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

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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 key microbial species that support animal growth under microbial transition.

INTRODUCTION

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; Zheng et al., 2020). 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; Su et al., 2022), 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; Pérez, 2021). Moreover, the composition of these complex microbiota tends to change over time (Moya & Ferrer, 2016; Qiu et al., 2021). 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, 2012; Grenier & Leulier, 2020; Lee & Brey, 2013). Furthermore, germ-free or gnotobiotic animals can be easily prepared (Ludington & Ja, 2020). 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; Storelli et al., 2011). 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). Consequently, the relationship between *Drosophila* and its associated microorganisms has often been classified as facultative (Gallo et al., 2022; Martino et al., 2018), 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). Germ-free larvae cannot grow on fresh fruits alone, while inoculation with certain species of yeasts or bacteria promotes pupariation (Anagnostou et al., 2010; Pais et al., 2018). 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; Quan & Eisen, 2018; Solomon et al., 2019), 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, Enterobacterales 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 key 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 larval foods, fermented bananas, that had been fed on by wild larvae (Figure 1A, Tables S1A and S1B). 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 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 food samples, respectively (Figures 1B and S1; Tables S2A and S2B).

In most food samples, yeasts consistently accounted for a major proportion of the fungal communities (top of Figure 1B; Figure S1A; 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 Enterobacterales predominating at the early stage and lactic acid bacteria (LAB) and acetic acid bacteria (AAB) predominating at the late stage (bottom of Figure 1B; Figure S1B; 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 bananas and wild *Drosophila*” section in MATERIALS AND METHODS for further details). We analyzed food samples from this trap for comparison. The microbiota in the foods significantly differed from that of other foods, with a lower percentage of yeast in the fungal community and a consistently high abundance of Enterobacterales 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; Tables S1A and S1C). 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 bananas and examined whether the microbial composition shifts occurred in them. Remarkably, the composition shifts occurred similarly, irrespective of the presence of larvae, as indicated by the decreased proportions of Saccharomycodaceae yeast and Enterobacterales bacteria, along with the increased proportions of Pichiaceae yeasts 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; Tables S1A and S1D). 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 PCR to quantify the copy numbers of fungal and bacterial rDNA in each food sample presented in Figures 1B, 1D, and 1F (Figure S2). 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 bananas, 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 the early-stage foods on larval development, we focused on yeast and bacterial strains belonging to microbial species dominated in the food samples shown in Figure 1B (see also Table 1 and Figure S1). The two characteristic microbial species in the early-stage foods were the yeast *Hanseniaspora uvarum* and the bacteria *Pantoea agglomerans* (Table 1; Figure S1). These two species along with *Pichia kluyveri*, the second dominant yeast in most of the early-stage foods, were selected for this experiment (Table 1; Figure S1A). We investigated whether each strain or a combination of the strains promotes larval

development (Figures 2A, 2B, S3A, and S3B). When inoculated individually, *H. uvarum* and *Pantoea agglomerans* effectively promoted larval growth ("Y2" and "E" in Figures 2A and 2B, respectively; "Y1" and "E" in Figures S3A and S3B, respectively), whereas *Pichia kluyveri*, another yeast species, had almost no promoting effect ("Y1" in Figures 2A and 2B; "Y2" in Figures S3A and S3B). 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 strains of the late-stage microbes (Figures 2C, 2D, S3C, and S3D; Table 1): two dominating late-stage yeast species, the most predominant LAB and AAB species, and *Pantoea agglomerans*, which persisted from the early stage, albeit in a smaller proportion (Table 1; Figure S1). When fed individually, none of the yeast or bacterial species efficiently supported larval growth, except for *H. uvarum* and *Pantoea agglomerans* (lines and symbols with different brightness of gray in Figure 2C and S3C). 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 and S3C). 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 S3F). 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 *Pichia kluyveri*) or a LAB (*Leuconostoc mesenteroides* or *Lactiplantibacillus plantarum*) (Figures 2E, 2F, S3E and S3F). 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 S4).

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 (Figures 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 "non-supportive" 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, S5A, and S5B). 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).

231 Interestingly, although larvae fed on AAB alone largely failed to pupariate ("A" in Figures
232 2C-2F), their gene expression profile after the 15-hr feeding period closely resembled that of the
233 supportive conditions (compare "AAB" with "Yeast" and "LAB+AAB" in Figure 3A). This result
234 implies that AAB possessed the ability to induce a growth-promoting response, but the effect likely
235 did not persist until pupariation. We observed that the growth rate of AAB on banana agar was
236 noticeably lower compared to those of other microbes. This led us to speculate that this lower
237 growth rate might result in a shortage of AAB, leading to undernutrition during the later stage of
238 larval development. To test this hypothesis, we investigated whether larval development could
239 recover when AAB was constantly available. Daily supplementation of AAB enabled larvae to
240 pupariate as effectively as the initial co-inoculation of AAB and LAB did (Figures 3E and 3F),
241 demonstrating that AAB can promote larval growth if it is available throughout the course of
242 development.

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

251 As described in the previous section, none of AAB, LAB, or the yeast in the late-stage foods
252 strongly promoted larval growth individually, while mixing AAB with LAB or the yeasts did
253 (Figures 2C-2F and S3C-S3F). All of our results so far strongly suggest that these interspecies
254 interactions among the microbes underlie the promotion of larval growth by late-stage microbiota.

255

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

258 We found that the early-stage yeast *H. uvarum* facilitated larval development, whereas the
259 dominant yeasts in the late stage, *Pichia kluyveri* and *Starmerella bacillaris*, did not (Figures 2A-
260 2D and S3A-S3D). To elucidate the underlying mechanisms behind these diverse outcomes, we
261 conducted comprehensive comparative analyses with the respective yeasts. We used six yeast
262 species that originated from the fermented bananas (*Kazachstania humilis*, *Martiniozyma asiatica*,
263 and *Saccharomycopsis crataegensis* in addition to the aforementioned three species) and a
264 laboratory strain of the model species *Saccharomyces cerevisiae*, which was rarely detected in our
265 food samples (Figure 4).

266 When we provided each species to germ-free larvae, the larvae fed on *H. uvarum* showed the
267 highest percentage of pupariation, while larvae fed on *K. humilis* or *M. asiatica* also pupariated at
268 relatively high rates, albeit with a slower timing for *M. asiatica* (Figures 4A and 4B). We classified
269 these three species as the "supportive" yeast species. In contrast, larvae fed on *Pichia kluyveri* or
270 *Starmerella bacillaris*, which are the dominant species during the late stage, showed notably low
271 percentage of pupariation, leading to their classification as "non-supportive" (Figures 4A and 4B).
272 Inoculation with *Saccharomycopsis crataegensis* or a laboratory strain of *Saccharomyces*
273 *cerevisiae* BY4741 resulted in reduced percentages and delayed timing of pupariation compared

to the inoculation with the 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, S5C, and S5D; cf. Figures 3A-3D, S5A, and S5B). 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 *Pichia kluyveri* and *Starmerella 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 (p-hydroxy-benzoic acid and propionic acid), and observed similar growth promotion by *Pichia kluyveri* and *Starmerella 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 (Figures 4I and 4J). This medium contains dry yeast and enables larvae to pupariate even without live yeasts ("GF" in Figures 4I and 4J). Under these conditions, larvae fed with *Starmerella bacillaris* pupariated as efficiently and nearly as rapidly as those without yeasts or with other yeast species, suggesting that *Starmerella bacillaris* did not impede larval growth (Figures 4I and 4J). In contrast, larvae fed with *Pichia 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 *Pichia kluyveri* on this food (Figure S5E). Nevertheless, none of these yeast species reproduced the low percentage of pupariation of larvae observed on banana agar. Therefore, the inhibitory effects of *Pichia kluyveri* and *Starmerella 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 non-supportive species (*Pichia kluyveri* and *Starmerella bacillaris*) and analyzed not only the yeast

317 cells but also two additional samples anticipated to contain metabolites released from the cells:
318 yeast-conditioned banana-agar plates and cell suspension supernatants (Figures 5A-5F and S6,
319 Table S6).

320 The yeast-conditioned banana-agar plates had been expected to contain yeast-derived nutrients.
321 On the contrary, the result revealed a depletion of various metabolites originally present in the
322 sterile banana agar (Figure 5A). This result prompted us to focus on the metabolites in the
323 chemically defined (holidic) medium for *D. melanogaster* (Piper et al., 2014, 2017). This medium
324 contains ~40 known nutrients, and supports the larval development to pupariation, albeit at the
325 half rate compared to that on a yeast-containing standard laboratory food (Piper et al., 2014, 2017).
326 Therefore, the holidic medium could be considered to contain the minimal essential nutrients
327 required for larval growth. Our analysis indicated a substantial reduction of these known nutrients
328 in the yeast-conditioned plates compared to their original quantities (Figure 5B).

329 The quantities of metabolites within the cells varied markedly among the species (Figures 5C
330 and 5D); while the supportive *H. uvarum* contained numerous known metabolites, it was intriguing
331 that *K. humilis*, another supportive species, did not possess as much essential nutrients as other
332 species, including the non-supportive *Pichia kluyveri* and *Starmerella bacillaris* (Figure 5D). This
333 result, in conjunction with the observations from feeding heat-killed yeasts (Figures 4G and 4H),
334 raises the possibility that the interspecies disparities in metabolites within living yeast cells do not
335 directly influence larval growth. On the other hand, our analysis of the cell suspension supernatant
336 revealed distinct variations in the known nutrients in the holidic medium between the supportive
337 and non-supportive species (Figures 5E and 5F). Among these, we focused on branched-chain
338 amino acids (BCAAs), leucine and isoleucine (Figure 5F). Previous studies have demonstrated
339 that bacteria associated with *Drosophila* provide these essential amino acids to the hosts feeding
340 on artificial diets lacking them (Henriques et al., 2020; B. Kim et al., 2021). In our analysis,
341 suspension supernatants of supportive yeasts had concentrations of both leucine and isoleucine
342 that were at least four-fold, on average, higher than those of non-supportive yeasts (Figures 5F-
343 5H; see also Table S6B).

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

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

370 Regarding the mechanism of nutrient release, one possibility is their secretion from live cells,
371 while another possibility is their leakage from dead cells. To address the latter hypothesis, we
372 collected cells of the four yeast species from banana-agar plates and stained dead cells using
373 Phloxine B (Figures 6D-6G). Noticeably more dead cells were found in the supportive *K. humilis*
374 compared to other species (Figure 6E), implying that nutrients leak from dead *K. humilis* cells into
375 the food, which is subsequently utilized by the larvae.
376

DISCUSSION

In this study, we have shown that the microbial composition of larval foods of domestic *Drosophila* species shifts dramatically as fermentation progresses. Individual microbial species play 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, from the initial predominance of *H. uvarum* to the compositional shifts leading to the dominance of AAB, LAB, and the late-stage yeasts. This shift coincides with alterations in the species serving as nutrient sources. Importantly, most of the microbes we isolated and investigated in this study are generally recognized as associates of *Drosophila*. For instance, yeasts such as *H. uvarum*, *Pichia kluyveri*, and *Starmerella bacillaris*, or bacteria belonging to the genera *Pantoea*, *Lactiplantibacillus* (formerly *Lactobacillus*), *Leuconostoc*, and *Acetobacter* have also been detected in or isolated from *Drosophila* spp. collected in North America, Europe, or Oceania (Chandler et al., 2011, 2012; Pais et al., 2018; Solomon et al., 2019). These findings suggest the universality of the *Drosophila*-microbe relationships revealed in our study. 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 plausibly influenced by factors such as interspecies interactions among microbes. These discrepancies may be due in part to differences in experimental conditions possibly including larval densities, food sample volumes, or culture vessels.

