Figures and figure supplements

Metabolite exchange between microbiome members produces compounds that influence Drosophila behavior

Caleb N Fischer et al
Figure 1. *Drosophila* detection of microbe-microbe metabolite exchange. (A) T-maze setup and

Figure 1 continued on next page
Figure 1 continued

(B) Drosophila behavior toward yeasts (blue), acetic acid bacteria (red), and lactic acid bacteria (brown) (Supplementary file 2). 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]). The one-sample t-test was used to assess the mean 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 microorganism was graphed according to microbial group. The means were compared by one-way ANOVA with Tukey’s post-hoc comparison. (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 hr; Drosophila preference for the three- versus two-membered community is the last column. Mean ± SEM of 12–18 replicates (n = 2–3 experiments). The one-sample t-test assessed the mean 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 hr. Mean ± SEM of 18–30 replicates (n = 5 experiments). A one-way ANOVA followed by post-hoc Tukey’s multiple comparison correction test evaluated whether the means of the experimental groups were different from one another.

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The following source data is available for figure 1:

Source data 1. Raw Drosophila preference data for Figure 1B,C.
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Source data 2. Raw Drosophila preference data for Figure 1D.
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Source data 3. Raw Drosophila preference data for Figure 1E.
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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, 10 μl 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 5 μl of AJM mixed with 5 μl of water. Data points represent the mean +/- SEM combined from three experiments (n = 12 per experimental group). A one-sample t-test assessed the mean deviance from 0. NS p > 0.05; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 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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The following source data is available for figure 1:

**Figure supplement 1—Source data 1.** Raw Drosophila preference data for *Figure 1—figuresupplement 1*. DOI: 10.7554/eLife.18855.008
Figure 2. *Drosophila* temporal preference for metabolite exchange. (A) *S. cerevisiae* and *A. malorum* viable populations. Mean ± SEM of 2–3 experiments with one pooled replicate (2–3 cultures from the same colony) per experiment. Limit of detection is 20 CFU/mL. A curve was fitted to the data with 40 values. Subsequently, an exponential plateau equation was compared between the individual cultures from 0 to 72 hr. The null hypothesis that the k values are the same was not rejected (p>0.05). A separate analysis compared a slope of 0 between *S. cerevisiae* alone and *S. cerevisiae* with *A. malorum* from 48–127 hr. The null hypothesis that the slopes were the same was rejected (p=0.0205). (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. Two statistical tests were run. First, a one-sample t-test assessed whether *Drosophila* was attracted, neutral, or repelled by the test arm by evaluating mean deviance from 0. Symbols: NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. Second, a one-way ANOVA followed by Dunnet’s post-hoc multiple comparison test evaluated whether *Drosophila* was attracted to the co-culture aged 96 hr differently than other aged co-cultures. The results are shown in pink; unique letters indicate difference (p<0.05) from 96 hr. (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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The following source data is available for figure 2:

Source data 1. Raw *Drosophila* preference data for Figure 2B & Figure 2—figure supplement 1C.
DOI: 10.7554/eLife.18855.010

Source data 2. Raw *Drosophila* preference data, microbial population data, and pH data for Figure 2A,C,D & Figure 2—figure supplement 1A,B.
DOI: 10.7554/eLife.18855.011
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 behavioral 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 hr – 127 hr). Mean RI ± SEM of three experiments with 16–18 total replicates. A one-sample t-test assessed whether the group means were different from 0. NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001 Significance is denoted beside or within bars of each experimental group. ACV = apple cider vinegar (25% in water); PO = paraffin oil. (D) Behavior of Drosophila toward the co-culture grown for 72 hr versus S. cerevisiae alone, A. malorum alone, or the separate-culture mixture grown for different periods of time that correspond to different stages of growth (e.g. late log, stationary; see [E]). Mean ± SEM of 11–12 replicates in two experiments. A one-sample t-test compared the experimental group means to 0. (E) Viable populations of conditions in (D) Mean ± SEM of 2 pooled replicates where each replicate contains two replicate cultures.

