Journal of High School Science

Analyzing the long-term physiological effects of probiotic microbiota manipulation in the model species *Drosophila melanogaster*

Prazak I

Submitted: June 7, 2024, Revised: version 1, July 7, 2024, version 2, July 9, 2024, version 3, July 10, 2024
Accepted: July 13, 2024

Abstract

Probiotic treatments, a form of microbiota manipulation, are often used in the short term to address and treat illness and disease as a form of reactive medicine. Instead, these probiotics can be administered over the long term to enable preventative prophylaxis. This approach intuitively involves less risk and is more conducive to the host's physiology. In this study, isolated bacterial species *Escherichia coli* K-12, *Bifidobacterium infantis*, and *Akkermansia muciniphila* were administered and tested for their long-term health span effects on model species *Drosophila melanogaster* starting from the first instar larval phase. The effect of these bacteria relative to a control group under similar conditions was compared. To evaluate the bacteria's suitability for long-term microbiota manipulation, statistical analysis was performed on physiological traits, including average lifespan, fecundity rates, temperature stress, and movement/activity. Utilizing a model organism such as *D. melanogaster* in this study served as a crucial step toward translating these findings into practical applications for preventative prophylactic human health. While the direct experimentation of microbiota manipulation in humans poses unknown risks, leveraging model organisms offers researchers a controlled environment that minimizes risk and is an important step toward manipulating microbiota as a practical preventative medicine in humans.

Keywords

Microbiota manipulation, Microbiota engineering, Drosophila melanogaster, Preventative medicine, Microbiome, Host-microbe relationships, Probiotics, Prophylaxis, Dysbiosis, Bacterial colonization

Ian Prazak, Keystone School, 119 E Craig Pl, San Antonio, TX 78212, USA.
iprazak@keystoneschool.org

Introduction

Introducing specific microorganisms into a host species, thereby altering the composition and functions of resident microbes, is known as microbiota manipulation. This field has garnered significant attention due to advancements in isolating bacterial strains, the demonstrated effect of dysbiosis on the etiology and progression of a variety of diseases; including neurodegenerative disorders, metabolic disease and cancer. Microbiome engineering therefore holds potential for improving the health of hosts that undergo this process.

Currently, the most practical applications of microbiota manipulation are short-term treatments for adverse health consequences such as irritable bowel syndrome. However, the long-term application of microbiota manipulation over the lifespan of an organism remains limited to the lab with little clinical application. Still, there is the potential to extend this practice to humans as a practical means of preventing disease and maintaining positive physiological change. Although this prospect is years away from coming into practice, getting accurate knowledge and results from experiments will only accelerate its coming.

Performing experiments that interfere with human physiology carries significant risk. Even when attempting to improve human health through experiments favored to be physiologically beneficial, there is always the probability for adverse consequences. In response, researchers have preferred using model organisms such as *Drosophila melanogaster* (also known as the fruit fly) before testing applications in humans. Experimentation on invertebrates is not as ethically challenging. Furthermore, the significantly shorter lifespan of *D. melanogaster* allow researchers to observe the onset of physiological changes in a shorter time. Additionally, procuring *D. melanogaster* is cost-efficient and accessible, and its relatively simple microbiota composition allows for the effects of microbiota manipulation to be more easily observed (1). Specifically, wild-type (WT) *D. melanogaster*, the natural variant of the species with no artificial genetic changes (2), is dominated by solely three microbiota families; Acetobacteraceae, Lactobacillaceae, and Enterobacteriaceae (3). Thus, introducing just a singular other species can potentially cause a lasting and compounding effect on the longevity of the *D. melanogaster*.

In addition, *D. melanogaster* contains organs that anatomically resemble and function similarly to those of humans. For instance, the alimentary canal of *D. melanogaster* can serve as a surrogate for the gastrointestinal tract of humans. The alimentary canal, specifically in one of its parts known as the midgut, hosts one of the highest concentrations of microorganisms compared to other organs (4) and thus serves as a suitable candidate organ for experimental microbiota manipulation.

To investigate the long-term health span effects of microbiota manipulation, this study focused on introducing three specific bacterial species into *D. melanogaster*: *Escherichia coli* K-12, *Bifidobacterium infantis*, and *Akkermansia muciniphila*. These three bacterial species are not naturally present in WT *D. melanogaster* and were selected due to their significant physiological roles observed in other host species for maintaining cellular structural integrity. This study's results are not intended to suggest whether these probiotics can be directly translated to humans. Instead, it serves as a preliminary study to guide more relevant

research performed with similar probiotics in vertebrates, and eventually, in humans.

The first candidate, *E. coli* K-12, is a lab-grown strain of *E. coli* that has demonstrated past success in increasing overall health quality, improving widespread resistance to heat stress, locomotive activity, and increasing lifespan in alternative model organisms such as *Caenorhabditis elegans*. The source of these changes is linked to indole (5), which is released as a byproduct of tryptophanase catalysis. Indole regulates immune responses to infection and prevents gut inflammation by modulating aryl hydrocarbon (AhR) and progesterone X receptors. This stabilization occurs due to preserving the structural integrity of epithelial midgut cells and diminishing the activity of genes associated with virulence in pathogenic bacterial strains (6).

Bifidobacterium infantis is a bacterial strain commonly found in mammals and, like *E. coli* K-12, maintains cellular integrity by activating AhR pathways. However, unlike *E. coli* K-12, *B. infantis* does not rely on indole production for AhR activation. Instead, *B. infantis* promotes AhR pathways by synthesizing short-chain fatty acids, such as acetate and propionate. AhR activation triggers Nrf2, a transcription factor responsible for promoting antioxidant responses by activating cytoprotective genes such as the GSH pathway. These antioxidant species help combat reactive oxygen species (ROS) and maintain tissue structural integrity within the host's digestive system (7).

A. muciniphila is a recently discovered bacterial species primarily found as a constituent in mammalian gut microbiomes. It assists in structural maintenance primarily by influencing the release of cytokines and altering metabolism by interacting with the immune and digestive systems. For example, *A. muciniphila* promotes the secretion of anti-inflammatory cytokine interleukin-10 (IL-10) while inhibiting TNF- α production (8). TNF- α is responsible for inducing apoptosis and for signaling responses that intensify pro-inflammatory cytokines and ROS (9). In contrast, IL-10 plays a fundamental role in promoting tissue restoration and suppressing alternative pro-inflammatory cytokines such as interleukin-6 and interleukin-8. Moreover, *A. muciniphila* has been associated with increased production of short-chain fatty acids for maintaining metabolic homeostasis, much like the action of *B. infantis*. Additionally, there have been associations, albeit limited, with *B. infantis*' potential to reduce aging (8). The link between *B. infantis* and lifespan extension may be due to this bacterial species potential for mitigating tissue and digestive damage.

Previous experiments involving the expression of *B. infantis* and *A. muciniphila* in alternative model species have successfully utilized microbiota manipulation. Although few publications explicitly mention using *E. coli* K-12 for microbiota manipulation, due to its lack of presence in nature, there is compelling evidence that this bacterial strain can play a significant role in microbiota manipulation (10).