Another implication from our microbial composition analysis is a possible composition shift occurring in the adult intestine. The microbiota of late-third instar larvae resemble those of the late-stage foods they fed on, whereas those of trap-caught adults are similar to those of the corresponding early-stage foods. This result suggests a potential change in microbial composition during pupal stages or after adult eclosion. Unlike developing larvae, which continually consume fermented foods throughout the feeding stages, adults move among a number of food sources, which could lead to the acquisition of new microbes. Additionally, some bacteria have recently been demonstrated to colonize the adult intestine (Dodge et al., 2023; Pais et al., 2018), possibly contributing to the formation of adult microbiota through the dominance of microbes with a propensity to colonize the intestine.

As for the origin of food microbiota, the initial microbial inoculation to foods is assumed to be mediated by adult flies (Broderick & Lemaitre, 2012). Our study revealed the presence of common microbes between our early-stage foods and adult fly samples (Figure 1F; Tables S2C and S2E). Furthermore, the foods from the “no-fly” trap harbored distinct microbiota compared to those from other traps. The distinction was marked by the presence of *Colletotrichum musae*, which is the causal agent of banana anthracnose and might have been present in fresh bananas, and filamentous fungi, which could have originated from airborne spores (Figures 1B and S1A; Table S2A). These results support the previously proposed microbial inoculation to the foods by adult

flies. However, alternative sources of microbes should also be considered. For example, microbes may have been infiltrated in unpeeled bananas through peel injuries, as microbial rDNA was detected in bananas that had not undergone microbial inoculation ("Blank" samples in Figures S2C and S2D). In addition, they could be introduced by other insects such as rove beetles and sap beetles, which were found in some of the traps, sharing the same habitat with *Drosophila* spp. Further investigation is required to determine the inoculation routes of the microbes in larval foods.

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, Akherraz, 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 branched-chain amino acids (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 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, *Pantoea agglomerans* and *A. orientalis* did promote larval growth, but most bacteria, including the two aforementioned species, lack the steroid biosynthesis pathway (Hoshino & Gaucher, 2021). 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). Therefore, microbial nutrients that promote larval growth, whose functions have not yet been identified in this study, may be components other than lipids.

466 Despite the well-recognized importance of yeast as a source of nutrients, limited studies have
467 reported the detailed process of how these nutrients are utilized by larvae. Our results suggest that
468 for yeasts to serve as nutritional sources, they should not only produce the nutrients, but also
469 release them outside the cells. The amount of nutrients released extracellularly varied among yeast
470 species. This could be explained by varying abilities to secrete metabolites, although yeasts are
471 unlikely to release the nutrients that are also beneficial to themselves. Another possibility
472 suggested for *K. humilis* was that this species is more prone to death on the culture medium, thus
473 releasing more nutrients. Indeed, yeast autolysates are reported to contain various known nutrients,
474 such as proteins, lipids, and B-group vitamins (Rakowska et al., 2017). More thorough
475 investigations should be conducted on another supportive yeast, *H. uvarum*, to assess its mortality
476 on banana-agar plates.

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

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

492 **MATERIALS AND METHODS**

493 ***Drosophila melanogaster* strains and culture**

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

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

502

503 **Generation of germ-free larvae**

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

509

510 **Collection of fermented bananas and wild *Drosophila***

511 Larval foods, fermented bananas, were collected using traps placed outdoors in human residential
512 areas to collect domestic *Drosophila* species such as *D. melanogaster* or *D. simulans*. The
513 sampling places, near laboratory members' apartments or houses in Kyoto and Osaka prefectures,
514 Japan, or outside the laboratory at Kyoto University, are listed in Table S1A. Bananas were
515 selected as bait considering their use in previous *Drosophila* studies (Anagnostou et al., 2010;
516 Consuegra, Grenier, Akherraz, et al., 2020; Stamps et al., 2012). They are also affordable and
517 available year-round, which would be advantageous for subsequent analyses requiring large
518 amounts of fruit-based culture media. Peels of ripening bananas were treated with 70% ethanol
519 and then removed carefully so as not to touch the fruit inside to avoid contamination. The bananas
520 were subsequently cut into pieces with an autoclaved sterile spatula, and placed in a sterile 100
521 mL centrifuge tube (Iwaki). Each trap was made of an empty milk carton, and contained one tube
522 of banana bait.

523 Traps were set up for 2.5 days, which duration was determined from pilot experiments; a
524 shorter collection time resulted in a lower likelihood of obtaining traps visited by adult flies,
525 whereas a longer collection time caused overcrowding of larvae as well as deaths of adults from
526 drowning in the liquid seeping out of the fruits. When the traps were collected, wild flies or their
527 eggs/larvae were found in all traps or foods, except for one (the "no-fly" trap). In the sampling
528 shown in Figures 1C-F, rove beetles (Staphylinidae) and sap beetles (Nitidulidae) were found in
529 several foods. These insects appeared to share a niche with wild *Drosophila* in nature, suggesting
530 that the presence of these insects did not interfere with our goal of obtaining larval food samples.
531 The non-drosophilid insects were removed from the foods immediately after the trap collection. 3-
532 5 mL of the foods (early-stage foods) were sampled, and the remaining foods were incubated at

533 25°C. 4-5 days later, when late 3rd instar larvae were seen in all foods except for the one from the
534 no-fly trap, the foods were collected (late-stage foods). At the first sampling, we added germ-free
535 embryos of GFP-expressing *D. melanogaster* laboratory strain (*Cg-Gal4, UAS-mCD8:GFP*) to
536 obtain *D. melanogaster* larvae that were reared in the fermented food, but we could not sample
537 them because most animals apparently drowned in the juice that seeped out of the fermented
538 bananas.

539 To obtain early-stage-food suspensions, newly obtained early-stage foods were crushed in
540 autoclaved sterile PBS, and after vigorous mixing, the liquid was collected carefully without taking
541 bananas, embryos, or larvae. 200 µL of this microbial suspension (or sterile PBS for Blank) was
542 added to 100 mL tubes containing fresh ripening banana with or without ~200 germ-free embryos
543 (*Cg-Gal4, UAS-mCD8:GFP*). The bananas were incubated at 25°C for 4 days. Late-stage foods
544 were collected as described above. Larvae collected from the food were examined under a
545 fluorescent microscope to make sure they were derived from the embryos we added and not from
546 contaminating wild embryos. When sterile PBS was added to bananas instead of microbial
547 suspensions in Figure 1C, the food showed no apparent change in appearance or odor during the
548 4-day incubation, and larvae in the food remained at the first instar stage. The amounts of microbes
549 detected in such foods were less than those obtained for the food inoculated with the microbial
550 suspension by 2-4 orders of magnitude ("Blank" in Figures S2C and S2D; see details in the
551 legend)."

552 All the food samples were collected after removing embryos and larvae, and snap-frozen.
553 Larvae were surface-sterilized using the same procedure as embryo samples before snap-freezing.
554 Adult flies in the traps were collected following different procedures depending on whether their
555 microbiota were analyzed. When the adult samples were solely utilized for species identification,
556 the whole bodies were stored in 100% or 70% ethanol. When we also required samples for
557 microbial analysis, after washing the whole bodies in 70% ethanol, the head, external genitalia,
558 and wings of each fly were removed and stored in 70% ethanol and subsequently used to identify
559 the species. The rest of the body was snap-frozen and later used for the microbial composition
560 analysis. Adult flies collected in each sampling are listed in Tables S1B-D. For the traps where
561 adult flies were caught, the species of the drosophilids in them were identified, confirming the
562 presence of either or both *D. melanogaster* and *D. simulans*.

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

565

566 **ITS or 16S rDNA sequencing analysis**

567 Food, larval, or adult samples were freeze-dried for 2-3 days, followed by homogenization with 5
568 mm stainless beads using Shake Master Neo (Biomedical Science). Microbial DNA was extracted
569 using a QIAamp DNA Stool Mini Kit (QIAGEN). 3 mm and 0.1 mm zirconium beads were used
570 for the initial lysis step.

571 Library preparation, sequencing, and data analyses were performed by Macrogen Japan
572 (Tokyo, Japan). For fungal analysis, the ITS region was amplified with primers ITS3 (5'-
573 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCATCGATGAAGAACGCAGC-3')
574 and ITS4 (5'-
575 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTCCTCCGCTTATTGATATGC-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), followed by pre-processing and clustering using CD-HIT-OTU (Fu et al., 2012; Li & Godzik, 2006) with a clustering cutoff of 99%. Taxonomy was assigned using QIIME (Caporaso et al., 2010) using the UNITE database (Nilsson et al., 2019) for fungal ITS, and the Ribosomal Database Project (RDP) database (Cole et al., 2014) 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; Johnson et al., 2008). 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."

qPCR 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 bananas was 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)

ITS3: 5'-GCATCGATGAAGAACGCAGC-3'

ITS4: 5'-TCCTCCGCTTATTGATATGC-3'

For bacterial 16S (Hugerth et al., 2014)

341F: 5'-CCTACGGGNGGCWGCAG-3'

805R: 5'-GACTACHVGGGTATCTAATCC-3'

Banana and *Drosophila* rDNAs were expected to be amplified in this qPCR, as they were in the PCR for the ITS and 16S sequencing analysis. The proportion of microbial rDNA within the total amplification products was assumed to remain consistent between the qPCR and the corresponding sequencing analysis, because the template DNA samples and amplified regions were shared between the analyses. Based on this, the copy number of microbial rDNA was estimated by multiplying the qPCR results with the microbial rDNA ratio observed in the ITS or 16S sequencing analysis of each sample.

616

617 **Microbial culture**

618 Microbes were cultured on MRS, YPD, PDA, or banana agar. MRS, YPD, and PDA were prepared
619 using MRS Broth (Merck Millipore), YPD medium (Clontech), and Potato Dextrose Broth
620 (Sigma-Aldrich), respectively, following the manufacturers' protocol. Banana agar was prepared
621 as previously described (Anagnostou et al., 2010), with the addition of autoclaving after making
622 the banana-agar mixture. Cavendish bananas (*Musa acuminata*) were used. 10% p-hydroxy-
623 benzoic acid in 70% ethanol (prepared as described in Kanaoka et al., 2023), propionic acid
624 (Nacalai Tesque), cycloheximide (Wako), or ampicillin (Nacalai Tesque) were added after
625 autoclaving. Food-derived microbes were cultured at 25°C for 2 days, while *Saccharomyces*
626 *cerevisiae* was cultured at 30°C for 2 days before use, unless otherwise noted. Images of single
627 colonies were acquired with a digital camera (Visualix STD2) attached to a stereo microscope
628 (Olympus SZX10).

