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Acetobacter pomorum in the *Drosophila* gut microbiota buffers against host metabolic impacts of dietary preservative formula and batch variation in dietary yeast

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Abstract

Gut microbiota are fundamentally important for healthy function in animal hosts. *Drosophila melanogaster* is a powerful system for understanding host-microbiota interactions, with modulation of the microbiota inducing phenotypic changes that are conserved across animal taxa. Qualitative differences in diet, such as preservatives and dietary yeast batch variation, may affect fly health indirectly via microbiota, and may potentially have hitherto uncharacterized effects directly on the fly. These factors are rarely considered, controlled, and are not standardized among laboratories. Here we show that the microbiota's impact on fly triacylglyceride (TAG) levels - a commonly-measured metabolic index - depends on both preservatives and yeast, and combinatorial interactions among the three variables. In studies of conventional, axenic and gnotobiotic flies, we found that microbial impacts were apparent only on specific yeast-by-preservative conditions, with TAG levels determined by a tripartite interaction of the three experimental factors. When comparing axenic and conventional flies, we found that preservatives caused more variance in host TAG than microbiota status, and certain yeast-preservative combinations even reversed effects of microbiota on TAG. Preservatives had major effects in axenic flies, suggesting either direct effects on the fly or indirect effects via media. However, *A. pomorum* buffers the fly against this effect, despite the preservatives inhibiting growth, indicating that this bacterium benefits the host in the face of mutual environmental toxicity. Our results suggest that antimicrobial preservatives have major impacts on host TAG, and that microbiota modulates host TAG dependent on the combination of the dietary factors of preservative formulation and yeast batch.

Importance

Drosophila melanogaster is a premier model for microbiome science, which has greatly enhanced our understanding of the basic biology of host-microbe interactions. However, often overlooked factors such as dietary composition, including yeast batch variability and preservative formula, may cofound data interpretation of experiments within the same lab and lead to different findings when comparing between labs. Our study supports this notion; we find that the microbiota does not alter host TAG levels independently. Rather, TAG is modulated by combinatorial effects of microbiota, yeast batch, and preservative formula. Specific preservatives increase TAG even in germ-free flies, showing that a commonplace procedure in fly husbandry

47 alters metabolic physiology. This work serves as a cautionary tale that fly rearing methodology
48 can mask or drive microbiota-dependent metabolic changes, and also cause microbiota-
49 independent changes.
50

51 Background

52 Fruitflies are a preeminent model for understanding fundamental host-microbiome
53 biology, thanks to experimental tractability, powerful genetic tools, and a simple microbiota
54 dominated by culturable *Lactobacillaceae* and *Acetobacteraceae* (1, 2). Flies can be routinely
55 made germ-free (axenic), or selectively reassociated with defined cultures of physiologically-
56 and ecologically-relevant microbiota (gnotobiotic). The fly microbiota is less complex than in
57 vertebrates, yet effects on a plethora of host traits are conserved (3-14), potentially indicating
58 common mechanisms that can be characterized rapidly in the fly.
59 The microbiota affect fly nutrition, and so variation in microbiota and diet have mutually-
60 interdependent effects (15). Brewer's yeast is included ubiquitously in fly diets (16). Importantly,
61 yeast is supplied commercially in lots originating from distinct production batches, with
62 potentially variable chemical composition. This potentially introduces nutritional inconsistencies
63 among distinct lots (16), that may modify response to microbiota manipulation.
64 Fly diets also commonly contain antimicrobial preservatives. Preservative formulae vary
65 both in composition and concentration, and in some microbiota studies they are omitted entirely
66 (10, 13, 14, 17). The commonly-used preservative nipagin (methylparaben) affects the density of
67 *Acetobacter* (18), which may alter growth in fly food, and thereby modify physiological impact.
68 Further, nipagin is dissolved in ethanol, which interacts with variation in the microbiota (19).
69 Acid preservatives are also used, which may modulate fly function through effects on the
70 microbiota (e.g. density, metabolic substrate provision), diet (e.g. pH and nutrient solubility (14,
71 20-22)) and direct effects on the fly (23).
72 Here we test whether physiological impact of altering the fly microbiota depends on
73 dietary yeast batch and preservatives. We used two lots of one supplier's yeast, denoted A or B.
74 We either omitted preservatives, or added (**1**) phosphoric acid and propionic acid (15), or (**2**)
75 nipagin and propionic acid (13). These ingredients were incorporated into an otherwise identical
76 sucrose-yeast-agar (SYA) diet (24). We measured triacylglyceride (TAG) levels, the main
77 storage lipid, which are commonly measured as a metabolic index due to interest in the
78 microbiome's role in human obesity (25). Within each experiment we normalized TAG to the
79 mean of axenic flies without preservatives, giving a measure of relative TAG.
80

81 Results

82

83 Host TAG is subject to a microbiota*yeast*preservative interaction

84 First, we applied a simple microbiome manipulation, comparing relative TAG in
85 conventionally-reared and axenic females, three days after adult emergence. We analyzed data
86 with ANOVA (Table 1) and *post-hoc* tests with Tukey corrections, implemented in the R
87 “emmeans” package (Table 2). TAG response to bacterial elimination depended on the
88 interaction of yeast batch and preservative formula (ANOVA:
89 bacteria*yeast*preservative $F_{2,106}$=3.73, p=0.03; Table 1). This interaction obscured the
90 anticipated main effect of increased TAG in axenics (ANOVA: bacteria $F_{1,106}$=0.54, p=0.46, Table
91 1), suggesting that microbial capacity to modulate TAG depends on a yeast*preservative
92 interaction. To examine specifically how, we stratified our analysis per yeast*preservative

combination. Without preservatives, on both yeasts, TAG was elevated in axenics (Table 2). Surprisingly, this response was reversed by a specific yeast*preservative combination, with conventionals showing higher TAG than axenics on yeast A and with preservative formula 2 (Table 2, Figure 1A). Furthermore, microbial manipulation did not affect TAG in any other condition including preservatives, on either yeast (Table 2). Interestingly, preservative formula 2 increased TAG even in axenic flies, but only on yeast B (Table 2), suggesting effects via fly or food. Further, the TAG levels were typically more variable when preservatives were present on both yeasts, and this variability was most pronounced on yeast B with preservative set 2 (Figure 1A).

Having identified significant interactions among experimental factors, we asked which of these effects were large and which were small, i.e. what was the relative contribution of each experimental factor and their higher-order interactions to overall variance? We calculated a measure of effect size (partial Eta^2) for each experimental variable and their interactions (Figure 1B). This indicated that preservatives were the biggest source of variance (Figure 1B). Confidence intervals overlapped for all other significant terms, suggesting equivalent contributions to overall variation. These results indicated that variation in preservatives, and their interaction with yeast batch, are a hitherto unappreciated factor that affect fly TAG, which can both eclipse and determine effects of microbiota.

