Microbiota-dependent elevation of Alcohol Dehydrogenase in *Drosophila* is associated with changes in alcohol-induced hyperactivity and alcohol preference

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Summary

The gut microbiota impacts diverse aspects of host biology including metabolism, immunity, and behavior, but the scope of those effects and their underlying molecular mechanisms are poorly understood. To address these gaps, we used Two-dimensional Difference Gel Electrophoresis (2D-DIGE) to identify proteomic differences in male and female *Drosophila* heads raised with a conventional microbiota and those raised in a sterile environment (axenic). We discovered 22 microbiota-dependent protein differences, and identified a specific elevation in Alcohol Dehydrogenase (ADH) in axenic male flies. Because ADH is a key enzyme in alcohol metabolism, we asked whether physiological and behavioral responses to alcohol were altered in axenic males. Here we show that alcohol induced hyperactivity, the first response to alcohol exposure, is significantly increased in axenic males, requires ADH activity, and is modified by genetic background. While ADH activity is required, we did not detect significant microbe-dependent differences in systemic ADH activity or ethanol level. Like other animals, *Drosophila* exhibit a preference for ethanol consumption, and here we show significant microbiota-dependent differences in ethanol preference specifically in males. This work demonstrates that male *Drosophila's* association with their microbiota affects their physiological and behavioral responses to ethanol.

Introduction

The human microbiota, the community of microorganisms including bacteria and fungi that resides in and on our bodies, contribute to metabolism, immunity, and defense against pathogens [1], [2]. Surprisingly, recent evidence suggests that the bacterial microbiota of the gut can also influence learning, memory, anxiety, depression, and autism-associated behaviors in some animals [3]–[6]. The number of connections being made between symbiotic bacteria and host physiologies and behaviors is rapidly increasing, making it likely that more associations await discovery. Furthermore, we understand relatively little about the molecular mechanisms that mediate any of these host-microbe interactions.

Drosophila is emerging as an excellent model to dissect the role of the microbiota in animal physiology and behavior. Bacteria in the order *Lactobacillales* are found in both the *Drosophila* and human microbiota [7]–[9], and links between the microbiota and host physiology and behavior are also present in *Drosophila*. Fly behaviors such as egg laying, feeding, male competition, and kin recognition all respond to changes in the microbiota [10]–[18]. The fly microbiota can modulate insulin, insulin-like growth factor, and Target Of Rapamycin (TOR)

signaling thereby affecting systemic homeostasis in the fly [19], [20]. Furthermore, host-pathogen studies in *Drosophila* have proven invaluable to unraveling the mechanisms of human innate immunity [21], [22]. Thus, *Drosophila* provides an excellent model for host-microbe interactions.

Proteome analysis provides valuable information about protein abundance and post-translational modifications (PTMs) that can be missed at the transcriptome level [14]–[17]. Importantly, it has been shown that there is little correlation between mRNA expression and protein abundance [23]–[27]. While several studies have focused on microbe-dependent transcriptome changes in the *Drosophila* gut or in the whole fly [28]–[34], no proteomic analysis has been done. Two-Dimensional Difference Gel Electrophoresis (2D-DIGE) is a powerful technique to reveal proteomic changes between two or three protein samples simultaneously run on the same gel [35]–[38]. Protein differences detected by 2D-DIGE are then identified using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Here we used 2D-DIGE to identify *Drosophila* proteins that are responsive to the microbiota. We focused on the *Drosophila* head proteome to search for proteins with potential roles in neural function and behavior, as this aspect of host-microbe interactions is not well understood. By comparing the head proteomes of male or female flies raised with a conventional microbiota (CV) to those raised in a sterile environment (axenic, AX), we identified 22 proteins with altered abundance. Interestingly, several of these differences were sex specific. One of the male-specific difference-proteins is Alcohol Dehydrogenase (ADH), a key enzyme in ethanol metabolism in all animals, which was increased in AX males and reversed by reintroducing the conventional microbiota. ADH elevation suggested that AX males may have altered physiological and behavioral responses to alcohol. Indeed, we found that AX males exhibited significantly enhanced alcohol-induced hyperactivity (AIH), a response that is ADH dependent, male specific, and sensitive to host genetic background and dietary conditions. Using different measures of ethanol preference, we found that when offered a choice, AX males preferred to consume food containing alcohol significantly more than their CV siblings. Taken together, our work demonstrates a novel connection between the microbiota and host physiological and behavioral responses to alcohol in *Drosophila* that may have implications for our understanding of the microbiota's role in alcohol use disorders (AUD).

Results

The *Drosophila* head proteome is responsive to microbial condition

To identify proteome changes in the heads of *Drosophila* with or without gut microbiota, we compared head lysates (Fig. 1A) of CV and AX siblings (SFig. 1) from a wild type strain called "Top Banana" (see methods). Lysates from CV and AX fly heads were independently labeled with either Cy3 or Cy5 2D-DIGE dyes. The labeled protein lysates were then combined and run on the same 2D-DIGE gel (Fig. 1A). We detected and quantified the protein spots using an open-source astronomy software package called SourceExtractor, as previously described [35], [36], [39]. Because we saw variability in host protein expression similar to what has been observed in host microbiota-dependent mRNA expression studies [40], [41], we used two criteria to determine whether a protein was different in CV vs AX heads. First, we used a 20% difference in protein abundance (1.2-fold) as a cut off for a significant protein expression difference; this is approximately three standard deviations above the technical noise in a standard 2D-DIGE experiment [36]. Second, significant proteins must be different in at least two of the three biological replicates. Using these criteria, we identified 22 and 16 reproducible difference-proteins in male and females, respectively (Fig. 1B,C, SFig. 2A&B). All of these difference-proteins exhibited protein abundance changes. While some of the differences were shared between males and females, most appeared to be sex-specific: four were male-specific (Fig. 1B&C, blue shaded region) and six were female specific (Fig. 1B&C, grey shaded region). Among the shared proteins, three changed abundance in the same direction in AX male and female flies (Fig. 1B&C, orange shaded region) and 3 changed abundance in the opposite direction (Fig. 1B&C, red shaded region). Additionally, six protein differences detected in the male head gels did not resolve in the female head gels (Fig. 1B, "did not compare" group). Together, our 2D-DIGE analyses revealed microbiota-dependent sexually dimorphic changes in the *Drosophila* head proteome.

Alcohol Dehydrogenase protein level is elevated in the heads of AX male flies

LC-MS/MS identified spot #4 (SFig. 2A&B) as the metabolic enzyme Alcohol Dehydrogenase (ADH). We confirmed the protein identity by immunoblotting for *Drosophila* ADH after 2DE separation of CV head lysate (Fig. 2A). 2D-DIGE analysis showed that the loss of microbiota leads to elevated ADH protein in the heads of AX males, but not AX females (Fig. 1B and 2B). On average, ADH protein was elevated 1.8-fold in AX males. To confirm that the microbiota influences ADH protein levels, microbes were reintroduced to 0-1 day old AX adult males by exposure to CV fecal deposits (referred to as Reconstituted flies, RC; Fig. 1A). ADH

protein levels were restored to CV levels in RC males (Fig. 2B). One potential mechanism for the elevation of ADH protein is an increase in gene expression, but we did not find a consistent elevation of *Adh* transcripts in either AX male or female heads (Fig. 2C). Previous work had shown that ADH is not significantly expressed in fly brain [42], and consistent with this we find detectable ADH protein in the head capsule only (the head tissue without the brain; Fig. 2D). Because high levels of ADH are expressed in the abdominal fat body [42], the most likely site of ADH expression in the head is in the fat body that lies immediately anterior to the brain. Together, these data suggest a model in which the microbiota affect the level of ADH protein in the male head, likely in the fat body, through a mechanism that regulates ADH protein stability or translation efficiency.

