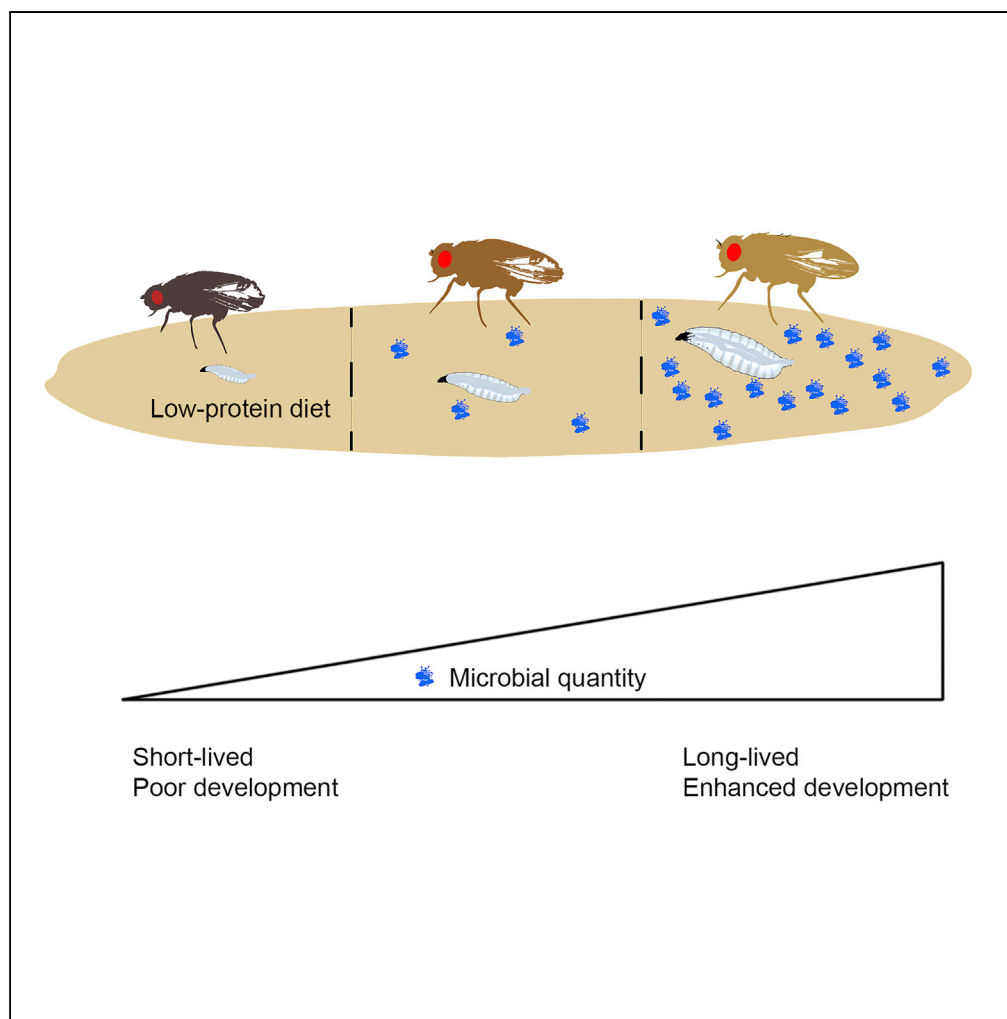**Article**

Microbial Quantity Impacts *Drosophila* Nutrition, Development, and Lifespan

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HIGHLIGHTS

Microbial association promotes fly longevity and development on low-protein diet

A wide range of microbes can serve as a source of protein during undernutrition

The extent of effects correlates with microbiota quantity and biomass

The most impactful microbial species simply thrive on fly culture medium

Article

Microbial Quantity Impacts *Drosophila* Nutrition, Development, and Lifespan

Erin S. Keebaugh,[1,2,4] Ryuichi Yamada,[1,2,4,5] Benjamin Obadia,[3] William B. Ludington,[3] and William W. Ja[1,2,6,*]

SUMMARY

In *Drosophila*, microbial association can promote development or extend life. We tested the impact of microbial association during malnutrition and show that microbial quantity is a predictor of fly longevity. Although all tested microbes, when abundantly provided, can rescue lifespan on low-protein diet, the effect of a single inoculation seems linked to the ability of that microbial strain to thrive under experimental conditions. Microbes, dead or alive, phenocopy dietary protein, and the calculated dependence on microbial protein content is similar to the protein requirements determined from fly feeding studies, suggesting that microbes enhance host protein nutrition by serving as protein-rich food. Microbes that enhance larval growth are also associated with the ability to better thrive on fly culture medium. Our results suggest an unanticipated range of microbial species that promote fly development and longevity and highlight microbial quantity as an important determinant of effects on physiology and lifespan during undernutrition.

INTRODUCTION

Host-microbe associations influence human health and disease. Altered microbiota can have a profound effect on host metabolism (Kau et al., 2011), and several studies have correlated imbalanced gut microbiota with cardiovascular disease, insulin resistance, diabetes, and aging (Claesson et al., 2012; Nicholson et al., 2012; Qin et al., 2012; Turnbaugh et al., 2009). Clearly, gut microbes are a promising target for metabolic disease intervention (Holmes et al., 2012), but further studies are needed to delineate the precise interplay between microbes and their hosts.

Studies in both humans and rodents have suggested that distinct microbial populations can act as a key signature of obesity (Jumpertz et al., 2011). Colonization of germ-free (axenic) mice with intestinal microbiota from obese donors induces a substantial increase in body weight (Turnbaugh et al., 2006). However, axenic rodents are protected against diet-induced obesity and display greater fatty acid oxidation (Bäckhed et al., 2007). It is unclear whether specific microbes or general traits such as microbial presence and number have the greatest effect on host metabolism—interestingly, microbial quantification has been useful in characterizing Crohn's disease-associated microbial changes (Vandeputte et al., 2017) and it has been suggested that microbial quantity is influential on *Drosophila* lifespan on undernutrition diets (Yamada et al., 2015). Together, these reports indicate that quantifying microbial abundance may be critical in future host-microbe studies.

Unfavorable microbial populations have also been implicated in the pathogenesis of kwashiorkor, the clinical manifestation of protein-specific malnutrition (Ahmed et al., 2009; Williams, 1963). Transfer of patient-derived microbiota into mice can induce symptoms of kwashiorkor, although only in the context of a low-protein diet (Smith et al., 2013). Although specific bacterial communities markedly impact the metabolic phenotype of the host, it remains uncertain whether such pathogenic microbial populations depend on the nutritional environment (Claus, 2013).

The interaction between host and microbe is multifaceted, involving host genetics (Spor et al., 2011), the immune system (Hooper et al., 2012), and diet (Faith et al., 2011; Hibberd et al., 2017). Identifying the functional basis of these interactions remains challenging owing to the complexity of mammalian microbial communities (Qin et al., 2010), high inter-individual variation (Turnbaugh et al., 2009), intra-individual fluctuations over time in response to disturbances such as antibiotics (Dethlefsen and Relman, 2011), and changes in diet over the course of life (Claesson et al., 2012). Defined models are beginning to reveal the impact of specific nutrients on microbial communities (Hibberd et al., 2017), yet further simplified models would greatly advance our understanding of nutrition-microbe-host interactions.

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One such model, *Drosophila*, has already provided useful insights into host-microbe relationships, including microbial pathogenicity and host immunity (Chambers and Schneider, 2012; Lazzaro, 2008). Although similarities exist between mammalian- and *Drosophila*-associated microbes, flies typically harbor a considerably simpler microbiota with ~200-fold fewer bacterial species (Broderick and Lemaitre, 2012; Chandler et al., 2011; Corby-Harris et al., 2007; Cox and Gilmore, 2007; Wong et al., 2011) that are distributed differently throughout the digestive tract (Keebaugh and Ja, 2016). Nonetheless, as in mammals, changes in the *Drosophila* diet can rapidly shift bacterial communities (Chandler et al., 2011) and the composition and quantity of gut microbes can shift with age (Claesson et al., 2011; Wong et al., 2011). These changes are associated with a decline in intestinal barrier function, the presence of microbes in non-canonical areas, and increased systemic inflammation (Clark et al., 2015; Guo et al., 2014; Li et al., 2016; Thevaranjan et al., 2017). These parallels highlight *Drosophila*, with its short lifespan and powerful genetic tools, as an ideal simplified model in which to determine the precise interplay between microbes, diet, metabolism, and lifelong health.

