Two gut microbes are necessary and sufficient for normal cognition in *Drosophila melanogaster*

Michael DeNieu, Kristin Mounts, and Mollie Manier*

Department of Biological Sciences, The George Washington University, Washington, DC, U.S.A.

*Correspondence: manier@gwu.edu

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Abstract

2 It is widely accepted that the gut microbiome can affect various aspects of brain function, includ-
 ing anxiety, depression, learning, and memory. However, we know little about how individual
 4 microbial species contribute to communication along the gut-brain axis. Vertebrate microbiomes
 are comprised of hundreds of species, making it difficult to systematically target individual mi-
 6 crobes and their interactions. Here, we use *Drosophila melanogaster* as a simple model organism
 to tease apart individual and combined effects of gut microbes on cognition. We used an aver-
 8 sive phototactic suppression assay to show that two dominant gut commensals in our lab stock,
Lactobacillus and *Acetobacter*, are necessary and sufficient for normal learning and short-term
 10 memory relative to flies with a conventional microbiome. We also demonstrate that microbes
 did not affect their hosts' ability to detect the aversive learning stimulus (quinine), suggesting
 12 that our results were due to decreased cognition and not sensory deficits. We thus establish
Drosophila as a model for elucidating mechanisms of gut-brain communication at the level of
 14 individual bacterial species.

Introduction

16 Within the last decade, there has been an explosion of studies showing that gut microbes affect
critical organismal functions, particularly communication along the gut-brain axis [1]. Early
18 studies found a link between the gut microbiota and anxiety [2–4], depression [5], learning [6],
and memory [7] suggesting that gut microbes impact more than just their immediate surround-
20 ings. Since then, other studies have found that disrupting the gut microbiota through diet [7],
pathogens [8], antibiotics [9, 10], or probiotics [11–14] can all affect learning. Moreover, disease
22 models for inflammatory bowel disease [15, 16], diabetes [17], and schizophrenia [18] have associ-
ated the loss of homeostasis of the gut community (dysbiosis) with learning and memory deficits
24 in experimental animals. These deficits were ameliorated with probiotic treatment, suggesting
that symptoms experienced by patients may be treated with probiotics as well.

26 If probiotic therapy is to become an effective treatment for dysbiosis of the microbial commu-
nity, we need a better understanding of how individual community members influence cognition
28 alone and in combination. We will then be able to target specific microbes or species combina-
tions to more effectively address underlying issues in brain function. We know that diversity of
30 the gut microbiota plays a role (some beneficial species have been identified), but it is not fully
understood how individual members of the microbiota contribute, because significantly variable
32 communities can nonetheless be healthy [19–21].

We chose to test learning and memory in *Drosophila*, because the microbiota in flies is much
34 simpler than in vertebrates [22], allowing us to generate single and combinatorial microbial as-
sociations that comprise the bulk of the total microbiota. In this way we can begin to dissect
36 community function and develop this system to further probe mechanisms of gut-brain axis com-
munication. We isolated two strains of bacteria from our *D. melanogaster* wild type strain and
38 created populations of flies that were axenic, monoassociated with each microbe, biassociated
with two microbes, and a conventional control with the entire microbiota. We then used the
40 phototaxis suppression assay to dissect the individual and combined effects of each microbe in
a test of learning and memory. Our assay combined both visual and gustatory cues, whereas

most previous studies focused primarily on memory using the novel object recognition test [8–10, 13–15, 18], and only a few studies tested a non-visual sensory modality [12, 14]. We found that removing the microbiota decreased performance in both learning and memory, and that association with a single microbe was not sufficient to restore wild type behavior. However, the biassociation of the two microbes was sufficient, suggesting that effects may be additive or interactive.

Materials and Methods

We began by characterizing the major taxa in the gut microbiomes of our wild type lab population (LHm). Previous work has shown that *D. melanogaster* laboratory strains typically host relatively few OTUs, dominated by *Lactobacillus* and *Acetobacter* [23]. We homogenized approximately 20 whole flies in 200 μ L of mMRS broth [24], plating the resulting solution onto 100 mm mMRS plates and incubating at 30°C for 48 hours. We found that *Lactobacillus* and *Acetobacter* also dominated our lab stock, with no other bacteria detectable by our culturing methods. We visually identified and isolated these bacteria by growing individual colonies in 20 mL of mMRS broth for an additional 48 hours and freezing the cultures at -80°C in 50% glycerol and distilled H₂O. These cultures were used later to generate a panel of gnotobiotic flies.