The increase in ADH protein could result in increased ADH activity and increased ethanol metabolism. To test this, we quantified ADH enzymatic activity and ethanol levels in CV, AX, and RC males after exposing them to ethanol vapor. Because of assay limitations, it was not technically feasible to do these measurements with only heads. We thus characterized whole fly ADH activity and ethanol metabolism. These assays did not indicate any significant microbiota-dependent differences (SFig. 3A,B). While this does not rule out the possibility of tissue or cell type specific increases in ADH activity and ethanol metabolism, it does indicate that there is no significant systemic change.

AX male flies are more responsive to alcohol

ADH catalyzes the oxidation of ethanol to acetaldehyde, which is the first step of ethanol metabolism. Thus, ADH influences several physiological responses to ethanol, including locomotor-hyperactivity [43]–[45]. Given the elevated ADH protein in AX male fly heads, we predicted that they may exhibit altered physiological responses to alcohol. To test this, we assessed alcohol induced hyperactivity (AIH) and sedation, two phases of alcohol induced responses common to all animals and well described in *Drosophila* [46], [47].

To assess AIH, we monitored locomotor activity of CV, AX, and RC males using the *Drosophila* activity monitor 2 (DAM2). This automated system uses infrared beams to quantify fly motility in the absence or presence of ethanol (Fig. 3A). Because ADH activity is required for AIH (SFig. 4A; [45]), we reasoned that AX males could have increased AIH due to elevated ADH protein. After monitoring baseline locomotion for 60 minutes, we exposed the flies to a low concentration of ethanol vapor (10:1 air to ethanol vapor) and continued monitoring for 120 minutes. Shortly after being exposed to ethanol vapor, CV males entered a period of hyperactivity peaking at an average of 3.4 beam passes/10 min (Fig. 3B). AX males entered the

hyperactivity phase during the same time frame with a peak of 9.5 laser passes/10 min. This was restored to CV levels with microbial reconstitution in RC males (peak of 3.4 laser passes/10 min; Fig. 3B). Consistent with the lack of ADH elevation in AX female heads, there was no difference in AIH between CV and AX females (SFig. 4B).

To understand the connection between increased ADH protein and AIH elevation, we asked whether AX males require ADH activity for their elevated AIH by treating CV and AX males with an ADH inhibitor, 4-Methylpyrazole [48]. If the elevated AIH in AX males requires ADH enzymatic activity, inhibiting ADH should reduce hyperactivity to the lower levels observed in CV. Indeed, AX males aged in the presence of the inhibitor for five days exhibited reduced AIH compared to control untreated AX males (Fig. 3C). Interestingly, the same inhibitor treatment of CV males did not significantly decrease their AIH, suggesting that the inhibitor treatment did not completely abolish ADH activity, or that there is a component of AIH that is ADH-independent (Fig. 3C).

Because diet and host genetic background are important for alcohol induced physiological changes, and influence the microbiota and its downstream effects [43], [49]–[53], we asked whether these factors influence microbiota-dependent AIH. First, we decreased protein availability to the adults by removing the autoclaved yeast supplement and found that this completely abolished the elevated AIH in AX males (Fig. 3D). To test if genetic background influences microbiota-dependent AIH, we examined AIH in two additional wild type lab strains, Canton S and Oregon R. Interestingly, there was no difference in AIH between Canton S CV and AX males (SFig. 4C). We did observe a significant increase in AIH in Oregon R AX compared to CV males (SFig. 4D), but the magnitude was less than what we found in Top Banana males (Fig. 3B). These data support the idea that diet and host genetic background interact with the microbiota to influence AIH in male flies.

Next, we asked whether the microbiota influences alcohol induced sedation, a physiological response that is largely independent of ADH activity [54]–[56]. To test this, we exposed groups of CV, AX, and RC males to ethanol vapor in fly vials and assessed the time to immobilization for the population [57]. The rate of sedation, assessed by comparing the time at which 50% of the population was immobilized (ST50), differed significantly between CV and AX males (Fig. 3E,F). AX males had a significantly higher ST50 (ST50=15.9 min) compared to CV males (ST50=11.7 min), and this was restored to CV levels in RC males (ST50=13.2 min). Overall, these data demonstrate that males have microbiota-dependent changes to AIH and sedation, and that the elevation in AIH requires ADH activity.

197 **AX males flies exhibit altered alcohol preference**

198 Human studies have shown that differences in physiological responses to alcohol can
199 influence alcohol consumption behavior and are a predictor of future alcohol addiction [58]. To
200 determine whether the microbiota affects alcohol consumption and preference, we asked
201 whether AX males have an altered alcohol preference. We first assessed alcohol feeding
202 preference using the well-established Two-choice Capillary Feeder (CAFÉ; SFig. 5A) assay
203 [59], and observed that both AX and RC males exhibited significantly altered alcohol food
204 preference compared to CV males (SFig. 5B-D). However, approximately 15% of the flies in
205 each condition died by the end of day 5 (data not shown), and some have suggested that flies
206 are experiencing starvation conditions in the assay [60]–[62]. This is particularly problematic for
207 assessing alcohol preference because it is difficult to separate a preference for alcohol due to
208 its pharmacological effects from a preference driven by its increased caloric content. To address
209 this potential caveat to the CAFÉ results, we used BARCODE, a new starvation-independent
210 alcohol preference paradigm that uses a sectioned stage providing unlimited food access, and
211 promoting a more natural feeding behavior of roaming and sampling [63]. The sectioned stage
212 contains alternating squares of solid fly food with and without ethanol in a large chamber (Fig.
213 4A). BARCODE permits measurement of both positional preference and food consumption
214 preference. Positional preference is determined by counting the average number of flies on
215 ethanol food squares versus non-ethanol food squares normalized to the total number of flies on
216 the stage. CV and RC males had an aversion to the ethanol squares (average PI = -0.10),
217 which decreased toward neutral by day 2 (Fig. 4B). In contrast, AX males exhibited a positional
218 preference for ethanol that did not change significantly during the assay (average PI = +0.10;
219 Fig. 4B,C) compared to both CV and RC males.

220 To quantify alcohol food consumption preference, the ethanol squares and the non-
221 ethanol squares were spiked with two different oligonucleotides. Following the two days of the
222 assay, qPCR was used to quantify these sequences in lysates of surface-washed flies, and
223 these values were used to calculate the alcohol consumption PI. Although CV and RC males
224 spent less time on the ethanol squares (Fig. 4C), they consumed more ethanol food than non-
225 ethanol food (PI = 0.12 and 0.07 respectively; Fig. 4D). Consistent with the increase in
226 positional preference, AX males consumed significantly more ethanol food than CV or RC males
227 (PI = 0.40; Fig. 4D). Together, the BARCODE assays indicate that AX males have a
228 significantly stronger preference for food containing ethanol than their CV or RC siblings.
229
230

Discussion

The microbiota's ability to profoundly influence host physiology and behavior has important implications for understanding normal biology and disease states, and we are only beginning to discover the scope of that influence and the underlying molecular mechanisms. Using a gel-based proteomic screen, we identified both generic and sex-specific microbiota-dependent proteome changes in the *Drosophila* head. In humans, mice, and flies, the composition of the microbiota appears to be different in males and females [64]–[66], and in mice this difference is mediated by sex hormones, and sex-specific differences in bile acids and immunity [67]–[69]. The interactions between host sex and gut microbiota appear to contribute to sex-specific differences in Type 1 diabetes [67], and liver carcinogenesis [68]. While these connections are being made, there is much to learn about the mechanisms that control sex-specific differences in the microbiota and sex-specific responses to the microbiota. To date only one transcriptomic study has examined microbe-dependent gene expression changes in both male and female adult *Drosophila* tissues [32]; all other studies have focused exclusively on females [40], [70]. Thus, sex-specific microbe-dependent changes to host gene expression and protein levels have not been thoroughly investigated. We anticipate that comprehensive identification and analysis of the sex-specific difference proteins we identified in this study could yield important insight into the underlying molecular mechanisms connecting sex, the microbiota, and host biology.