Materials and Methods

In this study, the change in physiological parameters was assessed by measuring lifespan, fecundity (reproductive) rates, responses to temperature stress to test metabolic homeostasis and movement assays. The effectiveness of all three bacteria was tested individually, in a control group and an experimental group that contained all three bacteria simultaneously. It was hypothesized that microbiota manipulation with each bacterial species and the combination of all three would lead to increased lifespans,

higher fecundity rates, improved survival rates after exposure to heat and cold stress, and enhanced climbing ability.

D. melanogaster preparation and feed

The probiotics were administered by mixing aqueous solutions containing the probiotics with the agar medium consumed by the *D. melanogaster* as sustenance. The following calculations were performed to determine the quantity of probiotics to administer to the experimental groups. First, all calculations were based on the ratio between the mass of an average human and that of a *D. melanogaster*. *B. infantis* and *A. muciniphila* were sourced in capsule form (from Desert Harvest, Ellsworth, ME, USA and Lifeatlas, Amazon, USA respectively), and their human daily dosages were used to determine the daily dosage required for *D. melanogaster*.

$$\frac{Quantity\ of\ Capsules\ for\ Humans}{Mass\ of\ Average\ Human\ (80kg)} = \frac{Quantity\ of\ Capsules\ for\ D.melanogaster}{Mass\ of\ Average\ D.melanogaster\ (2mg)} \quad (1)$$

Table 1. Calculated dosages for *B. infantis* and *A. muciniphila* from equation 1.

	Human Daily Dosage	*D. melanogaster* Dosage
B. infantis	1 capsule	2.5×10^{-5} capsules
A. muciniphila	2 capsules	5.0×10^{-5} capsules

All *D. melanogaster* groups were tested in vials of height 10 cm and a diameter of 3 cm. Each vial received approximately 13 mL of Formula 4-24® Instant Drosophila Medium (Carolina Biological, Burlington, NC, USA). To determine the quantity of probiotics required for the food medium, it was necessary to calculate the total number of daily servings in 13 ml of food medium. Given that the Drosophila medium has a density of about 0.98 g/mL, and a *D. melanogaster* consumes approximately three times its body weight each day (11), it was concluded that there were approximately 21,233 daily probiotic servings in 13 mL of food medium.

$$13mL\ of\ food\ medium \times \frac{0.98g}{1mL} \times \left(\frac{1}{3(2\times10^{-4}g\ of\ daily\ consumption}\right) = 21233\ daily\ servings \quad (2)$$

Multiplying the number of probiotic servings by each respective *D. melanogaster*, daily dosage yields 5.31×10^{-4} capsules of *B. infantis* per vial and 1.07×10^{-3} capsules of *A. muciniphila* per vial. Each *B. infantis* supplement weighs 0.28 g, excluding the capsule casing, so each vial containing *B. infantis* received approximately 1.49×10^{-4} g of capsule content. For *A. muciniphila*, with 0.62 g of probiotic content per capsule, each vial containing *A. muciniphila* received approximately 6.63×10^{-4} g of capsule content.

To administer such small quantities to the vials, approximately 0.02 g of capsule powder were measured out from each capsule using a balance with a precision of 0.01 g (Navigator™ NV222 AM, OHAUS, Parsippany, NJ, USA) and dissolved in 50 mL of distilled water. The resulting solution had a concentration of approximately 4.00×10^{-4} g/mL. To find out how many mL of probiotic solution needed to be administered and account for all the daily required dosages, a proportion was calculated:

$$\frac{Required\ \#\ of\ mL\ of\ probiotic\ solution}{Grams\ of\ probiotic\ received\ per\ container} = \frac{1\ mL\ of\ probiotic\ solution}{4.00\times10^{-4}g} \quad (3)$$

Table 2. Calculated mL of probiotic solution from equation 3.

B. infantis required mL of solution	1.49 mL
A. muciniphila required mL of solution	6.63 mL

The *E. coli* K-12 (Carolina biological) was obtained in colony form and stored within a vial. The *E. coli* K-12 was incubated in a Luria Bertani (LB) broth solution. In preparing the LB broth solution, 250 grams of distilled water was mixed with 6.25 grams of LB broth powder (Grainger, Lake Forest, IL, USA). The resulting solution was then transferred into an Erlenmeyer flask and tightly sealed with aluminum foil. Next, the solution was autoclaved (EZ10 Autoclave Steam Sterilizer, Tuttnauer, Hauppauge, NY, USA) at 121°C for 60 minutes. Once the autoclaving process ended, the autoclave was turned off and left open to allow the solution to cool for 30 minutes. The seal was removed after the solution was sufficiently cooled, and the *E. coli* sample was carefully inoculated into the solution with a swab. The top of the Erlenmeyer flask was then sealed again, and the flask was placed in an incubator set at approximately 37°C, where it was left overnight for incubation.

Next, to determine the concentration of the incubated *E. coli* K-12 solution, a sample was placed inside a spectrophotometer (Thermo Scientific Spectronic 20D+, Waltham, MA, USA) to perform an optical density (OD) reading. For *E. coli* K-12 cells, an OD value of 1 equals approximately 5×10^8 cells/mL (Technical Note 8 – OD600. Implen GmbH, 2020. https://www.implen.de/wp-content/uploads/docs/technical-note-8-OD600-implen-nanophotometer.pdf). The OD was measured to be approximately 0.626, or approximately 3.13×10^8 cells/mL.

Like *B. infantis* and *A. muciniphila*, the quantity of *E. coli* K-12 solution was established based on a mass ratio comparison between a human-designed *E. coli* supplement (Mutaflor probiotic, Prospect, SA, Australia) with approximately 25×10^9 CFU per daily serving) and the corresponding amount needed for *D. melanogaster*.

$$\frac{25\times10^9}{80\ kg} = \frac{D.melanogaster\ daily\ CFU\ amount}{2\ mg} \quad (4)$$

Given that there are 21,233 servings of Drosophila medium per vial, approximately 1,330,000 CFU worth of solution is needed. To calculate this, a proportion was established to determine the amount of *E. coli* K-12 solution required, which was calculated to be approximately 0.0424 mL.

$$\frac{1{,}333{,}000\ CFU\ required}{Required\ mL\ of\ solution} = \frac{1\ mL\ solution}{3.13\times10^8\ CFU} \quad (5)$$

To distribute this small volume of fluid uniformly, it was diluted in approximately 22.6 mL of distilled water, allowing it to be dispensed in a 1 mL sample.

Survival and fecundity assays

In preparation for the experiment's survival and fecundity analysis phase, 300 WT first instar *D. melanogaster* larvae (Carolina Biological) were randomly and evenly assigned into ten groups of thirty. These groups comprised two sets, each consisting of five different experimental conditions: control, *B. infantis*, *A. muciniphila*, *E. coli* K-12, and the group receiving all three probiotics simultaneously. Ten vials were prepared for each group and filled with 13 mL of Drosophila medium with their respective probiotic solutions. Then, the larvae were delicately placed into the vials and sealed with foam lids. The date and experimental treatment label were all recorded on the vials. Steps for maintaining the *D. melanogaster* were referenced from guides (12).

