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Title: Metabolite exchange within the microbiome produces compounds that influence *Drosophila* behavior

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Abstract:

Animals host multi-species microbial communities (microbiomes) whose properties may result from inter-species interactions; however current understanding of host-microbiome interactions is derived mostly from studies in which is it is difficult to elucidate microbe-microbe interactions. In exploring how *Drosophila melanogaster* acquires its microbiome, we found that a microbial community influences *Drosophila* olfactory and egg-laying behaviors differently than individual members. *Drosophila* prefers a *Saccharomyces-Acetobacter* co-culture to the same microorganisms grown

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23 individually and then mixed, a response mainly due to the conserved olfactory receptor,
24 *Or42b*. *Acetobacter* metabolism of *Saccharomyces*-derived ethanol was necessary, and
25 acetate and its metabolic derivatives were sufficient, for co-culture preference.
26 Preference correlated with three emergent co-culture properties: ethanol catabolism, a
27 distinct volatile emission profile, and yeast population decline. We describe a molecular
28 mechanism by which a microbial community affects animal behavior. Our results
29 support a model whereby emergent metabolites signal *Drosophila* to acquire its
30 preferred multispecies microbiome.
31
32 **Introduction:**
33 Multispecies microbial communities (microbiomes) influence animal biology in
34 diverse ways (1): microbiomes modulate disease (2), metabolize nutrients (3),
35 synthesize vitamins (4), and modify behavior (5). A central goal in host-microbiome
36 studies is to understand the molecular mechanisms underpinning these diverse
37 microbiome functions.
38 Some aspects of microbial community function are the product of interspecies
39 interactions (6-9). For example, microorganisms modulate the metabolomes of
40 neighboring species (10) and microbial metabolites (e.g., antibiotics) alter bacterial
41 transcriptional responses (11). Despite current understanding of microbial inter-species
42 interactions *in vitro*, some of which has been elucidated in exquisite detail, the
43 consequences of microbial interspecies interactions within host-associated microbiomes
44 are just beginning to be explored experimentally.

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Insight into host-associated microbiome function has stemmed mostly from whole-microbiome (e.g., re-association of germ-free hosts with whole microbiomes (12)) and modeling microbiome function based on gene annotation (13) or single-microorganism (e.g., re-association of germ-free hosts with a single microorganism (14)) studies. However, these approaches tend to reveal only limited insight into interspecies microbial interactions, which can provide hosts with essential services. For example, termite symbionts carry genes necessary for metabolism of different parts of complex carbohydrates (15), yet their function has not been demonstrated *in vivo*; co-occurring human gut symbionts share polysaccharide breakdown products cooperatively (16, 17), but the consequences of such interactions for the host are unknown; inter-species bacterial interactions protect *Hydra* from fungal infection (18), but the mechanism of host protection is unclear. The need to understand the effects of inter-species microbiome interactions motivated our current work.

Attractive model systems in which to study the outcomes of inter-species microbial interactions for host biology would include a tractable host that harbors a simple multispecies microbiome. Here, we report the use of *Drosophila melanogaster* to study interactions in a simple microbiome and their consequences for host behavior.

The *Drosophila* microbiome consists largely of yeasts, acetic acid bacteria, and lactic acid bacteria (19-22). *Drosophila* ingests microbiome members from the environment (e.g., fermenting fruit, (22-26)), a behavior posited as a mechanism for *Drosophila* to select, acquire, and maintain its microbiome (21, 25). *Drosophila* behavior toward environmental microorganisms has focused on yeasts (27-31). While yeasts

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attract *Drosophila* via ester production (28, 29), lactic and acetic acid bacteria produce metabolites (e.g., acids) that may repel *Drosophila*, based on olfactory avoidance of high acid concentrations (32). One motivation of our study was to analyze *Drosophila* behavior toward the yeast and bacteria that dominate the *Drosophila* microbiome.

Yeast and bacteria are largely studied within separate *Drosophila* sub-disciplines, despite their shared habitat (21). Yeasts serve as food, providing *Drosophila* vitamins, sterols, and amino acids (21). Lactic and acetic acid bacteria are gut microbiome members (33) promoting larval development (34, 35), increasing resistance to pathogens (25), inducing intestinal stem cell proliferation (36), and reducing adult sugar and lipid levels (37, 38). Since microorganisms that are traditionally considered 'food' co-exist with those considered "microbiome' in fruit fermentations and the two groups provide *Drosophila* with different services, we hypothesized that *Drosophila* might detect a beneficial community via metabolites that are produced cooperatively by the desirable symbionts. Alternatively, *Drosophila* might detect a different metabolite as the signal for each symbiont.

Fruit undergoes a well-characterized ripening process in which cell-wall degrading enzymes and amylases convert the firm, starchy tissue into soft, sugar-rich fruit (39-41). The high sugar content supports microbial colonization and fermentation by *Drosophila*-associated microorganisms, including yeasts, lactic acid bacteria, and acetic acid bacteria (23, 42). *Drosophila* avoids 'green' fruit and is attracted to 'overripe' fruit (43), yet it is unclear whether *Drosophila* behavior is influenced by the fruit microbiome and its metabolic properties. To this end, we developed a model fruit

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fermentation system that afforded measurement of microbial populations, microbial metabolites, and *Drosophila* behavior.

Here we demonstrate the importance of emergent microbiome metabolism—quantitatively different or unique metabolites produced by the microbiome, but not by any of its members in isolation—on behavior, suggesting that *Drosophila* modulates its chemical intake by selecting a beneficial microbiome.

Results:

To determine whether *Drosophila* responds to emergent microbial community metabolites, we used the T-maze olfactory assay to analyze *Drosophila* behavioral responses to several *Drosophila* microbiome members grown individually or in communities (Figure 1A, Table S1, Figure 1-figure supplement 1). When the strains were grown individually, *Drosophila* was strongly attracted to yeasts, moderately attracted to acetic acid bacteria, and neutral or slightly repelled by lactic acid bacteria (Figure 1B, C). Because strains within a microbial group attracted *Drosophila* similarly, a representative yeast, acetic acid bacterium, and lactic acid bacterium were used to test the effect of interactions between microbiome members on *Drosophila* behavior. *Drosophila* preferred microbial communities grown together to microorganisms grown individually and then mixed prior to analysis (defined throughout as a separate-culture mixture, Figure 1D). Focusing on a model *Saccharomyces cerevisiae* and *Acetobacter malorum* community, we found that when tested against apple juice medium (AJM), *Drosophila* preferred the community to the separate-culture mixture or individual

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111 members (Figure 1E). In sum, *Drosophila* detects, and prefers, microorganisms growing
112 together to a mixture of the same strains combined after they had completed growth.

113 We next measured the attractiveness and other properties of the co-culture over
114 time. When grown alone, the microorganisms had similar growth profiles (Figure 2A).
115 However, when grown with *A. malorum, S. cerevisiae* populations first increased, then
116 decreased between 60 and 72 h, and were undetectable by 96 h (Figure 2A). The
117 decrease in *S. cerevisiae* viable counts mirrored a decrease in pH (Figure 2-figure
118 supplement 1A). *Drosophila* did not prefer the co-culture relative to the separate-culture
119 mixture at 34 h, slightly preferred the co-culture at 48 and 54 h, and strongly preferred
120 the co-culture at 60 through 96 h (Figure 2B). *Drosophila* preference for the co-culture
121 correlated with lower pH and *S. cerevisiae* populations, despite *Drosophila* olfactory
122 avoidance of acid (32) and reliance on yeast for nutrition (44) (Figure 2C, D). *Drosophila*
123 preference did not correlate with viable *A. malorum* populations (Figure 2- figure
124 supplement 1B). *Drosophila* preference for the co-culture increased relative to sterile
125 media during 34-96 h of growth, which is consistent with the increase in *Drosophila*
126 attraction being due to a property of the co-culture rather than to a decrease in
127 attraction to the separate-culture mixture (Figure 2-figure supplement 1C). In sum,
128 several properties of the microbial community (e.g. *S. cerevisiae* density, pH) parallel
129 *Drosophila* detection of, and preference for, the co-culture.

130 Mutants in broadly and narrowly tuned ionotropic and olfactory receptors (Irs and
131 Ors, respectively, (45, 46)) were used to evaluate the role of *Drosophila* olfactory
132 reception in discriminating the co-culture from the separate-culture mixture during and

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133 immediately following peak attraction (Figure 3-figure supplement 1). During the most
134 attractive phase of the co-culture (e.g. 67-115 h), Or83b and Or42b showed significant
135 attraction to the co-culture, whereas no role was detected for Irs or Or35a (Figure 3). As
136 the co-culture proceeded (e.g. 139-163 h), attraction decreased and the role of Or42b
137 and Or83b waned (Figure 3). Or83b is a required co-receptor for all other Or gene
138 products (47) and Or42b, one of the most conserved olfactory receptors, detects esters
139 and 1,1-diethoxyethane (48-50).These results suggest that Or42b enables *Drosophila* to
140 distinguish the co-culture from the separate-culture mixture. Our results suggest that a
141 non-Or83b factor explains ~40% of *Drosophila* co-culture preference (Figure 3).
142 Previous work found that Or83b is fully responsible for *Drosophila* attraction to apple
143 cider vinegar (51), suggesting that the behavioral circuit activated by inter-species
144 interactions of the co-culture is distinct from the circuit activated by apple cider vinegar.
145 We speculated that the emergent property of co-culture attractiveness might
146 arise from a distinct metabolic profile of the co-culture. Using gas chromatography-mass
147 spectrometry (GC-MS), we identified volatiles unique to or differentially produced in the
148 co-culture compared to the separate-culture mixture. Five co-culture volatiles (ethanol,
149 isobutanol, isoamyl alcohol, acetic acid, isoamyl acetate) were confirmed with standards
150 (Table 1-figure supplements 1-2) and quantified with standard curves (Table 1-figure
151 supplement 3). The alcohol concentrations were lower, and acetic acid and isoamyl
152 acetate were unique in the co-culture relative to the other experimental groups (Table
153 1). The molecular profile was reminiscent of ethanol catabolism as the unique co-culture

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154 metabolic process. We therefore hypothesized that ethanol catabolism was the
155 emergent metabolic process.

156 We next measured ethanol and acetic acid levels over time (24-156 h) and
157 compared the chemical dynamics to *Drosophila* preference. Consistent with a
158 relationship between ethanol catabolism, acetic acid anabolism, and *Drosophila*
159 attraction, ethanol levels declined and acetic acid accumulated in the co-culture at a
160 similar time of *Drosophila* co-culture preference (Figure 4A). Furthermore, as ethanol
161 catabolism and acetic acid anabolism proceeded (36-96 h), *Drosophila* attraction toward
162 the co-culture increased until 96 h, at which point it decreased, consistent with lower
163 turnover of ethanol at the end of ethanol catabolism (Figure 4A, black line).

164 We hypothesized that *Drosophila* preferred the community during peak ethanol
165 turnover (e.g. co-cultures at ~72 h of growth) compared with the community pre-ethanol
166 catabolism (e.g. co-culture at ~36 h of growth) or during late-stage ethanol catabolism,
167 in which ethanol turnover is low (e.g. co-culture at ~144 h of growth; Figure 4A).
168 Consistent with our hypothesis, *Drosophila* preferred the co-culture in the middle stage
169 of ethanol catabolism to earlier or later stages of ethanol catabolism (Figure 4B).

170 We hypothesized that *Drosophila* preference for communities during peak
171 ethanol turnover reflected fitness benefits derived from ingesting metabolites associated
172 with different staged communities. To test this hypothesis, we measured *Drosophila*
173 survival when given ethanol and acetic acid concentrations characteristic of microbial
174 cultures at different stages of ethanol catabolism. *Drosophila* survival was highest when
175 given metabolites associated with middle-staged ethanol catabolism compared with pre-

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or end-stage ethanol catabolism (Figure 4C). In sum, our results are consistent with *Drosophila* preference providing benefits associated with consumption of microbial community-generated metabolites.

To test directly whether ethanol catabolism underpinned *Drosophila* co-culture preference, we evaluated *Drosophila* preference for the co-culture harboring a mutant in *adhA*, which encodes pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH-I), the enzyme that converts yeast-derived ethanol into acetaldehyde on path to acetic acid (34). Co-cultures using either *A. malorum* or *A. pomorum* wild-type (WT) along with *S. cerevisiae* were equally attractive to *Drosophila* (Figure 5-figure supplement 1). *Drosophila* preferred the co-culture containing *A. pomorum* WT versus a separate-culture mixture; however, *Drosophila* did not prefer the co-culture containing *A. pomorum adhA* versus a separate-culture mixture (Figure 5A), suggesting that ethanol catabolism is necessary for *Drosophila* to discriminate between the two conditions. *Drosophila* preferred the co-culture containing *A. pomorum* WT to the co-culture containing *A. pomorum adhA* (Fig. 5A). Moreover, *Drosophila* preferred to lay eggs in the co-culture containing *A. pomorum* WT to the co-culture containing *A. pomorum adhA*, further substantiating the importance of ethanol catabolism in *Drosophila* biology, as egg-laying decisions are crucial to reproductive success (Figure 5B).

We next identified additional metabolites unique to the co-culture using solid-phase microextraction gas chromatography-mass spectrometry (SPME GC-MS). Acetic acid, six acetate esters, an acetaldehyde metabolic derivative (acetoin), a putative

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198 acetaldehyde metabolic derivative (2,4,5-trimethyl-1,3-dioxolane), and two unknown
199 metabolites were more abundant in the co-culture relative to the separate-culture
200 mixture or co-culture with *A. pomorum* lacking *adhA* (Table 2, Table 2- figure
201 supplements 1-2). To determine the molecular basis for *Drosophila* co-culture
202 preference, select metabolites were added to the co-culture containing *A. pomorum*
203 *adhA*. Esters and acetic acid, but not esters alone, were sufficient to fully restore *A.*
204 *pomorum adhA* attractiveness to *A. pomorum* WT levels (Figure 5A).

205 Although acetate and its metabolic derivatives were sufficient for *Drosophila* co-
206 culture preference, acetaldehyde is a reactive intermediate during ethanol catabolism
207 whose metabolic derivatives might be increased in microbial communities compared
208 with individual microbial cultures. Consistent with this idea, acetoin was moderately
209 increased in the co-culture compared with the separate-culture mixture (Table 2-figure
210 supplement 1); strikingly, acetoin was increased ~27-fold in the tri-culture (*S. cerevisiae*,
211 *A. malorum*, and *L. plantarum*) compared to the co-culture (Figure 6A, B, Figure 6-
212 figure supplement 1) and was attractive to *Drosophila* (Figure 6-figure supplement 2). In
213 sum, emergent metabolites from 2- and 3-membered communities, including
214 acetaldehyde metabolic derivatives, attract *Drosophila*.

