Chronic ethanol ingestion impairs *Drosophila melanogaster* health in a microbiome-dependent manner

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Summary

Ethanol is one of the worlds most abused drugs yet the impacts of chronic ethanol consumption are debated. Ethanol is a prevalent component in the diets of diverse animals and can act as a nutritional source, behavior modulator, and a toxin. The source of ethanol is microbes, which can both produce and degrade ethanol, and the gut microbiome has been associated with differential health outcomes in chronic alcoholism. To disentangle the various and potentially interacting roles of bacteria and ethanol on host health, we developed a model for chronic ethanol ingestion in the adult fruit fly, *Drosophila melanogaster*, which naturally consumes a diet between 0 and 5% ethanol. We took advantage of the tractability of the fly microbiome, which can be experimentally removed to separate the direct and indirect effects of commensal microbes. We found that moderate to heavy ethanol ingestion decreased lifespan and reproduction, without causing inebriation. These effects were more pronounced in flies lacking a microbiome, but could not be explained by simple bacterial degradation of ethanol. However, moderate ethanol ingestion increased reproduction in bacterially-colonized flies, relative to bacteria-free flies. Ethanol decreased intestinal stem cell turnover in bacterially-colonized flies and decreased intestinal barrier failure and increased fat content in all flies, regardless of microbiome status. Analysis of host gene expression finds that ethanol triggers the innate immune response, but only in flies colonized with bacteria. Taken together we show that, chronic ethanol ingestion negatively impacts fly health in a microbiome-dependent manner.

Introduction

Ethanol is common in the diets of many animals and is also among the most abused drugs in the world. Naturally fermenting diets can contain appreciable amounts of ethanol, which is consumed by a variety of animals including primates, birds, bats, treeshrews, and insects (Hockings et al. 2015; Mazeh et al. 2008; Wiens et al. 2008; Sánchez et al. 2004). The common fruit fly, *Drosophila melanogaster*, naturally consumes ethanol and has long been used as a model for investigating the effects of ethanol on animals (Devineni & Heberlein 2013). Flies have an attraction to ethanol (Devineni & Heberlein 2009; Ja et al. 2007) and display 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). While the developmental effects of ethanol have been studied in fly larvae (McClure et al. 2011; Logan-Garbisch et al.

2015), the role of long-term oral ingestion of moderate ethanol in adult flies has not been investigated as previous studies focused on larval development and adult intoxication through ethanol vapor. To fill this gap, we developed a *Drosophila* model of chronic ethanol ingestion.

Drosophila is a powerful model system for investigating the commensal animal microbiome (Broderick & Lemaitre 2012; Douglas 2018). Flies can be cleared of their microbial communities so that both direct and indirect effects of the microbiome can be investigated (Koyle et al. 2016). Experiments investigating the role of the microbiome can be done on a large scale in flies, testing many variables in parallel (Wong et al. 2014; Gould et al. 2018). Studies have shown that commensal bacteria affect many components of fly fitness and physiology and many of these effects are seen only in a diet-specific context (Shin et al. 2011; Storelli et al. 2011).

Ethanol can be both produced and consumed by microbes, and therefore play a key role in host exposure. In humans, the microbiome is implicated in many of ethanol's negative consequences, although the relative roles of direct ethanol-induced damage and indirect damage through ethanol's ability to change the microbiota composition are unclear (Chen & Schnabl 2014; Hartmann et al. 2015). Here we use flies deconstruct the complex interplay between host, microbiome, and ethanol. We find that fly fitness in the presence of ethanol is heavily influenced by the microbiome, and that many aspects of fly biology, including intestinal homeostasis, lipid content, and the immune response, are mediated by microbes following ethanol ingestion. Taken together, our newly developed model of chronic ethanol ingestion in flies provides insight into how the animal microbiome modulates the effects of dietary ethanol.

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 25ºC with 60% humidity and 12-hour light/dark cycles on autoclaved glucose-yeast medium (10% glucose, 5% Red Star brand active dry yeast, 1.2% agar, 0.42% proprionic acid). Flies were three to six days old before bacterial or ethanol treatments were applied (i.e. all flies were bacteria-free and raised on 0% ethanol diets at birth). 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 for about 10 minutes, removing these flies, and then introducing bacteria-free flies. 0% to 15% ethanol media was made by adding 100% ethanol to autoclaved glucose-yeast medium after it had cooled to 50ºC. 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 in an attempt to create an isocaloric diet (except where indicated, Figures 2C and 2D). Flies were transferred to fresh media every three to four days, except for the experiment which controlled for ethanol evaporation by transferring every day (Figure 2C).

93 *Ethanol concentrations of fly diets*
94 Evaporation and bacterial metabolism may decrease the effective ethanol concentration of the fly
95 diets. Using a clinical grade breathalyzer, we developed a method to measure ethanol vapor
96 within the headspace of a vial and use this a proxy for dietary ethanol concentration [following
97 (Morton et al. 2014)]. Briefly, a 14-gauge blunt needle attached to 50 mL syringe is used to
98 sample the headspace of vial. The sampled air is then pushed through the mouthpiece of an
99 Intoximeters Alco-Sensor® III breathalyzer. Using 2.5%, 5%, and 10% ethanol media, with
100 either 20 bacterially-colonized or bacteria-free flies, we checked ethanol concentration once per
101 day for four days. Four (2.5% and 5%) or five (10%) replicate vials of each of the ethanol
102 treatments were used. Preliminary experiments show that ethanol vapor concentration in the
103 headspace stabilizes within two hours of opening a vial or taking a measurement (data not
104 shown).
105
106 *Inebriation Assay*
107 Inebriation was measured using an established method (Sandhu et al. 2015). Briefly, vials were
108 gently tapped and the number of individuals that were able to stand up 30 seconds later was
109 recorded. Inebriation was measured on bacterially-colonized and bacteria-free flies on diets
110 containing 5%, 10%, 12.5% and 15% ethanol, with four independent replicates per ethanol and
111 bacterial treatment. As a positive control, one mL of 85% ethanol was added to a cellulose
112 acetate plug that was pushed into the middle of a vial, and this vial was capped tightly with a
113 rubber stopper. Within 30 minutes, this method leads to inebriation in approximately 50% of
114 flies under a variety of experimental conditions (Sandhu et al. 2015). To measure ethanol vapor
115 in the positive control, a valve was attached to the rubber stopper and the headspace was sampled
116 at 30 minutes using the breathalyzer method described above.
117
118 *Internal ethanol concentration of flies*
119 To quantify the ethanol concentration to which fly tissues are exposed, we measured the internal
120 ethanol concentration using a colorimetric enzymatic assay (Sigma-Aldrich MAK076). This
121 approach measures the combined effects of ethanol uptake and internal metabolism. We
122 measured ethanol concentration in individual flies fed 0% or 10% ethanol diets for 15 days. As a
123 positive control, a group of flies not previously exposed to ethanol were enclosed in a rubber-
124 stoppered vial with a cotton ball soaked with two ml of 35% ethanol [similar to (Fry 2014)]. A
125 dry cotton ball was added above the ethanol soaked one so that flies were unable to ingest
126 ethanol, while still being exposed to ethanol vapor. After 60 minutes, individuals that could not
127 stand, but still showed leg movements were selected. To calculate final internal concentration per
128 fly, ethanol was considered to be primarily located in the hemolymph and the hemolymph
129 volume was assumed to be 85 μL per fly (Troutwine et al. 2016; Cowmeadow et al. 2005).
130
131 *Measurement of fecundity and lifespan*
132 Lifespan and fecundity were measured simultaneously during the same experiment. Four
133 replicate vials of 20 females each were created for the two bacterial treatments (bacterially-
134 colonized and bacteria-free) and the seven ethanol treatments (0% to 15%, in 2.5% increments)
135 resulting in 56 total vials for the 14 treatments. Survival was checked each day and dead flies
136 were removed with each transfer. Fecundity was calculated by the number of adults that emerge
137 per transfer to new diet, divided by the number of females alive at the start of that transfer,
138 summed over the entire experiment. Approximately 90% of all pupae that formed survived to

adulthood with no differences in eclosion rate between ethanol or microbial treatments (Figure S6) and thus only adult emergence data is shown. Development rate was measured as the day the first pupae formed following a transfer to a new vial. In a follow-up fecundity experiment that controlled for ethanol evaporation, flies were transferred to new freshly-inoculated media every day. This experiment also included a diet that was isocaloric with the 2.5% ethanol diet. The isocaloric diet was created by the addition of 4.4% glucose (to the 10% glucose added to all diets) and assumes ethanol is 7 kcal/g and glucose is 4 kcal/g (Ja et al. 2007).