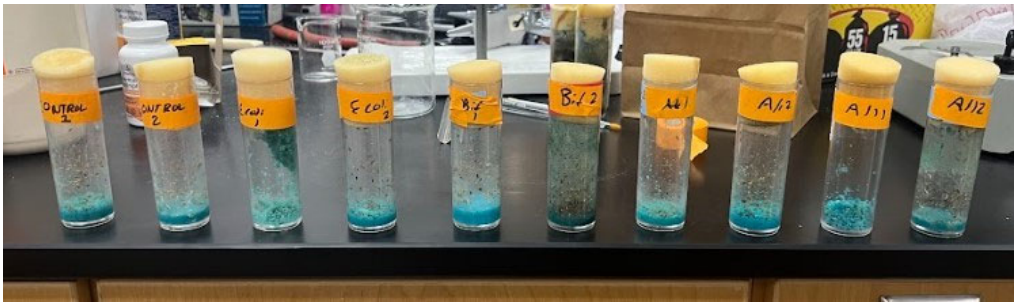


Figure 1. Experimental setup.

Every week, an additional set of ten vials was prepared using the Drosophila medium and probiotic solution to replenish the food medium. FlyNap® (Carolina Biological) was employed as an anesthetic to facilitate the transfer of the *D. melanogaster*, including the control group, to their new respective vials. FlyNap® was not used during the first two weeks when the original experimental units were still in the larval stage.

To use FlyNap®, a swab was dipped into the solution. The dipped end was gently inserted into the vial with the foam lid still in place. Then, the vial was tipped on its side and left for at least five minutes. After transferring *the D. melanogaster*, the number of units that were still alive was counted. The living *D. melanogaster* were unconscious against the vial's walls, while the deceased units were embedded in the medium. While they were still unconscious, the units were *D. melanogaster* transferred to their new vials and sealed. Subsequently (if applicable), the newly produced offspring were recorded, most of which were within the Drosophila medium. By the end of a given week, these offspring were in the larval or pupal stage, distinguishing them from the original experimental units. This process continued for the rest of the experiment, which concluded at week twelve.

Temperature stress/Homeostasis assays

For the temperature stress analysis, 200 first instar *D. melanogaster* larvae were randomly assigned into ten groups of twenty. Five groups were designated for heat stress analysis, while the remaining half were assigned for cold stress analysis. Similar to the survival and fecundity analysis, the cold and heat stress analysis groups consisted of two sets, each exposed to the five experimental conditions. In addition, these groups were assigned vials with 13 mL of Drosophila medium and given ample time to feed on the probiotic solutions.

Subsequently, the groups assigned to the heat stress analysis experiment were placed into an incubator at 37°C for approximately one hour. The number of live experimental units was counted. These groups were subsequently transferred to new vials containing Drosophila medium treated with their respective probiotic solutions. The number of live experimental units was recorded daily for the first three days, followed by counts every two days until day seven. Survival rates were measured in half-week intervals from week one to week two. After the second week, the counting and transfer of live experimental units followed the same procedure as the survival analysis. The cold stress assay was identical to the heat stress assay, except for the experimental units, which were exposed to a temperature of 4°C to 8°C in a refrigerator for one hour.

Climbing/Mobility assay

One hundred first-instar *D. melanogaster* larvae were randomly assigned into five groups of twenty for the climbing assay with their respective probiotic groups. When the larvae matured to adults by week two, the following procedure was adopted for each week: The *D. melanogaster* from each group were anesthetized and individually placed into separate, sealed vials. After regaining consciousness, the lights were turned off for thirty minutes to enable the flies to become dark-adapted. This allowed the *D. melanogaster* to settle towards the bottom of the vial. Next, a timer was initialized at the end of the thirty-minute period, and the lights were turned on. The vials were lightly tapped to prompt the *D. melanogaster* to start climbing, and the timer was started simultaneously. The timer was stopped once the flies reached the top of the vial. The trials continued until week ten when the *D. melanogaster* began exhibiting a lack of mobility that would hinder statistical analysis. Portions of this procedure were adapted from Manjila et al. experiment on performing climbing assays (13). This experiment adhered to the same transfer procedures as the previous assays.

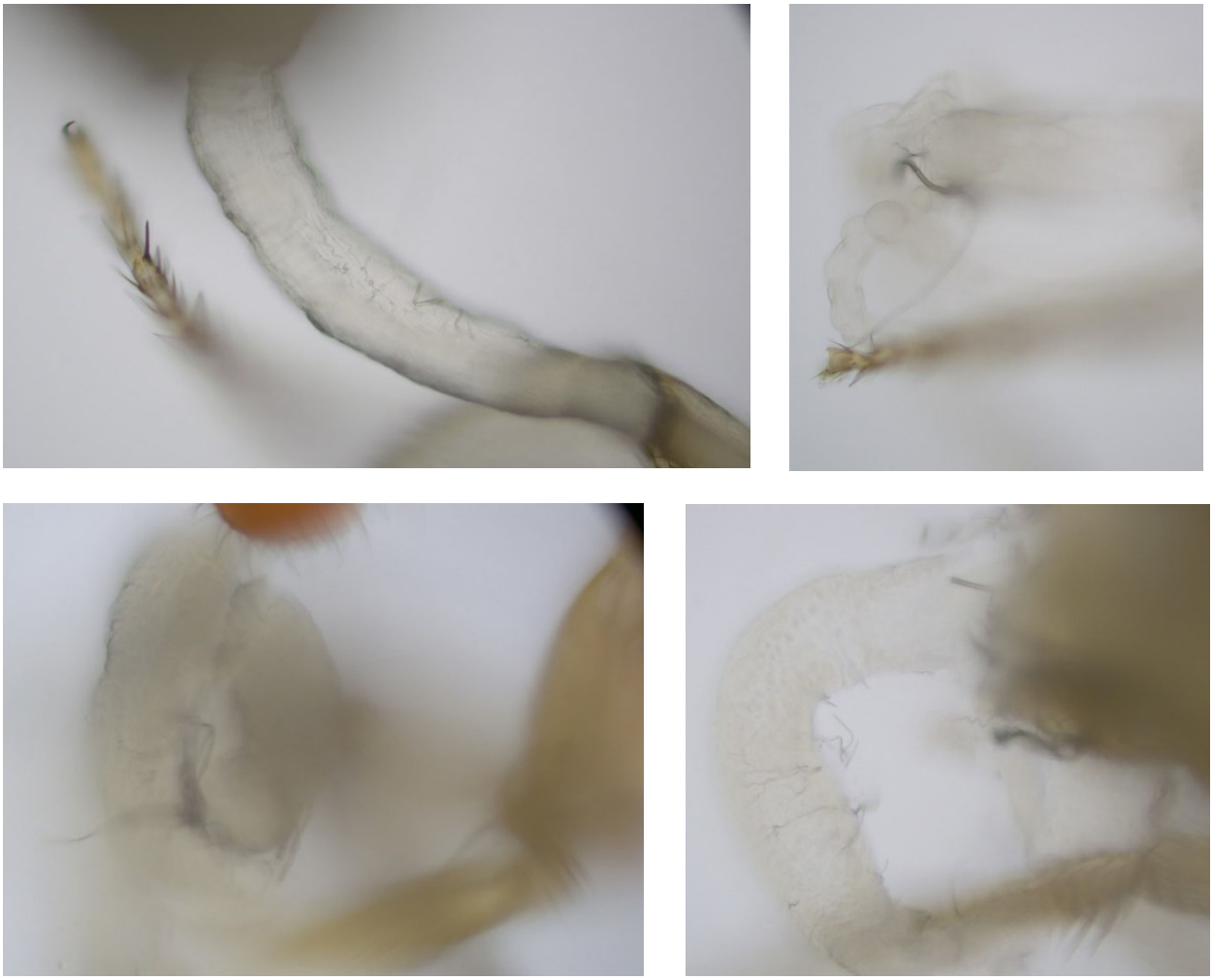
Additional qualitative research (Dissections)

The alimentary canal of the *D. melanogaster* was photographed (separate groups from the rest of the experiment) at eight weeks under a microscope (AmScope B400 Series Compound Microscope, Irvine, CA, USA) with a 100x objective. To do so, a petri dish was prepared with an anesthetized *D. melanogaster* along with tweezers and phosphate-buffered saline as a buffering agent to preserve the *D. melanogaster* tissue structure while examined. To access the alimentary canal, the center of the thorax was pierced and gently separated from the body. The elongated, transparent tube grasped by the tweezers was recognized as the alimentary canal and subsequently placed in the PBS to view under the 100x objective. To confirm its identity as the alimentary canal, its position and structure relative to the surrounding anatomy of the *D. melanogaster* were observed and noted.