In nature, many host-microbe symbioses are characterized by nutritionally challenging or limited food substrates; microbes generally provide hosts with access to essential nutrients (Adams et al., 2011; Dillon and Dillon, 2004; Douglas, 2009, 2011; Leitão-Goncalves et al., 2017; Muhammad et al., 2017; Sannino et al., 2018). *Drosophila*-associated microbes promote larval development (Anagnostou et al., 2010; Bakula, 1969; Liu et al., 2017; Meshrif et al., 2016; Starmer, 1982)—an effect mimicked by dietary nutrients (Sang, 1956). More recently, host-associated microbes have been shown to modulate Tor and insulin signaling pathways and intestinal peptidase expression to promote growth and development (Erkosar et al., 2015; Shin et al., 2011; Storelli et al., 2011); similar effects were revealed in a mammalian model (Schwarzer et al., 2016). It remains unclear whether bacterial strains activate these pathways and enhance host nutrition by actively aiding host digestion/absorption or by serving directly as a source of micro- or macronutrients.

Transcriptomic, physiological, and nutritional studies indicate that microbes can alter *Drosophila* development, metabolism, and nutritional status in a manner consistent with the idea that microbes contribute to host nutrition (Broderick et al., 2014; Ridley et al., 2012; Wong et al., 2014). Larvae exposed to chronic nutritional stress for over 170 generations are less dependent on nutrition and microbial association (Erkosar et al., 2017), suggesting that microbes and nutrition might serve overlapping purposes. Recent studies also show that microbes are a source of protein for *Drosophila suzukii*, which consume fresh fruit (Bing et al., 2018), and that the abundance of *Issatchenkia orientalis* rescues *D. melanogaster* lifespan during undernutrition (Yamada et al., 2015). How other fly-associated microbes might contribute to nutrition is unclear. Here, we examine how individual microbial species influence *Drosophila* lifespan and larval development. Association with a single microbial species can significantly extend malnourished fly lifespan and promote larval development. Our results suggest that microbes act primarily as a critical source of protein regardless of the species to rescue metabolic health during malnutrition. Microbial abundance in the fly environment seems strongly tied to the impact on fly phenotypes—thus, it is the ability of a particular microbial species to thrive under experimental conditions that best predicts its impact on the fly host. This study reinforces the importance of considering both microbial quantity and quality in microbiota studies of aging and development and underscores the fly as a valuable model for understanding explicit nutrition-microbe-host relationships.

RESULTS

Microbes Extend Fly Lifespan on Undernutrition Diet

Previously, fly lifespan on a low-yeast, undernutrition diet was shown to be strongly influenced by *I. orientalis* compared with other tested microbes (Yamada et al., 2015). This effect is conserved in two commonly studied fly strains regardless of whether axenic animals were inoculated with microbes during development or adulthood (Figures 1 and S1). Flies maintained axenically until day 8 of adulthood and subsequently inoculated with *I. orientalis* were also long-lived (Figure 1B), excluding the first week of adult life as a critical period for microbial association in our paradigm (Brummel et al., 2004). Consistent with previous studies (Yamada et al., 2015), *Saccharomyces cerevisiae, Acetobacter indonesiensis,* and *Lactobacillus plantarum* influenced lifespan less than *I. orientalis* (Figures 1 and S1). Results from embryo or adult association of microbes were generally consistent (Figure 1), demonstrating that microbial association during adulthood is sufficient for extending fly life on a low-nutrient diet.
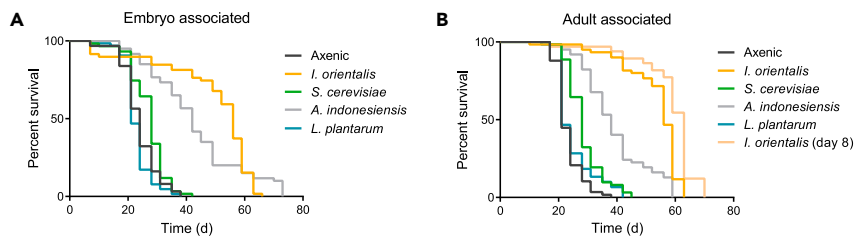
Figure 1. Association with Microbes Modulates Adult Lifespan on Undernutrition Diet

(A) Survival of axenic or monoxenic Canton-S male flies on 0.1% yeast extract (YE) malnutrition diet (axenic versus *L. plantarum*, p = 0.75, all other comparisons to axenic, p ≤ 0.03, log rank test). Axenic flies were associated as embryos with specific microbes. N = 59–64 flies.

(B) Survival of axenic or monoxenic Canton-S male flies on 0.1% YE diet (axenic versus *L. plantarum*, p = 0.28, all other comparisons to axenic, p ≤ 1.0×10^{-6}, log rank test). Axenic flies were associated with microbes 1 day after eclosion, except for the indicated trial with *I. orientalis*, which was inoculated 8 days after eclosion. N = 58–66 flies.

See also Figure S1.

Microbial Quantity and Biomass Correlate Positively with Fly Lifespan

What are the differences between microbial species that drive lifespan extension? We anecdotally observed that *I. orientalis* and *A. indonesiensis* grow better on fly medium than other microbes. Given our previous studies suggesting that *I. orientalis* serves as a protein-rich food source for flies (Yamada et al., 2015), we hypothesized that a broad range of microbes might have the potential to extend life, but that only certain species proliferate well enough on fly media to maintain sufficient numbers to maximize fly longevity. Thus, microbial abundance available in the environment, rather than the transient gut microbiota (Blum et al., 2013), would be the key metric for predicting the impact on fly phenotypes. We quantified environmental microbes in spent vials and found that fly lifespan extension resulting from a single inoculation with live microbes shows a strong correlation with exogenous microbe counts (Figure 2A). The correlation is strengthened when accounting for the larger mass of yeasts compared with bacteria (Figure 2B).

If microbes serve as a direct source of nutrition for flies, then lifelong association with microbes is likely needed for their impact on fly lifespan. To test this hypothesis, we manipulated microbe growth with methylparaben (methyl 4-hydroxybenzoate, also known as Tegosept or Nipagin M), a common food preservative (Ashburner et al., 2005; Obadia et al., 2018). Methylparaben eliminated *I. orientalis*, *A. indonesiensis*, and *S. cerevisiae* in the fly environment and also abolished lifespan extension imparted by these microbes compared with our standard methylparaben-free diet (Figures 2C and 2D). Interestingly, *L. plantarum* had a small, positive effect on lifespan regardless of whether the food contained methylparaben—paralleling the lack of an effect of methylparaben on *L. plantarum* growth (Figures 2C, 2D, and S2).

The number of microbes present in the fly environment results from multiple factors including microbial growth rate on the food surface, the presence of larvae, ingestion by flies, and spreading through fly excretion and external contact. These factors are likely further complicated by parameters such as the number of flies, enclosure size, and food surface area. Importantly, given that animals are provided with fresh, sterile food in an axenic environment every few days, microbial presence throughout life must be dependent on their passage using the fly as a vector. Since the transfer of flies to fresh food dramatically reduces the immediate microbial load (Blum et al., 2013), we next asked whether shorter intervals between food changes throughout life would affect microbe numbers and therefore attenuate the effects of microbial association on longevity. Axenic adult flies were inoculated once with microbial cultures, and fresh food was provided daily or every 3–4 days. The food change interval had no effect on axenic fly lifespan (Figure 3A). Association with *I. orientalis*, and to some extent *A. indonesiensis*, extended life when food was changed every 3–4 days, but this effect was severely diminished when transfers occurred daily (Figure 3A). These results correspond with the number of microbes found associated with flies and the spent vials. The abundance of *I. orientalis* and *A. indonesiensis* was largely maintained throughout life with the 3-4–day food change interval (Figure S3). Compared with counts from 3-4–day transfers, daily transfers generally diminished microbe numbers with the exception of *A. indonesiensis* and *L. plantarum* in fly samples (Figures 3B and S3). Our cumulative results demonstrate that continued microbial presence is required to impact fly lifespan on a low-yeast, malnutrition diet and that microbial persistence is maintained by flies
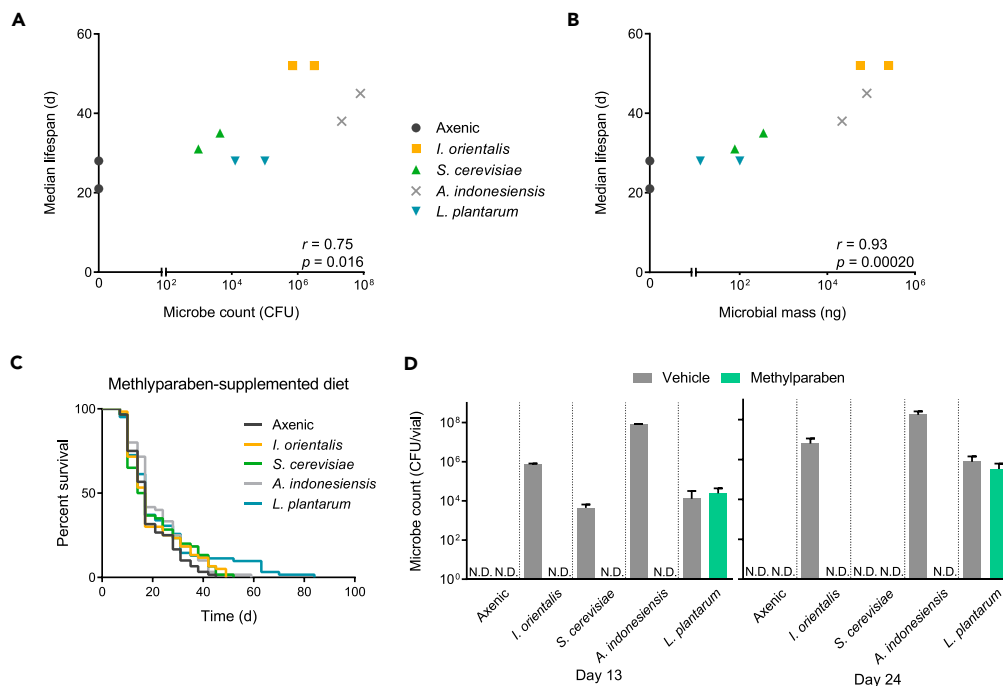
Figure 2. Microbial Growth in the Fly Environment Is Positively Correlated with Extended Life