Bacterial abundance within flies

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. Eight 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 (Supplementary Data X). 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 vitro

We tested *A. pasteurianus*, *L. plantarum*, and *L. brevis*, isolated in the bacterial abundance experiment, for sensitivity to ethanol. Isolates were grown overnight at 30ºC in an appropriate medium (MYPL for *A. pasteurianus* and MRS for *L. plantarum* and *L. brevis*) and diluted to a working OD of 0.01. For *A. pasteurianus* and *L. plantarum*, growth was measured in 0% to 15% ethanol media in a 96-well plate using a TECAN Infinite F200 PRO, set to 30ºC and 5 minutes of orbital shaking per 10 minutes. For *L. brevis,* which forms a pellet when grown in a 96-well plate, two mL of 0% to 15% ethanol MRS was inoculated with the overnight culture and shaken continuously in cell culture tubes at 30ºC. After 24 hours, maximum final OD was determined for each isolate, and a two-parameter Weibull function was fit to the normalized maximum ODs from the aggregate data for each strain (R package drc: Analysis of Dose-Response Curves). The inhibitory concentration for 50% growth (IC50) was calculated as the ethanol percentage that reduced normalized maximum OD by half.

Bacterial abundance on the diet

Experiments were set up as above, except only 0%, 7.5%, 10%, 12.5%, and 15% ethanol diets were used. On day three five flies from each of four replicates per treatment were individually homogenized, serially diluted in a 96-well plate, and pinned on selective media (MRS for *L. plantarum*, MRS+X-Gal for *L. brevis*, and MYPL for *A. pasteurianus*) using a 96-pin replicator (Boekel), (Obadia et al., 2017 and/or Gould et al., 2018). After fly removal from the vials, one mL of PBS and approximately ten glass beads were added. This was shaken gently on a Nutator at speed 3 for ten minutes, at which time 200 μL was serially diluted and pinned as above. To convert pinned colony growth to actual bacterial abundance, overnight cultures of *L. plantarum, L. brevis,* and *A. pasteurianus* were serially diluted in 96-well plates as above. These serial

185 dilutions were both plated onto agar plates (to determine actual abundance) and pinned (to
186 determine pinning efficacy). A standard curve was created relating actual abundance to growth
187 due to pinning.
188
189 *Intestinal Barrier Failure*
190 We measured the level of intestinal barrier failure (IBF) by supplementing fly diet with 2.5%
191 (w/v) FD&C Blue No. 1 (Rera et al. 2012). Two independent experiments were done, the first
192 with 0% and 5% ethanol diets and the second 0%, 5%, and 7.5% ethanol diets, each with
193 bacterially-colonized and bacteria-free treatments. For each, three or four vials of 10 flies were
194 monitored over their entire lifespan and degree of IBF determined by the amount of blue
195 coloration in tissues upon death. For statistical purposes, individuals in IBF categories 0 and 1
196 were considered IBF negative and individuals with IBF categories 2 and 3 were considered IBF
197 positive (Clark et al. 2015). No significant differences were found between experiment 1 and
198 experiment 2, so they were combined into a single dataset. Because the blue dye accumulates in
199 flies with IBF and increases mortality (Clark et al. 2015), we did not directly compare the
200 lifespan data from these IBF experiments with experiments lacking blue dye.
201
202 *Lipid content*
203 Bacterially-colonized or bacteria-free flies were reared on 0%, 5%, and 10% ethanol diets for 16
204 days, as described above. Four to ten individuals were pooled by sex (mean=9.5), with three to
205 five replicates for each bacteria-ethanol-sex treatment. The mass of pooled flies was determined
206 to the nearest 1/10 of a milligram on a Mettler Toledo microbalance. Free and total lipid content
207 was determined using established colorimetric methods (SIGMA F6428, T2449, and G7793),
208 (Wong et al. 2014; Tennessen et al. 2014).
209
210 *Measurement of gene expression*
211 We used NanoStrings profiling to quantify *D. melanogaster* gene expression changes due to
212 ethanol ingestion and bacterial colonization (NanoStrings Technologies, Inc. Seattle, WA, USA).
213 A custom NanoStrings probeset was designed to target genes related to ethanol metabolism,
214 innate immunity and inflammation, ethanol-mediated behavior, among others (A full list of
215 genes, raw counts, normalized counts, and P-Values are found in Supplementary Dataset S1).
216 Additionally, probes were designed to the bacterial 16S ribosomal RNA and alcohol
217 dehydrogenase A and B genes. Bacterially-colonized or bacteria-free flies were reared on 0%
218 and 10% ethanol diets for 11 days. Total RNA was obtained from individual whole flies using a
219 Trizol/Chloroform extraction [following (Elya et al. 2016)]. 50 to 75 ng of purified RNA per
220 sample was hybridized to the NanoString reporter and capture probesets following
221 manufacturing instructions, and profiled on an nCounter SPRINT machine (Laboratory of Greg
222 Barton, UC Berkeley). Raw counts were normalized to internal NanoStrings positive and
223 negative control probes and three housekeeping genes (Actin 5C, Gadph, and Ribosomal Protein
224 L32). The correlation between each of the three housekeeping genes and the final normalization
225 factor was always greater than 0.89. Treatment effects were determined with a two-way ANOVA
226 using ethanol and bacterial colonization as independent variables and normalized counts (i.e.
227 expression level) as the dependent variable (Supplementary Dataset S1). Significance was
228 determined using a 5% Benjamini-Hochberg False Discovery Rate. For all bacterial16S genes,
229 the normalized counts were greater than 10-fold higher in the bacterially-colonized treatments
230 compared to the bacteria-free treatments (Supplementary Dataset S1). For all bacterial ADH

genes, the normalized counts in the bacteria-free treatment were within three standard deviations of the negative control probes and were greater in the bacterially-colonized treatments (Supplementary Dataset S1).

Results and Discussion

Establishing a model for chronic ethanol ingestion in flies

Here we investigated the effects of chronic ethanol ingestion on *D. melanogaster* adults and if the microbiome can mediate these effects. We first developed an administration method in which ethanol is ingested directly from the media and asked how this method compares to established methods of ethanol administration in adult flies. Previous methods used liquid ethanol added to tightly capped vials, causing flies to absorb ethanol vapor through their cuticle.

For all subsequent experiments, we used two microbiome treatments: Bacteria-free and bacterially-colonized. Bacteria-free flies were generated using established protocols (Koyle et al. 2016) and bacterially-colonized flies were created by allowing approximately 50 normally-colonized adults (from unmanipulated lab stocks) to seed autoclaved media with their frass, removing these flies, and then introducing bacteria-free flies.

