

Lactobacillus rhamnosus GG does not increase *Caenorhabditis elegans* longevity

Maye Cheng, Rebecca Dos Remedios, Lucy Lu, Chris Tang

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY *Caenorhabditis elegans* is used as a model organism to study longevity. In recent years, *C. elegans* have helped advance knowledge regarding the human microbiome and host-pathogen interactions due to its characteristic as a bacterivore. Studies have reported that a strain of *Lactobacillus rhamnosus*, CNCM I-3690, enhances the lifespan of *C. elegans* by inducing the expression of genes under the control of the DAF-16 transcription factor. Although the effects of pathogens on *C. elegans* have been well-studied, the effects of a commonly used human probiotic, *L. rhamnosus* GG (LGG), on *C. elegans* are not well-characterized. We hypothesized that LGG would enhance longevity in *C. elegans* in a DAF-16-dependent manner, similar to previous observations of *L. rhamnosus* CNCM I-3690. Based on a series of survival assays, we observed the lifespan of the wild-type and *daf-16* *C. elegans* fed with *E. coli* OP50 or LGG. We determined that the lifespans of neither *C. elegans* strain increased when fed with LGG, which contradicts our hypothesis and contrasts with previous research that has demonstrated that *Lactobacilli* can enhance *C. elegans* lifespan. Interestingly, we also observed that starved *C. elegans* appear to avoid MRS media, which is used to culture LGG.

INTRODUCTION

Caenorhabditis elegans is a soil nematode used commonly as a model organism to study longevity (1). *C. elegans* have several features that allow it to be an appropriate model organism for this research. The organism reproduces rapidly and can be cultured easily in the laboratory (2). Its normal diet consists of readily-available bacterial strains, such as *Escherichia coli* (2). The transparent nature of the *C. elegans* body also allows visual observations to be made using fluorescence markers for studies, such as metabolism and embryogenesis, without harming the worm (2). Its small size allows for numerous assays to be conducted on a single petri dish and microtiter plate (3). The *C. elegans* genome also encodes orthologs for 60-80% of human genes, which are associated to signaling pathways such as the MAPK pathway, notch pathway and insulin/insulin-like signaling pathway (2). Finally, the ease of genetically modifying *C. elegans* makes it an attractive model to examine specific gene expression and molecular pathways (4).

The human gut microbiota has been a topic of extensive research in recent years due to the advancement of techniques and methods that permits the characterization of the commensals residing within the gut (5). The surge of knowledge regarding the species residing within our gut flora, their interactions with the unique environment and their influence on human physiology has provided substantial knowledge on host health and development. Disruption of the microbiome has been linked to gastrointestinal diseases and obesity (6,7). Animal models are useful tools to interrogate the complex mechanisms and interactions between host and microbiome. In particular, *C. elegans* is regarded as a unique model for studying gut commensals. Since *C. elegans* feeds on bacteria, it can be used to directly investigate the role of bacteria residing in the gut microbiota or the correlation between consumption and human health and lifespan (8). By substituting the regular feeding bacterium *E. coli* OP50 for a different bacterium in the *C. elegans* diet, the animal can be used as a simple model to study the interactions between different strains of bacteria and host physiology. Dietary composition is an important determinant of longevity in *C. elegans* (3). For example, feeding *C. elegans* a diet of pathogenic bacteria demonstrated decreased lifespans, impaired growth, and morphological defects in the organism (3). On the other hand, select species of bacteria, such as members of the genus *Lactobacillus*, have

Published Online: 17 September 2019

Citation: Cheng M, Dos Remedios R, Lu L, Tang C. 2019. *Lactobacillus rhamnosus* GG does not increase *Caenorhabditis elegans* longevity. UJEMI 24:1-9

Editor: Julia Huggins, University of British Columbia

Copyright: © 2019 Undergraduate Journal of Experimental Microbiology and Immunology. All Rights Reserved.

Address correspondence to:
<https://jemi.microbiology.ubc.ca/>

been found to enhance lifespan and improve host defense against pathogens in *C. elegans* when ingested. This further highlights the advantages of using *C. elegans* as an *in vivo* model for microbiome research (9-13).

