Microbiome-by-ethanol interactions impact *Drosophila melanogaster* fitness and physiology

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Abstract

The microbiome can modulate the interaction between animals and their environment. In particular, intestinal microbes play a strong role in shaping how animals respond to their diets, and especially dietary toxins. In this study, we investigated how the microbiome affects the interaction between the fruit fly *Drosophila melanogaster* and ingested ethanol. *D. melanogaster* naturally feeds on fermenting fruits and therefore commonly ingests ethanol. This dietary ethanol is generally considered to be a toxin, but its effect on adult fly fitness has yet to be shown. We found that the reproductive output of bacterially-colonized flies remains high with low amounts of dietary ethanol, while that of bacteria-free flies decreases precipitously after ethanol ingestion. This shows that bacteria protect *D. melanogaster* from the damaging effects of ingested ethanol, which has important implications for fitness under natural conditions. We also observed that bacterial colonization and ethanol both negatively affect fly lifespan. In particular, bacteria play a dominant role on fly lifespan and therefore the negative effects of ethanol are only observed in bacteria-free flies. We next asked how the bacterial microbiota changes in response to dietary ethanol. Contrary to our expectations, we found that total bacterial abundance stays relatively constant with increasing ethanol. *In vivo* survival of bacteria was well above the *in vitro* toxic dose of ethanol, demonstrating that the host is shielding the microbiome from the negative effects of ethanol. Next, we investigated several aspects of host physiology that may underlie bacterially-modulated fitness changes. We found that regardless of bacterial colonization, ethanol ingestion decreases the prevalence of intestinal barrier failure and increases fly body fat content, suggesting these mechanisms are not directly responsible for bacteria-dependent fitness differences. Finally, measurements of dietary ethanol content suggest that bacterial metabolism can only partially explain the observed fitness effects. Overall, we found significant bacteria-by-ethanol interactions on *D. melanogaster* and that bacteria ameliorate the negative effects of ethanol on host fecundity. Because of the central role of ethanol in the ecology of *D. melanogaster*, these results have important implications for our understanding of fruit fly natural history. More generally, they underscore the importance of the microbiome in shaping an animal's interaction with its environment.

Introduction

A complete understanding of animal biology requires acknowledging the contribution of the microbiota, the complex and diverse microbial communities that are associated with animals (McFall-Ngai et al. 2013). One area of particular interest is how the microbiota modulates the effect of ingested dietary toxins. For example, bean bugs, an important agricultural pest, gain resistance to the pesticide fenitrothion through a specific association with fenitrothion-degrading intestinal bacteria (Kikuchi et al. 2012). Likewise, in desert woodrats, the ability to specialize on the highly toxic creosote bush is dependent upon their gut microbes (Kohl et al. 2014). However, the presence of a microbial community may have negative effects as well. The efficacy of the agricultural insecticide BT toxin is dependent upon gut bacteria (Broderick et al. 2006) and acts via a mechanism in which BT toxin reduces gut integrity, allowing intestinal bacteria to enter the body cavity and induce sepsis (Mason et al. 2011). In this study, we investigated the role of the microbiota in modulating the effects of chronic ethanol ingestion in the fruit fly, *Drosophila melanogaster.*

Both wild and laboratory-raised *Drosophila* are commonly associated with the bacterial genera *Acetobacter* and *Lactobacillus*, although wild flies are associated with a higher overall diversity of bacteria [(Chandler et al. 2011; Corby-Harris et al. 2007; C. N. A. Wong et al. 2011; Staubach et al. 2013), reviewed in (Broderick & Lemaitre 2012)]. Bacteria affect many components of fly fitness and physiology and many of these effects are seen only in a diet-specific context (A. C.-N. Wong et al. 2014; Shin et al. 2011; Storelli et al. 2011). How these *Drosophila*-bacteria interactions affect the ecology and evolution of wild populations of flies is unknown: perhaps the transient nature of intestinal bacteria precludes a significant role of the microbiome in shaping *Drosophila* biology (Blum et al. 2013; Broderick et al. 2014)? However, because recent work has shown that certain strains of wild-collected bacteria stably colonize the *D. melanogaster* intestinal tract (Obadia et al. 2017; Téfit et al. 2017), there is the possibility of tight host-microbe associations that can significantly influence fitness under natural conditions.

In order to accurately study how animal biology is shaped by the microbiome, realistic environmental variables must be used. The natural habitat of *D. melanogaster,* fermenting fruit, often contains 1-2% ethanol and the unnatural but common habitat of vineyards can contain up to 10% ethanol (Gibson et al. 1981). Because of this, *D. melanogaster* has long been used for research into the effect of ethanol on animals [reviewed in (Devineni & Heberlein 2013)]. Ethanol exposure increases development time and decreases egg-to-adult survival in *D. melanogaster* larvae and this is mediated by molecular changes in the insulin, lipid metabolism, and oxidative stress pathways (McClure et al. 2011; Logan-Garbisch et al. 2014). Adult *D. melanogaster* show many hallmarks of human alcoholism including tolerance, addiction, and withdrawal (Kaun et al. 2011; Devineni & Heberlein 2009; Ghezzi et al. 2014; Robinson et al. 2012). The resistance of *D. melanogaster* adults to ethanol vapor has long been studied and the ethanol metabolism pathway has been identified as a key (but not complete) component in the ethanol resistance (Fry 2014). Two important gaps in our knowledge are how chronic ethanol ingestion, the natural way which flies acquire ethanol, impacts *D. melanogaster* adults and whether these impacts are mediated by the microbiome. The current study seeks to address these gaps.