One male-specific difference-protein we identified is ADH, elevated 1.8-fold in AX male heads (Fig. 2B). Because *Adh* mRNA was not responsive to microbiota elimination (Fig. 2B), we propose that ADH elevation results from increased translation or protein stability. Although ADH stability is affected by PTMs [71]–[73], we did not detect PTM changes in ADH by 2D-DIGE. Because the *Drosophila* abdominal fat body is a major site of alcohol metabolism [74], and ADH was not detectable in the brain (Fig. 2D), we propose that ADH elevation is most likely occurring in the fat body surrounding the brain. The fat body, together with the abdominal oenocytes, is functionally analogous to the liver [75], and reciprocally communicates with the brain to regulate physiology, including neural and immune activity [76]–[78]. AIH increase in AX males requires ADH activity (Fig. 3C), and ADH-dependent ethanol metabolism is required for AIH ([36] and SFig. 3A), but the precise mechanism by which ethanol metabolism mediates the elevated locomotor activity characteristic of AIH is not known. Interestingly, the lack of detectable systemic differences in ADH activity and ethanol metabolism between CV and AX flies (SFig. 3A,B) suggests that ADH elevation and activity increase in AX males is head specific. While little is known about the functional differences between head fat body and abdominal fat body,

several head fat body specific GAL4 drivers exist [79], arguing for some functional distinction. Elevated ADH activity in the head fat body could protect the brain from direct effects of ethanol, such as interference with neurotransmitters [80]–[84]. Alternatively, elevated ADH could indirectly affect the brain by changing fat body metabolism and consequently altering downstream neuropeptide or immunity signaling to the brain [85]–[87].

In addition, the increase in ADH that we observe in AX males could be a mechanism to compensate for the absence of microbial-derived byproducts, like short-chain fatty acids (scFAs), that normally promote host metabolic functions [88], and affect other aspects of host development, physiology, and behavior [89]–[94]. AX flies can have abnormally high triglycerides indicative of a change in lipid metabolism [95], [96]. In flies, ADH-dependent ethanol metabolism promotes lipid synthesis directly by converting ethanol to the scFA acetate [97]. Because *Drosophila* in the wild feed on fermenting fruit (which contains up to 5% EtOH), ethanol may contribute to normal lipid metabolism [98]. Acetic Acid is also produced by the *Drosophila* microbiota and impacts development and reproductive behaviors [99], [100]. Because sex, genetic background, and nutrition affect microbiota-dependent metabolic changes [95], [96], [101], ADH levels, and physiological and behavioral responses to alcohol (this work), a comprehensive understanding of the connections between the microbiota, host metabolism, and ADH necessitates that all of these variables be taken into account. An alternative hypothesis is that the microbiota may control host ADH and physiological and behavioral responses to alcohol to improve fitness through foraging. Recent work has demonstrated that the microbiota can promote optimal foraging and enhance fitness through multiple mechanisms [13], [16], [18]. Interestingly, *Drosophila's* responsiveness to microbe-derived alcohol and by-products of its microbe-dependent catabolism influences egg-laying behavior and promotes fitness [10].

While a microbe-dependent mechanism affecting *Drosophila's* response to alcohol may be adaptive, varying alcohol responsiveness in humans can have harmful effects. Multiple factors contribute to the risk of alcohol use disorder (AUD) in people including disinhibition/impulsivity, patterns of alcohol metabolism, a low level of response to alcohol, and increased alcohol preference [102]. Among these, a low level of responsiveness to alcohol is the most well studied, is a strong predictor of future alcoholism, and its heritability is as high as 60% [103]. We demonstrated that AX males have an increased preference for alcohol consumption (Fig. 4), as well as increased responsiveness to alcohol as reflected in elevated AIH (Fig. 3). The sex-specific effect on responsiveness in *Drosophila* is an interesting parallel to what has been found in human studies; while decreased alcohol responsiveness is strongly associated

299 with an increased risk of alcohol abuse in men, the same does not appear to be true in women
300 [103]. In addition, emerging evidence suggests that alcohol consumption can cause dysbiosis of
301 the gut microbiota observed in a subset of alcoholic patients [104], [105]. This dysbiosis appears
302 to contribute to the neuro-inflammatory withdrawal response [106], and to the emotional effects
303 of alcohol abuse [107]. Taken together, this accumulating evidence suggests that the microbiota
304 may be an important contributing factor to how animals respond to alcohol. Dissecting this
305 connection may impact our understanding of the risk factors for AUD as well.
306

307 Author contribution

308 Conceptualization, B.M.M.; Methodology, M.A.B., B.M.M., J.S.M., N.S.A., and N.L.H.;
309 Investigation, M.A.B., S.A.K., A.P., S.O., R.A.E., A.M.P., T.W.L., J.H.H., and H.M.K.; Writing –
310 Original Draft, M.A.B.; Writing – Review & Editing, M.A.B., B.M.M., N.L.H., J.S.M., and N.S.A.;
311 Funding Acquisition, B.M.M., J.S.M., N.L.H.; Resources, B.M.M., J.S.M., N.S.A., and N.L.H;
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322
323