215 To investigate further the potential role of acetaldehyde and its metabolic
216 derivatives in *Drosophila* behavior, we performed a dose response in which
217 acetaldehyde was added to the separate-culture mixture (Figure 6-figure supplement
218 3A) to evaluate its ability to induce attractiveness to co-culture levels. Even at the lowest
219 tested levels, acetaldehyde supplementation stimulated the separate-culture mixture to

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attractiveness levels equal to the co-culture (Figure 6-figure supplement 3A); moreover, acetaldehyde and its metabolic derivatives 'over-restored' preference of the separate-culture mixture beyond WT co-culture levels at its most attractive dose (Figure 6- figure supplement 3A). Finally, three acetaldehyde metabolic derivatives—acetoin, 1,1-diethoxyethane (an acetal), and 2,3-butanedione—were sufficient to induce the attractiveness of the separate-culture mixture to WT co-culture levels using concentrations of each metabolite at or below the physiological concentration of acetoin found in the tri-culture (Figure 6-figure supplement 3B). Creating a pure metabolite mixture comprised of key metabolic groups produced by microbial communities and identified in this study (esters, acetaldehyde metabolic derivatives, alcohols, acid) attracted *Drosophila* similarly to the co-culture (Figure 6-figure supplement 4A, B). Interestingly, the acetaldehyde metabolic derivatives alone were sufficient and the most attractive (Figure 6- figure supplement 4C, D), supporting acetaldehyde metabolic derivatives as potent *Drosophila* attractants.

Overall, our results suggest that both esters and acetaldehyde metabolic derivatives are keystone microbial community metabolites that attract *Drosophila*. We next created a simple 9-metabolite mixture in water (containing only 1 acid, 4 esters, and 4 acetaldehyde metabolic derivatives) and measured *Drosophila* preference toward this mixture in relation to the yeast-acetic acid bacteria co-culture, the yeast-acetic acid bacteria-lactic acid bacteria microbial community, or apple cider vinegar (ACV). The defined mixture used concentrations for each acetaldehyde metabolic derivative similar to the concentration of acetoin in the tri-culture and ester and acid concentrations that

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were in the range of detected in the co-culture. The defined 9-metabolite mixture was more attractive than all other conditions (Figure 6- figure supplement 5). In sum, acetaldehyde metabolic derivatives and esters are potent *Drosophila* attractants whose detection may signal the presence of actively metabolizing, multispecies microbial communities.

Discussion:

Here, we have demonstrated how emergent properties of a microbial community—volatile profile, population dynamics, and pH—influence *Drosophila* attraction, survival, and egg-laying behaviors. Our study is the first to identify the consequences of microbe-microbe metabolic exchange on animal behavior and discovers additional microbial interactions for further mechanistic study (Figure 1D).

Microbe-microbe metabolic exchange generates unique and quantitatively different volatiles from those resulting from individual microbial metabolism (Tables 1 & 2, Figure 7). *Acetobacter*-generated acetate coupled to *Saccharomyces*-derived alcohols spawn diverse acetate esters (Table 1 & Table 2). We hypothesize that more complex and diverse communities, comprising alcohol-producing yeasts, acetate-producing *Acetobacter*, and lactate-producing *Lactobacillus*, will generate a wider array of attractive esters (Figure 7). The community of *S. cerevisiae*, *A. malorum*, and *L. plantarum* emitted higher levels of acetoin and attracted *Drosophila* more strongly than the co-culture of *S. cerevisiae* and *A. malorum* (Figure 1D, Figure 6). Acetoin and 2,3-butanedione are formed by an α-acetolactate intermediate in bacteria and directly from

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acetaldehyde in yeast (52). We therefore hypothesize that communities of yeasts and bacteria may emit high levels of attractive acetaldehyde metabolic derivatives (Figure 7).

Drosophila behavioral studies have mostly focused on yeasts, even though *Drosophila* evolved in a bacterial-rich environment. Our results suggest that non-yeast microorganisms, especially when grown in microbial communities, affect *Drosophila* behaviors. We reason that additional investigations that couple chemical microbial ecology with *Drosophila* behavior will herald the discovery of microbe-influenced behaviors and microbial community-generated metabolites.

This study demonstrates the coordination of ethanol synthesis and catabolism by *S. cerevisiae* and *Acetobacter,* respectively, and the role of ethanol in *Drosophila* behavior and survival. Non-*Saccharomyces Drosophila* microbiome members also make ethanol (53) and diverse acetic acid bacteria catabolize ethanol, generalizing our findings to other microbial community combinations. Ethanol can have deleterious or beneficial fitness consequences for *Drosophila* depending on concentration (54, 55) and ecological context (56). Our results show that *Drosophila* uses products of inter-species microbiome metabolism to detect a community that titrates ethanol concentration optimally for the host. Work that further dissects the consequences of acetic acid and ethanol concentrations on *Drosophila* biology and investigates other community-level metabolic profiles will be of interest to enrich the chemical and ecological portrait of the *Drosophila* microbiome.

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Our work raises questions about the consequences of the observed behavior on microbiome assembly and stability in the *Drosophila* intestine. *Drosophila* possesses specific and regionalized gut immune responses to the microbiome (57-60) implying a tolerant environment in which privileged microbiome members are maintained and reproduce in the *Drosophila* intestine. Other work suggests that *Drosophila* acquires its adult microbiome from exogenous sources, that adult microbiome abundance drops without continuous ingestion of exogenous microorganisms, and that the microbiome can be shaped by diet (19, 25, 26). As such, a combination of internal mechanisms, exogenous factors, and host behavior likely sculpt the microbiome; determining the relative contribution of each will be important moving forward. Complicating our understanding of the contribution of these factors is the opaque distinction between 'microbiome' and 'food', since both are ingested from the environment (61). To dissect the formation and stability of the *Drosophila* microbiome, the fate of ingested microorganisms needs to be monitored and microbial intestinal reproduction needs to be surveyed as a function of *Drosophila* behavior, age, immune status, microbiome membership, and nutritional state (e.g. using synthetic diets without yeast; (34, 62)).

In sum, our results support a model in which the *Drosophila* olfactory system is tuned to fruity (e.g., esters) and buttery (several acetaldehyde metabolic derivatives, such as 2,3-butanedione) smelling metabolites promoted by microbe-microbe interactions. We anticipate that accounting for microbial interactions in diverse host-microbe studies will lead to new insights into diverse aspects of microbial-animal symbioses.

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Acknowledgements:

We thank Dr. Fabian Staubach (Stanford University, USA), Dr. Angus Chandler (University of California, Berkeley, USA), Dr. Matthew Goddard (The University of Auckland, New Zealand), Dr. Dan Jarosz (Stanford University, USA) and the Phaff Yeast Culture Collection (UC Davis, USA) for microbial strains. We thank Dr. Ryan Joseph and Dr. Karen Menuz (Yale University/University of Connecticut) for help with the *Drosophila* behavioral experiments; the Yale West Campus Analytical Core for use of the GC-MS; Dr. Terence Wu and Dr. Eric Patridge (Yale University, USA) for advice and experimental help with the GC-MS; Dr. Scott Strobel and Michelle Legaspi for use of and help with SPME GC-MS; Dr. John Carlson (Yale University, USA) for the T-maze and several *Drosophila* lines. We also thank Dr. Won Jae Lee (Seoul National University, S. Korea) for the *A. pomorum* WT and *adhA* strains, and John Carlson, Craig Crews, and Andrew Goodman (Yale University, USA) for helpful discussions. This work was supported by National Institutes of Health Grants NIDDKRC12T32GM007499-36, 5T32HG003198-10, 1R01GM099563, 7RC1DK086831. The raw data for the metabolomics experiments can be found at https://figshare.com/account/home#/projects/4735.

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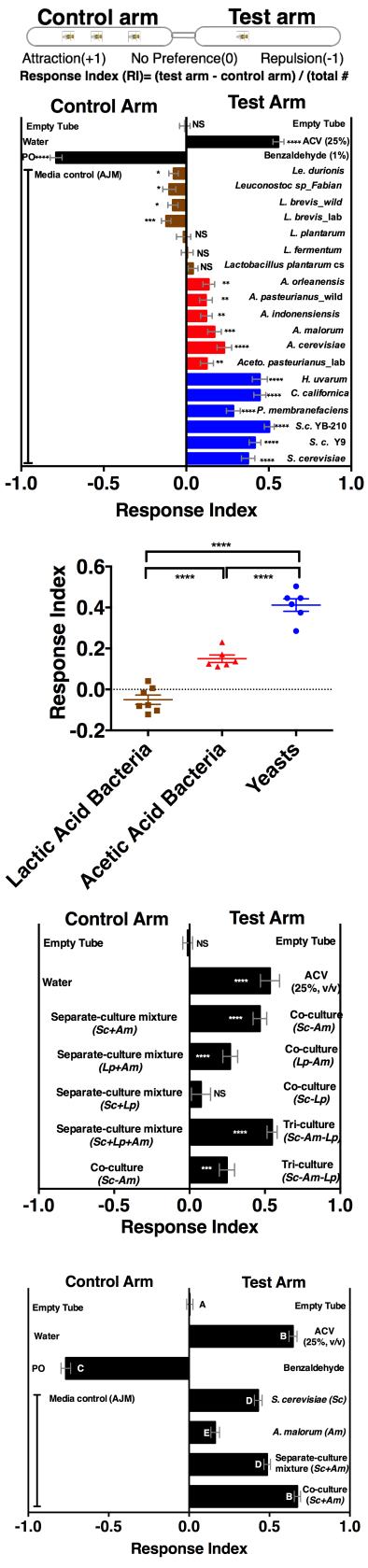

Fig. 1. *Drosophila* detection of microbe-microbe metabolic exchange. **(A)** T-maze setup and quantification. **(B)** *Drosophila* behavior toward yeasts (blue), acetic acid bacteria (red), and lactic acid bacteria (brown) (Table S1). Mean ± SEM of 12-36 replicates (n=2-6 experiments). Each T-maze replicate uses a technical replicate of a microbial culture and one cohort of *Drosophila* maintained in separate vials for 3-5 days. Mock (two empty tubes), ACV (25% apple cider vinegar versus water), and benzaldehyde (1% versus paraffin oil (PO)). A one-sample t-test assessed deviance from 0. Symbols: NS p >0.05; * p ≤0.05; ** p ≤0.01; *** p ≤0.001; **** p ≤0.0001. **(C)** Mean *Drosophila* behavior toward each microorganisms was graphed according to microbial group and analyzed by one-way ANOVA. **(D)** *Drosophila* behavior toward community combinations of a representative yeast, acetic acid bacterium, and lactic acid bacterium in relation to their separate-culture mixture (grown individually and mixed; *Sc = S. cerevisiae*; *Am= A. malorum; Lp = L. plantarum* cs) grown for 96 h; *Drosophila* preference for the three- versus two-membered community is the last column. Mean ± SEM of 12-18 replicates (n=2-3 experiments). A one-sample t-test assessed deviance from 0. **(E)** *Drosophila* olfactory behavior toward the *S. cerevisiae* and *A. malorum* community and its constituent parts relative to media grown for 48-60 h. Mean ± SEM of 18-30 replicates (n=5 experiments). Differences between groups were assessed by ANOVA. A Tukey's multiple comparison test was used to assess significant differences between groups (p≤0.05), as indicated by unique letters.

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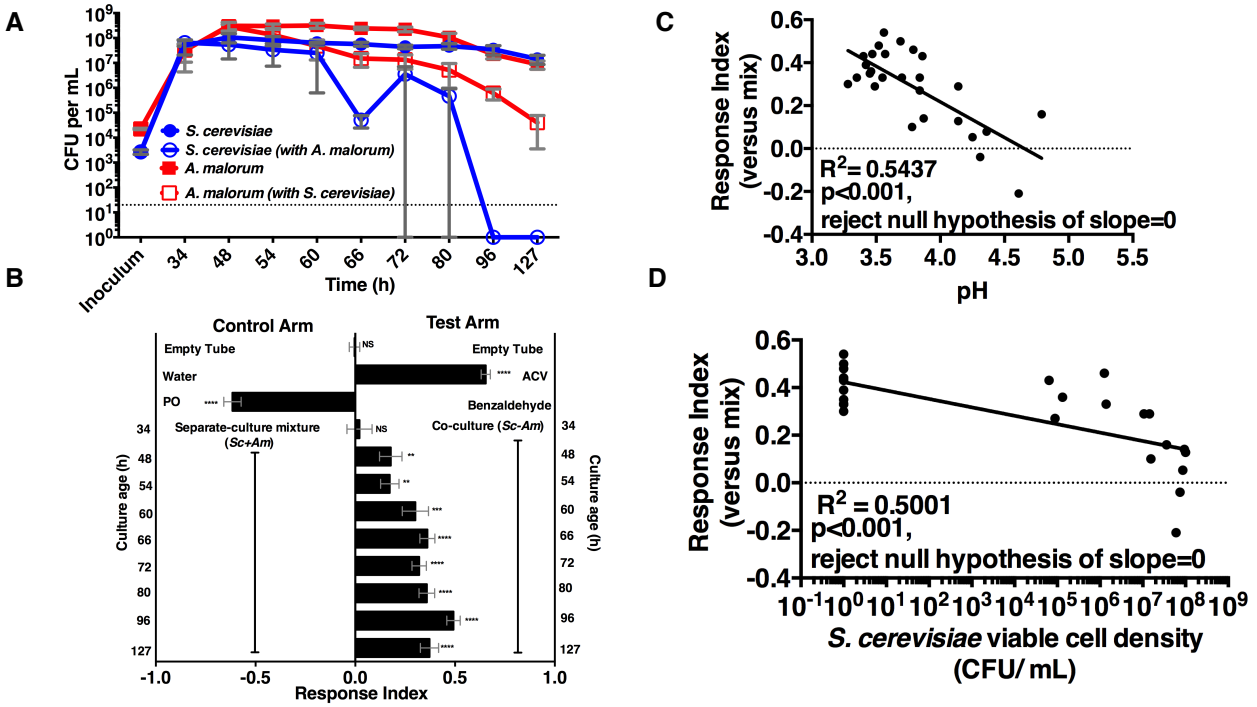

Fig. 2. *Drosophila* temporal preference for metabolic exchange. **(A)** *S. cerevisiae* and *A. malorum* viable populations. Mean ± SEM of 2-3 experiments with one pooled replicate (2-3 cultures derived from the same colony) per experiment. Limit of detection is 20 CFU/mL. **(B)** *Drosophila* olfactory behavior toward co-cultured *S. cerevisiae* and *A. malorum* versus its separate-culture mixture as a function of culture age. Mean ± SEM of 16-18 replicates from three experiments. A t-test against a hypothetical value of zero assessed deviance from 0. Symbols: NS p >0.05; * p ≤0.05; ** p ≤0.01; *** p ≤0.001; **** p ≤0.0001. **(C)** Relationship between pH and *Drosophila* preference for the *S. cerevisiae* and *A. malorum* co-culture versus the separate-culture mixture. Each data point represents the pH of a co-culture and the mean RI of *Drosophila* toward the same co-culture. A linear standard curve with an unconstrained slope was generated and compared to a null model with slope=0. The data fit to an unconstrained slope better than to the null model (p<0.0001, slope = -0.3295). **(D)** Relationship between *S. cerevisiae* populations and *Drosophila* preference for the co-culture versus the separate-culture mixture. Each data point represents viable *S. cerevisiae* populations of the culture along with the mean RI value toward the co-culture containing *S. cerevisiae*. A semilog standard curve with an unconstrained slope was generated and compared to a null model with slope=0. The data fit to an unconstrained slope better than to the null model (p<0.0001, slope = -0.0349).

