Research Paper

The role of commensal microbes in the lifespan of *Drosophila melanogaster*

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ABSTRACT

Commensal microbes have mutualistic relationships with their host and mainly live in the host intestine. There are many studies on the relationships between commensal microbes and host physiology. However, there are inconsistent results on the effects of commensal microbes on host lifespan. To clarify this controversy, we generated axenic flies by using two controlled methods – bleaching and antibiotic treatment – and investigated the relationship between the commensal microbes and host lifespan in *Drosophila melanogaster*. The removal of microbes by using bleaching and antibiotic treatments without detrimental effects increased fly lifespan. Furthermore, a strain of flies colonized with a high load of microbiota showed a greater effect on lifespan extension when the microbes were eliminated, suggesting that commensal bacteria abundance may be a critical determinant of host lifespan. Consistent with those observations, microbial flora of aged fly gut significantly decreased axenic fly lifespan via an increase in bacterial load rather than through a change of bacterial composition. Our elaborately controlled experiments showed that the elimination of commensal microbes without detrimental side effects increased fly lifespan, and that bacterial load was a significant determinant of lifespan. Furthermore, our results indicate the presence of a deterministic connection between commensal microbes and host lifespan.

INTRODUCTION

Commensal microbes with symbiotic relationships with their hosts have been actively investigated in several fields of research in attempts to elucidate their interaction with the host. In particular, many studies have been initiated since the development and wide application of metagenomic sequencing analysis. In recent years, studies on the treatment and prevention of diseases through the production of changes in the host's intestinal environment have been carried out, and many research teams have suggested that the intestinal microbiota can affect the host's metabolic diseases [1], immune diseases [2], development [3, 4], reproduction [5], biorhythms [6], and even behavior and mood [7].

Studies into the roles of microbiota as they relate to host aging and lifespan have also been conducted. In *Caenorhabditis elegans* grown axenically, developmental time and lifespan were about twice as long as those in control worms [8]. Furthermore, the use of microorganisms as food items and the secondary metabolites secreted by the commensal microbes are reported to affect the lifespan of *C. elegans* [9, 10]. The viability of axenic animals suggests that the presence of microbes in the gut is neither obligatory nor essential to the host's development, under appropriate conditions. However, conflicting results have been reported on the effects of commensal microbe presence on the lifespan of *Drosophila melanogaster*. Brummel *et al.* observed that the lifespan of flies reared axenically following egg

bleaching or antibiotic treatment was shorter than that of conventionally reared fly, and that effect could be recovered by exposure of the flies to microbes within 2–3 days from eclosion [11]. In contrast, Yamada *et al*. did not show an alteration of lifespan in axenic condition flies, while Clark *et al.*, Petkau *et al.*, Galenza *et al*., Tefit *et al.* and Obata *et al.* showed that the absence of commensal microbes extends the lifespan in *Drosophila* [14–19]. Ren *et al.*, Ridley *et al.*, and Iatsenko *et al.* showed increased lifespan of *Drosophila* following egg bleaching or antibiotic treatment, but the increases did not reach statistical significance [12, 13, 20]. To clarify the reasons for these inconsistencies, we generated axenic flies using highly elaborate well-controlled methods and observed that elimination of commensal microbes without detrimental side effects increased host lifespan. Moreover, we observed that an age-related increase in microbial load significantly decreased host lifespan, and a change in microbial load had a more critical effect than that from an age-related change in microbial composition.

RESULTS

Lifespan of axenic *D. melanogaster* from bleached eggs

To clarify the effect of an absence of commensal microbes on host lifespan, we generated axenic (Ax) flies of laboratory wild-type strain w^{1118} *D. melanogaster* by using a sodium hypochlorite-based bleaching method as previously reported [15]. The mean lifespan of the first generation (1G) Ax flies hatched from bleached eggs was shorter than that of conventionally reared (Conv) flies (Figure 1A and 1B, Conv fly 59.92 ± 1.52 days; 1G Ax fly 53.83 ± 1.31 days, 10.16% decrease, log-rank test, χ^2 = 38.85, p < 0.0001, Wilcoxon test, χ^2 = 20.81, p < 0.0001). Because the bleaching process might have a deleterious effect on the health of flies, we measured the lifespan of second- (2G) and third- (3G) generation flies after first-generation bleaching. Interestingly, the 2G and 3G Ax flies had increased lifespans compared to that of Conv flies (Figure 1A and 1B, Supplementary Table 1; 2G Ax fly 68.52 ± 0.94 days, 14.35% increase, log-rank test, χ^2 = 0.08, p = 0.77, Wilcoxon test, χ^2 = 8.91, p < 0.005; 3G Ax fly 70.73 ± 0.85 days, 18.04% increase, log-rank test, χ^2 = 8.20, p < 0.005, Wilcoxon test, χ^2 = 18.93, p < 0.0001), suggesting that the bleaching method may have a detrimental effect on *D. melanogaster*, but the effect diminished over subsequent generations. To confirm whether the lifespan extension by egg bleaching is due to the absence of commensal microbes, we introduced microbes from 10-day-old Conv flies to 3-day-old 3G Ax flies by using fecal transplantation. As expected, the longevity effect of bacterial removal in Ax flies was diminished by fecal microbe transplantation (Figure 1C and 1D, Supplementary Table 1; Ax 73.00 ± 1.40 days; Ax + $Feces^{Conv}$ 68.15 ± 1.30 days, 6.64% crease, log-rank test, χ^2 = 22.61, p < 0.0001). These results indicate that the bleaching of eggs can have an adverse effect on adult fly lifespan, but the elimination of commensal microbes increases the lifespan of subsequent generations of flies.

Lifespan of axenic *D. melanogaster* generated by antibiotic treatment

To confirm the effect of microbe removal on host lifespan, we supplied an antibiotic (AB) cocktail to Conv flies as described previously [12]. In our laboratory, bacteria were colonized into the guts of 10-day-old control flies at densities of up to 1.8×10^2 cells per fly; colonization was increased with fly age to densities of up to 2.2×10^5 cells per fly in 50-day-old flies (Figure 2A–2C). Treatment with the AB cocktail reduced the number of bacteria in the gut, and the AB effects were gradually decreased by dilution of the cocktail (Figure *2*A–2C). Following AB treatment (×1 AB), the mean lifespans of Conv flies were not significantly different from that of untreated (×0 AB) Conv flies (Figure 2D and 2F, Supplementary Table 1; Conv + ×0 AB 62.73 ± 1.32 days; Conv + ×1.0 AB 62.89 ± 1.21 days, log-rank test, χ^2 = 0.53, p = 0.47). However, AB cocktail treatment dramatically reduced the lifespan of Ax flies at the ×1 AB concentration (Figure 2E and 2F, Supplementary Table 1; Ax + ×0 AB 77.17 ± 1.32 days; Ax + ×1.0 AB 65.94 ± 1.34 days, 14.55% decrease, log-rank test, χ^2 = 64.01, p < 0.0001). We assumed that the AB cocktail concentration used might have had a toxic effect on fly health. To investigate this possibility, we treated flies with the AB cocktail at 2- and 10-fold concentration dilutions (×0.5 AB and ×0.1 AB, respectively). The ×0.5 AB and ×0.1 AB cocktail treatments did not significantly affect the lifespan of Ax flies (Figure 2E and 2F, Supplementary Table 1; Ax + ×0.1 AB 77.44 ± 1.34 days, log-rank test, χ^2 = 1.41, p = 0.23; Ax + ×0.5 AB 77.97 ± 1.06 days, log-rank test, χ^2 = 0.64, p = 0.42), whereas the lifespan of Conv flies was increased at these lower AB concentrations (Figure 2D and 2F, Supplementary Table 1; Conv + ×0.1 AB 68.93 ± 1.16 days, 9.88% increase, log-rank test, χ^2 = 12.33, p < 0.001; Conv + ×0.5 AB 67.84 ± 0.99 days, log-rank test, χ^2 = 2.86, p = 0.09). These results indicate that an AB treatment at a dosage that did not have toxic effects can increase the lifespan of *D. melanogaster*, which is consistent with the results obtained by using the bleaching method to produce Ax flies.

Strain-specific longevity effect of commensal microbe elimination

Han *et al.* (2017) reported that *Drosophila* with different genetic backgrounds had different microbial

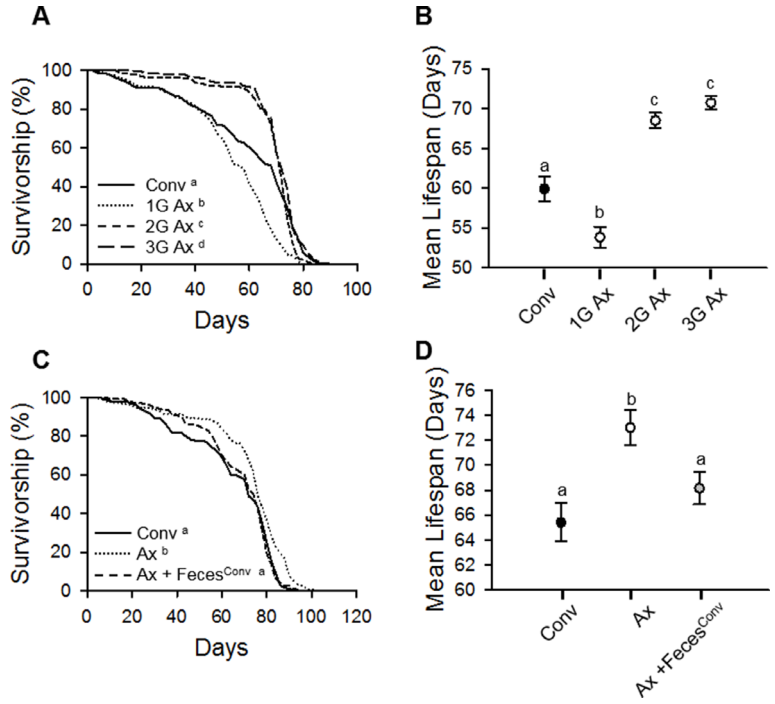

Figure 1. Elimination of commensal microbes by egg bleaching extends host lifespan. (**A**) Survival of first (1G), second (2G), and third (3G) generations of Ax flies from bleached eggs. (**B**) Mean lifespan of Conv fly and 1G, 2G, and 3G of Ax flies from bleached eggs. Filled dot, Conv flies; open dots, Ax flies. (**C**) Survival of Ax flies treated with feces from 10-day-old Conv flies. (**D**) Mean lifespan of Ax flies treated with feces from 10-day-old Conv flies. Black dot, Conv flies; white dot, Ax flies fed feces from Ax flies; gray dot, Ax flies fed feces from Conv (Ax + Feces^{Conv}) flies. Different letters indicate significant differences between groups. Error bars represent the SEM.

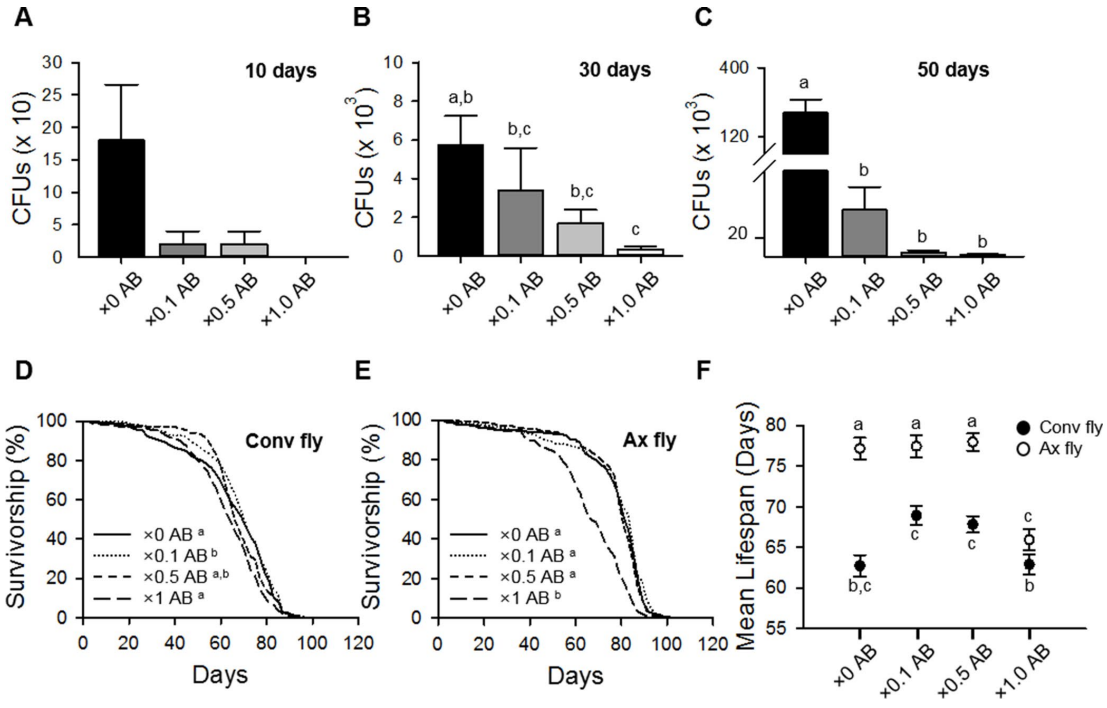

Figure 2. Elimination of commensal microbe by antibiotic treatment extends host lifespan. (**A**–**C**) Colony-forming units (CFUs) of Conv flies treated with ×0.1, ×0.5, or ×1 of the antibiotic cocktail AB for 10 days (**A**), 30 days (**B**), and 50 days (**C**). (**D**–**E**) Survival of flies treated with ×0.1, ×0.5, or ×1 AB in Conv fly (**D**) and Ax fly (**E**). (**F**) Mean lifespan of flies treated with ×0.1, ×0.5, or ×1 AB. Filled dots, Conv flies; open dots, Ax flies. Different letters indicate significant differences between groups. Error bars represent the SEM.

