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Supplemental Information

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

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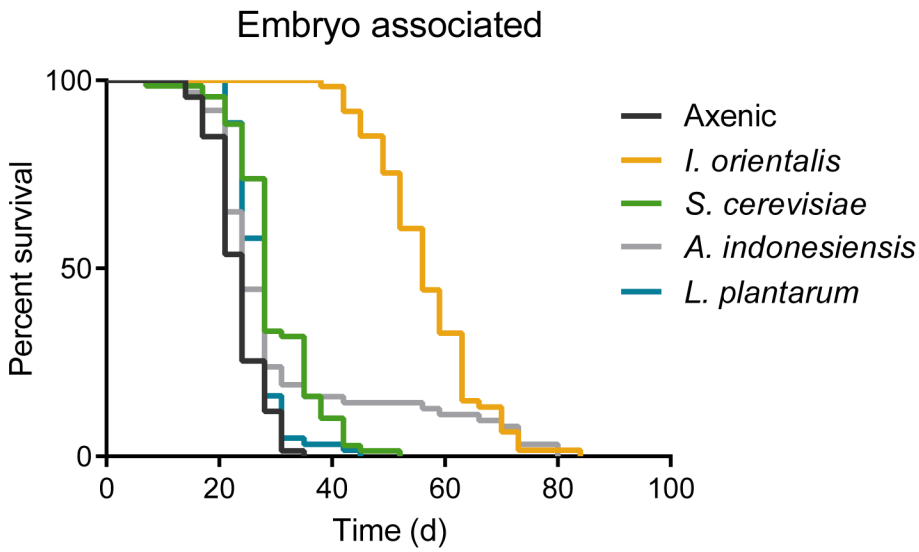

Figure S1. Association With Microbes Modulates Adult Lifespan on Undernutrition Diet, Related to Figure 1. Survival of axenic or monoxenic Dahomey male flies on 0.1% YE malnutrition diet (all comparisons to axenic, $p \leq 0.0076$, log-rank test). Axenic flies were associated as embryos with specific microbes. N = 59-69 flies.

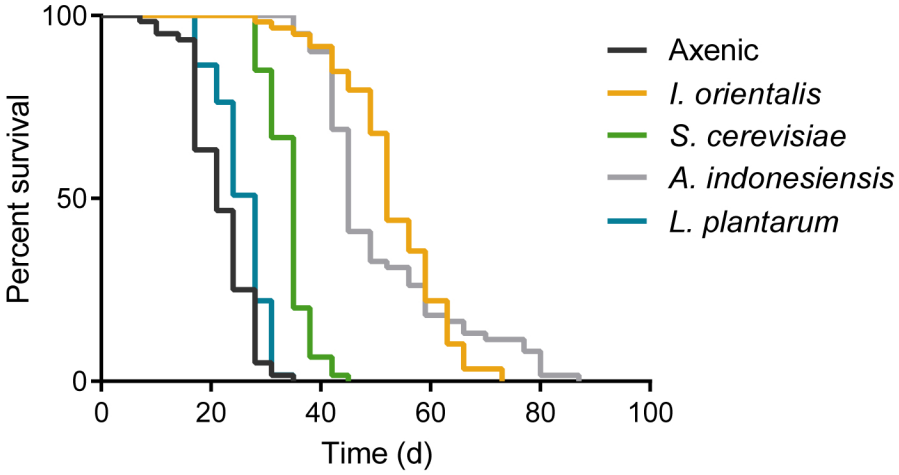

Figure S2. Association With Microbes Modulates Adult Lifespan on Undernutrition Diet, Related to Figure 2. Survival of axenic or monoxenic flies on our standard 0.1% YE malnutrition diet which includes our acid-based preservative (all comparisons to axenic, $p \leq 0.0012$, log-rank test). Axenic flies 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.