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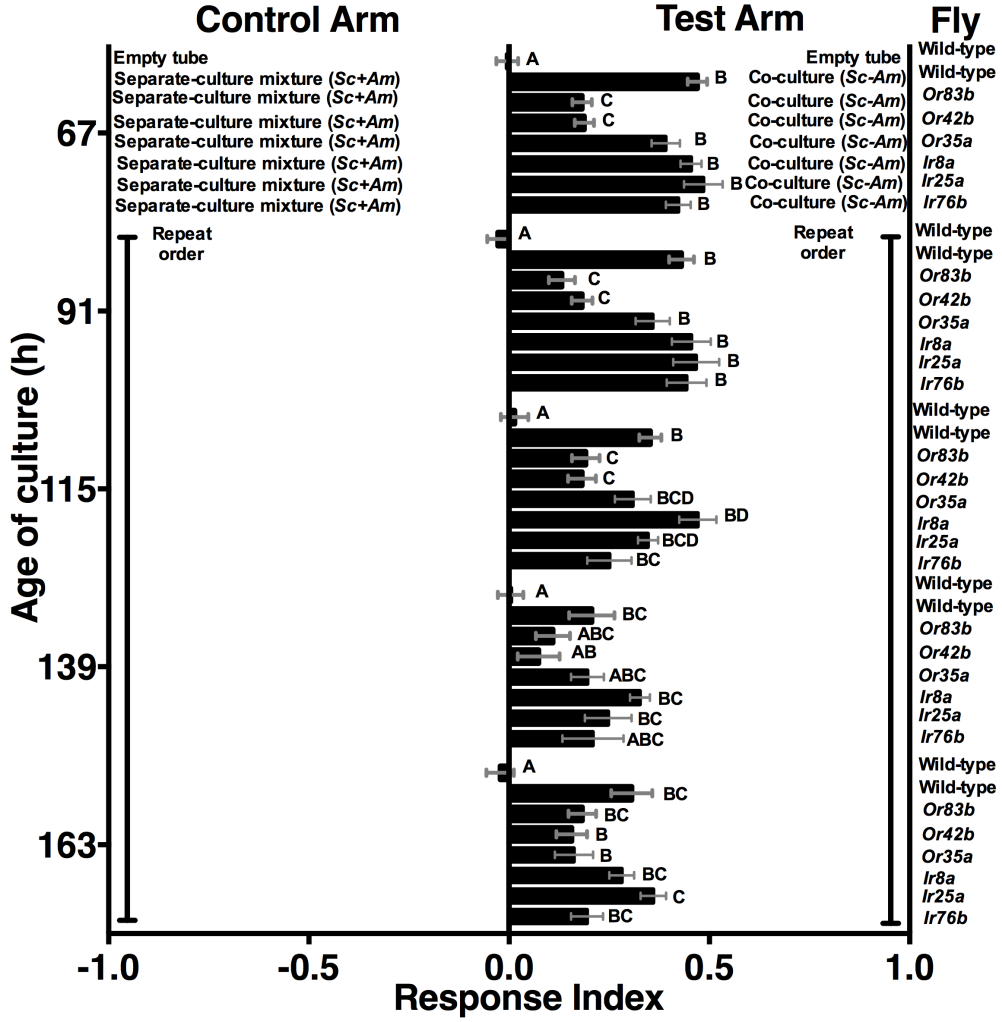

Fig. 3. Role of olfactory receptor mutants in *Drosophila* detection of inter-species microbial interactions. Culture were grown at and after peak *Drosophila* attraction. Differences were assessed by a one-way ANOVA within each culture age. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. Mean +/- SEM of 12-24 replicates per time point per fly condition (n=2-4 experiments per time point).

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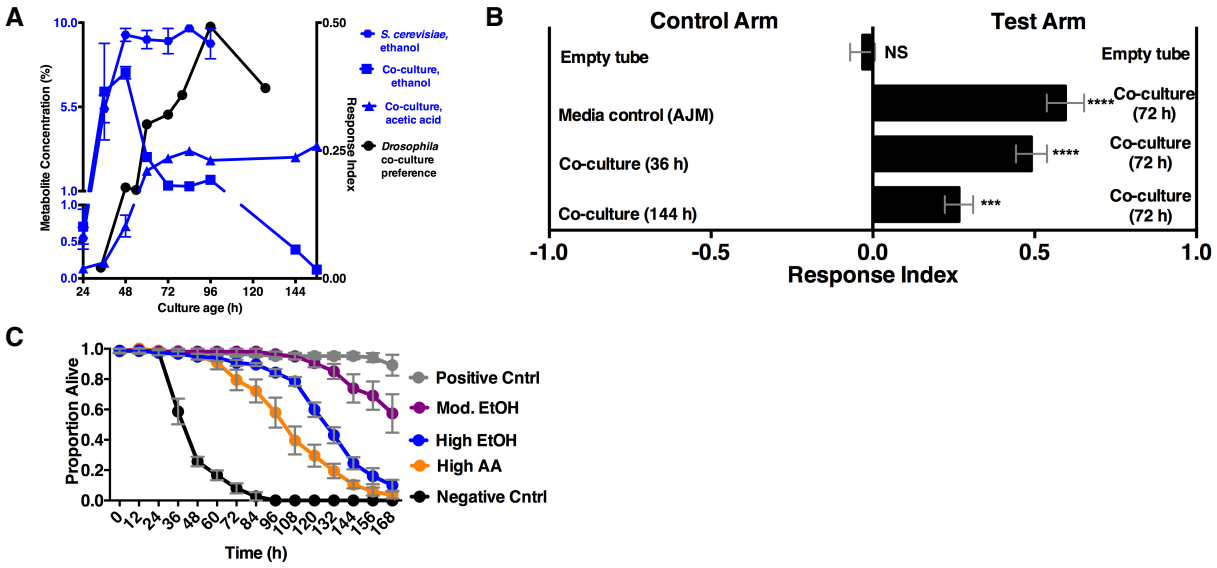

Fig. 4 *Drosophila* behavior, survival, and ethanol catabolism. **A)** Dynamics of ethanol, acetic acid and *Drosophila* co-culture preference. Acetic acid was only detected in the co-culture. The abundance was derived from a linear regression calculated from standards (figs. S6). Chemical data is the mean ± SEM of two values calculated from two experiments with three replicates per experiment (except acetic acid and ethanol concentrations at 144 and 156 h, which are from one experiment with three replicates). *Drosophila* co-culture preference is the mean value of the preference shown in Figure 2B**. B)** *Drosophila* preference for stages of ethanol catabolism. 72 h is 'mid' stage; 36 h is 'early' stage and 144 is 'late' stage. The co-culture contains *S. cerevisiae* and *A. malorum* grown for the time indicated. AJM= apple juice media. Data points represent the mean ± SEM of the combined results of two experiments with 8-10 total replicates per group. A t-test assessed whether the values were different from zero. C) *Drosophila* survival in the presence of acetic acid (AA), ethanol (EtOH) or the combination of the two in water. Groupings were based on concentrations of metabolites estimated from pre-ethanol catabolism (9.4% ethanol, High EtOH), middle-staged ethanol catabolism (1.4% ethanol, 2.8% acetic acid, Mod. EtOH) and post ethanol catabolism (3.42% acetic acid, High AA). Data represent mean +/- SEM of 5-6 replicates of one representative experiment of two. Mod. EtOH was significantly different from High EtOH condition from 108 h onward and High AA condition from 72 h onward (assessed by two-way ANOVA comparing time and condition, $p \leq 0.05$, Tukey's correction). Negative Cntrl is water and Positive Cntrl is Shield's & Sang M3 Insect Medium.

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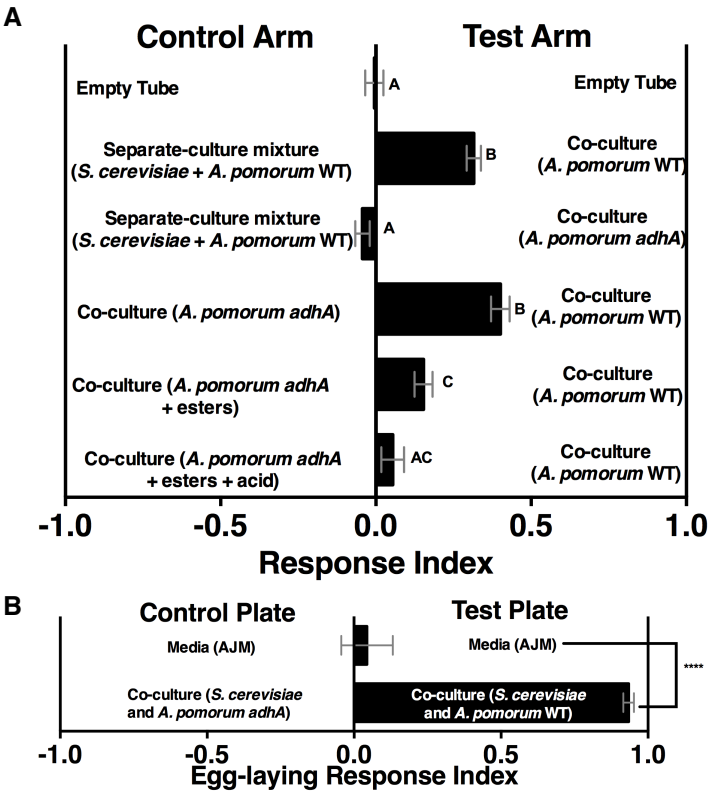

Fig. 5. Role of ethanol catabolism in *Drosophila* olfactory and egg-laying behaviors. **(A)** *Drosophila* olfactory behavior toward specified conditions. Mean ± SEM of 2-7 experiments with 10-42 total replicates. Differences were assessed by a one-way ANOVA. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. Esters include ethyl acetate, isoamyl acetate, 2-phenethyl acetate, isobutyl acetate, 2-methylbutyl acetate, and methyl acetate; acid is acetic acid. Amounts added are based on physiological amounts in co-cultures and are found in Table 2. The co-culture contains *S. cerevisiae* and the specified *A. pomorum* strain. acid= acetic acid **(B)** *Drosophila* was given a choice to lay eggs in a co-culture containing *S. cerevisiae* and *A. pomorum* wild-type (WT) or *S. cerevisiae* and *A. pomorum adhA*. The co-cultures were grown for 96 h and mixed 1:1 with a 1.6% agarose solution. *Drosophila* was allowed to lay eggs for eight hours. Difference between the two groups was assessed by an unpaired t-test (**** $p \leq 0.0001$). Mean ± SEM of 16-18 replicates from two experiments.

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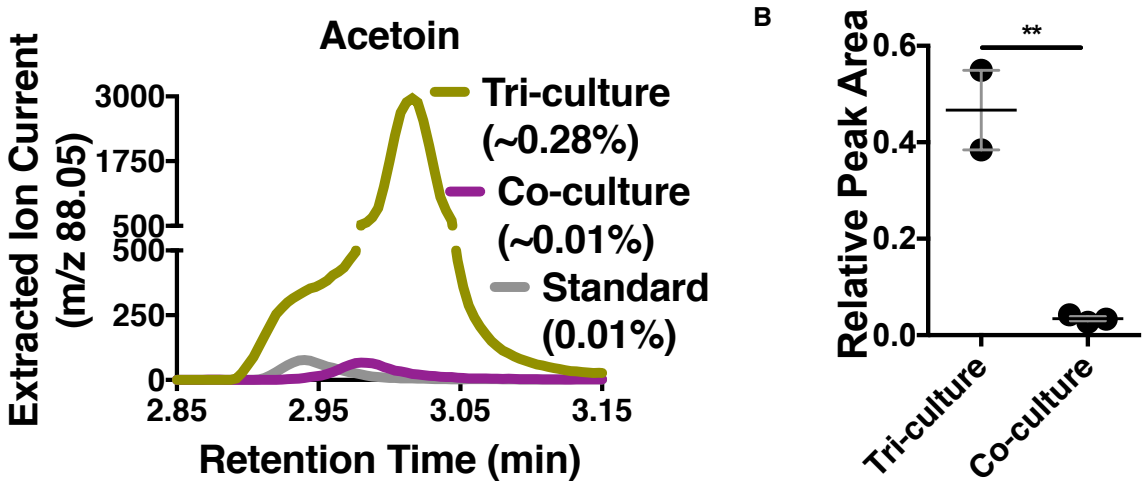

Fig. 6. Acetoin as an attractive metabolite increased in the 3-membered communitiys **A)** Representative chromatogram of the tri-culture (*S. cerevisiae-A. malorum-L. plantarum*) compared to the co-culture (*S. cerevisiae* and *A. malorum).* **B)** Estimated quantification of is based on a linear regression of acetoin (figure 6-figure supplement 1). Relative quantification of acetoin in the tri-culture (one replicate with *A. malorum* and one replicate with *A. pomorum* from separate days) and the co-culture (one replicate with *A. malorum* and two replicates with *A. pomorum* from separate days). Difference in peak areas was assessed by an unpaired two-tailed t-test (** p≤0.01).