(A) Microbe numbers in the fly environment correlate with median lifespan on malnutrition diet (r = 0.75, p = 0.016, Spearman's correlation coefficient). Average microbe counts from fly enclosures (N = 3), including the food surface, collected on day 8 or 13 from two independent survival studies with adult-associated live microbes.

(B) Microbial mass calculated from microbe counts (80 pg/cell for *I. orientalis* and *S. cerevisiae*, 1 pg/cell for *A. indonesiensis* and *L. plantarum*) correlates with median lifespan (r = 0.93, p = 0.00020, Spearman's correlation coefficient).

(C) Methylparaben abolishes the benefit of microbes on lifespan. None of the survival curves differ significantly from that of axenic flies (p > 0.05 for each comparison, log rank test). Median lifespan of microbe-associated flies also does not differ from that of axenic animals (p > 0.05 for each comparison, Fisher's exact test). N = 60–62 flies.

(D) Methylparaben eliminates *I. orientalis*, *A. indonesiensis*, and *S. cerevisiae* in the fly environment. Shown are average microbe counts (+ SD) from spent fly vials (N = 3) collected on days 13 and 24 (3–4 days after food change). N.D., not detected (in all replicates). Flies were maintained on 0.1% YE malnutrition diet with either our standard acid-based preservative or 0.3% methylparaben.

See also Figure S2.

inoculating their environment upon each passage to fresh food. The full benefit to fly lifespan is only conferred by microbes that are given sufficient time to grow on the new medium.

Heat-Killed Microbes Have a Dose-Dependent Effect on Fly Longevity

To more directly test the idea that microbial quantity broadly modulates fly lifespan, we assessed whether heat-killed microbes provided either one time in early adulthood or at every food change would promote longevity. A single and relatively low dose of heat-killed *I. orientalis* did not alter lifespan compared with axenic flies (Figure 4A). In contrast, a recurring supply of heat-killed *I. orientalis* extended life, although not to the extent of that from a single inoculation with live microbes (Figure 4A). Increasing the quantity of heat-killed *I. orientalis* given to flies throughout life revealed a dose-dependent effect on longevity that can match or exceed the lifespan extension observed with live microbe inoculation (Figure 4B). Surprisingly, providing an optimal dose of heat-killed *S. cerevisiae*, *A. indonesiensis*, or *L. plantarum* can also extend life beyond the effect of a single live inoculation. These results support the idea that microbes provide a broad benefit to flies and show that live, metabolically active microbes are not necessary for promoting fly longevity.

What common factor from various microbial species might be mediating the effects on fly lifespan? We previously suggested that *I. orientalis* serves broadly as a protein-rich food source for flies (Yamada et al.,
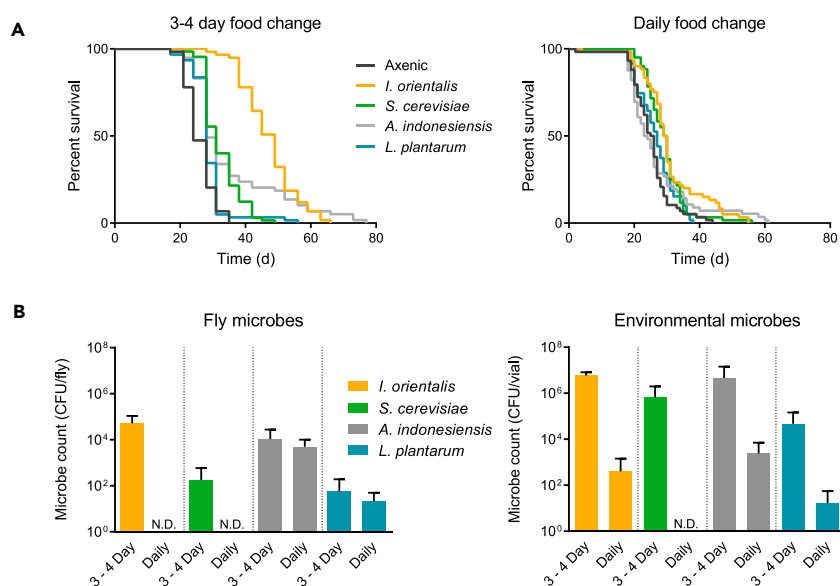
Figure 3. Food Change Interval Affects Lifespan and Microbial Abundance

(A) Survival of axenic or adult-associated, monoxenic flies on 0.1% YE malnutrition diet with fresh food provided every 3–4 days (left) or daily (right) (axenic versus microbes, 3-4–day transfers: p ≤ 0.01 for each comparison; daily transfers: p > 0.05 for axenic versus *A. indoensiensis* or *L. plantarum*, p ≤ 0.0029 for axenic versus *I. orientalis* or *S. cerevisiae*, log rank test). N = 56–65 flies.

(B) Average microbial counts (+SD) in adult flies (left) or spent vials (right) when flies were transferred to fresh vials every 3–4 days or daily (averaged over CFU counts from days 4, 8, 12, and 16 from the study shown in A). For each day sampled, CFU/fly is calculated from N = 6 flies each and CFU/vial was calculated from N = 3 enclosures each. N.D., not detected (in all replicates). CFU, colony-forming unit.

See also Figure S3.

2015). Our heat-killed microbial studies show that many species are likely sufficient to serve as this food source, and that it is the ability of that species to grow or thrive on fly media that directly contributes to its ability to promote fly longevity. Numerous studies have emphasized the importance of protein as a determinant in modulating *Drosophila* lifespan (Bruce et al., 2013; Grandison et al., 2009b; Ja et al., 2009; Skorupa et al., 2008), and yeast (or yeast extract [YE]) is the primary source of protein in most laboratory fly diets. Interestingly, if we estimate the microbial protein content for all the heat-killed microbe doses used, maximum lifespan extension is achieved when flies are provided with sufficient microbial protein (4–20 μg/fly/day) to rescue nutrient-deficient conditions (Figure 4C). These values are consistent with the lifespan differences observed between flies on 0.1% and 0.5% YE diets (Figure 4D), where food consumption measurements reveal a difference in protein intake of ~3 μg/fly/day (Bruce et al., 2013). Further increases in the dose of heat-killed microbes leads to shortened fly lifespan, reminiscent of the effect of high YE concentration diets (Figures 4C and 4D). These results are in strong agreement with previous studies demonstrating that longevity is maximized at a relatively low protein:carbohydrate ratio, and altering the ratio to cause protein deficiency or excess shortens life (Bruce et al., 2013; Galenza et al., 2016; Grandison et al., 2009a; Lee, 2015; Lee et al., 2008; Min and Tatar, 2006).