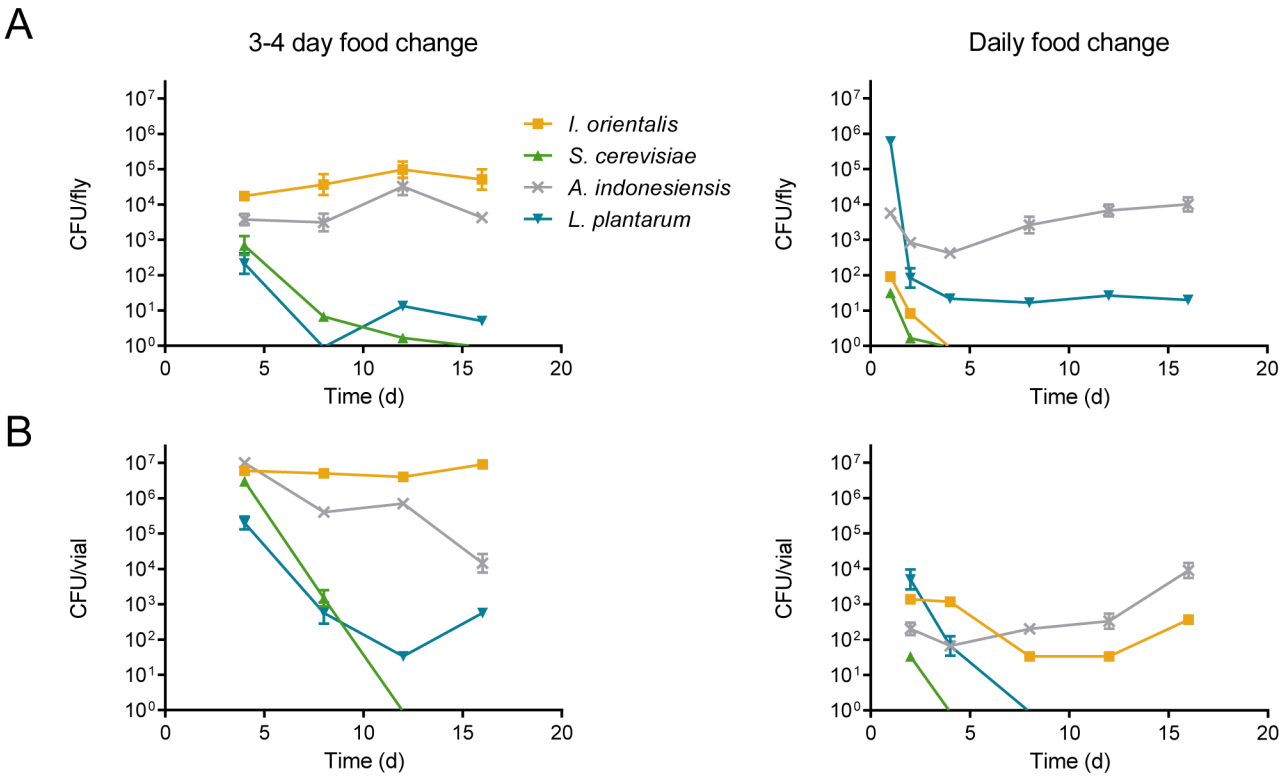

Figure S3: Food Change Interval Affects Microbial Counts, Related to Figure 3. (A) Microbial load in adult flies. Microbes were harvested on days 4, 8, 12 and 16 (3-4 day food change interval, left) or on days 1, 2, 4, 8, 12, and 16 (daily food change, right). N = 6 flies each. (B) Microbial load in 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.).

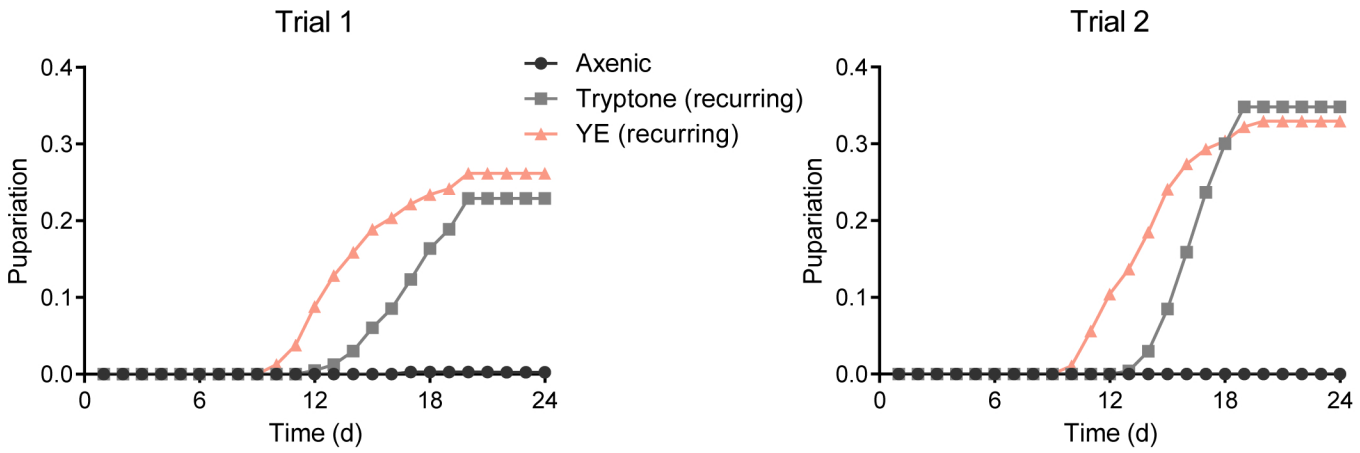

Figure S4: Tryptone and YE Rescue Pupariation on Undernutrition Diet, Related to Figure 6. Pupariation rate (pupal count sum/eggs seeded) on 0.5% YE diet supplemented with tryptone or YE (15 mg/vial, equivalent to 0.75% w/v, final) every 2 days. Two independent trials are shown.