Methods

Fly stocks, husbandry, and creation of ethanol media

All experiments used *Wolbachia*–free *D. melanogaster* Canton-S strain (Bloomington Line 64349) as previously described (Obadia et al. 2017). Flies were maintained at 25C with 60% humidity and 12 hr light/dark cycles on autoclaved glucose-yeast medium (10% glucose, 5% active dry yeast (Red Star Brand), 1.2% agar, 0.42% proprionic acid. Bacteria-free flies were

generated by sterilizing dechorionated embryos (Ridley et al. 2013). Bacteria-free stocks were kept for several generations and checked regularly for presence of yeasts, bacteria, and known viruses. Bacterially-colonized flies were created by allowing approximately 50 normally-colonized young adults (from unmanipulated lab stocks) to seed autoclaved media with their frass, removing these flies, and then introducing bacteria-free flies. Ethanol media was made by adding 100% ethanol to autoclaved glucose-yeast medium after it had cooled to 50C. Vials were stored under equivalent ethanol vapor pressure to reduce evaporation until use. Because we were interested in the toxic, rather than nutritional, effects of ethanol, and because the caloric value of ethanol is not easily comparable to that of sugars (Xu et al. 2012), we did not adjust amount of glucose to in an attempt to create an isocaloric diet. To limit the effect of evaporation, flies were transferred to fresh media every three to four days. For all experiments, three to six day old adults were used.

Measurement of fecundity and lifespan

Lifespan and fecundity were measured simultaneously during the same experiment. Four replicate vials of 20 females each were created for the 2 bacterial treatments (bacterially-colonized and bacteria-free) and the 7 ethanol treatments (0% to 15%, in 2.5% increments) resulting in 56 vials for the 14 treatments. Because of the high mortality rate for the higher ethanol treatments and because we used young (3 to 6 day old) flies, Day 0 is set as the start of the experiment rather than the date of birth. We used this convention for all experiments described in this study. Fecundity was determined as the number of pupae that form in the used vials after adults are transferred to fresh vials. Fecundity is measured as the total pupae that form from each bacteria/ethanol/replication combination over the entire experiment. Pupae per female was calculated by the number of pupae that emerge per flip, divided by the number of females alive at the start of the egg laying period. Approximately 90% of all pupae emerged as adults with no differences in eclosion rate between ethanol or microbial treatments (data is not shown). Development rate are measured as the day the first pupae formed following a transfer to a new vial. For the lifespan experiments, survival was checked each day and dead flies were removed with each transfer.

Bacterial abundance

This experiment was set up identical to the lifespan and fecundity experiment, except that only three replicate vials were used. On days 14, 21, 28, and 31, one to three individual flies from each replication and treatment were externally sterilized, homogenized, serially diluted, and plated onto MRS media (Obadia et al. 2017). For the 12.5% and 15% ethanol treatments, we did not sample flies on days 31, and 28 and 31, respectively, because of fly death before the end of the experiment. 8 to 16 individuals were plated per ethanol treatment (mean=11.5). Colony forming units (CFUs) were identified by visual comparison to laboratory stocks of various species of *Acetobacter* and *Lactobacillus*. Additionally, the identity of representative CFUs was confirmed using 16S rRNA sequencing (data not shown). In only one of 81 individual flies sampled was there a CFU that had neither *Acetobacter* nor *Lactobacillus* morphology. Because this CFU morphology represented less than 2% of the total bacterial community of this fly, it was disregarded as potential contamination.

Bacterial sensitivity to ethanol in vivo

We tested a set of ten representative *A. pasteurianus* and *L. plantarum* isolated from our lab for sensitivity to ethanol. Genetically identified isolates were grown overnight at 30C in an

appropriate medium (MYPL for *A. pasteurianus* and MRS for *L. plantarum*). Cultures were diluted to a working OD of 0.01 in media with 0% to 15% ethanol in a 96-well plate. Growth with measured in 10-minute intervals on a TECAN Infinite F2000 PRO, at 30C and 5 minutes of orbital shaking between measurements. Maximum OD was determined for each growth curve, and a two-parameter Gompertz function was fit to the normalized maximum ODs from the aggregate data for each strain. The inhibitory concentration for 50% growth (IC50) was calculated as the ethanol percentage that reduced normalized maximum OD by half.

Intestinal Barrier Failure

We measured the level of intestinal barrier failure (IBF) by supplementing fly diet with 2.5% (wt/vol) FD&C Blue No. 1 (Rera et al. 2012). Two independent experiments were done, the first with 0% and 5% ethanol diets and the second 0%, 5%, and 7.5% ethanol diets, each with bacterially-colonized and bacteria-free treatments. For each, three or four vials of 10 flies were monitored over their entire lifespan and degree of IBF determined by the amount of blue coloration in tissues upon death. For statistical purposes, individuals in IBF categories 0 and 1 were considered IBF negative and individuals with IBF categories 2 and 3 were considered IBF positive (Clark et al. 2015). No significant differences were found between experiment 1 and experiment 2, so they were combined into a single dataset. Because the blue dye accumulates in flies with IBF and increases mortality (Clark et al. 2015), we did not directly compare the lifespan data from these IBF experiments with experiments lacking blue dye.

Lipid content

Bacterially-colonized or bacteria-free flies were reared on 0%, 5%, and 10% ethanol diets for 16 days, as described above. Four to ten individuals were pooled by sex (mean=9.5), with three to five replicates for each bacteria-ethanol-sex treatment. The mass of pooled flies was determined to the nearest 1/10 of a milligram on a Mettler Toledo microbalance. Free and total lipid content was determined using established colorimetric methods (SIGMA F6428, T2449, and G7793), (A. C.-N. Wong et al. 2014; Tennessen et al. 2014).

Ethanol concentrations of fly diets

Evaporation and bacterial metabolism may decrease the effective ethanol concentration of the fly diets. Using a clinical grade breathalyzer, we developed a method to measure ethanol vapor within the headspace of a vial and use this a proxy for dietary ethanol concentration [following (Morton et al. 2014)]. Briefly, a 14 gauge blunt needle attached to 50 ml syringe is used to sample the headspace of vial. The sampled air is then pushed through the mouthpiece of an Intoximeters Alco-Sensor® III. Using 0%, 2.5%, 5%, and 10% ethanol media, with either 20 bacterially-colonized or bacterially free flies, we checked ethanol concentration once per day for four days. Four replicate vials of each of the ethanol treatments and two replicate vials of the 0% ethanol were used. Preliminary experiments show that ethanol vapor concentration in the headspace stabilizes within two hours of opening a vial or taking a measurement (data not shown).