We then cleared our LHm flies of intracellular symbionts, including *Wolbachia*, to ensure that we could generate truly germ-free populations. To do this, we reared LHm flies on sucrose and yeast medium at room temperature under ambient light conditions with tetracycline antibiotic for eight consecutive generations. The absence of *Wolbachia* was verified by PCR amplification of 16S ribosomal RNA. We then generated germ-free flies by sterilizing eggs in 0.6% hypochlorite solution and transferred approximately 100 eggs each into sterile 50 mL conical tubes in a laminar flow hood. Axenic flies were reared to adulthood without further manipulation. Monoassociation treatments were supplemented with 100 μ L of 48-hour liquid media from *Acetobacter* or *Lactobacillus* cultures, and Combined treatment tubes received 50 μ L from both cultures in a separate laminar flow hood to avoid contamination. Conventional flies were

68 produced by allowing 25 males from the base stock to inoculate each tube for 18-24 hours before
adding eggs. Shortly after eclosion, all gnotobiotic adults were transferred without anesthesia
70 into polyethylene vials with standard media that had been autoclave sterilized and aged 24-48
hours to verify sterility (lack of microbial growth). We thus generated gnotobiotic flies that car-
72 ried monoassociations with "*Lactobacillus*" or "*Acetobacter*", a biassociation with both strains
("Combined"), or neither ("Axenic"). These treatments were compared with "Conventional"
74 flies hosting the full gut microbiome. Because identifying individual bacterial species by cultur-
ing is a limited approach, we expect that Conventional flies will carry additional unidentified
76 gut microbes. All flies were aged 3-5 days post-eclosion when assayed.

We used the aversive phototactic suppression assay [25] to assess learning and memory of
78 individual flies. Individual testing eliminated the potential effect of social influence on decision-
based behaviors [26]. This assay tests the fly's ability to associate light with a bitter substance
80 (quinine) and suppress its innate positive phototaxis. To do this, we used a T-maze with a
central gate separating a foil-covered dark chamber and a light chamber with the bulb of a
82 goose-neck dissection lamp placed at the end. A 3 cm strip of filter paper was placed into the
light chamber near the light source as a vehicle for liquid stimuli that the fly would encounter
84 when moving toward the light. For a benign stimulus, we used 400 μ L distilled water, and for
the aversive stimulus we used 400 μ L of 100 mM quinine. We used separate, dedicated chambers
86 for each stimulus to avoid cross contamination. For each trial, a single fly was aspirated into
the dark chamber to acclimate for 30 seconds, after which the light was turned on, and the gate
88 lifted. Flies that entered the light chamber within 30 seconds were scored as phototactic, and
flies that remained in the dark chamber were scored as non-phototactic. Once the fly was scored,
90 it was tapped back into the dark chamber for another 30-second acclimation period, and the
process was repeated for a total of 16 trials. Flies entering the light chamber generally walked
92 all the way toward the light and encountered the filter paper. All assays were performed in a
dark room at 23°C under dim red light.

94 Flies from each microbiota treatment were subjected to one of three assays. The *phototaxis*

control assay consisted of 16 trials with water only in order to establish a baseline phototaxis response for each gnotobiotic treatment. In the *learning assay*, flies were exposed to quinine for all 16 trials to assess their ability to associate quinine with the light. In the *memory assay*, flies were exposed to the quinine for the first eight trials and water for the final eight trials to determine if they could remember the initial association and continue to avoid the light. For all assays, a phototactic response was scored as a binary variable (yes/no), with a positive response if the fly entered the light chamber. For all assays, the last eight trials represented the testing phase, in which we counted the number of phototactic responses. All flies were pre-screened for positive phototaxis in a separate, empty light chamber before each assay. Trials took place between 8 am and 6 pm in a dark room. Because of the large time range, experiments were performed in 30 blocks fully randomized by microbiota treatment and assays type.

For statistical analysis, we used the total number of phototactic trials during the testing phase as the response variable in a linear mixed effects analysis using the lmer function in lme4 v1.1-20 [27] in R v5.2.3 [28]. We fit a model with microbiota treatment (Conventional, Axenic, *Lactobacillus*, *Acetobacter*, or Combined), assay treatment (phototaxis, learning, or memory) and their interaction as fixed effects, with the date each block was performed as a random effect. All estimates report the mean, standard error. The p-values for model fixed effects were generated using a parametric bootstrap in the bootMer function in lme4 with 100000 simulations.