Microbes Can Serve as a Replacement for Dietary Protein

To further define the essential nutrients provided by microbes, we examined the effect of microbes on various minimal diets. We supplemented a simple agar medium lacking any nutritional value with either microbes or protein-rich ingredients. Live or heat-killed microbes were provided with every food change, ensuring an *ad libitum* supply of the additive regardless of microbial growth. Tryptone (digested casein) was used as a pure protein source. On the agar-only base medium, tryptone and YE at the highest concentration tested (0.75%) extended life, although median lifespan was still short (~10 days). Live and heat-killed *I. orientalis* and *S. cerevisiae* mimicked the effect of tryptone and YE (Figure 5A and Table 1). These results suggest that microbes or equivalent bulk macronutrients cannot supply sufficient nutrition to act as the sole, optimal food source for fly survival.
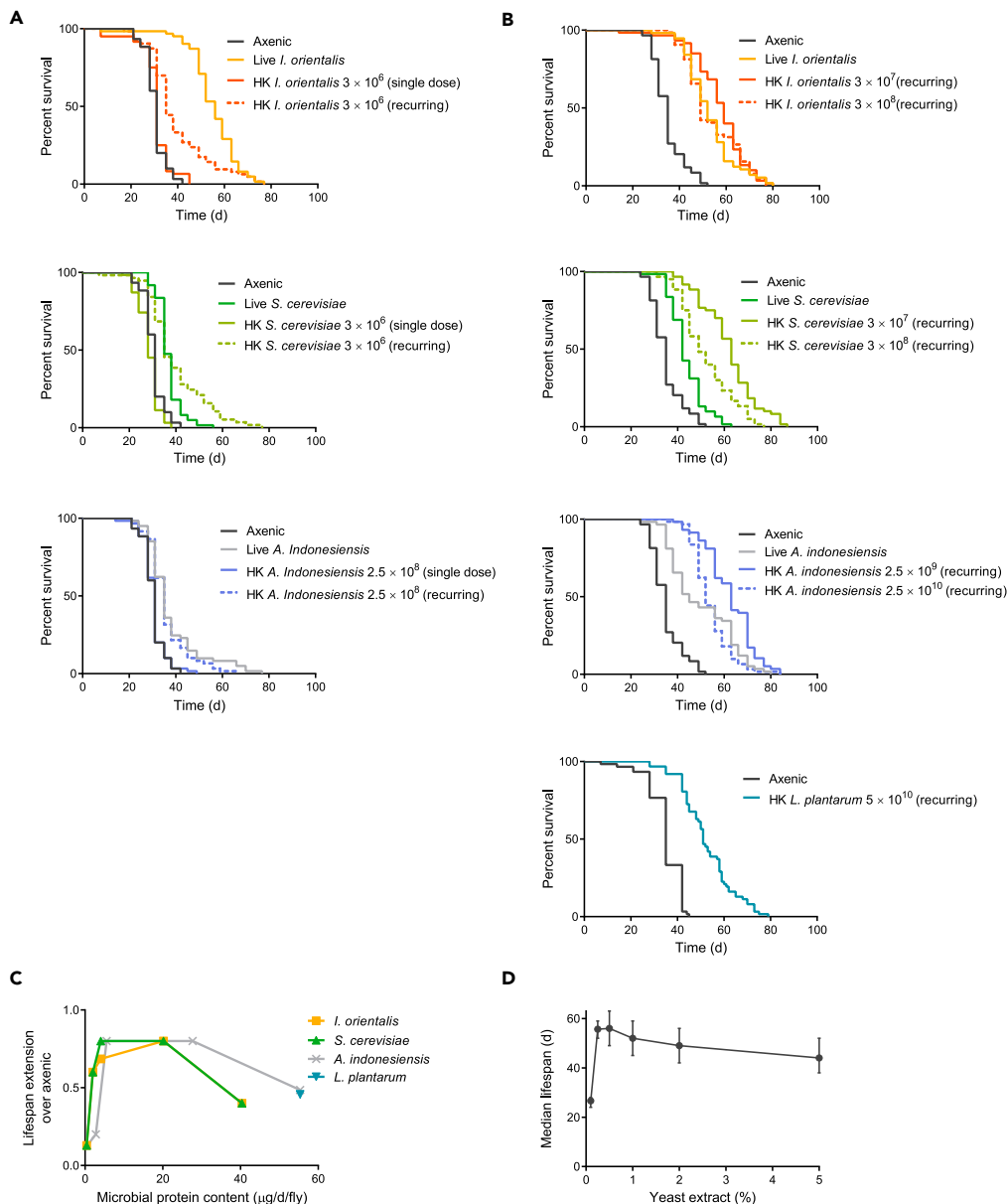
Figure 4. Microbes Extend Fly Lifespan in a Dose-Dependent Manner

(A) Survival of axenic or microbe-associated flies on 0.1% YE malnutrition diet. For microbe-associated conditions, 5 × 10^5 CFU of live *I. orientalis* or *S. cerevisiae* or 5 × 10^7 CFU of live *A. indonesiensis* was supplied one time in early adulthood, or the indicated quantity of autoclaved (heat-killed [HK]) microbes were provided either one time (single dose) or at every food change (twice/week) throughout life (recurring). A single dose of HK microbes resulted in no change in survival ($p > 0.05$, log rank test). Both live and recurring doses of HK microbes resulted in significant lifespan extension compared with the axenic control ($p \leq 4.3 \times 10^{-6}$ for each comparison, log rank test). N = 57–63 flies.

(B) HK microbes supplied throughout life can extend lifespan on a malnutrition diet (0.1% YE) (axenic versus live or HK microbes; $p \leq 1.5 \times 10^{-8}$, log rank test). Axenic flies were associated once with live microbes or continuously with HK microbes throughout life. The indicated CFU of HK microbes was supplied at every food change. N = 57–64 flies.

(C) Relationship between lifespan extension and the estimated protein content of different doses of HK microbes. Lifespan extension is calculated as the change in median lifespan between flies receiving lifelong HK microbe supplementation and axenic controls. Estimated protein content from each dose of HK microbes is divided by the initial number of flies per enclosure and number of days between transfers to fresh food to show the protein content available per fly per day. N = 57–64 flies.

(D) Effect of yeast extract (YE) concentration on lifespan. As YE is reduced from the highest concentration tested (5%), dietary restriction results in lifespan extension. From the point of maximal longevity (0.25–0.5% YE), further reduction in YE

Figure 4. *Continued*

results in malnutrition and drastically reduced lifespan. Median lifespan is plotted with whiskers representing the interquartile range (first and third quartiles). Diets were composed of the indicated YE (%, w/v) in a base medium containing 5% sucrose and 8.6% cornmeal (all w/v). N = 64–158 axenic flies. CFU, colony-forming unit.

Next, we added only sucrose to the pure agar diet. On 5% sucrose medium (0YE/5S, containing 0% YE and 5% sucrose), YE supplementation greatly extended lifespan, whereas tryptone only partially rescued longevity (Figure 5B and Table 1). These results are consistent with the idea that on a pure sucrose diet, both additional protein and other nutrients—likely micronutrients such as vitamins and minerals—are required for optimal health. This aligns with previous findings in Queensland flies that show that the major contribution of yeast to a diet is protein, except in very low yeast diets, wherein the micronutrient contribution becomes important (Fanson and Taylor, 2012). Interestingly, live or heat-killed *I. orientalis*, *S. cerevisiae,* and *L. plantarum* are capable of mimicking the effects of YE, whereas the effect of *A. indonesiensis* was more similar to that of tryptone supplementation (Figure 5B and Table 1).

When a marginal level of YE is added to the base diet (0.1YE/5S), supplementation of tryptone or YE extended fly lifespan similarly (Figure 5C and Table 1). These results suggest that, on a 0.1YE/5S diet, the main contribution of YE is protein. All microbes tested—dead or alive—also fully rescue longevity (Figure 5C and Table 1). Given that microbes can mimic tryptone or YE, our findings are consistent with the idea that microbes extend fly lifespan on malnutrition diet by directly contributing to protein nutrition.

Microbes Promote Larval Growth on Malnutrition Diet

Association with certain strains of *L. plantarum* and *Acetobacter pomorum* was previously shown to enhance larval growth on low-yeast or low-protein diets (Shin et al., 2011; Storelli et al., 2011), mimicking the effect of increasing dietary yeast concentration to shorten time to pupariation (Anagnostou et al., 2010). We hypothesized that enhanced larval growth on an undernutrition diet might be associated with higher microbial quantity of the growth-promoting strains of *Lactobacillus* and *Acetobacter* over non–growth-promoting controls. Our experimental 0.1% YE diet lacks sufficient nutrition for axenic larval development, so we increased the YE to 0.5%. Axenic larvae do not reach the pupal stage on this diet, but tryptone and YE supplementation can rescue pupariation (Figure S4), suggesting that this diet is undernutritious but suitable for determining if microbes can enhance larval development.

We next monocolonized embryos with *Lactobacillus plantarum*[WJL] or *A. pomorum*, which promote larval growth over two conspecific control strains, *Lactobacillus plantarum*[endoref] or *Acetobacter pomorum*[PQQ-ADH], respectively (Shin et al., 2011; Storelli et al., 2011). Similar to previous reports (Shin et al., 2011; Storelli et al., 2011), microbes generally promoted growth over the axenic state, and the two growth-promoting strains produced larger larvae compared with their respective controls after 6 days of association on the 0.5% YE diet (Figure 6A). There was a significant correlation between larval size and microbial counts washed from food surfaces (Figure 6B). Microbial counts on spent larval diets varied from larval-free conditions and also across diets, suggesting that a multitude of context-dependent factors can influence microbial growth (Figure S5)—this interpretation is consistent with recent studies showing that larval presence helps sustain *L. plantarum*[WJL] abundance in the environment (Storelli et al., 2018). Overall, microbial effects on monoxenic larval development are consistent with previous reports on *Lactobacillus* (Storelli et al., 2011) and *Acetobacter* (Shin et al., 2011), and the effects of microbial association on larval development mirror the apparent nutritional benefits in adult flies. These results suggest that the growth of larvae on a protein-poor diet is influenced by the abundance of associated bacteria.