Results and Discussion

In this study, we investigated the effect of chronic ethanol ingestion on *D. melanogaster* adults. We argue that this method of ethanol exposure is more natural than the standard method, in

which ethanol vapor is absorbed through the cuticle [reviewed in (Devineni & Heberlein 2013)]. In studies that do fed ethanol to adult flies, it is often under starvation conditions where ethanol is the only nutrient source (Xu et al. 2012) or in very low amounts [1%, (Galenza et al. 2016)]. By adding ethanol directly to the diet, we mimicked the route of natural ingestion for flies and increase the translational power of our model, as humans consume ethanol via their diet rather than through inhalation.

Bacterial ameliorate the negative effects of ethanol on fecundity

We found a strong effect of ethanol on fly fecundity that is mediated by the bacterial treatment (Figure 1; Figure S1; Table 1; Table S1). Without ethanol, the 20 females in both the bacterially-colonized and bacteria-free treatments produced approximately 360 pupae over their entire lifespans (Table 1). For bacterially-colonized flies, fecundity remained constant at 2.5% ethanol, but decreased by 60% at 5% ethanol and higher concentrations. Conversely, for bacteria-free flies, 2.5% ethanol led to a 60% drop in fecundity, with this number dropping to essentially zero at just 5% ethanol. At 2.5% ethanol bacterially-colonized flies had significantly higher fecundity (Table 1; $P = 1.2 \times 10^{-7}$). These fecundity results suggest significant ecological and evolutionary impacts of microbes in mediating ethanol toxicity effects on fly fitness. While the exact doses of ethanol which flies consume in the wild remains obscure, the concentration in fermenting fruit is typically 1-2%, but can rise to as much as 10% in vineyards (Gibson et al. 1981). Thus, at ecologically relevant concentrations of ethanol, bacterial colonization has a strongly positive effect on fecundity.

The observed effects on fecundity could be due a combination of maternal health and larval development. To explore this, we measured larval development time as a proxy for the effect of ethanol on larval survival. Although we found an increase in development time at the highest ethanol diets (Figure S2), we found no effect on development time at the focal 0%, and 2.5% ethanol treatments (all pairwise t-tests, $P > 0.2$). This finding agrees with a previous result that ethanol does not affect larval development except in the final larval stage at 5 days (McClure et al. 2011) at which point most of the ethanol has evaporated from the media (Figure 6). Therefore, under our experimental conditions, larvae are not exposed to ethanol during the critical time period. Furthermore, previous work has shown that 12% ethanol over the entire developmental period only reduces larval survival by 75% (McClure et al. 2011), but our results found that fecundity dropped to approximately zero at 5% ethanol for bacteria-free flies and at 10% ethanol for bacterially-colonized flies. Together, these observations strongly suggest that the effects of ethanol and bacteria that we measured on fly fecundity are more likely attributed to effects on the mother.

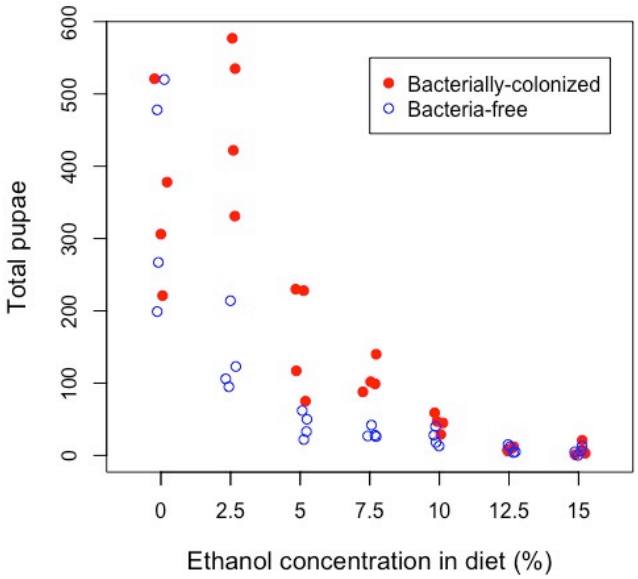

Figure 1: Bacteria ameliorate the negative effects of ethanol on fecundity: Total pupae is calculated as the sum of all pupae from 20 flies over their entire lifespan (each replicate began with 20 flies). Pupae per female data, which takes into account adult mortality, is show in Figure S1.

		Total Pupae		
Ethanol Treatment	Bacterial Treatment	Mean	SD	Pairwise t test
0%	B-	366	157	NS
	B+	357	127	
2.5%	B-	135	54	1.2×10^{-7}
	B+	466	111	
5%	B-	42	18	NS
	B+	163	78	
7.5%	B-	31	7.5	NS
	B+	108	23	
10%	B-	25	12	NS
	B+	45	13	
12.5%	B-	9	5.4	NS
	B+	9	2.6	
15%	B-	6	5.4	NS
	B+	8	9.0	

Table 1: Bacteria ameliorate the negative effects of ethanol on fecundity. Total pupae is calculated as the sum of all pupae from 20 flies over their entire lifespan (each replicate began with 20 flies). P values are calculated from a pairwise t test between bacterial treatments within an ethanol treatment and are Holm-Bonferonni corrected for multiple comparisons. B+, Bacterially-colonized. B-, Bacteria-free. Mean and SD are for four independent replicates. Pupae per female, which takes into account adult mortality, is shown in Table S1

Both bacterial colonization and ethanol negatively affect fly lifespan

Bacterially-colonized flies showed a shorter lifespan than germ-free flies (Pairwise t-test, corrected $P = 1.0 \times 10^{-12}$), in agreement with previous studies (Ridley et al. 2012; Clark et al. 2015; Steinfeld 1927). However, the shorter lifespan in bacterially-colonized flies was robust to dietary ethanol, with no significant ethanol-linked decrease in lifespan observed except at very high (and unnatural) levels of 12.5% ethanol or greater (Figure 2; Table 2; Data for individual flies is shown in Figures S2; Lifespan curves are shown in Figure S3). This was in sharp contrast to the bacteria-free flies that show a nearly linear and dose-dependent decrease in lifespan beginning at just 2.5% ethanol. Overall, these data suggest that two independent mechanisms interact to determine lifespan in this system: bacterial-colonization and ethanol exposure. First, the effect of bacterial colonization is dominant to the effect of ethanol at levels below 10%. Second, there is a clear negative effect of ethanol, but its effect is completely superseded by bacteria at low to moderate ethanol concentrations.