Lactobacillus rhamnosus GG (LGG) is one of the most extensively studied and characterized human probiotics found commonly in food products (14). A probiotic is defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” by the World Health Organization (15). LGG has been shown to have positive effects on the human body, such as preventing antibiotic-associated diarrhea (14) and boosting mucosal immunity (16). Although *C. elegans* has been used to identify a growing list of *Lactobacilli* that can promote host defense against pathogens, resistance against oxidative stress, and increase their lifespan (5-7), little research has been done to elucidate the impact of commercial probiotic strains on *C. elegans* physiology. Grompone *et al.* found that a similar strain of probiotic, *L. rhamnosus* CNCM I-3690, enhances the lifespan of *C. elegans* by inducing a differential expression of genes regulated by DAF-16 (11). DAF-16 is a transcription factor in the highly-conserved insulin-like pathway that plays a role in mediating host defense and responding to various types of stress in *C. elegans* (9, 11, 12). Studies has shown that it integrates signals from IIS pathway, TOR pathway, AMPK pathway, JNK signalling pathway and germline signaling pathway, which are all related to aging and longevity in *C. elegans* (17).

In this paper, we aimed to investigate the effects of LGG on the longevity of *C. elegans*. As LGG shares the same species classification as *L. rhamnosus* CNCM I-3690, we hypothesized that LGG would enhance longevity in *C. elegans* in a similar DAF-16-dependent manner. By analyzing another strain of *L. rhamnosus*, we hope to uncover a potential pattern in *L. rhamnosus* effects on *C. elegans* longevity. We performed lifespan assays on wild type (WT) and *daf-16* mutant *C. elegans* fed with OP50 or LGG. Our data suggest that LGG does not increase the longevity of *C. elegans*, and a subsequent experiment demonstrated that starved worms may be averse to the LGG growth media.

METHODS AND MATERIALS

Bacterial and *C. elegans* strains. *L. rhamnosus* GG and *E. coli* OP50 were acquired from the laboratories of Drs. Brett Finlay and Don Moerman respectively. The WT *C. elegans* strain (N2), and the *daf-16* knockout mutant strain (GR1307) were acquired from the Caenorhabditis Genetics Center through the Moerman lab (18). The identity of the LGG and OP50 strains were confirmed through Sanger sequencing of the whole 16s rRNA gene (not shown), a service provided by Genewiz[®].

Seeding Nematode Growth Media Plates. Overnight cultures of OP50 and LGG were prepared from isolated colonies grown on solid Nematode Growth Media (NGM) and De Man, Rogosa and Sharpe (MRS) media respectively. Well-formed, isolated colonies were used to inoculate 5 mL of either liquid LB (OP50) or MRS media (LGG). OP50 seeded NGM plates were prepared by spreading 150 μ L of overnight OP50 culture on to unseeded NGM plates, and incubating at 37 °C for ~24 hours. LGG seeded NGM plates were prepared by inoculating 5 mL of MRS broth with an isolated colony of LGG, and incubating the culture at 37 °C and 200 rpm for ~16 hours. After incubation, 100 μ L of LGG culture was spread on unseeded NGM plates and incubated at 20 °C for ~24 hours. Seeded OP50 and LGG NGM plates were stored at 4 °C until used.

Survival Assays. Survival assays were performed as previously described by Amrit *et al.* (19). Age-synchronized WT and *daf-16* worms were grown to the L4 phase on lawns of OP50. 30 L4 worms were transferred onto duplicate OP50 or LGG seeded NGM plates using a sterile platinum pick. Plates were stored at 20 °C. On day 1, worms from day 0 plates were counted as alive, dead or censored. Worms responding to gentle physical stress with a pick were counted as alive. Worms that were burrowed into the agar, desiccated on the wall of the agar plates, or killed by the operator were censored from analysis. Live worms were transferred daily onto fresh plates. This process was repeated every day until all 30 worms were dead or censored. All operators rotated in counting and transferring the

worms. To ensure that all operators were handling the worms with the appropriate care and precision, a pilot survival assay was performed on WT worms on OP50 (Fig. S1).

Avoidance Assay. The avoidance assay was modified from the *C. elegans* osmotic avoidance assay as described by Calahorra *et al.* (20). Two days prior to starting the assay L4 WT worms were transferred to unseeded NGM plates to produce starved worms, or new OP50 seeded plates to produce pre-fed worms. Overnight cultures were prepared by inoculating 5 mL of LB or MRS with OP50 or LGG respectively. The cultures were incubated at 37 °C and 200 rpm for ~16 hours. A 1 mL aliquot was taken from each overnight culture and spun at 21,130 x g for 1 min to separate the supernatant. To prepare the assay plates, 2 cm diameter circles were drawn on unseeded NGM plates. The circle was spread with 11 µL of LB media, MRS media, supernatant from the overnight cultures, the unspun overnight cultures, or left blank as a negative control.