flora, even though the flies were reared under the same conditions in the same laboratory [21]. Since a different microbial flora may lead to different lifespan results following bacterial removal, we compared the lifespan of Ax flies generated from different strains in our laboratory; wild-type Oregon-R, Canton-S, and w^{1118} strains. Under the same conditions, the load levels of commensal microbes residing in these strains were different; the number of colony-forming units (CFUs) of commensal microbes was lowest in the Canton-S strain and highest in the w^{1118} (Figure 3A, ANOVA, $p < 0.0001$; Tukey's HSD test, w^{1118} *vs.*OR, $p < 0.0001$; w^{1118} *vs.*CS, $p < 0.0001$; OR *vs.*CS, $p < 0.005$). In all three strains, the bleached egg effect on the longevity of 3G Ax flies was observed, but the extent of lifespan extension resulting from bacterial removal was different among the strains. Bacterial removal increased the lifespan of w^{1118} flies by 32.01%, whereas the lifespan increases were only 16.82% and 3.03% in the Oregon-R and Canton-S strains, respectively (Figure 3B and 3C, Supplementary Table 1, log-rank test, w^{1118}, $\chi^2 = 146.33$, $p < 0.0001$; Oregon-R, $\chi^2 = 37.43$, $p < 0.0001$; Canton-S, $\chi^2 = 6.60$, $p < 0.05$). Moreover, the extent of the lifespan extension was positively correlated with the abundance of commensal microbes residing in each strain (Spearman's correlation, rho = 0.88, $p < 0.0001$). To confirm that the longevity effect produced by axenic culture was related to bacteria abundance in flies, we performed an analysis of covariance (ANCOVA) test to adjust the bacterial loads among three strain groups. Following the ANCOVA test and after adjustment of bacterial abundance, we confirmed that the differences in the lifespan extension effect of axenic culture among the three strains disappeared (ANCOVA, $p = 0.99$). It showed the possibility that the difference in lifespan extension effect by bacteria removal among these three strains was the result of colonized bacterial abundance.

Taken together, the results showed that the elimination of commensal bacteria using a method that is not accompanied by detrimental side effects can increase the lifespan of flies. In addition, strains colonized with a high level of commensal bacteria exhibit a greater increase in lifespan following bacteria elimination.

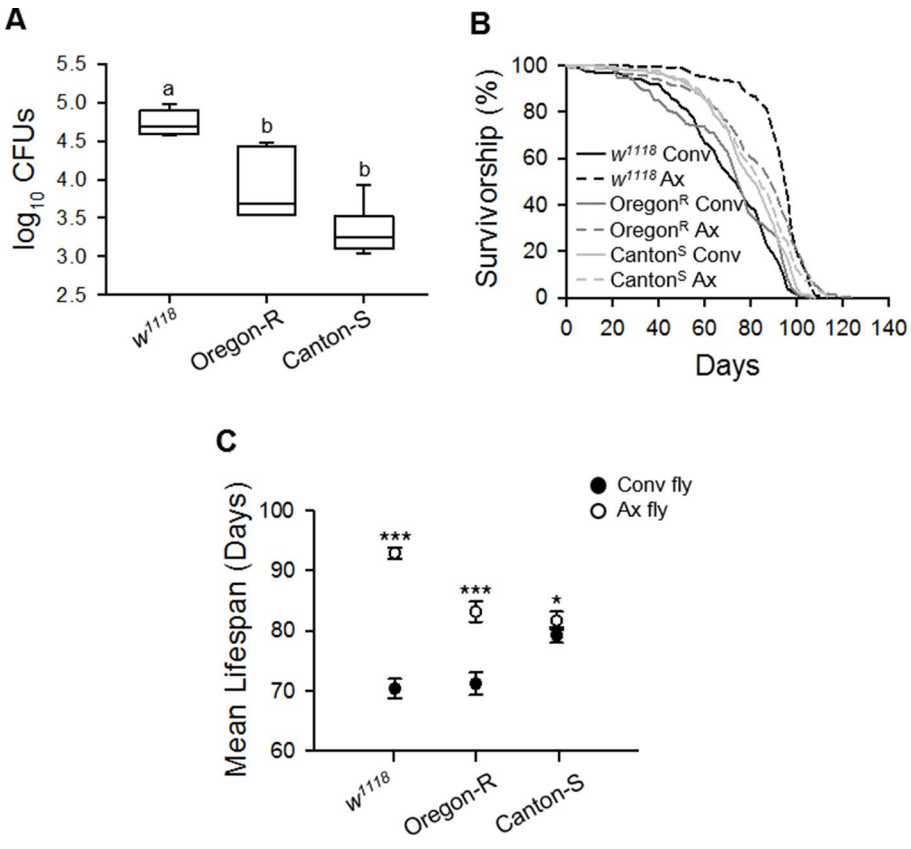

Figure 3. Lifespan extension effect of the elimination of commensal microbes differs among laboratory fly strains. (**A**) The total number of CFUs from 1-week-old flies in plate count agar (PCA) media. (**B**) Survival curve of laboratory wild-type w^{1118}, Oregon-R, and Canton-S strains with the elimination of microbes. Solid lines, Conv flies; dashed lines, Ax flies. (**C**) Mean lifespan of laboratory wild-type w^{1118}, Oregon-R, and Canton-S strains following the elimination of microbes. Filled dots, Conv flies; open dots, Ax flies. Asterisks indicate significant differences between Conv flies and Ax flies (log-rank test, $*p < 0.05$, $***p < 0.0001$). Error bars represent the SEM.

Age-related commensal microbe flora changes shorten the lifespan of axenic *D. melanogaster*

Since the *Drosophila* strain colonized with the higher level of commensal microbes showed a greater effect of bacterial elimination on longevity, we hypothesized that the commensal microbial load can determine host lifespan. To investigate this hypothesis, we supplied homogenates of flies of different ages to Ax flies and determined the effects on lifespan.

The commensal microbe floral abundance is widely reported to increase with host age [12, 15, 22, 23], and our 16S rRNA PCR and CFU results also showed that the abundance of commensal bacteria significantly increases with fly age (Supplementary Figure 1A and 1B). In addition, by using 454-pyrosequencing analysis, we observed that the composition of commensal bacteria also changes with age (Supplementary Figure 1C–1E). In 10-day-old (young) flies, there were 328 operational taxonomic units (OTUs) assigned; while, in 50-day-old (old) flies, 635 OTUs were assigned (CD-HIT 99% threshold) (Supplementary Figure 1C, Supplementary Table 2), indicating that the compositional richness of the microbial species in the gut flora increased with age. At the phylum level, *Proteobacteria* (including *Acetobacter* and *Komagataeibacter*) and *Firmicutes* (including *Lactobacillus* and *Leuconostoc*) comprised 99% of the microbiome in the *D. melanogaster* in this study (Supplementary Table 3). At the species level, *Acetobacter persici* JCM25330(T) (*Ap*, 45.29% of microbiome) and *Lactobacillus brevis* ATCC14869(T) (*Lb*, 34.25%) were dominant in young flies, while *Acetobacter malorum* LMG1746(T) (*Am*, 54.51%) and *Lactobacillus plantarum* ATCC14917(T) (*Lp*, 24.47%) were dominant in old flies (Supplementary Figure 1E, Supplementary Table 3). Moreover, the proportions of *Ap* (5.92%) and *Lb* (1.33%) were markedly reduced in old flies when compared with the levels in young flies. *Komagataeibacter medellinensis* (13.72%) and *Leuconostoc pseudomesenteroides* (11.33%) were detected in young and old flies, respectively (Supplementary Figure 1E, Supplementary Table 3).

To determine whether age-related microbial changes can affect host lifespan, we fed body homogenates from 10-day-old (young) or 50-day-old (old) Conv flies to 3-day-old Ax flies. Consistent with above results, the lifespan of the Ax flies was longer than that of Conv flies (Figure 4A and 4B, Supplementary Table 4; Conv fly 62.74 ± 2.08 days; Ax fly 82.64 ± 2.05 days, 31.72% increase, log-rank test, $\chi^2 = 45.98$, $p < 0.0001$). When the body homogenate from young flies was fed to Ax flies, the Ax fly lifespan was decreased by 8.12% compared to the lifespan of non-fed Ax flies, while the lifespan of Ax flies fed body homogenate from old flies was decreased by 22% (Figure 4A and 4B, Supplementary Table 4; Ax + Homogenate^{Co,Y} 75.93 ± 2.09 days, log-rank test, $\chi^2 = 4.61$, $p < 0.05$; Ax + Homogenate^{Co,O} 64.46 ± 2.32 days, log-rank test, $\chi^2 = 43.12$, $p < 0.0001$). A deleterious effect of body homogenate feeding on Ax fly lifespan was not observed when Ax flies were fed body homogenates of young or old Ax flies (Figure 4A and 4B, Supplementary Table 4; Ax + Homogenate^{Ax,Y} 82.39 ± 2.06 days, log-rank test, $\chi^2 = 2.43$, $p = 0.12$; Ax + Homogenate^{Ax,O} 79.14 ± 2.21 days, log-rank test, $\chi^2 = 0.49$, $p = 0.49$). We also observed a deleterious effect of commensal microbes in old flies by feeding them with gut homogenates from young or old Conv flies (Supplementary Figure 2 and Supplementary Table 4). These results indicate that the microbiota present in aged flies has a more adverse effect on lifespan than that present in young flies.

To elucidate whether the detrimental effect of microbiota from aged flies was due to the increased load of commensal microbes in old flies, we fed homogenates from 50-day-old (old) flies at several dilution rates to Ax flies. We observed that the Ax flies fed 10^2–10^3 times dilutions of old fly homogenate had a similar abundance of microbes to that of flies fed a 10-day-old (young) homogenate, indicating that the young (×1 Y) homogenate, the ×0.001 old (×0.001 O) homogenate, and the ×0.01 O homogenate contained microbial flora with similar microbial abundances but different compositions (Figure 4C). When the diluted old homogenates were fed to Ax flies, the fly lifespan was similar to that of flies fed with young homogenate, while the lifespan of Ax flies fed undiluted old (×1 O) homogenate was markedly decreased (Figure 4D and Supplementary Figure 3A, Supplementary Table 4; Ax + ×1 Y 76.96 ± 1.42 days; Ax + ×0.001 O 72.37 ± 1.71 days, log-rank test, $\chi^2 = 2.30$, $p = 0.13$; Ax + ×0.01 O 74.33 ± 1.32 days, 3.42% decrease, log-rank test, $\chi^2 = 8.35$, $p < 0.005$; Ax + ×0.1 O 70.37 ± 1.38 days, 8.56% decrease, log-rank test, $\chi^2 = 20.06$, $p < 0.0001$; Ax + ×1 O 66.74 ± 1.34 days, 13.28% decrease, log-rank test, $\chi^2 = 55.63$ $p < 0.0001$). These results indicate that the age-related increase in bacterial load is a strong determinant of host lifespan. To confirm the importance of bacterial load on host lifespan, the lifespan of Ax flies fed concentrated young homogenate was determined. We found that the level of colonized microbes in Ax flies was increased by feeding with concentrated young homogenates (Figure 4E), and the reduction of lifespan of Ax flies was worsened by feeding with a 2-fold concentrate of young fly homogenate (×2 Y) compared to the lifespans of flies fed ×0.5 Y and ×1 Y homogenates (Figure 4F, and Supplementary Figure 3B, Supplementary Table 4; Ax + ×1 Y 69.98 ± 1.87 days;

Ax 83.92 ± 1.82 days, 19.92% increase, log-rank test, χ^2 = 50.50, p < 0.0001; Ax + ×0.5 Y 70.52 ± 1.99 days, log-rank test, χ^2 = 0.61, p = 0.44; Ax + ×2 Y 67.28 ± 1.63 days, 3.86% decrease, log-rank test, χ^2 = 5.40, p < 0.05). Taken together, these results indicate that microbial abundance is a stronger determinant of host lifespan than microbial composition.