That bacterially-colonized flies have a reduced lifespan compared to bacteria-free flies has been reported before though never with the same magnitude we found here. Because the composition of the microbiome has significant effects on lifespan (Gould, Zhang, and Ludington, unpublished data) and the native microbiome of *D. melanogaster* varies significantly between laboratories (Chandler et al. 2011), we suggest that the flies in our lab are colonized with a lifespan-shortening consortium of bacteria.

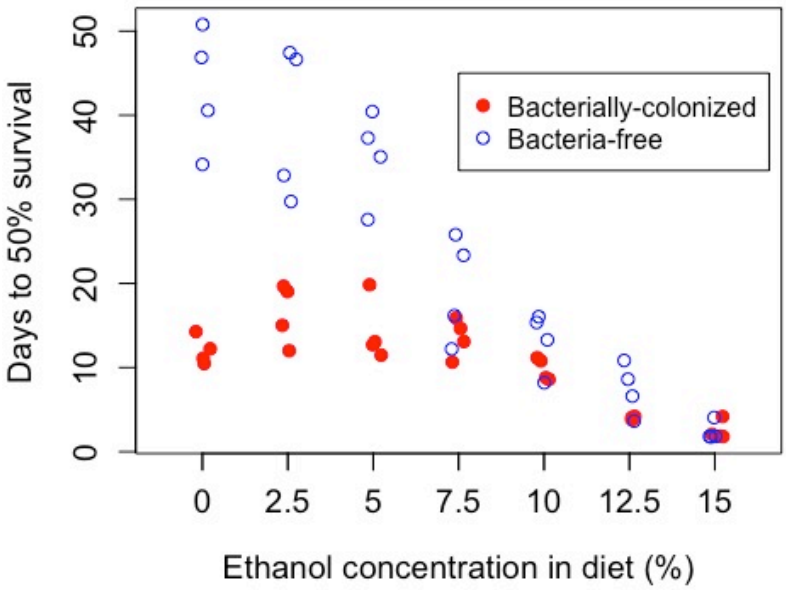

Figure 2: Bacterial colonization and ethanol negatively affect fly lifespan. Days to 50% survival is per replicate and calculated from the start of the experiment, not from birth (see methods). Data for individual flies is show in Figure S2 and Table 2.

		Days to 50% Survival			Lifespan of all individual flies				
Bacterial Treatment	Ethanol Treatment	Mean	SD	SEM	Mean	SD	Noise (SD/Mean)	SEM	Max
Bacteria-free	0%	43	7.3	3.7	41.4	19.4	0.5	2.2	67
	2.5%	39	9.2	4.6	38.4	18.6	0.5	2.1	62
	5%	35	5.5	2.8	34.9	16.3	0.5	1.8	57
	7.5%	19	6.3	3.2	22.3	14.9	0.7	1.6	54
	10%	13	3.5	1.8	17.6	14.6	0.8	1.6	48
	12.5%	7.4	3.1	1.6	11.0	9.8	0.9	1.1	36
	15%	2.4	1.1	0.6	3.6	4.1	1.1	0.5	27
Bacterially-colonized	0%	12	1.6	0.8	13.8	8.3	0.6	0.9	39
	2.5%	16	3.6	1.8	17.6	10.0	0.6	1.1	51
	5%	14	3.7	1.9	16.4	11.3	0.7	1.3	60
	7.5%	14	2.3	1.2	15.7	10.6	0.7	1.2	45
	10%	9.8	1.3	0.7	13.2	11.1	0.8	1.2	55
	12.5%	4	0.2	0.1	7.1	7.1	1.0	0.8	36
	15%	2.5	1.1	0.6	4.0	4.1	1.0	0.5	26

Table 2: Bacterial colonization and ethanol negatively affect fly lifespan. Days to 50% survival is per replicate and calculated from the start of the experiment, not from birth (see methods). The lifespan of all individual flies is calculated regardless of replicate vial the fly originated from (Figure S3).

Ethanol as a toxin

Regarding the fitness results overall, we argue that ingested ethanol is acting more like a toxin than as a source of calories. First, while increasing sugar does decrease reproductive output (Bass et al. 2007), the magnitude of decline is minimal compared to the near complete loss of fecundity observed here (Figure 1). Specifically, an addition of 10% sucrose reduces the number of eggs by less than 50% (Bass et al. 2007), whereas 10% ethanol drops fecundity to near zero in both bacterial treatments. Second, increasing dietary sugar typically increases fly lifespan (Galenza et al. 2016; Bruce et al. 2013; Lee et al. 2008), whereas our study found that ethanol has a negative effect on lifespan regardless of whether bacteria are present (although this trend is only apparent at high ethanol concentrations for the bacterially-colonized treatment). Third, we found that ethanol does not lead to the typical tradeoff between lifespan and fecundity observed by varying nutrients (Zera & Harshman 2001; Djawdan et al. 1996) – instead we found that ethanol decreases both components of fitness (Figures 1 and 2). Finally, previous studies show that while ethanol slightly increases lifespan relative to starvation conditions, its positive effects are minimal compared to an isocaloric amount of sucrose (Xu et al. 2012). This suggests that the potential calories provided by ethanol are less efficient than sugars. Thus, the effects of ethanol we observed here are most consistent with its role as a toxin, despite the fact that it likely provides some additional calories.