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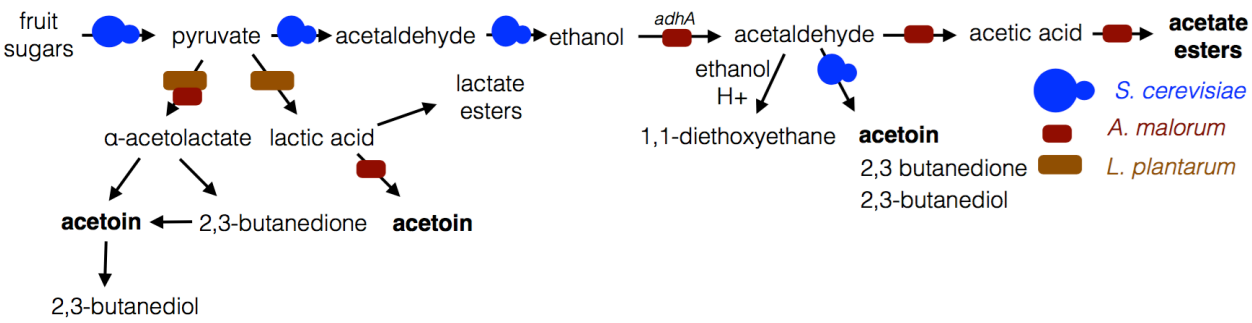

Figure 7. Model of microbe-microbe metabolite exchange. Bolded are metabolites increased due to microbe-microbe interactions.

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509 **Table 1.** Summary of volatiles detected using GC-MS

Identity	*Standard confirmation*	*Relative quantification (co-culture: separate-culture mixture)*
Ethanol	Y	5.0-12.6-fold reduced
Isobutanol	Y	7.3-24.7-fold reduced
Isoamyl acetate	Y	unique to co-culture
Isoamyl alcohol	Y	3.6-6.4-fold reduced
Acetic acid	Y	unique to co-culture

510 **Table 1.** Relative abundance of volatiles in the co-culture (*S. cerevisiae* and *A. malorum* grown together)
511 compared to the separate-culture mixture (*S. cerevisiae* and *A. malorum* grown separately, and their
512 quantities added in during anaylsis). GC-MS captured volatiles with XAD-4 beads suspended above the
513 cultures during growth; subsequently, beads were methanol-extracted (n=6 experiments, Table 1-figure
514 supplements 1-2). Quantification is based on two experiments in which a linear regression was computed
515 with standards (Table 1-figure supplement 3). Quantification is based on beads suspended above the
516 cultures between 84 and 96 h of culture growth.
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Table 2. Estimated concentrations of key metabolites in the co-culture using SPME GC-MS

Metabolite	Lin. reg. eq. 1	Lin. reg. eq. 2	Normalized Peak Area (single experiment)	Normalized Peak Area (Average, All experiments)	Estimated Concentration (%)
Isobutyl acetate	Y=4151X - 0.1319	Y=3252X - 0.07251	0.29	1.16	0.00023
Isoamyl acetate	y=8158X	Y = 7800X	0.78	3.8	0.00026
2-Phenethyl acetate	Y=5129X -0.04011	Y=6972X -0.2013	1.2	1.9	0.00028
2-Methylbutyl acetate	Y=8995X - 0.05042	Y=8087X-0.1307	0.56	3.1	0.00023
Methyl acetate	Y=75.22X +0.004457	NA	0.018	0.040	0.00033
Ethyl acetate	NA	NA	NA	NA	~0.02*
Acetic acid	NA	NA	NA	NA	~3.0*
Acetoin	NA	NA	NA	NA	~0.01*

Table 2. Estimated concentrations of differentially concentrated or unique metabolites in the co-culture. Linear regression equations (Lin. reg. eqs. 1 and 2) were estimated from individual experiments in which peak areas of different concentrations of metabolites were fitted with a linear regression (Table 2-figure supplement 2). Normalized peak areas correspond to the specified metabolites in co-cultures containing *S. cerevisiae* and *A. malorum*. Separate estimates were derived from a normalized peak area estimated from a single experiment (co-culture and standard samples were from a run with similar internal standard signal) or from the mean normalized peak area estimated from all experiments (co-cultures were run over four days, standards were run on two days). The final estimated concentration was an average of all estimated concentrations (n=4 estimates (two from each standard regression equation times two estimates of the normalized peak area), execpt for methyl acetate, n=2 estimates). The estimated concentrations (exept acetoin) were added to the co-culture containing *A. pomorum adhA* (Figure 5A). *Ethyl acetate, acetic acid and acetoin concentrations were estimated from standards in Table 2-figure supplement 1.

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549 **SUPPLEMENTARY MATERIALS**

Table S1. Microorganisms and their sources used in this study

Organism	Clade	Source
Saccharomyces cerevisiae	Yeast	*Drosophila pinicola,* Phaff culture collection
S. cerevisiae Y9	Yeast	Ragi (similar to Sake wine) fermentation (30)
S. cerevisiae YB-210	Yeast	Banana; ARSC culture collection (63)
Pichia membranefaciens	Yeast	*D. melanogaster* in Handelsman Laboratory
Candida californica	Yeast	Bananas and *D. melanogaster* habitat in Stamps laboratory; Phaff culture collection
Hanseniaspora uvarum	Yeast	Bananas and *D. melanogaster* habitat in Stamps laboratory; Phaff culture collection
Lactobacillus plantarum cs	LAB	*D. melanogaster* in Handelsman Laboratory (25)
*L. brevis*_lab	LAB	*D. melanogaster* in Handelsman Laboratory
L. fermentum	LAB	*D. melanogaster* in Handelsman Laboratory
*L. brevis*_wild	LAB	Wild-caught *D. melanogaster* (19)
Leuconostoc sp_Fabian	LAB	Wild-caught *D. melanogaster* (64)
L. plantarum	LAB	*D. melanogaster* in Handelsman Laboratory
Leuconostoc durionis	LAB	Wild-caught *D. melanogaster* (64)
*Acetobacter pasteurianus*_lab	AAB	*D. melanogaster* in Handelsman Laboratory
A. cerevisiae	AAB	*D. melanogaster* in Handelsman Laboratory
A. malorum	AAB	*D. melanogaster* in Handelsman Laboratory
A. indonensiensis	AAB	*D. melanogaster* in Handelsman Laboratory
*A. pasteurianus*_wild	AAB	Wild-caught *D. melanogaster* (19)
A. orleanensis	AAB	Wild-caught *D. melanogaster* (19)
A. pomorum	AAB	*D. melanogaster* in Won-Jae Lee Laboratory (34)
A. pomorum adhA	AAB	Mutant derivative of *A. pomorum* (34)

550 LAB = lactic acid bacteria; AAB = acetic acid bacteria

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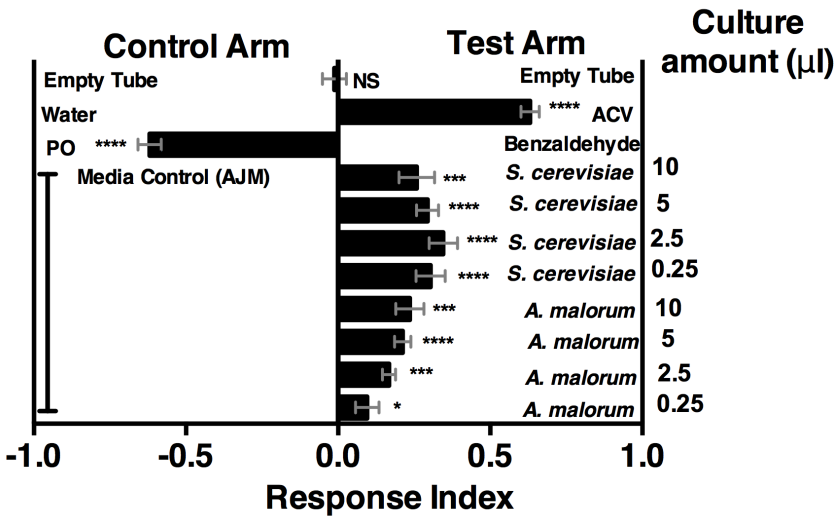

Figure 1-figure supplement 1. *Drosophila melanogaster* olfactory behavior toward different culture volumes of *Saccharomyces cerevisiae* and *Acetobacter malorum*. The top three experimental groups are controls: Mock (empty tube versus empty tube) recapitulates alternating of test and control arms, as in all experimental groups; apple cider vinegar (ACV (25% in water)) is the positive control and tested against water only; Benzaldehyde (1%) is the negative control and is a 100-fold dilution of benzaldehyde in paraffin oil (PO) tested against paraffin oil only. In all experimental groups, 10μl of total volume was used; the culture amount is specified, when appropriate, on the right-hand portion of the plot. The remaining volume in the microbial groups is water. Media control (AJM) is always 5μl of AJM mixed with 5μl of water. Data points represent the mean +/- SEM combined from three experiments (n=12 per experimental group). A t-test was performed against a hypothetical value of 0 to test for significance and is denoted by the following system (consistent throughout manuscript): NS $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. Based on this analysis, we used a total microbial culture volume of 5 μl throughout the manuscript, unless otherwise noted.

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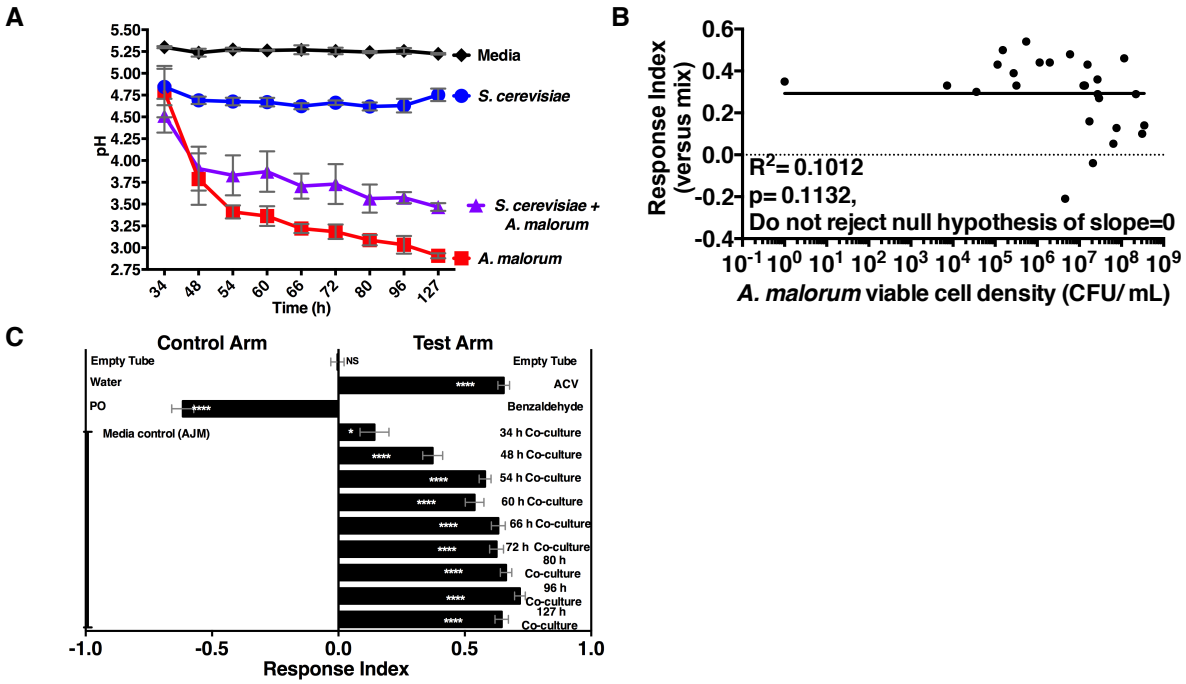

Figure 2-figure supplement 1. Properties of the co-culture and its relationship to *Drosophila* preference **(A)** pH of experimental groups as a function of microbial growth time. Mean pH ± SEM of three experiments with one pooled replicate per experiment. **(B)** Relationship between *A. malorum* populations and *Drosophila* preference for the co-culture versus the separate-culture mixture. Each data point represents viable *A. malorum* populations in the co-culture along with the mean RI value toward the co-culture containing *A. malorum*. A semi-log standard curve with an unconstrained slope was generated and compared to a null model with slope=0. The data do not fit to an unconstrained slope better than to the null slope= 0 model (p=0.1132). **(C)** *Drosophila* attraction to the co-culture versus sterile media as a function of co-culture age (grown 34 h – 127 h). Mean RI ± SEM of three experiments with 16-18 total replicates. T-test against a hypothetical value of 0 was performed. NS p >0.05; * p ≤0.05; ** p ≤0.01; *** p ≤0.001; **** p ≤0.0001 Significance denoted beside or within bars of each experimental group. ACV = apple cider vinegar (25% in water); PO = paraffin oil.

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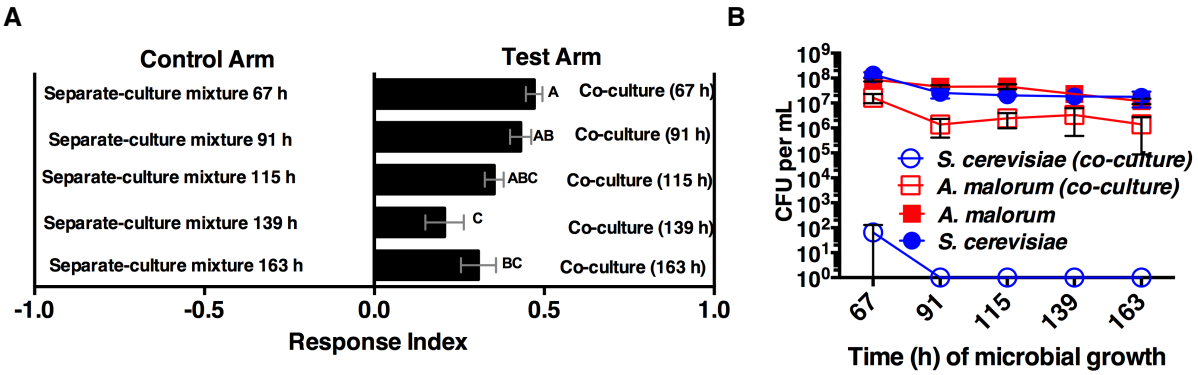

Figure 3-figure supplement 1. A) Attraction of wild-type *Drosophila* to different aged co-cultures (grown 67-163 h, *S. cerevisiae* and *A. malorum*). Mean ± SEM of 12-24 replicates per group (n=2-4 experiments). One-way ANOVA assessed significance between groups. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. **B)** Corresponding viable counts at different times of microbial growth. Mean ± SEM of 2-3 replicates.

