

Identification of Core Yeast Species and Microbe-Microbe Interactions Impacting Larval Growth of *Drosophila* in the Wild

Reviewed Preprint

Published from the original preprint after peer review and assessment by eLife.

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Reviewed preprint posted

September 13, 2023 (this version)

Posted to bioRxiv

August 3, 2023

Sent for peer review

July 24, 2023

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Abstract

Microbiota consisting of various fungi and bacteria have a significant impact on the physiological functions of the host. However, it is unclear which species are essential to this impact and how they affect the host. This study analyzed and isolated microbes from natural food sources of *Drosophila* larvae, and investigated their functions. *Hanseniaspora uvarum* is the predominant yeast responsible for larval growth in the earlier stage of fermentation. As fermentation progresses, *Acetobacter orientalis* emerges as the key bacterium responsible for larval growth, although yeasts and lactic acid bacteria must coexist along with the bacterium to stabilize this host-bacterial association. By providing nutrients to the larvae in an accessible form, the microbiota contributes to the upregulation of various genes that function in larval cell growth and metabolism. Thus, this study elucidates the core microbial species that support animal growth under microbial transition.

eLife assessment

This is an **important** study that addresses a significant question in microbiome research. The authors provide **convincing** evidence that certain bacterial groups within the fly microbiome have critical functions for host development. Additionally, dietary aspects such as microbial community progression in a natural food source are integrated into their host-microbe interaction analyses.

In nature, animals live in association with a diverse community of microorganisms. These associated microbes, especially fungi and bacteria, perform a range of beneficial functions for their host, such as nutrient provision and immune modulation (Ikeda-Ohtsubo et al., 2018 [DOI](#); Zheng et al., 2020 [DOI](#)). Some of these host-microbe associations are facultative and dispensable, while others are more important or even essential for host growth or survival under specific circumstances. Compared to symbioses between a limited number of specific species (Shigenobu & Wilson, 2011 [DOI](#); Su et al., 2022 [DOI](#)), however, those that encompass a larger number of species are intricate and analytically challenging. Additionally, despite a growing number of reports documenting the presence of fungi in the mammalian intestine, including humans, our comprehension of their roles is still limited, with the exception of a handful of pathogenic fungi (Hallen-Adams & Suhr, 2017 [DOI](#); Pérez, 2021 [DOI](#)). Moreover, the composition of these complex microbiota tends to change over time (Moya & Ferrer, 2016 [DOI](#); Qiu et al., 2021 [DOI](#)). Therefore, we focused on how the host sustains its life processes in such unstable environments.

Drosophila melanogaster has made remarkable contributions to our comprehension of the regulatory mechanisms governing development, growth, and metabolism, which are highly conserved among animals. More recently, the fly has emerged as a valuable model for investigating animal-associated microbes. The microbiota associated with *Drosophila* comprise relatively few species, most of which can be cultured aerobically (Chandler et al., 2011 [DOI](#), 2012 [DOI](#); Grenier & Leulier, 2020 [DOI](#); Lee & Brey, 2013 [DOI](#)). Furthermore, germ-free or gnotobiotic animals can be easily prepared (Ludington & Ja, 2020 [DOI](#)). The aforementioned advantages of analysis enabled a thorough exploration of the role of *Drosophila*-associated microbes. Notably, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) demonstrate the ability to enhance larval growth under nutrient scarcity (Shin et al., 2011 [DOI](#); Storelli et al., 2011 [DOI](#)). However, most researchers have examined only a single species or a limited number of bacteria. Additionally, most previous studies did not investigate the association with yeasts. Possibly, this is because live yeasts are either absent or present in very small quantities, due to antifungal agents added to the *Drosophila* laboratory foods. Instead of live yeasts, the foods typically contain heat-killed budding yeast *Saccharomyces cerevisiae* as a major nutrient source, but this yeast species rarely coexists with *Drosophila* in the wild (Hoang et al., 2015 [DOI](#)). Consequently, the relationship between *Drosophila* and its associated microorganisms has often been classified as facultative (Gallo et al., 2022 [DOI](#); Martino et al., 2018 [DOI](#)), and the role played by associated fungi has been largely overlooked.

In the wild, the presence of microbes is crucial for the developmental growth of *Drosophila* larvae that feed on fruit-based food sources. *D. melanogaster* in its natural habitat feeds on fruits fermented by its associated microbes (Watanabe et al., 2019 [DOI](#)). Germ-free larvae cannot grow on fresh fruits alone, while inoculation with certain species of yeasts or bacteria promotes pupariation (Anagnostou et al., 2010 [DOI](#); Pais et al., 2018 [DOI](#)). These findings suggest that, in the wild, microbes provide vital nutrients for larval growth. While the bacterial roles have been extensively investigated, few reports have focused on yeasts; some studies described interspecies variation of yeasts with regard to their effects on larval growth (Anagnostou et al., 2010 [DOI](#); Quan & Eisen, 2018 [DOI](#); Solomon et al., 2019 [DOI](#)), but the underlying mechanisms have not been thoroughly explored. We therefore set out to address the following questions: (1) which species play a central role in food microbiota? (2) what are the essential traits that these species possess? (3) what microbe-derived nutrients are necessary for host growth?

To address these questions, we sampled fermented bananas that had been fed upon by wild *Drosophila* species. We collected the foods at two different timepoints, referred to as “early-stage” and “late-stage” foods, and demonstrated a dramatic shift in fungal and bacterial taxonomic compositions during fermentation. Regarding fungi, we observed that yeasts predominated in both stages, but the dominant species changed between the stages. Among bacterial species, Enterobacteriales accounted for a large proportion in the early stage, whereas LAB and AAB

dominated in the late stage. We then isolated yeast and bacterial strains from the food samples and tested their ability to support larval development on a banana agar. *Hanseniaspora uvarum*, the predominant yeast species in the early stage, was able to support larval growth by itself. In contrast, most of the late-stage microbes tested did not efficiently promote larval growth when inoculated individually. However, we found that when the acetic acid bacteria *Acetobacter orientalis* coexisted with either LAB or late-stage yeast species, it effectively promoted larval growth. Our analyses of larvae under different microbial environments, including transcriptomic analyses of first-instars, strongly suggest that *A. orientalis* is potentially able to promote larval growth, although it requires interactions with other late-stage microbes. Finally, we investigated the molecular basis underlying the distinct larval growth-promoting effects among yeast species, including the supportive *H. uvarum* from the early-stage foods and non-supportive *Pichia kluyveri* and *Starmerella bacillaris* from the late-stage foods. Surprisingly, all the yeast species strongly promoted larval growth upon heat killing. This and additional results indicate that all species produce sufficient nutrients for larval development, but larvae cannot utilize those produced by the live non-supportive species. Our metabolomic analysis and metabolite supplementation assay suggest that only the supportive yeast cells can release critical metabolites for larval growth, including branched-chain amino acids, leucine and/or isoleucine. Collectively, our findings detail the core microbial species, their interactions, and the yeast species-dependent supply of nutrients that contribute to the development of *Drosophila* larvae in nature.

Results

The composition of both yeast and bacteria in the food microbiota shifts as fermentation progresses, independently of the presence of larvae

To examine the community structure of the microbiota associated with *Drosophila* larvae in nature, we collected and analyzed fermented foods that had been fed on by wild larvae (**Figure 1A** [↗](#), Table S1A). Because *D. melanogaster* and its related species are often found near human settlements, we set traps containing freshly peeled and sliced bananas outside of our residences so that wild flies would lay eggs on the foods. A portion of each fermented food sample was collected as “early-stage” food, while the remaining food was incubated in the laboratory. When the larvae reached the late third-instar stage, we collected further fermented “late-stage” food samples (refer to MATERIALS AND METHODS for experimental definitions of early- or late-stage foods). We performed sequencing of the fungal internal transcribed spacer (ITS) region and the bacterial 16S rRNA region to analyze the composition of fungi and bacteria in individual samples, respectively (**Figure 1B** [↗](#); Tables S2A and S2B).

In most samples, yeasts consistently accounted for a major proportion of the fungal communities (top of **Figure 1B** [↗](#); Table S2A), as previously reported ([Chandler et al., 2012](#) [↗](#)). At the family level, the compositions showed dramatic differences between the early and late stages; while Saccharomycodaceae dominated in most of the early-stage foods, Pichiaceae, *Starmerella*, and Saccharomycetaceae became dominant in the late-stage foods. The dominant bacteria also differed between the two stages, with Enterobacteriales predominating at the early stage and lactic acid bacteria (LAB) and acetic acid bacteria (AAB) predominating at the late stage (bottom of **Figure 1B** [↗](#); Table S2B). Henceforth, we refer to the transition from early-to-late stages in microbial composition as the microbial composition shift or simply the composition shift. In this sampling, we obtained, by chance, a “no-fly” trap; no adult flies were caught in the trap, and no eggs, larvae, or pupae were found in the food at either stage (“No-fly” in **Figure 1B** [↗](#); “No-fly trap” in Tables S2A and S2B; refer to “Collection of fermented foods and wild *Drosophila* samples” section in MATERIALS AND METHODS for further details). We analyzed food samples from this

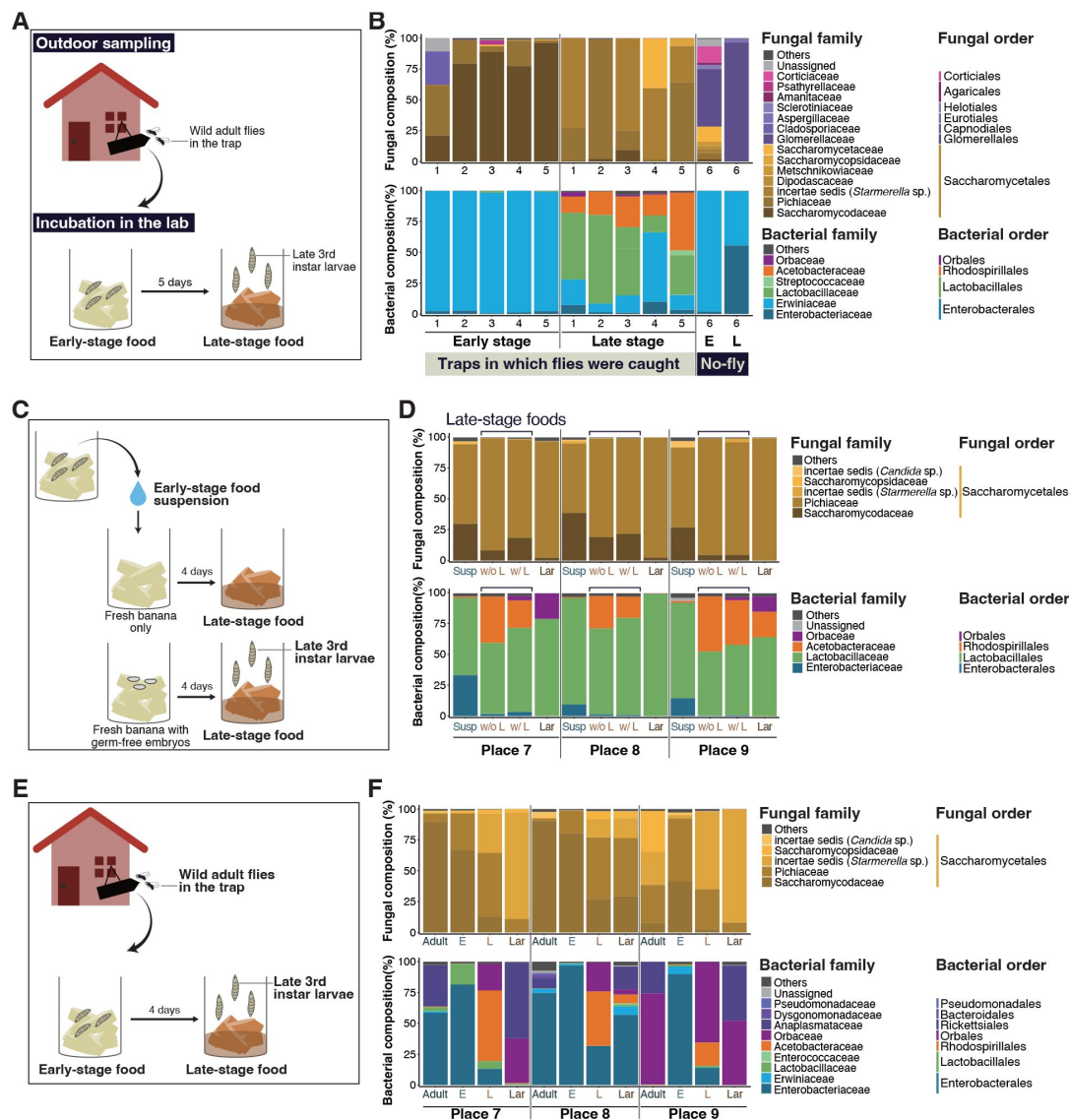


Figure 1.

The composition of both yeast and bacteria in the food microbiota shifts as fermentation progresses, independently of the presence of larvae

(A-F) Sampling designs and results for the analyses of microbial compositions in natural food sources of *Drosophila* (A and B), effects of larval presence on the microbial composition shift (C and D), or microbial compositions in adult flies, larvae, and their foods (E and F). (A, C, and E) Designs of the samplings for microbial analyses. Traps with bananas were set up outdoors near human habitations in order to collect samples on which wild *Drosophila* spp. lay eggs. Fermented food samples were collected from the traps at two time points: the early stage (just after trap collection), and the late stage (when larvae in the foods developed into late 3rd instars). Collected samples are indicated in bold letters. (C) In this sampling, the collected early-stage food was crushed in PBS, and the liquid portion (microbial suspension) was added to fresh bananas with or without germ-free embryos (*Cg-Gal4, UAS-mCD8:GFP*). After incubation, late-stage food and surface-sterilized larvae were collected. (E) In addition to the food samples, adult flies in the traps and larvae from the late-stage foods were collected. This sampling was conducted independently from that shown in (C) and (D), although the sampling places were in common. Both adults and larvae were surface-sterilized. Note that all of the adults in the traps at Place 7 and Place 8 were collected, while 20 out of 37 were collected at Place 9. (B, D, and F) The relative abundances of fungi or bacteria in the fermented banana or fly samples. The compositions were analyzed using primer sets amplifying the fungal ITS region or bacterial 16S rDNA region. Operational taxonomic units (OTUs) accounting for >1% were grouped by families, as shown. The ratio of those accounting for <1% was summed and is indicated as "Others." The genera belonging to uncertain families (incertae sedis) are indicated by their genus name in parentheses. (B) The result corresponding to the sampling depicted in (A). The numbers on the horizontal axis indicate each numbered sampling location. (D and F) The result corresponding to the sampling depicted in (C) and (E), respectively. E, early-stage food; L, late-stage food; Susp, microbial suspension; w/o L, late-stage food without larvae; w/ L, late-stage food with larvae; Lar, larvae.

trap for comparison. The microbiota in the foods significantly differed from that of other samples, with a lower percentage of yeast in the fungal community and a consistently high abundance of Enterobacteriales in the bacterial community.

A previous study showed that the presence of larvae influences the community structures of food microbiota (Stamps et al., 2012). Therefore, we sought to determine whether the larvae contributed to the composition shift (Figure 1C; Table S1B). We prepared microbe-containing suspensions using newly collected early-stage foods and introduced them to fresh bananas, with or without germ-free embryos. We then incubated the samples and examined whether the microbial composition shifts occurred in the bananas. Remarkably, the composition shifts occurred similarly, irrespective of the presence of larvae, as indicated by the decreased proportions of Saccharomycodaceae yeast and Enterobacteriales bacteria, along with the increased proportions of Pichiaceae yeast as well as LAB and AAB (compare “Susp” with “w/o L” or “w/L” in Figure 1D; see also Tables S2C and S2D). These findings suggest that the composition shift observed in the food microbiota is unlikely to be solely attributed to the presence of larvae but more plausibly influenced by factors such as interspecies interactions among microbes.

Furthermore, we analyzed the microbial composition of adult flies captured in traps, as well as the food and larval samples (Figure 1E; Table S1C). This analysis revealed a similar composition of the yeast species in adult flies and early-stage foods, and a similar composition of yeast species in larvae and late-stage foods (top of Figure 1F; Table S2C). However, notable dissimilarities were observed in the bacterial compositions between the *Drosophila* samples and the early-stage foods, primarily attributed to the conspicuous presence of *Wolbachia* sp. (Anaplasmataceae) and *Gilliamella apicola* (Orbaceae) in the *Drosophila* samples (bottom of Figure 1F; Table S2E). Anaplasmataceae and Orbaceae are intracellular and gastrointestinal symbionts of insects, respectively (Kwong & Moran, 2013; Werren et al., 1995), which plausibly accounts for their relatively lower abundance in the food samples.

In addition to the community structures, we investigated whether there were alterations in the overall abundance of yeasts and bacteria between the early- and the late-stage foods. For this purpose, we performed quantitative RT-PCR to quantify the copy numbers of fungal and bacterial rDNA in each food sample presented in Figures 1B, 1D, and 1F (Figure S1). The analysis indicated that there were no dramatic increases or decreases in copy numbers in most food samples. Note that measuring rDNA copy numbers in the microbiome does not necessarily reflect actual cell numbers due to variations in the genomic rDNA copy numbers among species (Lofgren et al., 2019; Stoddard et al., 2015). Nonetheless, these results suggest that the quantities of microbes did not undergo substantial changes between the two stages.

Prominent acceleration of larval development is observed with early-stage-dominant yeast alone, as well as in combination with late-stage-dominant acetic acid bacteria and other microbes

As described in the previous section, we documented the presence of various yeast and bacterial species in fermented larval foods, the populations of which underwent notable compositional shifts over time. Previous studies suggest that different yeast or bacterial species contribute to larval development to varying degrees (Anagnostou et al., 2010; Consuegra, Grenier, Baa-Puyoulet, et al., 2020; Pais et al., 2018; Quan & Eisen, 2018; Solomon et al., 2019). Continuing this line of investigation, we undertook to identify which of the dominating microbial species in the early- or late-stage foods, either individually or as mixtures, promote larval development. To this end, we isolated fungal and bacterial strains from the food samples (refer to Table S3 and the “Isolation and species identification of microbes” section in MATERIALS AND METHODS for further details). We subsequently inoculated the isolated strains, either individually or in combinations, into a sterile banana-based food (banana agar), and introduced germ-free larvae. Thereafter, we evaluated the effects of the microbial species on the percentage and the timing of

pupariation (see details in “Quantification of larval development” in MATERIALS AND METHODS). Note that on this food, larval development critically depends on associated microbes, as the larvae failed to pupariate on banana agar without microbes (“germ-free (GF)” in **Figure 2A** and subsequent figures).

To assess the impact of microbes found in early-stage foods on larval development, we employed yeast strains belonging to *Hanseniaspora uvarum*, which dominated in the early-stage foods, or *Pichia kluyveri*, which was also detected in the foods (Table S2A; also represented by the darkest and the second darkest brown bars in the top of **Figure 1B**), along with a bacterial strain of *Pantoea agglomerans*, which constituted the sole dominant bacterial genus (Table S2B; also represented by cyan bars at the bottom of **Figure 1B**). We investigated whether each strain or a combination of the strains promotes larval development (**Figures 2A**, **2B**, **S2A**, and **S2B**). When inoculated individually, *H. uvarum* and *Pa. agglomerans* effectively promoted larval growth (“Y₂” and “E” in **Figures 2A** and **2B**, respectively; “Y₁” and “E” in **Figures S2A** and **S2B**, respectively; see also **Table 1**), whereas *Pi. kluyveri*, another yeast species, had almost no promoting effect (“Y₁” in **Figures 2A** and **2B**; “Y₂” in **Figures S2A** and **S2B**). When the strains were combined, we observed a significantly accelerated larval development in the presence of *H. uvarum* (“1: Y₁Y₂” and “3: Y₁Y₂E” in **Figures 2A** and **2B**) than in its absence (“2: Y₁E” in **Figures 2A** and **2B**). Hence, in the early-stage foods, *H. uvarum* played a critical role in promoting larval development.