Ethanol shifts the composition of bacteria associated with D. melanogaster

Diet is a strong determinant of microbiome composition in flies and other animals. In particular, fruit feeding flies, which are exposed to naturally produced dietary ethanol, have significantly different bacterial and yeast communities than flies collected from other substrates (Chandler et al. 2011; Chandler et al. 2012). We hypothesized that the bacterial communities associated with flies would shift in response to ethanol ingestion. In particular, we expected that ethanol would strongly decrease the total abundance of bacteria in high ethanol treatments and these shifts would favor the abundance of bacteria with low sensitivity to ethanol.

We found that total bacterial load per fly was between 9×10^3 and 3×10^6 for the 0% ethanol containing diets (mean=7×10^5). This is comparable to previous studies of *D. melanogaster* (Blum et al. 2013; Obadia et al. 2017). While mean total bacterial abundance decreased 10 fold from 0% to 2.5% ethanol, we found (contrary to our expectations) that total load was still relatively constant from 2.5% up until the highest ethanol treatment (Figure 3). Because the bacterial load remained high in 12.5% and the 15% treatments (means = 1.9×10^{-5} and 1.9×10^{-5}, respectively), this shows that that the drop in lifespan above 10% ethanol in bacterially-colonized flies is not simply due to them becoming effectively "bacteria-free".

We next asked how the bacterial composition changes in response to ethanol. In agreement with the previous work in our laboratory and that of others, our flies are dominated by species in the genera *Acetobacter* and *Lactobacillus* [reviewed in (Broderick & Lemaitre 2012)]. Different bacteria had different responses to dietary ethanol. *Acetobacter pasteurianus* concentrations decreased 10 fold from 0% to 2.5% ethanol and remained constant until 12.5% ethanol where they dropped to essentially 0 (Figure 3). The decrease at 12.5% is notable because this is the first ethanol percentage when the lifespan of bacterially-colonized flies was significantly less than on 0% ethanol, suggesting that *A. pasteurianus* may mitigate the negative effects of ethanol. The decreasing abundance of *A. pasteurianus* with increasing ethanol is in agreement with an independent experiment performed on pooled samples of fly intestines (Figure S4).

Conversely, we found that the response of the *Lactobacilli* to ethanol was remarkably different than *A. pasteurianus.* The abundance of *L. brevis* increased with dietary ethanol and this was the only species that was present in all flies at 15% ethanol. *L. plantarum* was most abundant at intermediate concentrations of ethanol, but like *L. brevis,* it did not appear as sensitive to high levels of ethanol as *A. pasteurianus.* Because *Lactobacilli* ameliorate the negative effects of ethanol in mouse models of alcoholic liver disease (Forsyth et al. 2009; Bull-Otterson et al. 2013; Li et al. 2016) our data suggest that a similar effect is worth exploring in flies.

These *in vivo* experiments suggest differential sensitivity to ethanol amongst the bacterial species, with *Acetobacter pasteurianus* more sensitive to ethanol than *Lactobacilli*. To test this hypothesis, we measured the *in vitro* growth response to ethanol of a representative set of different *A. pasteurianus* and *L. plantarum* strains isolated in our lab. These experiments confirmed the sensitivity of *A. pasteurianus* to ethanol and showed that they are more sensitive to ethanol than are *L. plantarum* (Figure S5), with *A. pasteurianus* averaging an inhibitory concentration for 50% growth (IC50) of 6% ethanol while the *Lactobacilli* averaging 10%. These results indicate that the bacterial composition of flies varies, at least in part, according the *in*

vitro ethanol sensitivities of the bacterial strains. However, because the fly bacterial abundance of *L. plantarum* remains high despite dietary ethanol levels that should completely inhibit this bacterium's growth (Figure S6), it suggests that the host mediates the effect of ethanol on the bacteria.