Results and Discussion

Dissection observations

The *D. melanogaster* dissected alimentary canals are shown in Figures 2-5. Without advanced staining techniques, discerning the precise physiological effects on the morphology of the *D. melanogaster* digestive system was challenging. However, especially in the *B. infantis* group, the common motif of distinct patterns of digestive folding was rather prominent compared to the other experimental groups. In addition, the extracted midgut was reminiscent of dissections pictured in published literature.



Figures 2-5. Dissected alimentary canals of the *D. melanogaster*: control (top left), *B. infantis* (top right), *E. coli* K-12 (bottom left), *A. muciniphila* (bottom right).

To assess the morphology of the midgut more accurately, one approach would be to stain the *D. melanogaster* tissue using methods such as hematoxylin and eosin staining. This technique would enable researchers to analyze the tissue morphology more precisely and better observe characteristics of epithelial tissue, such as tightly packed single layers of cells attached to a basal membrane (14).

Survival analysis

The results of the survival assay and its standard errors are shown in the Kaplan-Meier chart illustrated in Figure 6. The Kaplan-Meier (KM) chart is a survival analysis estimator that uses computed data to predict the probability of survival for a given experimental group. For more information about analyzing Kaplan-Meier charts, refer to Rich's published guide (15).

The KM chart predicted a 40% chance of survivability between days 49 and 56 for the control group. The observation period spanned 12 weeks, during which two sets of five experimental groups were combined to form a more extensive set, resulting in a sample size of 60 per group. This consolidation helped minimize the potential for statistical inference errors. Each trial was conducted independently, and a significance level of 0.05 was set to determine statistical significance. The survival

statistical significance values relative to the control group were calculated using a chi-squared log-rank test, with results presented in Table 3.

The log-rank tests were computed in Python to assess the significance of differences in survival distributions between the experimental groups in the *D. melanogaster* study. With the respective p-values for each probiotic group falling below the significance level, it was concluded that there was a statistically significant difference in survival among the various probiotic groups.

A notable observation from Figure 6 was the control group's exit from the shaded region, known as the Kaplan-Meier Estimate. This estimate illustrates the overall survival trend range for the experimental groups, offering a visual interval that outlines the expected survival probabilities for *D. melanogaster*. The divergence of the control group from the Kaplan-Meier Estimate suggests a significant deviation in survival probability compared to the other groups that were fed the probiotics.

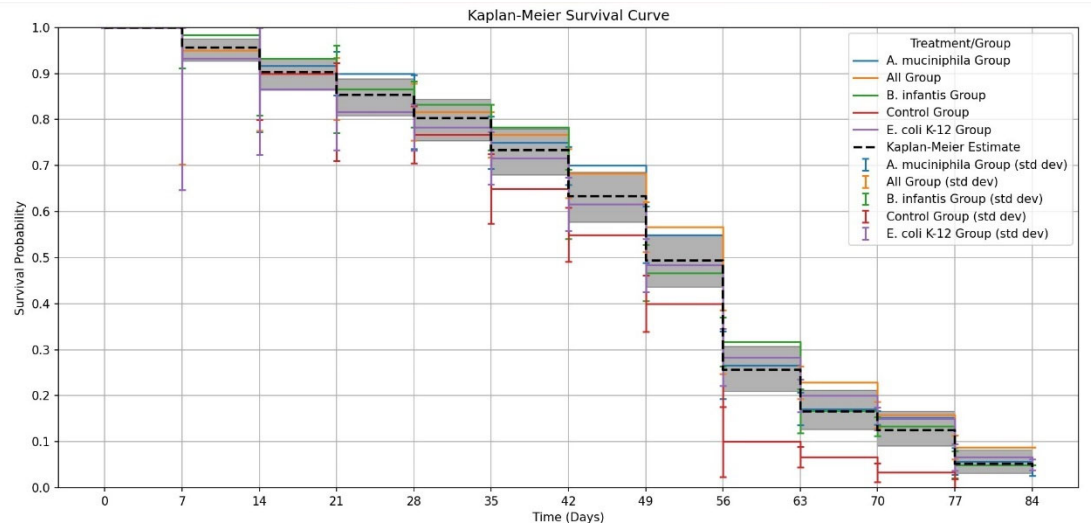


Figure 6. Kaplan-Meier chart of the results of the survival assay and standard errors.

Table 3. Calculated log-rank results relative to the control group.

Probiotic Species	P-Value (Relative to Control Group)
E. coli K-12	0.04
B. infantis	0.02
A. muciniphila	0.01
All	<0.005

It was also noted that a small percentage of the probiotic-treated *D. melanogaster* outlasted the twelve-week testing duration. In contrast, the control groups only survived up to week 11, demonstrating the potential that probiotic manipulation may have for increasing lifespan. Past studies have supported the notion that these microbes are present since birth in other host species. For example, the presence of *B. infantis* in infant humans has been associated with mitigating consequences originating from potential congenital disabilities (16). Furthermore, *A. muciniphila* has been observed to stabilize blood sugar levels in mammals and prevent inflammation over the long term (17). While probiotics may exhibit varying functions in different host species, these probiotics demonstrate long-standing potential in

improving the overall health span of diverse populations.

Moreover, previous studies have demonstrated the potential for genetically modified *E. coli* strains to induce positive physiological changes. Mice treated with the genetically modified *E. coli* strain exhibited greater glucose tolerance over the long term compared to the control group (10). While *E. coli* K-12 is not often assessed for its application in probiotic manipulation, its status as a well-engineered lab strain suggests potential applications in clinical probiotic manipulation (18), particularly in addressing the effect of ROS.

Fecundity analysis

The results from the fecundity analysis can be visualized using the time graph and its standard errors, as shown in Figure 7. This time graph displays the per-capita averages of the offspring that the *D. melanogaster* produced in the study.