A similar feeding experiment was conducted using a total of five stocks of the late-stage microbes (**Figures 2C**, **2D**, **S2C**, and **S2D**; **Table 1**): two dominating late-stage yeast species, the most predominant LAB and AAB species (Table S2B; see also green and orange in the bottom of **Figure 1B**, respectively), and *Pa. agglomerans*, which persisted from the early stage, albeit in a smaller proportion (**Table 1**). When fed individually, none of the yeast or bacterial species efficiently supported larval growth, except for *Pa. agglomerans* (lines and symbols with different brightness of gray in **Figure 2C**). Conversely, when larvae were fed mixtures, their development was almost equally efficient as long as LAB and AAB coexisted (color-coded in blues in **Figure 2C**). In this experiment, the coexistence effects of yeast and acetic acid bacteria were not tested. Therefore, we repeated the experiment using only one strain from each of the most dominant yeast, LAB, and AAB species (see the tables in **Figures 2F** and **S2F**). Larval development, in terms of both the pupariation rate and timing, was promoted when the AAB *A. orientalis* was inoculated together with either a yeast (*Starmerella bacillaris* or *Pi. kluyveri*) or a LAB (*Leuconostoc mesenteroides* or *Lactiplantibacillus plantarum*) (**Figures 2E**, **2F**, **S2E** and **S2F**). This result suggests that, in the late-stage foods, the coexistence of AAB and other microbial species, yeasts or LAB, was critical for larval development. Additionally, we investigated the eclosion of pupae under different microbial conditions and confirmed that a majority of the pupae successfully eclosed, albeit with some variation observed across experiments (**Figure S3**).

To summarize these results, strong promotion of larval development was observed when the larvae were associated with an early-stage yeast *H. uvarum*, or a combination of *A. orientalis* and other late-stage microbes.

During the late stage, acetic acid bacteria play a crucial role in supporting larval development through interspecies interactions among the microbes

To investigate how larvae respond to the different microbial conditions, we conducted whole-body RNA-seq of gnotobiotic first-instar larvae that were subjected to a 15-hour feeding period on banana agar (Table S4). The agar was either kept sterile or inoculated with yeast *H. uvarum*, LAB, or AAB individually, or a combination of LAB and AAB (**Figure 3A-D**). Remarkably distinct gene expression profiles were observed between the “supportive” conditions, where larvae efficiently pupariated in the previous experiments (“Yeast” and “LAB+AAB” in **Figure 3A**), and the

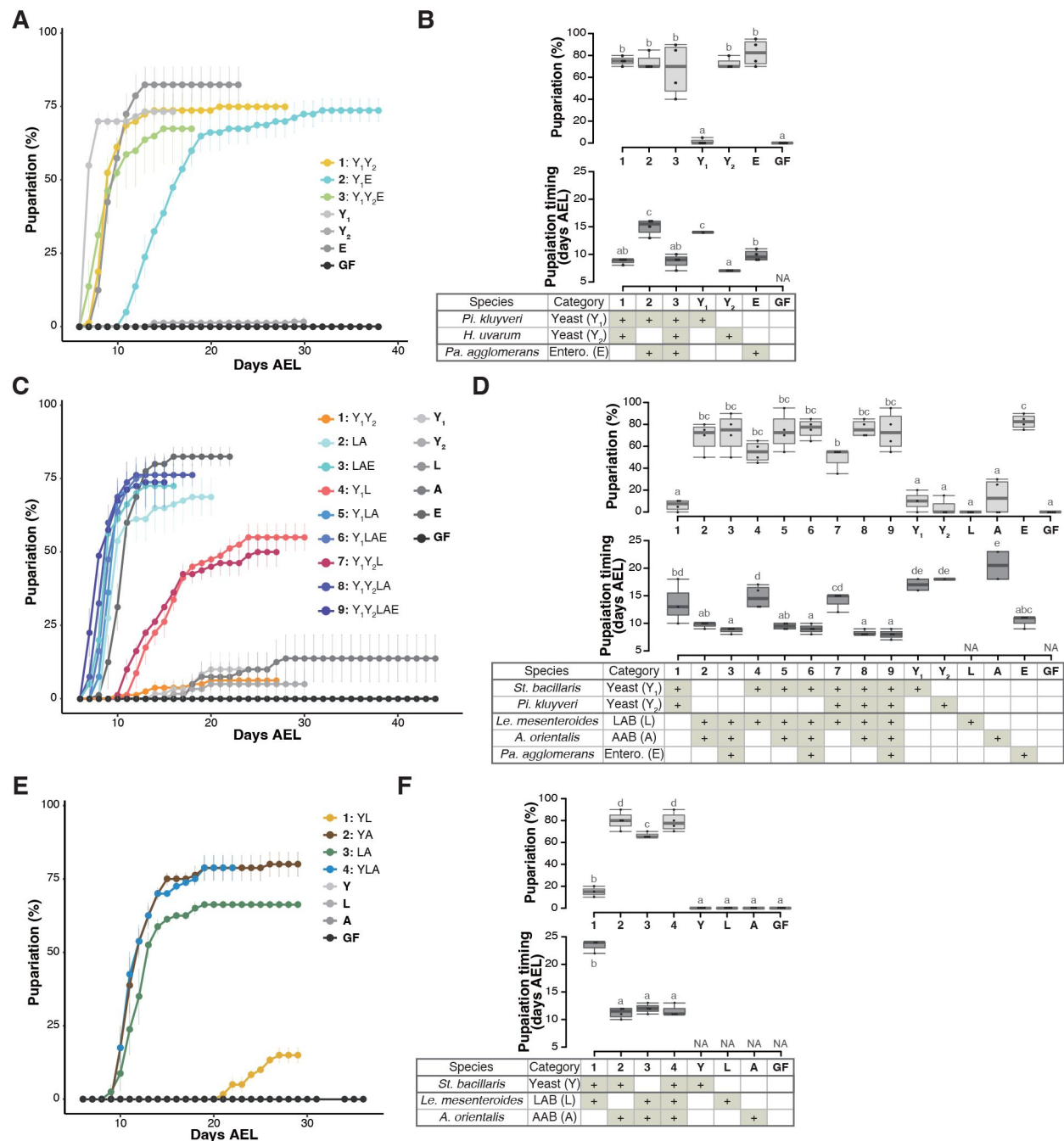


Figure 2.

Prominent acceleration of larval development occurred with early-stage-dominant yeast alone, as well as in combination with late-stage-dominant acetic acid bacteria and other microbes

(A-F) The percentage and timing of pupariation of larvae feeding on microbes detected in the early-stage food (A and B), or five (C and D) or three (E and F) microbes detected in the late-stage food from Sampling Place 1 in **Figure 1B**. (A, C, and E) Each data point represents the average percentage of individuals per tube that pupariated by each day. $n=3-4$. Colored lines are for mixed conditions, while gray lines are for mono-associated or germ-free conditions. The codes for individual species are provided in the chart at the bottom of (B), (D), and (E), respectively. (C) The color coding for the respective foods is as follows: includes yeasts only (orange); includes yeast(s) and LAB but no AAB (red); includes LAB and AAB (blue). (B, D, and F) Percentage of the larvae pupariated (pupariation ratio; upper) and the timing at which 50% of the pupariation ratio was achieved (lower). Boxes represent upper and lower quartiles, while the central lines indicate the median. Whiskers extend to the most extreme data points, which are no more than 1.5 times the interquartile range. Unique letters indicate significant differences between groups (Tukey-Kramer test, $p < 0.05$). GF, germ-free; days AEL, days after egg laying.

Yeast or bacterium	Fermentation stage	Family	Species	Sampling place 1 (early stage)	Sampling place 1 (late stage)	Sampling place 2 (early stage)	Sampling place 2 (late stage)
Yeast	Early	Saccharomycodaceae	<i>Hanseniaspora uvarum</i>	20.93%		79.28%	2.11%
Yeast	Late	Pichiaceae	<i>Pichia kluyveri</i>	40.09%	26.26%	19.07%	97.14%
Yeast	Late	incertae sedis	<i>Starmerella bacillaris</i>		72.97%		
Bacterium	Early	Erwiniaceae	<i>Pantoea agglomerans</i>	45.31%	12.81%	90.56%	6.17%
Bacterium	Late	Lactobacillaceae	<i>Leuconostoc mesenteroides</i>		54.17%		
Bacterium	Late	Lactobacillaceae	<i>Lactiplantibacillus plantarum</i>				60.84%
Bacterium	Late	Acetobacteraceae	<i>Acetobacter orientalis</i>		8.25%		15.48%

Table 1.

List of microbial species used in the feeding experiments.

Percentages indicate the relative abundance of each species in the foods shown in Figure IB.

“nonsupportive” conditions, where larval development was markedly impaired (“LAB” and “GF” in **Figure 3A**). Notably, a multitude of genes involved in metabolism and cell growth displayed significant upregulation in response to the AAB and LAB mixtures when compared to LAB alone (**Figures 3B**, 3C, S4A, and S4B). Moreover, differentially expressed genes under the supportive and the non-supportive conditions exhibited strikingly similar profiles to those reported in a previous investigation of fed and starved conditions (Zinke et al., 2002; **Figure 3D**).

Interestingly, although larvae fed on AAB alone largely failed to pupariate (“A” in **Figures 2C**–**2F**), their gene expression profile after the 15-hr feeding period closely resembled that of the supportive conditions (compare “AAB” with “Yeast” and “LAB+AAB” in **Figure 3A**). This result implies that AAB possessed the ability to induce a growth-promoting response, but the effect likely did not persist until pupariation. We observed that the growth rate of AAB on banana agar was notably lower compared to those of other microbes. This led us to speculate that this lower growth rate might result in a shortage of AAB, leading to undernutrition during the later stage of larval development. To test this hypothesis, we investigated whether larval development could recover when AAB was constantly available. Daily supplementation of AAB enabled larvae to pupariate as effectively as the initial co-inoculation of AAB and LAB did (**Figures 3E** and **3F**), demonstrating that AAB can promote larval growth if it is available throughout the course of development.

The aforementioned observations have prompted us to assume that yeast and LAB contribute to the stable coexistence of AAB and larvae. To investigate this hypothesis, we raised larvae on a diet containing AAB either alone or in conjunction with the other species. After a 4-day incubation, we quantified the abundance of AAB in each tube, including those in the food and those inside the larval body. In most of the combinations, co-inoculation with other species led to a 5–19 fold increase in the number of AAB colonies compared to the monoculture conditions (**Figures 3G** and **3H**). These findings suggest that AAB plays a crucial role in providing nutrients during the late stages of larval development, while other microbial species support a stable association between AAB and larvae.

Thus, none of AAB, LAB, or the yeast in the late-stage foods strongly promoted larval growth individually, while mixing AAB with LAB or the yeasts did (**Figures 2C**–**2F** and **S2C**–**S2F**). All of our results so far strongly suggest that these interspecies interactions among the microbes underlie the promotion of larval growth by late-stage microbiota.

The isolated yeast species promote varying degrees of larval development, but all support larval development upon heat killing

We found that the early-stage yeast *H. uvarum* facilitated larval development, whereas the dominant yeasts in the late stage, *Pi. kluyveri* and *St. bacillaris*, did not (**Figure 2A**–**2D** and **S2A**–**S2D**). To elucidate the underlying mechanisms behind these diverse outcomes, we conducted comprehensive comparative analyses with the respective yeasts. We used six yeast species that originated from the fermented foods (*Kazachstania humilis*, *Martiniozyma asiatica*, and *Saccharomycopsis crataegensis* in addition to the aforementioned three species) and a laboratory strain of the model species *Saccharomyces cerevisiae*, which was not detected in our food samples (**Figure 4**).

When we provided each species to germ-free larvae, the larvae fed on *H. uvarum* showed the highest percentage of pupariation, while larvae fed on *K. humilis* or *M. asiatica* also pupariated at relatively high rates, albeit with a slower timing for *M. asiatica* (**Figures 4A** and **4B**). We classified these three species as the “supportive” yeast species. In contrast, larvae fed on *Pi. kluyveri* or *St. bacillaris*, which are the dominant species during the late stage, showed notably low percentage of pupariation, leading to their classification as “non-supportive” (**Figures 4A** and **4B**). Inoculation with *Saccharomycopsis crataegensis* or an laboratory strain of *Saccharomyces*

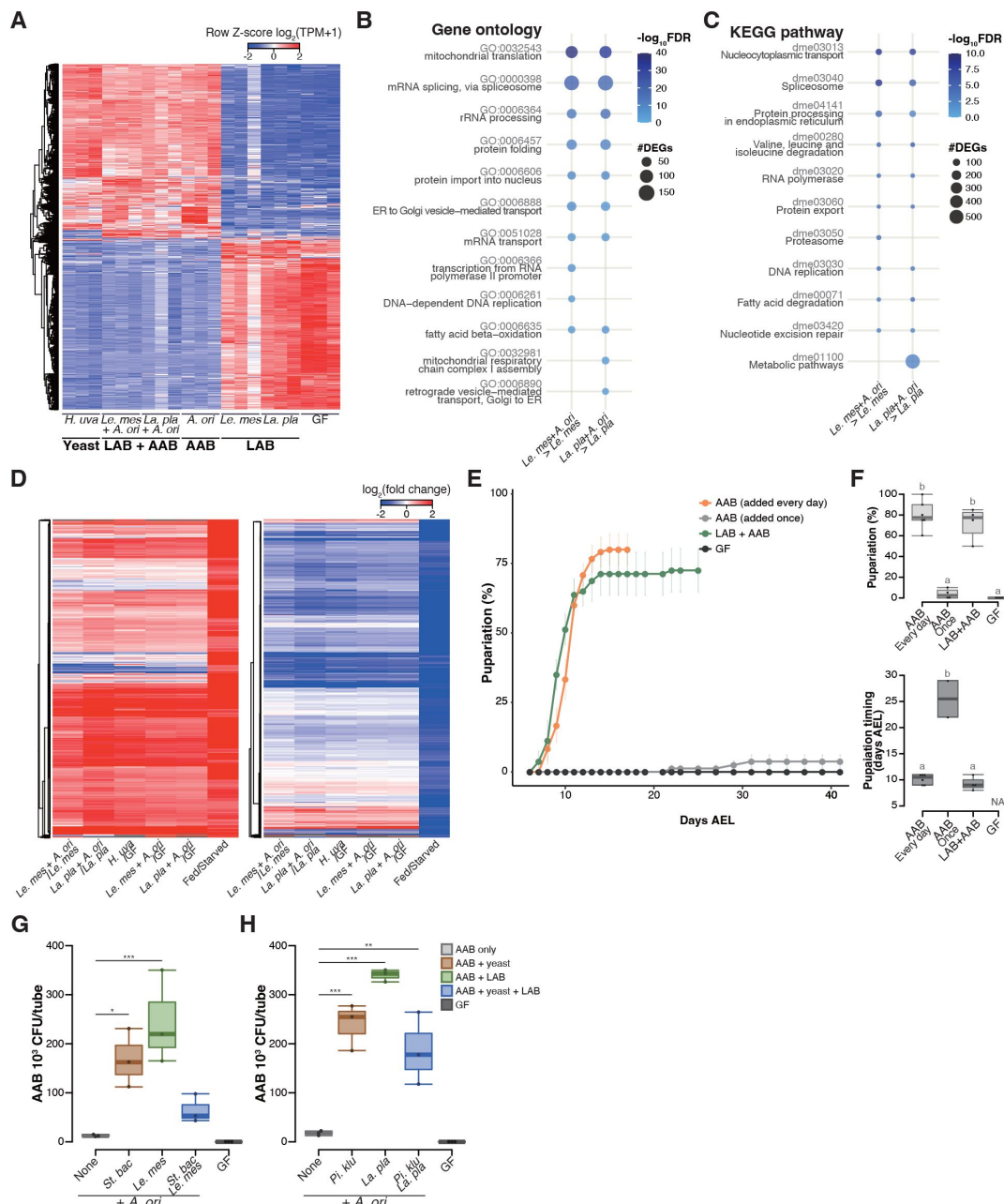


Figure 3.

During the late stage, acetic acid bacteria play a crucial role in supporting larval development through interspecies interactions among the microbes

(A) Heat map of gene expression values for first instar larvae fed *H. uvarum* or bacteria. Freshly hatched germ-free larvae were placed on banana agar inoculated with each microbe and collected after 15 h feeding to examine gene expression of the whole body. (B and C) Plots showing the result of GO term (B) or KEGG Pathway (C) enrichment analysis of the RNA-seq data. 10 terms/pathways that showed the smallest FDR in each analysis are shown. (D and E) Heat maps showing the similarity between our RNA-seq data and microarray data in a previous study (Zinke et al., 2002). Zinke et al. used larvae at 47–49 h AEL and fed them yeast paste or starved them for 12h before comparing the gene expression profiles. The data from Zinke et al., 2002 are labeled as “Fed/Starved” in the righthand column of each heat map. Only the genes exhibiting more than 2-fold changes in their analysis are shown. Our data were calculated using TPM of each gene in the larvae on the supportive conditions versus those on the non-supportive conditions. (E and F) The percentage and timing of pupariation of the larvae feeding on AAB on banana agar. Graphs are presented as in Figure 2. $n=3-4$. (G and H) AAB load of foods inoculated with AAB, with or without other microbial species. Boxplots are depicted as in Figure 2 (Dunnett’s test, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). *La. pla*, *Lactiplantibacillus plantarum*; *Le. mes*, *Leuconostoc mesenteroides*; *A. ori*, *Acetobacter orientalis*; *H. uva*, *Hanseniaspora uvarum*; *St. bac*, *Starterella bacillaris*; *Pi. klu*, *Pichia kluyveri*; GF, germ-free; days AEL, days after egg laying.

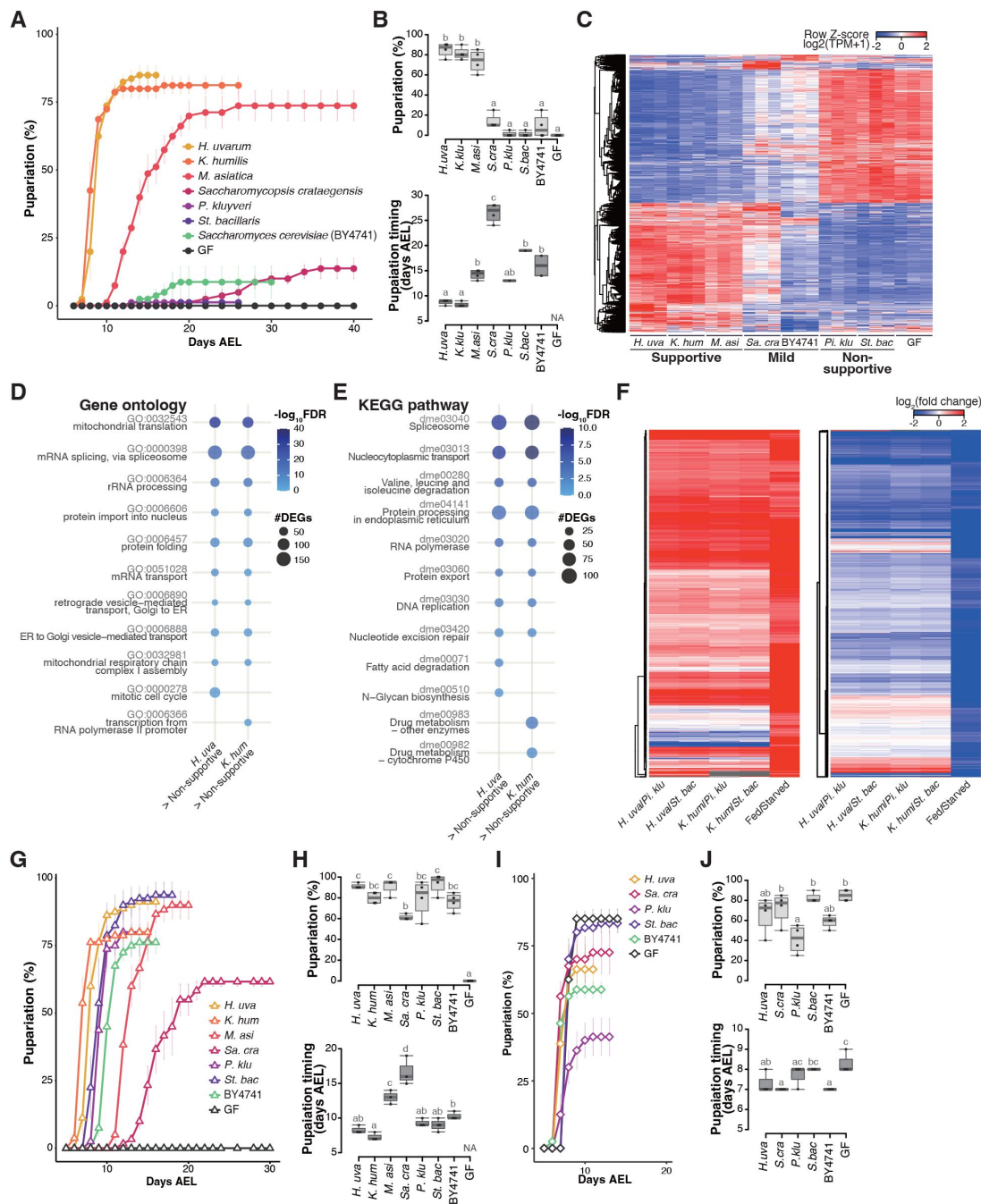


Figure 4.