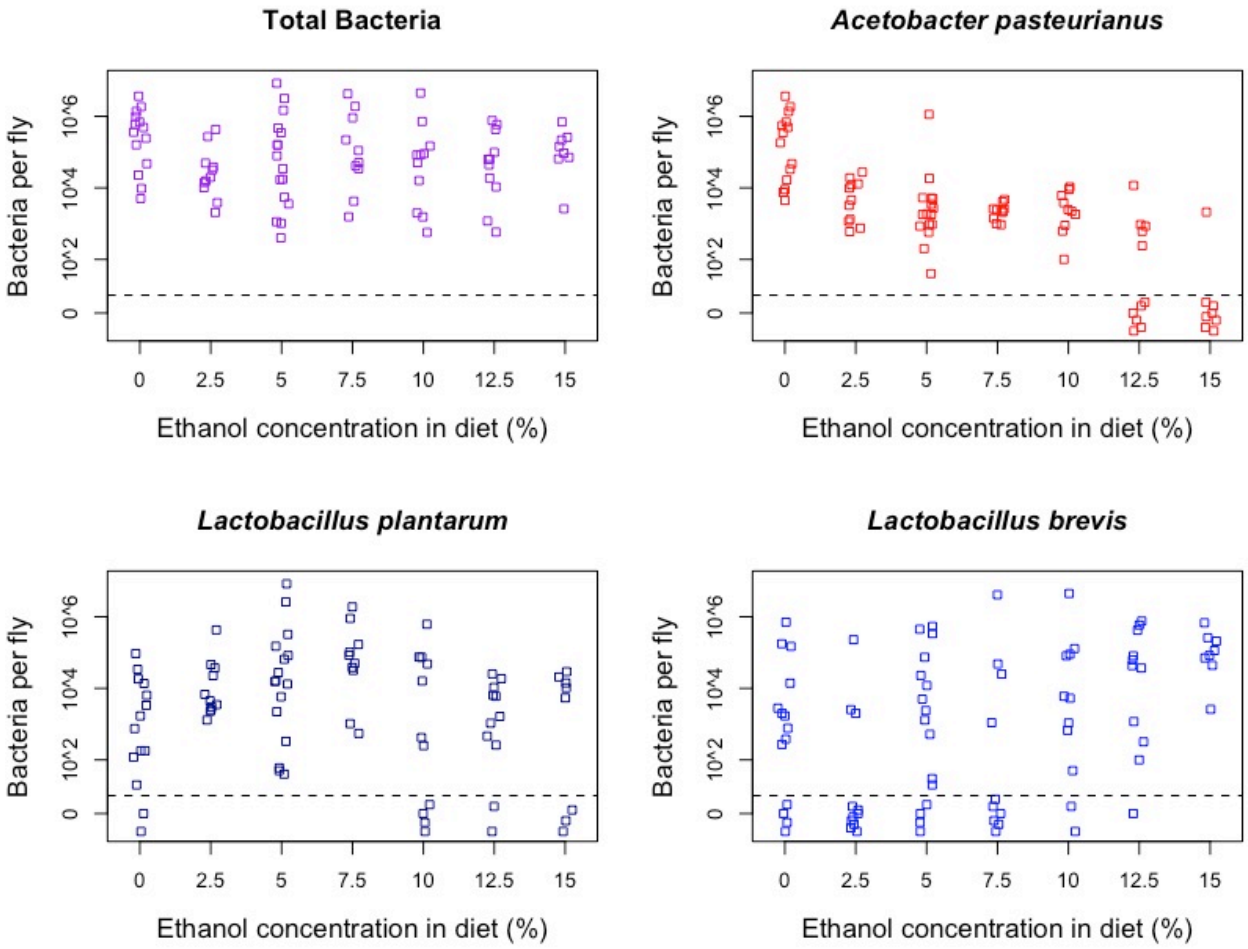

Figure 3: Bacterial community dynamics in response to ethanol diets. Each point represents an individual fly. All points below the dashed line are 0 and are expanded for clarity. Number of individual flies per treatment: 0%-14; 2.5%-11; 5%-16; 7.5%-10; 10%-11; 12.5%-11; 15%-8. We found no effect of fly age [multivariate ANOVA (Adonis, package vegan in R; P = 0.159)] and therefore all four timepoints are considered together (see methods).

Intestinal Barrier Failure is not linked to ethanol-related mortality

To explore a potential mechanism underlying mortality following ethanol ingestion, we examined intestinal barrier failure (IBF), which is strongly linked to alcoholic liver disease in humans [reviewed in (Chen & Schnabl 2014)] and is a hallmark of aging-related death in flies (Rera et al. 2012; Clark et al. 2015). In humans, excessive ethanol consumption damages the intestinal barrier, which leads to translocation of microbial byproducts into the bloodstream and

hepatic inflammation, injury, and eventual failure. Consistent with previous results (Rera et al. 2012; Clark et al. 2015), we found that nearly all flies not ingesting ethanol show IBF upon death, which is interpreted as IBF being a normal process during aging. However, on ethanol diets, we found a significant decrease in the proportion of flies that show IBF (Figure 4; Table 3). This suggests that any mechanism of potential ethanol-induced injury is not mediated by IBF. Furthermore, bacteria-free flies show significantly less IBF than bacterially-colonized flies on ethanol diets (Figure 4, Table 4). Because bacteria-free flies showed a significant decrease in lifespan at these ethanol concentrations (Figure 2), this further supports the conclusion that, in contrast to humans, ethanol-induced pathology is not mediated by IBF. That some individuals in the ethanol treatments nonetheless showed IBF can be explained by the normal background aging process, which presumably occurs regardless of ethanol or microbial treatment.

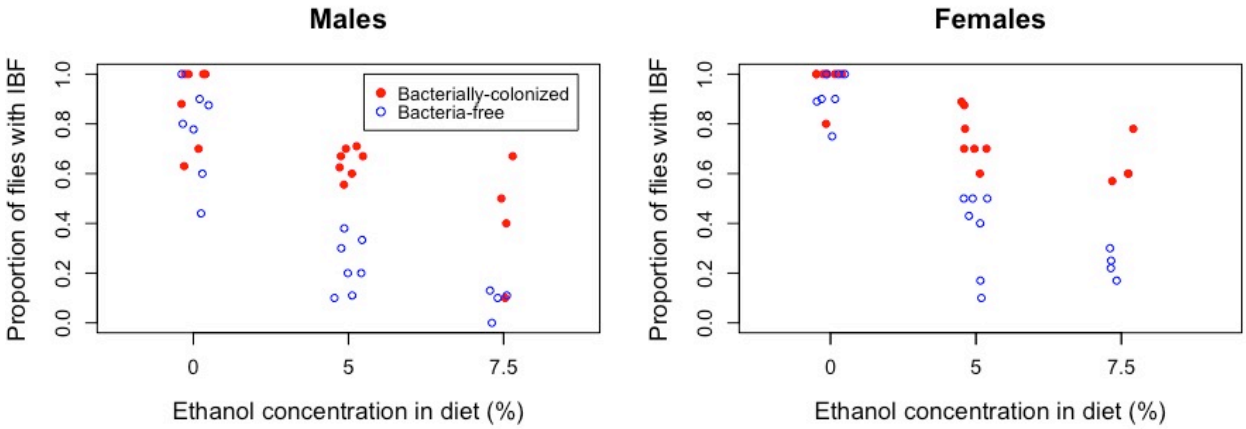

Figure 4: The prevalence of intestinal barrier failure (IBF) decreases in with dietary ethanol and this decrease is greater in bacteria free flies. Each point represents the average from a replicate vial.

	Effect of Ethanol	Effect of Bacteria	Interaction
Males	6.4×10^{-11}	2.3×10^{-6}	0.025
Females	4.3×10^{-13}	6.2×10^{-8}	2.8×10^{-4}

Table 3: Ethanol and bacterial treatment both decrease the proportion of flies exhibiting intestinal barrier failure upon death. Values represent the results of a two-way ANOVA.