To ensure that the results of the learning assay were due to differences in cognition rather than taste perception, we tested each gnotobiotic treatment's gustatory response to quinine in colored agar. To do this, we used three different taste assays: no quinine, quinine in red agar, and quinine in blue agar. The control treatment contained no quinine, in order to assess a color preference. The red or blue quinine treatments had 100 mM quinine in the red or blue 1% agar (with McCormick food coloring), respectively, controlling for any inherent color preference. A solution of live yeast in water was added to the surface of each well to stimulate feeding. We reared flies on our standard sterile media, collected flies 3-5 days old under light CO₂ anesthesia into vials at densities of 25 flies per vial. After 24 hours acclimation, 50 flies

(equal sex ratio) were bumped into a sealed plastic container with a 16-well microtiter plate filled in alternating patterns with red or blue agar. This experiment was repeated for each microbiota and quinine treatment group, such that approximately 100 flies were assayed for each combination. Containers were enclosed in a cardboard box to exclude light and placed in a 23°C incubator for 24 hours. Flies were then anesthetized with CO₂, and abdomen color recorded as either blue, red, or purple. Because fewer than 6% flies in the control assay were purple, and no flies were purple in either quinine assay, we removed them from the analysis. For statistical analysis, we calculated proportions of flies consuming each color in each gnotobiotic treatment in the control assay. We used those proportions to generate matching expected count values for each gnotobiotic treatment in chi-squared goodness of fit tests for the assays with the quinine in the blue agar and the red agar respectively. Reported p-values were generated using Monte Carlo simulation with 100000 replicates.

Results

All gnotobiotic treatments showed significant levels of learning, as measured by reductions in phototaxis in the presence of quinine relative to the phototaxis control assay (Figure 1a). In the learning assay, control flies had the largest reduction in phototaxis as predicted ($\Delta_{\text{phototaxis}} = -3.66 \pm 0.55, p < 0.0001$). However, the Axenic ($\Delta_{\text{phototaxis}} = -1.73 \pm 0.77, p = 0.015$), *Lactobacillus* ($\Delta_{\text{phototaxis}} = -1.67 \pm 0.77, p = 0.011$) and *Acetobacter* treatments ($\Delta_{\text{phototaxis}} = -1.60 \pm 0.76, p < 0.009$) had reduced learning relative to Conventional flies. This result suggests that an intact gut microbiome is required for normal cognition, and neither *Lactobacillus* nor *Acetobacter* alone were sufficient to improve learning above that of Axenic flies. A biassociation of both species in the Combined treatment, however, rescued learning ($\Delta_{\text{phototaxis}} = -3.10 \pm 0.77; p = 0.51$), suggesting that both bacterial species are necessary and sufficient for normal cognition.

A similar outcome was found for the memory assay (Figure 1b). All treatments had significant reductions in phototaxis (stats), but the Axenic ($\Delta_{\text{phototaxis}} = -1.13 \pm 0.77; p = 0.001$),

Figure 1. Reduction in phototactic trials during the testing phase of the (a) learning (quinine only) and (b) memory (quinine, then water) assays relative to the phototaxis control assay (water only), with 95% confidence intervals. Asterisks represent significant differences relative to the Conventional treatment.

148 *Lactobacillus* ($\Delta\text{phototaxis} = -1.57 \pm 0.77$; $p = 0.008$), and *Acetobacter* ($\Delta\text{phototaxis} =$
 -1.09 ± 0.77 ; $p = 0.0001$) treatments performed more poorly relative to Conventional flies.
150 Again, memory was rescued in the Combined treatment ($\Delta\text{phototaxis} = -2.63 \pm 0.77$; $p = 0.21$).
Though all treatments were similarly positively phototactic with water ($p_{\text{microbiota}} = 0.37$), there
152 were minor differences among the microbiota groups, with *Acetobacter* flies showing the largest
difference as compared to the conventional flies (Table 1). To account for this, we used this assay
154 as a baseline level of phototaxis specific to each treatment (a relative value of 0 in Figure 1),
against which cognition was compared. Thus, minor differences in phototaxis among treatments
156 were accounted for in the learning and memory assessments.

	Conv	Axenic	Lacto	Aceto	Comb
Control	5.69 (0.43)	4.91 (0.54)	4.82 (0.54)	4.68 (0.54)	5.19 (0.54)
Learning	2.09 (0.54)	3.19 (0.76)	3.15 (0.76)	3.09 (0.77)	2.09 (0.76)
Memory	2.09 (0.55)	3.79 (0.77)	3.25 (0.77)	3.59 (0.77)	2.55 (0.77)

Table 1. Average number of phototactic trials in the testing phase for the control, learning, and memory assays for each microbiota treatment, with standard errors in parentheses.