***A. pomorum* buffers flies against a TAG-promoting effect of preservative set 2.**

The fly microbiota is dominated by two bacterial genera, with *Acetobacter* and *Lactobacilliaceae* exhibiting strain-specific effects on fly physiology (10). Monoassociation with *Acetobacter spp*., but not *Lactobacilliaceae,* recapitulates conventional fly TAG levels (10). In conventional flies, the effects of yeast and preservative could potentially be driven by either compositional changes in the microbiota, or bacterial physiological changes. We reasoned that compositional changes can be excluded if effects of yeast and preservatives are apparent in gnotobiotic flies monoassociated with a single strain, in which case strain-specific physiological effects might be expected because growth of *Acetobacter* but not *Lactobacilliaceae* is impacted by nipagin (18). Could yeast*preservative*microbiota effects on the fly be driven by particular bacterial strains?

We made gnotobiotic flies with *A. pomorum* (DmCS004) and *L. brevis* (DmCS003), and axenic controls, and modulated yeast and preservatives, to determine strain*yeast*preservative effects (Figure 1C), and analyzed TAG levels with ANOVA (Table 3) and *post-hoc* analyses (Tables 4-5). We used the same yeast and preservative set as in the first experiment. We also confirmed that there were no significant differences in standard curves for assays between the two experiments (Supplementary Text, Figure S2), confirming that our technical detection capacity was the same for the two different experiments. TAG response to bacterial elimination again depended on the interaction of yeast batch and preservative formula (ANOVA: bacteria*yeast*preservative $F_{4,162}$=4.96, p=0.0008; Table 3). Across all preservative and yeast conditions, *A. pomorum* gnotobiotes had lower average TAG than axenics and *L. brevis* gnotobiotes (Figure 1C).

We again calculated Partial Eta^2 (effect size), to indicate impact of experimental variables on overall variation in the experiment, i.e. which effects were significant and large, and which were significant but smaller. Partial Eta^2 indicated that preservative formula and bacterial strain were the leading contributors to TAG variation in this experiment (Figure 1D). The preservative*bacterial strain interaction had a substantially-sized (and statistically significant:

$p<2.2e^{-16}$, Table 3) effect, suggesting that variation in bacterial strain and preservatives conspired to produce sizeable variation. Altogether, these results indicated that (**1**) impacts of varying microbiota strains depend on yeast*preservative variation, (**2**) the lower-order preservative*bacterial strain interactions was a particularly large source of variation, and (**3**) the effect of changing preservatives is equivalent to the effect of perturbing the microbiota.

To assess strain-specific impacts of yeast*preservative, we stratified our ANOVA analysis by bacteria (Table 4), revealing yeast*preservative effects in gnotobiotes with *L. brevis* ($F_{2,162}$=9.577, p=0.0001), but not with *A. pomorum* ($F_{2,162}$=1.072, p=0.3446) or in axenic flies ($F_{2,162}$=1.623, p=0.2005). Preservative variation had a significant effect across all microbial conditions (Table 4), while yeast had no significant effect in any microbial condition (Table 4).

Why would a bacteria*yeast*preservative effect arise? We reasoned it could occur either because (A) a given bacterial strain modulates host TAG only on specific yeast*preservative conditions i.e. indirect effects of preservatives and yeast, or (B) yeast*preservative conditions affect host TAG, but this effect is buffered by specific bacteria, i.e. direct effects of preservatives and yeast, dependent on microbiota. The finding that yeast*preservative effects were apparent in axenic and *L. brevis*-associated flies suggested that *A. pomorum* may indeed buffer an effect of yeast*preservative variation that is apparent in axenic and *L. brevis*-associated flies. We noted that preservative set 2 appeared to elevate TAG levels in axenic and *L. brevis*-associated flies, but not *A. pomorum*-associated flies (Figure 1C: noting that in the first experiment Axenic TAG was elevated on Yeast B but not Yeast A), suggesting that *A. pomorum* may buffer a TAG-promoting effect of these preservatives, in which case TAG should be significantly elevated by these preservatives in axenic or *L. brevis*-associated flies, but not in *A. pomorum*-associated flies. We tested this prediction using *post hoc* pairwise tests (Table 5), and found that indeed these preservatives significantly elevated TAG in axenic or *L. brevis*-associated flies, but not in *A. pomorum*-associated flies: in fact, in the presence of *A. pomorum*, the effect of these preservatives was reversed, moderately decreasing TAG. This suggested that *A. pomorum* abrogates a TAG-promoting effect of nipagin and propionic acid contained in preservative set 2.

Elevated TAG in axenic or *L. brevis*-associated flies suggested that the impact of varying microbial association may be contingent on preservatives and yeast. Specifically, we predicted that the impact of *A. pomorum* would be greater on preservative set 2, because the starting TAG levels in axenic flies were elevated, and these effects are not rescued by *L. brevis*. We ran F tests for the effect of microbiota status on each yeast*preservative combination (Table 6), and found that indeed F ratios (a measure of effect size) were markedly greater on preservative set two (yeast A, F=58; yeast B F=106), than either set one or no preservatives (all <10.5). To confirm that this was due to *A. pomorum*, we ran a series of post-hoc tests. We stratified the analysis by yeast and preservatives, and measured pairwise differences in TAG levels among the microbial conditions. As anticipated, t-ratios for the difference between *A. pomorum* and *L. brevis* conditions, or *A. pomorum* and axenic conditions, were greater on medium containing preservative set 2 than either set 1 or no preservatives (Table 7). Therefore, we expected that the overall effect of preservative variation would be lesser in the presence of *A. pomorum* than in the presence of *L. brevis*, or in axenic flies. As expected, when we stratified the analysis by yeast and bacteria, F-ratios for effect of preservatives in were substantially reduced by *A. pomorum* association, relative to axenic flies (~3x lower on yeast A, ~8x lower on yeast B), and relative to *L. brevis*-associated flies (~3x lower on yeast A, ~14x lower on yeast B) (Table 8). Previous reports suggested that *Acetobacter* are nipagin-sensitive (18), however our present results indicated any that *A. pomorum* nipagin sensitivity did not translate into impaired modulation of

host TAG: rather, this strain rescued flies from a TAG-promoting effect of the nipagin-containing preservative set 2.