Isolated yeast species promote larval development to varying degrees, but all support larval development upon heat killing

(A and B) The percentage and timing of pupariation of larvae feeding on live yeasts on banana agar. Graphs are presented as in [Figure 2](#). *n* = 3-4. (C) Heat map of gene expression values for first instar larvae fed on each yeast species. Freshly hatched germ-free larvae were placed on banana agar inoculated with each microbe and collected after 15 h feeding to examine gene expression of the whole body. (D and E) Plots showing the result of GO term (D) or KEGG Pathway (E) enrichment analysis of the RNA-seq data. Genes that showed significantly higher expression on each of the supportive species than on both the non-supportive species were analyzed. 10 terms/pathways that showed the smallest FDR in each analysis are shown. (F) Heat maps showing the similarity between our RNA-seq data and microarray data in [Zinke et al., 2002](#), shown as described in [Figure 3](#). (G-J) The percentage and timing of pupariation of the larvae feeding on heat-killed yeasts on banana agar (H and I) or live yeasts on a nutritionally rich medium (J and K). Graphs are depicted as in [Figure 2](#). *n* = 3-4. *H. uva*, *Hanseniaspora uvarum*; *K. hum*, *Kazachstania humilis*; *M. asi*, *Martiniozyma asiatica*; *Sa. cra*, *Saccharomycopsis crataegensis*; *Pi. klu*, *Pichia kluyveri*; *St. bac*, *Starmerella bacillaris*; GF, germ-free; days AEL, days after egg laying.

cerevisiae BY4741 resulted in reduced percentages and delayed timing of pupariation compared to the inoculation with supportive species, thus earning the designation of “mild.” These results underscore the variable abilities of individual yeast species to promote larval development.

To investigate the response of larvae to different yeast species, we conducted whole-body RNA-seq analysis of larvae (Table S5). The overall findings closely resembled those obtained from various bacterial species (Figures 4C–4F, S4C, and S4D; cf. Figures 3A–3D, S4A, and S4B). Gene expression profiles exhibited marked differences between larvae fed on the supportive yeasts and those fed on the non-supportive yeasts, with intermediate responses observed in larvae fed on the mild yeasts (Figure 4C). Feeding on the supportive yeasts upregulated genes involved in metabolism and cell growth (Figures 4D and 4E), and the expression profiles under the supportive and the non-supportive yeast diets resembled those observed in fed and starved conditions, respectively (Figure 4F).

One potential factor determining the ability of larvae to develop on specific yeast species might be the production or secretion of adequate nutrients for larval growth. To assess whether each yeast species produced sufficient essential nutrients internally, we administered heat-killed yeasts to the larvae. Somewhat surprisingly, strong growth enhancement was observed in all seven yeast species upon heat killing, with *Pi. kluyveri* and *St. bacillaris* promoting larval development nearly as effectively as the supportive yeasts (Figures 4G and 4H). We further explored the effect of killing the yeasts using banana agar supplemented with antifungal agents (10 mL/L 10% p-hydroxy-benzoic acid in 70% ethanol and 6 mL/L propionic acid, following the concentration described in Kanaoka et al., 2023), and observed similar growth promotion by *Pi. kluyveri* and *St. bacillaris* (data not shown). This finding suggests that all of these yeasts do indeed produce the requisite nutrients for larval development; however, it is likely that the nutrients produced by the live non-supportive yeasts are inaccessible to larvae (further analyzed in the subsequent section).

We also considered the possibility that the non-supportive yeasts somehow inhibited the host growth. To test this possibility, we cultured the yeast species on a nutrient-rich sterile medium and fed them to the larvae (Figure 4I and 4J). This medium contains dry yeast and enables larvae to pupariate even without live yeasts (“GF” in Figure 4I and 4J). Under these conditions, larvae fed with *St. bacillaris* pupariated as efficiently and nearly as rapidly as those without yeasts or with other yeast species, suggesting that *St. bacillaris* did not impede larval growth (Figures 4I and 4J). In contrast, larvae fed with *Pi. kluyveri* exhibited a significantly reduced percentage of pupariation compared to larvae grown with the other yeast species or without yeasts. This could be related to the extensive growth of *Pi. kluyveri* on this food (Figure S4E). Nevertheless, none of these yeast species reproduced the low percentage of pupariation of larvae observed on banana agar. Therefore, the inhibitory effects of *Pi. kluyveri* and *St. bacillaris* are less likely.

Supportive yeasts facilitate larval growth by producing nutrients, including branched-chain amino acids, and releasing them from their cells

Given that the non-supportive yeast species supported larval growth upon heat-killing, we hypothesized that the key distinction between the supportive and the non-supportive species lies in their ability to release nutrients contained within the cells. To test this hypothesis, we conducted a metabolomic analysis of the two supportive species (*H. uvarum* and *K. humilis*) and the two nonsupportive species (*Pi. kluyveri* and *St. bacillaris*) and analyzed not only the yeast cells but also two additional samples anticipated to contain metabolites released from the cells: yeast-conditioned banana-agar plates and cell suspension supernatants. (Figure 5A–5F, Table S6).

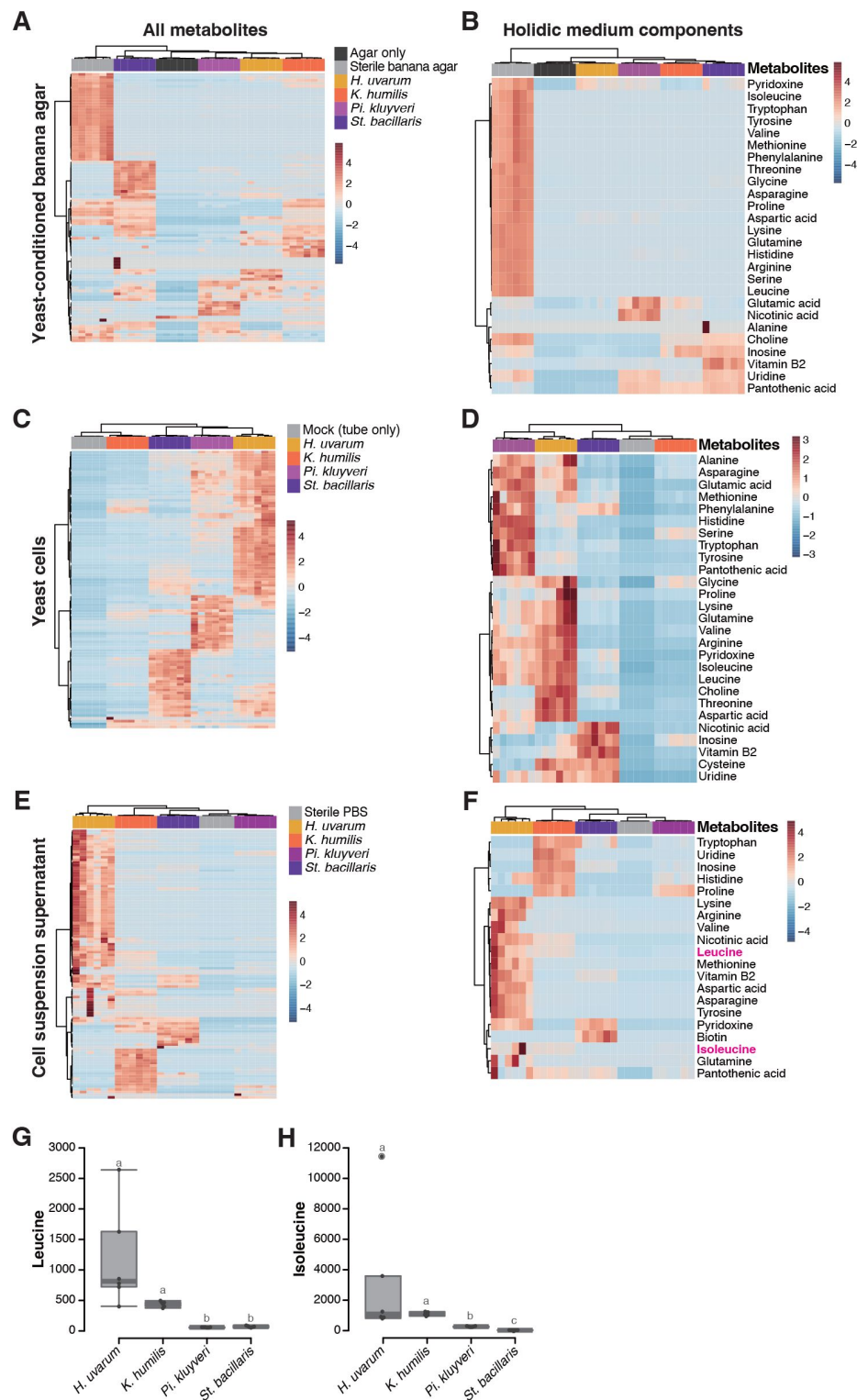


Figure 5.

Metabolomic analysis of yeast cells, yeast-conditioned banana-agar plates, or cell suspension supernatants

(A-F) Heat maps showing all detected metabolites (A, C, and E) or only the metabolites included in a chemically defined synthetic (holidic) medium for *Drosophila melanogaster* (Piper et al., 2014) (B, D, and F) detected from the metabolomic analysis of banana-agar plates (A and B), yeast cells (C and D), or yeast-cell suspension supernatants (E and F). (G and H) Normalized peak areas of leucine (G) and isoleucine (H) in yeast-cell suspension supernatants. Boxplots are presented as in Figure 2.

In the yeast-conditioned banana-agar plates, which were anticipated to contain yeast-derived nutrients, many well-known nutrients included in a chemically defined synthetic (holidic) medium for *Drosophila melanogaster* (Piper et al., 2014 [DOI](#), 2017 [DOI](#)) were not increased compared to the sterile banana-agar plates; instead, they exhibited drastic decreases irrespective of the yeast species (**Figures 5A** [DOI](#) and **5B** [DOI](#)). The quantities of metabolites within the cells varied markedly among the species (**Figures 5C** [DOI](#) and **5D** [DOI](#)); while the supportive *H. uvarum* contained numerous known metabolites, it was intriguing that *K. humilis*, another supportive species, did not possess as many essential nutrients as other species, including the non-supportive *Pi. kluyveri* and *St. bacillaris* (**Figure 5D** [DOI](#)). This result, in conjunction with the observations from feeding heat-killed yeasts (**Figure 4G** [DOI](#) and **4H** [DOI](#)), raises the possibility that the interspecies disparities in metabolites within living yeast cells do not directly influence larval growth. On the other hand, our analysis of the cell suspension supernatant revealed distinct variations in the known nutrients in the holidic medium between the supportive and non-supportive species (**Figures 5E** [DOI](#) and **5F** [DOI](#)). Among these, we focused on branched-chain amino acids (BCAAs), leucine and isoleucine (**Figure 5F** [DOI](#)). Previous studies have demonstrated that bacteria associated with *Drosophila* provide these essential amino acids to the hosts feeding on artificial diets lacking them (Consuegra, Grenier, Akherraz, et al., 2020; Henriques et al., 2020 [DOI](#); B. Kim et al., 2021 [DOI](#)). In our analysis, suspension supernatants of supportive yeasts had concentrations of both leucine and isoleucine that were at least four-fold higher than those of non-supportive yeasts (**Figures 5F** [DOI](#)–**5H** [DOI](#); see also Table S6B).

The above finding prompted us to explore whether leucine and isoleucine are supplied by the associated supportive yeasts, similar to bacterial symbiosis. To investigate this, we supplemented banana agar with these BCAAs and inoculated it with the non-supportive yeasts, subsequently evaluating the restoration of larval development (**Figures 6A** [DOI](#) and **6B** [DOI](#)). Remarkably, the supplementation of BCAAs elicited a significant improvement in larval development in the presence of the non-supportive yeasts, while it had no effect on larvae fed with the supportive yeasts (**Figures 6A** [DOI](#) and **6B** [DOI](#)). At 12 days after egg laying, a significant increase was observed in the proportion of individuals that had progressed to the second instar or later stages (**Figure 6B** [DOI](#)). These results suggest that the lack of BCAAs in bananas inoculated with the non-supportive yeast species is one of the causes of larval growth deficiency. Additionally, we explored the conservation of the biosynthetic pathways responsible for leucine and isoleucine among the isolated yeast strains. We performed *de novo* genome assembly and annotation for the six yeast species and examined the presence of orthologs (Table S7). This analysis, along with a subsequent RNA-seq analysis of the yeast species, revealed the conservation of the biosynthetic pathways of these amino acids across all the isolated yeasts (**Figure 6C** [DOI](#); Table S8). The result of this genomic analysis also strengthens the possibility that differences in the ability of yeasts to support larvae do not stem from variances in their ability to biosynthesize BCAAs, but rather in their ability to provide BCAAs in an available form to the larvae.

We noted, however, that the supplementation of BCAAs alone did not completely restore the growth of larvae that fed on the non-supportive yeasts. Despite attempts to enhance growth by increasing the BCAA concentration five- or ten-fold, no improvement was observed (data not shown). We also supplemented the banana agar with other metabolites, including nicotinic acid and/or lysine and asparagine, which were detected in higher amounts within the suspension supernatants of the supportive yeasts, individually or in combination with the BCAAs. None of these additions had any effect on larval growth (data not shown). These results suggest that other crucial nutrients are provided by the supportive yeasts.

Regarding the mechanism of nutrient release, one possibility is their secretion from live cells, while another possibility is their leakage from dead cells. To address the latter hypothesis, we collected cells of the four yeast species from banana-agar plates and stained dead cells using

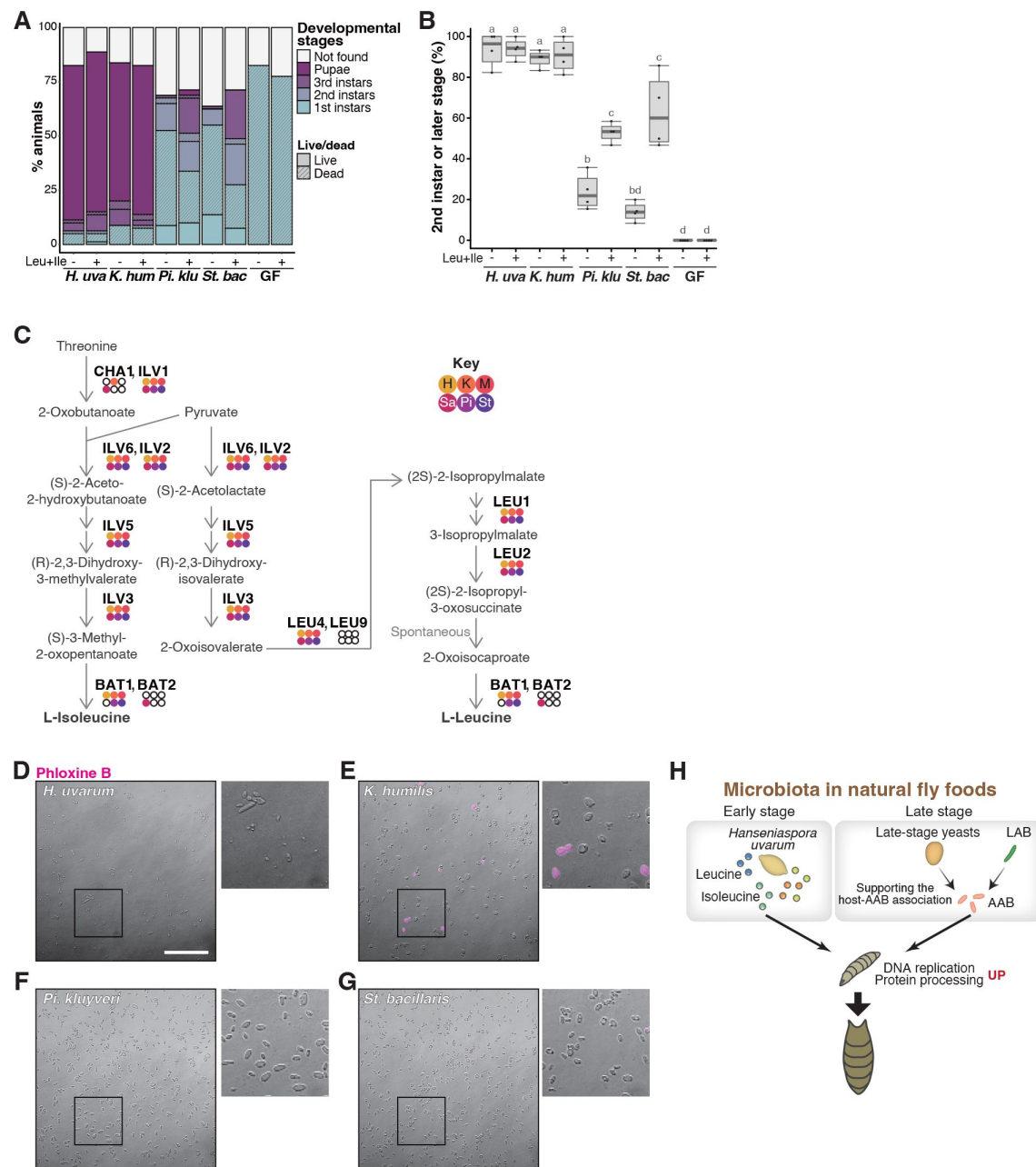


Figure 6.

Supportive yeasts facilitate larval growth by providing nutrients, including branched-chain amino acids, by releasing them from their cells

(A and B) Growth of larvae feeding on yeasts on banana agar supplemented with leucine and isoleucine. (A) The mean percentage of the live/dead individuals in each developmental stage. $n=4$. (B) The percentage of larvae that developed into second instar or later stages. The “Not found” population in **Figure 6A** was omitted from the calculation. Each data point represents data from a single tube. Unique letters indicate significant differences between groups (Tukey-Kramer test, $p < 0.05$). (C) The biosynthetic pathways for leucine and isoleucine with *S. cerevisiae* gene names are shown. The colored dots indicate enzymes that are conserved in the six isolated species, while the white dots indicate those that are not conserved. Abbreviations of genera are given in the key in the upper right corner. LEU2 is deleted in BY4741. (D-G) Representative image of Phloxine B-stained yeasts. The right-side images are expanded images of the boxed areas. The scale bar represents 50 μ m. (H) Summary of this study. *H. uvarum* is predominant in the early-stage food and provides Leu, Ile, and other nutrients that are required for larval growth. In the late-stage food, AAB directly provides nutrients, while LAB and yeasts indirectly contribute to larval growth by enabling the stable larva-AAB association. The host larva responds to the nutritional environment by dramatically altering gene expression profiles, which leads to growth and pupariation. *H. uva*, *Hanseniaspora uvarum*; *K. hum*, *Kazachstania humilis*; *Pi. klu*, *Pichia kluyveri*; *St. bac*, *Starmerella bacillaris*; GF, germ-free.

Phloxine B (**Figures 6D** [↗](#)-**6G** [↗](#)). Noticeably more dead cells were found in the supportive *K. humilis* compared to other species (**Figure 6E** [↗](#)), implying that nutrients leak from dead *K. humilis* cells into the food, which is subsequently utilized by the larvae.

Discussion

In this study, we have shown that the composition of the microbiota shifts dramatically as fermentation progresses. Each individual microbial species plays a crucial and indispensable role in promoting host growth, either through the provision of essential nutrients or by establishing a stable association between the host and the nutrient-providing species (**Figure 6H** [↗](#)). The capacity of yeasts to facilitate larval growth seems to rely on their ability to extracellularly release essential nutrients, such as leucine and/or isoleucine, thereby making these nutrients accessible to the host. These microbial functions have effectively empowered the host to grow on a nutritionally inadequate fruit.