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The following source data is available for figure 2:

Figure supplement 1—Source data 1. Raw Drosophila preference data and microbial population data for Figure 2—figure supplement 1D,E.

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Figure 3. Role of olfactory receptor mutants in Drosophila detection of inter-species microbial interactions. (A) The mean rank of the response index of the various Drosophila mutants toward the co-culture was compared with the wild-type fly. (B) The mean rank of the response index of the various Drosophila mutants toward the co-culture was compared with the wild-type fly.
Figure 3 continued

the mean rank of wild-type fly behavior toward the co-culture using the Kruskal-Wallis test followed by Dunn’s post-hoc multiple comparisons testing. Symbols: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. A lack of symbol indicates no difference when comparing each mutant group to the wild-type group. The behavioral responses of all Drosophila (wild-type and each mutant) toward the co-culture was greater than 0 (using the non-parametric Wilcoxon signed rank test in which the medians were compared to 0, p<0.05, no symbols shown). Mean +/- SEM of 12–24 replicates per time point per fly condition (n = 2–4 experiments per time point). (B) The mean rank of mutant fly behavior toward the co-culture was compared between wild-type and the specified conditions using the Kruskal-Wallis test followed by Dunn’s post-hoc host multiple comparisons testing. Mean +/- SEM of 11–12 replicates (n = 2 experiments).

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The following source data is available for figure 3:

**Source data 1.** Raw Drosophila preference data and microbial population data for Figure 3A and Figure 3—figure supplement 1.

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**Source data 2.** Raw Drosophila preference data for Figure 3B.

DOI: 10.7554/eLife.18855.016
Figure 3—figure supplement 1. Effect of co-culture age on Drosophila attraction and microbial density. (A) Attraction of wild-type Drosophila to different aged co-cultures (grown 67–163 hr, S. cerevisiae and A. malorum). Mean ± SEM of 12–24 replicates per group (n = 2–4 experiments). A one-way ANOVA with Tukey’s post-hoc multiple comparisons assessed the difference between all experimental groups. (B) Corresponding viable counts at different times of microbial growth. Mean ± SEM of 2–3 replicates.
DOI: 10.7554/eLife.18855.017
Figure 4. *Drosophila* behavior 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 (Table 1—source data 3). 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 hr, which are from one experiment with three replicates). *Drosophila* co-culture preference is the mean value of the preference shown in Figure 2B. The estimated ethanol concentrations in the co-culture and *S. cerevisiae* culture were compared with multiple t-tests and multiple comparisons correction by the Holm-Sidak method. Symbols: NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. (B) *Drosophila* preference for stages of ethanol catabolism. 72 hr is ‘mid’ stage; 36 hr 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. The one-sample t-test was used to assess the mean deviation from 0. (C) *Drosophila* olfactory behavior toward specified conditions. Mean ± SEM of 2–7 experiments with 10–42 total replicates. Two statistical tests were used to evaluate the behavior. First, a one-sample t-test assessed the mean deviation from 0. Symbols: NS p>0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Second, a one-way ANOVA with Tukey’s post-hoc comparison assessed whether the means of the bottom three experimental groups were different from one another (differences are denoted by unique pink letters). Esters include ethyl acetate, isoamyl acetate, 2-phenethyl acetate, isobutyln acetate, 2-methylbutyl acetate, and methyl acetate; acid is acetic acid. Amounts added are based on Figure 4 continued on next page.
Figure 4 continued

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.

DOI: 10.7554/eLife.18855.023

The following source data is available for figure 4:

**Source data 1.** Raw spectral abundance data associated with metabolites graphed in Figure 4A.
DOI: 10.7554/eLife.18855.024

**Source data 2.** Raw *Drosophila* preference data for Figure 4B.
DOI: 10.7554/eLife.18855.025

**Source data 3.** Raw *Drosophila* preference data for Figure 4C.
DOI: 10.7554/eLife.18855.026
Figure 4—figure supplement 1. Drosophila behavior toward the co-culture using A. malorum or A. pomorum. Drosophila behavior toward co-cultures grown for 96 hr 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 the difference between the co-cultures grown with A. malorum or A. pomorum.