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

635

636 **Isolation and species identification of microbes**

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

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

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

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

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

659

660 **Quantification of larval development**

661 The microbes were individually grown on banana agar twice before being suspended in PBS
662 (OD600 = 60). To mix multiple species, equal amounts of the suspensions prepared as described
663 were mixed together. 10 μL of each suspension was added to ~1.1 mL of autoclaved banana agar
664 in 1.5 mL tubes. Tubes were incubated at 25°C for 2 days before larvae were added. The following
665 procedure was performed as described in Watanabe et al., 2019, with a few modifications. Briefly,
666 20 germ-free larvae were added to each tube, and the tubes were kept in a moisturized incubator
667 (80-90% humidity) at 25°C. Pupariated individuals were removed from the tubes prior to eclosion,
668 and either discarded or transferred to vials for further incubation until eclosion occurred. The
669 number of pupae in each tube was counted until all larvae either pupariated or died. In experiments
670 that require daily addition of AAB, the bacterial cells (a lump of ~3 μL by volume per tube) were
671 added daily. As more individuals pupariated, less bacteria were consumed each day. Therefore, to
672 avoid the excessive accumulation of the bacteria, no AAB was added in a tube where the
673 previously added bacterial lump was visible to the naked eye.

674 The effect on larval development was quantified as the final percentage of the pupariated
675 individuals and timing of the pupariation. The developmental timing was quantified from the date
676 on which the percentage of pupariation exceeded 50% of the final percentage of pupariation. Molds
677 were occasionally seen on foods during incubation, in which case the tubes were removed from
678 the experiment. On a rare occasion when the number of samples for a single condition fell below
679 3, all the tubes for the condition were excluded from the results.

680 When feeding heat-killed yeasts to larvae, yeasts were added to the banana-agar tubes and
681 subsequently heated as following procedures. The yeasts were revived from frozen stocks on
682 banana-agar plates, incubated at 25°C, and then streaked on fresh agar plates. After 2-day
683 incubation, yeast cells were scraped from the plates and suspended in PBS at the concentration of
684 400 mg of yeast cells in 500 μL of PBS. 125 μL of the suspensions were added to banana-agar
685 tubes prepared as described, and after centrifugation at 3,000 x *g* for 5 min, the supernatants were
686 removed. The amount of cells in each tube is ~50x compared to that when feeding live yeasts,
687 which compensates for the reduced amount due to their inability to proliferate. The tubes were
688 subsequently heated at 80°C for 30 min before adding germ-free larvae.

689 When feeding yeasts on banana agar supplemented with antifungal agents, the yeasts were
690 individually grown on normal banana agar twice before being suspended in PBS at the
691 concentration of 400 mg of yeast cells in 500 μL of PBS. 125 μL of the suspensions was introduced
692 onto the anti-fungal agents (10 mL/L 10% p-hydroxy-benzoic acid in 70% ethanol and 6 mL/L
693 propionic acid, following the concentration described in Kanaoka et al., 2023)-containing banana
694 agar in 1.5 mL tubes. After centrifugation, the supernatants were removed. The amount of cells in
695 each tube is ~50x compared to that when feeding live yeasts.

696

697 **Sequencing and annotation of yeast genomes**

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

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

716 For RNA-seq analysis, equal amounts of yeast cells cultured under 6 conditions (2
717 temperatures: 25°C and 30°C, and 3 culture plates: MRS, YPD, or banana-agar plates) were mixed
718 and used for RNA extraction. RNA was extracted as previously described (Iida & Kobayashi,
719 2019) and purified using the RNeasy mini kit (QIAGEN). Libraries for RNA-seq were prepared
720 with NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB) and sequenced on
721 the NextSeq 500 platform (Illumina) to generate 300-base paired end sequences which were used
722 for annotation described below. A total of 23.48-26.75M reads were obtained for each yeast
723 (average; 25.63M reads).

724 Genome assembly was performed by MaSuRCA v3.2.6 (Zimin et al., 2013) using MiSeq and
725 MinION reads. In the heterozygosity rate estimation by GenomeScope based on MiSeq reads, the
726 *Kazachstania humilis* and *Pichia kluyveri* genomes showed higher heterozygosity rates (*K.*
727 *humilis*; 2.98%, *Pichia kluyveri*; 2.08%) while those of other genomes were 0.02% - 1.00%. In the
728 assessment of autoannotated genome assemblies using BUSCO (Simão et al., 2015), completeness
729 was over 86% in the four genomes other than the *H. uvarum* (64.58%) and *Starmerella bacillaris*
730 (75.21%) genomes. However, among the four genomes, those of *Pichia kluyveri* and *K. humilis*
731 showed a high proportion of "Duplicated complete" genes (*K. humilis*; 27.74%, *Pichia kluyveri*;
732 5.17%, respectively), which was apparently due to the high heterozygosity rate of these genomes.
733 Therefore, these two genomes were assembled using Platanus-allee v2.2.2 (Kajitani et al., 2019),
734 and by comparing it with the MaSuRCA assemblies using GenomeMatcher (Ohtsubo et al., 2008),
735 redundant sequences in the MaSuRCA assemblies were identified and removed from the
736 assemblies. As the proportions of "Duplicated complete" genes estimated by BUSCO were 5.51%
737 for the *K. humilis* genome and 0.74% for the *Pichia kluyveri* genome after removing redundant
738 sequences, these redundant sequence-removed genome assemblies were used as the final genome
739 assemblies of *Pichia kluyveri* and *K. humilis*.

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

747 Mitochondrial sequences in each genome assembly were identified by tblastx homology
748 search with fungal mitochondrial sequences obtained from the RefSeq database (O'Leary et al.,
749 2016). When multimerized mitochondrial sequences were generated in genome assemblies, they
750 were trimmed to the smallest unit.

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

756

757 **Quantification of AAB**

758 Microbes were inoculated onto 200 mL of banana agar in a tube included in a Biomasher II
759 homogenizer kit (NIP). After adding 5 germ-free larvae, the tubes were incubated at 25°C for 4
760 days. 150 µL of PBS was added to the tube, and food was crushed along with the larvae in it. More
761 PBS was subsequently added to bring the total volume of the suspension to 700 µL. 1/5 serial
762 dilutions were made, 50 µL of which were spread onto MRS plates supplemented with antibiotics
763 (10 µg/mL cycloheximide and 10 µg/mL ampicillin to inhibit the growth of yeasts and LAB,
764 respectively). The plates were incubated at 25°C, and 3 days later, colonies were counted to
765 calculate CFU.

766

767 **RNA sequencing for gene expression analyses**

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

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

777 For the heatmaps in Figures 3A and 4C, heatmap.2 in the gplots (Warnes et al., 2022) package
778 of R (R Core Team, 2020) was used. Database for annotation, visualization and integrated
779 discovery (DAVID) Functional Annotation Chart (Huang et al., 2009; Sherman et al., 2022) was
780 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 (fold change value) of the individuals at 12 h after feeding or starvation was used. For our data, $\log_2$(fold change) of gene expression was calculated for each supportive and non-supportive condition pair. 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. The genes that showed significantly different expression between fed ("normal" in the cited paper) and starved conditions, i.e., those with fold-change values of ≥1 or ≤ -1, are shown.

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. 22 mm^2 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 2,260 x *g* 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. Chromatogram peaks obtained with compound-specific MRM channels were integrated and manually confirmed. If more than one confirmatory MRM channel was available for the target compound, it was set to confirm the identification of the peak compound. Structural isomers were separated either by retention time on the column or by compound-specific MRM signals. Detailed MRM conditions are identical to the previously published study (Oka et al., 2017). To further confirm the signal specificity,

823 sample-derived chromatographic peaks were compared with the corresponding standards to
824 ensure that retention times were consistent. Peak quantification values obtained for each
825 compound were corrected for recovery due to L-Met in the IS. Data are presented as peak area
826 values for each metabolite normalized by the internal standard. Those for the yeast-conditioned
827 plates or cells were further normalized with sample wet weight. Heat maps were generated by
828 MetaboAnalystR 3.3.0. (Pang et al., 2020), using data for all of the detected metabolites or a
829 subset of metabolites that are included in the holidic medium of *Drosophila melanogaster* (Piper
830 et al., 2014).

831

832 **Developmental progression with BCAA supplementation**

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

840 Technically, it was difficult to match the amounts of leucine and isoleucine to be added to the
841 foods to the difference in amounts released by the supportive and non-supportive species. This was
842 because under our experimental condition, quantification of the microbe-derived BCAAs
843 accumulating in the culture medium, which was performed in previous studies (Consuegra,
844 Grenier, Akherraz, et al., 2020; Henriques et al., 2020), was impractical due to constant absorption
845 of the nutrients by the yeasts themselves. Given this constraint, we opted for the amino acid
846 concentrations in the holidic medium, which concentrations are sufficient to support larval growth
847 under axenic conditions and not detrimental to larval growth (Piper et al., 2014).

848

849 **Imaging of dead yeast cells**

850 Microbes were grown on banana-agar plates for 3 days, then collected and suspended in PBS at a
851 concentration of ~1x 10^8 cells/mL. The cells were subsequently incubated in 5 µg/mL Phloxine B
852 (Wako) for 10 min at room temperature. The cells were subsequently washed twice with PBS and
853 observed using a Nikon C1 laser scanning confocal microscope coupled to a Nikon Eclipse E-800
854 microscope. Note that the brightness of the images was adjusted with a gradation to achieve mostly
855 uniform brightness, because the bottom side of the original images was darker due to microscope
856 malfunction.

857

858 **Statistical analysis**

859 R (R Core Team, 2020) was used for all statistical analyses. P-values less than 0.05 were
860 considered statistically significant. For statistical analyses of RNA-seq data, see Tables S4, S5,
861 and S8. See Table S9, MATERIALS AND METHODS, and figure legends for analysis of other
862 experiments.

863

864 Data availability

865 All sequence data obtained in this study have been deposited in the DDBJ Sequence Read Archive.
866 The accession numbers for the sequence data are DRR212820-DRR212838 (BioProject accession
867 number: PRJDB9099) and DRR471830-DRR471964 (BioProject accession number:
868 PRJDB15711). Yeast genome annotation data have been deposited in DDBJ/EMBL/GenBank
869 under BioProject accession number PRJDB9099. The accession numbers are BTGD01000001-
870 BTGD01000029 (*Kazachstania humilis*), BTGA01000001-BTGA01000014 (*Hanseniaspora*
871 *uvarum*), BTFZ01000001-BTFZ01000021 (*Saccharomycopsis crataegensis*), BTGB01000001-
872 BTGB01000010 (*Pichia kluyveri*), BTFY01000001-BTFY01000014 (*Martiniozyma asiatica*),
873 and BTGC01000001-BTGC01000009 (*Starmerella bacillaris*).

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COMPETING INTERESTS

The authors declare no competing interests.