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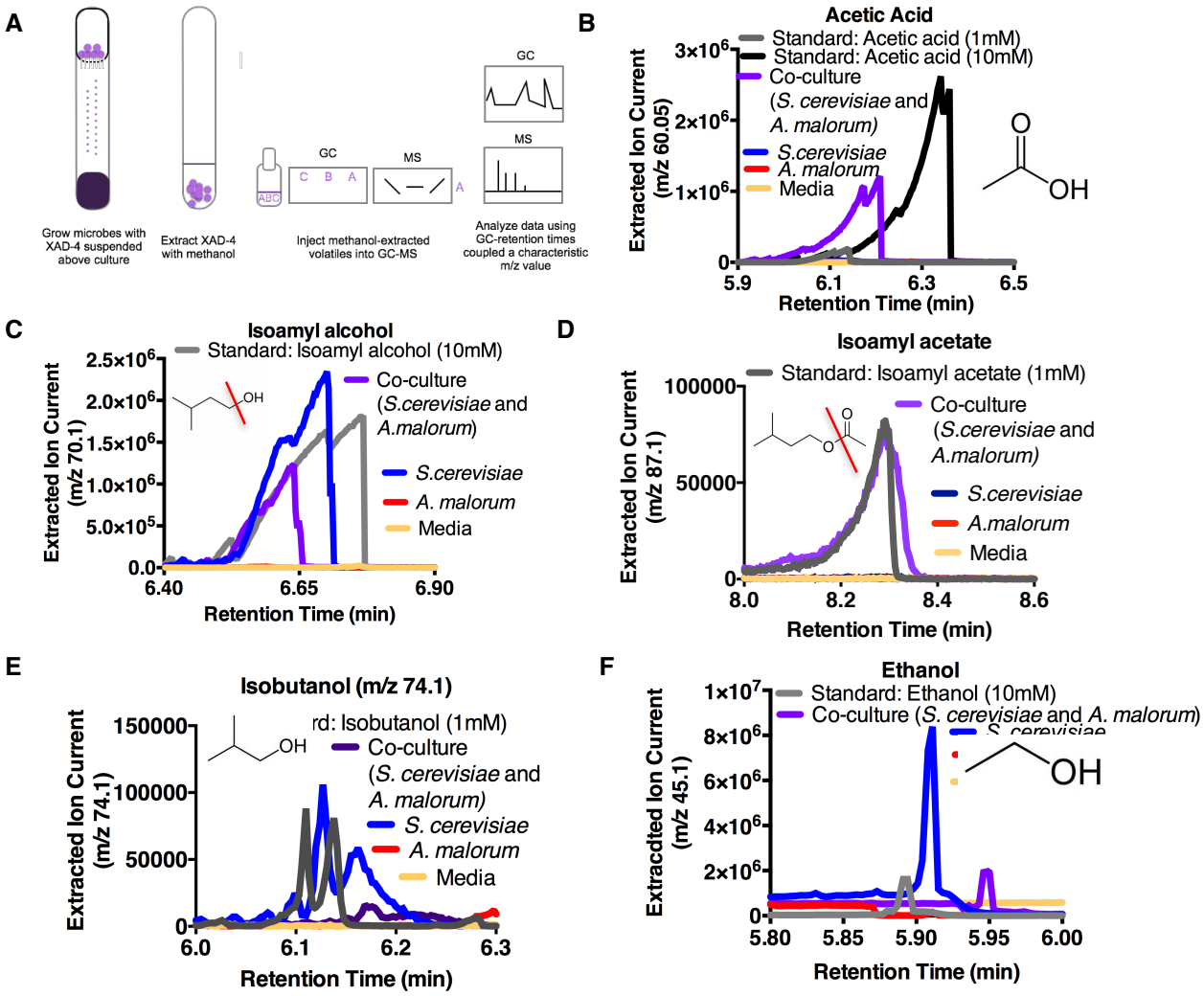

Table 1-figure supplement 1. Extracted ion chromatograms of the five metabolites detected by gas chromatography- mass spectrometry (GC-MS). **(A)** Schematic depicting the experimental setup **(B-F)** Representative extracted ion chromatograms from one replicate (out of three total) of one experiment (out of 3-4 total) of m/z values corresponding to major metabolites identified in the experimental conditions along with appropriate standards. Acetic acid **(B)**, isoamyl alcohol **(C)**, isoamyl acetate (**D)** isobutanol **(E)**, and ethanol **(F)** were identified as the five major metabolites in the co-culture (*S. cerevisiae* and *A. malorum*). Isoamyl alcohol **(C)**, ethanol **(E),** and isobutanol **(F)** were identified as the major metabolites in *S. cerevisiae* grown alone. Extracted ion chromatograms were constructed using the m/z value in the title of each graph. For acetic acid and isobutanol, the m/z value used corresponds to the molecular weight of the molecule. For ethanol, the m/z used corresponds to the molecular weight minus one (hydrogen). For isoamyl alcochol, the m/z used corresponds to the loss of the hydroxyl group (depicted), which may have picked up a hydrogen and been lost as water. For isoamyl acetate, the m/z value corresponds to the molecule shown within the graph. In all cases, figures showing the complete mass spectra between the metabolite and standard are found in Table 1-figure supplement 2. Microorganisms were grown 72-96 hours.

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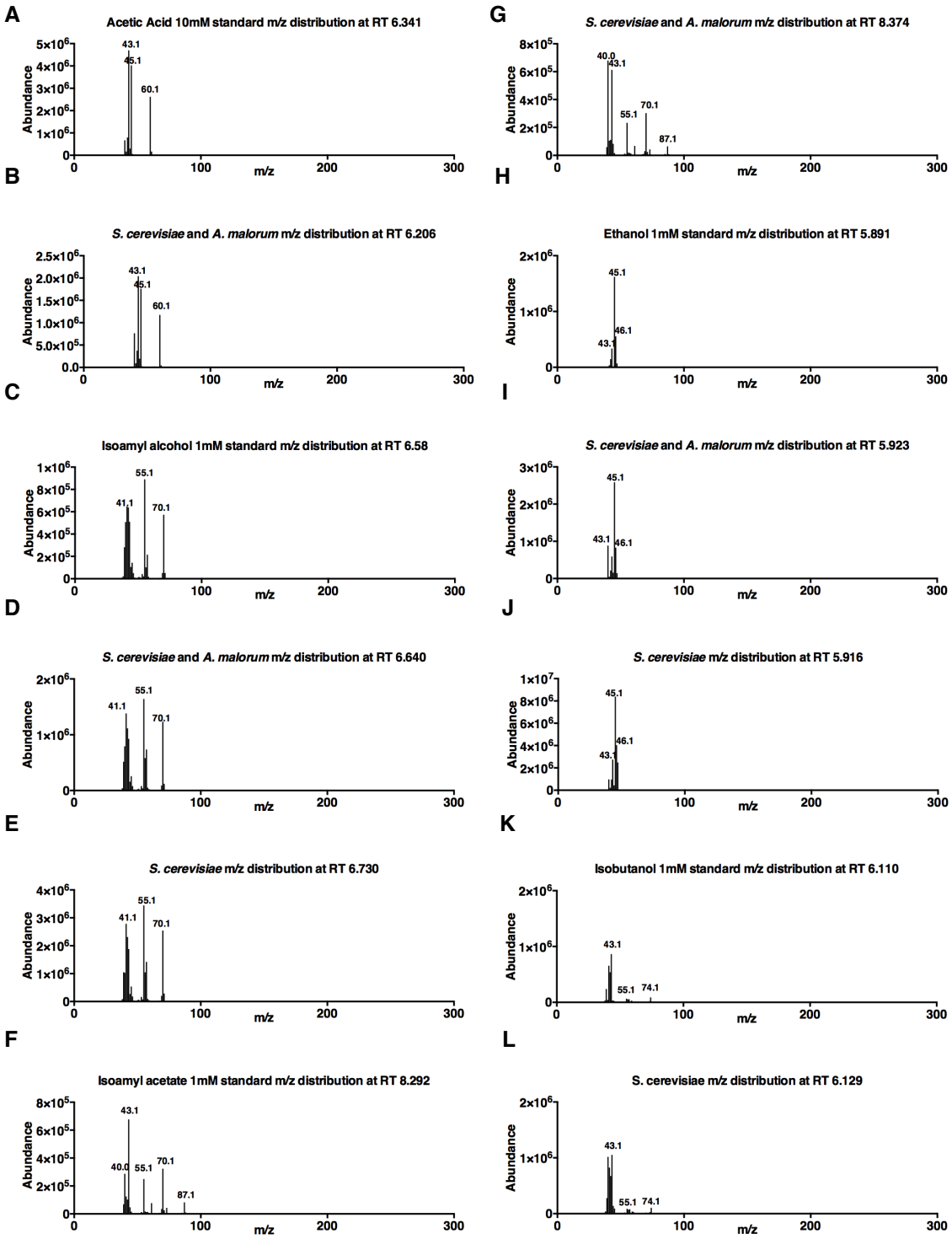

Table 1-figure supplement 2. Representative spectra of acetic acid (**A-B**), isoamyl alcohol (**C-E**), isoamyl acetate (**F-G**), ethanol **(H-J)** and isobutanol **(K-L)** in standard and experimental samples**.** Standard concentrations are denoted on individual graphs. All mass spectra are one replicate (out of 3-4 experiments with three replicates per experiment).

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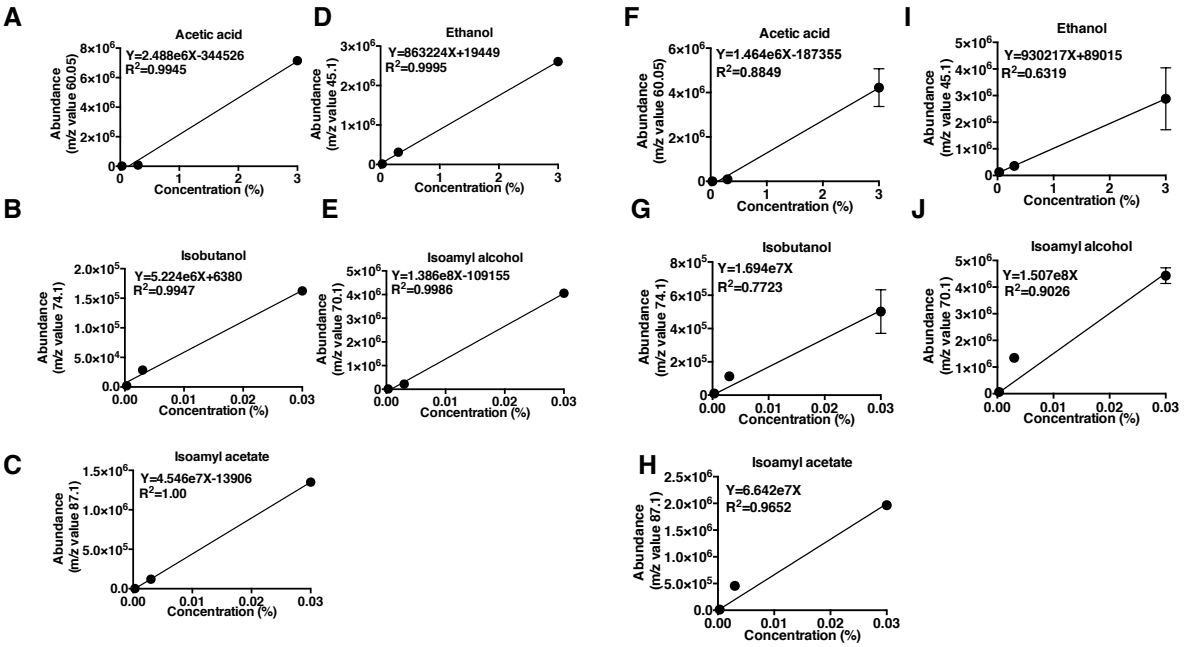

Table 1-figure supplement 3. Estimation of volatile quantity using GC-MS. Separate experiments are graphed in panels **(A-E)** and **(F-J)**. **(A-E)** Data points represent the value of a single replicate per concentration for each standard. The abundance of a single m/z value at a specific retention time was chosen for each standard. The values were fitted with a linear regression and the equation was used to estimate the concentration of the five metabolites in the experimental samples from the same experiment. (**F-J)** Data points represent the mean ± SEM of three replicates for a given concentration for each standard. The abundance of a single m/z value at a specific retention time was chosen for each standard. The values were fitted with a linear regression. The equation was used to estimate the concentration of the 5 metabolites in the experimental samples from the same experiment. When applicable an equation was calculated when the line was forced to go through X,Y=0,0; these equations were used to calculate the concentrations of isoamyl alcohol, isoamyl acetate, and isobutanol.

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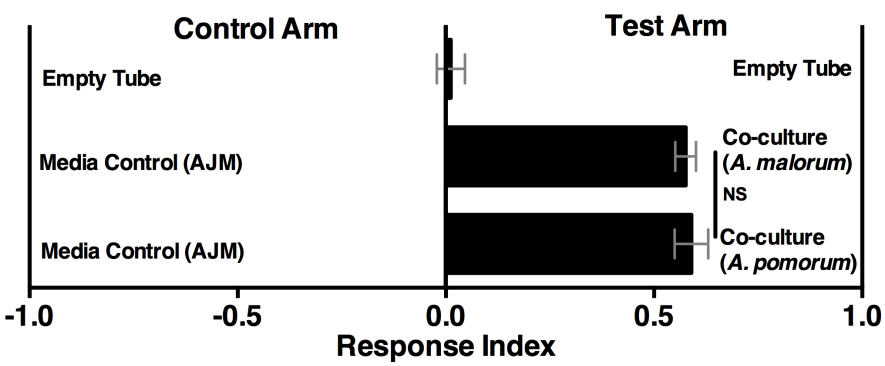

Figure 5-figure supplement 1. *Drosophila* behavior toward the co-culture using *A. malorum* or *A. pomorum*. *Drosophila* behavior toward co-cultures grown for 96 h using *A. malorum* or *A. pomorum* versus a media control (AJM = apple juice medium). Result of two experiments with six replicates each. Data points represent mean ± SEM. An unpaired two-tailed t-test assessed whether *Drosophila* behavior toward the co-cultures was different (NS $p > 0.05$).