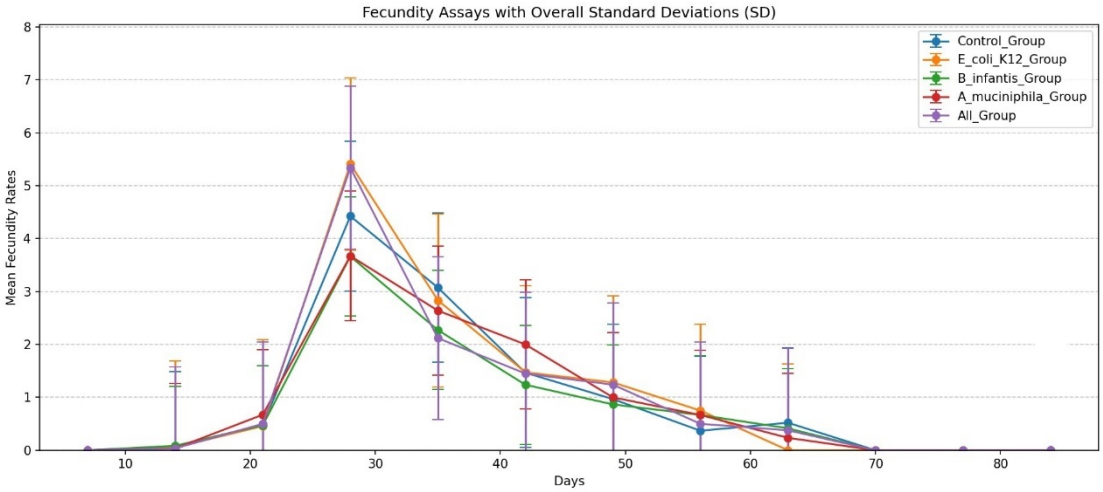


Figure 7. Fecundity analysis results visualized with time graph and standard errors.

Statistical analysis was conducted in Python using a repeated measures analysis of variance test (ANOVA). A repeated measures ANOVA test is a largely resilient statistical analysis tool that reduces the probability of Type I errors. ANOVA assumes equal measures of variance across all experimental groups, which was confirmed by results from Levene's test. Tests for the equal distribution of variance, confirmed this condition was valid with a p-value of 0.986. Thus, it was safe to assume that an ANOVA test was appropriate for this dataset. The ANOVA test itself yielded an f-value of 0.8451 and a p-value of 0.5042, suggesting that there was no significant difference in the fecundity rates of the *D. melanogaster* which were administered the probiotics compared to the control group.

With respect to these results, it appears that the probiotics did not affect the fecundity rates of the *D. melanogaster*. This outcome can be attributed to the probiotics primarily affecting the digestive system rather than the reproductive system. While the probiotics may enhance the metabolism of the host species, potentially leading to increased fertility, this effect was not seen in this study, especially considering the nuances involved in altering microbiota compositions and its impact on physiological function.

Across a variety of species, an imbalanced microbiota often leads to various complications, including reproductive hormone imbalance (19). Yet, considering the increase in survival rates for *D. melanogaster* treated with

probiotics, it is unlikely that dysbiosis occurred in their digestive systems. Additionally, no adverse effects on reproduction were observed from treating the *D. melanogaster* with probiotics; instead, there was minimal change.

If this assay were replicated, it would be interesting to investigate and compare the survival and other physiological traits of the offspring of initially treated *D. melanogaster*, as was done in previous experiments (20). This additional analysis could potentially find hereditary effects or long-term benefits from the initial probiotic treatment typically associated with survival analysis, the temperature stress, and how the probiotics mitigated such effects.

Temperature stress analysis

Figures 8 and 9 show the two time graphs for both cold and heat stress respectively, along with their standard errors. A fixed-effects model was used to analyze the statistical effects of probiotic manipulation when subject to temperature stress that significantly altered metabolic activity. The fixed-effects model was especially pertinent because of the irregularity in the reported time intervals. These datasets encompassed various time intervals, ranging from daily intervals initially to expanding into weeks. Opting for methods like log-rank tests would seem plausible given that the test is catered to survival analysis. However, those results would not be as resistant to inconsistent time intervals as they would in a fixed-effects model. In both the heat and cold assays, the control group presented with the highest mortality rates immediately after exposure to the harsher climates, at 10%. The long-term damage that the *D. melanogaster* control groups sustained presented in a decrease in their populations that was significantly greater than the decrease experienced by the flies that were treated with probiotics. Similar to the survival assay measured using a log-rank test, the no flies in the control groups survived past week 11.

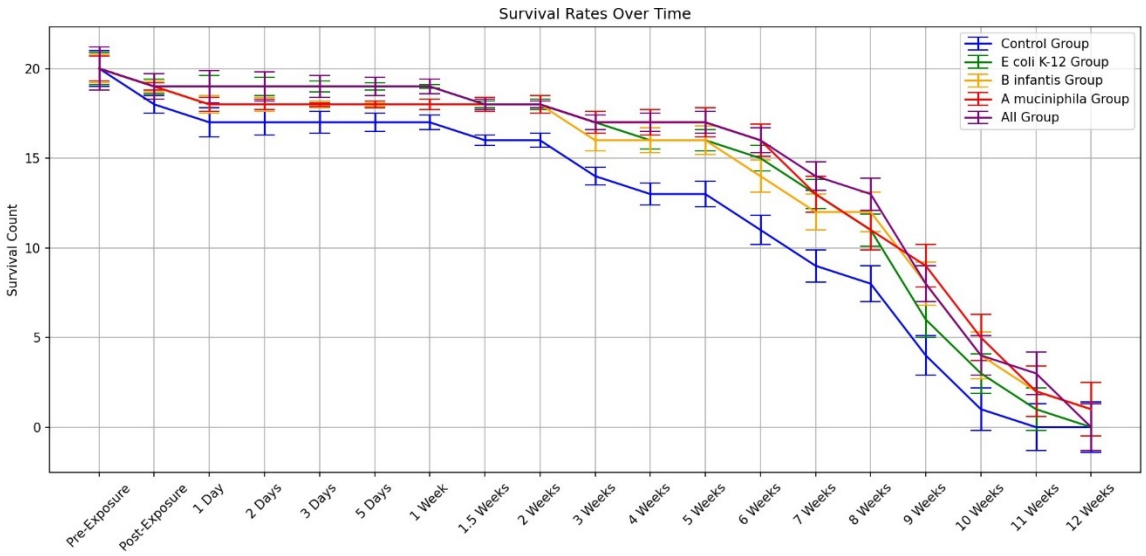


Figure 8. Time graph for cold stress and standard errors.

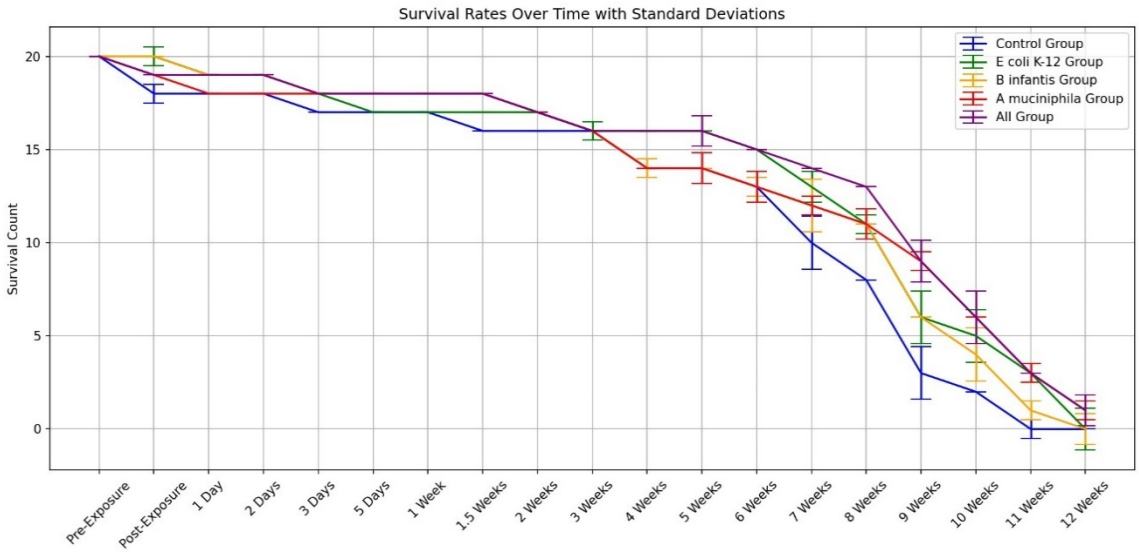


Figure 9. Time graph for heat stress and standard errors.