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Figures

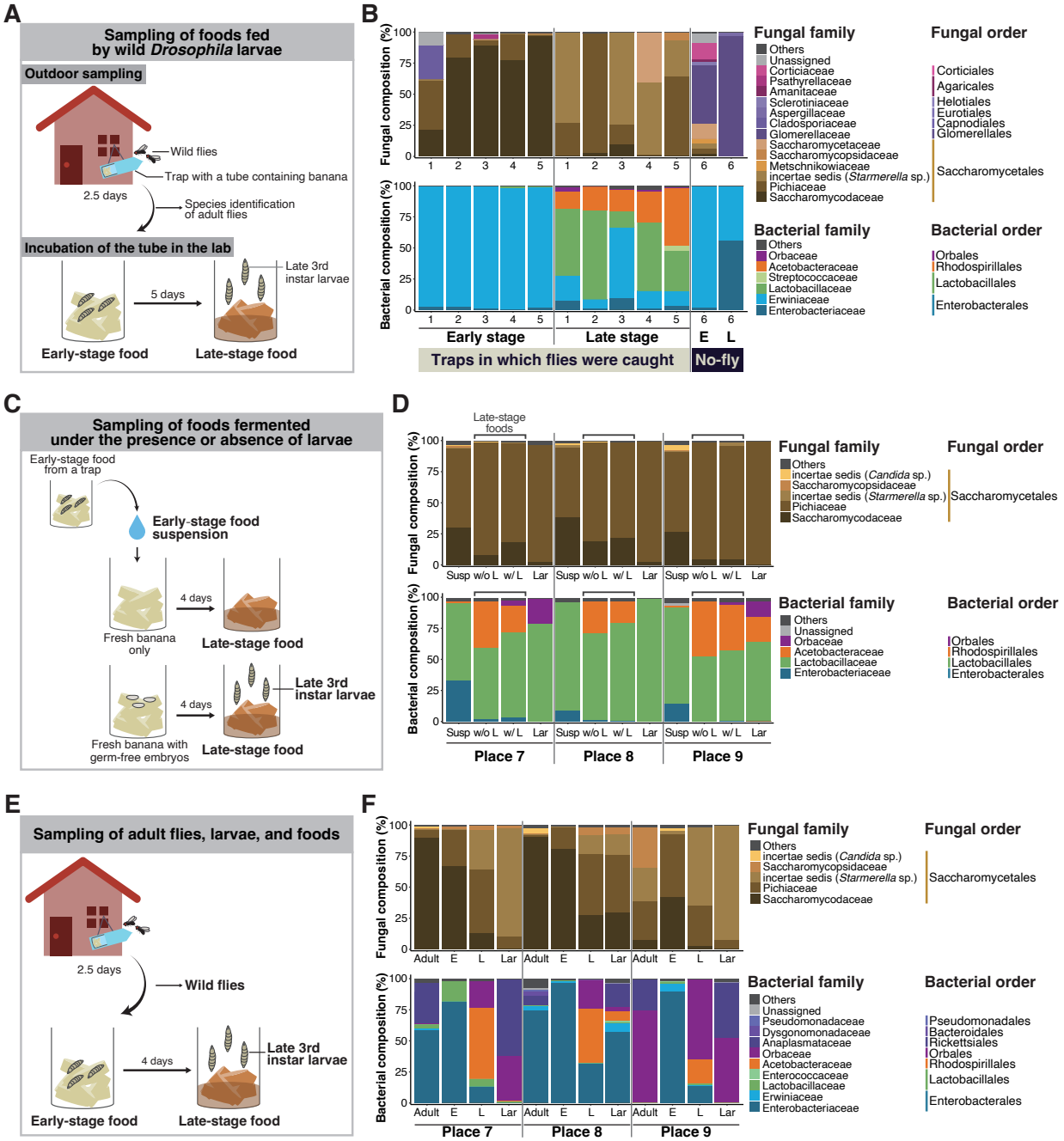

Figure 1. The composition of both yeast and bacteria in the food microbiota shifts as fermentation progresses, independently of the presence of larvae

(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)

1158 Designs of the samplings for microbial analyses. Traps with bananas were set up outdoors near
1159 human habitations in order to collect food samples on which wild *Drosophila* spp. lay eggs.
1160 Fermented food samples were collected from the traps at two time points: the early stage (just after
1161 trap collection), and the late stage (when larvae in the foods developed into late 3rd instars).
1162 Collected samples are indicated in bold letters. (C) In this sampling, the collected early-stage food
1163 was crushed in PBS, and the liquid portion (microbial suspension) was added to fresh bananas
1164 with or without germ-free embryos (*Cg-Gal4, UAS-mCD8:GFP*). After incubation, late-stage food
1165 and surface-sterilized larvae were collected. (E) In addition to the food samples, adult flies in the
1166 traps and larvae from the late-stage foods were collected. This sampling was conducted
1167 independently from that shown in (C) and (D), although the sampling places were in common (see
1168 Table S1A). Both adults and larvae were surface-sterilized. Note that all of the adults in the traps
1169 at Place 7 and Place 8 were collected for the microbial analysis, while 20 out of 37 were collected
1170 at Place 9. (B, D, and F) The relative abundances of fungi or bacteria in the fermented banana or
1171 fly samples. The compositions were analyzed using primer sets amplifying the fungal ITS region
1172 or bacterial 16S rDNA region. Operational taxonomic units (OTUs) accounting for >1% in any of
1173 the samples were grouped by families, as shown. The ratio of those accounting for <1% was
1174 summed and is indicated as "Others." The genera belonging to uncertain families (incertae sedis)
1175 are indicated by their genus name in parentheses. (B) The result corresponding to the sampling
1176 depicted in (A). The numbers on the horizontal axis indicate each numbered sampling location.
1177 "No-fly" indicates the food samples from sampling place 6, where no fly or larva was found in the
1178 trap or the foods, respectively. (D and F) The result corresponding to the sampling depicted in (C)
1179 and (E), respectively. E, early-stage food; L, late-stage food; Susp, microbial suspension; w/o L,
1180 late-stage food without larvae; w/ L, late-stage food with larvae; Lar, larvae.

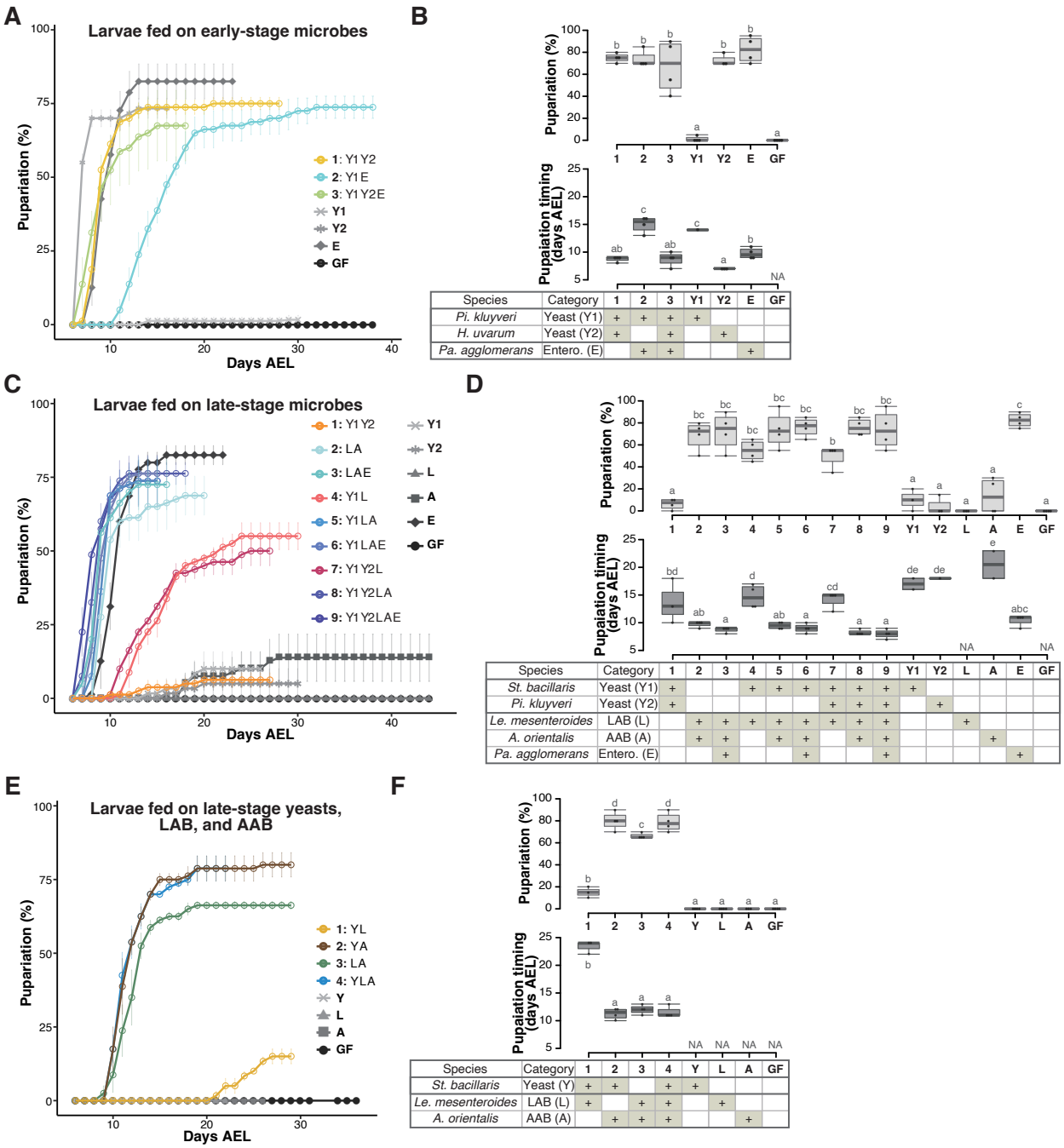

Figure 2. Prominent acceleration of larval development occurred with early-stage-dominant yeast alone, as well as in combination with late-stage-dominant acetic acid bacteria and other microbes

(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

1190 conditions, while gray lines are for mono-associated or germ-free conditions. The codes for
1191 individual species are provided in the chart at the bottom of (B), (D), and (E), respectively. Error
1192 bars represent SEM. (C) The color coding for the respective foods is as follows: includes yeasts
1193 only (orange); includes yeast(s) and LAB but no AAB (red); includes LAB and AAB (blue). (B,
1194 D, and F) Percentage of the larvae pupariated (pupariation ratio; upper) and the timing at which
1195 50% of the pupariation ratio was achieved (lower). Boxes represent upper and lower quartiles,
1196 while the central lines indicate the median. Whiskers extend to the most extreme data points, which
1197 are no more than 1.5 times the interquartile range. Unique letters indicate significant differences
1198 between groups (Tukey-Kramer test, $p < 0.05$). *Pi. kluyveri*, *Pichia kluyveri*; *Pa. agglomerans*,
1199 *Pantoea agglomerans*; *St. bacillaris*, *Starmerella bacillaris*; *Le. mesenteroides*, *Leuconostoc*
1200 *mesenteroides*; Entero., Enterobacterales; GF, germ-free; NA, not applicable; days AEL, days after
1201 egg laying.