To determine if our strains were indeed differentially sensitive to the two preservative formulae, we quantified bacterial colony forming units (CFU) from gnotobiotic adult flies (Figure S1). One implication of the yeast effects we have documented is that experiments within a given laboratory will be confounded when a given yeast batch is exhausted. In our case, we ran out of yeasts A and B, and could not obtain any more. Therefore, we used three new yeast batches (C-E), to quantify CFU over a wide range of yeast conditions, asking whether CFUs vary by yeast*preservatives and whether these effects are strain-specific. We confirmed that there was indeed a bacteria*yeast*preservative effect (Table 9, GLM with negative binomial distribution, joint tests: $F=11.63$, $p=1.18e^{-07}$). Next, we applied *post-hoc* tests to assess impacts of preservatives, per yeast and per bacterium, and determined that both preservative sets reduced *A. pomorum* CFUs relative to no preservatives, but this effect was consistently bigger with nipagin-containing set 2 (Table 10). T-ratios for the preservative set 2 vs. no preservative comparison for each yeast batch were lower than those comparing set 1 vs. no preservatives per each yeast batch for *A. pomorum* CFUs. Further, there were consistently significantly more *A. pomorum* CFUs on preservative set 1 than set 2 (Figure S1), supplementing previous findings that nipagin limits *Acetobacter* growth. Taken together, these findings along with fly food preservatives can affect fly physiology directly, the nature of this effect can depend on batch variation in dietary yeast, but specific bacteria can abrogate these deleterious effects despite themselves enduring negative effects of the preservatives.

Discussion

Our study suggests that microbial regulation of fly TAG is highly dependent not only on media preservatives and constituent yeast batch, but also the yeast*preservative interaction. A specific combination of yeast and preservative formula was even sufficient to reverse the effect of microbial elimination in conventionally-reared flies, producing a distinct experimental outcome. Preservative formula interfered with microbial effects particularly strongly, with potential to block microbial regulation of host TAG. The data suggest that these effects are mediated by an impact of nipagin and propionic acid, either directly on the fly or via fly food, which is safeguarded against by *A. pomorum* (but not *L. brevis*), despite a cost to the bacteria themselves of compromised growth on the preservatives. These overlooked factors appear to be significant determinants of microbiota-dependent fly phenotypes and bacterial strain colonization densities, as well as major causes of microbiota-independent variation. Factors that we have not measured, such as dietary sugar (15) may further influence these complex interactions.

Our results have implications for future fly research, and not only in the microbiota field. Sparse methodological detailing of diet is a persistent problem, e.g. with methods reporting "standard media", when media can in fact vary widely among labs. Preservatives are sometimes not reported, and yeast batch variation receives little attention in the lab or literature. Yet our results indicate that these variables can determine experimental outcomes, with implications for repeatability. Our results are consistent with the suggestion that variability among labs may result from yeast batch variation (26). We suggest that diet standardization (e.g. chemically-defined diet, or chemostat-cultured yeast) may mitigate these potential confounding factors. Further studies are required to systematically determine how experimental contexts determine outcomes of manipulating the microbiota.

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236 for helpful comments on the manuscript.
237
238 **Materials and methods**
239 **Fly rearing and bacterial culturing**
240 All flies, were from the Dahomey background, which were originally collected in Dahomey, now
241 Benin. They bore the *w1118* mutation and were free of the endosymbiont *Wolbachia*. All flies
242 (conventional, axenic, and gnotobiotes) were maintained at 25°C on a 12hr light/dark cycle. SYA
243 fly food was composed of 5% sucrose (Fisher), 10% yeast (MP Biomedicals), and 1.5% agar
244 (Sigma). For the first two experiments, six different SYA diets were used, varying in yeast batch,
245 either lot number S4707 (Yeast A) or SR03010 (Yeast B). From each batch, no preservative food
246 was made, or food containing preservative set 1 (0.04% phosphoric acid and 0.4% propionic
247 acid), or preservative set 2 (0.3% nipagin and propionic acid). This was repeated for the next set
248 of experiments looking at the bacterial densities in each fly. For these experiments the following
249 yeast lot numbers were used: S6853 (Yeast C), S7760 (Yeast D), and U1122284494-1 (Yeast E).
250 *Levilactobacillus brevis* DmCS003 was grown and maintained in YPD medium at 30°C without
251 shaking, while *Acetobacter pomorum* DmCS004 was grown and maintained in M9 medium with
252 0.5% DL-lactic acid at 30°C with shaking at 250 rpm.
253
254 **CFU counts**
255 Flies were anesthetized 3 days post eclosion. For each condition, 6 replicates of 8 females were
256 aseptically collected and transferred to a sterile Eppendorfs containing 500µL 1X PBS. The flies
257 were homogenized using a sterile pestle, and subsequently serially diluted and plated from the
258 10^0 to the 10^3 dilutions. Plates that had 30-300 colonies were counted for CFU determination.
259 The CFUs were then calculated per fly and $\log_{10}$ transformed.
260
261 **Generation of axenic and gnotobiotic flies**
262 Flies were put in laying cages containing juice agar, transferred to a fresh cage, and allowed to
263 lay eggs for <18hrs. Eggs were collected using PBS and a brush into a sterile chamber with
264 netting. The chamber was incubated in 10% bleach for 3m, followed by 1m in sterile dH_2O, then
265 3m in 10% bleach, 1m in 100% ethanol, and lastly 1m in sterile dH_2O. Eggs were collected in
266 sterile 1X PBS and 20µL was pipetted into sterile T75 flasks with filter caps containing 60 mL of
267 each variation of theSYA diets. Those without bacteria added remained axenic. To generate
268 gnotobiotes, overnight bacterial cultures' OD_{600} were measured, normalized to an $OD_{600} = 1$, and
269 pelleted. The pellet was washed with sterile 1X PBS, resuspended to an $OD_{600} = 1$ in sterile 1X
270 PBS, and then diluted 1:5 to a final concentration of $OD_{600} = 0.2$. 200µL of each bacterium was
271 aseptically added to the surface of the SYA containing the sterilized eggs.
272
273 **TAG experiments**
274 The eggs were incubated for 10 days, by which adult flies emerged. They were then transferred
275 to sterile T75 flasks containing the appropriate diet. After 2 days on the diet (3 days post-
276 eclosion), flies were collected, sorted by sex, and females were collected. 10 groups of 5 females