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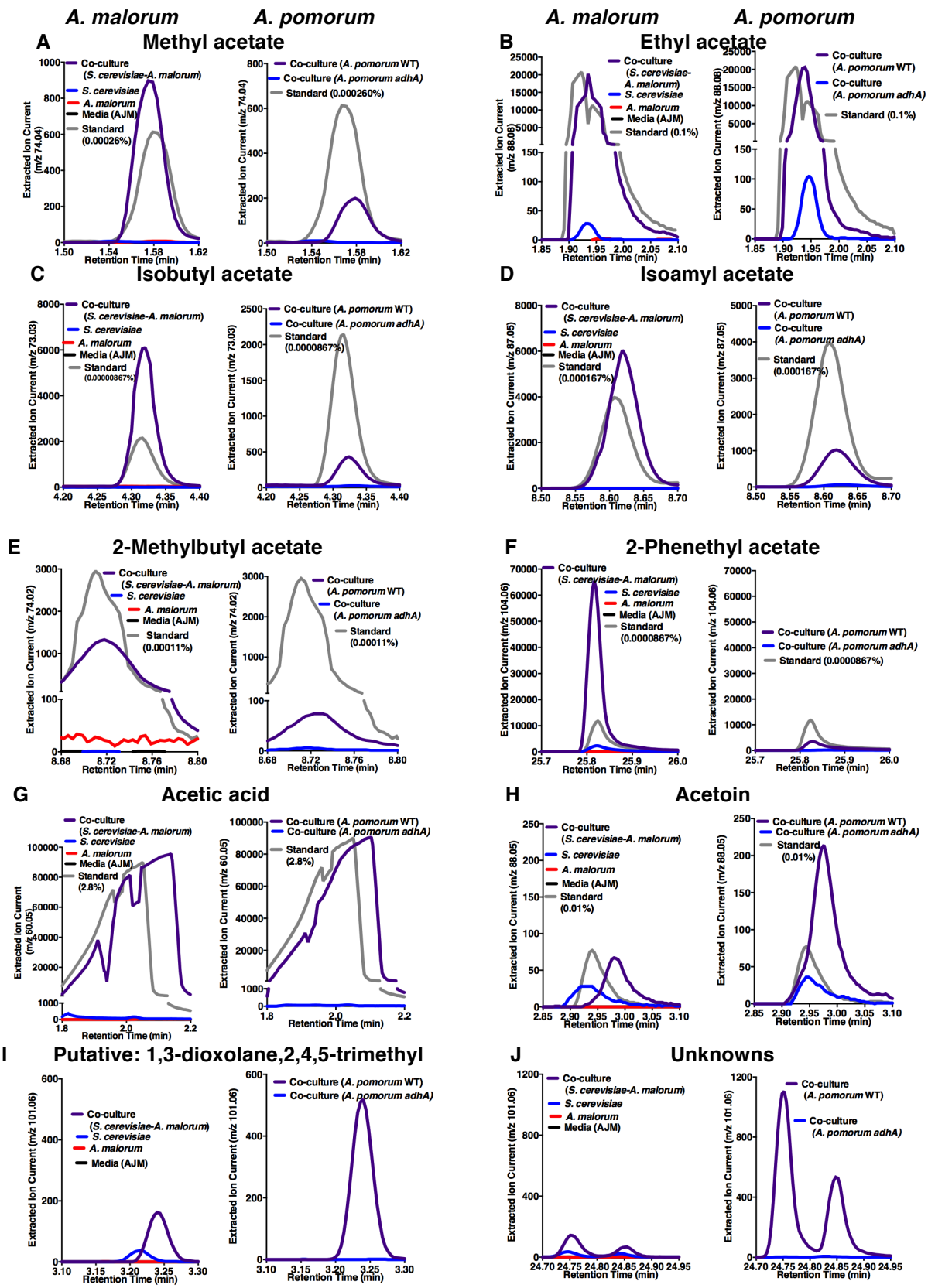

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Table 2-figure supplement 1. Extracted ion chromatograms of differentially emitted or unique metabolites in the co-culture according to solid phase microextraction gas chromatography-mass spectrometry (SPME GC-MS). Specific metabolites are displayed above each panel. For each panel, the left-most plot compares the co-culture containing *S. cerevisiae* and *A. malorum* to *S. cerevisiae* grown alone, *A. malorum* grown alone, or media (AJM (apple juice medium)); the right-most plot compares the co-culture containing *S. cerevisiae* and *A. pomorum* wild-type to the co-culture containing *S. cerevisiae* and *A. pomorum adhA*, since *A. pomorum adhA* is required for *Drosophila* co-culture preference (Figure 5A). The two plots within the same panel contain the same standard. The y-axis for each plot is the ion current for a m/z value that discriminates the metabolite of interest over a specific retention time window. The following m/z values were chosen for each metabolite based on standards or, in the cases of putative and unknown metabolites (**I** and **J)** were chosen from the experimental groups: **(A)** m/z 74.04 **(B)** m/z 88.08 **(C)** m/z 73.03 **(D)** 87.05 **(E)** 74.02 **(F)** 104.04 **(G)** 60.05 **(H)** 88.05 **(I)** 101.06 **(J)** 101.06. Each panel is one representative replicate of 1 experiment (out of 3-5 total replicates in three experiments).

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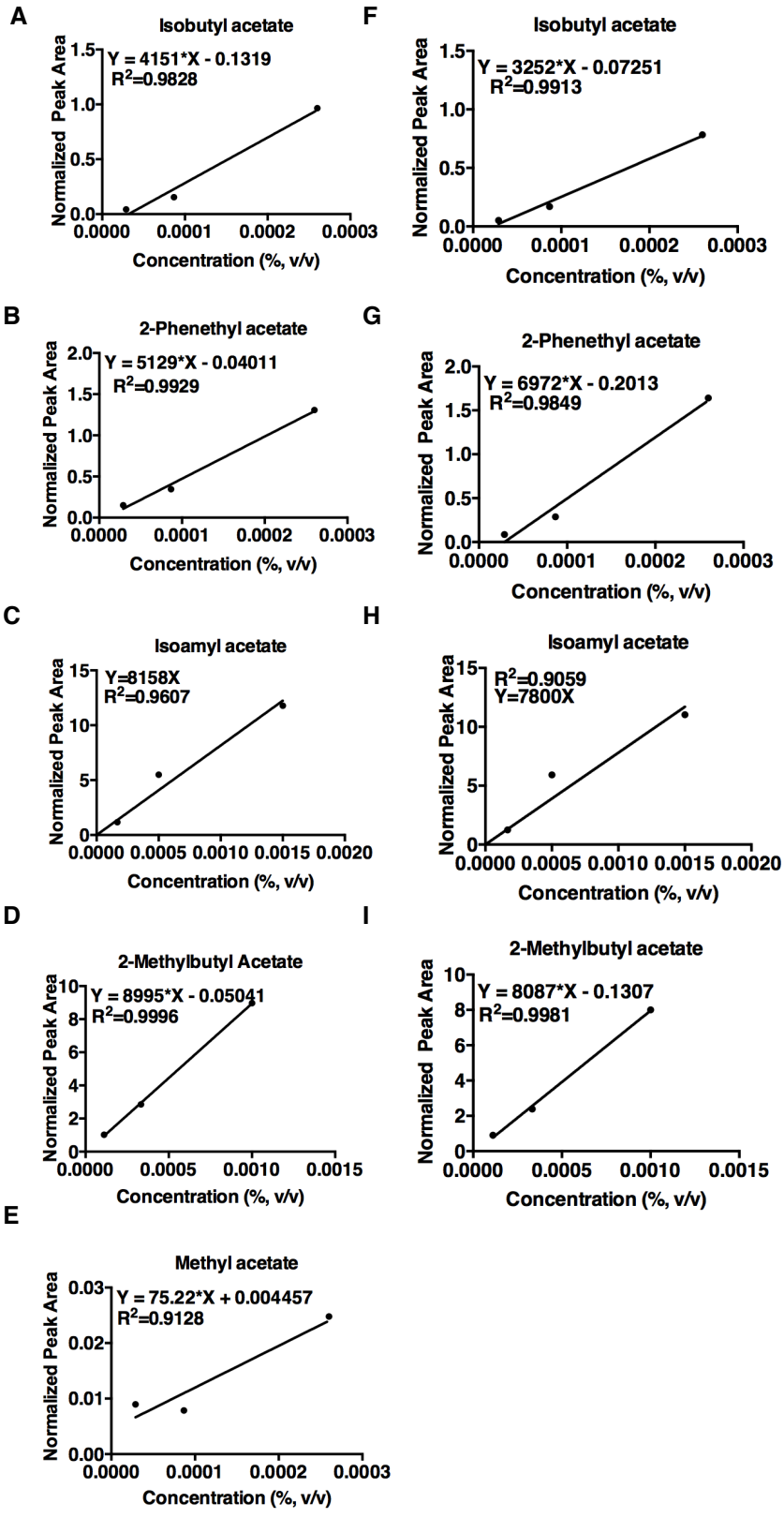

Table 2-figure supplement 2. Normalized peak areas corresponding to metabolites in a defined metabolite mixture (from SPME GC-MS). A linear regression was calculated to quantify the metabolites in the co-culture. Each concentration is from one replicate. **A-E** and **F-I** are two separate experiments. Linear regression was used to estimate the concentration of the metabolites in the co-culture containing *S. cerevisiae* and *A. malorum* (Table 2) and to complement the co-culture containing *A. pomorum adhA* (Figure 5A).

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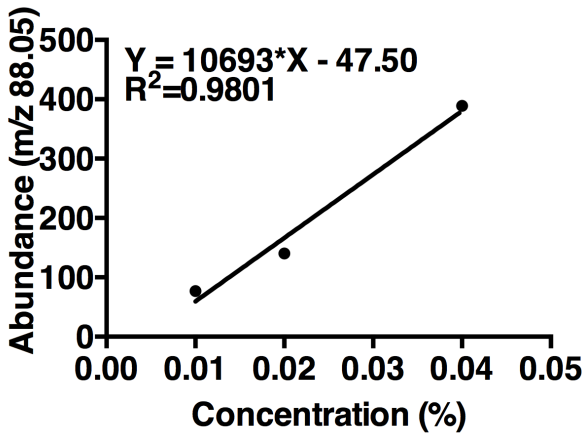

Figure 6-figure supplement 1. Acetoin linear regression. The curve is based on maximum m/z values (88.05) of three concentrations of acetoin. One replicate per concentration (n=1 experiment). The linear regression was used to estimate acetoin concentrations in the tri-culture and the co-culture (Figure 6B).

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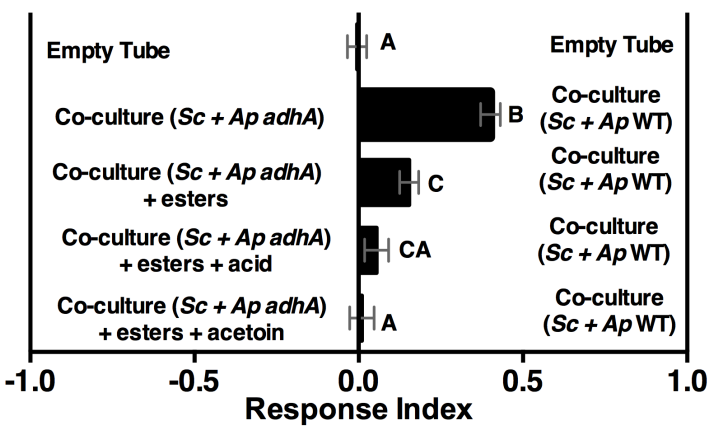

Figure 6-figure supplement 2. Role of esters alone or esters with acetoin in complementing co-culture with *A. pomomrum adhA*. Mean +/- SEM of three experiments with 16-18 total replicates. A one-way ANOVA assessed whether groups were different. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. Esters include ethyl acetate, isoamyl acetate, 2-phenethyl acetate, isobutyl acetate, 2-methylbutyl acetate, and methyl acetate. Esters added are based on physiological amounts in co-cultures and are calculated in Table 2 and Table 2-figure supplement 2). Acetoin is added in a similar amount as the tri-culture (Figure 6A, 0.3%). *Sc = S. cerevisiae, Ap = A. pomorum*.

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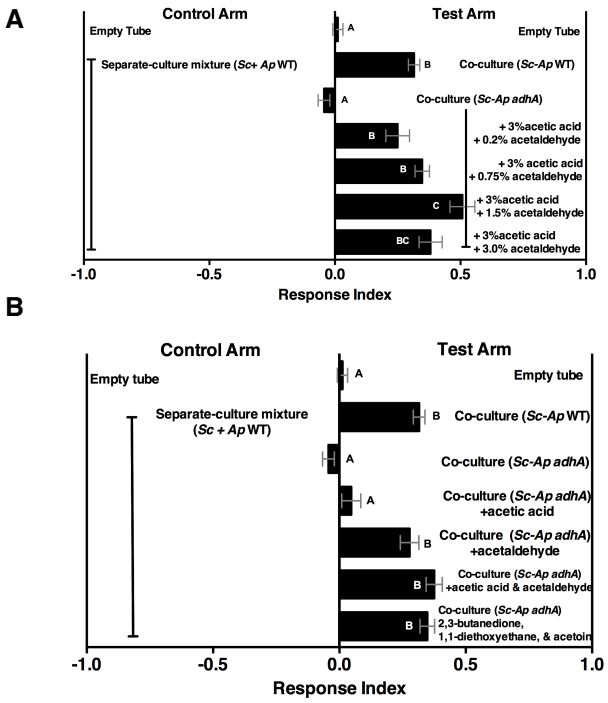

Figure 6-figure supplement 3. Acetaldehyde metabolic derivatives can complement the co-culture containing *A. pomorum adhA*, although their physiological concentrations are unknown. **(A)** Dose response of acetaldehyde was given to the co-culture containing *A. pomorum adhA* (along with a constant dose of 3.0% acetic acid). Metabolite additions were added to the culture in the noted percentages, allowed to sit 35 minutes at room temperature, mixed 1:1 in water, and assessed for *Drosophila* attractivess. Mean ± SEM of 1-7 experiments with 12-42 total replicates per group. The experimental groups from one experiment were run in two separate groups during the day. Differences between groups were tested by one-way ANOVA. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. **(B)** Role of acetic acid, acetaldehyde, or specified acetaldehyde metabolic derivatives in complementing the co-culture containing *A. pomorum adhA.* Acetic acid (3.0%) and acetaldehyde (0.75%) were added and allowed to sit at room temperature for 35 minutes, mixed 1:1 in water, and *Drosophila* attraction was assayed. 2,3-butanedione (0.15%), 1,1-diethoxyethane (0.01%), and acetoin (0.15%) were added to the culture, mixed 1:1 with water, and *Drosophila* behavior was assayed. Mean ± SEM of 2-7 experiments with 5-6 replicates per experiment. Differences between groups were tested by one-way ANOVA. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. *Sc = S. cerevisiae, Ap = A. pomorum*.