DOI: 10.7554/eLife.18855.027

The following source data is available for figure 4:

Figure supplement 1—Source data 1. Raw Drosophila preference data for Figure 2—figure supplement 1.

DOI: 10.7554/eLife.18855.028
Figure 5. Acetaldehyde metabolic derivatives as attractive microbial community generated metabolites. (A) Representative chromatogram of m/z 88.05 in the tri-culture (S. cerevisiae-A. malorum-L. plantarum) compared to the co-culture (S. cerevisiae and A. malorum). (B) Estimated quantification 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). (C) Mean ± SEM of three experiments with 16–18 total replicates. A one-way ANOVA with Tukey’s post-hoc multiple comparisons correction assessed the differences between Drosophila behavior toward the co-culture with A. pomorum adhA and esters to various groups in which individual molecular groups were removed or added (p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001). 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—source data 2). Acetoin is added in a similar amount as the tri-culture. Sc = S. cerevisiae, Ap = A. pomorum.

DOI: 10.7554/eLife.18855.036
The following source data is available for figure 5:

Source data 1. Extracted ion current for m/z 88.05 in Figure 5A.
DOI: 10.7554/eLife.18855.037

Source data 2. Peak areas associated with acetoin for Figure 5B.
DOI: 10.7554/eLife.18855.038

Source data 3. Raw Drosophila preference data for Figure 5C.
DOI: 10.7554/eLife.18855.039
Figure 5—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 5B).
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The following source data is available for figure 5:

Figure supplement 1—Source data 1. Extracted ion current for Figure 5—figure supplement 1.
DOI: 10.7554/eLife.18855.041
Figure 6. Drosophila behavior toward 21 metabolite mixtures. (A) Supplementary file 5 contains the concentrations of all mixtures (in 50% AJM). The co-culture was grown for 96 hr. Mean ± SEM of 4–6 replicates per
Figure 6 continued

experimental group. Groups were tested over five days. (B) Drosophila attraction to a co-culture grown for 96 hr and metabolite mixture #21. Mean ± SEM of three experiments with 17–18 replicates per group. A Mann-Whitney test compared the median values of the co-culture and metabolite mixture #21, the Wilcoxon Signed-Rank test compared the median value of fly behavior toward the co-culture relative to metabolite mixture #21 to 0. (C) Sufficiency of metabolite groups to attract Drosophila. The individual groups are: acetaldehyde 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). Mean ± SEM of 6 replicates of 1 experiment (except the acetaldehyde metabolic derivative group which is 12 replicates from two experiments). A one-way ANOVA followed by Dunnet’s post-hoc comparison assessed the difference between the co-culture and all experimental groups. NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤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 in the same manner as in C. Mean +/- SEM of 6 replicates from one experiment.

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The following source data is available for figure 6:

Source data 1. Concentrations of mixtures and raw Drosophila preference data for Figure 6.

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Figure 6—figure supplement 1. 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 min at room temperature, mixed 1:1 in water, and assessed for Drosophila attractiveness. Mean ± SEM of 1–7 experiments with 12–42 total replicates per group. The mean rank of fly behavior toward all experimental conditions was compared using the Kruskal-Wallis test followed by Dunn’s post-hoc host multiple comparisons testing. Unique letters indicate difference (p<0.05) (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 min, 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. The mean rank of fly behavior toward all experimental conditions was compared using the Kruskal-Wallis test followed by Dunn’s post-hoc host multiple comparisons testing. Unique letters indicate difference (p<0.05).


DOI: 10.7554/eLife.18855.044

The following source data is available for figure 6:

Figure supplement 1—Source data 1. Raw Drosophila preference data for Figure 6—figure supplement 1.

DOI: 10.7554/eLife.18855.045
Figure 6—figure supplement 2. *Drosophila* behavior toward water amended with nine metabolites (9-metabolite mixture) versus three 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 hr 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-sample t-test assessed whether the mean values of the experimental groups were different from 0. NS > 0.05; *p* ≤ 0.05; **p** ≤ 0.01; ***p** ≤ 0.001; ****p** ≤ 0.0001. The acetoin concentration was similar to that calculated from the tri-culture (Figure 5A, 0.3%). The concentrations of all nine metabolites can be found in the materials and methods.