FIGURE 1

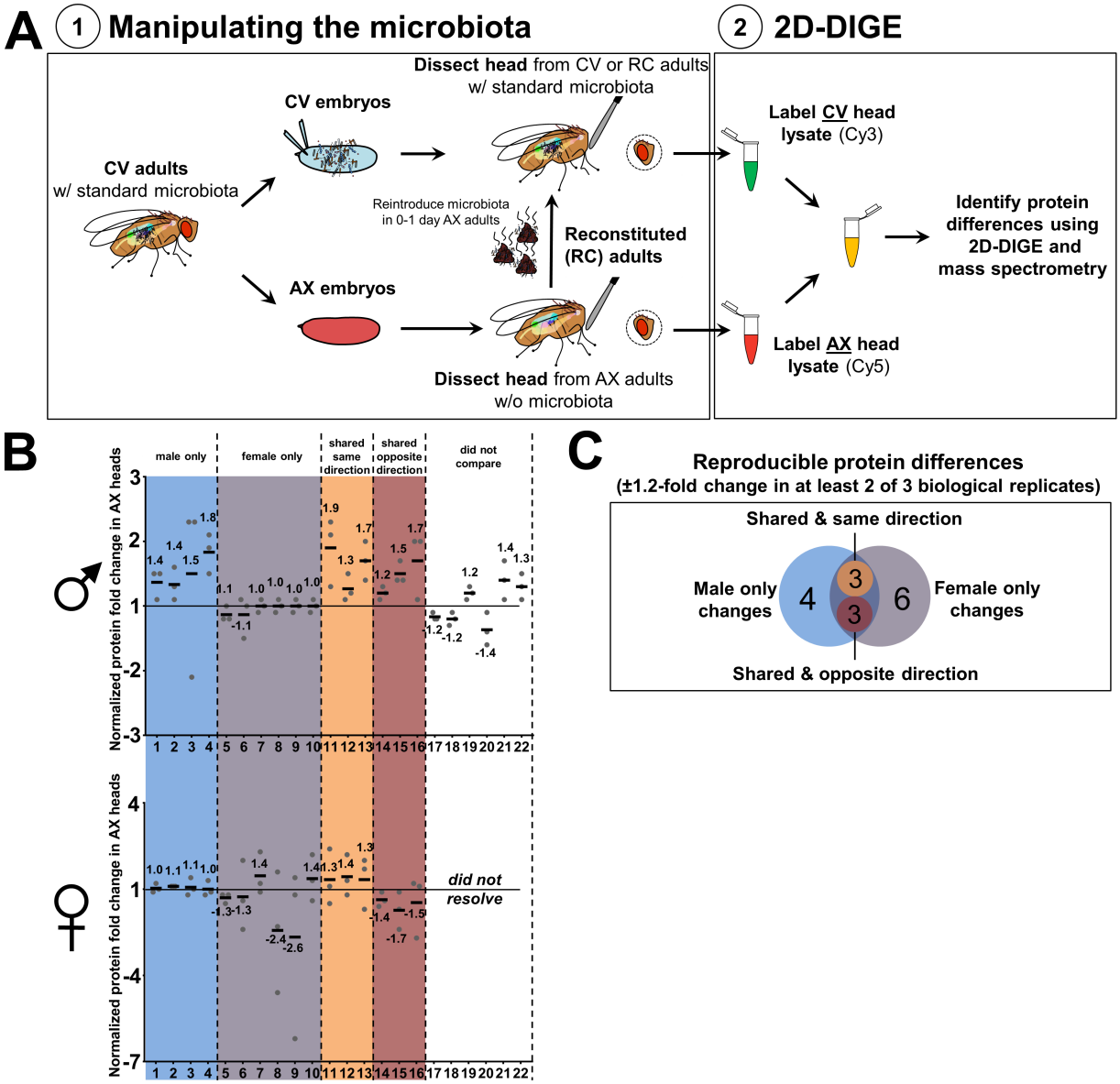

Figure 1. Identification of microbe-dependent differences in Drosophila male and female head proteomes.

(A) Manipulating the microbiota and 2D-DIGE screen experimental design. (1) CV, AX, and RC siblings were derived from cohorts of embryos with the same parents. CV embryos were not manipulated, while AX embryos were dechorinated and reared under sterile conditions (see methods). 0-1 day old CV and AX adult flies were transferred to vials and aged for 5-6 days. For microbial reconstitution (RC), 0-1 day old AX flies were transferred to vials conditioned with feces from CV males and aged for 5-6 days. (2) Protein lysates were prepared from dry dissected heads, covalently labeled with either propyl-Cy3 or methyl-Cy5, combined, and co-

334 electrophoresed on a 2DE gel. Difference proteins were identified by LC-MS/MS. **(B)** Measured
335 fold-change for each protein difference was calculated using the Cy3 and Cy5 raw fluorescence
336 intensities. Each dot represents a biological replicate containing 40 fly heads. Solid black bars
337 indicate the mean. Six difference proteins identified in males (17-22) could not be resolved in
338 the gels with female samples. **(C)** The Venn diagram shows a summary of the 16 reproducible
339 protein differences for male and female data sets across three biological replicates. The color
340 code matches that of panel B.
341

FIGURE 2

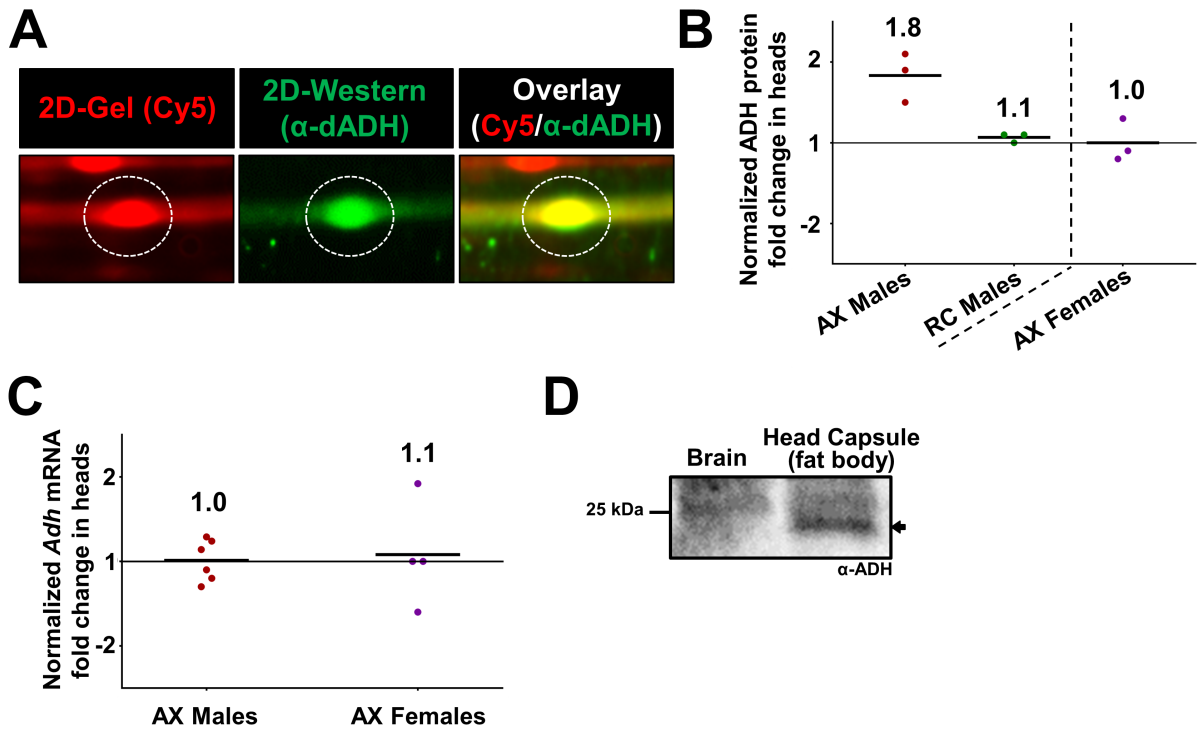

Figure 2. Alcohol Dehydrogenase (ADH) protein, but not mRNA, is elevated in AX male heads and decreased with microbial reconstitution.

(A) 2D-Western blot of the ADH protein in the CV male head proteome. The overlay of the Cy5 labeled protein (red) with the signal from the anti-ADH immunoblot (green) confirmed the protein identification made by LC-MS/MS. **(B)** Reconstitution (RC) of the microbiota in AX adult males restored ADH to CV levels. AX male and female data shown were re-plotted from Fig. 1B. **(C)** RT-qPCR revealed no change in *Adh* transcription in AX compared to CV heads when normalized to *rpl32*. **(D)** Anti-ADH immunoblot of isolated CV male brains and head capsules (containing fat body) shows detectable ADH only in the head capsule (arrow).