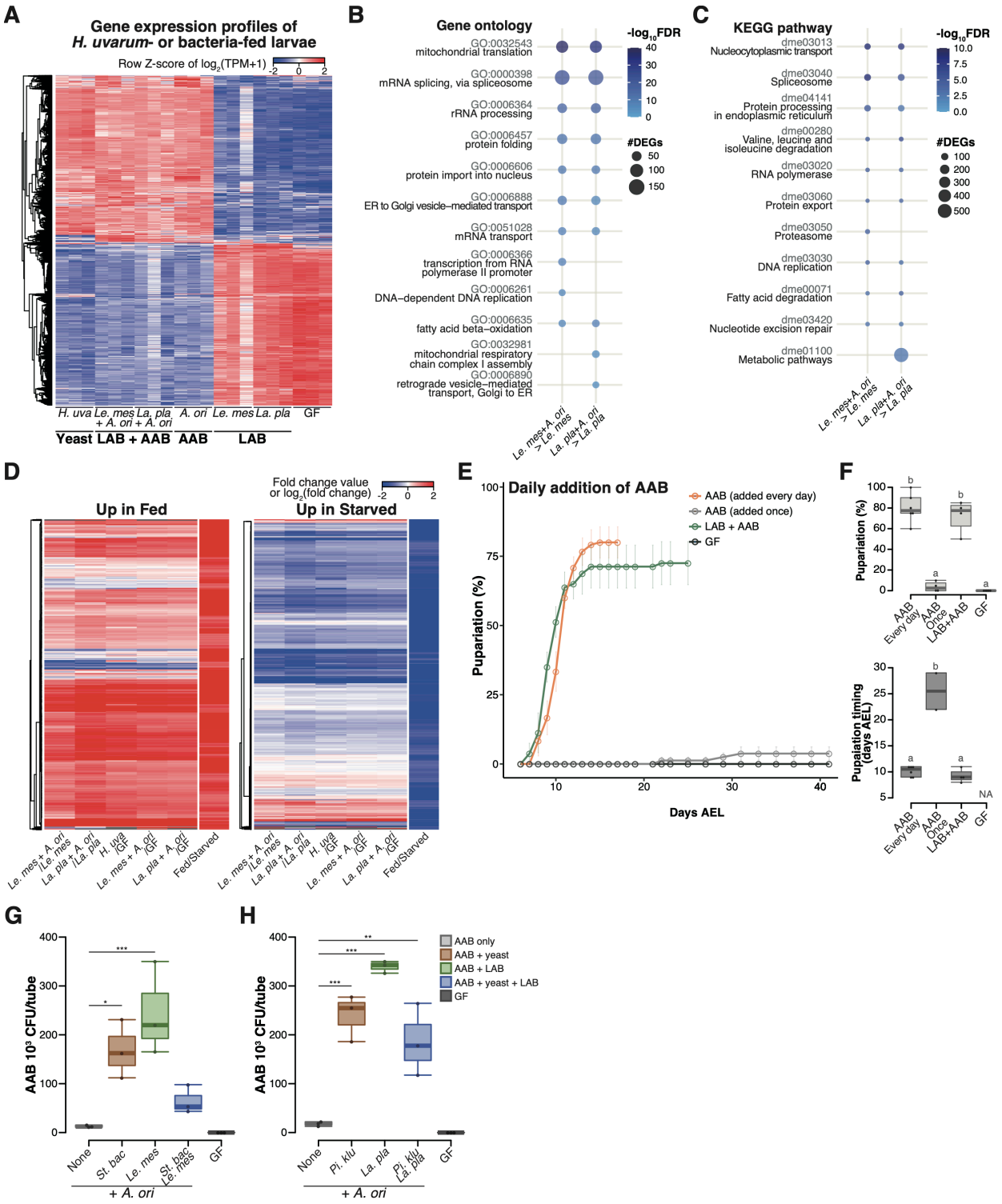

Figure 3. During the late stage, acetic acid bacteria play a crucial role in supporting larval development through interspecies interactions among the microbes

1206 (A) Heat map of gene expression values for first instar larvae fed *H. uvarum* or bacteria. Freshly
1207 hatched germ-free larvae were placed on banana agar inoculated with each microbe and collected
1208 after 15 h feeding to examine gene expression of the whole body. The data of the genes that were
1209 differentially expressed between "LAB"-fed conditions and the respective "LAB + AAB"
1210 conditions are shown. (B and C) Plots showing the result of GO term (B) or KEGG Pathway (C)
1211 enrichment analysis of the RNA-seq data. 10 terms/pathways that showed the smallest FDR in
1212 each analysis are shown. (D) Heat maps showing the similarity between our RNA-seq data and
1213 microarray data in a previous study (Zinke et al., 2002). Zinke et al. used larvae at 47-49 h AEL
1214 and fed them yeast paste or starved them for 12h before comparing the gene expression profiles.
1215 The data from Zinke et al., 2002. are labeled as "Fed/Starved" in the righthand column of each
1216 heat map, which show the fold change value of each gene. Only the genes exhibiting significant
1217 differences in their analysis are shown. For our data, $\log_2$(fold change) were calculated using TPM
1218 of each gene in the larvae on the supportive conditions versus those on the non-supportive
1219 conditions. (E and F) The percentage and timing of pupariation of the larvae feeding on AAB on
1220 banana agar. Graphs are presented as in Figure 2. n=3-4. (G and H) AAB load of foods inoculated
1221 with AAB, with or without other microbial species. Boxplots are depicted as in Figure 2 (Dunnett's
1222 test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). *La. pla*, *Lactiplantibacillus plantarum*; *Le. mes*,
1223 *Leuconostoc mesenteroides*; *A. ori*, *Acetobacter orientalis*; *H. uva*, *Hanseniaspora uvarum*; *St.*
1224 *bac, Starmerella bacillaris*; *Pi. klu*, *Pichia kluyveri*; GF, germ-free; NA, not applicable; days AEL,
1225 days after egg laying.

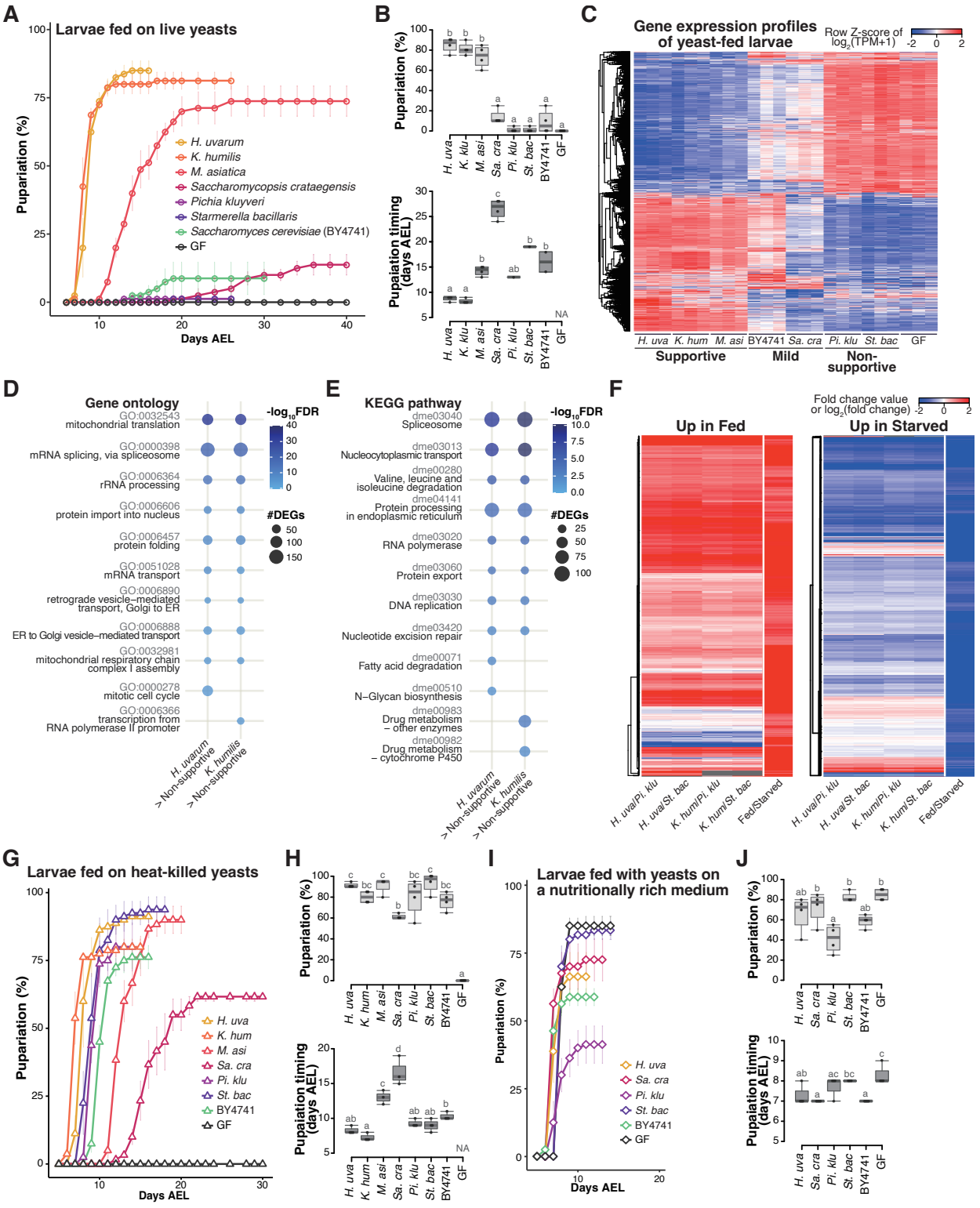

Figure 4. Isolated yeast species promote larval development to varying degrees, but all support larval development upon heat killing

1230 (A and B) The percentage and timing of pupariation of larvae feeding on live yeasts on banana
1231 agar. Graphs are presented as in Figure 2. n= 3-4. (C) Heat map of gene expression values for first
1232 instar larvae fed on each yeast species. Freshly hatched germ-free larvae were placed on banana
1233 agar inoculated with each microbe and collected after 15 h feeding to examine gene expression of
1234 the whole body. The data of the genes that were differentially expressed between the *H. uvarum*-
1235 fed larvae and the *Starmerella bacillaris*-fed larvae are shown. (D and E) Plots showing the result
1236 of GO term (D) or KEGG Pathway (E) enrichment analysis of the RNA-seq data. Genes that
1237 showed significantly higher expression on each of the supportive species than on both the non-
1238 supportive species were analyzed. 10 terms/pathways that showed the smallest FDR in each
1239 analysis are shown. (F) Heat maps showing the similarity between our RNA-seq data and
1240 microarray data in Zinke et al., 2002, shown as described in Figure 3. (G-J) The percentage and
1241 timing of pupariation of the larvae feeding on heat-killed yeasts on banana agar (G and H) or live
1242 yeasts on a nutritionally rich medium (I and J). Graphs are depicted as in Figure 2. n=3-4. *H. uva*,
1243 *Hanseniaspora uvarum*; *K. hum*, *Kazachstania humilis*; *M. asi*, *Martiniozyma asiatica*; *Sa. cra*,
1244 *Saccharomycopsis crataegensis*; *Pi. klu*, *Pichia kluyveri*; *St. bac*, *Starmerella bacillaris*; BY4741,
1245 *Saccharomyces cerevisiae* BY4741 strain; GF, germ-free; NA, not applicable; days AEL, days
1246 after egg laying.