277 were weighed and flash frozen in 2mL screw cap tubes containing 125µL of TEt Buffer (TE
278 buffer with 0.1% triton X-100). Flies were homogenized for 30s using a Bead Ruptor Elite bead
279 mill homogenizer at speed 6.5, incubated at 72ºC for 15m to inactivate endogenous lipases, and
280 spun down for 5m at 4°C at 12000xg. In a 96 well plate, 3µL of supernatant or standard was
281 mixed with 300µL of Infinity™ Triglycerides reagent (Thermo Scientific), and plates were
282 covered in foil and incubated at 37ºC for 15 minutes. The absorbance at 540nm was taken using
283 a ThermoScientific Multiscan FC plate reader. Standard curves were generated using an array of
284 9 glycerol standards ranging from 1-0µg/µL, and TAG levels were calculated from the best fit
285 line equation. TAG levels were normalized to the weight of the 5 flies.
286
287 **<u>Statistical analysis</u>**
288 All data were analyzed in R v4.2.1. Violin plots were produced using ggplot2.
289
290 For ANOVA analyses, linear models of the form
291
292 $$TAG \sim Bacteria * Yeast * Preservative$$
293
294 were fit using the base function lm, where TAG represented µg TAG normalized to mg fly mass,
295 Yeast represented yeast batch, and preservative represented preservative formula. In the first
296 experiment, Bacteria coded whether flies were axenic or conventionally-reared. In the second
297 experiment, Bacteria coded whether flies were reared axenically, or gnotobiotically with either
298 *A. pomorum* or *L. brevis*. All contrasts were set to "contrast sum". ANOVA tests were applied
299 with car::Anova, test type set to type-3. Post-hoc comparisons were applied using
300 emmeans::pairs, specifying comparisons within levels of Yeast and Preservatives.
301
302 Effect sizes were calculated using effectsize::eta_squared.
303
304 **<u>Data availability</u>**
305 R script and data are freely available at https://github.com/dobdobby/preservatives-microbes-
306 yeast
307
308
309 **Figure 1. Metabolic impact of microbiota depends on combination of yeast batch and**
310 **preservative formula.** **(A)** and **(C)** show relative TAG levels in two different experiments,
311 separated by preservative conditions (columns, shown at top), and yeast batch (rows, shown at
312 side). In both experiments, relative TAG was calculated by normalizing TAG density (µg per mg
313 fly wet weight) to the mean of axenic flies without preservatives on yeast A. **(B)** and **(D)** show
314 effect size calculations for main effects and interaction terms in the two experiments, color-
315 coded by statistical significance. **(A)** Comparisons between axenic (Ax) and conventional (Cv)
316 flies show that, on yeasts used in this experiment, relative TAG is reduced only in conventional
317 flies when no preservatives are present. On yeast A, adding preservative set 2 reversed the sign
318 of the effect of eliminating the microbiota. **(B)** In the experiment shown in panel A, comparing
319 axenic and conventional flies, preservatives are the biggest source of variance in relative TAG,
320 with both a statistically significant effect ($p<0.05$), and the biggest-sized effect. The bacteria-by-
321 preservative interaction is the next biggest-sized effect, suggesting that impacts of eliminating
322 the microbiota are contingent on preservatives. **(C)** Comparisons of relative TAG between

323 axenic (Ax), *Levilactobacillus brevis* DmCS003 (Lb), and *Acetobacter pomorum* DmCS004
324 (Ap) associated flies show that *A. pomorum* reduces TAG levels relative to axenic flies in most
325 conditions. Preservative set 2 elevated TAG on both yeasts (noting that it only did so on Yeast B
326 in the first experiment), but *A. pomorum* abrogated this effect. **(D)** In the experiment shown in
327 panel C, comparing axenic to monoassociated flies, bacteria and preservatives are equally major
328 contributors to the variance in TAG observed, with their interaction being another significant
329 contributor: again this indicates that the impact of variation in microbiota is contingent on
330 preservatives.

331

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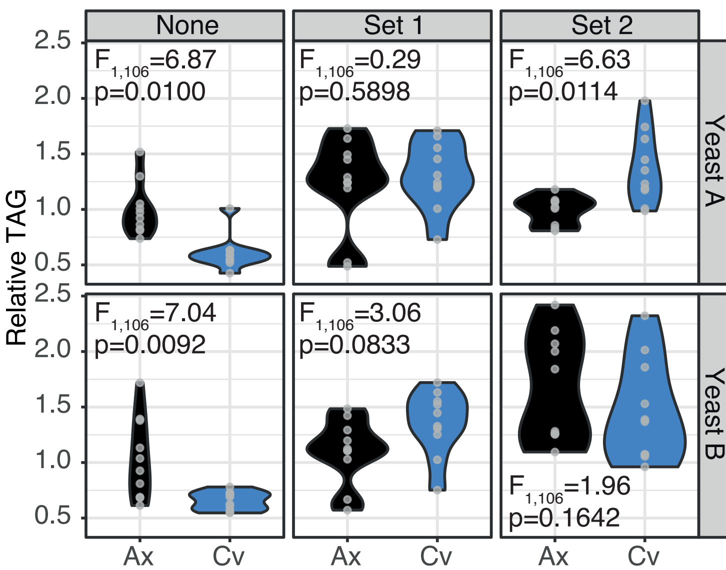
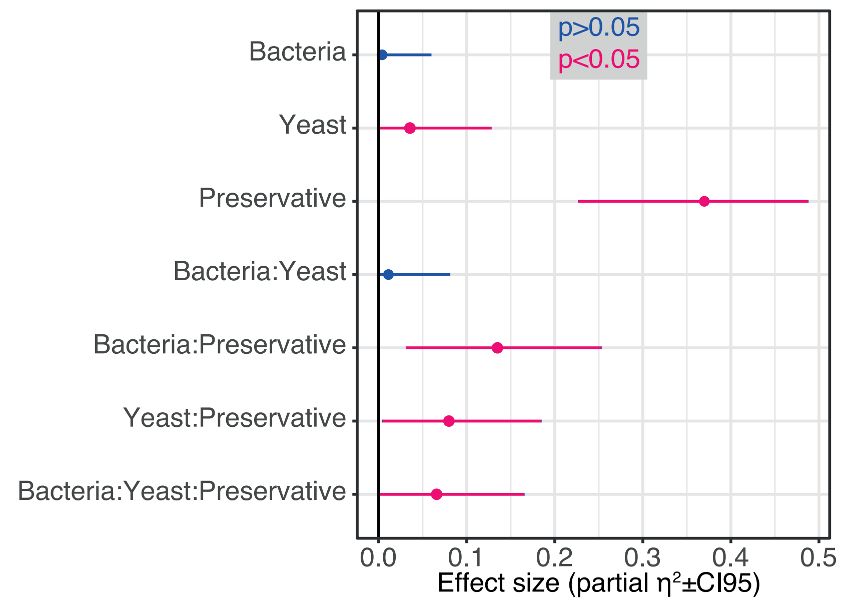
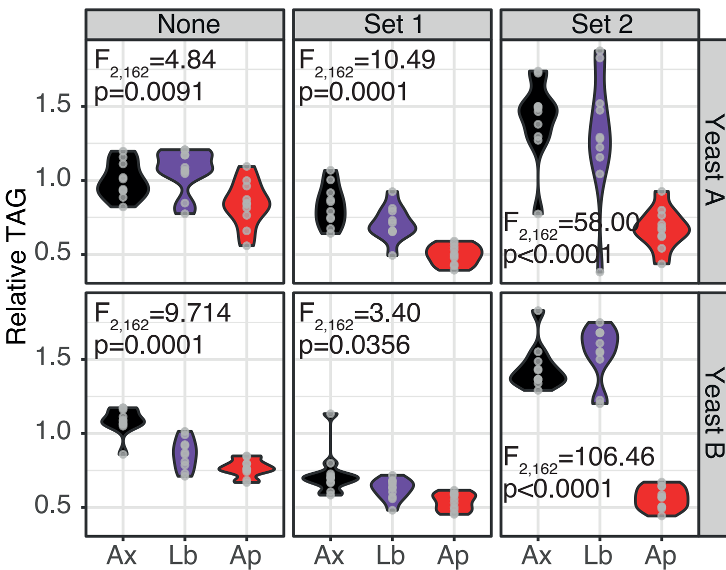
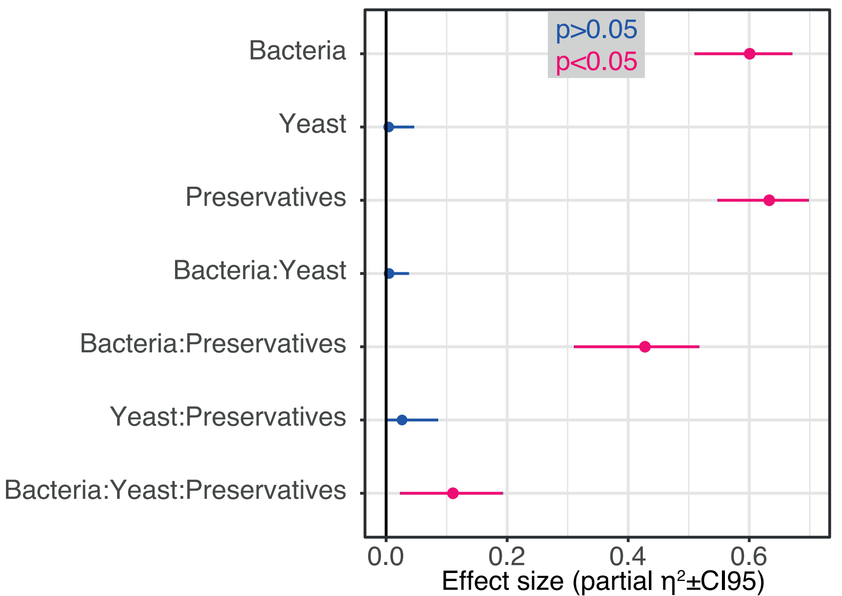