We first wanted to know how ethanol headspace vapor in our experiments compares with the vapor levels of ethanol used in established ethanol inebriation studies and if headspace vapor serves as a good proxy for dietary ethanol. To measure ethanol content, we developed a low-cost and rapid method to measure ethanol in the vapor headspace of the fly vial using a breathalyzer and used this as a proxy for dietary ethanol content [following (Morton et al. 2014)]. We sampled the headspace vapor of freshly prepared vials with 0% to 15% dietary ethanol added. Additionally, we used two methods to expose flies to ethanol vapor. In the first, we soaked a cotton ball with 2 mL of 35% ethanol and covered with a dry cotton ball so flies could not ingest the ethanol [similar to (Fry 2014)]. In the second, we added 1 mL of 85% ethanol to a cellulose acetate plug (Sandhu et al 2015). We found that headspace vapor accurately measures dietary ethanol (Figure 1A and S1). We also found that these methods lead to ethanol vapor levels many times greater than our dietary ethanol method (Figure 1A). Therefore, our chronic ingestion model exposes flies to a much lower headspace vapor than previously established acute inebriation models, and suggests the main source of ethanol uptake is ingestion.

To confirm that flies effectively uptake ethanol when it is mixed directly in the media, we measured the internal ethanol concentrations of flies fed ethanol diets. We found that flies fed 10% ethanol diets contain higher internal concentrations of ethanol than flies fed 0% ethanol diets, which shows that in our treatment conditions flies successfully ingest dietary ethanol (Figure 1B). We were also interested in how internal ethanol concentrations in flies fed ethanol compare to flies exposed to ethanol vapor. We found that inebriated flies (exposed to 35% ethanol vapor, which causes most flies to become immobile within an hour) have even higher internal ethanol levels. This suggests that our dietary regime exposes flies to sub-inebriating levels of ethanol.

276 We next asked if flies show behavioral signs of intoxication using the inebriation assay of
277 Sandhu et al. 2015, which measures inebriation as the inability for flies to stand after gently
278 tapping the vials. We found that, after 30 minutes, less than 5% of flies show signs of
279 inebriation, even on the highest ethanol diets, while half of flies exposed to 85% ethanol vapor
280 are inebriated (Figure 1C). Importantly, there was no effect of bacterial treatment on inebriation,
281 consistent with results from a previous study that used antibiotics to clear flies of their bacterial
282 communities (Sandhu et al 2015).
283
284 Finally, we asked how dietary ethanol concentration changes over time and hypothesized that
285 both evaporation and bacterial metabolism reduce ethanol content. As expected, the ethanol
286 vapor decreases over time (Figures 1D-1E). For 10% ethanol media, approximately half the
287 ethanol remained after 3 days. Colonization of vials with fly gut bacteria reduced the ethanol
288 levels further, particularly at low concentrations. For example, in the 2.5% ethanol treatment,
289 there is no detectable ethanol in the bacterially-colonized treatment on day two, but for the 10%
290 ethanol treatment there was no difference in the ethanol concentrations until day four. These
291 results suggest ethanol loss by two mechanisms. First, evaporation decreases ethanol
292 concentration. Second, bacterial metabolism consumes ethanol. The lag in bacterial ethanol
293 degradation presumably occurs because bacterial populations in the vials start off small. All vials
294 are initially sterile and are inoculated with the transfer of bacterially-colonized flies. Thus
295 bacterial abundance only becomes great enough to affect the measured ethanol on day two or
296 later.
297
298 Taken together, we have established an experimental model of chronic ethanol ingestion in adult
299 *D. melanogaster.* Ethanol remains in the media long enough for flies to uptake, ethanol is
300 detectable internally after ingestion, and flies do not show overt signs of inebriation. By adding
301 ethanol directly to the diet, we mimicked the route of natural ingestion for flies and increase the
302 translational power of our model, as humans consume ethanol via their diet rather than through
303 inhalation.
304

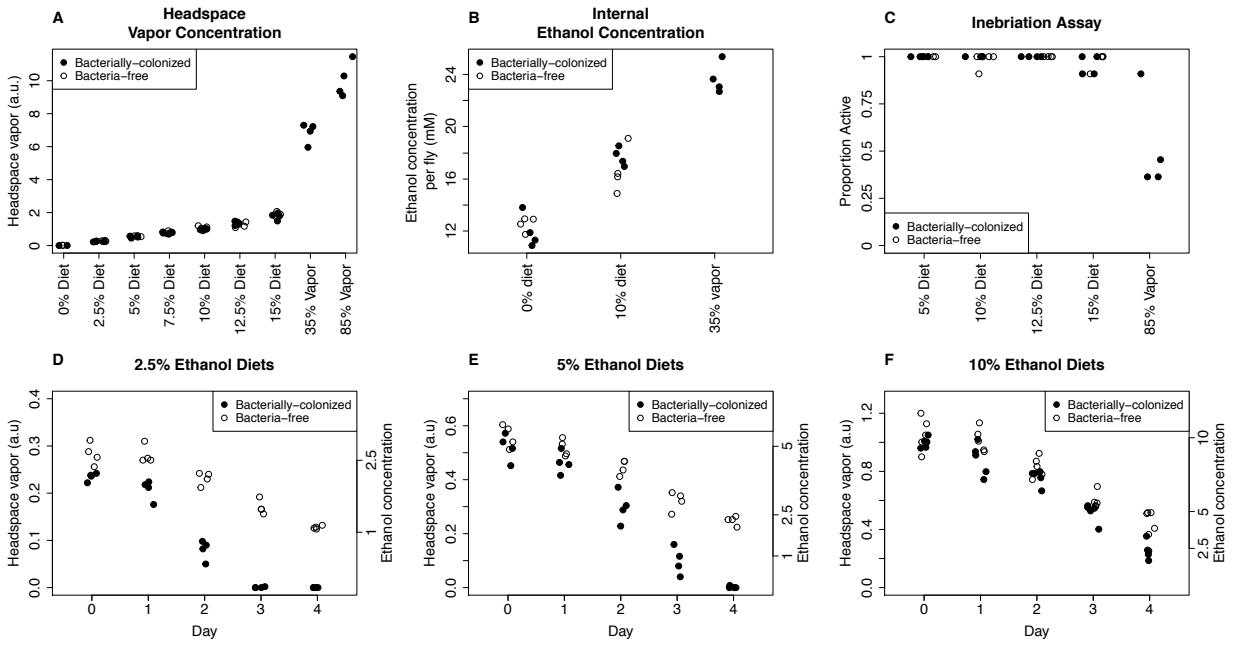

Figure 1: Establishing a chronic ethanol ingestion model in *Drosophila*.
A: Dietary ethanol leads to less headspace ethanol vapor than standard methods for ethanol inebriation. Headspace vapor was determined by sampling the vial headspace with a syringe and forcing this mixture through a medical-grade breathalyzer. For the 35% and 85% ethanol treatments, either a cotton ball (35%) or a cellulose acetate plug (85%) was soaked with liquid ethanol in a tightly capped vial (Fry 2014; Sandhu et al. 2015).
B: Flies uptake ethanol from their diet, but this still leads to lower internal ethanol concentrations than inebriating levels of ethanol vapor. Internal ethanol concentration was assayed enzymatically on individual flies fed either 0% or 10% ethanol diets or exposed to 35% ethanol vapor (Fry 2014).
C: Dietary ethanol does not lead to inebriation by standard assay. Proportion active is the proportion of 11 individual flies that can stand up after gently tapping the vial 30 minutes after initial exposure to ethanol. 85% ethanol vapor (final column) robustly leads to inebriation in about half of the individuals at this timepoint (Sandhu et al. 2015).
D, E and F: Dietary ethanol content decreases over time with greater loss in bacterially-colonized treatments. Ethanol concentration (right axis) was calculated from day 0 measurements (Figure S1). Measurements from 0% ethanol media are always below 0.02 and are therefore not shown. Note that flies are transferred to fresh vials on day 3 or 4 (Figure 2A and B, 3, 4, and 5) or day 1 (Figure 2C).