The plates were incubated at 20 °C until used. To begin the assay 10 adult worms, either starved or pre-fed, were transferred to the center of each circle. The number of worms inside each circle were counted at 10 min intervals for 1 hour after the transfer. The next day, worms inside each circle were counted once more.

Growth Curve. To confirm that OP50 and LGG were capable of comparable rates of growth on NGM media, growth curves of the two bacteria strains in liquid NGM media were measured (Fig. S2). Overnight cultures were prepared as described above. A 1:100 dilution of either OP50 or LGG overnight culture in liquid NGM media (provided by the Moerman lab) was plated in triplicate in a 96-well plate. The OD600 was read every 10 mins in a BioTek Epoch plate reader at 37 °C over 16 hours. The growth curves were produced using liquid media at 37 °C, while the OP50 and LGG used in the previously mentioned experiments were grown on solid media at 20 °C. The results from the produced growth curve may give an estimate of how OP50 and LGG grow, but in future experiments growth curves run under conditions similar to the previously run experiments would give more representative results to determine if OP50 and LGG actually had comparable rates of growth.

Statistical analysis. All analyses and graphs were created using GraphPad Prism version 7.0a. Survival rates of *C. elegans* were illustrated using Kaplan-Meier survival curves. Differences in survival rates were tested for significance using the log-rank test. Post hoc analyses of mean lifespans were performed using the Mann-Whitney U test (non-parametric t-test). The chi-square independence test was used to examine the relationship between food source and censoring.

RESULTS

***Lactobacillus rhamnosus* GG does not increase the lifespan of WT or *daf-16* *C. elegans*.**

To determine whether the probiotic bacterium LGG influenced the lifespan of *C. elegans*, we compared the lifespans of WT worms grown on *E. coli* OP50 (OP50) versus *L. rhamnosus* GG (LGG). In a parallel experiment, we compared the lifespans of *daf-16* (GR1307) worms grown on OP50 versus LGG to determine whether the potentially lifespan-enhancing effects of LGG are dependent on DAF-16. Thirty L4 worms grown on NGM plates seeded with OP50 were transferred onto duplicate plates seeded with either OP50 or LGG, passaged daily, and maintained at 20°C.

The lifespans of WT and *daf-16* *C. elegans* did not increase when the worms were fed

TABLE 1 Lifespan characteristics of WT and *daf-16* *C. elegans* grown on OP50 and LGG.

Experimental condition	Median lifespan (days)	Maximum lifespan (days)	Death events	Censored events	% worms censored
WT + OP50	19	26	44	16	26.67
WT + LGG	15	25	22	38	63.33
<i>daf-16</i> + OP50	13	19	55	5	8.33
<i>daf-16</i> + LGG	13	18	39	21	35

on LGG (Fig. 1A). Non-significant decreases in mean lifespans were observed in both WT ($p = 0.0620$) and *daf-16* ($p = 0.1139$) worms grown on LGG compared to those grown on OP50 (Figure 1B). Compared to OP50, LGG shortened the median lifespan of WT *C. elegans*, but did not change the median lifespan of *daf-16 C. elegans* (Table 1). These data suggest that LGG has a minimal impact on the lifespans of both WT and *daf-16 C. elegans*.

Of note, a greater frequency of worms that were grown on LGG were censored compared to worms that were grown on OP50. 63% of WT and 35% of *daf-16* worms grown on LGG were censored, compared to 26% of WT and 8% of *daf-16* worms grown on OP50. Food source and censorship distribution were significantly related in both WT ($X^2(2, N=60) = 16.30, p < 0.0001$) and *daf-16* ($X^2(2, N=60) = 12.57, p < 0.0001$) worms. This indicates that censorship was not independent of whether the worms were grown on LGG or OP50, suggesting that censoring may be obscuring actual death events in both WT and *daf-16* worms grown on LGG. Thus, our survival curves may not accurately model the survival characteristics of *C. elegans* worms that are grown on LGG.

Starved *C. elegans* may be averse to food sources containing MRS media. The high frequency of censorship events observed in the worms grown on LGG led us to examine whether *C. elegans* is averse to LGG or the MRS media used to cultivate the bacteria. *C. elegans* have been reported to exhibit dietary preferences when given choices between different qualities of bacteria (quality defined as the ability to support growth (21)). Previous feeding behaviours are also known to affect dietary preferences in *C. elegans* (21). To test whether *C. elegans* is averse to LGG or MRS media, we developed a basic avoidance assay based on the osmotic avoidance assay described by Calahorra *et al.* (20). Ten WT L4 worms grown on NGM plates seeded with OP50 were starved, or allowed to continue feeding on OP50, for two days prior to transfer onto a single plate seeded in the center with one of six food sources. Worms were also transferred onto one plate with an unseeded center as a negative control. The number of worms remaining in the center were measured, and an endpoint count of all worms remaining in the center was performed the following day (Fig. 2). Due to time constraints, only a single trial per food source was performed.