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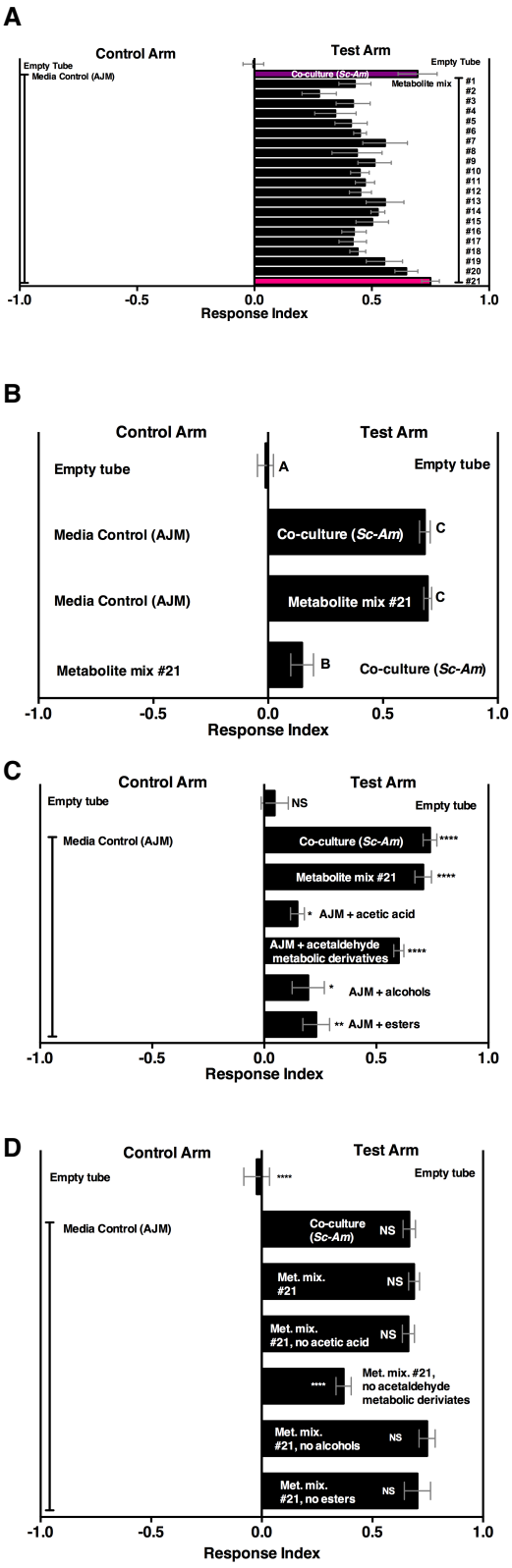

Figure 6-figure supplement 4. *Drosophila* behavior toward metabolite mixtures and their component parts **(A)** *Drosophila* behavior toward 21 metabolite mixtures versus AJM (50% in water). Methods Table 4 contains the concentrations of all mixtures (in 50% AJM). The co-culture (*S. cerevisiae* and *A. malorum*, purple bar) was grown for 96 h. The pink bar is metabolite mixture #21 and most closely resembled the co-culture. Mean ± SEM of 4-6 replicates per experimental group. Groups were run over five different days. **(B)** Relative *Drosophila* attraction to a co-culture grown for 96 h and metabolite mixture #21. Mean ± SEM of three experiments with 17-18 replicates per group. One-way ANOVA assessed whether the groups were different. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. **(C)** The sufficiency of individual groupings within metabolite mixture #21 to attract *Drosophila*. The individual grouping are as follows: acetaldaldehyde metabolic derivatives (1,1-diethoxyethane; acetoin; 2,3-butanedione); alcohols (ethanol; isobutanol; isoamyl alcohol; 2-methyl, 1-butanol; benzeneethanol); esters (isoamyl acetate; ethyl acetate; isobutyl acetate; 2-phenethyl acetate; butyl acetate; 2-methylbutyl acetate; methyl acetate; phenethyl benzoate; propyl acetate; ethyl isobutyrate; ethyl hexanoate; isovaleric acid; butyl ester; ethyl octanoate; ethyl decanoate; ethyl laurate); and acetic acid (acetic acid). Concentrations are in Methods Table 4**.** Mean ± SEM of six replicates of one experiment (except the acetaldehyde metabolic derivative group which is 12 replicates from 2 experiments). A t-test against a hypothetical value of 0 was performed. $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$ **(D)** The same groups used in **C** were used and removed from metabolite mix #21. The difference between the co-culture (*Sc-Am*) and each group was assessed by one-way ANOVA. A Tukey's multiple comparison test was used to assess significant differences between groups ($p \leq 0.05$), as indicated by unique letters. Mean ± SEM of six replicates of one experiment are shown.

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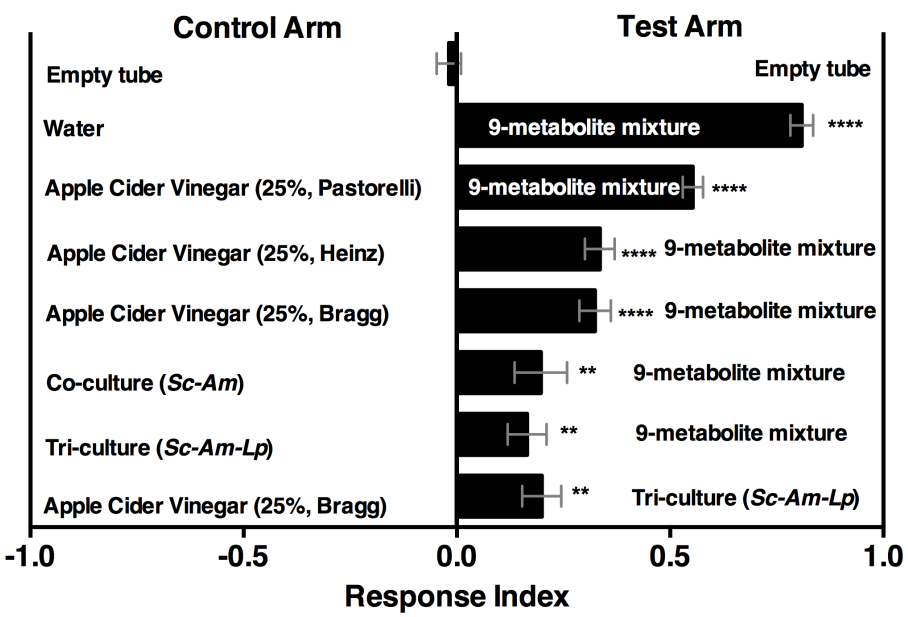

Figure 6-figure supplement 5. *Drosophila* behavior toward water amended with 9 metabolites (9-metabolite mixture) versus 3 different apple cider vinegars (ACV), a co-culture (*Sc-Am = S. cerevisiae* and *A. malorum*), or tri-culture (*Sc-Am-Lp = S. cerevisiae, A. malorum*, *L. plantarum* cs). Cultures were grown for 72 h and mixed 1:1 with water, as in all other experiments. Data points represent the Mean ± SEM of two experiments with twelve total replicates. A one-way ANOVA assessed whether each group was different from the mock condition (empty tube versus empty tube). $p > 0.05$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$. The acetoin concentration was similar to that calculated from the tri-culture (Figure 6A, 0.3%). The concentrations of all 9 metabolites can be found in the materials and methods.

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MATERIALS AND METHODS

Fly maintenance

Fly stocks, genotypes, and sources are listed in Methods Table 1. *Drosophila melanogaster* was reared at 25°C on a 12h:12h light: dark cycle on autoclaved food (5% yeast, 10% dextrose, 7% cornmeal, 0.6% propionic acid, 0.7% agar).

Microbial strains

Microorganisms used in this study are listed and described in table S1. Microorganisms were streaked onto yeast-peptone dextrose (YPD; 1% yeast extract (Becton Dickinson, and Company, Franklin Lakes, NJ, USA), 2% peptone (Becton Dickinson, and Company, Franklin Lakes, NJ, USA), and 2% dextrose (Avantor Performance Materials, Center Valley, PA, USA)) or Man, de Rosa, Sharpe (MRS, Fisher Scientific, Waltham, MA, USA) plates from a freezer stock.

T-maze olfactory attraction assays

The T-maze apparatus was a kind gift of the Carlson Laboratory. Flies were wet-starved for 15-26 hours prior to T-maze olfactory experiments by placing flies into vials containing Kimwipes (Kimberly Clark, Dallas, TX, USA) soaked with 2 mL of milliQ water. Flies were collected within 4 days (<65 flies per vial) of emergence and matured on autoclaved food. Flies between 3 and 10 days-old were used in experiments.

Single microbial colonies were picked from rich media (MRS and YPD) plates and grown overnight. Cultures were washed 1X in PBS, diluted 100-fold, and 10μl was aliquoted into 3mL of apple juice media (AJM, apple juice (Martinelli's Gold Medal, Watsonville, CA, USA), pH adjusted to 5.3 with 5M NaOH, with 0.5% yeast extract). Media was filtered with a 0.22 μM-size pore attached to a 250-mL polystyrene bottle (Corning, NY, USA). For co-culture experiments, 1e3-1e5 CFU of each microorganism was placed simultaneously into AJM. Microorganisms were grown in 14-mL round bottom polypropylene tubes (Corning Science, Tamaulipas, Mexico) at 28°C, 200rpm for the time noted in individual experiments. The

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983 microbial culture was diluted 1:1 with sterile milliQ water (0.22 μM filter (Millipore, Billerica, MA, USA))
984 and placed directly onto autoclaved 10 mM round Whatman filter paper (GE Healthcare Life Science,
985 Pittsburgh, PA, USA) placed near the bottom of 15mL CentriStar centrifuge tubes (Corning, NY). A total
986 volume of 10 μl was used for all experiments.
987
988 Tubes containing microorganisms and *Drosophila* were placed into the behavioral room (20-25°C, 50-
989 70% humidity maintained by a humidifier (Sunbeam Tower Humidifier, Boca Raton, FL, USA) and
990 equilibrated for 10 minutes prior to the beginning of the experiment. Flies (~40-130) were knocked into the
991 T-maze apparatus, rested for ~1 min, and then allowed to choose from the test and control arms for 2 min
992 in the dark. The test arm was alternated from one side of the apparatus to the other every experimental
993 replicate. A Response Index (RI) was computed to analyze preference for the test arm (flies in test arm-
994 flies in control arm)/(total flies).
995
996 *Chemicals*
997 Chemicals can be found in Methods table 2**.**
998
999 *Microbial populations and pH*
1000 Selective plates were used to distinguish *S. cerevisiae* from *A. malorum*. MRS containing 50 μg/mL
1001 cycloheximide selected for *A. malorum* while MRS containing 10 μg/mL chloramphenicol and 20 μg/mL
1002 tetracycline selected for *S. cerevisiae*. pH of filtered cultures (0.22 μM) was measured using a Beckman
1003 Coulter pH meter (Model Phi510, Fullerton, CA, USA).
1004
1005 *Gas chromatography- Mass spectrometry*
1006 Microbial samples were grown in AJM for a specified amount of time in 14-mL round bottom tubes fitted
1007 with an autoclaved tissue strainer (250 μM nylon mesh (Thermo Scientific Pierce, Grand Island, NY)
1008 holding between 0.03 and 0.05 grams of autoclaved Amberlite XAD-4 resin (Sigma-Aldrich, St Louis, MO,
1009 USA) prewashed in water and methanol. After microbial growth, XAD-4 from two cultures was dumped

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1010 into an autoclaved glass vial. XAD-4 was swirled with 900μl methanol for 30 seconds. 500-750μl of
1011 methanol was removed for GC-MS analysis. Quantification for table S2 was derived from beads
1012 suspended above the cultures from 84-96 h of growth. Quantification for Fig. 2D was derived from beads
1013 suspended above the culture every 12 h; time points on the graph refer to the end point of the 12-h span
1014 (e.g. 84 h corresponds to beads suspended from 72-84 h of growth).
1015
1016 Samples (5 μl of methanol-extracted samples) were injected into the GC-MS (Agilent 7890A/5975C) at
1017 250 °C using helium as the carrier gas at a flow rate of 1.1 mL per minute (column head pressure 13 psi).
1018 The following chromatography temperature program was used for experiments to initially identify
1019 metabolites in the co-culture and individually grown microorganisms: 40°C for 3 min ramped at 1.7°C per
1020 minute to 200 °C (held for 3 min) then to 220°C at 3 °C per min and held for a further 5 min. The total run
1021 time was 111.78 min. For experiments focused on the five major metabolites, a shorter program was used
1022 that maintained the same first 10 min of the previous method (all 5 volatiles eluted within 9 min). The
1023 chromatography temperature program was 40°C for 3 min, ramped at 1.7°C per min to 46.8°C and held
1024 for 3 min, then ramped at 60°C per min until 220°C and held for 5 min. The total run time was 17.9 min.
1025
1026 The mass spectrometer was run in electron-impact (EI) mode at 70 eV. The temperatures of the transfer
1027 line, quadrupole, and ionization source were 220°C, 180°C, and 230°C respectively. The ionization was
1028 off during the first 4 min to avoid solvent overloading with a source temperature of 230°C. Mass spectra
1029 were recorded in the range of 35-300 m/z. Full scan mode was used at a scan rate of 6 scans/sec. The
1030 electron multiplier voltage was set in the relative mode to autotune procedure.
1031
1032 In the initial experiments peaks were manually picked using Agilent Chemstation Software. Volatiles
1033 associated with peaks were searched against the National Institute of Standards (NIST) 11 database.
1034 Subsequent experiments focused on the 5 major volatiles identified in the initial experiments by
1035 performing extracted ion chromatograms using an ion that successfully identified a standard at a specific
1036 retention time. Quantification was performed by tabulating the maximum abundance of the ion at a