FIGURE 3

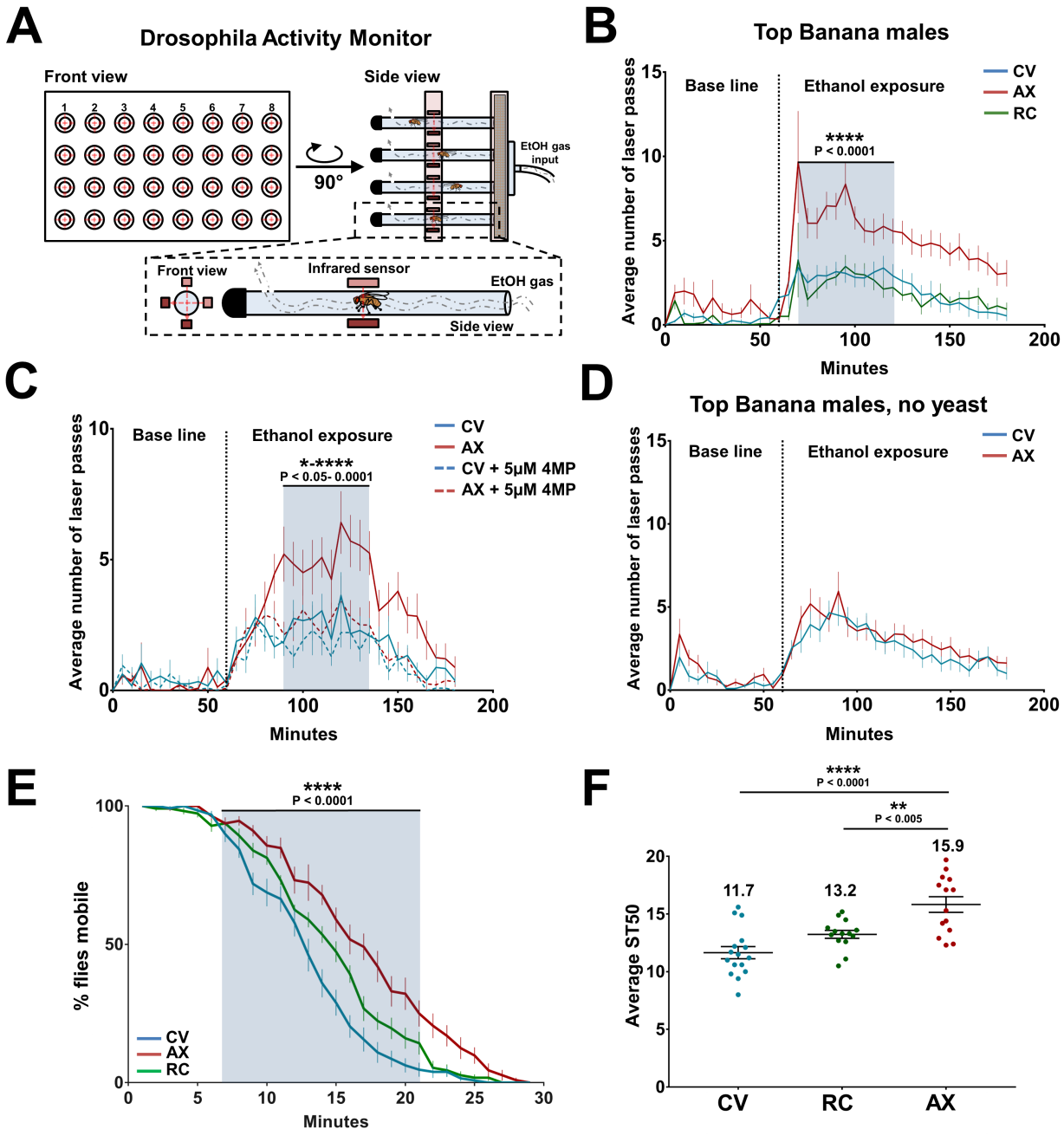

Figure 3. Microbe-dependent differences in hyperactivity and sedation responses to alcohol vapor exposure.

(A) A schematic of the *Drosophila* Activity Monitor used to test alcohol induced hyperactivity (AIH). **(B-D)** In all experiments examining AIH, the flow ratio was H_2O:EtOH (10:1) and the dotted lines indicate start of ethanol exposure. The error bars indicate standard error of the mean and statistical significance (blue shaded region) was assigned by a two-way ANOVA

using **(B)** Tukey's multiple comparisons post hoc test or **(C)** Dunnett's multiple comparisons post hoc test. **(B)** AX males exhibited significantly elevated AIH compared to their CV and RC siblings. n = 32 flies in 4 trials/condition. **(C)** AIH in CV and AX males with and without 5 μM 4-Methylpyrazole demonstrated that ADH activity is necessary for the elevated AIH in AX males. n = 24 flies in 3 trials/condition. **(D)** AIH in CV and AX males raised in the absence of autoclaved inactive yeast granules shows that nutrient deprivation suppresses the elevated AIH in AX males. n = 32 flies in 4 trials/condition. **(E-F)** AX males exhibit delayed alcohol induced sedation compared to their CV and RC siblings. **(E)** Alcohol induced sedation curves for CV (n=128 in 16 vials), AX (n=112 in 14 vials), and RC (n=112 flies in 14 vials) males averaged from 4 trials. Bars indicate standard error of the mean. Statistical significance (blue shaded region, CV vs. AX comparison) was assigned by a two-way ANOVA (Dunnett's multiple comparisons post hoc test). **(F)** Average ST50 derived from the alcohol induced sedation curves in panel E. Each dot represents the ST50 for a single vial. Bars indicate standard error of the mean and statistical significance was assigned by a one-way ANOVA (Tukey's multiple comparisons post hoc test).