Drosophila has been known to co-exist with yeast and bacteria in nature (Chandler et al., 2011 [↗](#), 2012 [↗](#); Corby-Harris et al., 2007 [↗](#); Cox & Gilmore, 2007 [↗](#); Quan & Eisen, 2018 [↗](#); Shihata & Mrak, 1952 [↗](#)). Our investigation reveals substantial shifts in both fungal and bacterial compositions during fermentation, which coincides with alterations in the species serving as nutrient sources. A previous study reported that *Drosophila* larvae have profound effects on the community structure of yeasts in fermented bananas (Stamps et al., 2012 [↗](#)). Our experiment, however, suggests that the microbial composition shift observed in our food samples is unlikely to be solely attributed to the presence of larvae but is more plausibly influenced by factors such as interspecies interactions among microbes. These discrepancies may be due in part to differences in experimental conditions such as larval densities, food sample volumes, or culture vessels. The initial microbial inoculation to foods is assumed to be mediated by adult flies (Broderick & Lemaitre, 2012 [↗](#)). Our results imply that the inoculation leads to the dominance of *H. uvarum*, which can support larval growth on its own. Consequently, a microbially self-regulated transition occurred, leading to the dominance of microbial communities, including AAB, LAB, and the late-stage yeasts.

In the early stage of fermentation, both the predominant yeast and bacterial species were capable of supporting larval growth on their own. However, in stark contrast, at the late stage of fermentation, microbial interactions assumed a crucial role. While AAB provide sufficient nutritional resources to support larval growth, their growth rate on bananas is suboptimal. On the other hand, other associated microbes may lack essential nutrients for larvae but contribute to establishing a stable association between AAB and the larvae. Previous studies have reported interactions between AAB and LAB in artificial foods, where AAB act as a source of nutrients, including amino acids, while LAB provide lactic acid as a substrate for amino acid biosynthesis (Consuegra, Grenier, Akheraz, et al., 2020; Henriques et al., 2020 [↗](#)). Our findings have demonstrated that LAB also play a supportive role in the symbiotic relationship between larvae and AAB on a fruit, a natural food source of *Drosophila* larvae. It is worth noting that interactions may also occur between the AAB and the late-stage yeasts. We observed the presence of various metabolites not only in the suspension supernatant of the supportive yeast species but also in that of the non-supportive late-stage yeast species. Therefore, the growth of AAB on fruits could be enhanced through the utilization of such metabolites by the bacterium.

In laboratory settings, yeasts are recognized and employed as important nutritional sources for *Drosophila*, providing essential nutrients such as amino acids, vitamins, and fatty acids (Baumberger, 1917 [↗](#); Broderick & Lemaitre, 2012 [↗](#)). In our study, we have demonstrated that during larval growth on their natural food sources, the associated yeasts contribute to the provision of BCAAs, leucine and/or isoleucine. Of these two amino acids, the availability of leucine acts as an effective regulatory signal for the mTORC1 pathway, as established in both mammals and *Drosophila* (Fox et al., 1998 [↗](#); Gu et al., 2022 [↗](#); J. Kim & Guan, 2019 [↗](#); Zhang et al., 2021 [↗](#)).

The mTORC1 pathway governs metabolism and cell growth in response to nutritional conditions. Indeed, the expression levels of numerous genes involved in these processes exhibited significant up- or down-regulation in larvae fed on supportive microbes compared to those fed on non-supportive microbes or germ-free individuals. Nonetheless, supplementing these branched-chain amino acids (BCAAs) alone did not fully restore larval growth, suggesting that there should be other key nutrients. Our metabolomic analysis did not encompass some known metabolites, including lipids. Among these, sterols, substrates for ecdysone biosynthesis, are indispensable for larval growth. However, sterols are unlikely to be lacking in the fermented bananas. In our experiments, *Pa. agglomerans* and *A. orientalis* did promote larval growth, but most bacteria, including the two aforementioned species, lack the steroid biosynthesis pathway (Hoshino & Gaucher, 2021 [DOI](#)). Therefore, larvae can grow on bananas without bacterial-derived sterols. Deficiency in other lipids is also unlikely, because larvae can grow on the holidic medium for *D. melanogaster* that contains cholesterol as a sole lipid component, and the developmental delay on the medium is rescued by a “critical element” which is “not a lipid” (Piper et al., 2014 [DOI](#)). Therefore, microbial nutrients that promote larval growth, whose functions have not yet been identified in this study, may be components other than lipids.

Despite the well-recognized importance of yeast as a source of nutrients, limited studies have reported the detailed process of how these nutrients are utilized by larvae. Our results suggest that for yeasts to serve as nutritional sources, they should not only produce the nutrients, but also release them outside the cells. The amount of nutrients released extracellularly varied among yeast species, which could be explained by varying abilities to secrete metabolites. Another possibility suggested for *K. humilis* was that this species is more prone to death on the culture medium, thus releasing more nutrients. Our metabolomic analysis of yeast-conditioned culture medium also suggested that the released nutrients do not accumulate in the medium but are likely to be eventually taken up and utilized by the yeast themselves. Further investigation is required to elucidate the nutritional dependence and competition between yeast and larvae.

Our results indicate that yeast cells are not necessarily digested in the larval gut, which might contradict previous reports. For instance, vegetative cells of *Saccharomyces cerevisiae* have been reported to be digested in the gut of adult flies (Coluccio et al., 2008 [DOI](#)). However, the digestibility of yeast may vary depending on the species of yeast, diet composition, or developmental stage of the host. Indeed, a study has discussed the possibility that *St. bacillaris* is not digested in the larval gut (Solomon et al., 2019 [DOI](#)). In our study, by focusing on the non-model yeast species associated with wild flies, we revealed part of the mechanism by which wild *Drosophila* larvae utilize yeast as a nutrient source.

To date, the majority of studies on animal-associated yeasts have focused on a limited number of specific species that are either pathogenic or employed for fermenting foods. In this study, we have examined the species composition of yeasts that make up the symbiotic mycobiota of *Drosophila* and analyzed the roles of each predominant species by investigating their interactions with bacteria as well as with the host. To obtain a comprehensive and detailed understanding of the function of animal microbiota, future studies should further explore the contribution of associated yeasts.

Materials and methods

Drosophila melanogaster strains and culture

D. melanogaster Canton-Special (E-10002) strain was obtained from EHIME-Fly *Drosophila* Stocks of Ehime University. This strain was used for all the experiments unless otherwise noted. *Cg-GAL4* was a gift from H. Asha (Asha et al., 2003 [DOI](#)). The stocks were reared on a laboratory standard diet as previously described (Watanabe et al., 2017 [DOI](#)).

Yeast-based nutrient-rich diet was prepared following the instructions at Bloomington Stock Center (<https://bdsc.indiana.edu/information/recipes/germanfood.html>). Reagents used are described in Kanaoka et al., 2023. The preservatives (propionic acid and 10% p-hydroxy-benzoic acid methyl ester) were omitted.

Generation of germ-free larvae

Germ-free animals were prepared as previously described (Watanabe et al., 2019) with minor alterations. Briefly, embryos were collected on apple agar plates topped with yeast paste, and were incubated at 25°C for 12-15 or 14.5-17.5 h. They were subsequently dechorionated in 50% bleach, followed by washes with sterile water, 70% ethanol, and sterile water once more. The embryos were placed on sterile agar plates and incubated at 25°C until newly hatched larvae were obtained.

Collection of fermented foods and wild *Drosophila* samples

Fermented food samples were collected using traps placed outdoors, near the authors' apartments or houses in Kyoto and Osaka prefectures, Japan, or outside the laboratory at Kyoto University. Within each trap made from an empty milk carton, ripening bananas, freshly peeled and cut into small pieces, were placed in a sterile 100 mL centrifuge tube (Iwaki). 2.5 days later, wild flies were found in all traps except for one (no-fly trap). The traps were collected, and 3-5 mL of the foods (early-stage foods) were sampled. The remaining foods were incubated at 25°C, and 4-5 days later, when late 3rd instar larvae were seen in all foods except for the one from the no-fly trap, the foods were collected (late-stage). At the first sampling, we added germ-free embryos of GFP-expressing *D. melanogaster* laboratory strain (*Cg-Gal4, UAS-mCD8:GFP*) to obtain *D. melanogaster* larvae that were reared in the fermented food, but we could not sample them because most animals apparently drowned in the juice that seeped out of the fermented bananas.

To obtain early-stage-food suspensions, newly obtained early-stage foods were crushed in PBS, and after vigorous mixing, the liquid was collected carefully without taking banana, embryos, or larvae. 200 µL of this microbial suspension (or sterile PBS for Blank) was added to 100 mL tubes containing fresh ripening banana with or without ~200 germ-free embryos (*Cg-Gal4, UAS-mCD8:GFP*). The samples were incubated at 25°C for 4 days. Late-stage foods were collected as described above. Larvae collected from the food were examined under a fluorescent microscope to make sure they were derived from the embryos we added and not from contaminating wild embryos. When sterile PBS was added to bananas instead of microbial suspensions in **Figure 1C**, the food showed no apparent change in appearance or odor during the 4-day incubation, and larvae in the food remained at the first instar stage. The amounts of microbes detected in such foods were less than those obtained for the food inoculated with the microbial suspension by 2-4 orders of magnitude ("Blank" in **Figure S1C** and **S1D**; see details in the legend).

All the food samples were collected after removing embryos and larvae, and snap-frozen. Larvae were surface-sterilized using the same procedure as embryo samples before snap-freezing. Adult flies in the traps were collected following different procedures depending on whether their microbiota were analyzed. When the samples were solely utilized for species identification, the whole bodies were stored in 100% or 70% ethanol. When we also required samples for microbial analysis, after washing the whole bodies in 70% ethanol, the head, external genitalia, and wings of each fly were removed and stored in 70% ethanol and subsequently used to identify the species. The rest of the body was snap-frozen and later used for the microbial composition analysis. Adult flies collected in each sampling are listed in Table S1.

All tools were sterilized prior to use. Frozen samples were stored at -80°C. Adult or larval samples collected from a single trap or food, respectively, were stored as a pool.

ITS or 16S rDNA sequencing analysis

Food, larval, or adult samples were freeze-dried for 2-3 days, followed by homogenization with 5 mm and 3 mm zirconium beads. Microbial DNA was extracted using a QIAamp DNA Stool Mini Kit (QIAGEN).

Library preparation, sequencing, and data analyses were performed by MacroGen Japan (Tokyo, Japan). For fungal analysis, the ITS region was amplified with primers ITS3 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTCGATGAAGAACGCAGC-3') and ITS4 (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCTCCGCTTATTGATATGC-3'). For bacterial analysis, the V3-V4 region of 16S rRNA was amplified with primers 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') and 805R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'). The amplicons were sequenced on an Illumina MiSeq sequencer (300 bp paired-end).

The paired-end reads were assembled using FLASH (Magoč & Salzberg, 2011 [DOI](#)), followed by pre-processing and clustering using CD-HIT-OTU (clustering cutoff = 99%). Taxonomy was assigned using QIIME (Caporaso et al., 2010 [DOI](#)) using the UNITE database (Nilsson et al., 2019 [DOI](#)) for fungal ITS, and the Ribosomal Database Project (RDP) database (Cole et al., 2014 [DOI](#)) for bacterial 16S rDNA (outputs can be found in Tables S2F-S2J), followed by manual correction and reassignment, in which reads originating from *Drosophila* spp. or banana (including chloroplast) were removed, and fungal and bacterial taxonomy was reassigned by NCBI BLAST search against the Nucleotide collection (nr/nt) database or using the RDP sequence match tool, respectively (Cole et al., 2014 [DOI](#); Johnson et al., 2008 [DOI](#)). Sequences with the highest query cover and identity in blastn, or the highest S_ab score in RDP sequence match were considered as top hits. OTUs that had no top hits with an assigned species name or with top hits that belonged to more than one genus were marked as “Unassigned.”

qRT-PCR of ITS or 16S rDNA

Quantification of fungal ITS or bacterial 16S rDNA was performed by quantitative real-time PCR with THUNDERBIRD SYBR qPCR Mix (TOYOBO). DNA extracted from fermented food or wild *Drosophila* spp. samples were diluted 10-100 fold before use. After an initial denaturation at 95°C for 1 min, 45 cycles of PCR were carried out: 95°C for 15 sec, 55°C for 30 sec, and 72°C for 50 sec. ITS or 16S rDNA amplicons derived from *Hanseniaspora uvarum* or *Pantoea agglomerans*, respectively, were used for the generation of standard curves. The following primers were used:

For fungal ITS (White et al., 1990 [DOI](#))

ITS3: 5'-GCATCGATGAAGAACGCAGC-3'

ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

For bacterial 16S (Hugerth et al., 2014 [DOI](#))

341F: 5'-CCTACGGGNGGCWGCAG-3'

805R: 5'-GACTACHVGGGTATCTAATCC-3'

Microbial rDNA copy numbers were calculated by multiplying the RT-PCR results by the respective percentages of microbial reads from ITS or 16S amplicon sequencing.

Microbial culture

Microbes were cultured on MRS, YPD, PDA, or banana agar. MRS, YPD, and PDA were prepared using MRS Broth (Merck Millipore), YPD medium (Clontech), and Potato Dextrose Broth (Sigma-Aldrich), respectively, following the manufacturers' protocol. Banana agar was prepared as previously described (Anagnostou et al., 2010), with the addition of autoclaving after making the banana-agar mixture. 10% p-hydroxy-benzoic acid in 70% ethanol (prepared as described in Kanaoka et al., 2023), propionic acid (Nacalai Tesque), cycloheximide (Wako), or ampicillin (Nacalai Tesque) were added after autoclaving. Food-derived microbes were cultured at 25°C for 2 days, while *S. cerevisiae* was cultured at 30°C for 2 days before use, unless otherwise noted.

The *S. cerevisiae* BY4741 strain was obtained from NBRP-Yeast, Japan. To investigate underlying mechanisms of differential growth-promoting ability between AAB and LAB, we attempted to use *La. plantarum*^{WJL} and a genetically engineered BCAA-producing *Lacto*^{BCAA} strain (B. Kim et al., 2021), both of which were kind gifts from W.-J. Lee. However, growth of *Lacto*^{BCAA} strain on our banana agar was too slow, which made it impossible to obtain a sufficient quantity of cells for the feeding experiments.

Isolation and species identification of microbes

Microbial strains were isolated on MRS, PDA, or banana-agar plates. The fermented foods were spread on each of these plates. Single colonies were picked and re-streaked on a plate, and after repeating the process once more, uniform colonies were collected, suspended in MRS liquid medium containing 40% glycerol, and stored at -80°C.

To identify the species of each strain, PCR and sequencing were performed. Microbial colonies were directly suspended in water in PCR tubes and heated (96°C for 5 min) before the addition of other reagents. In the cases when DNA failed to amplify, DNA was extracted using InstaGene Matrix (BIO-RAD) according to the manufacturer's protocol. NL1/NL4 primers were used for fungi, while 27F/1492R primers were used for bacteria (Chandler et al., 2011, 2012). The amplicon was purified prior to sequencing using a Wizard SV Gel and PCR Clean-Up System (Promega).

Sequencing was performed using an Applied Biosystems 3130xl Genetic Analyzer, and primers were NL1 for fungi and 518F (5'-CCAGCAGCCGCGGTAATACG-3') for bacteria (https://www.macrogen-japan.co.jp/cap_seq_0104.php). See Table S3A for the sequencing results.

Species identification of isolated yeast strains was performed by NCBI BLAST searches against the Nucleotide collection (nr/nt) database (Johnson et al., 2008). Sequences with Percent Identity = 100%, E-value = 0 were considered as top hits, and the species name that appeared in more than two top hits was used to refer to the strain. See also Table S3B for the number of the top hits for each strain.

For bacteria, representative sequences of OTUs from the microbial composition analysis were compared with the sequences of isolated strains, and the strain with the closest 16S rDNA sequence was used as the strain corresponding to that OTU. See also Table S3C.

Quantification of larval development

The microbes were individually grown on banana agar twice before being suspended in PBS (OD600 = 60). To mix multiple species, equal amounts of the suspensions prepared as described were mixed together. 10 µL of each suspension was added to ~1.1 mL of autoclaved banana agar in 1.5 mL tubes. Tubes were incubated at 25°C for 2 days before larvae were added. The following procedure was performed as described in Watanabe et al., 2019, with a few modifications. Briefly, 20 germ-free larvae were added to each tube, and the tubes were kept in a moisturized incubator (80-90% humidity) at 25°C. Pupariated individuals were removed from the tubes prior to eclosion, and either discarded or transferred to vials for further incubation until eclosion.

occurred. The number of pupae in each tube was counted until all larvae either pupariated or died. In experiments that require daily addition of AAB, the bacterial cells (~3 mL by volume) were added daily except for those days when the cells added the previous days were still visibly left.

The effect on larval development was quantified as the final percentage of the pupariated individuals and timing of the pupariation. The developmental timing was quantified from the date on which the percentage of pupariation exceeded 50% of the final percentage of pupariation. Molds were occasionally seen on foods during incubation, in which case the tubes were removed from the experiment. On a rare occasion when the number of samples for a single condition fell below 3, all the tubes for the condition were excluded from the results.

Sequencing and annotation of yeast genomes

To extract genomic DNA from yeast cells for genome sequencing, yeasts were cultured on MRS plates. Collected cells were treated with Zymolyase-20T (Nacalai Tesque) to remove cell walls, and DNA was extracted using QIAGEN Genomic-tip 20G kit (QIAGEN).

The genomic DNA prepared from six strains was sequenced by both Illumina and Oxford Nanopore Technologies (ONT) technologies. For Illumina sequencing, libraries were prepared with QIAseq FX DNA Library Kit (QIAGEN) and sequenced on the Illumina MiSeq platform (Illumina, CA, USA) to generate 2 x 301 bp paired-end sequence reads. MiSeq reads were trimmed using Platanus_trim (http://platanus.bio.titech.ac.jp/platanus_trim [↗](#)). The thresholds for quality and read length were set at 15 and 25, respectively. After trimming, a total of 1.863 - 2.396 Gb sequence was obtained for each genome (average; 2.113 Gb). Sequence coverages estimated by GenomeScope (Vurture et al., 2017 [↗](#)) were over 100x for all genomes (103.42x - 210.48x). For ONT sequencing, libraries were prepared with Rapid Barcoding Kit (ONT) and sequenced using the R9.4.1 flow cell with the MinION platform, followed by base-calling using Albacore ver. 2.3.3. (ONT). Adapter sequences of MinION reads were trimmed with NanoFilt (De Coster et al., 2018 [↗](#)) or Porechop (<https://github.com/rrwick/Porechop> [↗](#)), followed by filtering with a quality threshold of 10 and a length threshold of 2000. After filtering, 84,598 - 197,215 reads (765 - 4,402 Mb in total sequence length) were obtained for each genome (average; 121,913 reads and 1,035 Mb). Estimated sequence coverages by MinION reads were 63.1x - 107.3x (average; 87.9x).

For RNA-seq analysis, equal amounts of yeast cells cultured under 6 conditions (2 temperatures: 25°C and 30°C, and 3 culture plates: MRS, YPD, or banana-agar plates) were mixed and used for RNA extraction. RNA was extracted as previously described (Iida & Kobayashi, 2019 [↗](#)) and purified using the RNeasy mini kit (QIAGEN). Libraries for RNA-seq were prepared with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB) and sequenced on the NextSeq 500 platform (Illumina) to generate 300-base paired end sequences which were used for annotation described below. A total of 23.48-26.75M reads were obtained for each yeast (average; 25.63M reads).

Genome assembly was performed by MaSuRCA v3.2.6 (Zimin et al., 2013 [↗](#)) using MiSeq and MinION reads. In the heterozygosity rate estimation by GenomeScope based on MiSeq reads, the *Kazachstania humilis* and *Pichia kluyveri* genomes showed higher heterozygosity rates (*K. humilis*; 2.98%, *Pi. kluyveri*; 2.08%) while those of other genomes were 0.02% - 1.00%. In the assessment of autoannotated genome assemblies using BUSCO (Simão et al., 2015 [↗](#)), completeness was over 86% in the four genomes other than the *H. uvarum* (64.58%) and *St. bacillaris* (75.21%) genomes. However, among the four genomes, those of *Pi. kluyveri* and *K. humilis* showed a high proportion of “Duplicated complete” genes (*K. humilis*; 27.74%, *Pi. kluyveri*; 5.17%, respectively), which was apparently due to the high heterozygosity rate of these genomes. Therefore, these two genomes were assembled using Platanus-alley v2.2.2 (Kajitani et al., 2019 [↗](#)), and by comparing it with the MaSuRCA assemblies using GenomeMatcher (Ohtsubo et al., 2008 [↗](#)), redundant sequences in the MaSuRCA assemblies were identified and removed from the assemblies. As the proportions of “Duplicated complete” genes estimated by BUSCO were 5.51% for the *K. humilis* genome and 0.74%

for the *Pi. kluyveri* genome after removing redundant sequences, these redundant sequence-removed genome assemblies were used as the final genome assemblies of *Pi. kluyveri* and *K. humilis*.