Increased microbial load may be a stronger determinant of host lifespan than age-related changes in microbial composition

As previously mentioned, both the abundance and composition of commensal microbes change with host age. To further elucidate whether the age-dependent

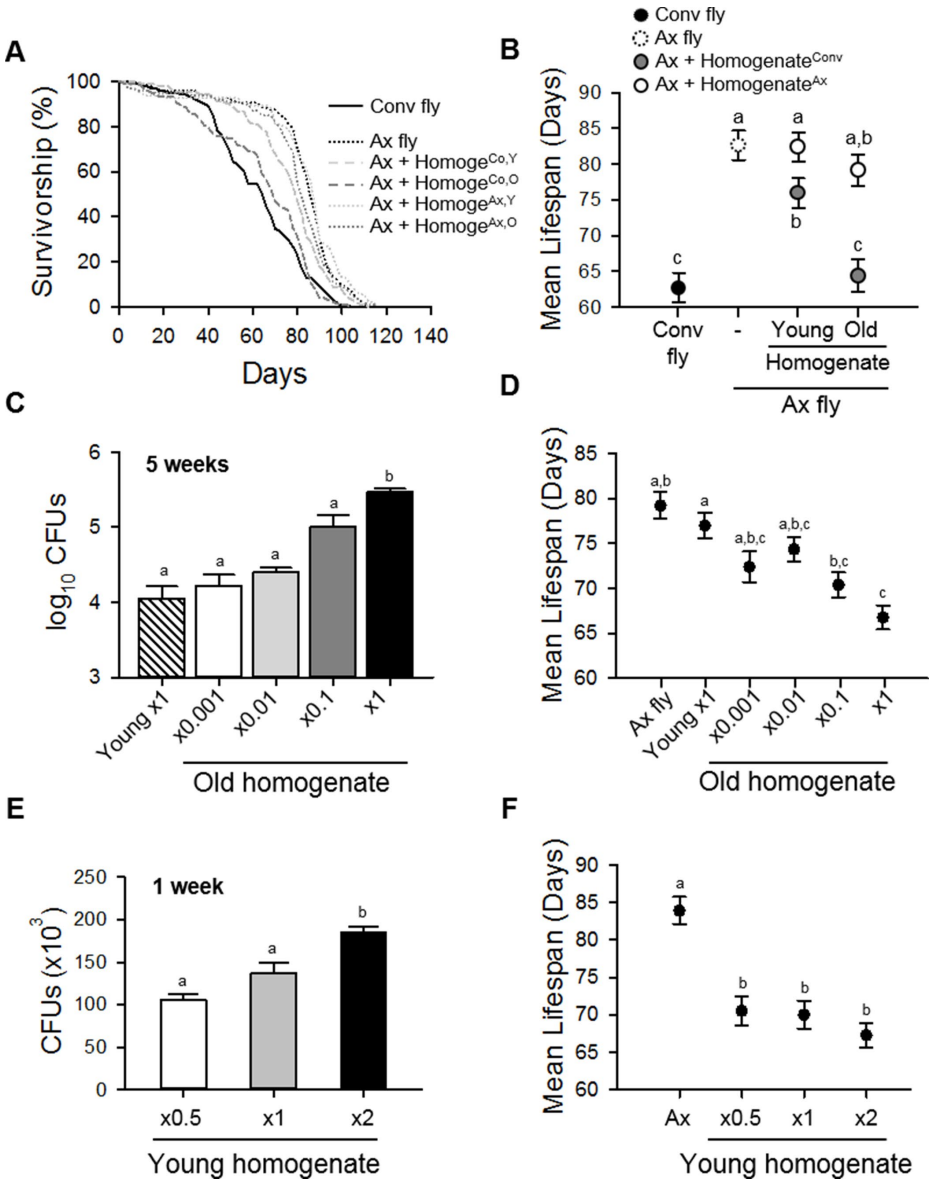

Figure 4. Homogenate of Conv fly decreases the lifespan of Ax fly. (**A**) Survival curves of Conv or Ax flies fed fly body homogenates from 10-day-old (young) or 50-day-old (old) flies. Solid lines indicate Conv flies, dotted lines indicate Ax flies or Ax flies fed Ax fly homogenate, and dashed lines indicate Ax flies fed Conv fly homogenate. Homoge^{Co,Y} indicates homogenate from young Conv flies, Homoge^{Co,O} indicates homogenate from old Conv flies, Homoge^{Ax,Y} indicates homogenate from young Ax flies, and Homoge^{Ax,O} indicates homogenate from old Ax flies. (**B**) Mean lifespan of Ax flies fed homogenate of young or old Conv or Ax flies. Black dot, Conv flies; dashed line, Ax flies; gray dots, Ax + Homogenate^{Conv}; white dots, Ax + Homogenate^{Ax}. (**C**) CFUs of Ax flies fed young fly homogenate or serially diluted old fly homogenate. CFUs were determined 5 weeks after the initial feeding. (**D**) Mean lifespan of Ax flies after feeding homogenates of young or serially diluted old Conv flies. (**E**) CFUs of Ax flies fed young fly homogenate concentrated at ×0.5, ×1, and ×2. CFUs were measured 1 week after the initial feeding. (**F**) Mean lifespan of Ax flies after feeding homogenates of young Conv flies. Homogenate from young Conv flies was diluted or concentrated up to two-fold. Different letters indicate significant differences between groups. Error bars represent the SEM.

change of microbial abundance is more critical to determining host lifespan than that of microbial composition, we generated gnotobiotic flies inoculated with four of the dominant species of commensal microbes in *Drosophila*: *Lb*, *Lp*, *Ap*, and *Am*. When Ax flies were inoculated with a single species of these microbes (monoxenic) at 10^3 CFUs, the mean lifespans were increased, but the changes did not reach statistical significance (Figure 5A, Supplementary Figure 4A, ANOVA, $p = 0.52$). In addition, Ax flies mono-inoculated with each bacterium at 10^8 CFUs lived for a shorter period than the control flies, but those changes also did not have statistical significance (Figure 5A, Supplementary Figure 4B, ANOVA, $p = 0.18$). Interestingly, the inoculated fly's lifespans were decreased with mono-inoculations of each of the four microbes (*Lb*, *Lp*, *Ap*, or *Am*) at 10^{14} CFUs, with the changes in the *Lb* and *Am* groups having statistical significance (Figure 5A, Supplementary Figure 4C, Supplementary Table 5, ANOVA, $p < 0.0001$; Tukey's HSD *vs.* Ax, *Lb*, $p < 0.005$; *Lp*, $p = 0.06$; *Ap*, $p = 0.08$; *Am*, $p < 0.0001$). These results indicate that the effect of commensal bacteria on host lifespan is dependent on the abundance of microbes colonized in the gut and that a high abundance of bacteria decreases the lifespan of flies.

To reveal whether a specific microbial composition can affect host lifespan, we measured the lifespan of Ax flies inoculated with 2^4 possible combinations of the four dominant commensal microbe species at two concentration levels (10^8 and 10^{14} CFUs). Similar to the results obtained with the monoxenic flies, the Ax flies inoculated with each microbe combination at 10^8 CFUs had lifespans similar to that of untreated Ax flies, whereas inoculation of a 10^{14} CFUs microbe combination decreased the inoculated flies mean lifespan (Figure 5B and 5C, 10^8 CFUs, Ax fly 85.59 days, gnotobiotic flies 82.35 days; 10^{14} CFUs, Ax fly 76.68 days, gnotobiotic flies 67.58 days, 11.87% decrease). In other words, the lifespan of flies inoculated with the higher concentration of bacteria was shorter than that of flies inoculated with the lower concentration of bacteria, indicating that microbial abundance is a strong determinant of lifespan in *Drosophila*.

To verify the effects of microbial abundance on fly lifespan, we measured the actual colonization level of the inoculated bacterial species. Regardless of the inoculated bacterial species composition, the colonized bacterial load was increased to a greater extent with 10^{14} inoculations than with 10^8 inoculations (Figure 5D). The results indicate the presence of a significant negative correlation between the CFUs in the fly's body and the lifespan of the fly (Figure 5E, Spearman's correlation, rho = −0.57, $p < 0.05$).

DISCUSSION

Commensal microbes in a symbiotic relationship are known to affect host health and aging. In this study, we observed that commensal microbes can affect host lifespan in *D. melanogaster*, and an increase in microbial load in aging flies can be a stronger determinant of lifespan than that from a compositional change in microbiota.

There is a growing body of evidence indicating that commensal microbes, directly and indirectly, affect the lifespan of a host. For example, supplementation with different strains of *Escherichia coli* can affect the lifespan of *C. elegans* through direct, metabolic, or species-specific signals [24]. Also, the lifespan of aged killifish can be improved when they are treated with transplanted feces from young fish [25]. Moreover, germ-free mice have lived longer than their germ-bearing counterparts [26]. Similar to those animal model studies, several studies have shown that commensal microbes have effects on lifespan in *Drosophila*; however, the effects reported have been contradictory.

In this study, we generated axenic flies by using two methods (egg bleaching and antibiotic treatment) and then determined their lifespans. Interestingly, the lifespan of Ax flies was shorter than that of Conv flies after egg bleaching (Figure 1), which indicates that bleaching can have a detrimental effect on lifespan since the effect was diminished as the number of generations of Ax flies increased. In addition, the lifespan of Ax flies was similar to that of Conv flies after antibiotic treatment at a dosage equal to that used by Ren *et al*. (2007) (Figure 2). However, that antibiotic dose appeared to have a toxic effect on Ax flies since dilution of the antibiotic cocktail increased the lifespan of Ax flies, resulting in a longer lifespan in Ax flies than that in Conv flies. Egg bleaching and antibiotic treatment are commonly used methods to generate Ax flies; regardless, our results suggest that the toxic effects of both of those methods should be considered when generating Ax flies. Most authors have not provided information about which fly generation after egg bleaching they used in their studies [11–13, 15, 27–29], and some tested antibiotics within a very narrow concentration window [11, 12, 17, 27, 28, 30] (see Supplementary Table 12). The inconsistencies in the results reported by different groups on the effect of eliminating microbes on host lifespan might be due to differences in the methods used for microbe elimination.