FIGURE 4

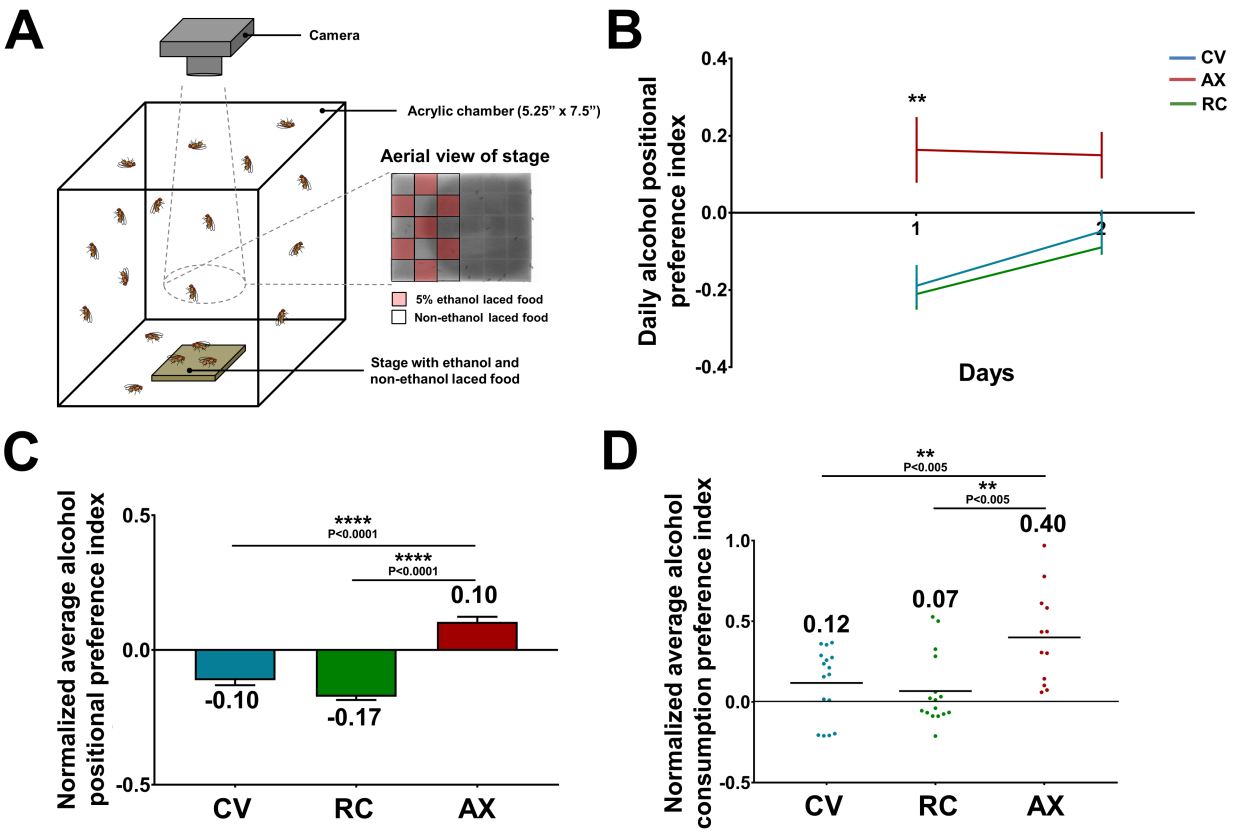

Figure 4. AX males exhibit a greater preference for alcohol compared to their CV or RC siblings.

(A) A schematic of the BARCODE assay to test alcohol preference. **(B)** Daily alcohol positional preference for CV, AX, and RC males. On day one, AX male flies were significantly different from CV and RC flies (**, P=0.0092 and P=0.0032, respectively). No significant difference was observed on day two. n = 50 flies/condition. Bars indicate standard error of the mean and statistical significance was assigned by a two-way ANOVA (Tukey's multiple comparisons post hoc test). **(C)** Normalized average alcohol positional preference index for CV, AX, and RC males over the two days of the assay. n = 50 flies/condition. Bars indicate standard error of the mean and statistical significance was assigned by a Kruskal-Wallis test (Dunn's multiple comparisons post hoc test). **(D)** Normalized average alcohol consumption preference index for CV, AX, and RC males over the two days of the assay reveals that AX males have a significantly greater preference for alcohol consumption than their CV or RC siblings. Each dot represents the average of five flies. Solid black bars indicate the mean and statistical significance was assigned by a one-way ANOVA (Holm-Sidak's multiple comparisons post hoc test).

Materials and methods

Drosophila stocks

Top Banana is a recent wild isolate (Seattle, WA) generously donated by M. Dickinson (CalTech). We used the following stocks from the Bloomington *Drosophila* Stock Center: Oregon R (Stock #5), Canton S (Stock #64349), and Adh^{N1} (Stock #3976). Canton S was obtained from the Bloomington *Drosophila* Stock Center before the w- and y- transgene contamination. *Wolbachia* PCR analysis was performed on the three wild-type stocks showing that Top Banana and Canton S were infected with *Wolbachia*, and Oregon R was *Wolbachia* free (not shown).

Creation of CV, AX, and RC cultures

CV and AX cultures were derived from embryos obtained from the same parents. A 4 hour collection of embryos was transferred in standard embryo wash (120 mM NaCl and 0.04% Triton X-100) to a plastic petri dish. For CV cultures, approximately 150 embryos were transferred to a fresh culture bottle containing autoclaved molasses fly food (8.5% molasses, 7% cornmeal, 1.1% brewer's yeast, 0.86% agar, supplemented with 0.27% propionic acid, 0.23% methyl-parahydroxybenzoate, and 0.23% ethanol). Autoclaved inactive yeast was added to the bottle cultures (0.2-0.3g per bottle). Embryos for AX cultures were prepared as previously described [101], [102] with a few adaptations. Embryos were transferred to a separate microcentrifuge tube and treated with filtered sterilized 50% bleach for two minutes, then rinsed in sterilized 70% ethanol twice, and once with sterile water to dechorionate the embryos and eliminate microbes that were associated with the chorion. Approximately 250 AX embryos were transferred to an autoclaved culture bottle containing autoclaved yeast granules as above. The difference in embryo seeding density for the two culture conditions was necessary to ensure consistent larval density and comparable nutritional environments. All adult flies were collected 0-1 day post-eclosion into autoclaved food vials containing ~0.05g autoclaved inactive yeast granules. The flies remained in these collection vials for 5 days before being used for experiments. For RC flies, 0-1 day old AX adults were placed in preconditioned food vials that had housed ten CV males (~one week old) for 4 days. RC males were aged in preconditioned vials for 5 days. All fly cultures were reared and adult progeny maintained at 22-23ºC/70% relative humidity/12-12hr light-dark cycle.

Verification of AX cultures

For culture-dependent verification, ten 5-6 day post-eclosion CV and AX flies were homogenized manually with a sterile pestle in 100 μL 1X PBS and three serial ten-fold dilutions were prepared. Undiluted homogenates and homogenate dilutions were then plated on MRS agar (wt/vol: 1% peptone, 1% beef extract, 0.4% yeast extract, 2% glucose, 0.5% sodium acetate, 0.1% polysorbate 80, 0.2% dipotassium hydrogen phosphate, 0.2% triammonium citrate, 0.02% magnesium sulfate, 0.005% manganese sulfate, 1% agar), Ace agar (wt/vol or vol/vol: 0.8% yeast extract, 1.5% peptone, 1% dextrose, 1.5% agar, 0.3% acetic acid, 0.5% ethanol), and Nutrient agar (wt/vol: 0.5% peptone, 0.3% yeast extract, 0.5% sodium chloride, 1.5% agar). Plates were incubated at 30°C for 48-72 hours. Plated CV fly homogenate consistently yielded robust microbial growth, while plated AX fly homogenate consistently yielded no growth (Figure S1B).

For culture-independent characterization, two 0-1 day old males were homogenized manually in filter sterilized squishing buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, and 25 mM NaCl) with a fitted pestle until no obvious particulates could be seen. The homogenates were incubated with Proteinase K for 1 hour and boiled for 5 minutes. 150 ng of total DNA was used as template for PCR amplification. See Table 2 for primer information. Amplified DNAs were Sanger sequenced by Genewiz (South Plainfield, NJ).

Quantifying microbial load in CV and RC flies

Ten 5-6 day post-eclosion CV or RC flies were surface sterilized by sequential washes in 10% sodium hypochlorite and 70% ethanol. Flies were then washed three times with 1X PBS and mechanically homogenized in 125μL 1X PBS with ~125μL 1.0mm zirconia beads in a Mini-Beadbeater-16 (BioSpec Products) for 30 seconds. Five ten-fold serial dilutions were then prepared from fly homogenates and all dilutions were plated on both MRS and Ace agar plates. MRS plates were incubated at 37°C and Ace plates were incubated at 30°C for ~48 hours prior to counting colonies. *Lactobacillus brevis* and *Acetobacter* colonies were distinguished by characteristic colony morphologies. For each biological replicate, colonies were counted from all dilution plates with distinguishable colonies. CFU/fly values were calculated as follows: CFU/fly = ((C x D)/V) x (H / F), where C = colony counts, D = dilution factor, V = volume of diluted homogenate plated, H = volume in which flies were homogenized, and F = number of flies homogenized, as in Koyle et al. [101].