The results presented in Table 4 and Table 5 from the independent fixed-effects models indicate an overall positive physiological effect from the probiotic solutions, with the exception from *A. muciniphila*, during the heat assays. The purpose of analyzing the survival rates of the *D. melanogaster* was to observe potential damages incurred from exposure to harsh environmental conditions. These findings further support the notion that probiotic manipulation holds significant potential in long-term applications, particularly in limiting the effects of damage and in the maintenance of physiological function.

Table 4. Fixed-effects model results for the heat assays were reported compared to those of the control group.

Probiotic Type	Significance Level	Z-Value
E. coli K-12	0.01	2.582
A. muciniphila	0.942	0.781
B. infantis	0.026	2.225
All	0.048	1.349

Table 5. Fixed-effects model results for the cold assays were reported compared to those of the control group.

Probiotic Type	Significance level	Z-Value
E. coli K-12	<0.005	4.767
A. muciniphila	<0.005	4.302
B. infantis	0.026	2.085
All	<0.005	4.169

A potential explanation for the observed longer lifespans of *D. melanogaster* treated with probiotics under heat stress could be (speculatively) attributed to the heat shock proteins released by these probiotic species, such as σ32 found in *E. coli* K-12. It was previously reported that the activation of σ32 triggered a genetic response that alleviated the effects of denatured proteins on cellular stability, either by refolding them or by inhibiting their activity (22). Similarly, proteins like GroEL and GroES in *B. infantis* are known for their significant role in responding to cold stress. These molecular chaperones are

understood to function as binding regions that aid in refolding denatured proteins (23), potentially preventing structural damage induced by cold stress.

Although the probiotics generally found success when mitigating long-term damage sustained by sudden temperature stress, the probiotics had a more significant impact when it came to resisting cold stress when compared to heat stress. This was evident from the significantly greater z-scores observed in the cold assays. This could potentially be attributed to the colder temperatures, which, despite being relatively harsh for larval *D. melanogaster*, might benefit the host species by slowing their metabolism and decreasing energy consumption. In contrast, heat stress could potentially increase metabolic demands, placing additional strain on the host. To further this study, examining the physiological effects of temperature stress exposure for different periods of time could ensure a more thorough analysis of the effects of environmental stress on *D. melanogaster* larvae.

Climbing assay analysis

Similar to the fecundity analysis, a repeated measures ANOVA test was used to perform statistical analysis. Using Levene's test on the dataset that logs the average climb times per probiotic group, it was found that the Levene's test statistic was 0.8475 with a p-value of 0.5076. These results allowed for the assumption of homogeneity in variances and thus made using a repeated measures analysis ANOVA test an acceptable choice.

The repeated measures ANOVA test yielded an f-statistic of 34.791 and a p-value of < 0.005, indicating that the time it took to climb the 10 cm tall vial significantly differed between the control group and the flies fed with the probiotics, when evaluated at a significance level of 0.05.

The time graph for climb times and its standard errors are shown in Figure 10. Figure 10 shows that the probiotic groups climbed significantly faster when it came to this time-based analysis. The reported points on the plot refer to the cumulative average times the individual *D. melanogaster* took to climb the vials, even after accounting for sampling variability. There were no reported values up to day 14 due to the *D. melanogaster* in the experimental groups still being in the larval phase.

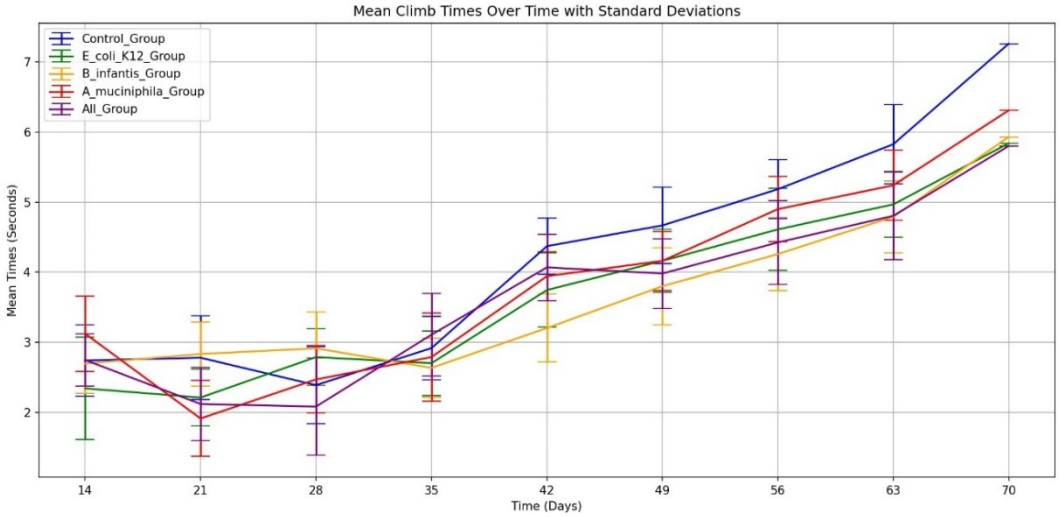


Figure 10. Time graph for climb times and its standard errors.

Once again, the effects of microbiota manipulation were most observable during the later stages of this experiment, from weeks six and onward. Between weeks two and six, climbing speeds between all experimental groups appeared to be relatively similar. After week six, the control group exhibited a significant decline in climbing speeds, falling notably behind the performance levels observed in the groups receiving the probiotic treatments. This visual analysis aligned with the results of the repeated measures ANOVA test, in which the distribution of climbing times of the control group significantly differed from that of the treated groups.

The results of this assay can likely be (speculatively) attributed to GLP-1. GLP-1 is a hormone primarily associated with appetite regulation and glucose metabolism and may have indirect implications for motor control and locomotion. Its activation, often triggered by the production of short-chain fatty acids, could influence energy levels by regulating glucose metabolism, contributing to more rapid and well-controlled movement. Although this pathway is more significant in vertebrate species with more complex nervous systems, it is plausible that the activation of GLP-1 potentially contributed to the observed enhancements in the probiotic-treated species that produce short-chain fatty acids (24).

Consequently, the physiological consequences caused by long-term microbiota manipulation allowed the groups treated with probiotics to climb more rapidly than the control group. To better gauge the influence of microbiota manipulation on the mobility of *D. melanogaster*, alternative movement assays, such as crawling or flight assays, should also be considered when conducting this experiment again (25).