DISCUSSION

As with mammals, insects are associated with important microbial symbionts (Douglas, 2011; Vega and Dowd, 2005) and can form stable intestinal symbioses with microbes (Inamine et al., 2018; Obadia et al., 2017). *Drosophila melanogaster* has an important relationship with microbes—flies consume microbes in rotting vegetation and microbes benefit from fly transmission to new environments. The capacity for *Drosophila* to vector microbes has been demonstrated in the wild (Ganter, 1988; Gilbert, 1980; Morais et al., 1994) and detailed in the laboratory (Stamps et al., 2012). Larvae and adult flies influence the density and composition of yeast growing on the nutritional substrate (Stamps et al., 2012) to the extent that microbial communities within the fly are similar to those in the nutritional environment (Tefit et al., 2018; Wong et al., 2015). Microbes support larval development on some minimal substrates (Anagnostou et al., 2010;
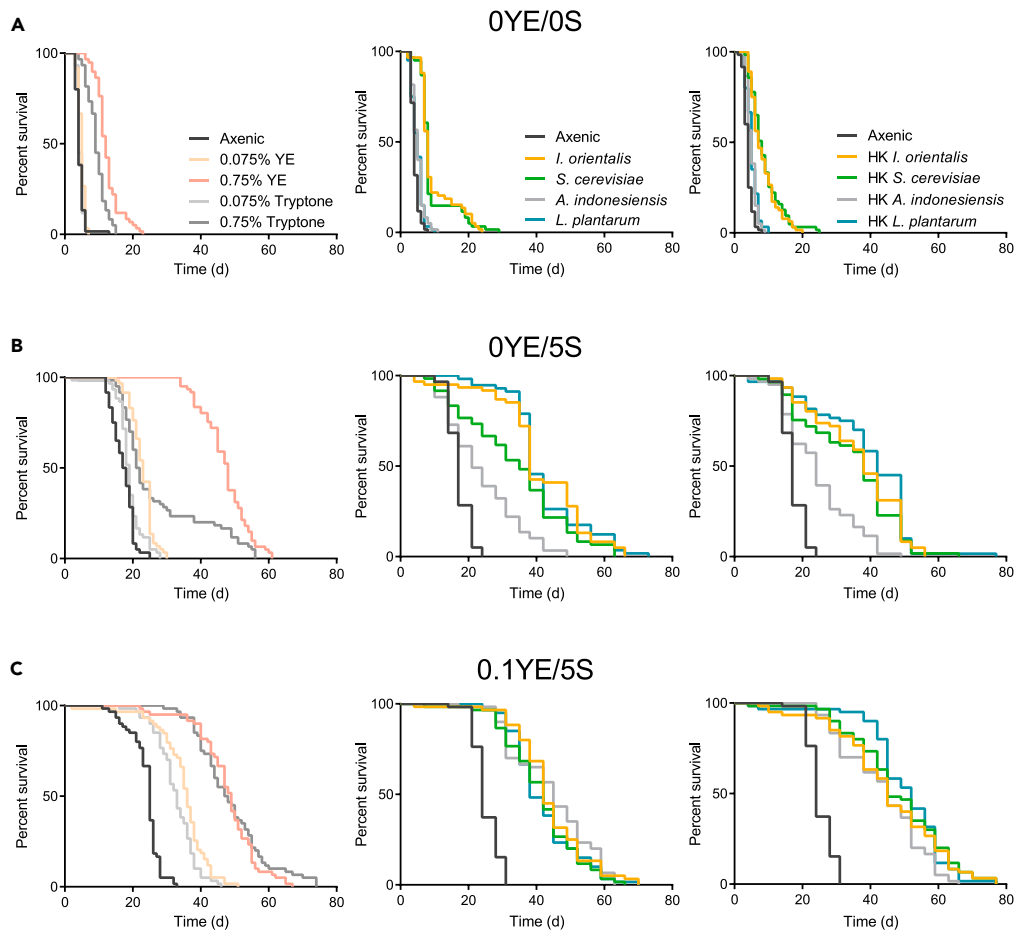
Figure 5. Microbes Provide Specific Nutritional Benefits under Different Malnutrition Diets

(A) On a starvation diet (0YE/0S = 0% yeast extract +0% sucrose), lifelong supplementation of live or autoclaved (heat-killed [HK]) microbes, YE, or tryptone has limited impact on longevity (left: axenic versus 0.075% tryptone, p > 0.05, all other comparisons, p ≤ 0.033; middle: all comparisons to axenic, p ≤ 0.015; right: all comparisons to axenic, p ≤ 0.0002, log rank test). At every food change, live or HK microbes (3×10^8 CFU/vial of *I. orientalis* or *S. cerevisiae* or 2.5×10^{10} CFU/vial of *A. indonesiensis* or *L. plantarum*), tryptone (equivalent to 0.075% or 0.75%, w/v, final), or YE (0.075% or 0.75%) were supplied. N = 59–64 flies.

(B) On a sucrose-only diet (0YE/5S), only lifelong supplementation of YE or certain microbes extends lifespan maximally (left: all comparisons to axenic, p ≤ 0.037; middle: all comparisons to axenic, p ≤ 5.0×10^{-6}; right: all comparisons to axenic, p ≤ 5.4×10^{-8}, log rank test). N = 56–66 flies.

(C) On a low yeast medium (0.1YE/5S), all additives supplied throughout life (tryptone, YE, or live or HK microbes) strongly extend lifespan (left: all comparisons, p < 1.0×10^{-10}; middle: all comparisons, p < 1.0×10^{-10}; right: all comparisons, p < 1.0×10^{-10}, log rank test). N = 59–60 flies. All flies used were initially axenic. CFU, colony-forming unit.

Becher et al., 2012; Liu et al., 2017; Meshrif et al., 2016; Starmer, 1982), indicating that the quality of the nutritional environment itself is an integral factor in fly-microbe symbioses (Shin et al., 2011; Storelli et al., 2011; Tefit et al., 2018; Yamada et al., 2015). The interplay between diet, microbe, and host translates into vertebrates as well (Dalby et al., 2017). Recent studies of kwashiorkor—protein-specific malnutrition coupled with dysbiosis—have demonstrated that the interaction between diet and gut microbiota should be considered when designing interventions for maintaining long-term health following malnutrition (Smith et al., 2013).

Host microbiota can affect nutrition by aiding the digestion of food or altering assimilation of nutrients (Bäckhed et al., 2005). Microbial metabolism can provide essential nutrients such as vitamins and lipids, increase the bioavailability of dietary components, help process nutritional substrates (Adams et al., 2011; Dillon and Dillon, 2004; Douglas, 2009; Kesnerova et al., 2017; Muhammad et al., 2017; Suh et al., 2003), and promote *D. melanogaster* nutrition by modifying the culture medium (Huang and Douglas, 2015;

YE/S	Base Diet	YE	Tryptone	*I. orientalis*	*S. cerevisiae*	*A. indonesiensis*	*L. plantarum*
0/0	●	+	+	+	+	●	●
0/5	++	+++	++	+++	+++	++	+++
0.1/5	++	+++	+++	+++	+++	+++	+++

Table 1. The Impact of Yeast Extract (YE), Tryptone, and Microbes on Lifespan

The effect of recurring supplementation with the indicated additive is categorized using median lifespan values from Figure 5. Categories were defined as: ●, 0–7.5 days; +, 7.5–15 days; ++, 15–30 days; +++, >30 days.

Wong et al., 2014). Interestingly, some changes in host nutritional indices that are attributed to microbe-mediated dietary modifications can also be achieved by placing axenic flies on diets with higher protein content (Yamada et al., 2015), suggesting that microbes passively mimic a protein-rich diet for axenic flies. In fact, microbes can serve as a direct source of protein for some detritivorous animals (Steffan et al., 2017). Here, we show that in *D. melanogaster*, multiple bacterial and yeast species phenocopy the effect of tryptone or YE supplementation during malnutrition to restore lifespan and development. Our results are consistent with the idea that microbes serve directly as a protein-rich food source during undernutrition—thereby modifying the effective protein:carbohydrate ratio of the culture medium—to affect host physiology. The impact of microbes is observed even with a single, early-life inoculation, thereby linking the effects of specific microbial strains to their ability to thrive under experimental conditions associated with flies. The fly gut microbiota is generally transient under laboratory conditions (Blum et al., 2013), and the number of live microbes in the fly gut is likely reflective of the quantity in the fly environment—thus, it is a sufficient supply of environmental microbes that seems to be the critical determinant for fly lifespan during malnutrition. Although we also associate microbial abundance—and therefore increased nutrient availability—with enhanced larval growth, further studies are needed to quantify the nutritional contribution to larval development.