458 **Two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), imaging**
459 **analysis and protein quantification**

460 Forty 5-6 day old flies were dry dissected on a sterile CO_2 pad and the heads were
461 pooled in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 mM DTT and 10 mM Na-Hepes
462 pH 8.0) spiked with 1% protease inhibitor (Sigma-Aldrich, St. Louis MO, USA). The heads were
463 homogenized manually with a fitted pestle until no obvious particulates could be seen. Head
464 lysates were adjusted to 2 mg/ml protein concentration with lysis buffer using the Bradford
465 standardizing method. Protein lysate solutions containing a total of 100 µg of protein were
466 labeled with 2 µl of either 1mM propyl-Cy3-NHS or 0.83 mM methyl-Cy5-NHS (CyDye DIGE
467 Fluors; GE Healthcare) as described previously [103], resulting in fewer than one dye molecule
468 per protein to prevent changing protein migration in 2DE gels. Reciprocal labeling experiments
469 were performed to control for dye-dependent changes and to generate technical replicates of
470 each sample. Two-dimensional electrophoresis (2DE) was performed as previously described
471 [104]. After second dimension electrophoresis, the gels were fixed in a solution of 40%
472 methanol and 10% acetic acid overnight then imaged in a lab built imager [32]. Protein
473 differences were determined by quantifying grey scale images of each channel using Source
474 Extractor as previously described [32], [33]. To determine the fold-difference between CV and
475 AX expression of a protein, the intensity values of each channel were normalized to five "guide
476 star" proteins, protein spots that reliably do not change within the proteome (determined from
477 multiple biological replicates), and analyzed as previously described [28], [32], [105].
478
479 **Immuno-blotting of ADH protein**

480 For standard western blots, CV brain and head capsule lysates were prepared in 2X
481 Laemmli sample buffer treated with 0.1% Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis
482 MO, USA) and separated by SDS-PAGE. For 2D-Westerns, CV head lysate was labeled with
483 Cy5 dye and run on a 2DE gel, as described above. Gels were equilibrated in a Tris buffer at pH
484 7.5 with 20% glycerol for 30 minutes. Proteins were transferred to Protran nitrocellulose
485 membranes (Whatman, Little Chalfont Buckinghamshire, UK) overnight in carbonate transfer
486 buffer at pH 9.9 (100mM $NaHCO_3$ and 80uM Na_2HCO_3) at constant 25 V. Membranes were
487 immuno-blotted using a goat anti-*Drosophila* ADH antibody at 1:500 (Santa Cruz
488 Biotechnologies, Dallas TX, USA). Donkey anti-goat HRP secondary antibody (Jackson
489 Immunoresearch, West Grove PA, USA) was used at 1:2,000. Chemiluminescence (Pierce ECL
490 Western Blotting Substrate, Thermo Scientific) was detected using a ChemiDoc MP Imaging
491 System (Bio-rad).

492

493 **qRT-PCR analyses of *Adh* gene expression levels**

494 Five to six day old CV and AX fly heads were dry dissected under CO_2 sedation. Ten
495 heads per sample were immediately bead beaten with approximately 50µl of 0.5 mm
496 Zirconia/Silica beads in 500µl of Trizol (Invitrogen, Carlsbad CA, USA). The homogenates were
497 immediately frozen at -20ºC until RNA extraction (stored no longer than 2 weeks).

498 RNA was extracted from *Drosophila* heads with the Direct-zol RNA mini kit (Zymo, Irvine
499 CA, USA). 500 ng of high quality RNA ($A_{260/280}$ ~1.8-2.0) was used as template for the synthesis
500 of first strand of cDNA using the SuperScript VILO synthesis kit (Invitrogen, Carlsbad CA, USA).
501 After first strand cDNA synthesis, 100 ng of the cDNA product was directly used for qRT-PCR
502 using the PowerUp SYBR Green Master Mix in a 7300 Real Time PCR System (Applied
503 Biosystems, Foster City CA, USA) using Sequence Detection Software (v1.4.0.25, Applied
504 Biosystems, Foster City CA, USA).

505 Housekeeping gene *rpl32* (*ribosomal protein L32*) was used for normalization [106], and
506 data were analyzed using the Pfaffl-ΔΔCT method [107] in Fig. 2C. We determined the primer
507 efficiencies for all primer sets used to calculate the fold-change between CV and AX flies. Fold
508 changes presented are the mean results from six biological replicates for males and four for
509 females.

510

511 **Assessing alcohol induced hyperactivity**

512 Locomotor activity was analyzed with a *Drosophila* Activity Monitor 2 (DAM2; Trikinetics,
513 Waltham, MA, USA) that can accommodate a total of 32 flies in individual tubes (Fig. 3A). For
514 inhibitor conditions, CV and AX male flies were aged for five days in vials with sterile fly food
515 and sterile yeast paste containing 5µM 4-methylpyrazole (Sigma-Aldrich, product code:
516 286672). For all experiments using the DAM2, single 5-6 day old adults were placed under mild
517 CO_2 sedation and transferred into monitoring plastic tubes (5 mm diameter) capped at one end
518 with a rubber cap. Tubes were randomly loaded onto a Trikinetics exhaust manifold. Base line
519 activity was established for the first hour followed by a 2 hour ethanol exposure (PHARMCO-
520 AAPER, Shelby KT, USA) using a mixture of air to ethanol vapor at a 10:1 ratio (empirically
521 determined for max hyperactivity difference) delivered by a homemade vaporizer. Activity data
522 (crossings of an infrared beam) were collected by the Trikinetics computer software in bins
523 every 30 seconds and later converted to 5 minute bins. A total of 8 flies from each condition
524 were run in parallel in any given experiment. Data were pooled from different trials for each
525 condition to obtain average activity levels. Locomotor activity curves were generated and

526 statistical analysis was performed with GraphPad Prism 7. A two-way ANOVA was used to
527 compare the hyperactivity curves.
528
529 **Assessing alcohol induced sedation**
530 The alcohol sedation assay was performed as described in Maples and Rothenfluh
531 (2011) with minor adaptations. 0-1 day adult males were collected in batches of 8 under CO_2
532 and aged to 5-6 days. Fresh fly food vials were converted into ethanol chambers by creating a
533 flat cotton bed at the bottom of the vial and sealing the chamber with a cotton ball soaked in
534 100% ethanol [50] (PHARMCO-AAPER, Shelby KT, USA). The chamber size was
535 approximately 1.25" in height.
536 A typical alcohol sedation experiment contained 4-5 vials each of CV males and AX
537 males. The flies were transferred to ethanol chambers, conditions randomized, and numbered.
538 The vials were taped together in batches of 4-5 vials. An iPad was used to record videos of
539 each trial. Before recording, each dry ethanol chamber cotton ball was replaced with a cotton
540 ball soaked in roughly 1.2 mL of red dyed ethanol. Time zero started after all dry cotton balls
541 were replaced. During the experiment, the vials were tapped every minute and immobility was
542 assessed after a 15 second recovery period until full sedation was reached. After the 15 second
543 recovery period, the number of flies immobilized was recorded and the mobile fraction was
544 calculated. The ethanol cotton ball was readjusted after every tap series to ensure the chamber
545 was approximately 1.25" high throughout the experiment. The videos were analyzed by two
546 observers who were blinded to the microbial conditions of the flies. Flies were deemed immobile
547 if: (1) the fly traveled less than the radius of the vial (to account for postural struggle or
548 spontaneous jumps after immobilized), (2) the fly lost postural control and flipped orientation, or
549 (3) the fly was completely motionless or stationary with tremors while maintaining postural
550 control. Statistical significance was determined by performing a two-way ANOVA comparing the
551 sedation curves and a one-way ANOVA for the ST50 values using GraphPad Prism 7.
552
553 **Assessing alcohol food preference using the Two-choice Capillary Feeder (CAFE) assay**
554 CV, AX, and RC male sibling flies were tested in the CAFE assay as previously
555 described [54]. Each vial housed 8 flies and contained 4 capillaries. The capillaries contained a
556 liquid food comprised of 5% yeast extract and 5% sucrose with either no ethanol (2) or 10%
557 ethanol (2). Measurements of food levels in the capillaries were taken daily by four observers.
558 Death counts for each vial were also noted per day. The assay was carried out for 5 days and