Genome annotation was performed using FunGAP (Min et al., 2017) with the above-mentioned RNA-seq data obtained from each yeast. Over 91% of RNA-seq reads obtained was mapped to the final assemblies of each genome using Hisat2 (D. Kim et al., 2015). In the assessment of annotated genomes using BUSCO, completeness was >86% for the four genomes other than those of *H. uvarum* (65.95%) and *St. bacillaris* (76.92%). It appears that the low completeness of these genomes are attributable to genome reduction. The final statuses of genome assembly and annotation of each genome are summarized in Supplementary Table S7.

Mitochondrial sequences in each genome assembly were identified by tblastx homology search with fungal mitochondrial sequences obtained from the RefSeq database (O'Leary et al., 2016). When multimerized mitochondrial sequences were generated in genome assemblies, they were trimmed to the smallest unit.

To determine gene orthology between *S. cerevisiae* and each of the isolated strains, blastp was performed. *S. cerevisiae* protein sequence data (GCF_000146045.2_R64_protein.faa) was downloaded from NCBI genome database (<https://www.ncbi.nlm.nih.gov/genome>). Reciprocal best hits with an e-value < 10^{-10} were defined as orthologs.

Quantification of AAB

Microbes were inoculated onto 200 mL of banana agar in a tube included in a Biomasher II homogenizer kit (Funakoshi). After adding 5 germ-free larvae, the tubes were incubated at 25°C for 4 days. 150 µL of PBS was added to the tube, and food was crushed along with the larvae in it. More PBS was subsequently added to bring the total volume of the suspension to 700 µL. 1/5 serial dilutions were made, 50 µL of which were spread onto MRS plates supplemented with antibiotics (10 µg/mL cycloheximide and 10 µg/mL ampicillin to inhibit the growth of yeasts and LAB, respectively). The plates were incubated at 25°C, and 2 days later, colonies were counted to calculate CFU.

RNA sequencing for gene expression analyses

For larval RNA-seq analysis, microbes were inoculated on banana agar as previously described and freshly hatched germ-free larvae were added. After 15 h feeding, 20 larvae were collected and snap-frozen. RNA extraction, sequencing, and data analysis were performed as previously described (Kanaoka et al., 2023).

Yeast RNA was extracted as described above, and sequenced as previously described in Kanaoka et al., 2023. Data obtained from the FunGAP analysis were used for mapping of reads obtained for the isolated strains, and the *S. cerevisiae* S288C genome R64-1-1, retrieved from Illumina iGenomes (https://jp.support.illumina.com/sequencing/sequencing_software/igenome.html) for *S. cerevisiae* BY4741.

For the heatmaps in Figures 3A and 4C, heatmap.2 in the gplots (Warnes et al., 2022) package of R (R Core Team, 2020) was used. Database for annotation, visualization and integrated discovery (DAVID) Functional Annotation Chart (Huang et al., 2009; Sherman et al., 2022) was used to find significantly enriched GO terms or KEGG Pathways in each analysis. For comparisons with the microarray analysis result of Zinke et al., 2002, their gene expression data of the individuals at 12 h after feeding or starvation was used. Genes that were represented by more than one probes were omitted. The FlyBase IDs in the data of Zinke et al., 2002 were

converted to current IDs using FlyBase ID Validator (<https://flybase.org/convert/id>). Only the genes that had one-to-one correspondence with current FlyBase IDs were included in the heat maps.

Preparing samples for LC-MS analysis

The yeasts were individually grown on banana agar three times. 100 mg of cells were scraped from the surface of the plates and snap-frozen. To prepare yeast-conditioned plates, yeasts were grown as described and thoroughly removed by scraping; in addition, 22 mm² chunks of agar from the plates (~100–160 mg) were collected and snap-frozen. 15 g/L agar plates or sterile banana-agar plates were prepared as controls and collected using the same procedure. To prepare cell suspension supernatants, ~250 mg of yeast cells were collected as described, and sterile PBS was added at a ratio of 5 µL PBS per 1 mg of yeast cells. After suspending the cells, the suspensions were centrifuged at 5,000 rpm for 5 min. The supernatants were further filtered using a 0.45 µm Millex-HA filter (Merck) to completely remove any remaining yeast cells. 700 µL of each supernatant was collected and snap-frozen.

Each sample was suspended or diluted to 500 µL of methanol containing internal standard; 100 mM MES and 100 mM L-Met. After mixing with 250 µL of water and 400 µL of chloroform, the samples were centrifuged and the upper layer was collected and filtered using an UltrafreeMC-PLHCC for Metabolome Analysis column (Human Metabolome Technologies, #UFC3LCCNB-HMT). The samples were dried completely using nitrogen gas and resuspended in water before injection into the LC-MS system.

LC-MS/MS measurement

Cationic metabolites including amino acids and nucleosides are quantified using a triple-quadrupole mass spectrometer equipped with an electrospray ionization (ESI) ion source (LCMS-8060, Shimadzu Corporation) in the positive and negative-ESI and multiple reaction monitoring (MRM) modes. The samples were resolved on the Discovery HS F5-3 column (2.1 mm ID × 150 mm, 3-µm particle, Sigma-Aldrich), using a step gradient with mobile phase A (0.1% formate / water) and mobile phase B (0.1% formate / acetonitrile) at ratios of 100:0 (0–5 min), 75:25 (5–11 min), 65:35 (11–15 min), 5:95 (15–20 min) and 100:0 (20–25 min), at a flow rate of 0.25 ml min⁻¹ and a column temperature of 40°C.

Data are presented as peak area values for each metabolite normalized by the internal standard. Those for the yeast-conditioned plates or cells were further normalized with sample wet weight. Heat maps were generated by MetaboAnalystR 3.3.0. (Pang et al., 2020), using data for all of the detected metabolites or a subset of metabolites that is included in the holidic medium of *Drosophila melanogaster* (Piper et al., 2014).

Developmental progression with BCAA supplementation

Yeasts were cultured as described on autoclaved banana agar supplemented with 2.03 g/L of leucine (Nacalai Tesque) and 1.12 g/L of isoleucine (Peptide Institute), both at the concentrations included in the holidic medium with exome-matched FLYAA (Piper et al., 2017). 20 larvae were added to each tube, and after 11 days of feeding, live or dead individuals in each developmental stage were counted. Larval developmental stages were determined based on tracheal morphology, as previously described (Niwa et al., 2010). The percentage of the larvae that could not be found are indicated as “Not found.”

Imaging of dead yeast cells

Microbes were collected from banana-agar plates, suspended in PBS at a concentration of ~1 × 10⁸ cells/mL, and incubated in 5 µg/mL Phloxine B (Wako) for 10 min at room temperature. The cells were subsequently washed twice with PBS and observed using a Nikon C1 laser scanning confocal

microscope coupled to a Nikon Eclipse E-800 microscope. Note that the brightness of the images was adjusted with a gradation to achieve mostly uniform brightness, because the bottom side of the original images was darker due to microscope malfunction.

Statistical analysis

R (R Core Team, 2020 [link](#)) was used for all statistical analyses. P-values less than 0.05 were considered statistically significant. For statistical analyses of RNA-seq data, see Tables S4, S5, and S8. See Table S9, Materials and Methods, and figure legends for analysis of other experiments.

Data availability

All sequence data obtained in this study have been deposited in the DDBJ Sequence Read Archive. The accession numbers for the data are DRR212820-DRR212838 (BioProject accession number: PRJDB9099) and DRR471830-DRR471964 (BioProject accession number: PRJDB15711).

Acknowledgements

We thank T. Kondo and Y. Sando for performing RNA sequencing; M. Umeda and T. Suito for technical advice on microbial isolation and identification; K. Furuya for technical advice on yeast genomic DNA extraction and Phloxine B staining; T. Iida and T. Kobayashi for technical advice on yeast RNA extraction; J. Hejna for polishing the manuscript; H. Imai, K. Oki, and M. Futamata for technical and secretarial assistance; T. Kuraishi, A. Hori, Y. Degawa, Y. Yoshihashi, R. Niwa, T. Ito for technical advice and discussion; W.-J. Lee for providing bacterial strains; and members of the Uemura laboratory for technical advice, discussion, and kind cooperation in obtaining fermented food samples. This work was supported by the Japan Society for the Promotion of Science (JSPS; 21K06186, 17K15039 and 16H06279 [PAGS] to Y.H., 17KT0018 to T.U., and 20J23332 to A.M.); AMED-CREST (JP18gm1110001 to T.U.); and JST FOREST Program (JPMJFR2051 to Y.H.).

Competing interests

The authors declare no competing interests.

Supplementary Files

Table S1. List of adult flies caught in the traps.

Table S2. Microbial composition analysis results.

Table S3. Microbial isolation and species identification.

Table S4. Table S3. RNA-seq data of whole bodies of 1st instar larvae fed on *H. uvarum* or bacteria.

Table S5. RNA-seq data of whole bodies of 1st instar larvae fed on yeasts

Table S6. Results of metabolomic analysis of yeast samples.

Table S7. Final genome assembly and annotation statuses of six yeast strains.

Table S8. RNA-seq data of isolated yeasts.

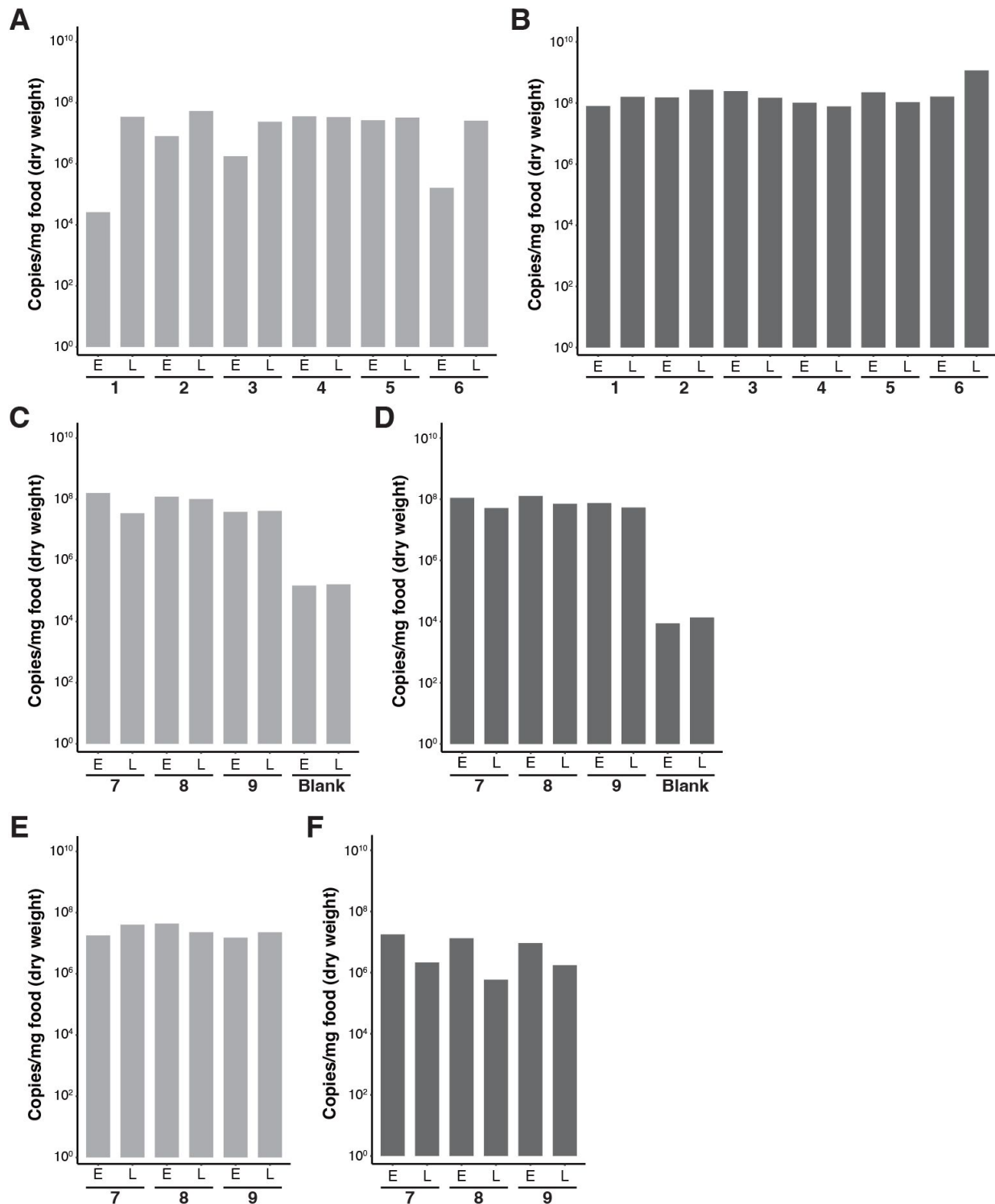


Figure S1.

Copy numbers of microbial rDNA in fermented banana samples.

(A-F) Graphs showing the copy numbers of the fungal ITS region (A, C, and E) or bacterial 16S rRNA region (B, D, and F) in the fermented food samples shown in [Figures 1A](#) and [1B](#) (A and B), [Figures 1C](#) and [1D](#) (C and D), and [Figures 1E](#) and [1F](#) (E and F). Numbers below each pair of foods indicate the sampling places, which correspond to those indicated in [Figure 1](#). (C and D) Yeast and bacteria were also detected in Blank samples, in which sterile PBS instead of microbial suspensions was added. They might have invaded the fruit by passing through wounds on the peel (Oyewole, 2012). E: early-stage food; L: late-stage food.

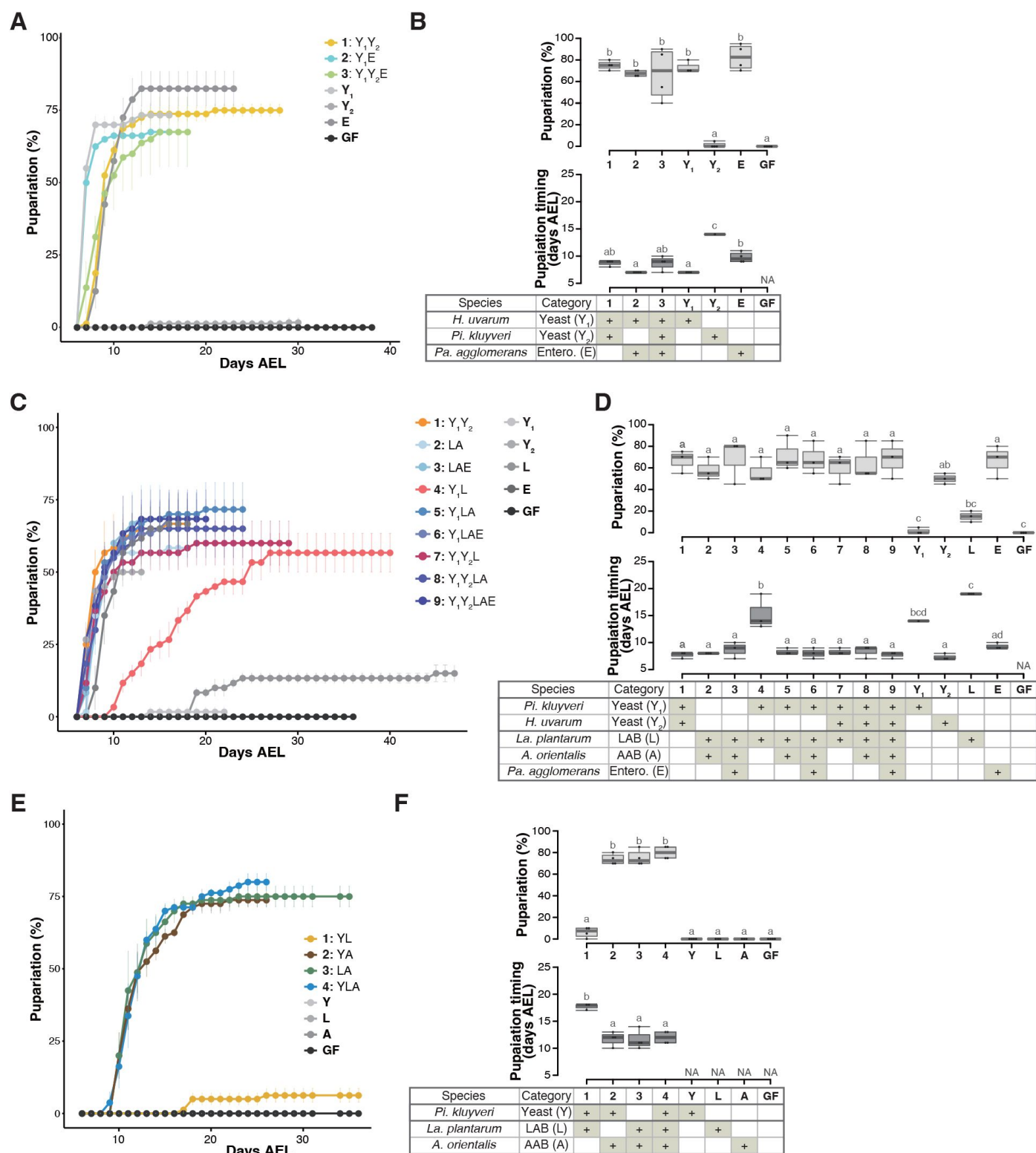


Figure S2.

Larvae feeding on mixtures of isolated microbial species detected in the samples from Sampling Place 2 in Figure 1B

(A-F) The percentage and timing of pupariation of larvae feeding on (A and B) microbes derived from the early-stage food, or (C and D) five, or (E and F) three microbes derived from the late-stage food. (A, C, and E) The codes for individual species are provided in the chart at the bottom of (B), (D), and (F), respectively. (B, D, and F) Graphs are presented as in **Figure 2**. $n=3-4$. GF, germ-free; days AEL, days after egg laying.

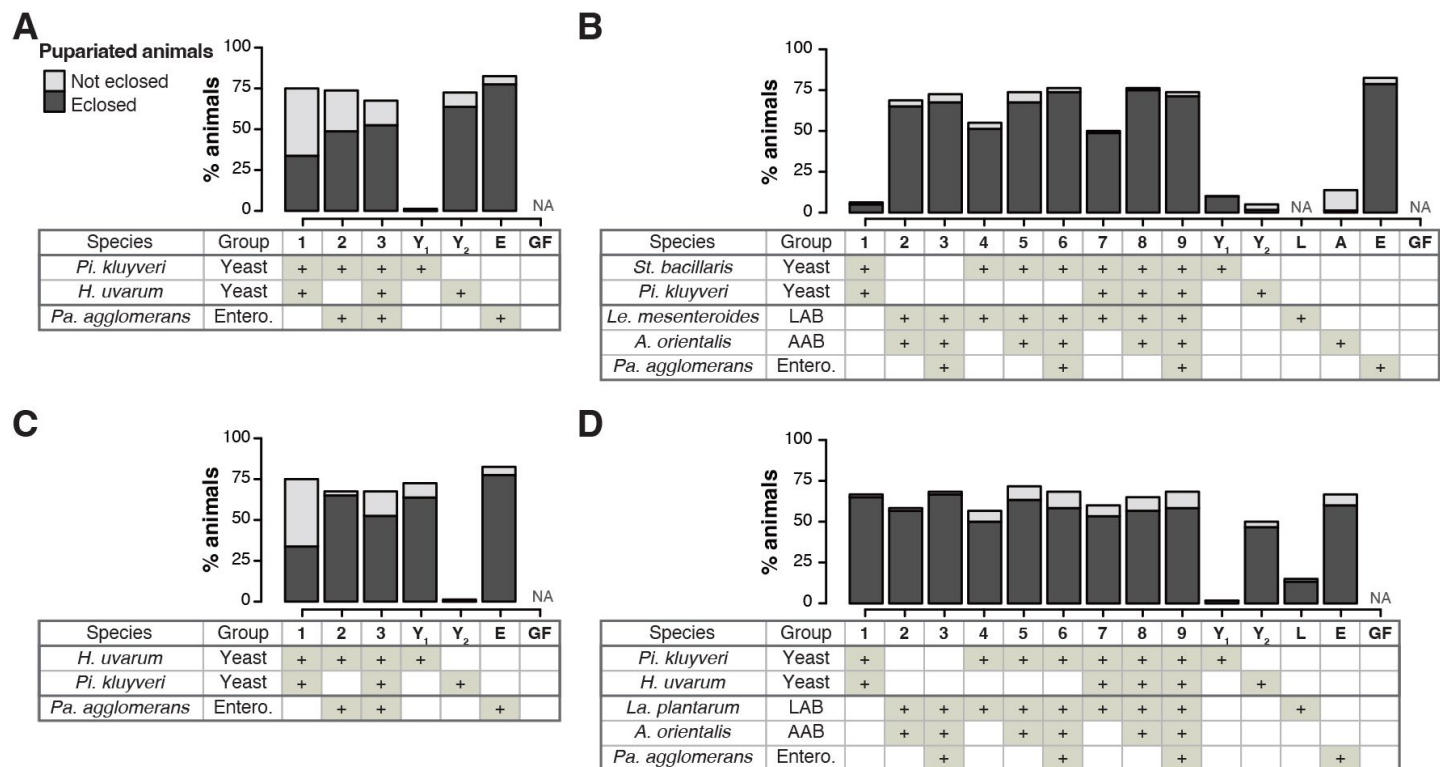


Figure S3.