Bacterial colonization of flies masks the negative effects of ethanol on lifespan

We sought to determine the effects of ethanol and the microbiome on fly fitness, focusing on lifespan and fecundity (Gould et al. 2018), which have not been investigated in a fly ethanol model. While the results in humans are conflicting at very low ethanol consumption, in general ethanol consumption is associated with shorter lifespan (Wood et al. 2018). The natural habitat of *D. melanogaster,* fermenting fruit, often contains 1-5% ethanol and the unnatural but common habitat of vineyards can contain up to 10% ethanol (Gibson et al. 1981). We therefore tested dietary ethanol concentrations from 0% to 15%, which spans from ecologically relevant concentrations to concentrations above those to which flies are normally exposed.

We measured lifespan, fecundity, and microbiome composition (see next section) in the same experiment. Four replicate vials of 20 flies were used for each ethanol and bacterial treatment. In our first experiment, we transferred flies to fresh food every three to four days to balance between maintaining dietary ethanol concentration, which decreases over time, (see Figure 1D-F) and maintaining bacterial colonization, which requires less frequent transfers (Blum et al. 2013)].

Bacterially-colonized flies consistently showed a shorter lifespan than bacteria-free flies, in agreement with previous studies (Figure 2A; Table S1; Data for individual flies is shown in Figure S2; Lifespan curves are shown in Figure S3), (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-induced decrease in average or maximum lifespan observed except at levels above those experienced in nature (12.5% and 15%), This was in sharp contrast to the bacteria-free flies, which overall live longer, but show a nearly linear and dose-dependent decrease in average and maximum 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 masked 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, suggesting that the flies in our lab may be colonized with a particularly lifespan-shortening consortium of bacteria. However, an equally plausible explanation would be that our media, which lacks the commonly-used microbial growth inhibitor tegosept, may have greater bacterial loads than other studies (Obadia et al. 2018). This would lead to a greater difference in dietary bacterial load between the bacterially-colonized and bacteria-free treatments which could explain the more drastic lifespan reduction that we observe.

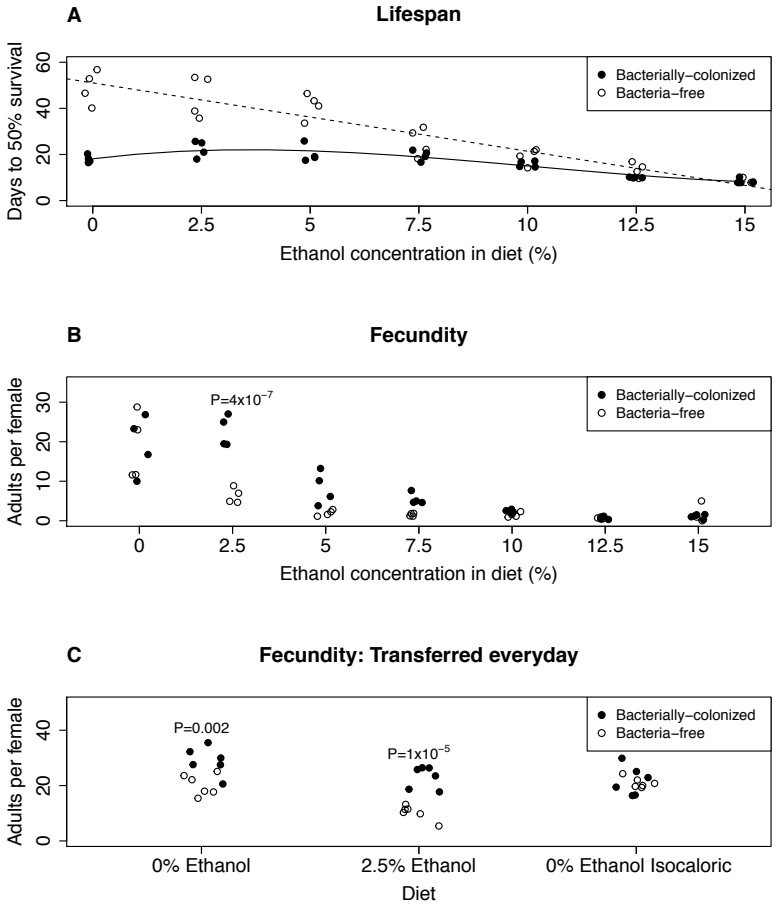

Figure 2: Bacteria mediate the effect of ethanol on fly fitness

A: Bacterial colonization and ethanol negatively affect fly lifespan, with the negative effect of ethanol being unmasked in bacteria-free flies. Days to 50% survival is per replicate and calculated from birth (see methods). Each replicate began with 20 female flies. Lines show the best fit lines for each bacterial treatment (Solid: bacteria-free, linear, R^2=0.874; Dashed: bacterially-colonized, third order, R^2=0.832). Data for individual flies is shown in Figure S2 and Table S1.

B: Bacteria ameliorate the negative effects of ethanol on fly fecundity. Adults per female is calculated by the number of adults that emerge per flip, divided by the number of females alive at the start of the egg laying period. Each replicate began with 20 females. P-values are calculated from a pairwise t test between bacterial treatments for a given ethanol treatment and are Holm-Bonferroni corrected for multiple comparisons. Non-significant P-values are not shown. An independent replication of this experiment (with 0% and 2.5% ethanol) is shown in Figure S4.

C: The effect of 2.5% ethanol on fecundity is not due to ethanol evaporation or the caloric contribution of ethanol. Flies were transferred to fresh diets each day to reduce the effect of ethanol evaporation (Figure 1D). The isocaloric diets have added glucose so they contain identical calories as the 2.5% ethanol diets. Adults per female was calculated as in Figure 2B, however in this experiment each replicate began with 10 females per replicate. P-values are calculated from a pairwise t test between bacterial treatments for a given dietary treatment and are Holm-Bonferroni corrected for multiple comparisons. Non-significant P-values are not shown.