Sex	Ethanol Treatment	Bacterial Treatment	IBF	SD	P - Value
Males	0%	B-	0.77	0.19	NS
		B+	0.89	0.16	
	5%	B-	0.23	0.11	1.0×10^{-6}
		B+	0.65	0.06	
	7.5%	B-	0.085	0.06	0.002
		B+	0.42	0.24	
Females	0%	B-	0.92	0.09	NS
		B+	0.97	0.08	
	5%	B-	0.37	0.18	6.0×10^{-6}
		B+	0.75	0.10	
	7.5%	B-	0.24	0.05	2.2×10^{-4}
		B+	0.64	0.10	

Table 4: Bacterial-colonization affects the incidence of intestinal barrier failure, but only in ethanol diets. B-, Bacteria-free. B+ , Bacterially-colonized. P values are calculated from a pairwise t test between bacterial treatments within an ethanol treatment and are Holm-Bonferroni corrected for multiple comparisons.

Ethanol ingestion increases stored triglycerides in flies, regardless of bacterial treatment

Increased fat deposits in the liver are a hallmark of human alcoholic liver disease.
We hypothesized that a similar process occurs with ethanol ingestion in flies, and that this process is stronger in the treatment that is more affected by ethanol (i.e. bacteria-free). In flies, triglycerides (TAG) are primary molecule for fat storage and are mainly found in adipocytes within the fat body, and organ analogous to the mammalian liver that is responsible for the majority of energy reserves in adult fly (Arrese & Soulages 2010). To test our hypothesis, we measured stored triglycerides (TAG) in bacteria-free and bacterially-colonized flies following ingestion of 0%, 5% or 10% ethanol. Contrary to our hypothesis, we found that dietary ethanol increases TAG in both bacterial treatments, with no effect on either total fly mass or free glycerides (Figure 5; Table 5). Because dietary sugars increase TAG content in flies (Skorupa et al. 2008), our finding is consistent with ethanol acting as a nutrient and suggests the toxic effect of ethanol described above is not mediated through body composition. However, it is worth noting that increased hepatic fat deposits are the first step in human alcoholic liver disease and therefore increased TAG content of flies ingesting ethanol may be indicative of a similar process occurring later in life and perhaps only in the bacteria-free flies. Following fat body inflammation and injury may shed light on the mechanism of ethanol-induced fitness effects observed in flies. Finally, because we found no ethanol-by-bacteria interaction in TAG content (Table 5), it suggests that the ethanol content experienced by both bacterial treatments is equivocal. Therefore, at least for this measure of fly physiology, the role of evaporation or bacterial metabolism of media ethanol (discussed in the next section) is minimal.

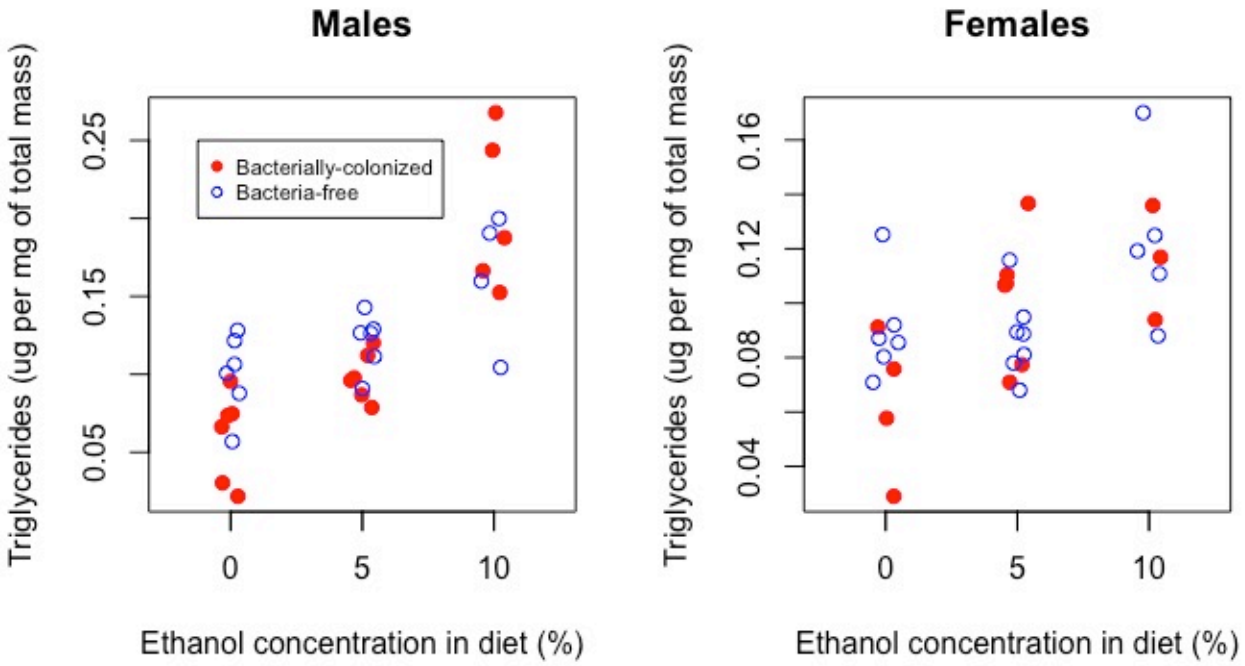

Figure 5: Ethanol ingestion increased stored triglycerides in flies, regardless of bacterial treatment. Each point represents a pooled sample of 4 to 10 flies.

	Sex	Effect of Ethanol	Effect of Bacteria	Interaction
Mass	Males	NS	NS	NS
	Females	NS	NS	NS
Free Glycerol / Mass	Males	NS	NS	NS
	Females	NS	NS	NS
Stored Triglycerides / Mass	Males	1.0x10-7	NS	NS
	Females	0.019	NS	NS

Table 5: Ethanol ingestion increased stored triglycerides in flies, regardless of bacterial treatment. Values represent the results of a two-way ANOVA.