The percentage of pupariation and eclosion of the larvae feeding on microbes.

(A-D) Graphs showing the percentage of larvae that pupariated in the experiments shown in [Figures 2A](#) and [2B](#) (A), [Figures 2C](#) and [2D](#) (B), [Figures S2A](#) and [S2B](#) (C), or [Figures S2C](#) and [S2D](#) (D). The mean percentages of the pupariated and eclosed (dark gray) or pupariated and not eclosed (light gray) individuals are shown. n=3-4.

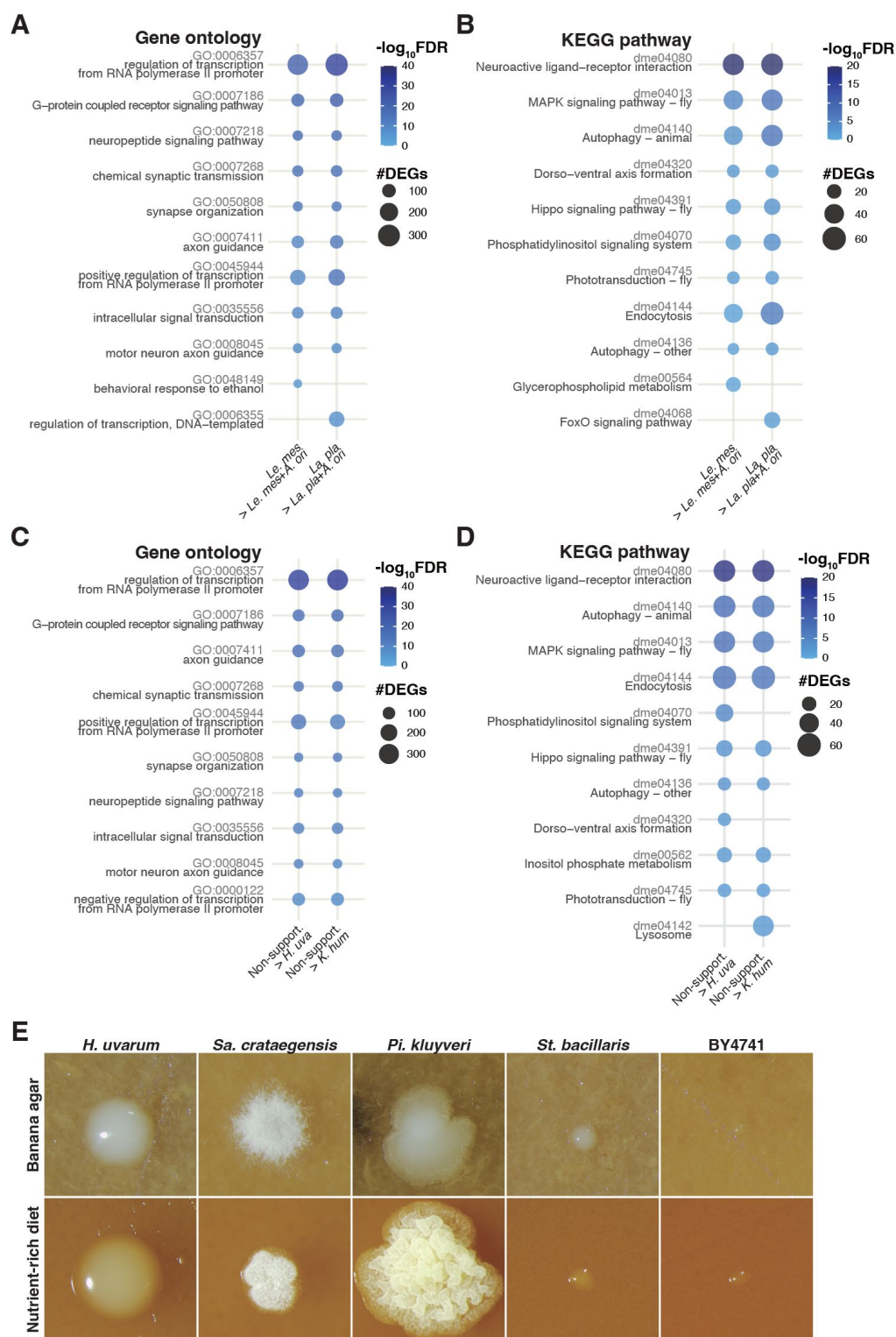


Figure S4.

Enrichment analyses of the genes upregulated in non-supportive conditions and morphology of yeast colonies grown on a nutrient-rich diet.

(A-D) Plots showing the result of GO term (A and C) or KEGG Pathway (B and D) enrichment analysis of the RNA-seq data shown in [Figure 3A](#) (A and B) and [Figure 4C](#) (C and D). Plots were generated as described in [Figure 3](#). (E) Images of single yeast colonies grown on banana agar or the nutrient-rich diet. The images were taken after a 2-day incubation at 25°C.

References

1. Anagnostou C., Dorsch M., Rohlf M (2010) **Influence of dietary yeasts on *Drosophila melanogaster* life-history traits** *Entomologia Experimentalis et Applicata* **136**:1–11 <https://doi.org/10.1111/j.1570-7458.2010.00997.x>
2. Asha H., Nagy I., Kovacs G., Stetson D., Ando I., Dearolf C. R (2003) **Analysis of Ras-induced overproliferation in *Drosophila* hemocytes** *Genetics* **163**:203–215
3. Baumberger J. P (1917) **The Food of *Drosophila Melanogaster* Meigen** *Proceedings of the National Academy of Sciences of the United States of America* **3**:122–126 <https://doi.org/10.1073/pnas.3.2.122>
4. Broderick N. A., Lemaitre B (2012) **Gut-associated microbes of *Drosophila melanogaster*** *Gut Microbes* **3**:307–321 <https://doi.org/10.4161/gmic.19896>
5. Caporaso J. G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F. D., Costello E. K., Fierer N., Peña A. G., Goodrich J. K., Gordon J. I., Huttley G. A., Kelley S. T., Knights D., Koenig J. E., Ley R. E., Lozupone C. A., McDonald D., Muegge B. D., Pirrung M., Knight ... (2010) **QIIME allows analysis of high-throughput community sequencing data** *Nature Methods* **7**:335–336 <https://doi.org/10.1038/nmeth.f.303>
6. Chandler J. A., Eisen J. A., Kopp A (2012) **Yeast communities of diverse *Drosophila* species: Comparison of two symbiont groups in the same hosts** *Applied and Environmental Microbiology* **78**:7327–7336 <https://doi.org/10.1128/AEM.01741-12>
7. Chandler J. A., Lang J., Bhatnagar S., Eisen J. A., Kopp A (2011) **Bacterial communities of diverse *Drosophila* species: Ecological context of a host-microbe model system** *PLoS Genetics* **7** <https://doi.org/10.1371/journal.pgen.1002272>
8. Cole J. R., Wang Q., Fish J. A., Chai B., McGarrell D. M., Sun Y., Brown C. T., Porras-Alfaro A., Kuske C. R., Tiedje J. M (2014) **Ribosomal Database Project: Data and tools for high throughput rRNA analysis** *Nucleic Acids Research* **42** <https://doi.org/10.1093/nar/gkt1244>
9. Coluccio A. E., Rodriguez R. K., Kernan M. J., Neiman A. M (2008) **The Yeast Spore Wall Enables Spores to Survive Passage through the Digestive Tract of *Drosophila*** *PLoS ONE* **3** <https://doi.org/10.1371/journal.pone.0002873>
10. Consuegra J., Grenier T., Akherraz H., Rahioui I., Gervais H., da Silva P., Leulier F. (2020) **Metabolic Cooperation among Commensal Bacteria Supports *Drosophila* Juvenile Growth under Nutritional Stress** *IScience* **23** <https://doi.org/10.1016/j.isci.2020.101232>
11. Consuegra J., Grenier T., Baa-Puyoulet P., Rahioui I., Akherraz H., Gervais H., Parisot N., da Silva P., Charles H., Calevro F., Leulier F. (2020) ***Drosophila*-associated bacteria differentially shape the nutritional requirements of their host during juvenile growth** *PLOS Biology* **18** <https://doi.org/10.1371/journal.pbio.3000681>
12. Corby-Harris V., Pontaroli A. C., Shimkets L. J., Bennetzen J. L., Habel K. E., Promislow D. E. L (2007) **Geographical Distribution and Diversity of Bacteria Associated with Natural Populations of *Drosophila melanogaster*** *Applied and Environmental Microbiology* **73**:3470–3479 <https://doi.org/10.1128/AEM.02120-06>

13. Cox C. R., Gilmore M. S (2007) **Native Microbial Colonization of *Drosophila melanogaster* and Its Use as a Model of *Enterococcus faecalis* Pathogenesis** *Infection and Immunity* **75**:1565–1576 <https://doi.org/10.1128/IAI.01496-06>
14. De Coster W., D’Hert S., Schultz D. T., Cruts M., Van Broeckhoven C. (2018) **NanoPack: Visualizing and processing long-read sequencing data** *Bioinformatics* **34**:2666–2669 <https://doi.org/10.1093/bioinformatics/bty149>
15. Fox H. L., Pham P. T., Kimball S. R., Jefferson L. S., Lynch C. J (1998) **Amino acid effects on translational repressor 4E-BP1 are mediated primarily by l-leucine in isolated adipocytes** *American Journal of Physiology* **275**:C1232–C1238 <https://doi.org/10.1152/ajpcell.1998.275.5.C1232>
16. Gallo M., Vento J. M., Joncour P., Quagliariello A., Maritan E., Silva-Soares N. F., Battistolli M., Beisel C. L., Martino M. E (2022) **Beneficial commensal bacteria promote *Drosophila* growth by downregulating the expression of peptidoglycan recognition proteins** *IScience* **25** <https://doi.org/10.1016/j.isci.2022.104357>
17. Grenier T., Leulier F (2020) **How commensal microbes shape the physiology of *Drosophila melanogaster*** *Current Opinion in Insect Science* **41**:92–99 <https://doi.org/10.1016/j.cois.2020.08.002>
18. Gu X., Jouandin P., Lalgudi P. V., Binari R., Valenstein M. L., Reid M. A., Allen A. E., Kamitaki N., Locasale J. W., Perrimon N., Sabatini D. M (2022) **Sestrin mediates detection of and adaptation to low-leucine diets in *Drosophila*** *Nature* **608** <https://doi.org/10.1038/s41586-022-04960-2>
19. Hallen-Adams H. E., Suhr M. J (2017) **Fungi in the healthy human gastrointestinal tract** *Virulence* **8**:352–358 <https://doi.org/10.1080/21505594.2016.1247140>
20. Henriques S. F., Dhakan D. B., Serra L., Francisco A. P., Carvalho-Santos Z., Baltazar C., Elias A. P., Anjos M., Zhang T., Maddocks O. D. K., Ribeiro C (2020) **Metabolic cross feeding in imbalanced diets allows gut microbes to improve reproduction and alter host behaviour** *Nature Communications* **11** <https://doi.org/10.1038/s41467-020-18049-9>
21. Hoang D., Kopp A., Chandler J. A (2015) **Interactions between *Drosophila* and its natural yeast symbionts-Is *Saccharomyces cerevisiae* a good model for studying the fly-yeast relationship?** *PeerJ* **3** <https://doi.org/10.7717/peerj.1116>
22. Hoshino Y., Gaucher E. A (2021) **Evolution of bacterial steroid biosynthesis and its impact on eukaryogenesis** *Proceedings of the National Academy of Sciences* **118** <https://doi.org/10.1073/pnas.2101276118>
23. Huang D. W., Sherman B. T., Lempicki R. A (2009) **Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources** *Nature Protocols* **4** <https://doi.org/10.1038/nprot.2008.211>
24. Hugerth L. W., Wefer H. A., Lundin S., Jakobsson H. E., Lindberg M., Rodin S., Engstrand L., Andersson A. F (2014) **DegePrime, a program for degenerate primer design for broad-taxonomic-range PCR in microbial ecology studies** *Applied and Environmental Microbiology* **80**:5116–5123 <https://doi.org/10.1128/AEM.01403-14>
25. Iida T., Kobayashi T (2019) **RNA Polymerase I Activators Count and Adjust Ribosomal RNA Gene Copy Number** *Molecular Cell* **73**:645–654 <https://doi.org/10.1016/j.molcel.2018.11.029>

26. Ikeda-Ohtsubo W., Brugman S., Warden C. H., Rebel J. M. J., Folkerts G., Pieterse C. M. J (2018) **How Can We Define “Optimal Microbiota?”: A Comparative Review of Structure and Functions of Microbiota of Animals, Fish, and Plants in Agriculture** *Frontiers in Nutrition* **5**
27. Johnson M., Zaretskaya I., Raytselis Y., Merezhuk Y., McGinnis S., Madden T. L (2008) **NCBI BLAST: A better web interface** *Nucleic Acids Research* **36**:W5–9 <https://doi.org/10.1093/nar/gkn201>
28. Kajitani R., Yoshimura D., Okuno M., Minakuchi Y., Kagoshima H., Fujiyama A., Kubokawa K., Kohara Y., Toyoda A., Itoh T (2019) **Platanus-allee is a de novo haplotype assembler enabling a comprehensive access to divergent heterozygous regions** *Nature Communications* **10** <https://doi.org/10.1038/s41467-019-09575-2>
29. Kanaoka Y., Onodera K., Watanabe K., Hayashi Y., Usui T., Uemura T., Hattori Y (2023) **Inter-organ Wingless/Ror/Akt signaling regulates nutrient-dependent hyperarborization of somatosensory neurons** *eLife* **12** <https://doi.org/10.7554/eLife.79461>
30. Kim B., Kanai M. I., Oh Y., Kyung M., Kim E.-K., Jang I.-H., Lee J.-H., Kim S.-G., Suh G. S. B., Lee W.-J (2021) **Response of the microbiome–gut–brain axis in *Drosophila* to amino acid deficit** *Nature* **593** <https://doi.org/10.1038/s41586-021-03522-2>
31. Kim D., Langmead B., Salzberg S. L (2015) **HISAT: A fast spliced aligner with low memory requirements** *Nature Methods* **12**:357–360 <https://doi.org/10.1038/nmeth.3317>
32. Kim J., Guan K.-L (2019) **MTOR as a central hub of nutrient signalling and cell growth** *Nature Cell Biology* **21** <https://doi.org/10.1038/s41556-018-0205-1>
33. Kwong W. K., Moran N. A (2013) **Cultivation and characterization of the gut symbionts of honey bees and bumble bees: Description of *Snodgrassella alvi* gen. nov., sp. nov., a member of the family Neisseriaceae of the Betaproteobacteria, and *Gilliamella apicola* gen. nov., sp. nov., a member of Orbaceae fam. nov., Orbales ord. nov., a sister taxon to the order ‘Enterobacteriales’ of the Gammaproteobacteria** *International Journal of Systematic and Evolutionary Microbiology* **63**:2008–2018 <https://doi.org/10.1099/ijs.0.044875-0>
34. Lee W.-J., Brey P. T (2013) **How microbiomes influence metazoan development: Insights from history and *Drosophila* modeling of gut-microbe interactions** *Annual Review of Cell and Developmental Biology* **29**:571–592 <https://doi.org/10.1146/annurev-cellbio-101512-122333>
35. Lofgren L. A., Uehling J. K., Branco S., Bruns T. D., Martin F., Kennedy P. G (2019) **Genome-based estimates of fungal rDNA copy number variation across phylogenetic scales and ecological lifestyles** *Molecular Ecology* **28**:721–730 <https://doi.org/10.1111/mec.14995>
36. Ludington W. B., Ja W. W (2020) ***Drosophila* as a model for the gut microbiome** *PLOS Pathogens* **16** <https://doi.org/10.1371/journal.ppat.1008398>
37. Magoč T., Salzberg S. L (2011) **FLASH: Fast length adjustment of short reads to improve genome assemblies** *Bioinformatics* **27**:2957–2963 <https://doi.org/10.1093/bioinformatics/btr507>
38. Martino M. E., Joncour P., Leenay R., Gervais H., Shah M., Hughes S., Gillet B., Beisel C., Leulier F (2018) **Bacterial Adaptation to the Host’s Diet Is a Key Evolutionary Force Shaping *Drosophila*-*Lactobacillus* Symbiosis** *Cell Host & Microbe* **24**:109–119 <https://doi.org/10.1016/j.chom.2018.06.001>

39. Min B., Grigoriev I. V., Choi I.-G (2017) **FunGAP: Fungal Genome Annotation Pipeline using evidence-based gene model evaluation** *Bioinformatics* **33**:2936–2937 <https://doi.org/10.1093/bioinformatics/btx353>
40. Moya A., Ferrer M (2016) **Functional Redundancy-Induced Stability of Gut Microbiota Subjected to Disturbance** *Trends in Microbiology* **24**:402–413 <https://doi.org/10.1016/j.tim.2016.02.002>
41. Nilsson R. H., Larsson K.-H., Taylor A. F. S., Bengtsson-Palme J., Jeppesen T. S., Schigel D., Kennedy P., Picard K., Glöckner F. O., Tedersoo L., Saar I., Kõljalg U., Abarenkov K (2019) **The UNITE database for molecular identification of fungi: Handling dark taxa and parallel taxonomic classifications** *Nucleic Acids Research* **47**:D259–D264 <https://doi.org/10.1093/nar/gky1022>
42. Niwa R., Namiki T., Ito K., Shimada-Niwa Y., Kiuchi M., Kawaoka S., Kayukawa T., Banno Y., Fujimoto Y., Shigenobu S., Kobayashi S., Shimada T., Katsuma S., Shinoda T (2010) **Non-molting glossy / shroud encodes a short-chain dehydrogenase/reductase that functions in the ‘Black Box’ of the ecdysteroid biosynthesis pathway** *Development* **137**:1991–1999 <https://doi.org/10.1242/dev.045641>
43. Ohtsubo Y., Ikeda-Ohtsubo W., Nagata Y., Tsuda M (2008) **GenomeMatcher: A graphical user interface for DNA sequence comparison** *BMC Bioinformatics* **9** <https://doi.org/10.1186/1471-2105-9-376>
44. O’Leary N. A., Wright M. W., Brister J. R., Ciufio S., Haddad D., McVeigh R., Rajput B., Robbertse B., Smith-White B., Ako-Adjei D., Astashyn A., Badretdin A., Bao Y., Blinkova O., Brover V., Chetvernin V., Choi J., Cox E., Ermolaeva O., Pruitt K. D. (2016) **Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation** *Nucleic Acids Research* **44**:D733–D745 <https://doi.org/10.1093/nar/gkv1189>
45. Oyewole O. A (2012) **Microorganisms associated with deterioration of stored banana fruits** *Frontiers in Science* **2**:86–91 <https://doi.org/10.5923/j.fs.20120205.01>
46. Pais I. S., Valente R. S., Sporniak M., Teixeira L (2018) **Drosophila melanogaster establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria** *PLOS Biology* **16** <https://doi.org/10.1371/journal.pbio.2005710>
47. Pang Z., Chong J., Li S., Xia J (2020) **MetaboAnalystR 3.0: Toward an Optimized Workflow for Global Metabolomics** *Metabolites* **10** <https://doi.org/10.3390/metabo10050186>
48. Pérez J. C (2021) **Fungi of the human gut microbiota: Roles and significance** *International Journal of Medical Microbiology* **311** <https://doi.org/10.1016/j.ijmm.2021.151490>
49. Piper M. D. W., Blanc E., Leitão-Gonçalves R., Yang M., He X., Linford N. J., Hoddinott M. P., Hopfen C., Soultoukis G. A., Niemeyer C., Kerr F., Pletcher S. D., Ribeiro C., Partridge L (2014) **A holidic medium for Drosophila melanogaster** *Nature Methods* **11**:100–105 <https://doi.org/10.1038/nmeth.2731>
50. Piper M. D. W., Soultoukis G. A., Blanc E., Mesaros A., Herbert S. L., Juricic P., He X., Atanassov I., Salmonowicz H., Yang M., Simpson S. J., Ribeiro C., Partridge L (2017) **Matching Dietary Amino Acid Balance to the In Silico-Translated Exome Optimizes Growth and Reproduction without Cost to Lifespan** *Cell Metabolism* **25**:610–621 <https://doi.org/10.1016/j.cmet.2017.02.005>