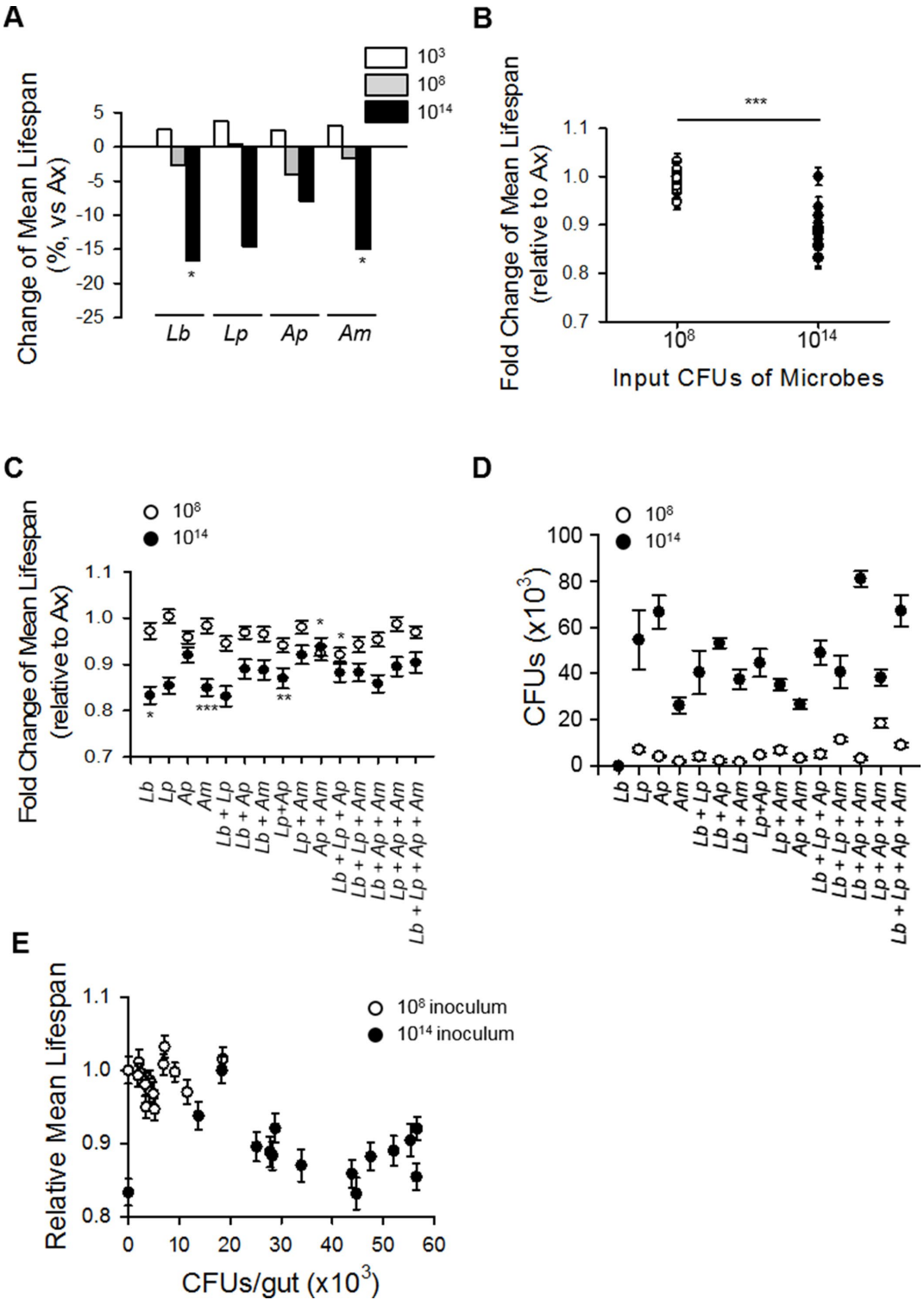

Figure 5. Increased microbial abundance shortens fly lifespan regardless of microbe composition. (**A**) Change in mean lifespan of Ax flies inoculated with single species of the dominant microbes at 10^3, 10^8, or 10^{14} CFUs. Asterisks indicate significant differences compared to Ax flies. (**B**) Mean lifespan of flies inoculated with combinations of four dominant microbes at 10^8 or 10^{14} CFUs. Asterisk indicates significant differences between 10^8 CFUs and 10^{14} CFUs (Spearman's correlation, rho = −0.79, $p < 0.0001$) (**C**) The mean lifespan of Ax flies inoculated with combinations of four dominant species. Asterisks indicate significant differences compared to Ax flies; all groups with 10^{14} CFUs are significantly reduced compared to Ax flies (log-rank test, *$p < 0.05$, ***$p < 0.0001$). (**D**) The CFUs of Ax flies inoculated with combinations of four dominant microbe species. (**E**) Mean lifespan of Ax flies inoculated with combinations of four dominant species as functions of the abundance of the colonized microbe. Abundance of microbes and mean lifespan of flies were negatively correlated (Spearman's correlation, rho = −0.57, $p < 0.05$). Error bars represent the SEM.

The above-mentioned inconsistencies among results might also be due to the distinct microbial flora within the flies of each laboratory. Flies reared under the same conditions in the same laboratory can have different microbial flora, depending on their genetic background [21]. In this study, we observed that microbial loads were different among the three tested *Drosophila* strains, and the strains colonized with a higher level of commensal bacteria exhibited a greater increase in lifespan following bacteria elimination than that in strains with a lower level of commensal bacteria (Figure 3), suggesting that differential bacterial loads within the flies of each laboratory can give rise to differences in lifespan changes after bacterial elimination.

Under our experimental conditions, abundance of commensal microbes was a more important factor than the composition of the microbiota in lifespan determination of *Drosophila.* Ingestion of diluted old fly homogenate had an effect on lifespan that was similar to that of undiluted young fly homogenate, and the ingestion of young fly homogenate decreased the lifespan of flies in a dose-dependent manner (Figure 4). In addition, gnotobiotic flies colonized with a combination of four dominant bacteria showed that the abundance of commensal microbes exerted a significant influence on fly lifespan, whereas the composition of the microbiota exerted only a moderate effect on lifespan (Figure 5). Consistent with our results, several studies have reported on the effect of commensal microbial load on host lifespan. Flies fed homogenate from old flies have been shown to have a decreased lifespan compared to that in flies fed young fly homogenate [15]. Moreover, an increase in microbiota diversity has been correlated with an increase in bacterial load, resulting in a lifespan decrease [31]. In addition, mutant flies lacking the POU domain transcription factor Nub-PD had a shorter lifespan than control flies with a high diversity and high abundance microbiome [30], while flies treated with rapamycin exhibited delayed microbial expansion with age and had an extended lifespan [32].

Our study was the first to undertake a comparative analysis of the relative effects of microbiota load and microbiota composition on *Drosophila* lifespan. Our results show that microbiota load has a greater influence than microbiota composition on the lifespan of *Drosophila*; however, we cannot exclude the possibility that a specific bacterial species proliferates faster than others which could affect the lifespan of axenic flies. In addition, we cannot exclude the possibility that other factors can also affect the host's lifespan, including microbial diversity and species specificity. There are several reports showing age-related increases in bacterial load and its deleterious effect on health, and there have been trials investigating the modulation of organismal health and lifespan by specific microbes. For example, *Bifidobacterium animalis lactis* has promoted longevity and reduced tumor incidence in mice [33], and *Lactobacillus salivarius* isolated from a centenarian's fecal samples extended the lifespan of *C. elegans* [34]. In addition, many bacteria have been shown to have species- or strain-specific effects on host health. *L. plantarum* was shown to affect systemic larval growth in *Drosophila* in a strain-specific manner [3]. Moreover, *L. plantarum* induces dNox dependent cellular ROS production, but that effect was not observed with other members of the microbiota [35]. In addition, *L. brevis* and *G. morbifer* can induce chronic DUOX activation via uracil production, while other commensal microbes cannot [36]. Consistent with those observations, our results showed that colonization of the most dominant microbes combinations at 10^8 CFUs did not have a significant effect on lifespan, but inoculation of *Ap+Am* or *Lb+Lp+Ap* decreased lifespan (Figure 5C, Supplementary Figure 5, Supplementary Table 5–7, ANOVA, $p < 0.0005$; Tukey's HSD *vs.* Ax, Ax 85.59 ± 0.97 days; *Ap+Am* 79.08 ± 1.31 days, 7.61% decrease, $p < 0.05$; *Lb+Lp+Ap* 78.80 ± 1.28 days, 7.93% decrease, $p < 0.05$), even though the final concentrations of the inocula were equal (Figure 5D, Supplementary Table 10, Tukey's HSD *vs*. each group, $p > 0.05$). Moreover, although the bacteria decreased lifespan in all species combinations at high concentration (10^{14} CFUs), the extent of the decrease was different with the different microbe combinations (Figure 5C, Supplementary Table 5, Supplementary Tables 8, 9, ANOVA, $p < 0.0005$; Ax 76.68 ± 1.58 days; *Lb* 63.89 ± 1.42 days, 16.80% decrease, Tukey's HSD test, $p < 0.05$; *Am* 65.17 ± 1.42 days, 15.01% decrease, Tukey's HSD test, $p < 0.005$; *Lp+Ap* 66.72 ± 1.70 days, 12.99% decrease, Tukey's HSD test, $p < 0.005$), indicating that there may be species-, strain-, or combination-specificity involved in the microbiota effects on lifespan regulation.

In addition, we cannot exclude the role of minor bacteria in lifespan regulation. In this study, we selected the four dominant species (the top two species in each age group) for our microbial combination study. Among the subdominant species, our 10-day-old flies contained *K. medellinensis*, and there was a high proportion presence of *L. pseudomesenteroides* in our 50-day-old flies. *K. medellinensis* (also called *Gluconacetobacter xylinus*) is reported to reduce triglyceride and glucose levels in *Drosophila* [37], and a reduced glucose level has been shown in flies with a low insulin-like peptide level, and such flies have been reported to live longer than control flies [38]. *Leuconostoc* negatively interacts with *Lactobacillus* indicating that the two genera occupy the same niche in *Drosophila* [39]; but whether

Leuconostoc regulates lifespan along with *Lactobacillus* has not been reported. Taken collectively, the species-specific or compositional effects of the microbiome on the flies in this study may have been overshadowed by the stronger lifespan effect associated with bacterial abundance.

The mechanism of how commensal bacteria can regulate host lifespan is a fascinating study topic. There are some studies that have described this mechanism in relation to intestinal barrier dysfunction, ROS generation, intestinal stem cell dysplasia, and several lifespan-regulating pathways. Clark *et al.* showed that deleterious changes in a microbiota can induce intestinal barrier dysfunction and is a primary cause of mortality [15, 40]. In addition, the intracellular ROS level has been considered a critical cause of aging [41], and ROS generation induced by an increase in commensal or pathogenic microbes, as a defensive response, has been suggested to affect host lifespan [36, 42]. Furthermore, microbes are reported to regulate intestinal stem cell (ISC) proliferation by inducing ROS generation [23, 43, 44], and the elimination of the microbiota has induced the quiescent stage in ISCs and reduced the number of progenitor cells [43, 44]. Lastly, lifespan-regulating signaling pathways, including the insulin/IGF-1 signaling pathway and the target of rapamycin pathway, have been reported to be regulated by commensal microbes [3, 4].

Taken together, we suggest that when an axenic fly is generated for use in host-microbe interaction study, it should be noted that both egg bleaching and antibiotic treatment can have an adverse effect on the health of the *Drosophila*, but these adverse effects can be eliminated by allowing trans-generation of flies hatched from bleached eggs or by using a suitably diluted antibiotics. In this study, we demonstrated that the age-related increase in bacterial load more strongly affects the lifespan of *Drosophila* than that associated with changes in microbiota composition. Our results present a basic but deterministic connecting point in the relationship between commensal microbes and host lifespan.

METHODS

Fly husbandry and generation of axenic *D. melanogaster*

All experiments, except those related to the strain-specific longevity effect of commensal microbe elimination, were conducted using flies of the *D. melanogaster* wild-type strain w^{1118} that were initially provided by the Bloomington Stock Center (Indiana University, USA) and have been adapting to our laboratory environment for 8 years. The enterobacteria *Wolbachia*, which can affect lifespan, was not present in the strains used in this study as was determined by PCR assay (data not shown). Flies were cultured and reared at 25ºC and 65% humidity on a 12:12 hour light:dark cycle. Sterile standard cornmeal-sugar-yeast (CSY) media (Supplementary Table 13) were used during culture and rearing of the flies. To produce the sterile CSY diet, the above-mentioned CSY medium was autoclaved at 120°C for 20 min, and all vials for food were exposed to UV light for 20 min on a clean bench. For the preparation of antibiotic-treated food, 640 μg/mL doxycycline (Sigma-Aldrich), 640 μg/mL ampicillin (Sigma-Aldrich), and 1 mg/mL kanamycin (Sigma-Aldrich) were added to sterile CSY media [12].

Axenic (Ax) flies were generated by bleaching the embryos. Embryos were collected for 12 h and were then dechorionated for 50 sec in 5% sodium hypochlorite solution (Wako, Japan), rinsed for 50 sec in 70% ethanol, and washed for 1 min in sterile distilled water [15]. Sterile embryos were transferred into sterile CSY food bottles on a clean bench. Eggs in an Ax condition were passed through repeated generations and became third-generation flies. All Ax flies were maintained on a clean bench and were transferred to fresh food every two days. The Ax conditions were confirmed by plating fly homogenate on plate count agar (PCA, Neogen Corporation, MI, USA) (Supplementary Table 13), and by 16S rRNA gene PCR using a bacterial 16S rRNA universal primer (27F and 1492R) provided by Macrogen (Seoul, South Korea).

Bacteria culture

Conventionally, bacteria were cultured on PCA medium. *Lactobacillus* was grown on 5.5% MRS media (*Lactobacilli* MRS Broth, BD & Difco, MD, USA) and *Acetobacter* was grown on *Acetobacter*-selective media (see Supplementary Table 13). All microbes were incubated at 29°C.

Quantitative analysis of bacteria

For CFU determination, 5 females were rinsed in 70% ethanol for 3 sec for surface decontamination and then homogenized in sterile distilled water. The homogenates were diluted as necessary and plated onto PCA media, MRS media, or *Acetobacter*-selective media. At least 5 replicates were established for each group. Data are presented as mean ± standard error of the mean (SEM) values. For 16S rRNA PCR, total genomic DNA from 45 surface-sterilized female flies was extracted by using a DNeasy Tissue Kit (Hilden, Germany) in accordance with the manufacturer's instructions. The PCR assays were performed at a 60°C annealing temperature and for 40–60 cycles using

taxon-specific 16S rRNA gene primers for *Lactobacillus* or *Acetobacter* designed using Primer3 software, as well as universal PCR primers. Sequences are presented in Supplementary Table 14.