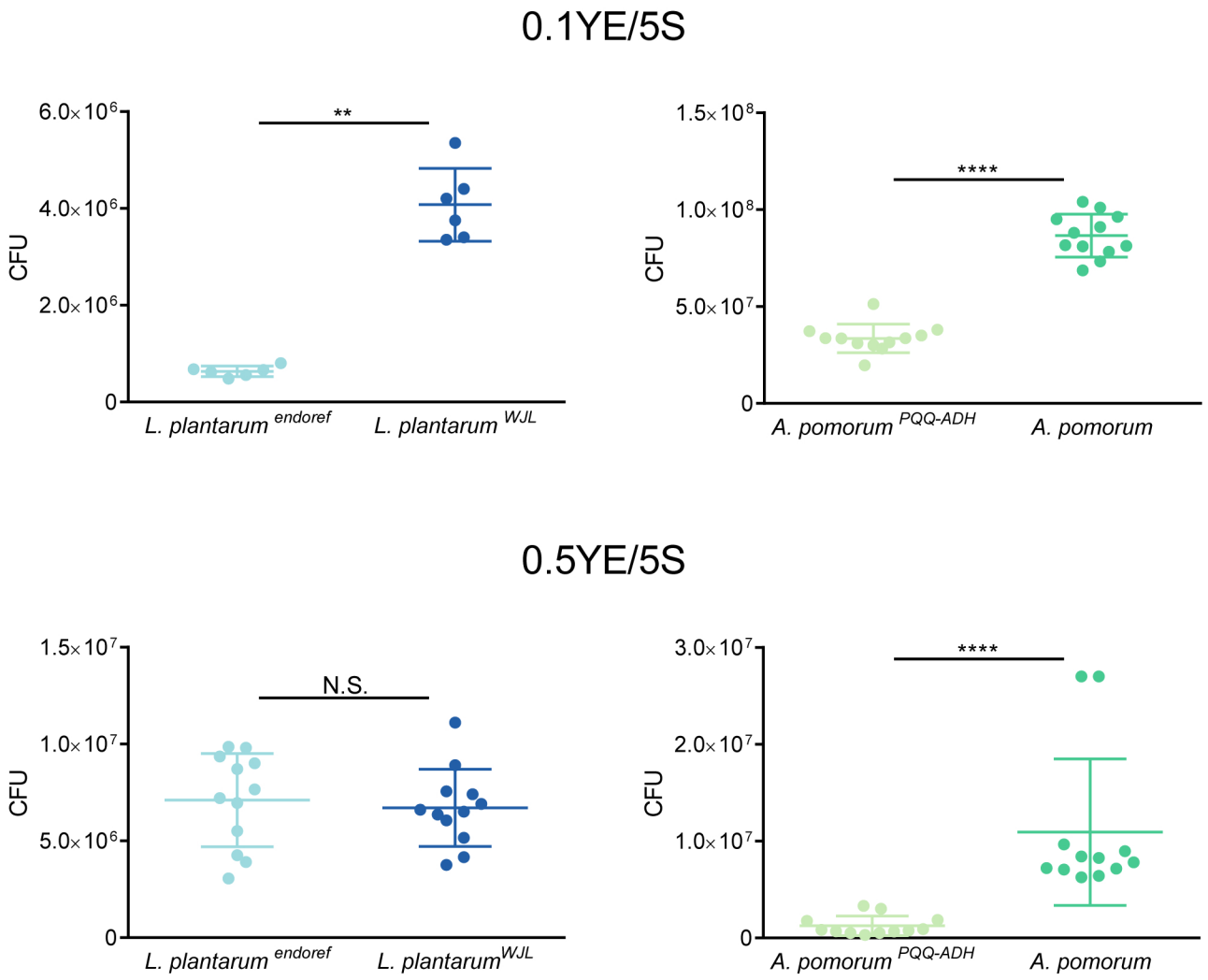

Figure S5. Fly- and Larval-Free Microbial Growth on 0.1% and 0.5% YE Medium, Related to Figure 6. CFU counts on day 4 after seeding 0.1% YE diet with approximately 5.8 × 10^6 CFU/well, and day 6 after seeding 0.5% YE medium with approximately 6.8 × 10^6 CFU/well of *L. plantarum*^{endoref}, *L. plantarum*^{WJL}, *A. pomorum*$^{PQQ\text{-}ADH}$, 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*$^{PQQ\text{-}ADH}$ 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*$^{PQQ\text{-}ADH}$, 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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