51. Qiu X., Yu L., Wang W., Yan R., Zhang Z., Yang H., Zhu D., Zhu B (2021) **Comparative Evaluation of Microbiota Dynamics and Metabolites Correlation Between Spontaneous and Inoculated Fermentations of Nanfeng Tangerine Wine** *Frontiers in Microbiology* **12**
52. Quan A. S., Eisen M. B (2018) **The ecology of the drosophila-yeast mutualism in wineries** *PLOS ONE* **13** <https://doi.org/10.1371/journal.pone.0196440>
53. R Core Team (2020) **R: A Language and Environment for Statistical Computing**
54. Sherman B. T., Hao M., Qiu J., Jiao X., Baseler M. W., Lane H. C., Imamichi T., Chang W (2022) **DAVID: A web server for functional enrichment analysis and functional annotation of gene lists (2021 update)** *Nucleic Acids Research* **50**:W216–W221 <https://doi.org/10.1093/nar/gkac194>
55. Shigenobu S., Wilson A. C. C (2011) **Genomic revelations of a mutualism: The pea aphid and its obligate bacterial symbiont** *Cellular and Molecular Life Sciences* **68**:1297–1309 <https://doi.org/10.1007/s00018-011-0645-2>
56. Shihata A. M. E.-T. A., Mrak E. M (1952) **Intestinal Yeast Floras of Successive Populations of Drosophila** *Evolution* **6**:325–332 <https://doi.org/10.1111/j.1558-5646.1952.tb01428.x>
57. Shin S. C., Kim S. H., You H., Kim B., Kim A. C., Lee K. A., Yoon J. H., Ryu J. H., Lee W. J (2011) **Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling** *Science* **334**:670–674 <https://doi.org/10.1126/science.1212782>
58. Simão F. A., Waterhouse R. M., Ioannidis P., Kriventseva E. V., Zdobnov E. M (2015) **BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs** *Bioinformatics* **31**:3210–3212 <https://doi.org/10.1093/bioinformatics/btv351>
59. Solomon G. M., Dodangoda H., McCarthy-Walker T. T., Ntim-Gyakari R. R., Newell P. D (2019) **The microbiota of Drosophila suzukii influences the larval development of Drosophila melanogaster** *PeerJ* **7** <https://doi.org/10.7717/peerj.8097>
60. Stamps J. A., Yang L. H., Morales V. M., Boundy-Mills K. L (2012) **Drosophila regulate yeast density and increase yeast community similarity in a natural substrate** *PLoS ONE* **7** <https://doi.org/10.1371/journal.pone.0042238>
61. Stoddard S. F., Smith B. J., Hein R., Roller B. R. K., Schmidt T. M (2015) **rrnDB: Improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development** *Nucleic Acids Research* **43**:D593–D598 <https://doi.org/10.1093/nar/gku1201>
62. Storelli G., Defaye A., Erkosar B., Hols P., Royet J., Leulier F (2011) **Lactobacillus plantarum Promotes Drosophila Systemic Growth by Modulating Hormonal Signals through TOR-Dependent Nutrient Sensing** *Cell Metabolism* **14**:403–414 <https://doi.org/10.1016/j.cmet.2011.07.012>
63. Su Y., Lin H.-C., Teh L. S., Chevance F., James I., Mayfield C., Golic K. G., Gagnon J. A., Rog O., Dale C (2022) **Rational engineering of a synthetic insect-bacterial mutualism** *Current Biology* **32**:3925–3938 <https://doi.org/10.1016/j.cub.2022.07.036>
64. Vurture G. W., Sedlazeck F. J., Nattestad M., Underwood C. J., Fang H., Gurtowski J., Schatz M. C (2017) **GenomeScope: Fast reference-free genome profiling from short reads** *Bioinformatics* **33**:2202–2204 <https://doi.org/10.1093/bioinformatics/btx153>

65. Warnes G. R., Bolker B., Bonebakker L., Gentleman R., Huber W., Liaw A., Lumley T., Maechler M., Magnusson A., Moeller S., Schwartz M., Venables B. (2022) **gplots: Various R Programming Tools for Plotting Data**
66. Watanabe K., Furumizo Y., Usui T., Hattori Y., Uemura T (2017) **Nutrient-dependent increased dendritic arborization of somatosensory neurons** *Genes to Cells* **22**:105–114 <https://doi.org/10.1111/gtc.12451>
67. Watanabe K., Kanaoka Y., Mizutani S., Uchiyama H., Yajima S., Watada M., Uemura T., Hattori Y (2019) **Interspecies comparative analyses reveal distinct carbohydrate-responsive systems among *Drosophila* species** *Cell Reports* **28**:2594–2607 <https://doi.org/10.1016/j.celrep.2019.08.030>
68. Werren J. H., Windsor D., Guo L (1995) **Distribution of *Wolbachia* among Neotropical Arthropods** *Proceedings: Biological Sciences* **262**:197–204
69. White T. J., Bruns T., Lee S., Taylor J (1990) **Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics** *PCR protocols: A guide to methods and applications* :315–322
70. Zhang S., Lin X., Hou Q., Hu Z., Wang Y., Wang Z (2021) **Regulation of mTORC1 by amino acids in mammalian cells: A general picture of recent advances** *Animal Nutrition* **7**:1009–1023 <https://doi.org/10.1016/j.aninu.2021.05.003>
71. Zheng D., Liwinski T., Elinav E (2020) **Interaction between microbiota and immunity in health and disease** *Cell Research* **30** <https://doi.org/10.1038/s41422-020-0332-7>
72. Zimin A. V., Marçais G., Puiu D., Roberts M., Salzberg S. L., Yorke J. A (2013) **The MaSuRCA genome assembler** *Bioinformatics* **29**:2669–2677 <https://doi.org/10.1093/bioinformatics/btt476>
73. Zinke I., Schütz C. S., Katzenberger J. D., Bauer M., Pankratz M. J (2002) **Nutrient control of gene expression in *Drosophila*: Microarray analysis of starvation and sugar-dependent response** *The EMBO Journal* **21**:6162–6173 <https://doi.org/10.1093/emboj/cdf600>

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Reviewer #1 (Public Review):

Summary:

This valuable study analyzes the contribution of fungal and bacterial microbiota species to the growth and development of *Drosophila*. The authors use bacterial and fungal species associated with *Drosophila* in the wild to analyze their respective contributions in promoting larval growth in a decaying banana, mimicking the natural niche of fruit flies. They found that some fungal species and some fungus/bacteria combinations effectively promote growth by supplementing key branched amino acids in the food substratum. Production of these amino acids by *Drosophila* itself is not sufficient, and only fungal species that secrete these amino acids into the medium can sustain *Drosophila* growth. Thus, the authors clarify how facultative symbionts contribute to host growth in a natural setting by changing the food substratum in a dynamic manner.

Strengths:

The natural setting developed by the authors to analyze the impact of the microbiota is clearly valuable, as is the focus on the role of fungal microbiota species. This complements studies of *Drosophila* microbiota that have previously focused on bacterial species and used a lab setting. While there has been an extensive focus on obligate endosymbionts or gut symbionts, this study analyzes how facultative symbionts shape the food substratum and influence host growth. A last strength of this study is that it analyzes the contribution of *Drosophila* microbiota over a dynamic timeframe, analyzing how microbial species change in decaying fruit over time.

Weaknesses:

1. The authors should better review what we know of fungal *Drosophila* microbiota species as well as the ecology of rotting fruit. Are the microbiota species described in this article specific to their location/setting? It would have been interesting to know if similar species can be retrieved in other locations using other decaying fruits. The term 'core' in the title suggests that these species are generally found associated with *Drosophila* but this is not demonstrated. The paper is written in a way that implies the microbiota members they have found are universal. What is the evidence for this? Have the fungal species described in this paper been found in other studies? Even if this is not the case, the paper is interesting, but there should be a discussion of how generalizable the findings are.
2. Can the authors clearly demonstrate that the microbiota species that develop in the banana trap are derived from flies? Are these species found in flies in the wild? Did the authors check that the flies belong to the *D. melanogaster* species and not to the sister group *D. simulans*?
3. Did the microarrays highlight a change in immune genes (ex. antibacterial peptide genes)? Whatever the answer, this would be worth mentioning. The authors described their microarray data in terms of fed/starved in relation to the Finke article. They should clarify if they observed significant differences between species (differences between species within bacteria or fungi, and more generally differences between bacteria versus fungi).
4. The whole paper - and this is one of its merits - points to a role of the *Drosophila* larval microbiota in processing the fly food. Are these bacterial and fungal species found in the gut of larvae/adults? Are these species capable of establishing a niche in the cardia of adults as shown recently in the Ludington lab (Dodge et al.)? Previous studies have suggested that microbiota members stimulate the Imd pathway leading to an increase in digestive proteases (Erkosar/Leulier). Are the microbiota species studied here affecting gut signaling pathways beyond providing branched amino acids?

Reviewer #2 (Public Review):

Summary:

In this manuscript, Mure et al investigated host-microbe interactions in wild-mimicked settings. They analyzed microbiome composition using bananas that had been fed on by wild larvae and found that the microbiota composition shifted from the early stage of feeding to the later stage of the fermentation process. They isolated several yeast and bacterial species from the food, and examined larval growth on banana-based food, mimicking a natural setting where germ-free larvae cannot grow on it. The authors found that a yeast, *Hanseniaspora uvarum*, can support larval growth sufficiently, and insisted that branched-chain amino acids (BCAAs) provided by the yeast may partly account for the growth support. Interestingly, in other isolated yeast species, some were non-supportive strains in terms of larval growth, which can assist larval development when they are heat-killed. Besides, they showed that acetic acid bacteria, isolated from well-fermented banana (later-stage food), is sufficiently supportive but their presence depended on other microbes, lactic acid bacteria or yeast.

Strengths:

So far, host-microbe studies using *Drosophila melanogaster* have focused relatively less on the roles of fungi, and many studies used only "model" yeasts. In the experimental setting where natural conditions may be well mimicked, the authors successfully isolated wild yeast species and convincingly showed that wild yeast plays a critical role in promoting host growth. In addition, the authors provided intriguing observations that all of the heat-killed yeast promoted larval growth even though some of the yeast never supported the development when they were alive, suggesting that wild yeasts produce the necessary nutrients for larval development, but the nutrients of non-supportive yeasts are not accessible to the host. This might be an interesting indication for further studies revealing host-fungi interactions.

Weaknesses:

The experimental setting that, the authors think, reflects host-microbe interactions in nature is one of the key points. However, it is not explicitly mentioned whether isolated microbes are indeed colonized in wild larvae of *Drosophila melanogaster* who eat bananas. Another matter is that this work is rather descriptive and a few mechanical insights are presented. The evidence that the nutritional role of BCAAs is incomplete, and molecular level explanation is missing in "interspecies interactions" between lactic acid bacteria (or yeast) and acetic acid bacteria that assure their inhabitation. Apart from these matters, the future directions or significance of this work could be discussed more in the manuscript.

Reviewer #3 (Public Review):

Summary:

In this manuscript, Mure et al. describe interactions between diet, microbiome, and host development using *Drosophila* as a model. By characterizing microbial communities in food sources and animals, the authors showed that microbial community dynamics in the food source are critical for host development.

Strengths:

This is a very interesting study where the authors managed to tackle a difficult question in an elegant manner. How the interactions between different microbial species within the microbiome shape host physiology is an area of great interest but equally challenging due to the complexity of intercellular interactions in complex, host-associated microbial

communities. By using a simplified model and interrogating not only microbe-microbe and host-microbe interactions, but also the role played by diet, authors were able to identify significant interactions during fly development.

Weaknesses:

Despite describing important findings, I believe that a more thorough explanation of the experimental setup and the steps expected to occur in the exposed diet over time, starting with natural "inoculation" could help the reader, in particular the non-specialist, grasp the rationale and main findings of the manuscript. When exactly was the decision to collect early-stage samples made? Was it when embryos were detected in some of the samples? What are the implications of bacterial presence in the no-fly traps? These samples also harbored complex microbial communities, as revealed by sequencing. Were these samples colonized by microbes deposited with air currents? Were they the result of flies that touched the material but did not lay eggs? Could the traps have been visited by other insects? Another interesting observation that could be better discussed is the fact that adult flies showed a microbiome that more closely resembles that of the early-stage diet, whereas larvae have a more late-stage-like microbiome. It is easy to understand why the microbiome of the larvae would resemble that of the late-stage foods, but what about the adult microbiome? Authors should discuss or at least acknowledge the fact that there must be a microbiome shift once adults leave their food source. Lastly, the authors should provide more details about the metabolomics experiments. For instance, how were peaks assigned to leucine/isoleucine (as well as other compounds)? Were both retention times and MS2 spectra always used? Were standard curves produced? Were internal, deuterated controls used?

Author Response

Reviewer #1 (Public Review):

Weaknesses:

- 1. The authors should better review what we know of fungal *Drosophila* microbiota species as well as the ecology of rotting fruit. Are the microbiota species described in this article specific to their location/setting? It would have been interesting to know if similar species can be retrieved in other locations using other decaying fruits. The term 'core' in the title suggests that these species are generally found associated with *Drosophila* but this is not demonstrated. The paper is written in a way that implies the microbiota members they have found are universal. What is the evidence for this? Have the fungal species described in this paper been found in other studies? Even if this is not the case, the paper is interesting, but there should be a discussion of how generalizable the findings are.*

The reviewer inquires as to whether the microbial species described in this article are ubiquitously associated with *Drosophila* or not. Indeed, most of the microbes described in this manuscript are generally recognized as species associated with *Drosophila* spp. For example, species such as *Hanseniaspora uvarum*, *Pichia kluyveri*, and *Starmerella bacillaris* have been detected in or isolated from *Drosophila* spp. collected in European countries as well as the United States and Oceania (Chandler et al., 2012; Solomon et al., 2019). As for the bacteria, species belonging to the genera *Pantoea*, *Lactobacillus*, *Leuconostoc*, and *Acetobacter* have also previously been detected in wild *Drosophila* spp. (Chandler et al., 2011). These elucidations will be incorporated into our revised manuscript.

Nevertheless, the term "core" in the manuscript title may lead to misunderstanding, as the generality does not ensure the ubiquitous presence of these microbial species in every

individual fly. Considering this point, we will replace the term with an expression more appropriate to our context.

1. *Can the authors clearly demonstrate that the microbiota species that develop in the banana trap are derived from flies? Are these species found in flies in the wild? Did the authors check that the flies belong to the *D. melanogaster* species and not to the sister group *D. simulans*?*

Can the authors clearly demonstrate that the microbiota species that develop in the banana trap are derived from flies? Are these species found in flies in the wild?

The reviewer asked whether the microbial species identified in the fermented banana samples were derived from flies. To address this question, additional experiments under more controlled conditions, such as the inoculation of specific species of wild flies onto fresh bananas, would be needed. Nevertheless, the microbes may potentially originate from wild flies, as supported by the literature cited in our response to the Weakness 1).

Alternative sources for microbial provenance also merit consideration. For example, microbial entities may be inherently present in unfermented bananas through the infiltration of peel injuries (lines 1141-1142 of the original manuscript). In addition, they could be introduced by insects other than flies, given that both rove beetles (Staphylinidae) and sap beetles (Nitidulidae) were observed in some of the traps. These possibilities will be incorporated into the 'MATERIALS AND METHODS' and 'DISCUSSION' sections of our revised manuscript.

*Did the authors check that the flies belong to the *D. melanogaster* species and not to the sister group *D. simulans*?*

Our sampling strategy was designed to target not only *D. melanogaster* but also other domestic *Drosophila* species, such as *D. simulans*, that inhabit human residential areas. After adult flies were caught in each trap, we identified the species as shown in Table S1, thereby showing the presence of either or both *D. melanogaster* and *D. simulans*. We will provide these descriptions in MATERIALS AND METHODS and DISCUSSION.

1. *Did the microarrays highlight a change in immune genes (ex. antibacterial peptide genes)? Whatever the answer, this would be worth mentioning. The authors described their microarray data in terms of fed/starved in relation to the Finke article. They should clarify if they observed significant differences between species (differences between species within bacteria or fungi, and more generally differences between bacteria versus fungi).*

Did the microarrays highlight a change in immune genes (ex. antibacterial peptide genes)? Whatever the answer, this would be worth mentioning.

Regarding the antimicrobial peptide genes, statistical comparisons of our RNA-seq data across different conditions were impracticable because most of them showed low expression levels (refer to Author response table 1, which exhibits the RNA-seq data of the yeast-fed larvae; similar expression profiles were observed in the bacteria-fed larvae). While a subset of genes exhibited significantly elevated expression in the non-supportive conditions relative to the supportive ones, this can be due to intra-sample variability rather than due to distinct nutritional environments. Therefore, it would be difficult to discuss a change in immune genes in the paper. Additionally, the previous study that conducted larval microarray analysis (Zinke et al., 2002) did not explicitly focus on immune genes.

Author Response Table 1.

Antimicrobial peptide genes are not up-regulated by any of the microbes Antimicrobial peptides gene expression profiles of whole bodies of first-instar larvae fed on yeasts. TPM values of all samples and comparison results of gene expression levels in the larvae fed on supportive and non-supportive yeasts are shown. Antibacterial peptide genes mentioned in Hanson and Lemaitre, 2020 are listed. NA or na, not available.