Identification of commensal microbes

For commensal microbe isolation, homogenates from 10- or 50-day-old female flies were plated on a PCA media plate. After incubation of a single colony at 29°C for 3 days, each colony was transferred to *Acetobacter*-selective or *Lactobacillus*-selective media broth. After culturing for 24 h, the cell walls of isolated microbes were broken down by bead beating using 0.1 mm diameter glass beads (BioSpec Products, Bartlesville, OK, USA). PCR assays were performed with a 55°C annealing temperature and 45 cycles with universal primers 27F and 1492R. PCR products were sequenced by using 16S sequencing (Macrogen Inc., South Korea) with universal primers 518F and 800R and then analyzed by using EzTaxon BLAST and NCBI BLAST. To determine the dominant commensal bacteria species in the gut of flies, 454 pyrosequencing analysis of the 16S rRNA gene was performed. The 16S rRNA gene amplicons from 100 dissected guts (comprising the Malpighian tubules but excluding the crop) from surface-sterilized females were analyzed by pyrosequencing using the 454 GS FLX Titanium Sequencing System (Roche, Brandford, CT, USA) at Chunlab Inc (South Korea). Phylogenetic relationships were determined by using EzTaxon BLAST and NCBI BLAST.

Introduction of commensal microbes to axenic *D. melanogaster*

To feed fly homogenate to Ax fly, fly homogenates from surface sterile flies with 70% ethanol were seeded in CSY food vials. The Ax flies were transferred to new homogenate-containing vials three times for a week. To generate adult flies carrying a predetermined composition of bacteria (gnotobiotic flies), 100 μL of commensal bacterial cultures (10^3, 10^8, or 10^{14} CFUs) were added to sterilized food vials containing 2-day-old Ax flies. Every 2 days for 1 week, the flies were transferred to new sterile CSY food vials seeded with experiment-specific compositions of commensal bacteria. For commensal bacteria compositions of more than one species, each species component was added in equal parts to make up the total inoculum. The abundances of colonized microbes were identified *via* CFU testing 10 days after the initial infection.

Lifespan assay

Newly eclosed adult female flies were collected for 2 days and were provided with a stabilizing time of 1 day with male flies. Mated female flies were randomly assigned to sterile CSY food vials to a final density of 20 flies per vial. Vials were changed every 2 days for new vials containing fresh sterile CSY food; at that time, dead flies were removed and the number was recorded. In the case of gnotobiotic flies, counting of dead flies was performed after bacterial seeding for 1 week. Ten replicate vials were established for each group (n = 200). Once a month, vials were spot checked for contamination by swabbing the food in the spent vials and plating on PCA-bearing culture plates.

Statistical analysis

Log-rank tests were carried out to determine the statistical significance of the results of the survival analysis. The JMP statistical package (SAS, NC, USA) was used for the analyses. The statistical probabilities of the obtained CFU and OTU numbers were determined by using the two-sample t-test. ANOVA, Tukey's HSD test, and Spearman's correlation coefficients were derived by using R 3.5.1 software.

AUTHOR CONTRIBUTIONS

H.Y., S.H., and K.J. designed the research; H.Y., S.H., and J.H. performed the research; H.Y. and S.H. analyzed the data; H.Y., S.H., W.J., and K.J. wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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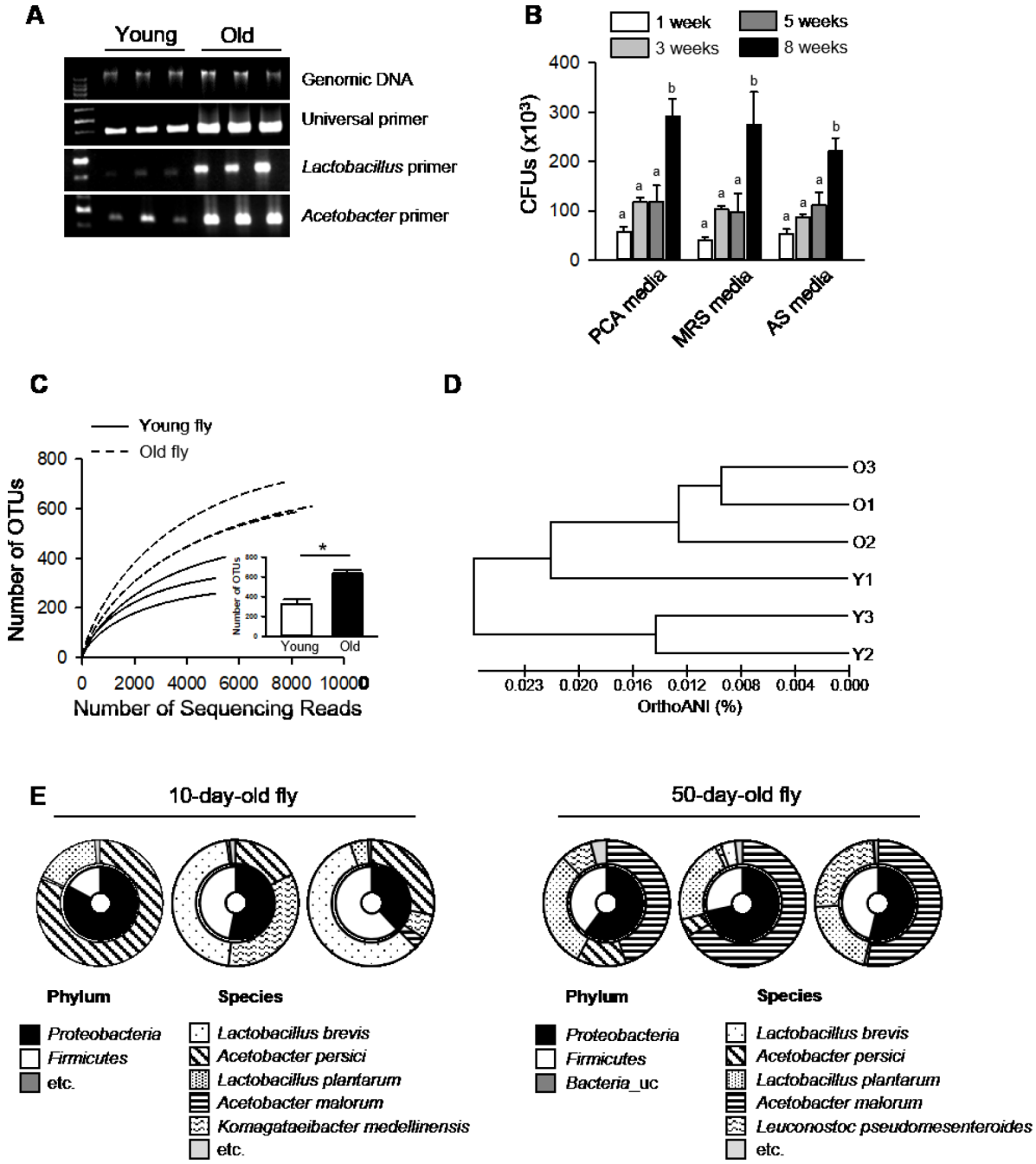
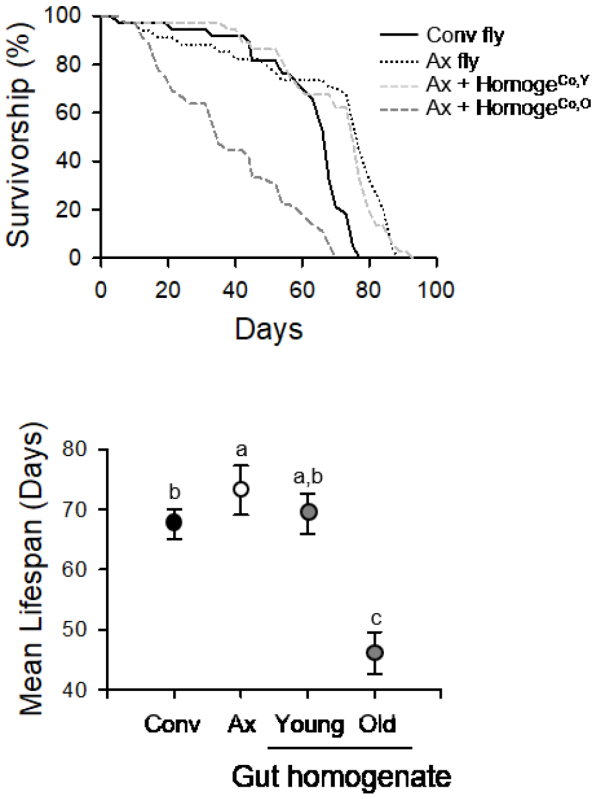
SUPPLEMENTARY MATERIALS

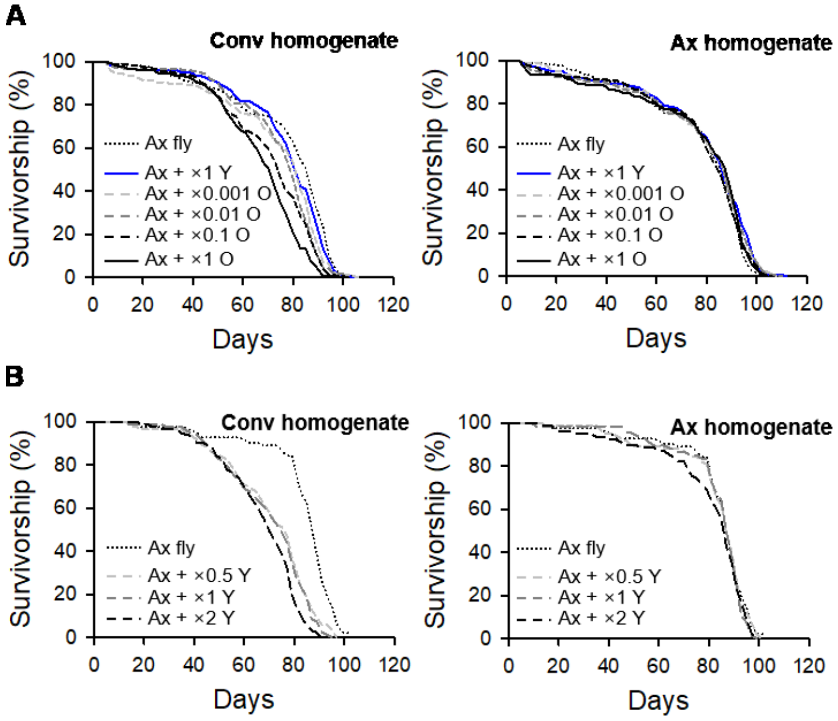
Supplementary Figures



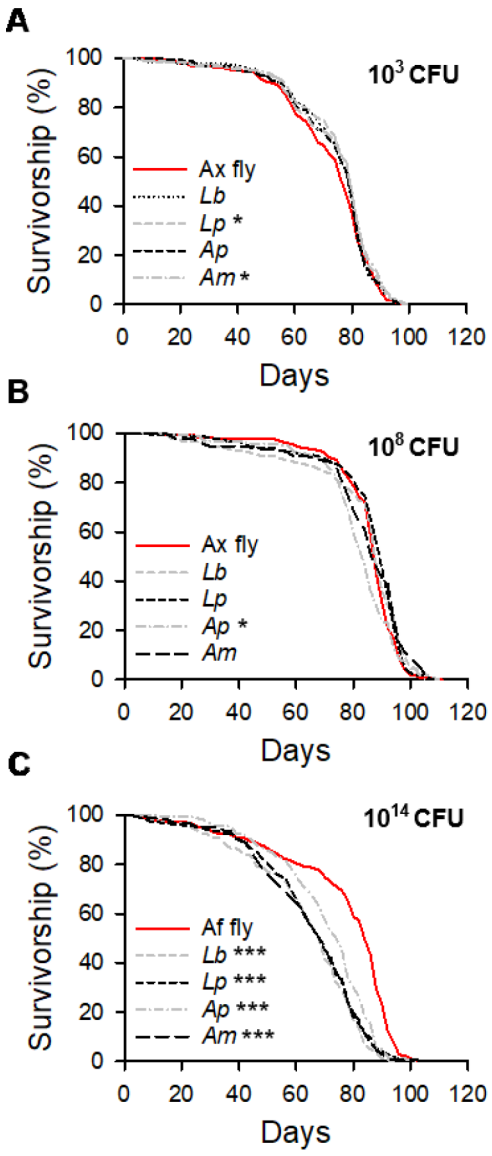
Supplementary Figure 1. Age-related changes in intestinal microbe flora. (**A**) PCR assay of microbial 16S rRNA amplified gene using universal (27F, 1492R), *Acetobacter*-, or *Lactobacillus*-specific primers. Microbial 16S rDNA gene sequences were amplified from genomic DNA extracted from 10-day-old flies (young; lanes 2 to 4) or 50-day-old flies (old; lanes 5 to 7). (**B**) The total number of CFU from 1-, 3-, 5-, and 8-week-old flies in PCA, MRS, or *Acetobacter*-selective media plates. Error bars represent the SEM. Different letters indicate significant differences between groups (ANOVA test, tukey's HSD test). (**C**) Rarefaction curves and the assigned number of operational taxonomic units (OTUs) from 454-pyrosequencing data. The inset shows the average number of OTUs in each group. Error bars represent the SEM. Asterisk indicates significant difference between the numbers of OTUs of young fly and old fly (*t*-test, $*p < 0.05$). (**D**) Dendrogram using microbial flora analyzed by 454-pyrosequencing of 16S rRNA gene. (**E**) Double pie charts of three replicate about bacteria compositions of young or old fly guts. These charts show major phylum and species analyzed by 454-pyrosequencing of 16S rRNA gene.