A. Conventional/axenic TAG
None
Set 1
Set 2
Yeast A
Yeast B
Relative TAG
F1,106=6.87
p=0.0100
F1,106=0.29
p=0.5898
F1,106=6.63
p=0.0114
F1,106=7.04
p=0.0092
F1,106=3.06
p=0.0833
F1,106=1.96
p=0.1642
Ax
Cv


B. Conventional/axenic TAG effect size
p>0.05
p<0.05
Bacteria
Yeast
Preservative
Bacteria:Yeast
Bacteria:Preservative
Yeast:Preservative
Bacteria:Yeast:Preservative
Effect size (partial η2±CI95)


C. Gnotobiotic/axenic TAG
None
Set 1
Set 2
Yeast A
Yeast B
Relative TAG
F2,162=4.84
p=0.0091
F2,162=10.49
p=0.0001
F2,162=58.00
p<0.0001
F2,162=9.714
p=0.0001
F2,162=3.40
p=0.0356
F2,162=106.46
p<0.0001
Ax
Lb
Ap


D. Gnotobiotic/axenic TAG effect size
p>0.05
p<0.05
Bacteria
Yeast
Preservatives
Bacteria:Yeast
Bacteria:Preservatives
Yeast:Preservatives
Bacteria:Yeast:Preservatives
Effect size (partial η2±CI95)

Supplemental text

We noted some differences between our first (conventional versus axenic) and second (gnotobiote versus axenic) experiments. Specifically, (A) preservative set two caused TAG gain in both experiments, but this effect appeared more pronounced in the axenic vs. gnotobiote experiment, and (B) the preservative elevated TAG on both yeasts in the axenic vs. gnotobiote experiment, but only on yeast B in the axenic vs. conventional experiment. To confirm that this was not due to measurement error we compared standard curves from TAG assays in both experiments (Fig S2).

We did not identify any differences in standard curves (Table S1), suggesting that any variation between the experiments was biological, perhaps due to stochastic differences between these two experiments conducted some months apart.

Table S1. Tests for variation between distinct TAG assays (96-well plates)*

	Estimate	Std error	t value	Pr(>|t|)
(Intercept)	6.68E-02	2.09E-02	3.194	0.00179
std.quant	6.06E-01	1.20E-02	50.35	<2.00E-16
id1.2	-1.24E-16	2.96E-02	0	1
id2.1	7.21E-03	2.97E-02	0.242	0.8089
id2.2	1.82E-03	2.96E-02	0.062	0.95097
id2.3	6.65E-03	2.96E-02	0.225	0.82265
std.quant:id1.2	6.17E-17	1.70E-02	0	1
std.quant:id2.1	2.18E-03	1.78E-02	0.122	0.90292
std.quant:id2.2	2.53E-02	1.70E-02	1.485	0.14015
std.quant:id2.3	2.15E-02	1.70E-02	1.261	0.20975

*All values relative to experiment 1, plate 1. Plate IDs denote experiment/plate, e.g. ID2.2 = experiment 2, plate 2. Experiment 1=conventional vs. axenic; experiment 2=gnotobiote vs. axenic.

Supplemental figure legends

Figure S1. Yeast batch and preservatives repress *A. pomorum* growth in the fly on a range of dietary yeasts. Panels show CFUs isolated from flies reared axenically, with *A. pomorum*, or with *L. brevis,* separated by preservative conditions (columns, shown at top), and yeast batch (rows, shown at side). For both species, preservative set 2 reduces CFU levels in comparison to no preservatives, with yeast E having the largest log decrease in CFUs for *A. pomorum*.

Figure S2. Technical repeatability between experiments 1 and 2. Panels show standard curves for each 96-well plate of assays in the two experiments presented in Figure 1. Rows

28 denote two different experiments (1=data from Figure 1A-B; 2=data from Figure 1C-D). No
29 significant differences in curves were detected (see Supplemental Text).