Yeast fed		TPM values																								Statistical analyses									
		H. uvarum (H. uva)			K. humilis (K. hum)			M. asiatica			Saccharomyces cerevisiae			Pichia kluyveri (P. klu)			Starmerella bacillaris (S. bac)			BY4741			Germ-free			Significant difference between conditions				log2(fold change)					
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	H. uva or P. klu	H. uva or S. bac	K. hum or P. klu	K. hum or S. bac	H. uva /P. klu	H. uva /S. bac	K. hum /P. klu	K. hum /S. bac		
Sample number		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	H. uva or P. klu	H. uva or S. bac	K. hum or P. klu	K. hum or S. bac	H. uva /P. klu	H. uva /S. bac	K. hum /P. klu	K. hum /S. bac		
Flybase ID	symbol																																		
FBgn0004240	Dpi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	51	0	na	na	na	na	NA	NA	NA	NA
FBgn0034407	DpiB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	43	0	0	0	0	0	0	25	1	na	na	na	na	NA	NA	NA	NA
FBgn0012042	AtaA	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	11	0	0	0	0	0	0	6	1	na	na	na	na	NA	NA	NA	NA
FBgn0041581	AtaB	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	26	0	0	0	0	0	0	15	1	na	na	na	na	NA	NA	NA	NA
FBgn0041579	AtaC	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	24	0	0	0	0	0	0	0	1	na	na	na	na	NA	NA	NA	NA
FBgn0038530	AHD	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0010388	Dro	0	0	2	0	0	1	1	1	0	1	0	1	3	0	0	0	0	10	0	2	4	1	7	2	na	na	na	na	na	NA	NA	NA	NA	
FBgn0000276	CecA1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4	0	0	0	0	0	0	15	1	na	na	na	na	NA	NA	NA	NA
FBgn0000277	CecA2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0000278	CecB	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0000279	CecC	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0000094	Anp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0010385	Def	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0010381	Dns	1	1	3	0	1	2	0	0	0	1	1	7	19	0	0	0	0	1	25	2	5	13	8	0	5	na	na	na	na	na	NA	NA	NA	NA
FBgn0002274	Dns1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0002278	Dns2	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0002282	Dns4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0002283	Dns3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0005434	Dns5	2	0	1	1	2	1	1	0	1	4	1	1	0	1	1	0	0	0	1	2	2	0	0	1	na	na	na	na	na	NA	NA	NA	NA	
FBgn0005288	Dns6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0014865	Mk	1	1	2	2	0	1	2	1	2	2	0	2	5	1	2	2	2	51	1	1	3	2	2	6	na	na	na	na	na	NA	NA	NA	NA	
FBgn0040653	IM4	5	4	4	1	1	2	4	2	4	0	0	3	63	1	1	1	1	23	1	1	3	1	1	27	na	na	na	na	na	0.28	-0.16	NA	NA	
FBgn0067905	IM14	1	1	2	0	1	0	0	0	1	0	0	2	41	0	0	0	0	27	0	0	0	0	0	0	1	23	na	na	na	na	NA	NA	NA	NA
FBgn0034512	CG18067	3	4	7	3	4	6	5	5	5	10	11	14	43	17	17	15	17	27	9	13	14	19	22	31	H. uva <P. klu	H. uva <S. bac	K. hum <P. klu	K. hum <S. bac	-1.54	-1.37	-1.59	-1.41		
FBgn0034329	IM1	0	0	1	0	0	0	0	0	0	0	0	0	8	131	1	1	1	2	116	1	1	0	1	3	76	na	na	na	na	na	NA	NA	NA	NA
FBgn0034330	CG18107	1	1	1	0	0	1	1	1	1	4	4	18	6	7	14	17	16	2	6	2	10	18	16	na	H. uva <S. bac	na	K. hum <S. bac	na	NA	-2.66	NA	-3.79		
FBgn0002583	IM2	0	0	1	0	0	0	0	0	0	0	0	2	113	0	0	0	0	86	0	0	0	0	0	0	43	na	na	na	na	NA	NA	NA	NA	
FBgn0040736	IM3	0	0	0	0	0	0	0	0	0	0	0	9	125	1	1	1	3	101	0	0	0	0	0	0	2	2	na	na	na	na	NA	NA	NA	NA
FBgn0040734	CG15065	2	0	8	2	1	3	2	3	2	1	1	12	49	2	0	1	2	24	3	3	15	1	0	20	na	na	na	na	na	NA	NA	NA	NA	
FBgn0040733	CG15068	0	0	2	0	2	1	0	0	1	0	1	4	56	7	5	17	21	36	0	5	2	14	31	44	na	H. uva <S. bac	na	K. hum <S. bac	NA	-3.02	NA	-3.25		
FBgn0026838	CG43202	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	na	na	na	na	NA	NA	NA	NA
FBgn0040735	CG16836	0	0	0	0	0	0	0	0	0	0	0	1	19	0	0	0	0	5	0	0	0	0	0	0	5	na	na	na	na	NA	NA	NA	NA	
FBgn0038930	CG5778	0	0	0	0	0	0	0	0	0	0	0	1	31	1	1	2	1	7	0	1	0	1	1	10	na	na	na	na	na	NA	NA	NA	NA	
FBgn0034328	IM23	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	10	0	0	0	0	0	0	3	na	na	na	na	NA	NA	NA	NA	
FBgn0034331	CG15067	0	0	0	0	0	0	0	0	0	0	0	1	29	0	0	0	0	1	19	0	0	0	1	1	20	na	na	na	na	na	NA	NA	NA	NA
FBgn0040582	CG5791	0	0	0	0	0	1	0	1	1	1	1	1	1	1	1	1	1	2	0	1	1	1	4	na	na	na	na	na	NA	NA	NA	NA	NA	

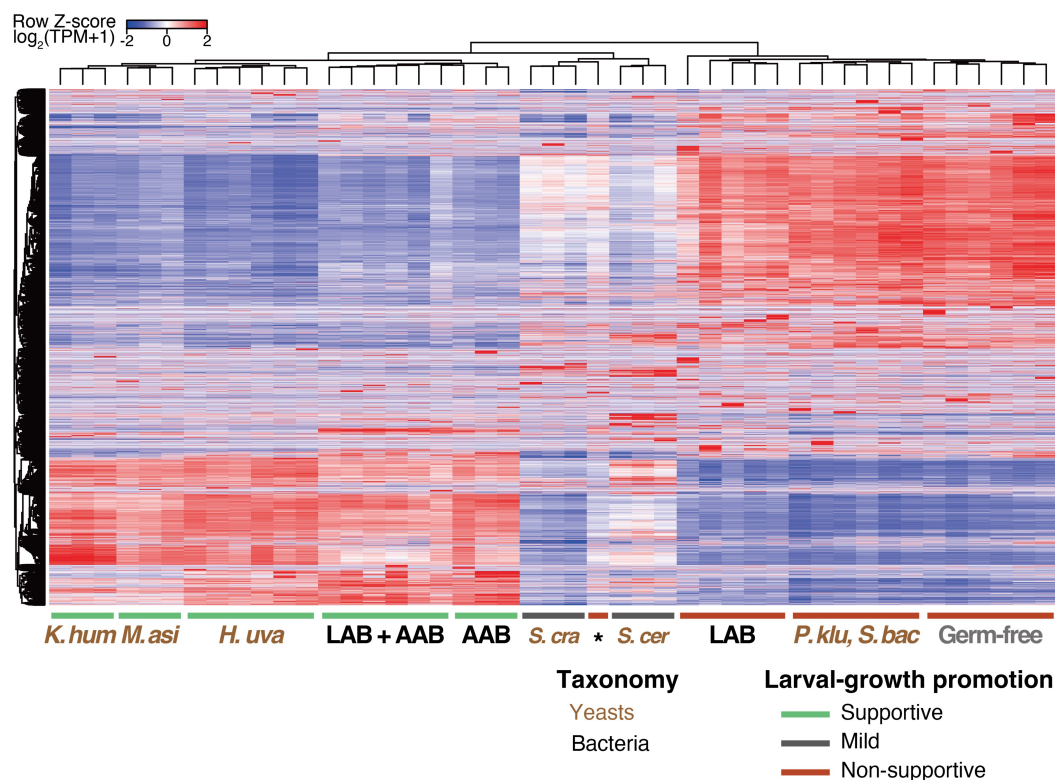
They should clarify if they observed significant differences between species (differences between species within bacteria or fungi, and more generally differences between bacteria versus fungi).

We did not observe significant differences between species within bacteria or fungi, or between bacteria and fungi. For example, the gene expression profiles of larvae fed on the various supporting microbes showed striking similarities to each other, as evidenced by the heat map showing the expression of all genes detected in larvae fed either yeast or bacteria (Author response image 1). Similarities were also observed among larvae fed on distinct non-supporting microbes.

Author response image 1.

Gene expression profiles of larvae fed on the various supporting microbes show striking similarities to each other. Heat map showing the gene expression of the first-instar larvae that fed on yeasts or bacteria. Freshly hatched germ-free larvae were placed on banana agar inoculated with each microbe and collected after 15 h feeding to examine gene expression of the whole body. Note that data presented in Figures 3A and 4C in the original manuscript, which are obtained independently, are combined to generate this heat map. The labels under the heat map indicate the microbial species fed to the larvae, with three samples analyzed for each condition. The lactic acid bacteria ("LAB") include Lactiplantibacillus plantarum and

Leuconostoc mesenteroides, while the lactic acid bacterium (“AAB”) represents *Acetobacter orientalis*. “LAB + AAB” signifies mixtures of the AAB and either one of the LAB species. The asterisk in the label highlights a sample in a “LAB” condition (*Leuconostoc mesenteroides*), which clustered separately from the other “LAB” samples. Brown abbreviations of scientific names are for the yeast-fed conditions. *H. uva*, *Hanseniaspora uvarum*; *K. hum*, *Kazachstania humilis*; *M. asi*, *Martiniozyma asiatica*; *S. cra*, *Saccharomycopsis crataegensis*; *P. klu*, *Pichia kluyveri*; *S. bac*, *Starmerella bacillaris*; *S. cer*, *S. cerevisiae* BY4741 strain.



Only a handful of genes showed different expression patterns between larvae fed on yeast and those fed on bacteria, without any enrichment for specialized gene functions. Thus, it is challenging to discuss the potential differential impacts, if any, of yeast and bacteria on larval growth.

1. The whole paper - and this is one of its merits - points to a role of the *Drosophila* larval microbiota in processing the fly food. Are these bacterial and fungal species found in the gut of larvae/adults? Are these species capable of establishing a niche in the cardia of adults as shown recently in the Ludington lab (Dodge et al.,)? Previous studies have suggested that microbiota members stimulate the Imd pathway leading to an increase in digestive proteases (Erkosar/Leulier). Are the microbiota species studied here affecting gut signaling pathways beyond providing branched amino acids?

The whole paper - and this is one of its merits - points to a role of the *Drosophila* larval microbiota in processing the fly food. Are these bacterial and fungal species found in the gut of larvae/adults? Are these species capable of establishing a niche in the cardia of adults as shown recently in the Ludington lab (Dodge et al.,)?

Although we did not investigate the microbiota in the gut of either larvae or adults, we did compare the microbiota within surface-sterilized larvae or adults with those in food samples. We found that adult flies and early-stage food sources, as well as larvae and late-stage food sources, harbor similar microbial species (Figure 1F). Additionally, previous examinations of

the gut microbiota in wild adult flies have identified microbial species or taxa congruent with those we isolated from our foods (Chandler et al., 2011; Chandler et al., 2012). We have elaborated on this in our response to Weakness 1).

While we did not investigate whether these species are capable of establishing a niche in the cardia of adults, we will cite the study by Dodge et al., 2023 in our revised manuscript and discuss the possibility that predominant microbes in adult flies may show a propensity for colonization.

Previous studies have suggested that microbiota members stimulate the Imd pathway leading to an increase in digestive proteases (Erkosar/Leulier). Are the microbiota species studied here affecting gut signaling pathways beyond providing branched amino acids?

The reviewer inquires whether the supportive microbes in our study stimulate gut Imd signaling pathways and induce the expression of digestive protease genes, as demonstrated in a previous study (Erkosar et al., 2015). According to our RNA-seq data, it seems unlikely that the supportive microbes stimulate the signaling pathway. Figures contained in Author response image 2 provide the statistical comparisons of expression levels for seven protease genes between the supportive and the non-supportive conditions. These genes did not exhibit a consistent upregulation in the presence of the supportive microbes (H. uva or K. hum in Author response image 2A; Le. mes + A. ori in Author response image 2B). Rather, they exhibited a tendency to be upregulated under the non-supportive microbes (St. bac or Pi. klu in Author response image 2A; La. pla in Author response image 2B).

Author response image 2.

Most of the peptidase genes reported by Erkosar et al., 2015 are more highly expressed under the non-supportive conditions than the supportive conditions. Comparison of the expression levels of seven peptidase genes derived from the RNA-seq analysis of yeast-fed (A) or bacteria-fed (B) first-instar larvae. A previous report demonstrated that the expression of these genes is upregulated upon association with a strain of *Lactiplantibacillus plantarum*, and that the PGRP-LE/Imd/Relish signaling pathway, at least partially, mediates the induction (Erkosar et al., 2015). H. uva, *Hanseniaspora uvarum*; K. hum, *Kazachstania humilis*; P. klu, *Pichia kluyveri*; S. bac, *Starmerella bacillaris*; La. pla, *Lactiplantibacillus plantarum*; Le. mes, *Leuconostoc mesenteroides*; A. ori, *Acetobacter orientalis*; ns, not significant.

A

FlyBase ID	symbol	gene_name	Significant difference between conditions				log2(fold change)			
			<i>H. uva</i> (H) or <i>P. klu</i> (P)	<i>H. uva</i> (H) or <i>S. bac</i> (S)	<i>K. hum</i> (K) or <i>P. klu</i> (P)	<i>K. hum</i> (K) or <i>S. bac</i> (S)	<i>H. uva</i> / <i>P. klu</i>	<i>H. uva</i> / <i>S. bac</i>	<i>K. hum</i> / <i>P. klu</i>	<i>K. hum</i> / <i>S. bac</i>
FBgn0035887	Jon66Cii	Jonah 66Cii	H>P	H>S	ns	ns	0.47	0.50	-0.24	-0.21
FBgn0035886	Jon66Ci	Jonah 66Ci	H<P	H<S	K<P	K<S	-1.27	-0.91	-1.31	-0.94
FBgn0001285	Jon44E	Jonah 44E	H<P	H<S	K<P	K<S	-0.66	-0.52	-0.96	-0.82
FBgn0035667	Jon65Ai	Jonah 65Ai	H<P	H<S	K<P	K<S	-0.51	-0.59	-0.60	-0.68
FBgn0003358	Jon99Ci	Jonah 99Ci	H>P	H>S	K>P	K>S	0.77	0.97	0.67	0.87
FBgn0036023	CG18179	-	H<P	H<S	K<P	K<S	-1.38	-0.55	-1.38	-0.56
FBgn0036024	CG18180	-	H<P	H<S	K<P	K<S	-0.84	-0.69	-0.53	-0.38

B

FlyBase ID	Symbol	Gene name	Significant difference between conditions		log2(fold change)	
			<i>Le. mes</i> + <i>A. ori</i> (LA) or <i>Le. mes</i> (L)	<i>La. pla</i> + <i>A. ori</i> (LA) or <i>La. pla</i> (L)	[<i>Le. mes</i> + <i>A. ori</i>] / <i>Le. mes</i>	[<i>La. pla</i> + <i>A. ori</i>] / <i>La. pla</i>
FBgn0035887	Jon66Cii	Jonah 66Cii	LA<L	LA<L	-0.99	-0.54
FBgn0035886	Jon66Ci	Jonah 66Ci	LA<L	LA<L	-2.15	-1.42
FBgn0001285	Jon44E	Jonah 44E	LA<L	LA<L	-0.70	-1.52
FBgn0035667	Jon65Ai	Jonah 65Ai	LA<L	LA<L	-0.65	-0.91
FBgn0003358	Jon99Ci	Jonah 99Ci	LA>L	ns	0.97	-0.13
FBgn0036023	CG18179	-	LA<L	LA<L	-3.36	-4.53
FBgn0036024	CG18180	-	LA<L	LA<L	-1.97	-1.84

Reviewer #2 (Public Review):

Weaknesses:

The experimental setting that, the authors think, reflects host-microbe interactions in nature is one of the key points. However, it is not explicitly mentioned whether isolated microbes are indeed colonized in wild larvae of Drosophila melanogaster who eat bananas. Another matter is that this work is rather descriptive and a few mechanical insights are presented. The evidence that the nutritional role of BCAAs is incomplete, and molecular level explanation is missing in "interspecies interactions" between lactic acid bacteria (or yeast) and acetic acid bacteria that assure their inhabitation. Apart from these matters, the future directions or significance of this work could be discussed more in the manuscript.

The experimental setting that, the authors think, reflects host-microbe interactions in nature is one of the key points. However, it is not explicitly mentioned whether isolated microbes are indeed colonized in wild larvae of Drosophila melanogaster who eat bananas.

The reviewer asks whether the isolated microbes were colonized in the larval gut. Previous studies on microbial colonization associated with Drosophila have predominantly focused on adults (Pais et al. PLOS Biology, 2018), rather than larval stages. Developing larvae continually consume substrates which are already subjected to microbial fermentation and abundant in live microbes until the end of the feeding larval stage. Therefore, we consider it difficult to discuss microbial colonization in the larval gut. We will add this point in the DISCUSSION of the revised manuscript.

Another matter is that this work is rather descriptive and a few mechanical insights are presented. The evidence that the nutritional role of BCAAs is incomplete, and molecular

level explanation is missing in "interspecies interactions" between lactic acid bacteria (or yeast) and acetic acid bacteria that assure their inhabitation.

While recognizing the importance of comprehensive mechanistic analysis, this study includes all experimentally feasible data. Elucidation of more detailed molecular mechanisms lies beyond the scope of this study and will be the subject of future research.

Regarding the nutritional role of BCAAs, the incorporation of BCAAs enabled larvae fed with the non-supportive yeast to grow to the second instar. This observation suggests that consumption of BCAAs upregulates diverse genes involved in cellular growth processes in larvae. We have discussed the hypothetical interaction between lactic acid bacteria (LAB) and acetic acid bacteria (AAB) in the manuscript (lines 402-405): LAB may facilitate lactate provision to AAB, consequently enhancing the biosynthesis of essential nutrients such as amino acids. To test this hypothesis, future experiments will include the supplementation of lactic acid to AAB culture plates and the co-inoculating LAB mutant strains defective in lactate production with AABs, to assess both larval growth and continuous larval association with AABs. With respect to AAB-yeast interactions, metabolites released from yeast cells might benefit AAB growth, and this possibility will be investigated through the supplementation of AAB culture plates with candidate metabolites identified in the cell suspension supernatants of the late-stage yeasts.

Apart from these matters, the future directions or significance of this work could be discussed more in the manuscript.

We appreciate the reviewer's recommendations and will include additional descriptions regarding these aspects in the DISCUSSION section.

Reviewer #3 (Public Review):

Weaknesses:

Despite describing important findings, I believe that a more thorough explanation of the experimental setup and the steps expected to occur in the exposed diet over time, starting with natural "inoculation" could help the reader, in particular the non-specialist, grasp the rationale and main findings of the manuscript. When exactly was the decision to collect early-stage samples made? Was it when embryos were detected in some of the samples? What are the implications of bacterial presence in the no-fly traps? These samples also harbored complex microbial communities, as revealed by sequencing. Were these samples colonized by microbes deposited with air currents? Were they the result of flies that touched the material but did not lay eggs? Could the traps have been visited by other insects? Another interesting observation that could be better discussed is the fact that adult flies showed a microbiome that more closely resembles that of the early-stage diet, whereas larvae have a more late-stage-like microbiome. It is easy to understand why the microbiome of the larvae would resemble that of the late-stage foods, but what about the adult microbiome? Authors should discuss or at least acknowledge the fact that there must be a microbiome shift once adults leave their food source. Lastly, the authors should provide more details about the metabolomics experiments. For instance, how were peaks assigned to leucine/isoleucine (as well as other compounds)? Were both retention times and MS2 spectra always used? Were standard curves produced? Were internal, deuterated controls used?

When exactly was the decision to collect early-stage samples made? Was it when embryos were detected in some of the samples?

We collected traps and early-stage samples 2.5 days after setting up the traps. This time frame was determined by pilot experiments. A shorter collection time resulted in a greater likelihood of obtaining no-fly traps, whereas a longer collection time caused larval overcrowding, as well as adults' deaths from drowning in the liquid seeping out of fruits. These procedural details will be delineated in the MATERIALS AND METHODS section of the revised manuscript.

What are the implications of bacterial presence in the no-fly traps? These samples also harbored complex microbial communities, as revealed by sequencing. Were these samples colonized by microbes deposited with air currents? Were they the result of flies that touched the material but did not lay eggs? Could the traps have been visited by other insects?

We assume that the origins of the microbes detected in the no-fly trap foods vary depending on the species. For instance, *Colletotrichum musae*, the fungus that causes banana anthracnose, may have been present in fresh bananas before trap placement. The filamentous fungi could have originated from airborne spores, but they could also have been introduced by insects that feed on these fungi. We will include these possibilities in the DISCUSSION section of the revised manuscript.

Another interesting observation that could be better discussed is the fact that adult flies showed a microbiome that more closely resembles that of the early-stage diet, whereas larvae have a more late-stage-like microbiome. It is easy to understand why the microbiome of the larvae would resemble that of the late-stage foods, but what about the adult microbiome? Authors should discuss or at least acknowledge the fact that there must be a microbiome shift once adults leave their food source.

We are grateful for the reviewer's insightful suggestions regarding shifts in the adult microbiome. We plan to include in the DISCUSSION section of the revised manuscript the possibility that the microbial composition may change substantially during pupal stages and that microbes obtained after eclosion could potentially form the adult gut microbiota.

Lastly, the authors should provide more details about the metabolomics experiments. For instance, how were peaks assigned to leucine/isoleucine (as well as other compounds)? Were both retention times and MS2 spectra always used? Were standard curves produced? Were internal, deuterated controls used?

We appreciate the reviewer's advice. Detailed methods of the metabolomic experiments will be included in our revised manuscript.