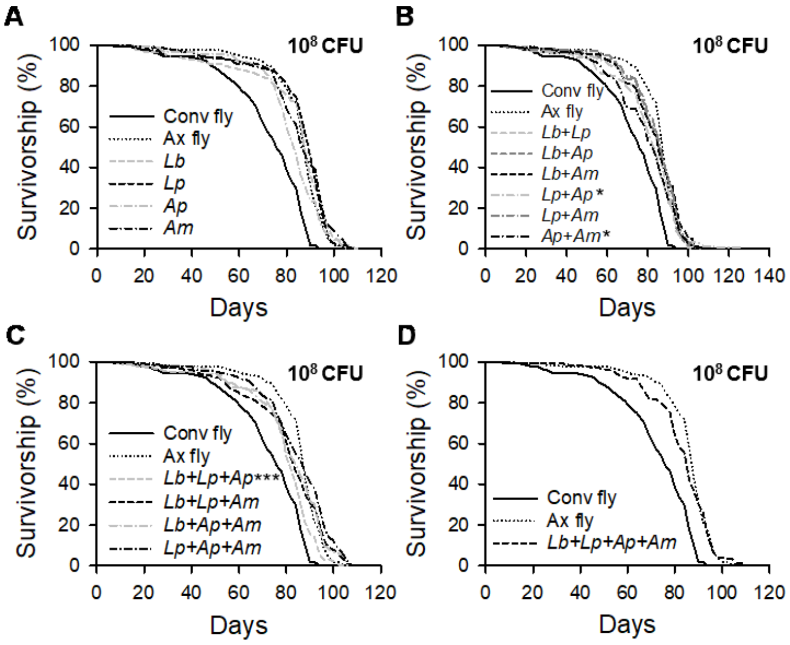
Supplementary Figure 2. Lifespan of flies fed gut homogenates from old conventional flies is shorter than that of flies fed gut homogenates from young conventional flies. The survival curve (upper) and mean lifespan (lower) of conventional (Conv) flies or axenic (Ax) flies fed gut homogenates from 10-day-old (young) or 50-day-old (old) flies. Solid line indicates Conv flies and dashed lines indicate Ax flies. Homoge^{Co,Y} indicates guts from young conventionally reared flies and Homoge^{Co,O} indicates guts from old conventionally reared flies. The lifespan of flies fed the gut from old flies was decreased more compared to that of flies fed the gut from young flies. Different letters indicate significant differences between groups (log-rank test, $p < 0.05$).



Supplementary Figure 3. Increased bacterial load shortens the lifespan of flies. (**A**) Survival curve of Ax flies after feeding diluted homogenate from old flies. (**B**) Survival curve of Ax flies after feeding concentrated homogenates from young flies.



Supplementary Figure 4. Survival curve of flies inoculated with four dominant bacterial species at 10^3,10^8, or 10^{14} CFUs. The survival curve of flies inoculated with mono-association at 10^3 (**A**), 10^8 (**B**), or 10^{14} CFU (**C**). Asterisks indicate significant differences compared to Ax flies (log-rank test, *$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$).



Supplementary Figure 5. Survival curve of flies inoculated with one of 2^4 combinations of the four dominant species at 10^8 CFUs. The survival curve of flies inoculated with mono- (**A**), dual- (**B**), triple- (**C**), and quadruple- (**D**) association at 10^8 CFU. Asterisks indicate significant differences compared to Ax flies (log-rank test, *$p < 0.05$, ***$p < 0.0001$).

Supplementary Tables

Supplementary Table 1. Lifespan of axenic flies.

Group		n	Mean Lifespan (Day)		Median Lifespan (Day)	Maximum Lifespan¶ (Day)	Log-rank		Wilcoxon	
Fly	Treat						χ^2	*p*-value	χ^2	*p*-value
Conv	DW†	191	59.92	± 1.52	70	76				
Ax	1G	179	53.83	± 1.31	58	66	38.85	<0.0001*	20.81	<0.0001*
	2G	190	68.52	± 0.94	72	74	0.08	0.7710	8.91	0.00288*
	3G	173	70.73	± 0.85	72	76	8.20	0.0042*	18.93	<0.0001*
Conv		180	65.41	± 1.54	72	80	20.42	<0.0001*	13.92	0.0002*
Ax†		191	73.00	± 1.40	76	84				
Ax + Feces^{Conv}		174	68.15	± 1.30	74	80	22.61	<0.0001*	13.72	0.0002*
Conv	×0 AB†	193	62.73	± 1.32	69	77				
	×0.1 AB	184	68.93	± 1.16	71	81	12.33	0.0004*	11.02	0.0009*
	×0.5 AB	186	67.84	± 0.99	69	77	2.86	0.0911	3.59	0.058
	×1.0 AB	180	62.89	± 1.21	64	73	0.53	0.4659	0.48	0.4898
Ax	×0 AB†	183	77.17	± 1.32	83	87				
	×0.1 AB	184	77.44	± 1.34	85	87	1.41	0.2347	0.71	0.4004
	×0.5 AB	192	77.97	± 1.06	81	87	0.64	0.4238	0.24	0.6254
	×1.0 AB	180	65.94	± 1.34	69	79	64.01	<0.0001*	63.64	<0.0001*
w^{1118}	Conv†	189	70.36	± 1.59	75	87	146.33	<0.0001*	146.66	<0.0001*
	Ax	188	92.88	± 0.94	96	100				
Oregon-R	Conv†	155	71.18	± 1.86	75	94	37.43	<0.0001*	26.41	<0.0001*
	Ax	182	83.15	± 1.69	90	98				
Canton-S	Conv†	188	79.26	± 1.30	82	92	6.60	0.0102*	2.22	0.136
	Ax	183	81.66	± 1.47	85	96				

† These letters indicate a control for statistical analysis.

¶ Maximum lifespan means average of the last 25% of surviving flies.

* Asterisks indicate significant differences compared to control.

Supplementary Table 2. Richness and diversity estimation of the 16S rRNA gene libraries from the 454-pyrosequencing analysis.

Sample	Valid reads	OTUs[a]	Species richness indices		Species diversity indices		
			Ace	Chao1	JackKnife	Shannon	Simpson
Young #1	5,092	258	301	279	318	3	0
Young #2	5,473	406	523	484	543	3	0
Young #3	5,093	320	371	347	395	3	0
Old #1	7,704	707	844	781	890	4	0
Old #2	8,781	610	739	685	776	4	0
Old #3	8,294	589	697	656	743	4	0

[a] The operational taxonomic units (OTUs) were defined with pairwise 97% ID.

Please browse Full Text version to see the data of Supplementary Table 3:

Supplementary Table 3. Bacteria listed by 454-Pyrosequencing.

Supplementary Table 4. Lifespan of Ax flies fed Conv homogenate.

Group		n	Mean Lifespan (Day)	Median Lifespan (Day)	Maximum Lifespan[¶] (Day)	Log-rank		Wilcoxon	
Fly	Treat					χ^2	*p*-value	χ^2	*p*-value
Conv	DW	101	62.74 ± 2.08	66	80	45.98	<0.0001*	51.93	<0.0001*
	DW[†]	95	82.64 ± 2.05	86	92				
Ax	Homogenate^{Co,Y}	90	75.93 ± 2.09	80	88	4.61	<0.0318*	9.77	0.0018*
	Homogenate^{Co,O}	103	64.46 ± 2.32	70	84	43.12	<0.0001*	42.91	<0.0001*
	Homogenate^{Ax,Y}	94	82.39 ± 2.06	86	92	2.43	0.1192	0.40	0.5281
	Homogenate^{Co,O}	99	79.14 ± 2.21	82	90	0.49	0.4851	2.72	0.0991
Conv	DW	38	67.55 ± 2.53	74	76	20.78	<0.0001*	11.26	0.0008*
	DW[†]	34	73.18 ± 4.11	83	90				
Ax	Gut Homogenate^{Co,Y}	38	75.47 ± 2.89	82	85	0.02	0.8786	0.10	0.7470
	Gut Homogenate^{Co,O}	36	44.22 ± 3.27	41	60	38.41	<0.0001	26.56	<0.0001
Ax	DW	193	77.69 ± 1.53	87	92	9.37	0.0022*	6.21	0.0127*
	×1 Young[†]	188	76.96 ± 1.42	83	92				
	×0.001 Old	199	72.37 ± 1.71	83	87	2.30	0.1293	2.57	0.1090
	×0.01 Old	199	74.33 ± 1.32	80	87	8.35	0.0038*	5.47	0.0193*
	×0.1 Old	195	70.37 ± 1.38	75	85	20.06	<0.0001*	17.46	<0.0001*
	×1 Old	189	66.74 ± 1.34	73	80	55.63	<0.0001*	45.34	<0.0001*
Ax	DW	83	83.92 ± 1.82	89	93	50.50	<0.0001*	45.52	<0.0001*
	×0.5 Young	86	70.52 ± 1.99	77	83	0.61	0.4356	0.13	0.7164
	×1 Young[†]	84	69.98 ± 1.87	75	83				
	×2 Young	95	67.28 ± 1.63	70	79	5.40	0.0202*	2.23	0.1356

[†] These letters indicate a control for statistical analysis.

[¶] Maximum lifespan means average of the last 25% of surviving flies.

* Asterisks indicate significant differences compared to control.

Supplementary Table 5. Lifespan of Ax flies inoculated with dominant microbe.

Group			n	Mean Lifespan (Day)		Median Lifespan (Day)	Maximum Lifespan¶ (Day)	Log-rank		Wilcoxon	
Conc	Fly	Inoculum						χ^2	*p*-value	χ^2	*p*-value
10^3 CFU	Ax	DW†	188	72.69	± 1.17	78	82				
		Lb	214	74.51	± 1.06	80	84	1.36	0.2428	1.95	0.1630
		Lp	193	75.40	± 1.14	80	84	4.99	0.0255*	4.66	0.0309*
		Ap	173	74.40	± 1.17	80	84	2.10	0.1475	1.69	0.1937
		Am	181	74.97	± 1.22	80	84	6.11	0.0134*	3.97	0.0463*
10^8 CFU	Ax	DW†	179	85.59	± 0.97	88	92				
	Mono	*Lb*	165	83.22	± 1.53	88	96	2.45	0.1173	0.42	0.5171
		Lp	154	85.89	± 1.27	90	94	3.50	0.0614	3.72	0.0539
		Ap	166	82.07	± 1.15	84	90	3.61	0.0575	11.59	0.0007*
		Am	173	84.14	± 1.38	88	96	2.89	0.0890	0.04	0.8468
	Dual	*Lb+Lp*	171	80.91	± 1.31	86	92	2.47	0.1162	5.73	0.0167*
		Lb+Ap	173	82.91	± 1.14	86	94	0.87	0.3515	2.57	0.1089
		Lb+Am	167	82.64	± 1.29	86	94	0.00	0.9579	2.40	0.1217
		Lp+Ap	173	80.54	± 1.30	84	90	5.72	0.0168*	15.50	<0.0001*
		Lp+Am	156	83.90	± 1.19	87	94	0.04	0.8477	0.97	0.3243
		Ap+Am	164	79.08	± 1.31	84	90	5.94	0.0148*	16.32	<0.0001*
	Triple	*Lb+Lp+Ap*	173	78.80	± 1.28	82	88	22.64	<0.0001	30.15	<0.0001*
		Lb+Lp+Am	168	80.72	± 1.41	84	94	0.25	0.6197	6.46	0.0111*
		Lb+Ap+Am	170	81.62	± 1.32	85	94	0.07	0.7988	4.72	0.0299*
		Lp+Ap+Am	156	84.49	± 1.32	88	96	3.65	0.0561	0.00	0.9577
	Quadruple	*Lb+Lp+Ap+Am*	169	83.00	± 1.11	86	94	0.95	0.329	4.78	0.0288
10^{14} CFU	Ax	DW†	180	76.68	± 1.58	84	90				
	Mono	*Lb*	185	63.89	± 1.42	68	78	92.74	<0.0001*	66.19	<0.0001*
		Lp	189	65.53	± 1.38	70	78	65.55	<0.0001*	54.68	<0.0001*
		Ap	176	70.56	± 1.26	76	84	39.09	<0.0001*	30.50	<0.0001*
		Am	173	65.17	± 1.42	70	78	65.64	<0.0001*	54.64	<0.0001*
	Dual	*Lb+Lp*	199	63.74	± 1.65	68	82	50.35	<0.0001*	46.33	<0.0001*
		Lb+Ap	190	68.25	± 1.56	76	84	39.02	<0.0001*	31.44	<0.0001*
		Lb+Am	187	68.11	± 1.62	76	84	33.92	<0.0001*	30.11	<0.0001*
		Lp+Ap	174	66.72	± 1.70	74	82	39.66	<0.0001*	36.25	<0.0001*
		Lp+Am	183	70.59	± 1.54	78	84	16.61	<0.0001*	19.05	<0.0001*
		Ap+Am	183	71.91	± 1.51	78	86	13.60	0.0002*	13.00	0.0003*
	Triple	*Lb+Lp+Ap*	192	67.64	± 1.51	72	82	52.62	<0.0001*	40.14	<0.0001*
		Lb+Lp+Am	189	67.72	± 1.50	74	84	41.68	<0.0001*	35.58	<0.0001*
		Lb+Ap+Am	183	65.84	± 1.45	70	80	62.75	<0.0001*	49.17	<0.0001*
		Lp+Ap+Am	181	68.67	± 1.56	74	84	17.92	<0.0001*	22.87	<0.0001*
	Quadruple	*Lb+Lp+Ap+Am*	190	69.34	± 1.67	78	86	20.53	<0.0001*	17.66	<0.0001*

† These letters indicate a control for statistical analysis.