397 *Bacteria ameliorate the negative effects of ethanol on fecundity*
398
399 We found a strong effect of ethanol on fly fecundity that is mediated by bacterial treatment
400 (Figure 2B). Without ethanol, there was no difference in fecundity between bacterially-colonized
401 and bacteria-free flies, which is consistent with previous work (Ridley et al. 2012). For both
402 bacterial treatments, ethanol reduced fecundity, but bacteria-free flies were more sensitive: at
403 2.5% ethanol, bacterially-colonized flies had significantly higher fecundity ($P=4x10^{-7}$, Figure
404 2B; P=0.03, Figure S4). The same trend was observed on both 5% and 7.5% ethanol diets
405 (though not statistically significant at $P<0.05$ after correction for multiple comparisons).
406 Interestingly, we found that ethanol does not lead to the typical tradeoff between lifespan and
407 fecundity observed by varying nutrients (Zera & Harshman 2001; Djawdan et al. 1996) – instead
408 we found that ethanol decreases both components of fitness (Figures 2A and 2B, Figure S5).
409 This suggests that ethanol, even at the low concentrations used in this study, is acting more like a
410 toxin than a source of calories.
411
412 On 2.5% ethanol media, ethanol content is significantly reduced by day two in the bacterially-
413 colonized treatment (Figure 1D). Therefore, the difference in fecundity observed in the 2.5%
414 ethanol treatment (Figure 2B) could simply be due to less dietary ethanol in the bacterially-
415 colonized treatment. Although the fecundity difference between bacterially-colonized and
416 bacteria-free flies remains even when accounting for differential ethanol loss (Supplementary
417 Dataset 2), we nonetheless repeated the fecundity experiment but transferred the flies to fresh
418 diets every day. To ensure the persistence of the intestinal bacterial communities, we seeded each
419 daily batch of media with the frass of bacterially-colonized flies. Also, to test whether the
420 calories added by the ethanol in the 2.5% treatment affect the flies, we added a 0% ethanol
421 treatment that is isocaloric with the 2.5% ethanol treatment.
422
423 The daily transfer experiments showed comparable results. In concordance with the 3-4 day
424 transfers, bacterially-colonized flies had greater fecundity in the 2.5% ethanol treatment relative
425 to bacteria-free flies ($P=1x10^{-5}$, Figure 2C). This strongly suggests that bacterial metabolism of
426 ethanol on the food does not cause the difference in fecundity between the bacterial treatments.
427 We also found no effect of bacterial treatment in the isocaloric diets suggesting that the
428 differences in fecundity cannot be attributed to ethanol's caloric contribution.
429
430 The observed fecundity differences could be due either to maternal egg production or larval
431 survival. To differentiate between these causes, we measured larval development time as a proxy
432 for larval survival because we could not directly count egg laying (and thus could not calculate
433 survival from egg to adulthood). Although development time increased on the highest ethanol
434 diets (Figure S6), we found no effect on development time between bacterially-colonized and
435 bacteria-free treatments at the 0%, and 2.5% ethanol treatments (all pairwise t-tests, $P>0.2$),
436 consistent with a previous result that ethanol does not affect larval development except in the
437 final larval stage at 5 days (McClure et al. 2011), when most of the ethanol has evaporated from
438 the media (Figures 1D,1E and 1F). Furthermore, previous work has shown that 12% ethanol over
439 the entire developmental period reduces larval survival to 25% (McClure et al. 2011). We find
440 that fecundity drops to near zero at 5% ethanol for bacteria-free flies versus 10% ethanol for
441 bacterially-colonized flies. Thus, maternal egg production, rather than larval survival, accounts
442 for fecundity effects seen in Figure 2B.

443
444 Taken together, these results suggest significant ecological and evolutionary impacts of microbes
445 in mediating the negative effects of ethanol toxicity on fly fecundity. While the exact doses of
446 ethanol that flies consume in the wild remains obscure, the concentration in naturally fermenting
447 fruit is typically 1-5% and can be as high as 10% in wineries (Gibson et al. 1981). In all cases for
448 flies fed 2.5% to 7.5% ethanol, we found that bacterially-colonized flies had higher fecundity
449 than bacteria-free flies and this effect persists even when controlling for ethanol metabolism by
450 daily transfers to fresh media. Thus, at ecologically relevant concentrations of ethanol, bacterial
451 colonization mitigates the negative effects on fecundity.
452
453 *Ethanol shifts the composition of bacteria associated with D. melanogaster*
454
455 Diet is a strong determinant of microbiome composition in flies and other animals. In particular,
456 fruit feeding flies, which are exposed to naturally produced dietary ethanol, have significantly
457 different bacterial and yeast communities than flies collected from other substrates (Chandler et
458 al. 2011; Chandler et al. 2012). We hypothesized that the bacterial communities associated with
459 flies would shift in response to ethanol ingestion. In particular, we expected that ethanol would
460 strongly decrease the total abundance of bacteria in high ethanol treatments and these shifts
461 would favor the abundance of bacteria with low sensitivity to ethanol. Thus, in a parallel
462 replicate of the lifespan-fecundity experiment (Figures 2A and 2B), we determined fly bacterial
463 load and composition by homogenizing individual flies and plating onto selective media. The
464 different bacterial strains were identified by colony morphology.
465
466 We found that total bacterial load per fly was between $9x10^3$ and $3x10^6$ colony forming units
467 (CFUs) for the 0% ethanol containing diets (mean=$7x10^5$). This is comparable to previous
468 studies of *D. melanogaster* (Blum et al. 2013; Obadia et al. 2017). Contrary to our expectations,
469 we found that total bacterial load was relatively constant up to the highest ethanol treatment
470 (Figure 3A). We next asked how the bacterial composition changes in response to ethanol. In
471 agreement with the previous work in our laboratory and that of others, our flies are dominated by
472 species in the genera *Acetobacter* and *Lactobacillus* (Broderick & Lemaitre 2012). Different
473 bacteria had different responses to dietary ethanol. *Acetobacter pasteurianus* concentrations
474 decreased 10-fold from 0% to 2.5% ethanol and remained constant until 12.5% ethanol where
475 they dropped to essentially 0 (Figure 3B). Conversely, we found that the response of the
476 *Lactobacilli* to ethanol was remarkably different than *A. pasteurianus.* The abundance of *L.*
477 *brevis* increased with dietary ethanol and this was the only species that was present in all flies at
478 15% ethanol (Figure 3C). *L. plantarum* was most abundant at intermediate concentrations of
479 ethanol, but like *L. brevis,* it did not appear as sensitive to high levels of ethanol as *A.*
480 *pasteurianus* (Figure 3D).
481
482 To confirm the direct effect of ethanol on the bacterial growth, we measured the *in vitro* growth
483 response to ethanol of *A. pasteurianus*, *L. plantarum* and *L. brevis* strains isolated during the
484 experiment in Figure 3A-3D. These experiments confirmed that *A. pasteurianus* is more
485 sensitive to ethanol than *L. brevis* and *L. plantarum* (Figure 3E). These results indicate that the
486 bacterial composition of flies varies, at least in part, according the ethanol sensitivities of the
487 bacterial strains. However, because these *in vitro* experiments show that these bacteria are more
488 sensitive to ethanol than is suggested by their *in vivo* abundances, we hypothesized that the fly

intestine protects bacteria from ethanol toxicity. In support of this, we found high abundance of *L. brevis* and *L. plantarum* within flies fed a 15% ethanol diet despite these bacteria being undetectable on this media (Figure S8). Similarly, *A. pasteurianus* is present within flies fed 12.5% ethanol despite this bacterium being absent on this media (Figure S8). This confirms that the host shields the effect of ethanol on the bacteria.