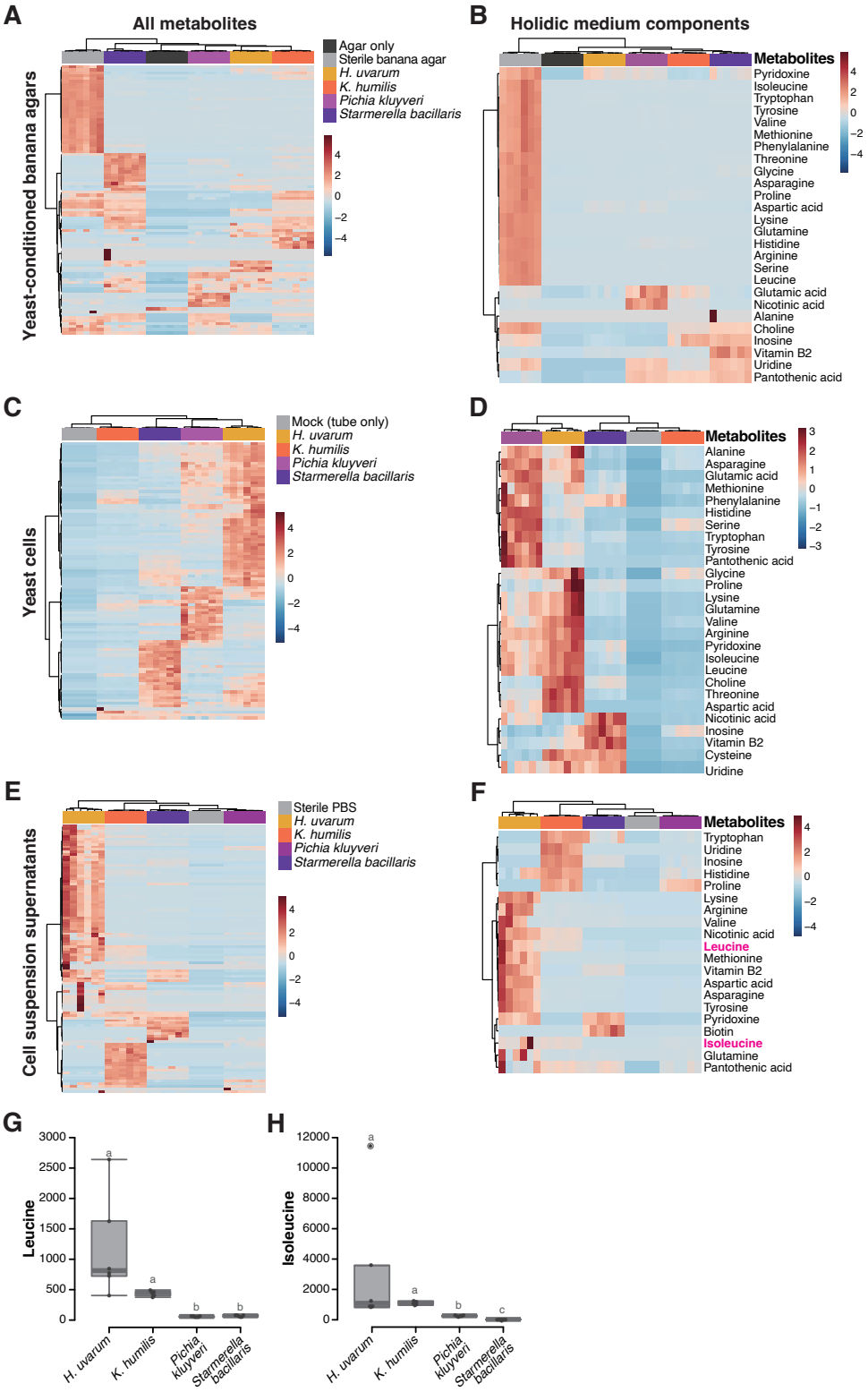

Figure 5. Metabolomic analysis of yeast cells, yeast-conditioned banana-agar plates, or cell suspension supernatants

1251 (A-F) Heat maps displaying all detected metabolites (A, C, and E) or only the metabolites included
1252 in a chemically defined synthetic (holidic) medium for *Drosophila melanogaster* (Piper et al.,
1253 2014) (B, D, and F) detected from the metabolomic analysis of banana-agar plates (A and B), yeast
1254 cells (C and D), or yeast-cell suspension supernatants (E and F). Row Z-scores of normalized peak
1255 areas are shown. (G and H) Normalized peak areas of leucine (G) and isoleucine (H) in yeast-cell
1256 suspension supernatants. Boxplots are presented as in Figure 2. Unique letters indicate significant
1257 differences between groups (Steel-Dwass test, $p < 0.05$).

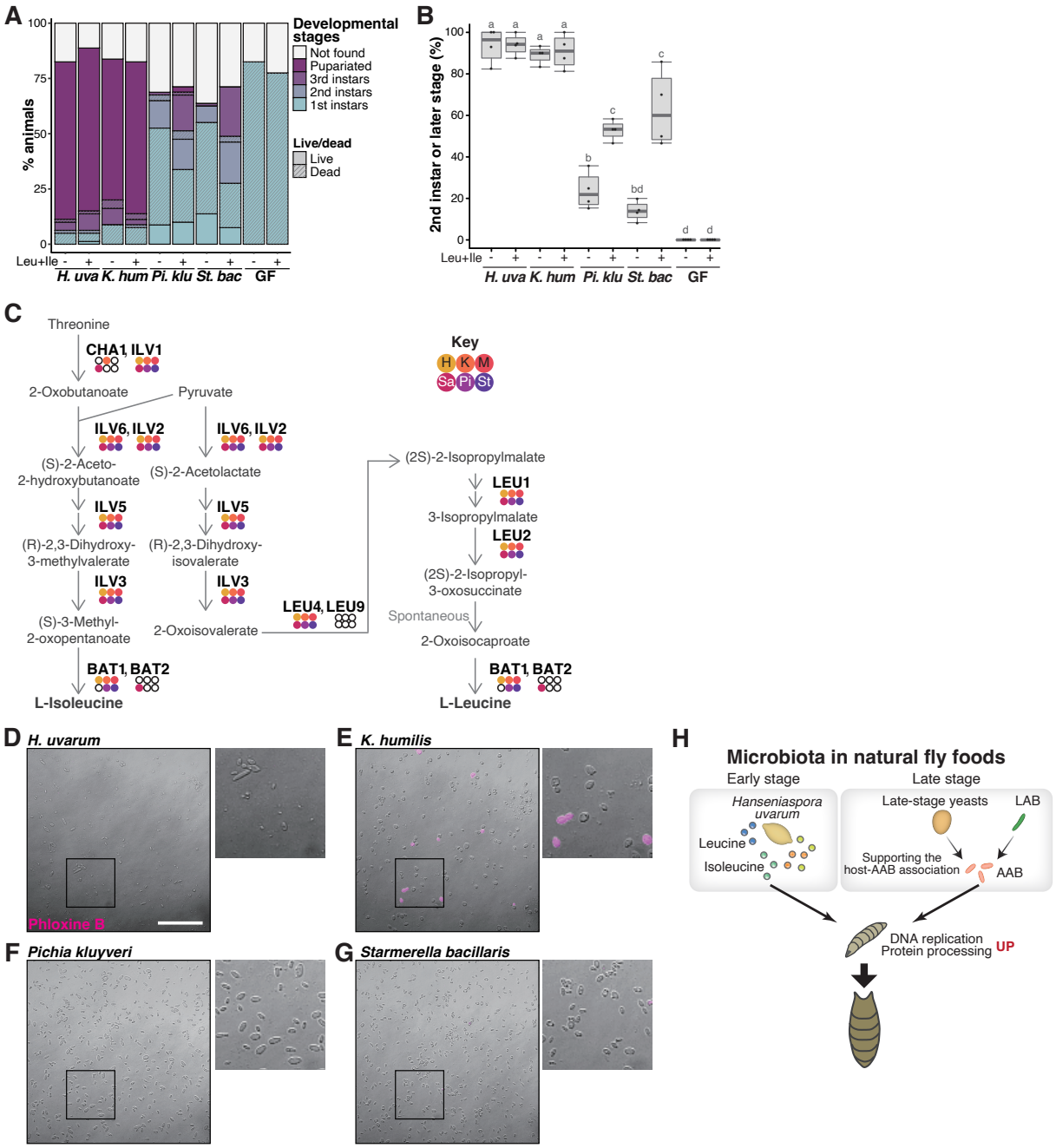

Figure 6. Supportive yeasts facilitate larval growth by providing nutrients, including branched-chain amino acids, by releasing them from their cells

(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 *Saccharomyces cerevisiae*

1268 gene names are shown. The colored dots indicate enzymes that are conserved in the six isolated
1269 species, while the white dots indicate those that are not conserved. Abbreviations of genera are
1270 given in the key in the upper right corner. LEU2 is deleted in BY4741. (D-G) Representative image
1271 of Phloxine B-stained yeasts. The right-side images are expanded images of the boxed areas. The
1272 scale bar represents 50 µm. (H) Summary of this study. *H. uvarum* is predominant in the early-
1273 stage food and provides Leu, Ile, and other nutrients that are required for larval growth. In the late-
1274 stage food, AAB directly provides nutrients, while LAB and yeasts indirectly contribute to larval
1275 growth by enabling the stable larva-AAB association. The host larva responds to the nutritional
1276 environment by dramatically altering gene expression profiles, which leads to growth and
1277 pupariation. *H. uva*, *Hanseniaspora uvarum*; *K. hum*, *Kazachstania humilis*; *Pi. klu*, *Pichia*
1278 *kluyveri*; *St. bac*, *Starmerella bacillaris*; GF, germ-free.