DOI: 10.7554/eLife.18855.046

The following source data is available for figure 6:

**Figure supplement 2—Source data 1.** Raw *Drosophila* preference data for Figure 6—figure supplement 2.

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Figure 7. *Drosophila* egg-laying preference, nutrition, and pathogen protection. (A) *Drosophila* was given a

Figure 7 continued on next page
choice to lay eggs in a co-culture containing \textit{S. cerevisiae} and \textit{A. pomorum} wild-type (WT) or \textit{S. cerevisiae} and \textit{A. pomorum} adhA. The co-cultures were grown for 96 hr and mixed 1:1 with a 1.6\% agarose solution. \textit{Drosophila} was allowed to lay eggs for eight hours. The Wilcoxon signed-rank test evaluated whether the median value of each experimental group was different from 0. Mean ± SEM of 16–18 replicates from two experiments. (B) \textit{Drosophila} (40 females and 15 males) deposited eggs in fly vials for 4 hr containing the co-culture of \textit{S. cerevisiae} and \textit{A. pomorum} WT (WT) or the co-culture of \textit{S. cerevisiae} and \textit{A. pomorum} adhA (adhA). Subsequently the number of pupae in each condition was monitored over time. Mean ± SEM of 5 replicates of 1 experiment. Between 12–16 d, larvae pupated in 3/5 WT replicates. Multiple unpaired t-tests were used to compare means at each time point. *p<0.05. (C) \textit{Drosophila} (40 females and 15 males) deposited eggs for 4 hr after which the total number of eggs were counted in the two experimental groups. Mean +/- SEM of 12 replicates of 1 of 2 representative experiments. A Mann-Whitney test compared the medians of each group. NS p>0.05; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. (D) three days after egg-laying the plates containing eggs quantified in (C) were exposed to the open environment and the consequence of exposure was the growth of unidentified fungi, as pictured. Control plates that were not exposed to the environment did not harbor any fungi. In experiment 1, 12/12 of the adhA plates harbored fungi and 0/12 plates of WT plates harbored fungi. In the second experiment 4/6 adhA plates harbored fungi and 0/6 of WT plates harbored fungi. (E, F) Following environmental exposure, the eggs were followed through pupation (E) and adulthood (F). Mean +/- SEM of 12 replicates of 1 of 2 representative experiments. The median values in E and F were compared the same way as in C.

Source data 1. Raw \textit{Drosophila} egg-laying preference data for Figure 7A.
DOI: 10.7554/eLife.18855.048

Source data 2. Raw developmental data for Figure 7B, C,E,F.
DOI: 10.7554/eLife.18855.050
Figure 7—figure supplement 1. Impact of co-culture metabolites on adult survival and yeast populations. (A) 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, Mod. AA) and post ethanol catabolism (3.42% acetic acid, High AA). Data represent mean +/- SEM of 5–6 replicates of 1 representative experiment of 2. Mod. EtOH, Mod. AA was different from High EtOH condition from 108 hr onward and High AA condition from 72 hr onward (assessed by two-way ANOVA comparing time and condition, p<0.05, Tukey’s correction). Negative Cntrl is water and Positive Cntrl is Shield’s and Sang M3 Insect Medium. (B) Photograph of yeast populations of a co-culture containing S. cerevisiae and A. malorum adhA (left) and S. cerevisiae and A. malorum WT (right) after growing for 72 hr. 50 ul of the culture was spread onto MRS plates containing a cocktail of antibiotics. Source Data Titles.

DOI: 10.7554/eLife.18855.051

The following source data is available for figure 7:

Figure supplement 1—Source data 1. Raw survival proportions for Figure 7-figuresupplement1A.

DOI: 10.7554/eLife.18855.052
Figure 8. Model of microbe-microbe metabolite exchange. Bolded are metabolites increased due to microbe-
microbe interactions.
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