Limitations

There is concern that the microbiome of *D. melanogaster* may not be representative of the human microbiome. To mitigate this concern, past studies have attempted to transfer microbiota normally present in humans into *D. melanogaster* so that the model organism's microbiome is more representative of the human microbiome. Such a manipulation was performed using antibiotics to first purge the *D. melanogaster* microbiota and subsequently exposing the *D. melanogaster* to a human fecal sample (26). However, this study reported a significant disparity between the microbial proportions between male and female *D. melanogaster*, potentially introducing biases and affecting the reliability of the experimental results.

Additionally, the uptake of microorganisms from human fecal matter does not guarantee that the composition of the host microbiota in *D. melanogaster* will adequately represent a human microbial population. For example, in the study treating *D. melanogaster* with feces, Lactobacillus emerged as one of the most prevalent genera, comprising approximately 30% and 75% in males and females, respectively. However, a genomic sequencing study evaluating the human microbiota complex did not identify Lactobacillus as one of the more prevalent species, with Ruminococcus and Bacteroides being more prominent instead (27). This disparity in microbiota composition implies that attempting to mimic the human microbiota complex inside of *D. melanogaster* may not accurately reflect the true microbiota configuration observed in humans.

Although direct evidence is limited, it is generally understood that the microbiota complex for most organisms, including humans (28), develops during the early stages of life.

Since the early development phase of Drosophila melanogaster spans just under two weeks, allowing enough time for the fecal-induced microbiota to establish in the fruit fly's digestive tract would limit the window available for effective microbiota manipulation using the three probiotic treatments.

Rather than introducing the human microbiota and treatment probiotics separately, an alternative approach would be doing so simultaneously. However, regardless of the timing, transferring microbiota through methods such as fecal matter does not allow for the dosage to be controlled. A method to control the dosages would be to meticulously measure every probiotic within the human microbiota complex to address this variability, which presents significant technical challenges. Although attempting to mimic the microbiota structure of humans can provide valuable insight for expediting the process of demonstrating the validity of these three probiotics in a real-world clinical setting, doing so would require further investigation to determine a feasible amount of human microbiota to introduce to *D. melanogaster* without disrupting the host's microbiota functionality as a whole.

Additionally, there is a significant difference in the magnitude of the number of microbiota taxa harbored in humans versus those present in *D. melanogaster*. This discrepancy is a concern, because the number of bacterial cells in the probiotic intake in *D. melanogaster* (expressed as a ratio to the number of cells present in the gut of *D. melanogaster*) may be significantly more amenable to colonization than a clinically appropriate ratio in humans; which may not colonize as effectively (because the ratio may be significantly lesser). Hence, there is no definitive means to quantify this effect on the calculated probiotic intake. However, this potential confounding variable may be less significant during the early stages of life for most organisms, as the microbiota complex is still developing and more adaptable to changes.

Another concern involves the weekly transfer of *D. melanogaster* into new vials. Several studies have documented that transferring *D. melanogaster* can affect the success of probiotic colonization (29). However, this effect was observed in these additional studies only when the flies were transferred to sterile vials that did not contain probiotics in the food medium. In this experiment, the vials used for transferring the flies always contained a food medium supplemented with the probiotic treatments, thereby mitigating this concern.

The precision of the balance used for measurements is an additional source of error. Given the 0.01 g precision of the balance used to measure out 0.02 g of the *B. infantis* and *A. muciniphila* powder, there is a chance of a standard error of ± 0.01 g or a percent change of approximately ± 50%. Assuming a range of 0.015 g to 0.024 g (± 0.009), the standard error is nearly equivalent to the entire range of measured weights, which indicates that the cases that vary the most from the mean may be prone to significant measurement error that may confound with the study's results.

However, it should be noted that the amount of probiotic supplement introduced to the *D. melanogaster* alone may not entirely determine the success of colonization. Although the quantity of microbiota introduced is likely one of the larger determinants for the probability of colonization, other factors such as environment, metabolism, and host physiology also play important roles (30). However, when mitigating potential confounding factors in the future, such

as the mass of the probiotic used, selecting a more precise balance could significantly enhance the accuracy of the dosage measurements and ensure greater reproducibility.

Conclusion

The host species, *D. melanogaster* exhibited positive physiological effects on the health span in all tested applications (lifespan, responses to temperature stress and movement assays) except for fecundity rates. Although the implementation of microbiota manipulation over the long term currently has limited practical implementation, it may be a viable solution for preventative medicine, especially for humans.

While species *B. infantis* and *A. muciniphila* are naturally present in humans, their absence in *D. melanogaster* used in this study highlights the potential scope of microbiota manipulation. This study aimed to broadly validate the concept of microbiota manipulation before its application in humans can be commercialized.

Additionally, considering that these strains may be scarce in specific individuals, the opportunity to introduce them into human systems remains, providing further routes for their beneficial application.

While *E. coli* K-12, a strain typically cultivated in laboratory settings, is not native to the human microbiota, it has shown promise in improving health span within its host organism. To further this study and continue the progression of probiotic manipulation in humans, these probiotics need to be tested on vertebrate species that more closely resemble human physiology. Given the successful and safe outcomes observed in *D. melanogaster*, expanding the application of these probiotics presents promising avenues for future success.

Abbreviations

AhR: Aryl Hydrocarbon, TNF- α: Tumor Necrosis Factor Alpha, ROS: Reactive Oxygen Species IL-10: Interleukin-10, WT: Wild-Type, LB: Luria Bertani, OD: Optical Density, PBS: Phosphate-Buffered Saline, GLP-1: Glucagon-Like Peptide 1, Nrf2: Nuclear factor erythroid-2-related factor 2, NR3C3 or PR: nuclear receptor subfamily 3, group C, member 3 or Progesterone receptor, GSH: Glutathione, GroEL and GroES: Phage growth mutation in phage head gene E, Large or Small subunit, Eukaryote equivalent is HSP60 or Heat Shock Protein 60, GLP-1: Glucagon like peptide 1

References

1. N. S. Tolwinski. Introduction: Drosophila—A Model System for Developmental Biology. Journal of Developmental Biology, 5(3), 9, 2017. https://doi.org/10.3390/jdb5030009

2. K. L. Adair, M. Wilson, A. Bost, A. E. Douglas. Microbial community assembly in wild populations of the fruit fly *Drosophila melanogaster*. ISME J, 4: 959-972, 2018. https://doi.org/10.1038/s41396-017-0020-x

3. A. Arias-Rojas, I. Iatsenko. The Role of Microbiota in *Drosophila melanogaster* Aging. Front Aging, 3:909-509, 2022. https://doi.org/10.3389/fragi.2022.909509

4. I. Miguel-Aliaga, H. Jasper, B. Lemaitre. Anatomy and Physiology of the Digestive Tract of *Drosophila melanogaster*. Genetics, 210, 2: 357-396, 2018. https://doi.org/10.1534%2Fgenetics.118.300224

5. R. Sonowal, A. Swimm, A. Sahoo, L. Luo, Y. Matsunaga, Z. Wu, et al. Indoles from commensal bacteria extend healthspan. Proceedings of the National Academy of Sciences of the United States of America, 114(36), 2017. https://doi.org/10.1073%2Fpnas.1706464114