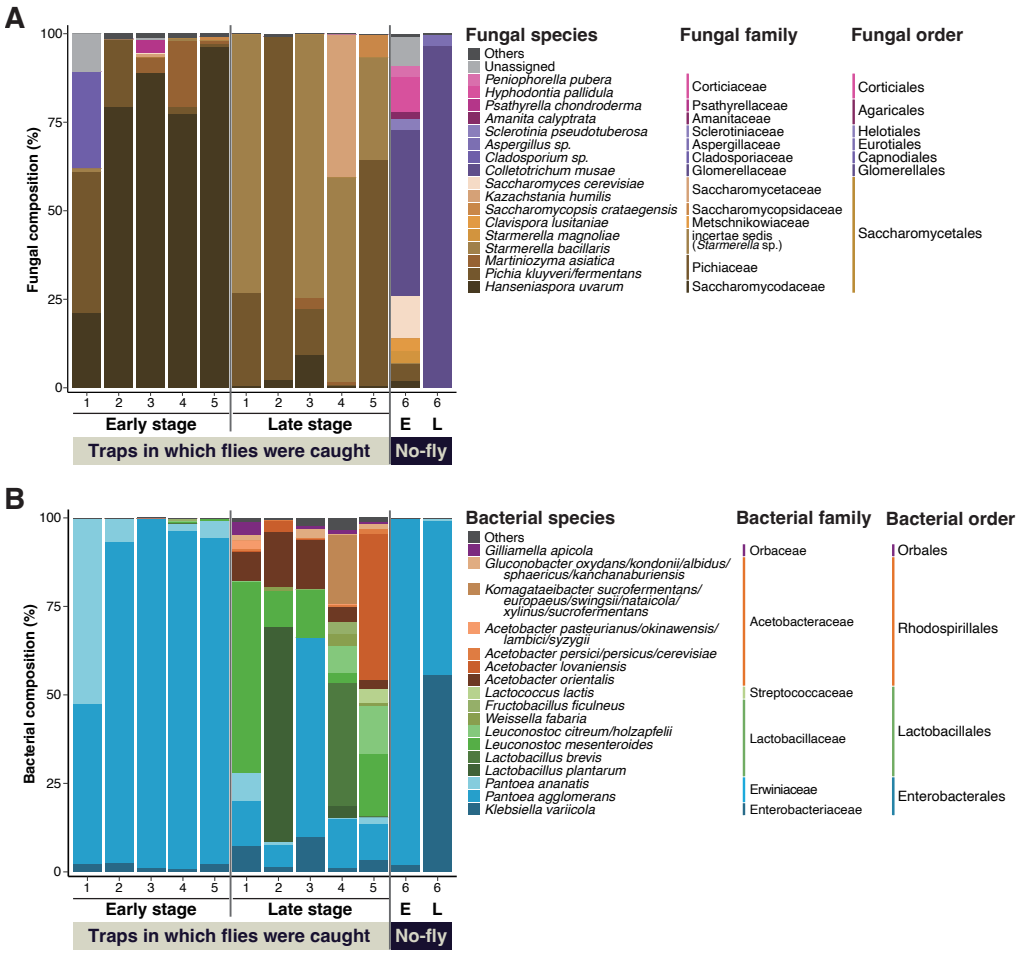

Figure S1. Fungal and bacterial species compositions in natural food sources of *Drosophila*.

(A and B) Results of microbial composition analysis corresponding to Figure 1B. The relative abundances of fungal (A) or bacterial (B) species in the fermented banana are shown. The compositions were analyzed using primer sets amplifying the fungal ITS region or bacterial 16S rDNA region. Operational taxonomic units (OTUs) accounting for >1% in any of the samples were grouped by species, as shown. The ratios of those accounting for <1% or those of unidentified species were summed and indicated as "Others." The genera belonging to uncertain families (incertae sedis) are indicated by their genus name in parentheses. The numbers on the horizontal axis represent each numbered sampling location, corresponding to those indicated in Figure 1B. "No-fly" highlights the food samples from sampling place 6, where no fly or larva was found in the trap or the foods, respectively. E, early-stage food; L, late-stage food.

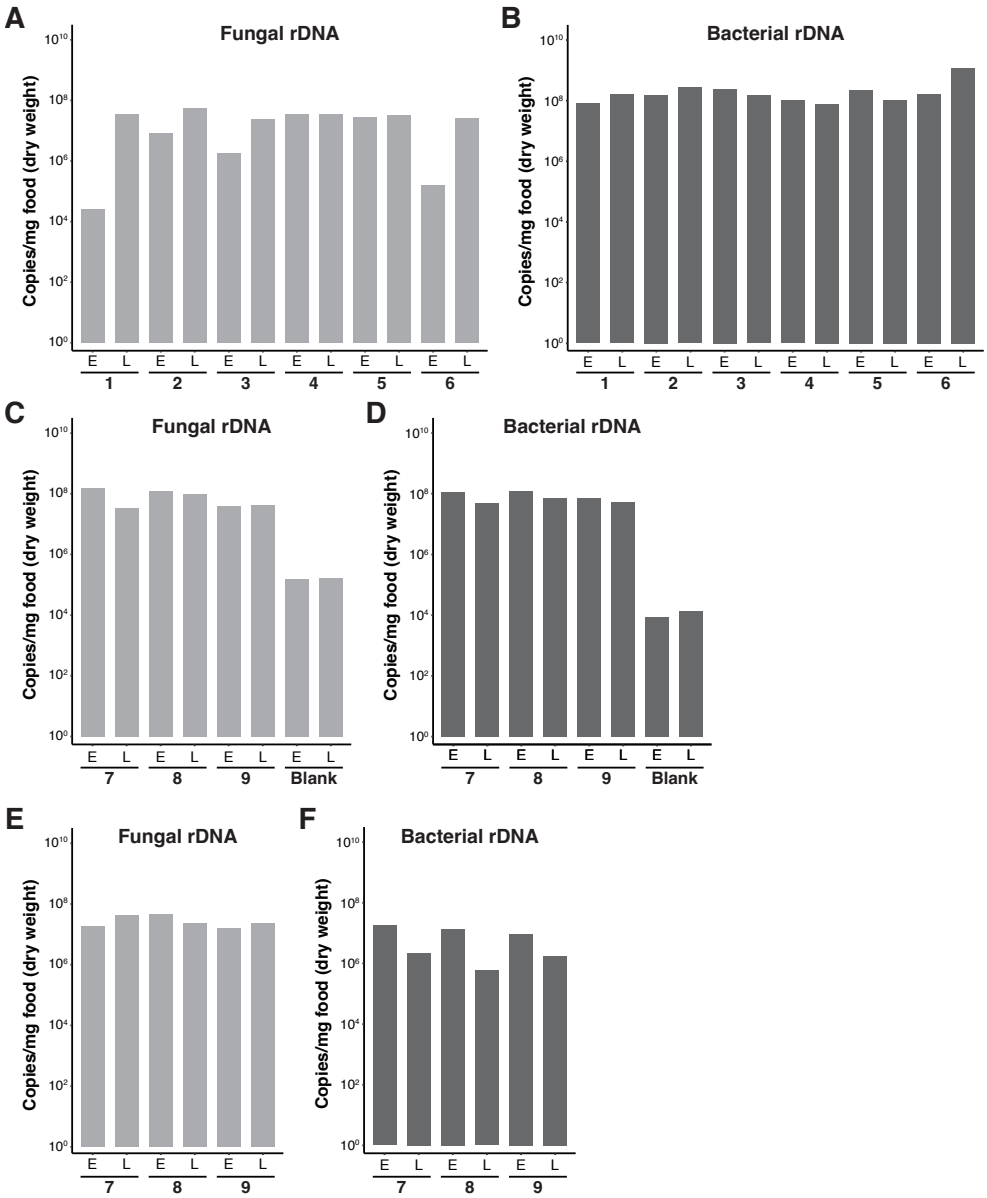

Figure S2. Copy numbers of microbial rDNA in fermented banana samples.

(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 banana 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 banana 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.

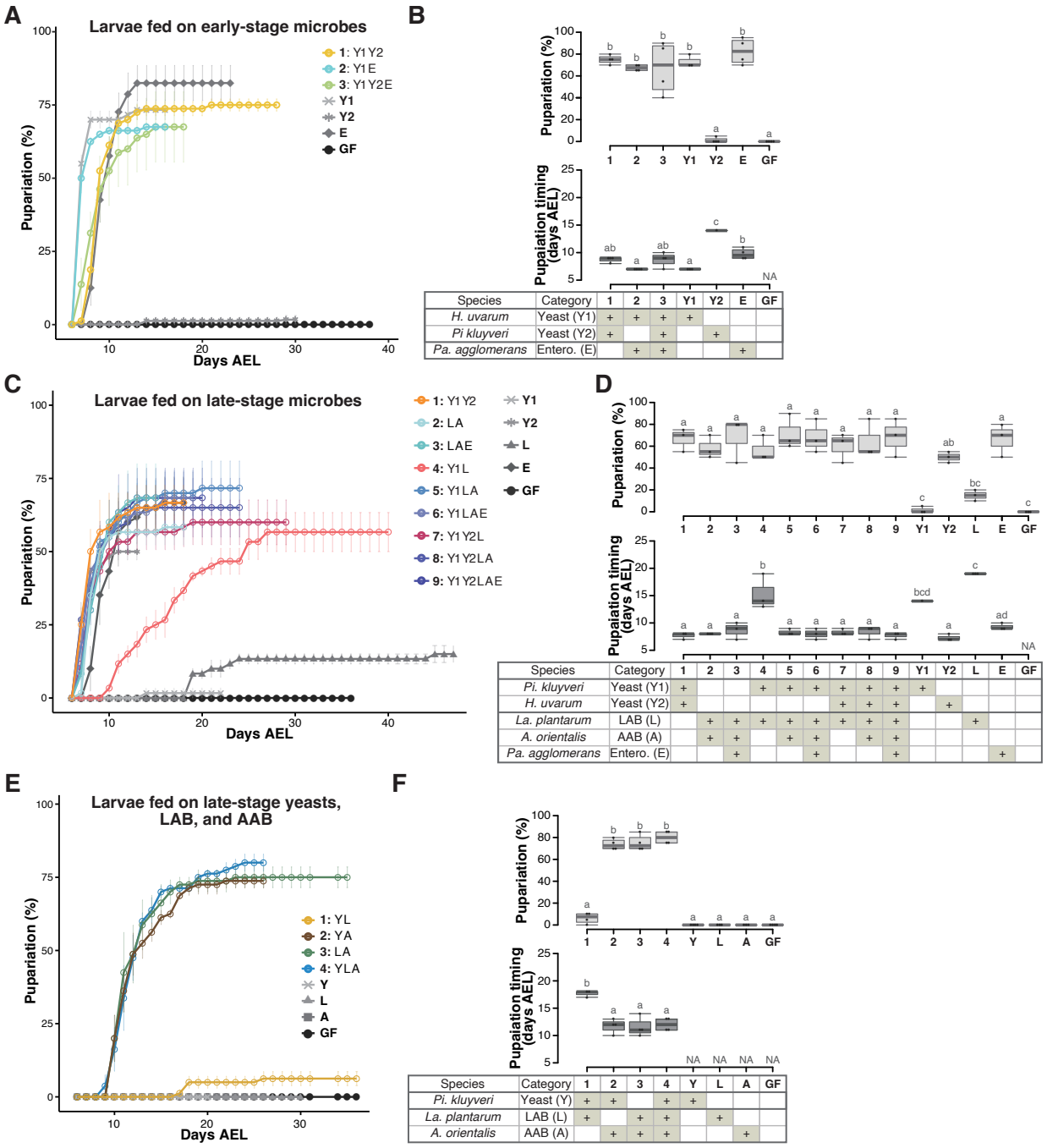

Figure S3. Larvae feeding on mixtures of isolated microbial species detected in the food samples from Sampling Place 2 in Figure 1B.