Protein metabolism is a key mechanism underlying microbial benefits on development and lifespan on low-protein diets, although additional nutrients or factors may contribute in other conditions. For instance, *A. indonesiensis* and tryptone showed limited effects on a carbohydrate-only diet, suggesting that *A. indonesiensis* and tryptone do not contribute micronutrients that YE, *I. orientalis*, *S. cerevisiae,* and *L. plantarum* provide. Interestingly, previous studies examining bacterial contributions to fly growth and development have implicated the roles of Tor and insulin signaling in the context of low-nutrient diets (Shin et al., 2011; Storelli et al., 2011). Although similar research identified an acid modification on bacterial cell walls that is important in *L. plantarum*-induced larval growth on low-protein diet (Matos et al., 2017), the involvement of key nutrient-sensing pathways and the requirement of low-nutrient conditions for bacteria-derived phenotypes is consistent with the hypothesis that microbes act as a critical macronutrient source to influence fundamental metabolic pathways during malnourished conditions. Microbial supplementation on more nutritious diets, however, can be neutral or detrimental (Brummel et al., 2004; Clark et al., 2015; Li et al., 2016; Yamada et al., 2015). Thus, it is under conditions in which dietary nutrients are scarce that microbial quantity may be more critical than microbial quality; indeed, we find that growth-promoting bacterial strains do grow better or are sustained at higher levels on an undernutrition diet.

More recent studies show that heat-killed *L. plantarum*[WJL] can also promote larval growth, but only in repeated and high doses—maximal growth is achieved with a constant supply of live microbes (Storelli et al., 2018). Further measurements of microbial growth kinetics in the fly environment or the use of *ad libitum* heat-killed microbe supplementation may help fully describe microbial influences in future research (i.e., to identify a direct nutritional contribution of microbes). Studies detailing microbial cell size or physiological characteristics across different nutritional environments may also reveal how these parameters affect the nutritional value of microbes (Bren et al., 2013; Sezonov et al., 2007; Storelli et al., 2018). It may be interesting to determine if microbial quantity impacts fly behavior, physiology, or longevity in nature, particularly under multi-microbial experimental conditions.

Although yeast is a common food source for wild and laboratory flies, the nutritional contribution of yeast and bacteria in the context of microbiota research seems largely ignored. It should be important to decouple the effects of microbivory or nutrition from specialized host-microbe interactions in future studies, especially in organisms that consume microbes for sustenance (Virk et al., 2016). Non-specific
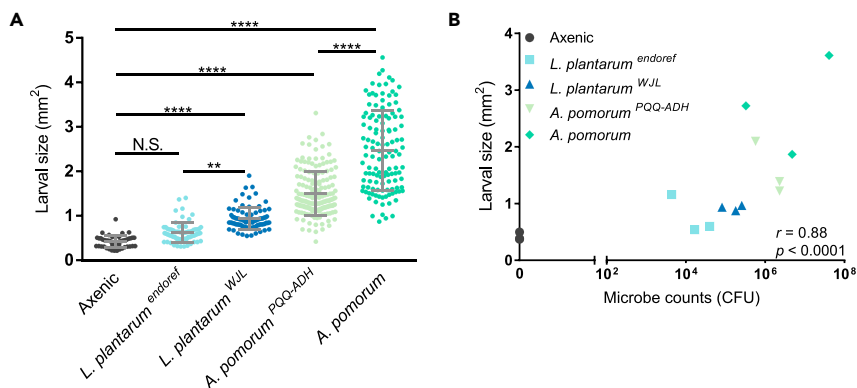
Figure 6. Microbial Quantity Is Associated with Enhanced Larval Development

(A) Larval size (mm^2) on 0.5% YE diet 6 days after axenic treatment or monoxenic exposure to approximately 5.4×10^6 CFU/well of *L. plantarum*endoref, *L. plantarum*WJL, *A. pomorum*PQQ-ADH, or *A. pomorum*. Live microbes were provided one time immediately following axenic processing of embryos (**$p \leq 0.01$, ****$p \leq 0.0001$, Dunn's multiple comparisons test). Average of all larvae from N = 3 arenas per group is represented by a horizontal line placed between error bars representing $\pm$ SD; individual arena averages are shown in (B). N = 46–121 measured larvae per strain.

(B) Average larval size (mm^2) compared with CFU from larval food surfaces in N = 3 arenas containing the cohorts of larvae displayed in A (*r* = 0.88, p < 0.0001, Spearman's correlation coefficient). CFU, colony-forming unit.

See also Figures S4 and S5.

effects might impact a range of physiological, metabolic, and behavioral phenotypes (Obadia et al., 2018), and recent studies on microbe communities reveal that some microbe-induced host phenotypes are general across a multitude of microbial strains (Faith et al., 2014). Measurement of bacterial growth rates or abundance in experimentally relevant conditions (Storelli et al., 2018), identifying stable bacterial colonizers (Inamine et al., 2018; Obadia et al., 2017), and carefully choosing diets to control for diet-induced changes in microbial composition or quantity (Dalby et al., 2017) will support studies on specialized interactions.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and five figures and can be found with this article online at https://doi.org/10.1016/j.isci.2018.06.004.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.S.K., R.Y., and W.W.J.; Investigation, E.S.K., R.Y., B.O., and W.B.L.; Writing – Original Draft, E.S.K., R.Y., and W.W.J.; Writing – Reviewing & Editing, E.S.K., R.Y., B.O., W.B.L., and W.W.J.; Supervision, W.B.L. and W.W.J.; Funding Acquisition, W.B.L. and W.W.J.

DECLARATION OF INTERESTS

W.B.L. was a co-founder of and is a shareholder in uBiome, Inc.