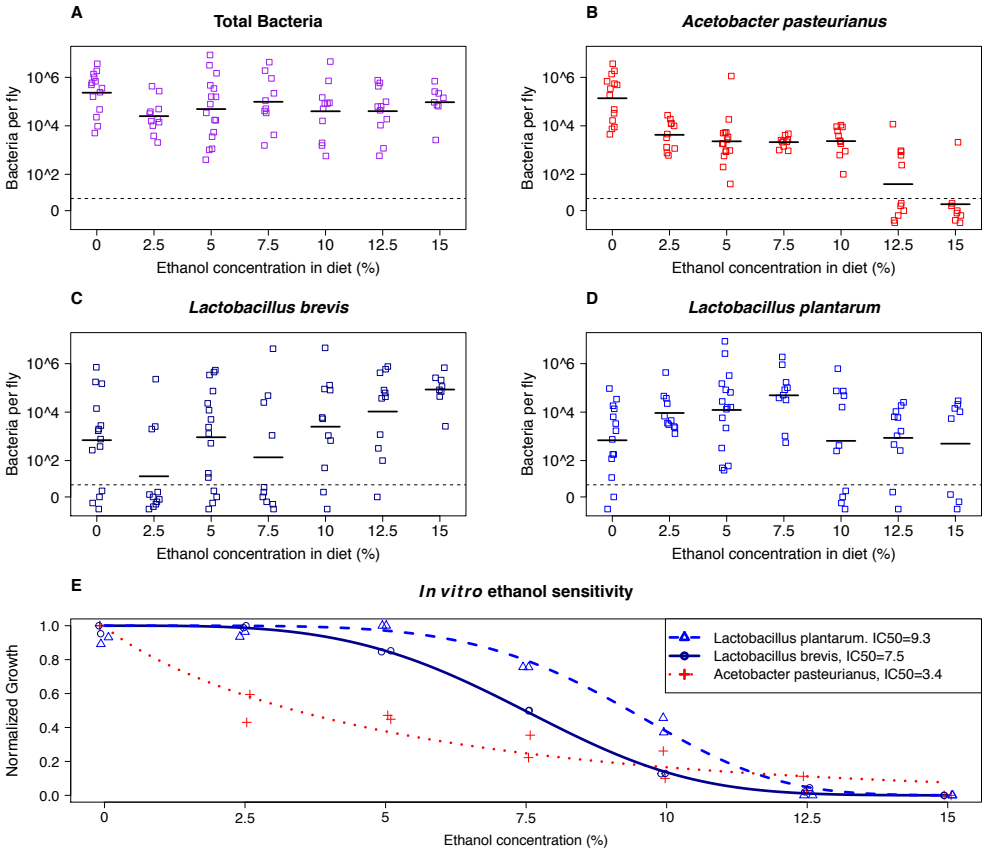

Figure 3: Bacterial community dynamics in response to ethanol diets.
A, B, C and D: The abundance of *Acetobacter pasteurianus* decreases with increasing dietary ethanol, while the abundance of *Lactobacillus plantarum* and *Lactobacillus brevis* remain high. Each point represents an individual fly. All points below the dashed line are 0 and are expanded for clarity. The black bars represent the mean of the log transformed bacterial load. Number of individual flies per treatment: 0% N=14; 2.5% N=11; 5% N=16; 7.5%N=10; 10%N=11; 12.5% N=11; 15%N=8. We found no effect of fly age [multivariate ANOVA (Adonis, package vegan in R; P = 0.159)] and therefore all four timepoints are pooled (see methods).
E. *A. pasteurianus* is more sensitive to ethanol than *L. plantarum* or *L. brevis in vitro.* Strains were isolated in the *in vivo* bacterial abundance experiment (Figure 4). Growth was measured using MRS or MYPL liquid media containing 0% to 15% ethanol in either a 96-well plate (*A. pasteurianus* and *L. plantarum*) or cell culture tubes (*L. brevis*) for 24 hours at 30C. Datapoints are the final normalized OD of two independent replicates. A two-parameter Weibull function was fit to the normalized ODs from the aggregate data for each strain (R package drc: Analysis of Dose-Response Curves). The inhibitory concentration for 50% growth (IC50) was calculated as the ethanol percentage that reduced normalized maximum OD by half.

513
514 *Dietary ethanol decreases intestinal barrier failure*
515
516 To explore the fly physiology underlying mortality following ethanol ingestion in flies, we
517 examined intestinal barrier failure (IBF), which is strongly linked to alcoholic liver disease in
518 humans (Chen & Schnabl 2014) and is a hallmark of aging-related death in flies (Rera et al.
519 2012; Clark et al. 2015). We used the Smurf assay (Rera et al. 2012), rearing flies on a diet
520 containing blue dye no. 1 and scoring them for a blue body coloration, which is indicative of a
521 permeabilized gut. Consistent with previous results (Rera et al. 2012; Clark et al. 2015), we
522 found that nearly all flies on a 0% ethanol diet show IBF upon death. Quite surprisingly, on
523 ethanol diets, we found a significant decrease in the proportion of flies that show IBF (Figures
524 4A and S9). Furthermore, bacteria-free flies (which are more sensitive to ethanol) show
525 significantly less IBF than bacterially-colonized flies on ethanol diets. Examining the effects of
526 bacteria and ethanol together, we detect a significant interaction ($P=1x10^{-4}$, Figure 4A). Taken
527 together, this suggests that IBF is not the causative mechanism of ethanol-induced lifespan
528 decline in flies. That some individuals in the ethanol treatments nonetheless showed IBF can be
529 explained by the normal background aging process (Rera et al. 2012; Clark et al. 2015).
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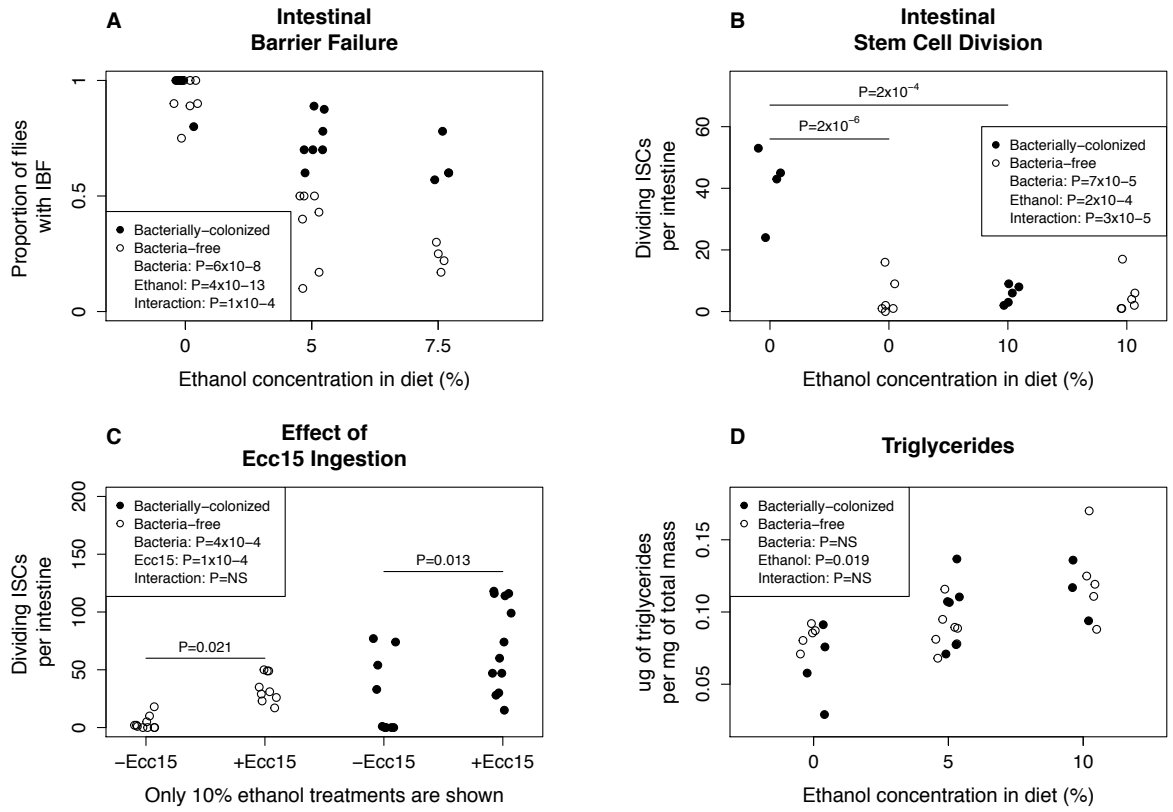


Figure 4: Bacteria mediate the effect of ethanol on fly physiology
Within each panel, values in the legend are the results from a two-way ANOVA. Comparison P values are calculated from a pairwise t test between treatments and are Holm-Bonferroni corrected for multiple comparisons within an experiment. All data shown is for females. Male data for IBF is shown in Figure S9 and for triglyceride content is shown in Figure S11.
A. The prevalence of intestinal barrier failure (IBF) decreases with dietary ethanol and this decrease is greater in bacteria free flies. Each point represents the average from a replicate vial. Flies were scored within 24 hours of death.
B. Ethanol reduces intestinal stem cell turnover in bacterially colonized, but not bacteria free, flies. Each datapoint indicates the number of pH3 stained cells in an individual intestine. Results from an independent experiment as shown in Figure S10.
C: **Ethanol does not inhibit the ability of ISCs to regenerate following oral ingestion of *Erwinia carotovora carotovora 15* (Ecc15).** Each datapoint indicates the number of pH3 stained cells in an individual intestine.
D: Ethanol ingestion increases stored triglycerides in flies, regardless of bacterial treatment. Each point represents a pooled sample of 4 to 10 flies.