¶ Maximum lifespan means average of the last 25% of surviving flies.

* Asterisks indicate significant differences compared to control.

Supplementary Table 6. Log-rank test result of survival with a combination of microbes (10^8 CFUs).

p-value (10^8 CFUs)		Conv	Ax	Mono-association				Dual-association						Triple-association				Quadruple-association
				Lb	*Lp*	*Ap*	*Am*	*Lb+Lp*	*Lb+Ap*	*Lb+Am*	*Lp+Ap*	*Lp+Am*	*Ap+Am*	*Lb+Lp +Ap*	*Lb+Lp +Am*	*Lb+Ap +Am*	*Lp+Ap +Am*	*Lb+Lp +Ap+Am*
Conv		1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Ax		<0.0001	1	0.1173	0.0614	0.0575	0.089	0.1162	0.3515	0.9579	0.0168	0.8477	0.0148	<0.0001	0.6197	0.7988	0.0561	0.329
Mono-association	*Lb*	<0.0001	0.1173	1	0.9847	0.0027	0.9243	0.0036	0.0138	0.1617	0.0019	0.0849	0.0006	<0.0001	0.0949	0.1308	0.573	0.0310
	Lp	<0.0001	0.0614	0.9847	1	0.0014	0.8059	0.0021	0.0071	0.1658	0.0002	0.0688	0.0004	<0.0001	0.0961	0.0969	0.4865	0.0163
	Ap	<0.0001	0.0575	0.0027	0.0014	1	0.0013	0.9144	0.4652	0.1869	0.8305	0.202	0.4248	0.0216	0.2611	0.1923	0.0022	0.3033
	Am	<0.0001	0.089	0.9243	0.8059	0.0013	1	0.0008	0.0044	0.0612	0.0017	0.052	0.0002	<0.0001	0.0948	0.1009	0.6675	0.0394
Dual-association	*Lb+Lp*	<0.0001	0.1162	0.0036	0.0021	0.9144	0.0008	1	0.577	0.0951	0.4639	0.1574	0.4751	0.0119	0.234	0.2266	0.0008	0.5315
	Lb+Ap	<0.0001	0.3515	0.0138	0.0071	0.4652	0.0044	0.577	1	0.1803	0.2086	0.4883	0.2246	0.0009	0.5509	0.5006	0.0047	0.8503
	Lb+Am	<0.0001	0.9579	0.1617	0.1658	0.1869	0.0612	0.0951	0.1803	1	0.0765	0.7014	0.0323	0.0001	0.9461	0.9233	0.0453	0.5918
	Lp+Ap	<0.0001	0.0168	0.0019	0.0002	0.8305	0.0017	0.4639	0.2086	0.0765	1	0.0943	0.6897	0.0683	0.2806	0.1923	0.0027	0.2054
	Lp+Am	<0.0001	0.8477	0.0849	0.0688	0.202	0.052	0.1574	0.4883	0.7014	0.0943	1	0.0571	0.0001	0.907	0.8301	0.0234	0.6719
	Ap+Am	<0.0001	0.0148	0.0006	0.0004	0.4248	0.0002	0.4751	0.2246	0.0323	0.6897	0.0571	1	0.1428	0.0908	0.0757	0.0002	0.1311
Triple-association	*Lb+Lp+Ap*	<0.0001	<0.0001	<0.0001	<0.0001	0.0216	<0.0001	0.0119	0.0009	0.0001	0.0683	0.0001	0.1428	1	0.0012	0.0007	<.0001	0.0009
	Lb+Lp +Am	<0.0001	0.6197	0.0949	0.0961	0.2611	0.0948	0.234	0.5509	0.9461	0.2806	0.907	0.0908	0.0012	1	0.9247	0.0548	0.9211
	Lb+Ap +Am	<0.0001	0.7988	0.1308	0.0969	0.1923	0.1009	0.2266	0.5006	0.9233	0.1923	0.8301	0.0757	0.0007	0.9247	1	0.0592	0.7937
	Lp+Ap +Am	<0.0001	0.0561	0.573	0.4865	0.0022	0.6675	0.0008	0.0047	0.0453	0.0027	0.0234	0.0002	<.0001	0.0548	0.0592	1	0.0338
Quadruple-association	*Lb+Lp +Ap +Am*	<0.0001	0.329	0.0310	0.0163	0.3033	0.0394	0.5315	0.8503	0.5918	0.2054	0.6719	0.1311	0.0009	0.9211	0.7937	0.0338	1

Supplementary Table 7. ANOVA and Tukey's HSD test results for fly lifespans treated with a combination of microbes (10^8 CFUs).

ANOVA

	Sum of sqrs	Df	Mean Square	F	*p*-value
Between groups	11103	15	740.2	2.734	0.000343
Residuals	720387	2661	270.7		

Tukey's HSD (Honestly Significant Difference) test

p-value (10^8 CFUs)		Ax	Mono-association				Dual-association						Triple-association				Quadruple-association
			Lb	*Lp*	*Ap*	*Am*	*Lb+Lp*	*Lb+Ap*	*Lb+Am*	*Lp+Ap*	*Lp+Am*	*Ap+Am*	*Lb+Lp +Ap*	*Lb+Lp +Am*	*Lb+Ap +Am*	*Lp+Ap +Am*	*Lb+Lp +Ap+Am*
Ax		1	0.994681	1.000000	0.833558	0.999983	0.357371	0.979056	0.955049	0.226635	0.999914	0.022988	0.010642	0.295254	0.659396	1.000000	0.985834
Mono-association	*Lb*	0.994681	1	0.987869	1	1	0.996339	1	1	0.982957	1	0.635314	0.492619	0.991913	0.999955	0.999999	1
	Lp	1	0.9878685	1	0.782016	0.999883	0.316062	0.961808	0.927661	0.199458	0.999595	0.020921	0.009965	0.260160	0.600623	0.999996	0.972496
	Ap	0.833558	0.9999995	0.782016	1	0.998904	0.999999	1	1	0.999973	0.999811	0.958406	0.904932	0.999995	1	0.995235	1
	Am	0.999983	1	0.999883	0.998904	1	0.909980	0.999998	0.999979	0.807035	1	0.257200	0.161460	0.867783	0.990172	1	1
Dual-association	*Lb+Lp*	0.357371	0.9963387	0.316062	0.999999	0.909980	1	0.999204	0.999875	1	0.960528	0.999755	0.998445	1	1	0.843913	0.998743
	Lb+Ap	0.979056	1	0.961808	1	0.999998	0.999204	1	1	0.99456	1	0.742384	0.606355	0.997838	0.999997	0.999965	1
	Lb+Am	0.955049	1	0.927661	1	0.999979	0.999875	1	1	0.99868	0.999999	0.842333	0.730064	0.999568	1	0.999778	1
	Lp+Ap	0.226635	0.9829566	0.199458	0.999973	0.807035	1	0.99456	0.998677	1	0.897808	0.999986	0.999836	1	1	0.715293	0.992377
	Lp+Am	0.999914	1	0.999595	0.999811	1	0.960528	1	0.999999	0.897808	1	0.383468	0.263260	0.936032	0.997351	1	1
	Ap+Am	0.022988	0.6353135	0.020921	0.958406	0.2572	0.999755	0.742384	0.842333	0.999986	0.383468	1	1	0.999942	0.990361	0.195019	0.715643
	Lb+Lp+Ap	0.010642	0.4926191	0.009965	0.904932	0.16146	0.998445	0.606355	0.730064	0.999836	0.26326	1	1	0.999514	0.970238	0.119008	0.577168
Triple-association	*Lb+Lp +Am*	0.295254	0.9919125	0.26016	0.999995	0.867783	1	0.997838	0.999568	1	0.936032	0.999942	0.999514	1	1	0.788867	0.996799
	Lb+Ap +Am	0.659396	0.9999554	0.600623	1	0.990172	1	0.999997	1	1	0.997351	0.990361	0.970238	1	1	0.972992	0.999993
	Lp+Ap +Am	1	0.9999985	0.999996	0.995235	1	0.843913	0.999965	0.999778	0.715293	1	0.195019	0.119008	0.788867	0.972992	1	0.999985
Quadruple-association	*Lb+Lp +Ap +Am*	0.985834	1	0.972496	1	1	0.998743	1	1	0.992377	1	0.715643	0.577168	0.996799	0.999993	0.999985	1

Supplementary Table 8. Log-rank test result of survival with a combination of microbes (10^{14} CFUs).

p-value (10^{14} CFUs)		Conv	Ax	Mono-association				Dual-association						Triple-association				Quadruple-association
				Lb	***Lp***	***Ap***	***Am***	***Lb+Lp***	***Lb+Ap***	***Lb+Am***	***Lp+Ap***	***Lp+Am***	***Ap+Am***	***Lb+Lp +Ap***	***Lb+Lp +Am***	***Lb+Ap +Am***	***Lp+Ap +Am***	***Lb+Lp +Ap+Am***
Conv		1	<0.0001	<0.0001	0.0062	0.8377	0.0058	0.1285	0.771	0.9889	0.5488	0.1516	0.0397	0.2226	0.5122	0.0247	0.408	0.1009
Ax		<0.0001	1	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Mono-association	*Lb*	<0.0001	<0.0001	1	0.2541	0.0002	0.3553	0.0330	<0.0001	<0.0001	0.0015	<0.0001	<0.0001	0.0043	0.0009	0.0780	<0.0001	<0.0001
	Lp	0.0062	<0.0001	0.2541	1	0.0098	0.8710	0.4144	0.0162	0.0067	0.0560	<0.0001	<0.0001	0.0959	0.0379	0.6300	0.0018	0.0002
	Ap	0.8377	<0.0001	0.0002	0.0098	1	0.0133	0.1600	0.9552	0.7885	0.6910	0.1304	0.0176	0.3251	0.6974	0.0486	0.3251	0.0494
	Am	0.0058	<0.0001	0.3553	0.8710	0.0133	1	0.2910	0.0081	0.0047	0.0451	<.0001	<0.0001	0.0949	0.0318	0.5329	0.0018	<.0001
Dual-association	*Lb+Lp*	0.1285	<0.0001	0.0330	0.4144	0.1600	0.2910	1	0.1752	0.0847	0.2913	0.0023	0.0002	0.5869	0.3166	0.5590	0.0153	0.0020
	Lb+Ap	0.771	<0.0001	<0.0001	0.0162	0.9552	0.0081	0.1752	1	0.7121	0.6413	0.0766	0.0171	0.2926	0.7354	0.0491	0.2346	0.1067
	Lb+Am	0.9889	<0.0001	<0.0001	0.0067	0.7885	0.0047	0.0847	0.7121	1	0.4702	0.1447	0.0546	0.1859	0.5345	0.0223	0.4177	0.2066
	Lp+Ap	0.5488	<0.0001	0.0015	0.0560	0.6910	0.0451	0.2913	0.6413	0.4702	1	0.0526	0.0091	0.5111	0.9218	0.1221	0.2115	0.0359
	Lp+Am	0.1516	<0.0001	<0.0001	<0.0001	0.1304	<0.0001	0.0023	0.0766	0.1447	0.0526	1	0.7537	0.0078	0.0421	0.0003	0.4952	0.8921
	Ap+Am	0.0397	0.0002	<0.0001	<0.0001	0.0176	<0.0001	0.0002	0.0171	0.0546	0.0091	0.7537	1	0.0006	0.0087	<.0001	0.3796	0.3732
Triple-association	*Lb+Lp+Ap*	0.2226	<0.0001	0.0043	0.0959	0.3251	0.0949	0.5869	0.2926	0.1859	0.5111	0.0078	0.0006	1	0.5501	0.2559	0.0688	0.0031
	Lb+Lp +Am	0.5122	<0.0001	0.0009	0.0379	0.6974	0.0318	0.3166	0.7354	0.5345	0.9218	0.0421	0.0087	0.5501	1	0.1200	0.1612	0.0472
	Lb+Ap +Am	0.0247	<0.0001	0.0780	0.6300	0.0486	0.5329	0.5590	0.0491	0.0223	0.1221	0.0003	<0.0001	0.2559	0.1200	1	0.0042	0.0002
	Lp+Ap +Am	0.408	<0.0001	<0.0001	0.0018	0.3251	0.0018	0.0153	0.2346	0.4177	0.2115	0.4952	0.3796	0.0688	0.1612	0.0042	1	0.7844
Quadruple-association	*Lb+Lp +Ap +Am*	0.1009	<0.0001	<0.0001	0.0002	0.0494	<0.0001	0.0020	0.1067	0.2066	0.0359	0.8921	0.3732	0.0031	0.0472	0.0002	0.7844	1

Supplementary Table 9. ANOVA and Tukey's HSD test result of lifespan with a combination of microbes (10^{14} CFUs).