30

Table 1. ANOVA (type 3) testing for preservative*bacteria interactions that determine TAG levels in conventionally-reared versus axenic flies.

Term	Sum Sq	Df	F	Pr(>F)
(Intercept)	1357.49	1	1484.85	<2.20E-16
Bacteria	0.49	1	0.54	0.46
Yeast	4.18	1	4.57	0.034
Preservative	56.17	2	30.72	2.90E-11
Bacteria:Yeast	1.17	1	1.28	0.26
Bacteria:Preservative	15.35	2	8.40	0.0004
Yeast:Preservative	8.64	2	4.73	0.011
Bacteria:Yeast:Preservative	6.83	2	3.73	0.027
Residuals	96.91	106		

Table 2. Effects of microbiota (conventional vs. axenic) on TAG levels of flies reared on specific combinations of dietary yeast and preservatives: ANOVA analysis stratified by yeast and preservatives (joint tests)

Yeast	Preservative	estimate	SE	df	t ratio	p value*
A	1	-0.231	0.428	106	-0.541	0.5898
B	1	-0.748	0.428	106	-1.748	0.0833
A	2	-1.131	0.439	106	-2.575	0.0114
B	2	0.599	0.428	106	1.401	0.1642
A	None	1.152	0.439	106	2.622	0.0100
B	None	1.135	0.428	106	2.653	0.0092

*Tukey corrected

Table 3. ANOVA (type 3) testing for preservative*bacteria interactions that determine TAG levels in flies reared either axenically, or in association with *A. pomorum* or *L. brevis*.

Term	Sum Sq	Df	F value	Pr(>F)
(Intercept)	149.958	1	5370.171	< 2.2e-16
Bacteria	6.829	2	122.2687	< 2.2e-16
Yeast	0.02	1	0.733	0.39319
Preservatives	7.877	2	141.05	< 2.2e-16
Bacteria:Yeast	0.023	2	0.4116	0.66328
Bacteria:Preservatives	3.393	4	30.3757	< 2.2e-16
Yeast:Preservatives	0.131	2	2.3448	0.09911
Bacteria:Yeast:Preservatives	0.554	4	4.9638	0.000844

Residuals	4.524	162

8 Table 4. Effects of yeast*preservative interactions on TAG levels under specific microbiota
9 conditions: ANOVA analysis stratified by microbiota status (joint tests)

Bacteria†	Term	df1	df2	F ratio	p value*
Ax	Yeast	1	162	0.02	0.8871
Ax	Preservatives	2	162	78.265	<.0001
Ax	Yeast:Preservatives	2	162	1.623	0.2005
Lb	Yeast	1	162	0.235	0.6289
Lb	Preservatives	2	162	107.785	<.0001
Lb	Yeast:Preservatives	2	162	9.577	0.0001
Ap	Yeast	1	162	1.301	0.2556
Ap	Preservatives	2	162	15.751	<.0001
Ap	Yeast:Preservatives	2	162	1.072	0.3446

10 † Ax = axenic, Ap = *Acetobacter pomorum* DmCS004, Lb = *Levilactobacillus brevis* DmCS003

11 *Tukey corrected

12

13 Table 5. Differences in TAG levels of flies reared on different preservatives (none, set 1, set 2)
14 on specific combinations of dietary yeast and microbiota: ANOVA analysis stratified by yeast
15 and microbiota.

Bacteria†	Yeast Batch	Contrast (Preservatives)	Estimate	SE	df	t ratio	p value*
Ax	A	None vs Set 1	0.173	0.0747	162	2.319	0.056
Ax	A	None vs Set 2	-0.417	0.0747	162	-5.575	<.0001
Ax	A	Set 1 vs Set 2	-0.59	0.0747	162	-7.894	<.0001
Lb	A	None vs Set 1	0.357	0.0747	162	4.774	<.0001
Lb	A	None vs Set 2	-0.244	0.0747	162	-3.27	0.0037
Lb	A	Set 1 vs Set 2	-0.601	0.0747	162	-8.043	<.0001
Ap	A	None vs Set 1	0.35	0.0747	162	4.682	<.0001
Ap	A	None vs Set 2	0.17	0.0747	162	2.278	0.0618
Ap	A	Set 1 vs Set 2	-0.18	0.0747	162	-2.404	0.0454
Ax	B	None vs Set 1	0.357	0.0747	162	4.782	<.0001
Ax	B	None vs Set 2	-0.367	0.0747	162	-4.909	<.0001
Ax	B	Set 1 vs Set 2	-0.724	0.0747	162	-9.691	<.0001
Lb	B	None vs Set 1	0.253	0.0747	162	3.383	0.0026

Lb	B	None vs Set 2	-0.687	0.0747	162	-9.188	<.0001
Lb	B	Set 1 vs Set 2	-0.939	0.0747	162	-12.571	<.0001
Ap	B	None vs Set 1	0.236	0.0747	162	3.156	0.0054
Ap	B	None vs Set 2	0.204	0.0747	162	2.727	0.0194
Ap	B	Set 1 vs Set 2	-0.032	0.0747	162	-0.429	0.9037

† Ax = axenic, Ap = *Acetobacter pomorum*, Lb = *Levilactobacillus brevis*

* P value adjustment: Tukey method for comparing a family of 3 estimates

Table 6. Effects of microbiota (axenic, *L. brevis, A. pomorum*) on TAG levels of flies reared on s pecific combinations of dietary yeast and preservatives: ANOVA analysis stratified by yeast and preservatives (joint tests)

Yeast	Preservatives	df1	df2	F ratio	p value*
A	None	2	162	4.764	0.0098
B	None	2	162	9.769	0.0001
A	Set 1	2	162	10.514	0.0001
B	Set 1	2	162	3.643	0.0283
A	Set 2	2	162	58.466	<.0001
B	Set 2	2	162	106.203	<.0001

*Tukey corrected

Table 7. Differences in TAG levels among gnotobiotic and axenic flies reared on specific combi nations of dietary yeast and preservatives.