The effect of ethanol on ISC turnover is microbiome dependent

Intestinal barrier function is maintained through controlled intestinal stem cell (ISC) turnover (Lemaitre & Miguel-Aliaga 2013). Because stem cell hyper-proliferation leads to loss of intestinal function (Li & Jasper 2016), we hypothesized that the reduction in IBF was due to a decrease in ISC turnover. To quantify stem cell turnover, we measured mitotic cells in the gut by

569 phospho-histone H3 antibody staining (Apidianakis & Rahme 2011). In two independent
570 experiments, we found that in the absence of ethanol, ISC division was significantly greater in
571 bacterially-colonized flies ($P=2x10^{-6}$, Figure 4B; $P=2x10^{-6}$, Figure S10), consistent with previous
572 work showing that ISC hyper-proliferation caused by commensal bacteria shortens fly lifespan
573 [(Figure 2A), (Buchon et al. 2009; Guo et al. 2014)].
574
575 In the presence of ethanol, we found that ISC division is significantly decreased in bacterially-
576 colonized flies, but unchanged in bacteria-free flies ($P=2x10^{-4}$, Figure 4B; $P=3x10^{-6}$, Figure
577 S10). These results are in accord with the IBF data presented in Figure 4A for bacterially-
578 colonized flies (specifically, that both IBF and ISC division, two processes linked to intestinal
579 homeostasis, are reduced), but suggest an additional mechanism reduces IBF in bacteria-free
580 flies fed ethanol. Why these two phenotypes are uncoupled in bacteria-free flies, in which
581 ethanol decreases IBF with no change in ISC turnover, remains unknown and suggests different
582 mechanisms of ethanol-induced pathology in bacterially-colonized and bacteria-free flies.
583
584 The decrease in dividing ISCs in bacterially-colonized flies led us to hypothesize that ethanol
585 might inhibit the ability of ISCs to regenerate following a biological or chemical challenge. To
586 test this hypothesis, we infected flies with *Erwinia carotovora carotovora 15* (Ecc15), a non-
587 lethal pathogen of *Drosophila*, which reliably induces ISC division following oral ingestion
588 (Buchon, Broderick, Poidevin, et al. 2009). In both bacteria-free and bacterially-colonized flies
589 ingesting ethanol, infection with Ecc15 increases ISC division (P=0.021 and P=0.013,
590 respectively, Figure 4C). Thus, ethanol does not inhibit the ability of ISCs to regenerate despite
591 the observed decrease in ISC division in bacterially-colonized and ethanol-fed flies (Figures 4B
592 and S10).
593
594 *Ethanol ingestion increases stored triglycerides in flies*
595
596 The maintenance of intestinal homeostasis with ethanol treatment may be due to a change in
597 overall fly metabolism. In flies, poor quality diets are linked to both ISC turnover and obesity
598 (Skorupa et al. 2008; Regan et al. 2016). In humans, increased fat deposits in the liver are a
599 hallmark of alcoholic liver disease (Diehl 2002). We hypothesized that ethanol ingestion is
600 leading to greater accumulation of stored triglycerides in flies. Triglycerides are a primary
601 molecule for fat storage in flies and are mainly found in adipocytes within the fat body, an organ
602 analogous to the mammalian liver that is responsible for the majority of energy reserves in adult
603 fly (Arrese & Soulages 2010). We therefore measured stored triglycerides in bacteria-free and
604 bacterially-colonized flies on 0%, 5% or 10% ethanol diets. Consistent with our hypothesis, we
605 found that dietary ethanol increases triglycerides regardless of bacterial colonization, with no
606 effect on either total fly mass or free glycerides (Figures 4D and S11, Table S2). Because dietary
607 sugars increase triglyceride content in flies (Skorupa et al. 2008), our finding is consistent with
608 ethanol acting as an energy source with regards to fat storage, despite the lack of tradeoff
609 between lifespan and fecundity due to ethanol ingestion (Figure S5). The finding that there is no
610 difference in triglyceride content between bacterially-colonized and bacteria-free flies is
611 consistent with the minimal role of bacterial metabolism on 5% and 10% ethanol diets (Figures
612 1E and 1F) and suggests that fat accumulation does not directly explain either the lifespan
613 (Figure 2A) or intestinal homeostasis (Figures 4A and 4B) results.
614

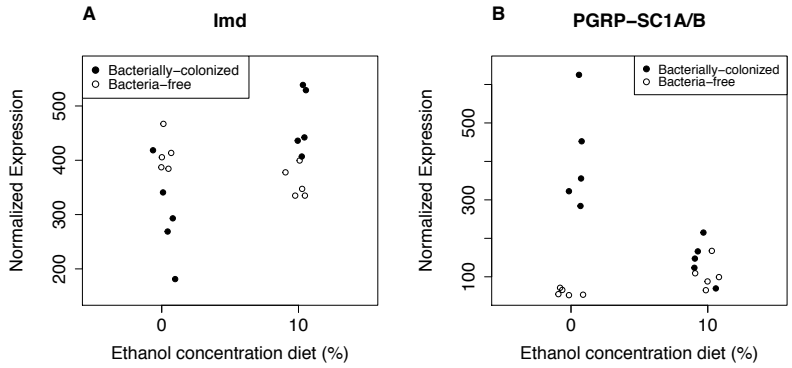
615 *Ethanol ingestion and bacterial colonization affect expression of innate immunity genes*
616
617 To understand the molecular mechanisms underpinning the differences in lifespan between
618 bacteria-free and bacteria-colonized flies ingesting ethanol (Figure 2A), we surveyed fly gene
619 expression using a custom NanoStrings probeset and selected candidate genes likely to be
620 influenced by ethanol or microbiome status. In concordance with previous work (Broderick et al.
621 2014), we found many immune system (e.g. lysozyme X and the PGRPs), stress related (e.g.
622 GstD5 and HSP23), and cell differentiation (e.g. upd3) genes to be upregulated in response to
623 bacterial colonization (Table 1). Likewise, and in agreement with Elya et al 2016, we found that
624 anti-microbial peptides (AMPs) as a group show increased expression in bacterially-colonized
625 treatments (Table S3).
626
627 We examined many genes and molecular pathways known to mediate the effects of ethanol
628 intoxication in flies, but we found that ethanol only subtle changes the expression of
629 neuropeptideF (Table 1). This is consistent with ethanol ingestion not leading to inebriation
630 (Figure 1C) and flies fed ethanol having a lower internal concentration of ethanol than inebriated
631 flies (Figure 1B).
632
633 There are two potential mechanisms that may contribute to the ethanol-induced lifespan
634 reduction in bacteria-free, but not bacterially-colonized, flies. First, host metabolism of ethanol
635 may be more efficient in bacterially-colonized flies, leading to a faster clearance of ingested
636 ethanol. Contrary to this hypothesis, we did not find that genes in the ethanol metabolism
637 pathway [alcohol dehydrogenase (Adh) and acetaldehyde dehydrogenase (Aldh)] were more
638 strongly induced in bacterially-colonized flies (Table 1), which is consistent with the equivalent
639 internal ethanol concentrations we observed for bacteria-free and bacterially-colonized flies fed
640 10% ethanol (Figure 1B). This result suggests that ethanol metabolism does not directly underpin
641 the differences in lifespan between bacteria-free and bacteria-colonized flies.
642
643 In the second mechanism, ethanol may be eliciting the innate immune response in bacteria-free,
644 but not bacterially-colonized, flies. Immune activation promotes shorter lifespan in flies
645 (Garschall & Flatt 2018; DeVeale et al. 2004; Eleftherianos & Castillo 2012) and resistance to
646 ethanol vapor in flies is linked to the innate immunity response (Troutwine et al. 2016). In
647 mammals, dysregulation of the immune response and persistent inflammation is linked to aging
648 (Gomez et al. 2008; Shaw et al. 2010). Two innate immunity genes showed a significant change
649 in expression due to the combination of ethanol and bacterial colonization (Figure 5): Immune
650 deficient (Imd) and Peptidoglycan recognition protein SC1A/B (PGRP-SC1A/B). Imd is a master
651 regulator of innate immunity in response to gram-negative bacteria (Lemaitre & Hoffmann
652 2007). PGRP-SC1A/B is a peptidoglycan scavenger that negatively regulates the IMD pathway
653 (Kurata 2014). Consistent with their known interaction pathway, PGRP-SC1A/B increased and
654 Imd decreased with ethanol ingestion in bacterially-colonized flies. However, both of these genes
655 show greater changes with ethanol ingestion in the bacterially-colonized treatment, with little or
656 no change in the bacteria-free treatment (Figure 5), and therefore do not directly explain the
657 lifespan differences identified in Figure 2A. We next examined whether antimicrobial peptide
658 (AMP) gene expression, which is a downstream target of Imd, was affected. We found no
659 ethanol dependence of AMP stimulation, (Table S3). This finding is consistent with literature
660 showing that the AMP expression can be muted when Imd is triggered in the absence of a