6. X. Ye, H. Li, K. Anjum, X. Zhong, S. Miao, G. Zheng, et al. Dual Role of Indoles Derived From Intestinal fimmu Microbiota on Human Health. Front Immunol. 13:903-526, 2022. https://doi.org/10.3389/.2022.903526

7. A. M. Ehrlich, A. R. Pacheco, B. M. Henrick, D. Taft, G. Xu, M. N. Huda, et al. Indole-3-lactic acid associated with Bifidobacterium-dominated microbiota significantly decreases inflammation in intestinal epithelial cells. BMC Microbiology, 20: 357, 2020. https://doi.org/10.1186%2Fs12866-020-02023-y

8. H. Jian, Y. Liu, X. Wang, X. Dong, X. Zou. *Akkermansia muciniphila* as a Next-Generation Probiotic in Modulating Human Metabolic Homeostasis and Disease Progression: A Role Mediated by Gut–Liver–Brain Axes? Int. J. Mol. Sci. 24(4), 3900, 2023. https://doi.org/10.3390/ijms24043900

9. Y. Luo, C. Lan, K. Xie, H. Li, E. Devillard, J. He, et al. Active or Autoclaved *Akkermansia muciniphila* Relieves TNF-α-Induced Inflammation in Intestinal Epithelial Cells Through Distinct Pathways. Front Immunol. 12:788-638, 2021. https://doi.org/10.3389/fimmu.2021.788638

10. B. J. Russell, S. D. Brown, N. Siguenza, I. Mai, A. R. Saran, A. Lingaraju, et al. Intestinal transgene delivery with native E. coli chassis allows persistent physiological changes. Cell 185(17):3263-3277, 2022. https://doi.org/10.1016/j.cell.2022.06.050

11. B. C. Shell, M. Grotewiel. Identification of additional dye tracers for measuring solid food intake and food preference via consumption-excretion in Drosophila. Scientific Reports, 12(1), 6201, 2022. https://doi.org/10.1038%2Fs41598-022-10252-6

12. G. R. Chaudhary, A. Pandey, A. Singh, V. Yadav, V. Dwivedi, R. Arya, et al. Experiments with Drosophila for Biology Courses, 2nd ed. https://bdsc.indiana.edu/pdf/Experiments_with_Drosophila_for_Biology_Courses.pdf

13. S. B. Manjila, G. Hasan. Flight and Climbing Assay for Assessing Motor Functions in Drosophila. Bio-protocol, 8(5), e2742, 2018. https://doi.org/10.21769%2FBioProtoc.2742

14. L. E. Morrison, M. R. Lefever, H. N. Lewis, M. J. Kapadia, D. R. Baeur. Conventional histological and cytological staining with simultaneous immunohistochemistry enabled by invisible chromogens. Lab Invest 102(5):545-553, 2022. https://doi.org/10.1038/s41374-021-00714-2

15. J. T. Rich, J. G. Neely, R. C. Paniello, C. C. J. Voelker, D. Phil, B. Nussenbaum, et al. A practical guide to understanding Kaplan-Meier curves. Otolaryngol Head Neck Surg. 143(3):331-336, 2010. https://doi.org/10.1016%2Fj.otohns.2010.05.007

16. M. A. Underwood, J. B. German, C. B. Lebrilla, D. A. Mills. *Bifidobacterium longum* subspecies infantis: champion colonizer of the infant gut. Pediatr Res. 77(1-2):229-35, 2014. https://doi.org/10.1038/pr.2014.156

17. X. Zhu, J. Shen, S. Feng, C. Huang, H. Wang, F. Huo, et al. *Akkermansia muciniphila*, which is enriched in the gut microbiota by metformin, improves cognitive function in aged mice by reducing the proinflammatory cytokine interleukin-6. Microbiome 11(1):120, 2023. https://doi.org/10.1186/s40168-023-01567-1

18. M. Chicklowski, N. Shah, J. L. Wampler, S. S. Wu, J. A. Vanderhoof. *Bifidobacterium longum* Subspecies infantis (*B. infantis*) in Pediatric Nutrition: Current State of Knowledge. Nutrients, 12(6), 1581, 2020. https://doi.org/10.3390%2Fnu12061581

19. N. Mih, J. M. Monk, X. Fang, E. Catoiu, D. Heckmann, L. Yang, et al. Adaptations of *Escherichia coli* strains to oxidative stress are reflected in properties of their structural proteomes. BMC Bioinformatics 21(1):162, 2020. https://doi.org/10.1186/s12859-020-3505-y

20. X. Qi, C. Yun, Y. Pang, J. Qiao. The impact of the gut microbiota on the reproductive and metabolic endocrine system. Gut Microbes, 13(1):1-21, 2021. https://doi.org/10.1080%2F19490976.2021.1894070

21. B. Nguyen, A. Than, H. Dinh, J. Morimoto, F. Ponton. Parental Microbiota Modulates Offspring Development, Body Mass and Fecundity in a Polyphagous Fruit Fly. Microorganisms, 8(9), 1289, 2020. https://doi.org/10.3390%2Fmicroorganisms8091289

22. T. Miwa, H. Taguchi. *Escherichia coli* small heat shock protein IbpA plays a role in regulating the heat shock response by controlling the translation of σ32. Proceedings of the National Academy of Sciences of the United States of America, 120, 32, 2023. https://doi.org/10.1073%2Fpnas.2304841120KP

23. K. P. Scott, J. M. Antoine, T. Midtvedt, S. van Hamert. Manipulating the gut microbiota to maintain health and treat disease. Microb. Ecol. Health 26:25877, 2015. https://doi.org/10.3402/mehd.v26.25877

24. M. Schöpping, A. A. Zeidan, C. J. Frazén. Stress Response in Bifidobacteria. Microbiology and Molecular Biology Reviews, 86(4), 2022. https://doi.org/10.1128%2Fmmbr.00170-21

25. T. T. Lai, Y. H. Tsai, C. W. Liou, C. H. Fan, Y. T. Hou, T. H. Yao, et al. The gut microbiota modulate locomotion via vagus-dependent glucagon-like peptide-1 signaling. NPJ Biofilms and Microbiomes, 10(1), 2, 2024. https://doi.org/10.1038%2Fs41522-024-00477-w

26. D. Ji, H. Sun, W. Yang, M. Gao, H. Xu. Transfer of Human Microbiome to Drosophila Gut Model. Microorganisms, 10(3), 553, 2022. https://doi.org/10.3390%2Fmicroorganisms10030553

27. A. Almeida, A. L. Mitchell, M. D. Boland, S. C. Forster. A new genomic blueprint of the human gut microbiota. Nature, 568(7753):1, https://doi.org/10.1038/s41586-019-0965-1

28. Y. Yao, X. Cai, Y. Ye, F. Wang, F. Chen, C. Zheng. The Role of Microbiota in Infant Health: From Early Life to Adulthood. Front Immunol., 12: 708472, 2021. https://doi.org/10.3389%2Ffimmu.2021.708472

29. I. S. Pais, R. S. Valente, M. Sporniak, L. Teixeira. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. Plos Biology, 16(7): e2005710, 2018. https://doi.org/10.1371/journal.pbio.2005710

30. G. Caballero-Flores, J. M. Pickard, G. Núñez. Microbiota-mediated colonization resistance: mechanisms and regulation. Nat Rev Microbiol., 21(6): 347-3z. https://doi.org/10.1038%2Fs41579-022-00833-7