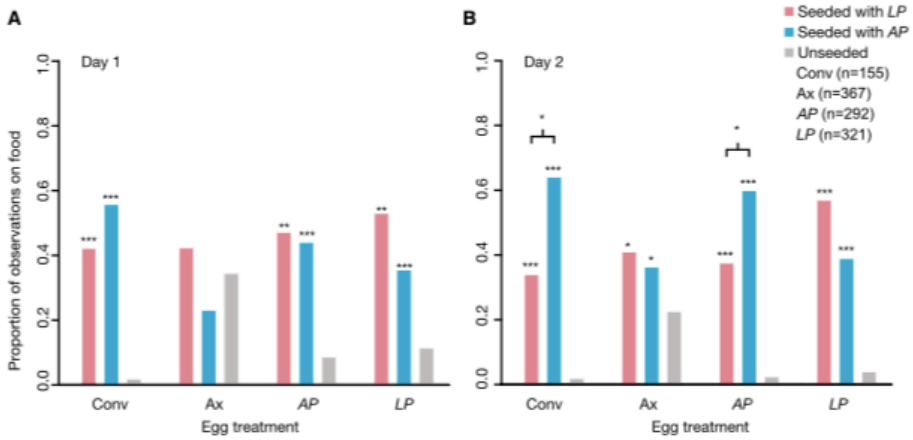

Figure 2. Early Microbial Exposure Influences Drosophila Larvae Microbial Preference

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.

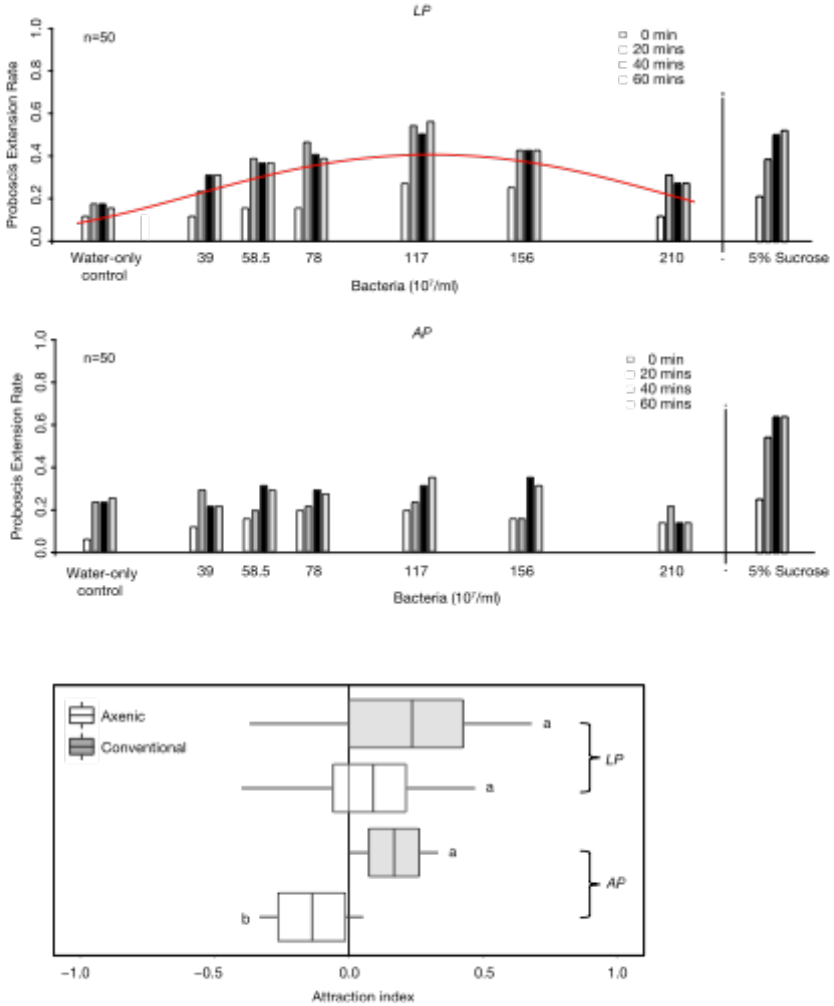

Figure 3. Drosophila Chemosensory Responses toward Beneficial Bacteria

(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.

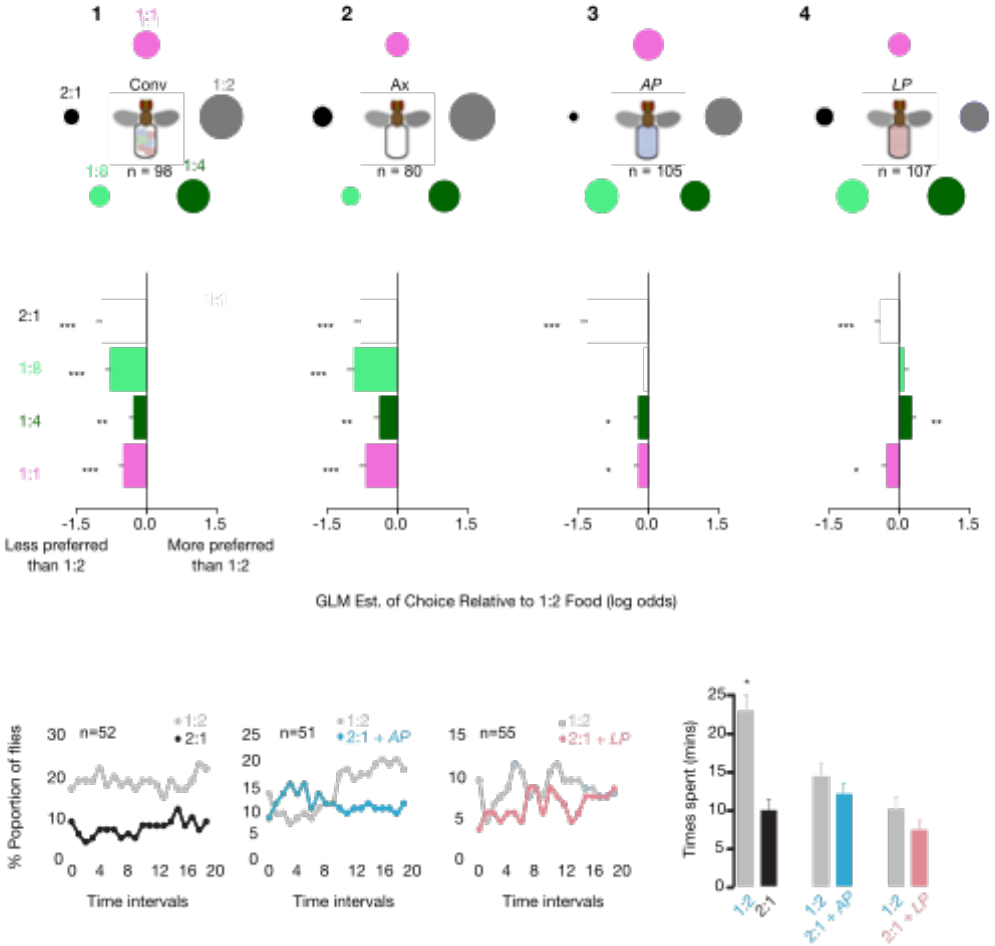

Figure 4. Drosophila Foraging Decisions Are Compounded by the Host and Food Microbiota

(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 % 0.05; **, 99% CI or p % 0.01; ***, 99.9% CI or p % 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