559 measurements were taken at the same time each day. Capillaries were replaced with fresh food
560 solution each day.
561
562 **BARCODE alcohol preference assay**
563 Fifty 5-6 day old CV, AX, RC flies were tested in parallel in three separate BARCODE
564 chambers. Standard molasses based fly food with agar was used for preference assays. Fly
565 food was liquified and additives were mixed in once the food was cooled to ~35 °C. The food
566 grid was filled in an alternating pattern with food containing 5% ethanol or non-ethanol food to
567 which a matching volume of water was added. The food type specific oligomers were added to
568 the corresponding food type at 3.5 ng/µl. After 2 days, the flies were collected from the chamber
569 using CO_2 and frozen immediately for future DNA extraction (see below).
570
571 Positional alcohol preference analysis
572 Preference was tested behaviorally in the BARCODE assay by capturing images of the
573 position of flies on the food pad in 5 minute intervals for 48 hrs. We used BTV Pro for Mac (Ben
574 Software) for automatic capture and analyzed using a custom Perl script and ImageMagick
575 (ImageMagick 7.0.5-0). The Preference Index (PI) was measured by counting the number of
576 flies on ethanol and non-ethanol food squares per day and calculated as PI = (N Flies on
577 Ethanol - N Flies on Non-Ethanol) / Total N Flies on Stage.
578
579 Consumptive alcohol preference analysis
580 Consumptive preference was measured following the conclusion of the behavioral
581 assay. Flies were washed using the washing protocol in [108] and homogenized in a squishing
582 buffer (10 mM Tris-HCl @ pH 8.2, 1 mM EDTA, 25 mMM NaCl). For each qPCR biological
583 replicate, we homogenized five flies per sample (n=5). Homogenates were then incubated with
584 Proteinase K (NEB, Ipswich, MA, Product No. P8107S) and spun down for 2 minutes at 10,000
585 G. Ten microliters of the supernatant was used for qPCR with the ThermoFisher Power SYBR™
586 Green PCR Master Mix reagents (Waltham, MA, Catalog No. 4367659) and analyzed with a
587 ThermoFisher Viia 7 Real-Time PCR System (Waltham, MA) with a T_m = 60 °C and 40 cycles
588 per run.
589
590 **Measuring alcohol levels**
591 20 males per biological replicate were pretreated with 10% ethanol vapor for 30 minutes
592 with a homemade vaporizer then frozen immediately. Flies were homogenized with glass

dounce homogenizers using 20 µL ddH_2O per fly. The homogenate was pipetted into 1.5 mL tubes and centrifuged at 10,0000 G for 3 minutes. A 10 µL aliquot of the supernatant was assessed using the Megazyme Ethanol Assay Kit (Bray, Co. Wicklow, Ireland, Product code: K-ETOH). Absorbance measurements were taken using a Nanodrop ND-1000 (Nano-drop Technologies, Inc., Wilmington, DE). The A_1 measurement was taken after 5 minutes following the addition of the ALDH enzyme and the A_2 measurement 15 minutes following the addition of the ADH. ALDH absorbance was background subtracted from ADH absorbance to calculate NADH produced.

Measuring alcohol dehydrogenase enzymatic activity

The Alcohol Dehydrogenase activity assay kit from Sigma-Aldrich (product code: MAK053) was used. Twenty males per biological replicate were pretreated with 10% ethanol vapor for 30 minutes with a homemade vaporizer then immediately homogenized with a fitted plastic pestle in 200 µL of ice cold ADH assay buffer, in a 1.5 mL microcentrifudge tube, until no particulates were visible. The homogenates were centrifuged at 10,0000 G for 10 minutes. Ten µL supernatant samples were assessed for ADH activity. Absorbance measurements were taken using a Tecan Safire 2 Plate Reader (Tecan Group Ltd., Männedorf, Zürich, Switzerland) and from these an NADH standard curve was generated. The initial absorbance measurement was taken two minutes following the addition of the enzyme mix (ADH assay buffer, developer, isopropanol substrate). The reaction was run at 27°C and absorbance measurements were recorded every minute for a total of 25 minutes. A ΔAbsorbance (ΔA) value was calculated for each sample by subtracting the initial absorbance from the final absorbance value. The ΔA was used to calculate the ADH activity using the following equation:

$$ADH\ activity = \frac{(\Delta A)\ x\ (sample\ dilution\ factor)}{(Reaction\ time) x\ (volume\ of\ reaction)}$$

Statistical analysis

All analyses were performed with GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). For comparisons with only two conditions, a Student's t test (or Mann-Whitney for non-normal distributed data) was performed. For experiments containing three or more conditions, an ANOVA analysis was used. Various posthoc analyses were performed depending on the type of comparison and sample distribution. For comparisons where experimental conditions were compared to only the control, a Dunnett's comparison was used. When all conditions were compared to each other and all samples had normal distributions, a Tukey's comparison was

626 used. When all conditions were compared to each other, and when at least one condition had a
627 non-normal distribution, a Sidak's comparison was used.

628 **Primers used in this study**

Primer	Sequence	Purpose	Reference
Universal forward (8F)	5'-AGAGTTTGATCMTGGCTCAG-3'	Testing microbes in fly cultures	[116]
Universal reverse (1492R)	5'-GGMTACCTTGTTACGACTT-3'	Testing microbes in fly cultures	[117]
Acetobacter forward	5'-TAGTGGCGGACGGGTGAGTA-3'	Testing microbes in fly cultures	[118]
Acetobacter reverse	5'-AATCAAACGCAGGCTCCTCC-3'	Testing microbes in fly cultures	
Lactobacillus forward	5'-AGGTAACGGCTCACCATGGC-3'	Testing microbes in fly cultures	
Lactobacillus reverse	5'-ATTCCCTACTGCTGCCTCCC-3'	Testing microbes in fly cultures	
DNA Oligomer 1	5'-ACCTACACGCTGCGCAACCGAGTCATGCCAATATAAGCAGATTAGCATTACTTTGAGCAACGTATCGGCGATCAGTTCGCCAGCAGTTGTAATGAGCCCC-3'	BARCODE assay	[63]
DNA Oligomer 2	5'-GGGCAGCAGGATAACTCGAATGTCTTAGTGCTAGAGGCTTGGGGCGTGT	BARCODE assay	

	AAGTGTATCGAAGAAGT TCGTGTTAAACGCTTTG GAATGACTGTAATGTAG-3’		
Forward qPCR Primer 1	5’-GCAACCGAGTCATGCCA ATA-3’	BARCODE alcohol consumption qPCR	
Reverse qPCR Primer 1	5’–TTACAACTGCTGGCGAA CTG-3’	BARCODE alcohol consumption qPCR	
Forward qPCR Primer 2	5’–CAGCAGGATAACTCGAA TGTCTTA–3’	BARCODE alcohol consumption qPCR	
Reverse qPCR Primer 2	5’–CAGTCATTCCAAAGCGT TTAACA–3’	BARCODE alcohol consumption qPCR	
cyp1 Forward qPCR primer	5’–ACCAACCACAACGGCAC TG–3’	BARCODE alcohol consumption qPCR	
cyp1 Reverse qPCR primer	5’–TGCTTCAGCTCGAAGTT CTCATC-3’	BARCODE alcohol consumption qPCR	

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