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1037 characteristic retention time and using a linear regression equation from a dose-response of the
1038 standards (fig. S6).
1039
1040 *Headspace solid phase microextraction (SPME) Gas chromatography- Mass spectrometry*
1041 A Waters GCT Premier gas chromatography time of flight mass spectrometer (Milford, MA) with a DB-
1042 5MS column (30m x 0.25 mm ID x 0.25 µm film thickness; Agilent) was used. Live cultures were
1043 transferred to autoclaved glass vials (20 mL, 23 X 75 mm, Supelco, Bellefonte, PA, USA) with screw caps
1044 (18mm, 35 Shore A, Supelco, Bellefonte, PA, USA) after growing for 72 h.
1045
1046 The glass vials containing live microbial cultures were analyzed via a 50/30µm
1047 carboxen/divinylbenzene/polydimethylsiloxane Stableflex solid-phase micro-extraction (SPME) fiber. The
1048 extraction methodology was based on previous studies using SPME to extract volatiles form vinegars (65,
1049 66). The syringe was inserted through the membrane of the caps and sampled the volatiles for 30 min at
1050 45°C; subsequently, metabolites were desorbed for 30 sec at 240C and baked for an additional 4.5 min in
1051 the injection port. The gas chromatograph was fitted with a microchannel plate (MCP) detector. The
1052 temperature program of the column was as follows: 40°C for 5 min, 2°C a min for 17.5 min followed by
1053 25°C a min for 10 min. A final hold time of 5 min at 325°C was used. The carrier gas was helium. A split
1054 ratio of 250 was used based on better peak resolution. An internal standard of cineole (Sigma-Aldrich, St.
1055 Louis, MO, USA) was run with each sample and used to compute relative abundances. The mass
1056 detector was in the range of 40 to 650 m/z.
1057
1058 To analyze the data, MassLynx software was used. The response threshold was set to an absolute area
1059 of 10.00. The software automatically picked out peaks and computed peak areas. To obtain a relative
1060 quantification, peaks were compared across samples and normalized to the internal standard. Peaks
1061 were first searched against the NIST5 database to identify potential hits. Most potential metabolites were
1062 confirmed by a standard mixture in 50% AJM. The standard mixtures are in Methods Table 3.
1063

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Chemical complementation of co-culture containing A. pomorum PQQ-ADH-I

A co-culture containing *S. cerevisiae* and *A. pomorum* WT or *A. pomorum PQQ-ADH-I* was grown for 72 h before use in the T-maze. For the physiological concentrations of acetate-derived metabolites, concentrations were added as in table S3 and then mixed 1:1 with water prior to behavioral analysis. For the acetaldehyde metabolic derivatives chemical complementation group, a 1:1 mixture of the mutant co-culture: water was supplemented with 1,1-diethoxyethane, 2,3-butanedione, and acetoin at final concentrations of 0.01%, 0.15% and 0.15%, respectively and used immediately in the T-maze. Acetic acid and/or acetaldehyde were added to the culture, allowed to sit at RT for 35 min, mixed 1:1 with water and then placed into the T-maze vials.

Standard curves were used to calculate the concentrations of individual metabolites (fig. S10). The standard curves were generated on 2 separate experiments in which 3 concentrations of each standard was used. The concentrations of the metabolites were independently calculated from the standard curve equations generated on the two separate days. Estimated concentrations from each standard curve equation were averaged (table S3). The experimental data is based on the peak areas of the *S.cerevisiae-A. malorum* co-culture.

Ester, acid, and acetaldehyde metabolic derivative mixture

The 9-metabolite mixture contains 1.5% acetic acid, 0.0003% isoamyl acetate, 0.0003% 2-phenethyl acetate, 0.01% ethyl acetate, 0.002% ethyl lactate, 0.3% 1,1-diethoxyethane, 0.3% 2,3-butanediol, 0.3% 2,3-butanedione, and 0.3% acetoin in filtered milliQ water.

Drosophila *survival in the presence of ethanol and acetic acid*

Adult male flies (0-3 d-old) were collected and matured for 1 day on fly food. Flies were then placed into vials containing kimwipes with 5 mL of either Shields & Sang Insect Medium (Sigma, St. Louis, MO; positive control), MilliQ water (negative control), or MilliQ water with ethanol (9.4%), acetic acid (3.42%), or ethanol and acetic acid (1.4 and 2.8% respectively). Survival was assessed every 12 h for 7 d. For

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each condition 5 mL was given at 0 and 12 h and every 24 h thereafter. Experimental replicates were considered separate vials (5-6 per group). Each replicate contained 8-31 flies.

Egg-laying preference assay

Egg-preference assay was adapted from Joseph *et al* 2009 (67). Microbial cultures grown for 96 h were heated to 65°C for 10 min, mixed 1:1 with 1.6% agarose and poured into a 35 mm petri dish separated in two by a straight-edge razor blade. Flies were starved for ~18 h prior to the experiment. The 35 mm petri dish was placed within clear flat top boxes with dimensions 2 5/16" X 2 5/16" X 5 1/16" (TAP plastics, San Leandro, CA, USA). The test and control sides were alternated for each replicate. *Drosophila* aged 4-10 days (n=50-100) was allowed to lay eggs for 8 h. After the assay, the number of eggs on deposited on each choice was tabulated and an egg-laying index was computed analogously to the olfactory response index.

Data analysis

Data analysis was performed in Prismv6.0b. Specific statistical tests are noted for individual experiments.

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Methods Table 1. *Drosophila melanogaster* stocks used in experiments

Notation	Gene Mutant	Genotype	Source
CS	NA	w+-Canton-S	John Carlson Laboratory
Or83b	*Or83b*	w-Canton-S;;orco2	John Carlson Laboratory
Ir64a	*Ir64a*	w[1118]; Mi{ET1}Ir64a[MB05283]	Bloomington Stock Center
Ir75a	*Ir75a*	y[1] w[67c23]; Mi{ET1}Ir75a[MB00253]	Bloomington Stock Center
W-CS	NA	w-Canton-S	John Carlson Laboratory
Balancer	NA	w-CS;Sp/Cyo;Dr/TM3B	John Carlson Laboratory
Ir25a	*Ir25a*	w^{1118}; Mi{ET1}$Ir25a^{MB09568}$	Bloomington Stock Center
Ir8a	*Ir8a*	$Ir8a^{1}$; Bl^{1} L^{2}/CyO	Bloomington Stock Center
Ir76b	*Ir76b*	y1 w67c23; Mi{ET1}Ir76bMB00216	Bloomington Stock Center
Or42b	*Or42b*	w; or42b/or42b; +/+	John Carlson Laboratory
Or35a	*Or35a*	W; or35a/or35a; +/+	John Carlson Laboratory

Chemical	**Company**	**Location**
Paraffin Oil	EMD chemicals	Gibbstown, NJ, USA
Apple Cider Vinegar	Heinz	Pittsburgh, PA, USA
Apple Cider Vinegar (unpasteurized)	Bragg (Fig. 3C only)	Santa Barbara, CA, USA
Apple Cider Vinegar	Pastorelli (Fig. 3C only)	Chicago, IL, USA
Benzaldehyde	Sigma Aldrich	St. Louis, MO, USA
2-Methyl-1-Propanol (Isobutanol)	Sigma Aldrich	St. Louis, MO, USA
Isopentyl Acetate (Isoamyl Acetate)	Sigma Aldrich	St. Louis, MO, USA

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Isoamyl Alcohol	Avantor Performance Materials	Center Valley, PA, USA
Acetic Acid, glacial	Avantor Performance Materials	Center Valley, PA, USA
Methanol	Avantor Performance Materials	Center Valley, PA, USA
Ethanol, 200 proof	Decon Laboratories	King of Prussia, PA, USA
Acetal (1,1, diethoxyethane)	Fisher	Pittsburgh, PA, USA
Acetaldehyde	Acros Organics	Grand Island, NY
Ethyl acetate	Sigma Aldrich	St. Louis, MO, USA
1-pentanol	Sigma Aldrich	St. Louis, MO, USA
Benzeneethanol (2-phenylethanol)	Fisher	Pittsburgh, PA, USA
Isobutyl acetate	Sigma Aldrich	St. Louis, MO, USA
2-phenethyl acetate	Sigma Aldrich	St. Louis, MO, USA
Butyl acetate	Sigma Aldrich	St. Louis, MO, USA
2-methylbutyl acetate	TCI	Tokyo, Japan
Methyl acetate	Sigma Aldrich	St. Louis, MO, USA
Acetoin acetate	Sigma Aldrich	St. Louis, MO, USA
Phenethyl formate	Sigma Aldrich	St. Louis, MO, USA
Phenethyl benzoate	Sigma Aldrich	St. Louis, MO, USA
acetoin	Sigma Aldrich	St. Louis, MO, USA
2,3-butanedione	Sigma Aldrich	St. Louis, MO, USA
2-methyl-1-butanol	Sigma Aldrich	St. Louis, MO, USA
Ethyl lactate	Sigma Aldrich	St. Louis, MO, USA
1-hexanol	Sigma Aldrich	St. Louis, MO, USA
Phenylacetic acid	Sigma Aldrich	St. Louis, MO, USA
2,3-butanediol	Sigma Aldrich	St. Louis, MO, USA
Ethyl octanoate	Sigma Aldrich	St. Louis, MO, USA
Ethyl decanoate	Sigma Aldrich	St. Louis, MO, USA

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Ethyl hexanoate	Sigma Aldrich	St. Louis, MO, USA
Ethyl laurate	Sigma Aldrich	St. Louis, MO, USA
Ethyl isobutyrate	Sigma Aldrich	St. Louis, MO, USA
phenylacetaldehyde	Sigma Aldrich	St. Louis, MO, USA
Butyl isovalerate	TCI	Tokyo, Japan
Propyl acetate	Sigma Aldrich	St. Louis, MO, USA

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Methods Table 3. Metabolite mixture concentrations used for identification and quantification in SPME GC-MS

Metabolite	**Met mix 1**	**Met mix 2**	**Met mix 3**
Ethanol	0.20%	1.00%	2.00%
Isobutanol	0.0040%	0.0010%	0.000250%
Acetic acid	2.80%	4.320%	1.920%
Isoamyl alcohol	0.00150%	0.0030%	0.0060%
Isoamyl acetate (1-butanol, 3-methyl acetate)	0.00050%	0.000167%	0.00150%
Acetal (1,1-Diethoxyethane)	0.020%	0.10%	0.0050%
Acetaldehyde	0.020%	NONE	0.0040%
Ethyl acetate	0.0050%	0.10%	0.020%
Benzeneethanol (2-phenylethanol)	0.00360%	0.00040%	0.00120%
Isobutyl acetate	0.000260%	0.0000867%	0.0000289%
2-Phenethyl acetate	0.000260%	0.0000867%	0.0000289%
2-Methylbutyl acetate (1- butanol 2-methyl acetate)	0.0010%	0.00033%	0.00011%
Methyl acetate	0.0000867%	0.0000289%	0.000260%
2-Methyl, 1-butanol	0.00030%	0.00150%	0.00450%
2,3-Butanedione	NONE	0.010%	0.0020%
Acetoin (1g/10mL h20, 10%)	0.01%	0.020%	0.040%

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2,3-Butanediol	NONE	0.10%	0.020%
Propyl acetate	0.000020%	0.0000020%	NONE*

*1/2 experiments had a concentration of 0.0000

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Methods Table 4. Composition of metabolite mixtures 1-21 used in figure S14

Volatile	Metabolite mixture																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Ethanol	0.5700	0.5700	0.5700	0.5700	0.5700	0.5700	0.5700	0.5700	0.5700	0.5700	0.2850	0.2850	0.2850	0.2850	0.2850	0.2850	0.5700	0.5700	0.5700	0.5700	0.5700
Isobutanol	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0004	0.0004	0.0004	0.0004	None	0.0004	0.0005	0.0007	0.0007	0.0007	0.0007
Acetic acid	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400	1.4400
Isoamyl alcohol	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0005	0.0005	0.0005	0.0005	0.0005	0.0002	0.0005	0.0005	0.0010	0.0010	0.0010	0.0010
Isoamyl acetate	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0010	0.0007	0.0005	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007	0.0007
1,1-diethoxyethane	0.0100	0.0300	0.0100	None	0.0033	0.0100	0.0100	0.0010	0.0200	0.0100	0.0050	0.0100	0.0300	0.0500	0.0500	0.0030	0.0030	0.0030	0.0100	0.0100	0.0100
acetaldehyde	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100	0.0100	0.0010	0.0100	0.0100	0.0050	0.0050	0.0050	0.0050	0.0025	0.0025	None	None	None	None	None
ethyl acetate	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0020	0.0010	0.0005	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
1-pentanol	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0005	0.0003	0.0005	0.0005	0.0005	0.0002	None	None	None	None	None	None
benzeneethanol	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0005	0.0003	0.0005	0.0005	0.0005	0.0002	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
isobutyl acetate	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0005	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
2-phenethyl acetate	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	None	0.0010	None	None	0.0005	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
butyl acetate	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	None	0.0010	None	None	0.0005	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
2-methylbutyl acetate	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0020	0.0010	0.0005	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
methyl acetate	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0005	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
2-acetoxy-3-butanone	None	None	0.0010	0.0010	0.0010	0.0010	None	0.0010	None	None	0.0005	0.0010	0.0010	0.0010	0.0010	None	None	None	None	None	None
phenethyl formate	None	None	0.0010	0.0010	0.0010	0.0010	None	0.0010	None	None	0.0005	None	None	None	None	None	None	None	None	None	None
phenethyl benzoate	None	None	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0010	0.0005	0.0010	0.0010	0.0010	0.0002	0.0010	0.0010	0.0007	0.0007	0.0007	0.0007
2-methy, 1-butanol	None	None	None	None	None	0.0010	0.0010	0.0010	0.0010	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005	0.0005
2,3butanedione	None	None	None	None	None	0.001	0.0010	0.0010	0.0010	0.0010	0.0005	0.0010	0.0030	0.0050	0.0050	0.0030	0.0030	0.0030	0.0100	0.0500	0.1500
acetion (10% m/v, H_20)	None	None	None	None	None	0.001	0.001	0.0010	0.0020	0.0010	0.0005	0.0010	0.0030	0.0050	0.0050	0.0030	0.0030	0.0030	0.0100	0.0500	0.150
propyl acetate	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0010	0.0007	0.0007	0.0007	0.0007
ethyl isobutyrate	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0010	0.0007	0.0007	0.0007	0.0007
ethyl hexanoate	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0010	0.0007	0.0007	0.0007	0.0007
isovaleric acid, butyl ester	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0007	0.0007	0.0007
ethyl octanoate	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0010	0.0007	0.0007	0.0007	0.0007
ethyl decanoate	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0010	0.0007	0.0007	0.0007	0.0007
ethyl laurate	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	None	0.0010	0.0007	0.0007	0.0007	0.0007

The percent composition (v/v) of the metabolite mixtures used in fig. S1

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