Preservatives	Yeast	Contrast (Bacteria)	estimate	SE	df	t.ratio	p.value*
None	A	Ax vs Lb	-0.0617	0.0747	162	-0.825	0.6881
None	A	Ax vs Ap	0.1617	0.0747	162	2.164	0.0807
None	A	Lb vs Ap	0.2233	0.0747	162	2.989	0.009
None	B	Ax vs Lb	0.2254	0.0747	162	3.016	0.0083
None	B	Ax vs Ap	0.3218	0.0747	162	4.307	0.0001
None	B	Lb vs Ap	0.0965	0.0747	162	1.291	0.4024
Set 1	A	Ax vs Lb	0.1218	0.0747	162	1.629	0.2362
Set 1	A	Ax vs Ap	0.3383	0.0747	162	4.527	<.0001
Set 1	A	Lb vs Ap	0.2165	0.0747	162	2.897	0.0119
Set 1	B	Ax vs Lb	0.1208	0.0747	162	1.617	0.2414
Set 1	B	Ax vs Ap	0.2003	0.0747	162	2.68	0.022
Set 1	B	Lb vs Ap	0.0795	0.0747	162	1.063	0.5383
Set 2	A	Ax vs Lb	0.1106	0.0747	162	1.48	0.3031
Set 2	A	Ax vs Ap	0.7486	0.0747	162	10.017	<.0001
Set 2	A	Lb vs Ap	0.638	0.0747	162	8.537	<.0001

Set 2	B	Ax vs Lb	-0.0944	0.0747	162	-1.263	0.4181
Set 2	B	Ax vs Ap	0.8925	0.0747	162	11.943	<.0001
Set 2	B	Lb vs Ap	0.9869	0.0747	162	13.206	<.0001

* P value adjustment: Tukey method for comparing a family of 3 estimates

Table 8. Effects of preservatives (none, set 1, set 2) on TAG levels of flies reared on specific combinations of dietary yeast and microbiota: ANOVA analysis stratified by yeast and microbiota (joint tests)

Bacteria	Yeast	df1	df2	F ratio	p value*
Ax	A	2	162	32.926	<0.0001
Ax	B	2	162	46.962	<0.0001
Lb	A	2	162	32.726	<0.0001
Lb	B	2	162	84.637	<0.0001
Ap	A	2	162	10.965	<0.0001
Ap	B	2	162	5.859	0.0035

Table 9. ANOVA (type 3) testing for preservative*yeast interactions that determine CFUs in gnotobiotic flies colonized with *A. pomorum* or *L. brevis*

Term	Sum Sq	Df	F value	Pr(>F)
(Intercept)	2205.28	1	54503.58	< 2.2e-16 ***
Bacteria	18.54	1	458.1797	< 2.2e-16 ***
Yeast	0.98	2	12.1356	2.157e-05 ***
Preservatives	24.37	2	301.1093	< 2.2e-16 ***
Bacteria:Yeast	0.01	2	0.1566	0.855265
Bacteria:Preservatives	0.35	2	4.2667	0.016970 *
Yeast:Preservatives	0.71	4	4.3905	0.002741 **
Bacteria:Yeast:Preservatives	1.88	4	11.6292	1.177e-07 ***
Residuals	3.64	90		

Table 10. Differences CFU of flies reared on different preservatives (none, set 1, set 2) on specific combinations of dietary yeast and microbiota: ANOVA analysis stratified by yeast and microbiota

Bacteria†	Yeast Batch	Contrast (Preservatives)	Estimate	SE	df	t ratio	p value*
Lb	C	Set 1 - Set 2	0.267	0.116	90	2.296	0.0614
Lb	C	Set 1 - None	-0.951	0.116	90	-8.189	<.0001
Lb	C	Set 2 - None	-1.218	0.116	90	-10.486	<.0001
Ap	C	Set 1 - Set 2	0.417	0.116	90	3.591	0.0015
Ap	C	Set 1 - None	-0.539	0.116	90	-4.645	<.0001
Ap	C	Set 2 - None	-0.956	0.116	90	-8.236	<.0001
Lb	D	Set 1 - Set 2	0.894	0.116	90	7.698	<.0001

Lb	D	Set 1 - None	-0.339	0.116	90	-2.92	0.0122
Lb	D	Set 2 - None	-1.233	0.116	90	-10.618	<.0001
Ap	D	Set 1 - Set 2	0.315	0.116	90	2.71	0.0217
Ap	D	Set 1 - None	-0.534	0.116	90	-4.595	<.0001
Ap	D	Set 2 - None	-0.848	0.116	90	-7.305	<.0001
Lb	E	Set 1 - Set 2	0.828	0.116	90	7.128	<.0001
Lb	E	Set 1 - None	-0.193	0.116	90	-1.659	0.2265
Lb	E	Set 2 - None	-1.02	0.116	90	-8.787	<.0001
Ap	E	Set 1 - Set 2	0.555	0.116	90	4.775	<.0001
Ap	E	Set 1 - None	-1.146	0.116	90	-9.867	<.0001
Ap	E	Set 2 - None	-1.7	0.116	90	-14.642	<.0001