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Supplemental Information

Microbial Quantity Impacts *Drosophila*

Nutrition, Development, and Lifespan

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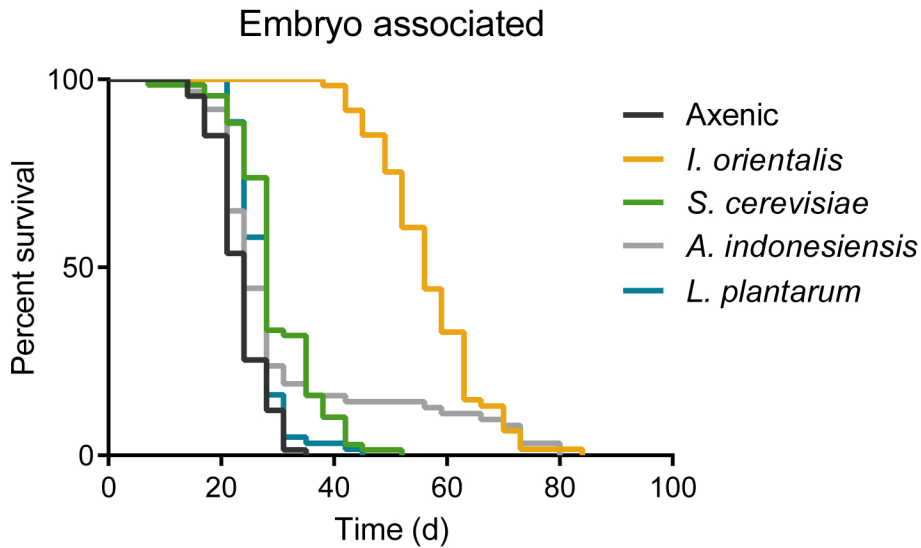
Figure S1. Association With Microbes Modulates Adult Lifespan on Undernutrition Diet, Related to Figure 1. Survival of axenic or monoxenic Dahomey male flies on 0.1% YE malnutrition diet (all comparisons to axenic, $p \leq 0.0076$, log-rank test). Axenic flies were associated as embryos with specific microbes. N = 59-69 flies.
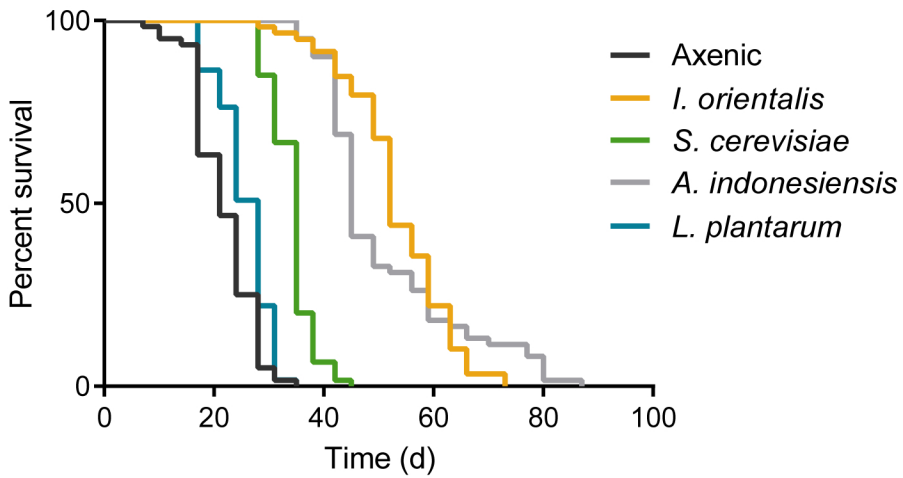
Figure S2. Association With Microbes Modulates Adult Lifespan on Undernutrition Diet, Related to Figure 2. Survival of axenic or monoxenic flies on our standard 0.1% YE malnutrition diet which includes our acid-based preservative (all comparisons to axenic, $p \leq 0.0012$, log-rank test). Axenic flies were associated once as adults with specific microbes. Experiment was performed at the same time as the experiment shown in Figure 2C,D. Microbial counts from spent vials during this lifespan study are shown in Figure 2D. N = 59-61 flies.
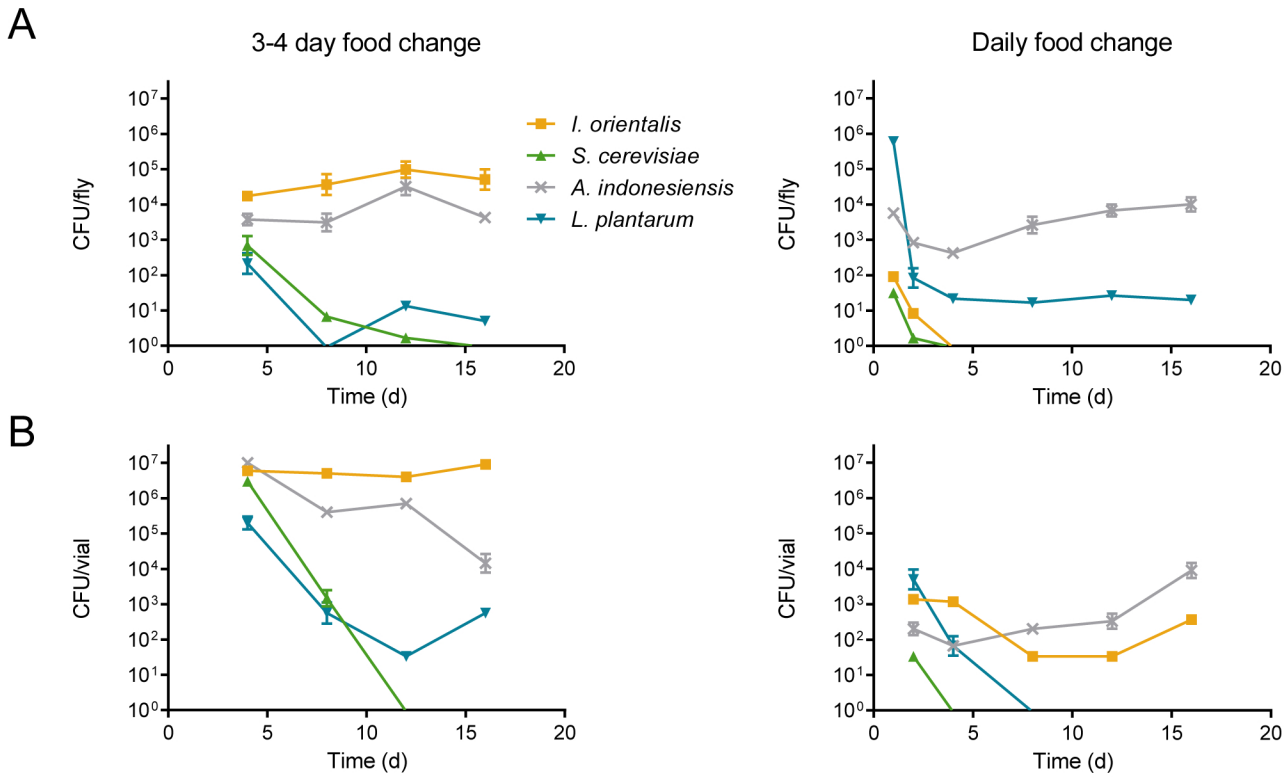
Figure S3: Food Change Interval Affects Microbial Counts, Related to Figure 3. (A) Microbial load in adult flies. Microbes were harvested on days 4, 8, 12 and 16 (3-4 day food change interval, left) or on days 1, 2, 4, 8, 12, and 16 (daily food change, right). N = 6 flies each. (B) Microbial load in fly enclosure. Microbes from spent vials were collected on days 4, 8, 12, and 16 (3-4 day food change interval, left panel) or on days 2, 4, 8, 12, and 16 (daily food change, right panel). N = 3 enclosures each. Data from the same experiment in Figure 3A. All data shown as average CFU per fly or per vial (± s.d.).
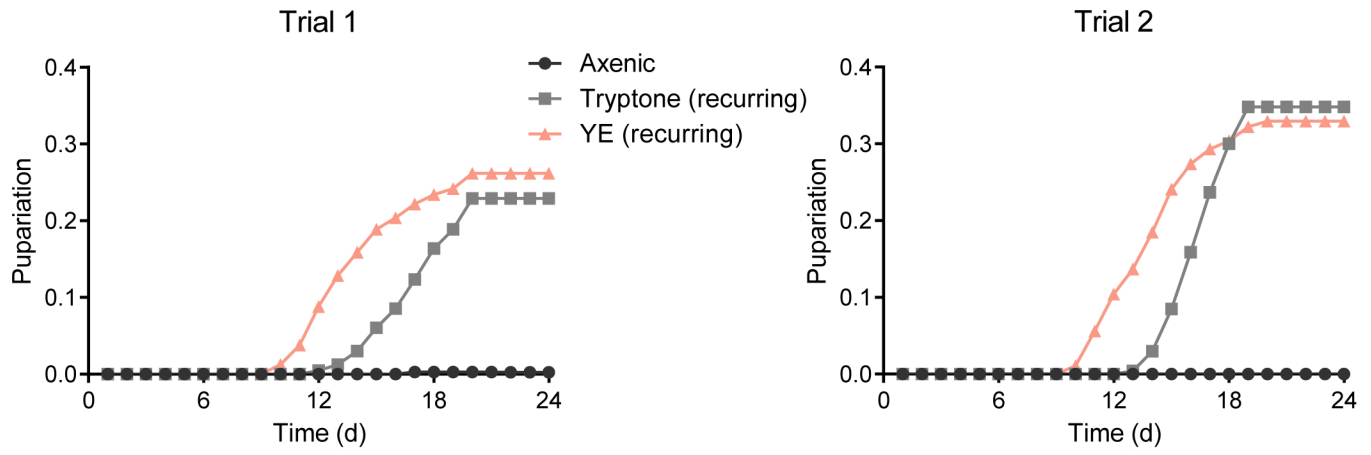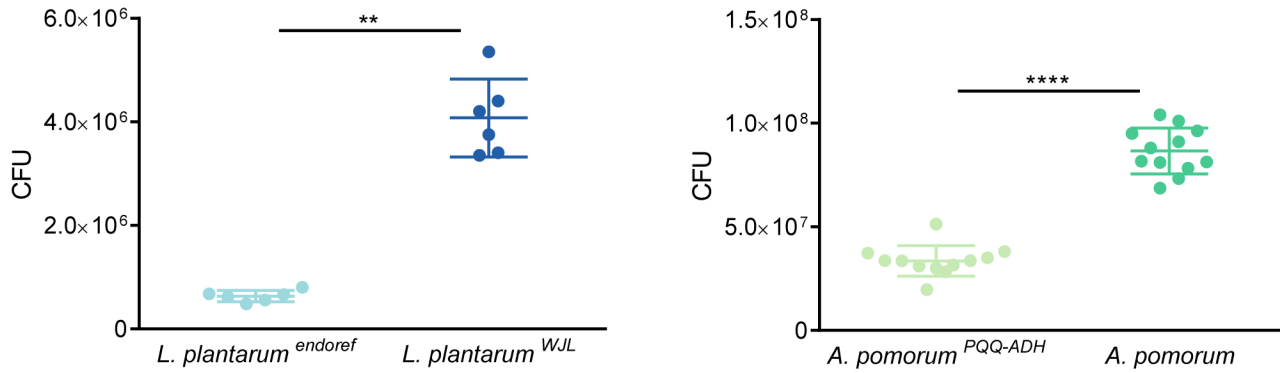
Figure S4: Tryptone and YE Rescue Pupariation on Undernutrition Diet, Related to Figure 6.
Pupariation rate (pupal count sum/eggs seeded) on 0.5% YE diet supplemented with tryptone or YE (15 mg/vial, equivalent to 0.75% w/v, final) every 2 days. Two independent trials are shown.
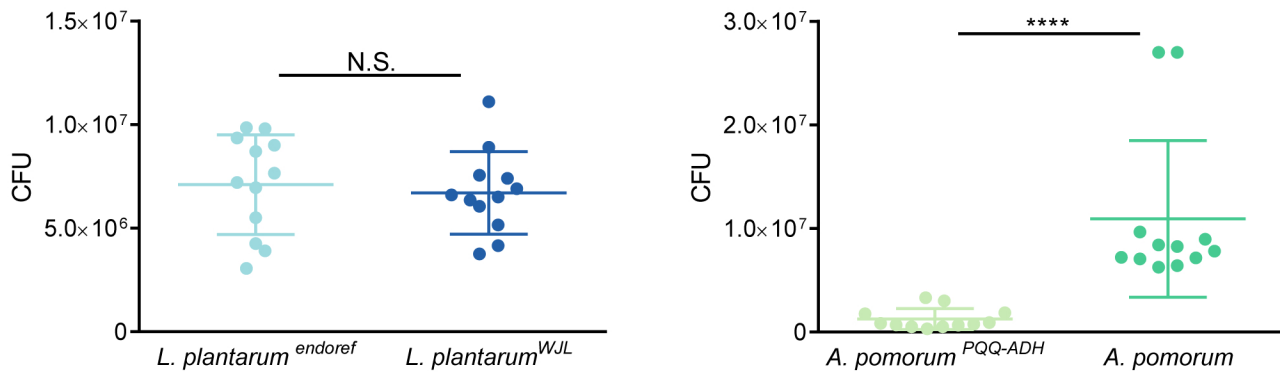
Figure S5. Fly- and Larval-Free Microbial Growth on 0.1% and 0.5% YE Medium, Related to Figure 6. CFU counts on day 4 after seeding 0.1% YE diet with approximately 5.8×10^6 CFU/well, and day 6 after seeding 0.5% YE medium with approximately 6.8×10^6 CFU/well of *L. plantarum*[endoref], *L. plantarum*[WJL], *A. pomorum*[PQQ-ADH], or *A. pomorum*. Timepoints were chosen to mimic our standard fly lifespan studies, which used 0.1% YE malnutrition diet and fresh food changes every 3-4 days, or our larval development studies, which used 0.5% YE diet for a 6-day period. ** $p \leq 0.01$, **** $p \leq 0.0001$, Mann-Whitney test. Average CFU count represented by a horizontal line placed between error bars representing ± s.d. N = 6-12 replicates per strain and diet.