In the taste assays, we detected no differences among the gnotobiotic treatments in quinine

158 taste perception. Axenic and *Acetobacter* flies did show a preference for red media, avoiding
the blue, while *Lactobacillus*, Combined, and Conventional flies showed no preference (Figure
160 2a). However, all treatments avoided the color containing the quinine, regardless of their base
preference (Figure 2b,c; $p \leq 0.01$ for all treatments except *Acetobacter* with the blue quinine
162 where avoidance of blue was already strong and only slightly increased). These results suggest
that the microbiome treatments were not disrupting taste perception, and that our results
164 reflected differences in cognition.

Figure 2. Proportion of flies eating the blue and red agar for (a) control with no quinine, (b) quinine in red agar only, and (c) quinine in blue agar only.

Discussion

166 Our findings demonstrate that *Drosophila melanogaster* can be used as a simple model organism
for isolating different components of the gut-brain axis, beginning here with individual members
168 of the gut community. We showed that learning deficits can be invoked by manipulating the gut
microbiome, and that multiple species are required to rescue normal learning. A significant role
170 of the gut microbiome in learning in both flies and vertebrates suggests that mechanisms of gut-
brain communication may be evolutionarily conserved. Indeed, there is widespread homology
172 in neurological mechanisms of learning [29], intestinal structure [30], and overlap in community

members between vertebrates and *Drosophila* (e.g., *Lactobacillus* and other Firmicutes; [23, 31]).

174 The neurological pathways controlling olfaction in *Drosophila* are both more complex [32] and
better understood than those for gustation, but there is substantial overlap in how both sensory
176 modalities feed into learning centers in the brain. Signals initiated by bitter taste receptors on
the legs are communicated to the brain by type 3 taste projection neurons (TPN3), with axons
178 terminating in the higher brain close to olfactory neural projections [33]. TPN3 neurons directly
activate PPL1 dopaminergic neurons [33] that are also required for aversive olfactory learning
180 and target the central complex and mushroom body [34], the site of learning. Indeed, dopamine,
serotonin, and GABA all contribute to regulating learning in flies [35–40], with similar functions
182 in vertebrate learning systems [41–43].

These neuroactive peptides can also be produced by gut bacteria themselves. For example,
184 *Lactobacillus brevis*, a common commensal of *D. melanogaster* [23], is a proficient producer
of GABA in culture [43], and other *Lactobacillus* species are known to produce dopamine,
186 serotonin, and GABA [44]. Probiotic treatment of mice with *Lactobacillus plantarum* (also
commonly found in *D. melanogaster*) increases dopamine and decreases serotonin in the brain
188 and ameliorates anxiety-like behaviors [45]. Additionally, gut commensals have been shown to
decrease symptoms of depression by catalyzing the conversion of tryptophan into kynurenine
190 and reducing its availability to produce serotonin [46, 47]. Short-chain fatty acids (SCFAs) are
another class of bacterially-derived metabolite that may be associated with brain function [48–
192 51]. In *Drosophila*, the SCFA acetate is produced by *Acetobacter* and regulates host growth and
development via the insulin signalling pathway [52–54]. If and how these metabolites directly
194 travel to the central nervous system is unclear, but in mice, acetate produced in the colon can
reach and cross the blood-brain barrier to control appetite, accumulating in the hypothalamus
196 and incorporating into GABA within GABAergic neurons [55].

Though the mechanism of action in our study has not yet been elucidated, our results under-
198 score the importance of gut community structure on host phenotype, with emergent properties
that exceed the sum of its parts. These results also suggest that probiotic treatments targeting

single microbial species may be less effective than treatments targeting the whole community (e.g., fecal transplant). At the same time, it is important to understand the nature of species interactions among gut microbes in order to design the most effective and personalized treatments. Future directions include characterizing metabolites produced by our *Lactobacillus* and *Acetobacter* strains in culture as well as in the guts of mono- and biassociated flies, especially since biogenic amine production by bacteria may be more strain-specific than species-specific [56]. Taken together, we show that the gut microbiota are important for the maintenance of learning and memory in *D. melanogaster*, a simple model organism in which the differences between mono- and biassociations can be easily probed. This approach may prove to be a valuable starting point for understanding the interactions between the microbiota and the host in more complex gut microbiomes.

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