40 † Lb = *L. brevis*, Ap = *A. pomorum*

41 *Tukey corrected

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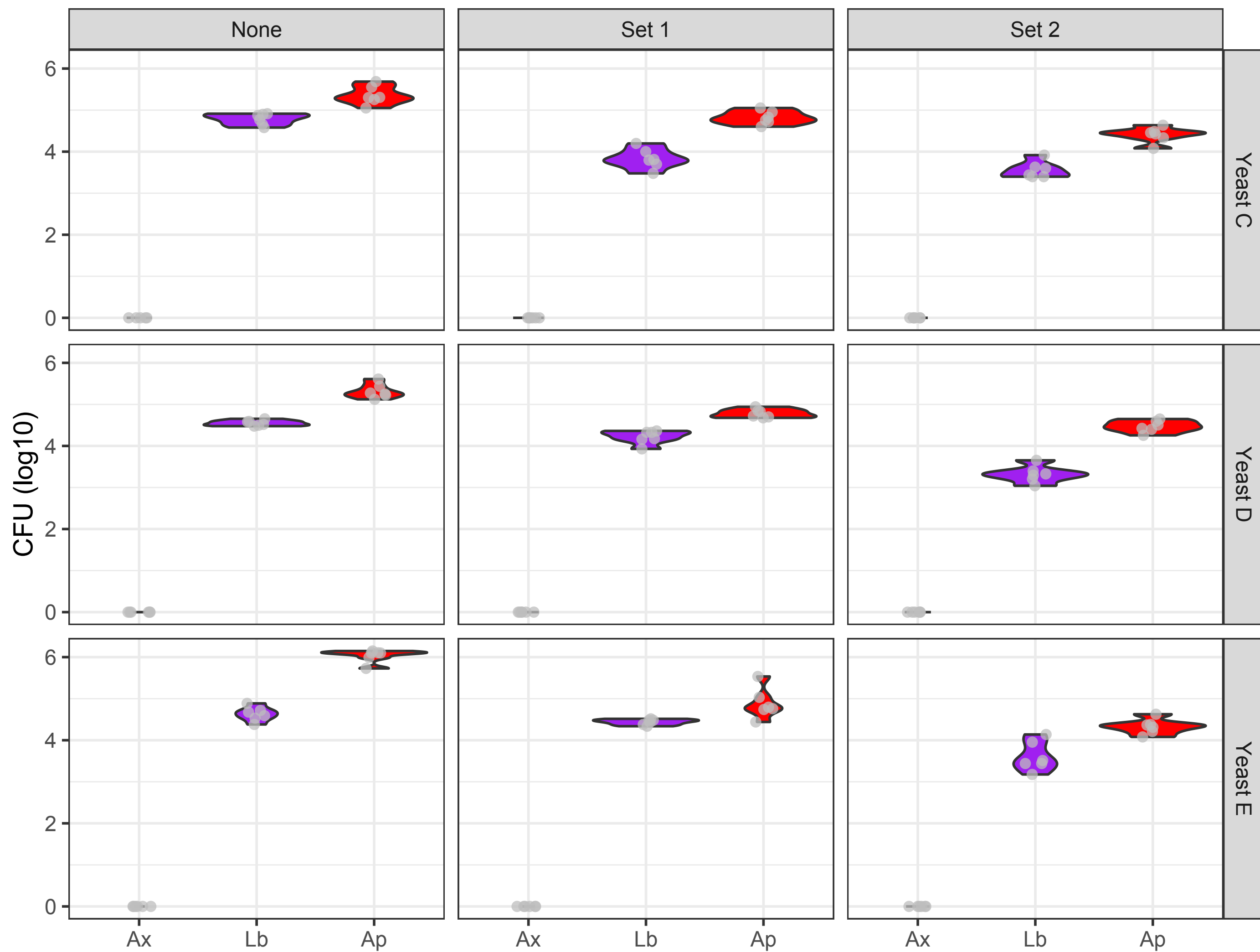
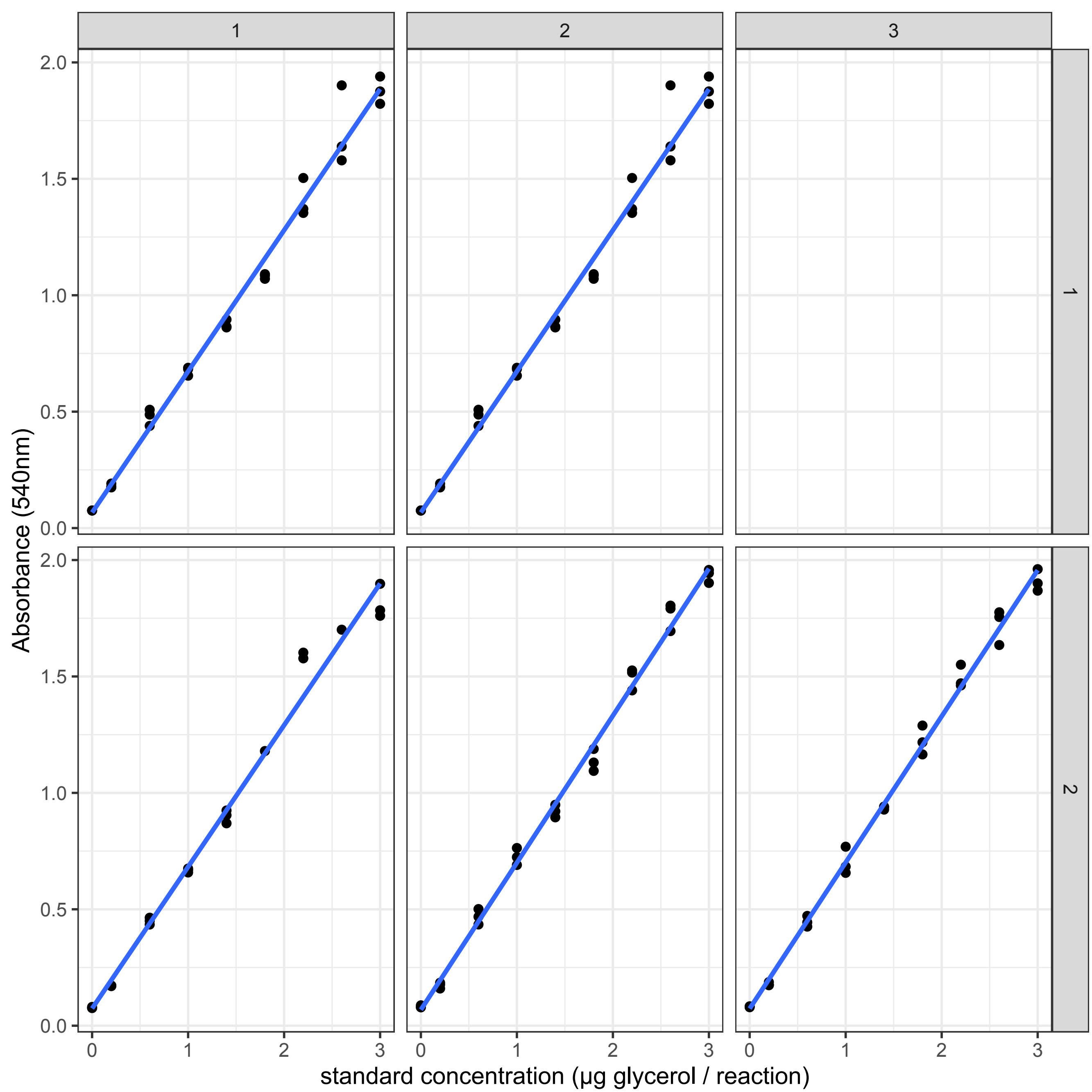

CFU
None
Set 1
Set 2
Yeast C
Yeast D
Yeast E
CFU (log10)
0
2
4
6
Ax
Lb
Ap

Figure S1. Yeast batch and preservatives repress *A. pomorum* growth in the fly on a range of dietary yeasts. Panels show CFUs isolated from flies reared axenically, with *A. pomorum*, or with *L. brevis,* separated by preservative conditions (columns, shown at top), and yeast batch (rows, shown at side). For both species, preservative set 2 reduces CFU levels in comparison to no preservatives, with yeast E having the largest log decrease in CFUs for *A. pomorum*.



1
2
3
1
2
Absorbance (540nm)
standard concentration (µg glycerol / reaction)
0.0
0.5
1.0
1.5
2.0
0
1
2
3

Figure S2. Technical repeatability between experiments 1 and 2. Panels show standard curves for each 96-well plate of assays in the two experiments presented in Figure 1. Rows denote two different experiments (1=data from Figure 1A-B; 2=data from Figure 1C-D). No significant differences in curves were detected (see Supplemental Text).