ANOVA

	Sum of sqrs	Df	Mean Square	F	*p*-value
Between groups	31614	15	2107.6	2.962	0.000102
Residuals	2254285	3168	711.6		

Tukey's HSD (Honestly Significant Difference) test

p-value (10^{14} CFUs)		Ax	Mono-association				Dual-association						Triple-association				Quadruple-association
			Lb	*Lp*	*Ap*	*Am*	*Lb+Lp*	*Lb+Ap*	*Lb+Am*	*Lp+Ap*	*Lp+Am*	*Ap+Am*	*Lb+Lp +Ap*	*Lb+Lp +Am*	*Lb+Ap +Am*	*Lp+Ap +Am*	*Lb+Lp +Ap+Am*
Ax		1	0.018132	0.355422	0.398274	0.000243	0.764823	0.973670	0.823822	0.003995	0.956063	0.998188	0.982943	0.882279	0.074832	0.486508	0.998588
Mono-association	*Lb*	0.018132	1	0.999582	0.999181	0.999752	0.963559	0.725966	0.940439	1	0.787346	0.456969	0.675366	0.901177	1	0.997407	0.437559
	Lp	0.355422	0.999582	1	1	0.774434	1	0.999428	1	0.986822	0.999808	0.987151	0.998802	1	1	1	0.984703
	Ap	0.398274	0.999181	1	1	0.733675	1	0.999717	1	0.980349	0.999914	0.991688	0.999367	1	0.999998	1	0.989958
	Am	0.000243	0.999752	0.774434	0.733675	1	0.365608	0.105301	0.301995	1	0.135908	0.034791	0.085921	0.235038	0.987473	0.647719	0.031852
Dual-association	*Lb+Lp*	0.764823	0.963559	1	1	0.365608	1	1	1	0.814224	1	1	1	1	0.998370	1	0.999933
	Lb+Ap	0.97367	0.725966	0.999428	0.999717	0.105301	1	1	1	0.433435	1	1	1	1	0.939380	0.999940	1
	Lb+Am	0.823822	0.940439	0.999999	1	0.301995	1	1	1	0.753798	1	1	1	1	0.996122	1	0.999984
	Lp+Ap	0.003995	1	0.986822	0.980349	1	0.814224	0.433435	0.753798	1	0.503566	0.209144	0.382443	0.672628	0.999984	0.960918	0.196513
	Lp+Am	0.956063	0.787346	0.999808	0.999914	0.135908	1	1	1	0.503566	1	1	1	1	0.962067	0.999985	1
	Ap+Am	0.998188	0.456969	0.987151	0.991688	0.034791	0.999954	1	0.99999	0.209144	1	1	1	1	0.772048	0.996793	1
	Lb+Lp+Ap	0.982943	0.675366	0.998802	0.999367	0.085921	1	1	1	0.382443	1	1	1	1	0.916661	0.999844	1
Triple-association	*Lb+Lp +Am*	0.882279	0.901177	0.999993	0.999998	0.235038	1	1	1	0.672628	1	0.999999	1	1	0.990479	1	1
	Lb+Ap +Am	0.074832	1	1	0.999998	0.987473	0.99837	0.93938	0.996122	0.999984	0.962067	0.772048	0.916661	0.990479	1	0.999985	0.755034
	Lp+Ap +Am	0.486508	0.997407	1	1	0.647719	1	0.99994	1	0.960918	0.999985	0.996793	0.999844	1	0.999985	1	0.995999
Quadruple-association	*Lb+Lp +Ap +Am*	0.998588	0.437559	0.984703	0.989958	0.031852	0.999933	1	0.999984	0.196513	1	1	1	0.999998	0.755034	0.996	1

Supplementary Table 10. ANOVA and Tukey's HSD test result of CFUs from fly's body with a combination of microbes (10^8 CFUs).

ANOVA

	Sum of sqrs	Df	Mean Square	F	*p*-value
Between groups	749375	14	53527	2.838	0.00257
Residuals	1131742	60	18862		

Tukey's HSD (Honestly Significant Difference) test

p-value (10^8 CFUs)		Mono-association				Dual-association						Triple-association			Quadruple-association	
		Lb	*Lp*	*Ap*	*Am*	*Lb+Lp*	*Lb+Ap*	*Lb+Am*	*Lp+Ap*	*Lp+Am*	*Ap+Am*	*Lb+Lp +Ap*	*Lb+Lp +Am*	*Lb+Ap +Am*	*Lp+Ap +Am*	*Lb+Lp +Ap+Am*
Mono-association	*Lb*	1	1	0.1774	0.9998	1	0.0051	0.9997	0.9999	0.9641	0.9981	0.3202	0.9999	0.9547	0.6564	0.8382
	Lp	1	1	0.2586	1	1	0.0091	0.9999	1	0.9880	0.9997	0.4342	1	0.9838	0.7749	0.9160
	Ap	0.1774	0.2586	1	0.7178	0.2978	0.9926	0.7470	0.5230	0.9754	0.8495	1	0.6875	0.9813	1	0.9984
	Am	0.9998	1	0.7178	1	1	0.0701	1	1	1	1	0.8809	1	1	0.9913	0.9994
Dual-association	*Lb+Lp*	1	1	0.2978	1	1	0.0114	0.9999	1	0.9929	0.9999	0.4845	1	0.9901	0.8172	0.9390
	Lb+Ap	0.0051	0.0091	0.9926	0.0701	0.0114	1	0.0788	0.0326	0.2946	0.1235	0.9547	0.0622	0.3202	0.7325	0.5283
	Lb+Am	0.9997	0.9999	0.7470	1	0.9999	0.0788	1	1	1	1	0..8994	1	0.9999	0.9938	0.9996
	Lp+Ap	0.9999	1	0.5230	1	1	0.0326	1	1	0.9998	0.9999	0.7276	1	0.9996	0.9529	0.9922
	Lp+Am	0.9641	0.9880	0.9754	1	0.9929	0.2946	1	0.9998	1	1	0.9971	1	1	1	1
	Ap+Am	0.9981	0.9997	0.8495	1	0.9999	0.1235	1	0.9999	1	1	0.9547	1	1	0.9987	1
Triple-association	*Lb+Lp+Ap*	0.3202	0.4342	1	0.8809	0.4845	0.9547	0.8994	0.7276	0.9971	0.9547	1	0.8605	0.9981	1	1
	Lb+Lp +Am	0.9999	1	0.6875	1	1	0.0622	1	1	1	1	0.8605	1	1	0.9880	0.9990
	Lb+Ap +Am	0.9547	0.9838	0.9813	1	0.9901	0.3202	0.9999	0.9996	1	1	0.9981	1	1	1	1
	Lp+Ap +Am	0.6564	0.7749	1	0.9913	0.8172	0.7325	0.9938	0.9529	1	0.9987	1	0.9880	1	1	1
Quadruple-association	*Lb+Lp +Ap +Am*	0.8382	0.9160	0.9984	0.9994	0.9390	0.5283	0.9996	0.9922	1	1	1	0.9990	1	1	1

Supplementary Table 11. ANOVA and Tukey's HSD test result of CFUs from fly's body with a combination of microbes (10^{14} CFUs).

ANOVA

	Sum of sqrs	Df	Mean Square	F	p-value
Between groups	668496	14	47750	4.901	< 0.0001
Residuals	584590	60	9743		

Tukey's HSD (Honestly Significant Difference) test

p-value (10^{14} CFUs)		Mono-association				Dual-association						Triple-association			Quadruple-association	
		Lb	*Lp*	*Ap*	*Am*	*Lb+Lp*	*Lb+Ap*	*Lb+Am*	*Lp+Ap*	*Lp+Am*	*Ap+Am*	*Lb+Lp +Ap*	*Lb+Lp +Am*	*Lb+Ap +Am*	*Lp+Ap +Am*	*Lb+Lp +Ap+Am*
Mono-association	*Lb*	1	1	0.1824	0.9255	0.9832	0.3352	0.9705	0.2053	0.8912	0.9999	0.9958	<0.0001	0.1450	0.1672	0.4407
	Lp	1	1	0.2531	0.9650	0.9945	0.4354	0.9890	0.2817	0.9438	1	0.9990	<0.0001	0.2053	0.2339	0.5506
	Ap	0.1824	0.2531	1	0.9917	0.9535	1	0.9718	1	0.9960	0.7046	0.8943	0.0652	1	1	1
	Am	0.9255	0.9650	0.9917	1	1	0.9995	1	0.9945	1	0.9999	1	0.0012	0.9832	0.9890	0.9999
Dual-association	*Lb+Lp*	0.9832	0.9945	0.9535	1	1	0.9930	1	0.9650	1	1	1	0.0004	0.9255	0.9438	0.9983
	Lb+Ap	0.3352	0.4354	1	0.9995	0.993	1	0.9968	1	0.9998	0.8779	0.9754	0.0278	1	1	1
	Lb+Am	0.9705	0.9890	0.9718	1	1	0.9968	1	0.9796	1	1	1	0.0006	0.9517	0.9650	0.9994
	Lp+Ap	0.2053	0.2817	1	0.9945	0.9650	1	0.9796	1	0.9976	0.7406	0.9150	0.0563	1	1	1
	Lp+Am	0.8912	0.9438	0.9960	1	1	0.9998	1	0.9976	1	0.9998	1	0.0016	0.9912	0.9945	1
	Ap+Am	0.9999	1	0.7046	0.9999	1	0.8779	1	0.7406	0.9998	1	1	0.0006	0.6344	0.6781	0.9374
Triple-association	*Lb+Lp+Ap*	0.9958	0.9990	0.8943	1	1	0.9754	1	0.9150	1	1	1	0.0002	0.8485	0.8779	0.9917
	Lb+Lp +Am	< 0.0001	<0.0001	0.0652	0.0012	0.0004	0.0278	0.0006	0.0563	0.0016	0.0006	0.0002	1	0.0850	0.0723	0.0170
	Lb+Ap +Am	0.1450	0.2053	1	0.9832	0.9255	1	0.9517	1	0.9912	0.6344	0.8485	0.0850	1	1	1
	Lp+Ap +Am	0.1672	0.2339	1	0.9890	0.9438	1	0.965	1	0.9945	0.6781	0.8779	0.0723	1	1	1
Quadruple-association	*Lb+Lp +Ap +Am*	0.4407	0.5506	1	0.9999	0.9983	1	0.9994	1	1	0.9374	0.9917	0.0170	1	1	1

Please browse Full Text version to see the data of Supplementary Table 12:

Supplementary Table 12. Summary of axenic fly lifespan data in previous reports.

Supplementary Table 13. Composition of fly husbandry food and bacteria incubation media.

Food	Composition
Cornmeal-sugar-yeast (CSY) media	5.2% cornmeal 11% sugar 2.5% instant yeast 0.5% propionic acid 0.04% methyl-4-hydroxybenzoate 1% agar
Plate count agar (PCA)	0.5% tryptone 0.25% yeast extract 0.1% glucose 1.5% bacto agar
Lactobacillus-selective (MRS) media	1% peptone 1% beef extract 0.5% yeast extract 2% dextrose 0.1% polysorbate 0.2% ammonium citrate 0.5% sodium acetate 0.01% magnesium sulfate 0.005% manganese sulfate 0.2% dipotassium phosphate 1.5% bacto agar
Acetobacter-selective media	1.5% bacto agar (BD & Difco) 2.5% D-mannitol (BD & Difco) 0.5% yeast extract (BD & Difco) 0.3% peptone (BD & Difco) 1.5% bacto agar

Supplementary Table 14. 16S rRNA PCR primer sequences.

	Forward (5'-3')	Reverse (5'-3')
Lactobacillus-selective primer	GCAAGGCTGAAACTCAAAGG	TTCATGTAGGCGAGTTGCAG
Acetobacter-selective primer	CCCTTATGTCCTGGGCTACA	TCACCGGCTTAAGGTCAAAC
Universal primer (27F, 1492R)	AGAGTTTGATCMTGGCTCAG	TACGGYTACCTTGTTACGACTT