Bacterial metabolism of ethanol partially explains fitness results

A simple hypothesis for protective effects of the microbiome is that microbes directly consume ethanol in the food. We developed methods to measure ethanol in the vapor headspace of the fly vial as a proxy for food ethanol content. We then tracked ethanol concentration in the vials over time, noting a decrease in ethanol content within all the vials over time regardless of bacteria or ethanol (Figure 6, see Figures S6 and S7 for an explanation of the conversion of headspace vapor concentration to dietary concentration and the uncorrected data). Consistent with our hypothesis, we observed that the ethanol content of the bacterially-colonized treatments decreased faster than

the bacteria-free treatment. However, this difference is amplified on later days and at lower initial ethanol treatments. Indeed, for the 2.5% ethanol treatment, there is no detectible ethanol in the bacterially-colonized treatment on day 2, but for the 10% ethanol treatment residual ethanol remains (approximately 2%) even on day 4. We propose there are two mechanisms occurring: First, in both vials evaporation slowly decreases ethanol concentration. Second, bacterial metabolism further decreases ethanol in the bacterially-colonized vials. Since all vials are initially sterile and only become inoculated with the transfer of bacterially-colonized flies, this latter process is insignificant until media bacterial abundance increases on day 2 or later.

Taken as a whole, the low rate of ethanol loss relative to the total ethanol concentration suggests that the bacterially-mediated fitness results can be partially attributed to different media concentrations. We found that the fecundity of bacterially-colonized flies on 2.5% ethanol remained high, while that of bacteria-free flies decreased, consistent with the drop in dietary ethanol due to bacterial activity. Conversely on a diet with 10% ethanol the lifespan of bacteria-free flies was reduced by 70% compared with the 0% ethanol diet. In contrast, bacterially-colonized flies had no change in lifespan (Figure 2; Table 2), despite both treatments having essentially the same ethanol content throughout the entire three to four day interval between transfers to fresh vials. Thus, microbial degradation of ethanol on the food can only partially explain the protective effects of the microbiome.

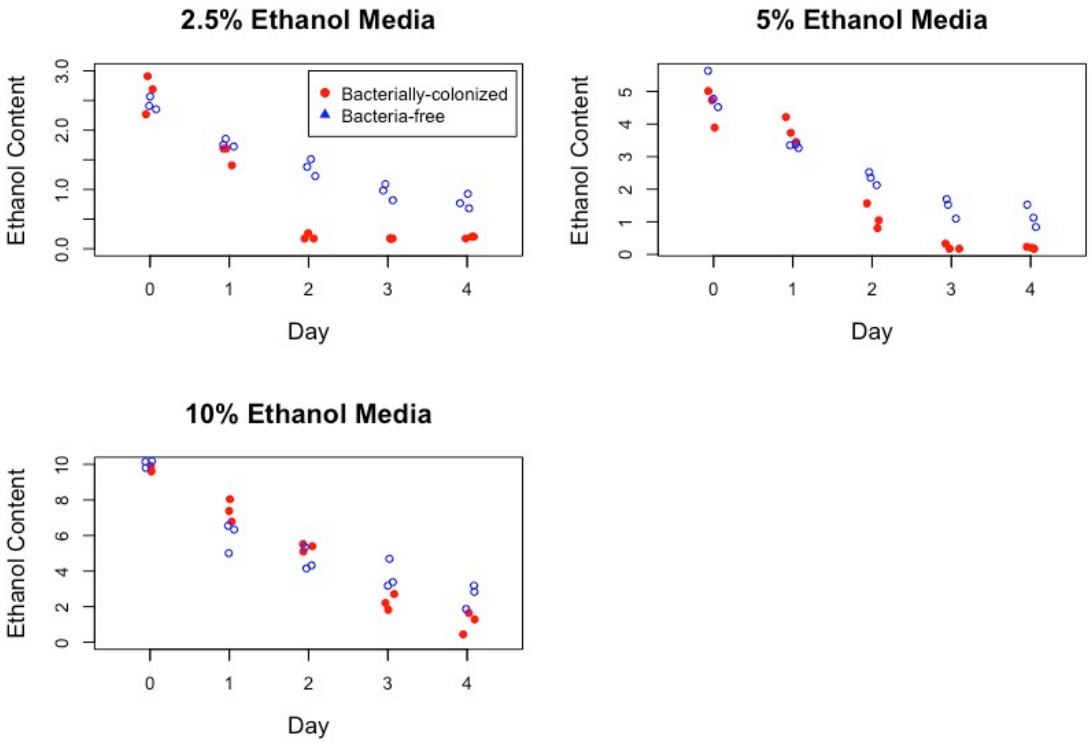

Figure 6: Dietary ethanol content decreases over time with greater loss in bacterially-colonized treatments. Each point represents an independent replicate. Measurements from 0% ethanol media are always before 0.2 and are therefore not shown. Note that in the fitness experiments (Figures 1 and 2) flies are transferred to fresh vials on day 3 or 4. Ethanol content was determined using separate standard curves for each bacterial treatment (Figure S6). Uncorrected values are available in Figure S7.

Conclusion

Although it is assumed that ethanol is important to the ecology and evolutionary history of the *Drosophila* genus, the effects of chronic ethanol ingestion have not previously been investigated. Furthermore, while the interaction between diet and the microbiome on fly physiology and fitness has been established under laboratory conditions, it remains unclear how these results translate to natural conditions. Here we have shown the toxic effect of ethanol is mediated by bacterial status (colonized or free) in both fecundity and lifespan, and we explored the physiological mechanisms that may contribute to these fitness effects. This system is well poised to further our understanding of the complex interplay between animals, their microbiomes, and dietary toxins. Given the wealth of genetics tools in *D. melanogaster,* the translational power of this model to explore the underlying molecular and cellular basis of human alcohol related pathology cannot be understated.

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