Transparent Methods

Fly strains
Dahomey and Canton-S fly stocks were maintained on a standard stock food at a constant temperature of 23-25°C under a 12/12-h light/dark cycle. The Dahomey and Canton-S lines tested negative for *Wolbachia* by PCR of the *wsp* gene using 81F and 691R primers (Braig et al., 1998). Axenic subjects were generated by bleach treatment of embryos, as described previously (Bakula, 1969; Brummel et al., 2004; Yamada et al., 2015). Briefly, embryos were dechorionated for 20 min in a 50% v/v bleach solution, placed in 70% ethanol for 5 min, and washed three times with sterile 1× PBS + 0.01% Triton X-100. Only first-generation axenic flies were used throughout this report.

Microbial strains
The *I. orientalis, S. cerevisiae, A. indonesiensis* SB003, and *L. plantarum* SB001 strains used in adult experiments were all grown in MRS broth and described previously (Yamada et al., 2015). *A. indonesiensis* SB003 and *L. plantarum* SB001 are available from the NRRL ARS Culture Collection (Stock B-65486 and B-65492, respectively). For larval experiments, the *L. plantarum*^{endoref} and *L. plantarum*^{WJL} (Storelli et al., 2011) strains were grown statically in MRS broth at 37°C, and the *A. pomorum*$^{PQQ-ADH}$ and *A. pomorum* (Shin et al., 2011) strains were grown with aeration in mannitol broth at 30°C. Heat-killed microbes were prepared as previously described (Yamada et al., 2015) by a 30-minute 121°C autoclave cycle. Autoclaved aliquots were stored at -20 °C for subsequent use.

Diets
Sterile standard stock food (5.0% sucrose, 1.5% dry active yeast, 5.0% cornmeal, and 1.5% agar [all w/v], supplemented with 0.4% [v/v] propanoic acid and 0.035% [v/v] phosphoric acid) was used to house axenic or gnotobiotic subjects until adult flies were placed on experimental diets for lifespan assays. The food cooking process (Bruce et al., 2013) kills active yeast, as verified by dilution plating on MRS agar. The standard experimental diet contained 5.0% sucrose, 0.1% YE, 8.6% cornmeal, and 0.5% agar (all w/v) supplemented with 0.4% propanoic acid and 0.035% phosphoric acid (both v/v). Variations in sucrose and YE concentration were used as indicated. Larval or pupal experiments used 5.0% sucrose and 0.5% YE. Methylparaben (Methyl 4-hydroxybenzoate) diets consisted of 0.3% (w/v) methylparaben in the absence of propanoic and phosphoric acids. Diets in Figure 5 did not contain cornmeal. Fly- and larval-free growth measures were performed in 6- or 12-well plates containing the indicated diet inoculated with 100 μL 1× PBS containing the indicated quantity of microbes.

Lifespan assay
Flies were developed on sterile standard stock food. Axenic or monoxenic adult males aged 3-5 days were transferred to vials containing experimental diets at a density of 20-25 flies/vial. Flies were maintained in incubators set at 25°C with controlled light (12/12-h light/dark cycle) and humidity (60%). Flies were typically transferred to fresh food every 3-4 days unless noted otherwise. Fly manipulations were conducted aseptically using 70% ethanol and axenic conditions were periodically verified using 16S rDNA gene PCR or plating swabs from spent fly vials on MRS agar. Figure S2, a portion of the *I. orientalis* and axenic data from Figures 2C and 4; and axenic, 0.75% YE, and 0.75% tryptone data in the left panel of Figure 5C were from Yamada et al. (2015).

Pupariation measurements
Pupariation was quantified by counting the number of pupae daily over a 24-day period. All subjects were axenic and placed on the standard experimental diet containing 0.5% YE without supplementation, or with supplements of 15 mg sterile yeast extract or 15 mg sterile tryptone every 2 days throughout the experiment. Data are represented as the total number of pupae divided by the total original number of seeded embryos in triplicate vials across two independent trials.

Larval size measurements
Adult Dahomey were allowed to lay eggs for a 4-hour period. Embryos were subsequently collected and processed through a bleach treatment as previously described (Yamada et al., 2015) and detailed above. Axenic embryos were then sorted into wells of a 12-well plate containing 0.5% YE experimental diet, and exposed to 100 µL of 1× PBS containing ~5.4 × 10^6 CFUs of the indicated microbial strain or 100 µL of sterile 1× PBS as a vehicle control. Each well was stoppered with a sterilized foam stopper (Droso-Plugs), and the plates were placed in a chamber containing water-soaked Kimwipes within an incubator held at 25°C and 60% humidity under a 12/12-h light/dark cycle. After 6 days, larvae were frozen and imaged for surface area analysis using ImageJ.

Microbial load measurements
Microbial seed amount was quantified by either O.D. measures or dilution plating, and microbial load was quantified by serial dilution plating. MRS agar was used for quantifying all microbes except for the *A. pomorum* strains, which were quantified on mannitol plates. *A. pomorum$^{PQQ-ADH}$*, a mutagenized strain, was also quantified on mannitol plates containing 30 µg/mL kanamycin to verify selection-free counts. To measure the internal microbial load of adults, flies were sterilized with 70% ethanol, washed three times with sterile 1× PBS, and single flies were homogenized in 100 µL 1× PBS for 10 s. To measure microbe growth in the fly or larval environment, spent food vials or larval arenas were rinsed with 1 mL 1× PBS and plated as described above. Fly-free growth was measured similarly using 6- or 12-well plates. For arenas containing axenic larvae, swabs were plated on either MRS or mannitol agar plates to check for microbial contamination since larvae were too small to reliably use PBS washes without losing subjects during the washing process. A portion of the *I. orientalis* data from Figure 2D was from Yamada et al. (2015).

Statistics
Spearman's correlations, Mann-Whitney tests, and Kruskal-Wallis tests with Dunn's corrections were performed using GraphPad Prism 6. OASIS 2 was used to analyze differences between survival curves using log-rank test and median lifespan using Fisher's exact test (Han et al., 2016). Bonferroni *p*-values were reported when applicable.

Supplemental References

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