(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. *Pi. kluyveri*, *Pichia kluyveri*; *Pa. agglomerans*, *Pantoea agglomerans*; *La. plantarum*,

1311 *Lactiplantibacillus plantarum*; Entero., Enterobacterales; GF, germ-free; NA, not applicable; days
1312 AEL, days after egg laying.

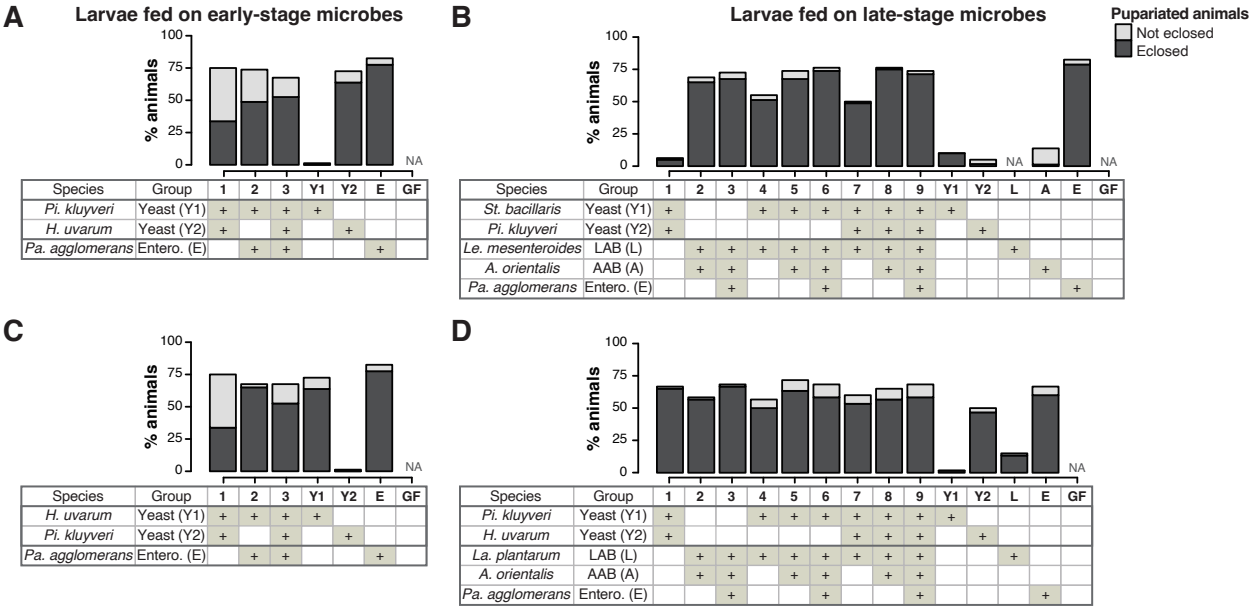

Figure S4. The percentage of pupariation and eclosion of the larvae feeding on microbes.

(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 S3A and S3B (C), or Figures S3C and S3D (D). The mean percentages of the pupariated and eclosed (dark gray) or pupariated and not eclosed (light gray) individuals are shown. n=3-4. *Pi. kluyveri*, *Pichia kluyveri*; *Pa. agglomerans*, *Pantoea agglomerans*; *St. bacillaris*, *Starmerella bacillaris*; *Le. mesenteroides*, *Leuconostoc mesenteroides*; *La. plantarum*, *Lactiplantibacillus plantarum*; Entero., Enterobacterales; GF, germ-free; NA, not applicable.

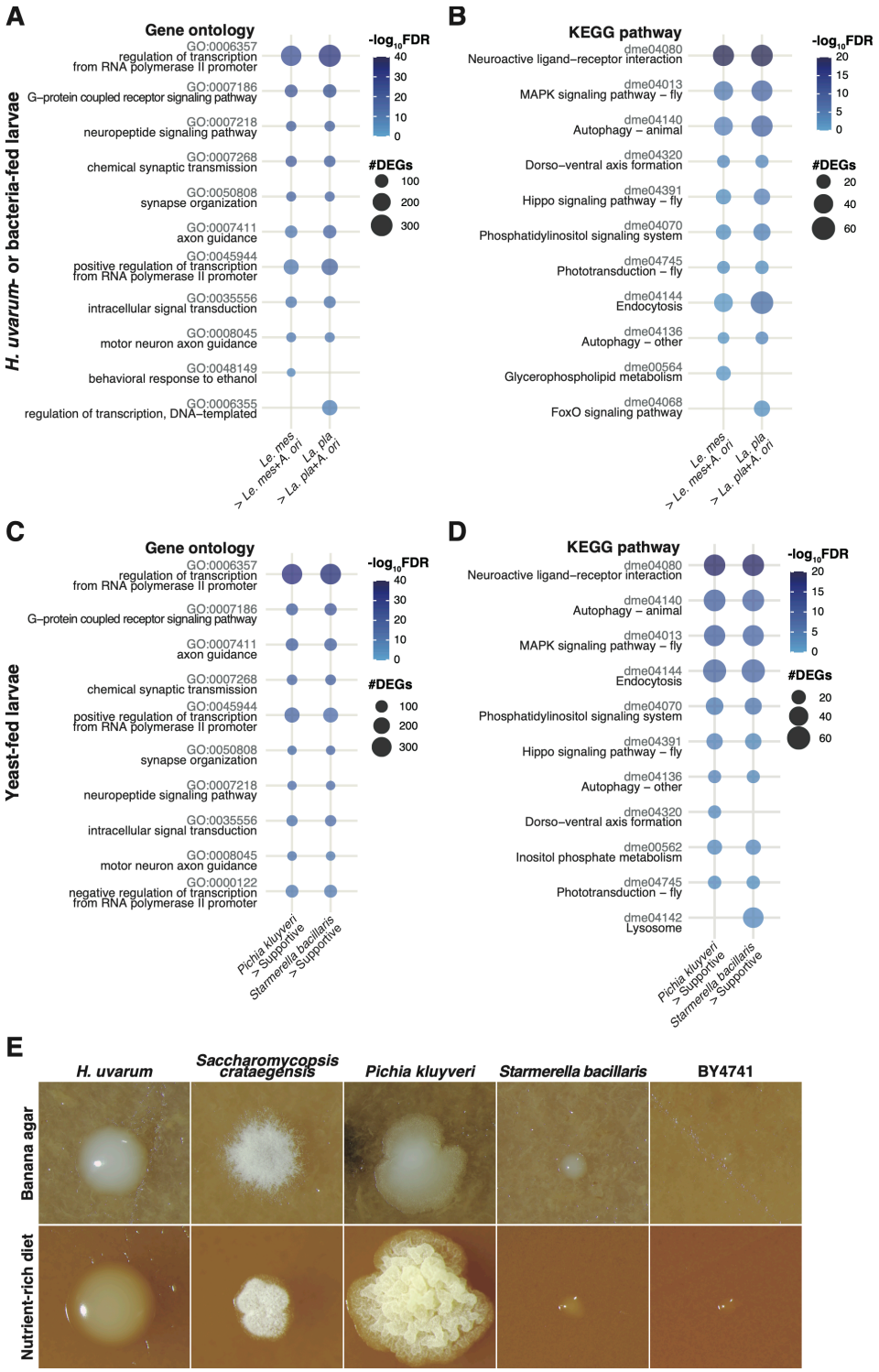

Figure S5. Enrichment analyses of the genes upregulated in non-supportive conditions and morphology of yeast colonies grown on a nutrient-rich diet.

1327 (A-D) Plots showing the result of GO term (A and C) or KEGG Pathway (B and D) enrichment
1328 analysis of the RNA-seq data shown in Figure 3A (A and B) and Figure 4C (C and D). Plots were
1329 generated as described in Figure 3. (E) Images of single yeast colonies grown on banana agar or
1330 the nutrient-rich diet. The images were taken after a 2-day incubation at 25°C. *La. pla*,
1331 *Lactiplantibacillus plantarum*; *Le. mes*, *Leuconostoc mesenteroides*; *A. ori*, *Acetobacter orientalis*.

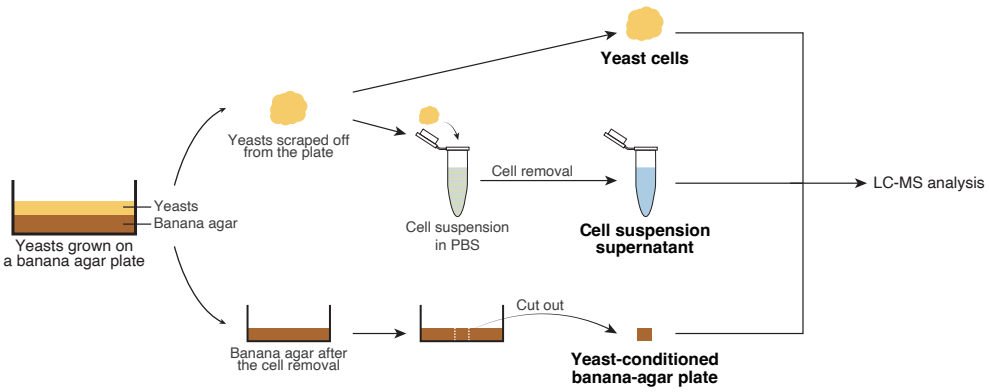

Figure S6. Sample preparations for the metabolomic analysis.

Sample preparation process for the metabolomic analysis. We suspected that the supportive live yeast cells may release critical nutrients for larval growth, whereas the non-supportive yeasts may not. To test this possibility, we made three distinct sample preparations of individual yeast strains (yeast cells, yeast-conditioned banana-agar plates, and cell suspension supernatants). Yeast cells were for the analysis of intracellular metabolites, whereas yeast-conditioned banana-agar plates and cell suspension supernatants were for that of extracellular metabolites. The samples were prepared as the following procedures. Yeasts were grown on banana-agar plates for 2 days at 25°C, and then scraped from the plates to obtain "yeast cells." Next, the remaining yeasts on the resultant plates were thoroughly removed, and a portion from each plate was cut out ("yeast-conditioned banana agar"). In addition, we suspended yeast cells from the agar plates into sterile PBS, followed by centrifugation and filtration to eliminate the yeast cells, to prepare "cell suspension supernatants."

1347 **Table**

1348 **Table 1. List of microbial species used in the feeding experiments.**

1349 Percentages indicate the relative abundance of each species in the foods shown in Figure 1B.

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

1350

1351 **Supplementary Files**

1352 Table S1. Details of the samplings of fermented bananas and wild *Drosophila*.

1353

1354 Table S2. Microbial composition analysis results.

1355

1356 Table S3. Microbial isolation and species identification.

1357

1358 Table S4. RNA-seq data of whole bodies of 1st instar larvae fed on *H. uvarum* or bacteria.

1359

1360 Table S5. RNA-seq data of whole bodies of 1st instar larvae fed on yeasts

1361

1362 Table S6. Results of metabolomic analysis of yeast samples.

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1364 Table S7. Final genome assembly and annotation statuses of six yeast strains.

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1366 Table S8. RNA-seq data of isolated yeasts.

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1368 Table S9. Statistical details of experiments.