661 pathogen (Lhocine et al. 2008). Regardless, because of the recognized role of the IMD pathway
662 and innate immunity in regulating fly lifespan, Imd and PGRP-SC1A/B are promising targets to
663 pursue to further our understanding of microbiome by ethanol fitness effects in *Drosophila*.
664

Genes affected by ethanol ingestion		
Gene	P-value	Fold-Change
neuropeptideF	0.015	1.5
Acetaldehyde Dehydrogenase	0.035	1.6
Acetyl-CoA synthetase	0.038	2.8
HSP70Bc	0.047	3.1
Alcohol Dehydrogenase	0.047	1.8

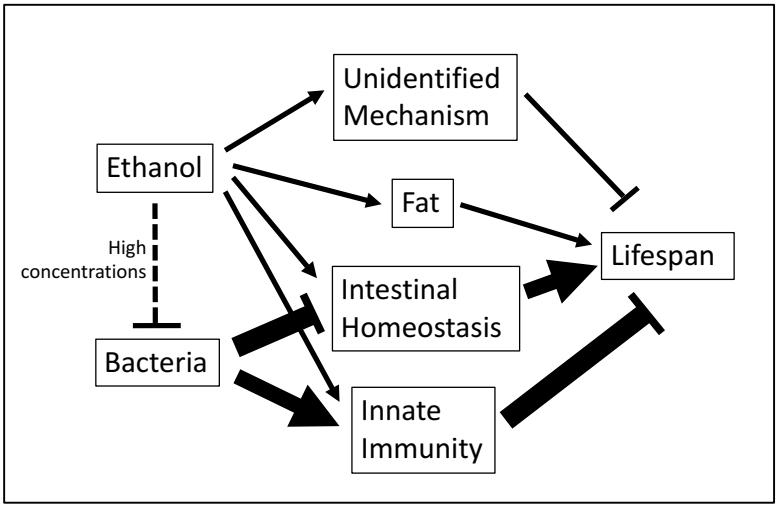
Genes affected by bacterial colonization		
Gene	P-value	Fold-Change
PGRP SC1A/B	0.0029	3.3
upd3	0.030	5.5
LysozymeX	0.030	18
upd2	0.030	69
Crys	0.030	9
Defensin	0.030	5.3
Charon	0.030	3.8
GstD5	0.030	2.7
PGRP SD	0.030	4.2
PGRP LB	0.030	2.2
Drosomycin-like 1	0.031	44
HSP23	0.040	2.3
PGRP LC	0.048	2.7

665 **Table 1: Genes showing significant expression changes in response to either ethanol**
666 **ingestion or bacterial colonization.** P-values are adjusted using a 5% Benjamini-Hochberg false
667 discovery rate correction. Genes with less than a 1.5 fold-change or with average normalized
668 counts within two standard deviations of the negative control probes are excluded.



669
670 **Figure 5. Ethanol induces the Imd response in bacterially-colonized, but not bacteria-free,**
671 **flies.** A two-way ANOVA finds a significant bacteria-by-ethanol interaction on the expression of
672 Imd (Panel A) and PGRP-SC1A/B (Panel B). For both genes, the P-value of the interaction is
673 0.023 after a 5% Benjamini-Hochberg false discovery rate correction.

674 *Conclusion: the interaction of bacteria and ethanol shape fly fitness and physiology*
675
676 Previous studies of the microbiome's role in alcoholic pathology have focused on alcoholic liver
677 disease, finding that specific bacteria can reduce alcoholic liver disease through a decrease in gut
678 permeability (Forsyth et al. 2009; Bull-Otterson et al. 2013). However, isolating experimental
679 variables has proven challenging due to the complex composition of the microbiome as well as
680 contextual effects that depend on an interaction between the microbiome and diet (Wong et al.
681 2014).
682
683 Our *Drosophila* model of chronic alcoholic pathology shows that ethanol's effects are mediated
684 by the microbiome. For ecologically relevant levels of dietary ethanol, fecundity was greater in
685 bacterially-colonized flies, highlighting the context-dependence of the microbiome in host
686 physiology. Despite the greater fecundity, bacterially colonized flies had much shorter lifespans
687 than bacteria-free flies, indicating that the microbiome mediates tradeoffs between physiological
688 states (Figure S5).
689
690 We were curious to understand how dietary ethanol and the microbiome interact to shape fly
691 lifespan. We propose that in the absence of ethanol or at low ethanol concentrations, the negative
692 effects of bacteria are dominant and reduce lifespan by disrupting intestinal homeostasis and
693 inducing innate immunity (Figure 6). In the absence of a microbiome, the negative effects of
694 ethanol are unmasked, which accounts for the dose-dependent decrease in lifespan of bacteria-
695 free flies. This effect is likely independent of overt inebriation (Figure 1C), disruption of
696 intestinal homeostasis (Figures 4A and 4B), or induction of the innate immune response (Figure
697 5 and Table S3). We speculate there is an unidentified mechanism of ethanol-induced health
698 decline in flies and this is chiefly observable in bacteria-free conditions.
699
700 For bacterially-colonized flies fed higher ethanol concentrations (which have the same lifespan
701 as flies not fed ethanol), two offsetting mechanisms are occurring: First, ethanol is reducing
702 lifespan through the unidentified mechanism described above. Second, ethanol is changing
703 microbiome composition to a less pathogenic state [potentially through the reduction in
704 *Acetobacter* abundance (Figure 3B)]. Future research will use gnotobiotic flies with defined
705 microbial communities to isolate the independent effects of the microbiome and dietary ethanol.
706



707

Figure 6: Model of microbiome-dependent ethanol-induced lifespan decline in flies. In the absence of ethanol or at low ethanol concentrations, the negative effects of bacteria are dominant and reduce lifespan by disrupting intestinal homeostasis and inducing innate immunity. At high ethanol concentrations, the microbiome changes composition (primarily through the reduction of *Acetobacter* abundance). This eliminates the bacteria-dependent lifespan reduction, but is offset via an unknown ethanol-dependent mechanism which is damaging to fly health (so the net result is no change in lifespan). This mechanism is primarily independent of intestinal homeostasis and the innate immune response. It is also likely different from known intoxication pathways in flies because our method of ethanol administration does not lead to overt inebriation.

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