At all timepoints within the first hour following transfer, the fractions of starved worms remaining in the center when it was seeded with food sources containing LB media were higher compared to when the center was seeded with food sources containing MRS media (Fig. 2A-B). Figure 2C indicates that worms pre-fed on OP50 were inclined to remain in the center seeded with LB and OP50 cell culture during the first hour following transfer. In

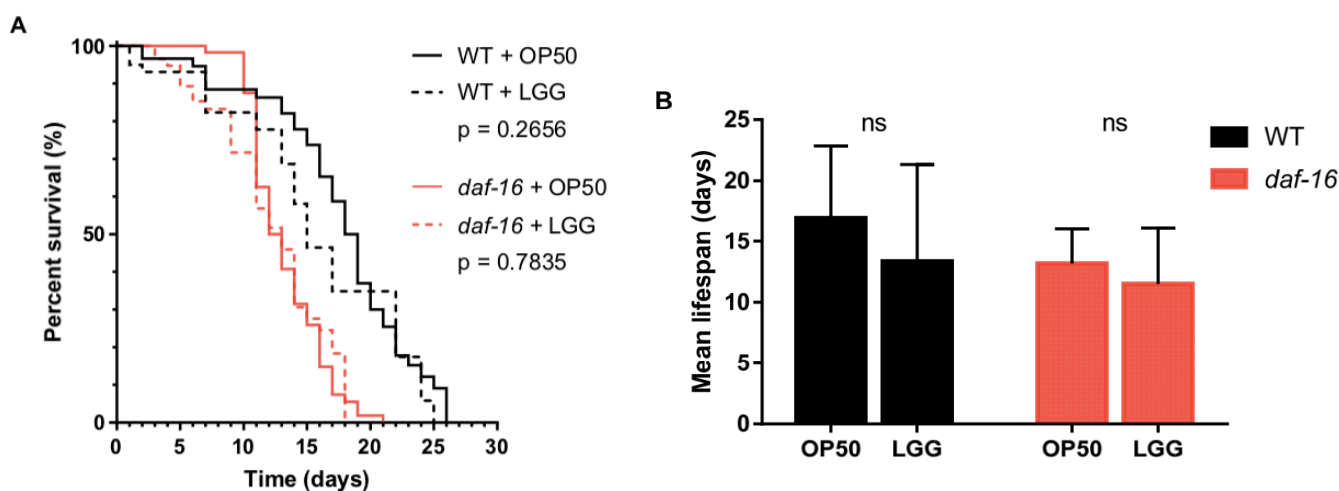


FIG. 1 Growth on LGG does not increase *C. elegans* lifespan compared to OP50. Thirty-day survival assays were performed with wild type (WT) and *daf-16* worms grown on OP50 or LGG seeded NGM plates. Worms were counted as alive, dead, or censored, then transferred to fresh plates daily. Kaplan-Meier survival estimates (A), and mean lifespans (B), were calculated for each of the four experimental conditions. Survival curves represent pooled data from duplicate plates of $n=30$ per plate. Mean lifespans data represent mean \pm standard deviation of $n=2$ plates per condition. Statistical analyses were performed using log-rank test for survival curves, and Mann-Whitney U test for mean lifespans.

contrast, the center seeded with MRS and LGG cell culture attracted a similar fraction of worms compared to the unseeded center (Fig. 2D) within the first hour. There were similar fractions of pre-fed worms that remained on centers seeded with LB alone versus MRS alone (Fig. 2C-D).

Figure 2E illustrates the endpoint fractions of worms remaining in the center seeded with each of the seven food sources, 24H after transfer. Both starved and pre-fed *C. elegans* showed a greater preference to remain in centers seeded with LB and OP50 cell culture, compared to MRS and LGG cell culture. Starved worms appeared to exhibit an aversion to MRS, with nearly no worms remaining in the center seeded with MRS alone. Visual observations (Fig. 3) demonstrate that the worms found outside of the center seeded with this treatment were found on the walls of the Petri dish. Pre-fed worms did not appear to share this aversion, as nearly the same fraction of worms chose to remain in the center seeded with MRS alone and in the center seeded with LB alone. Altogether, our preliminary results suggest that starved *C. elegans* may possess an aversion to MRS media, but pre-fed

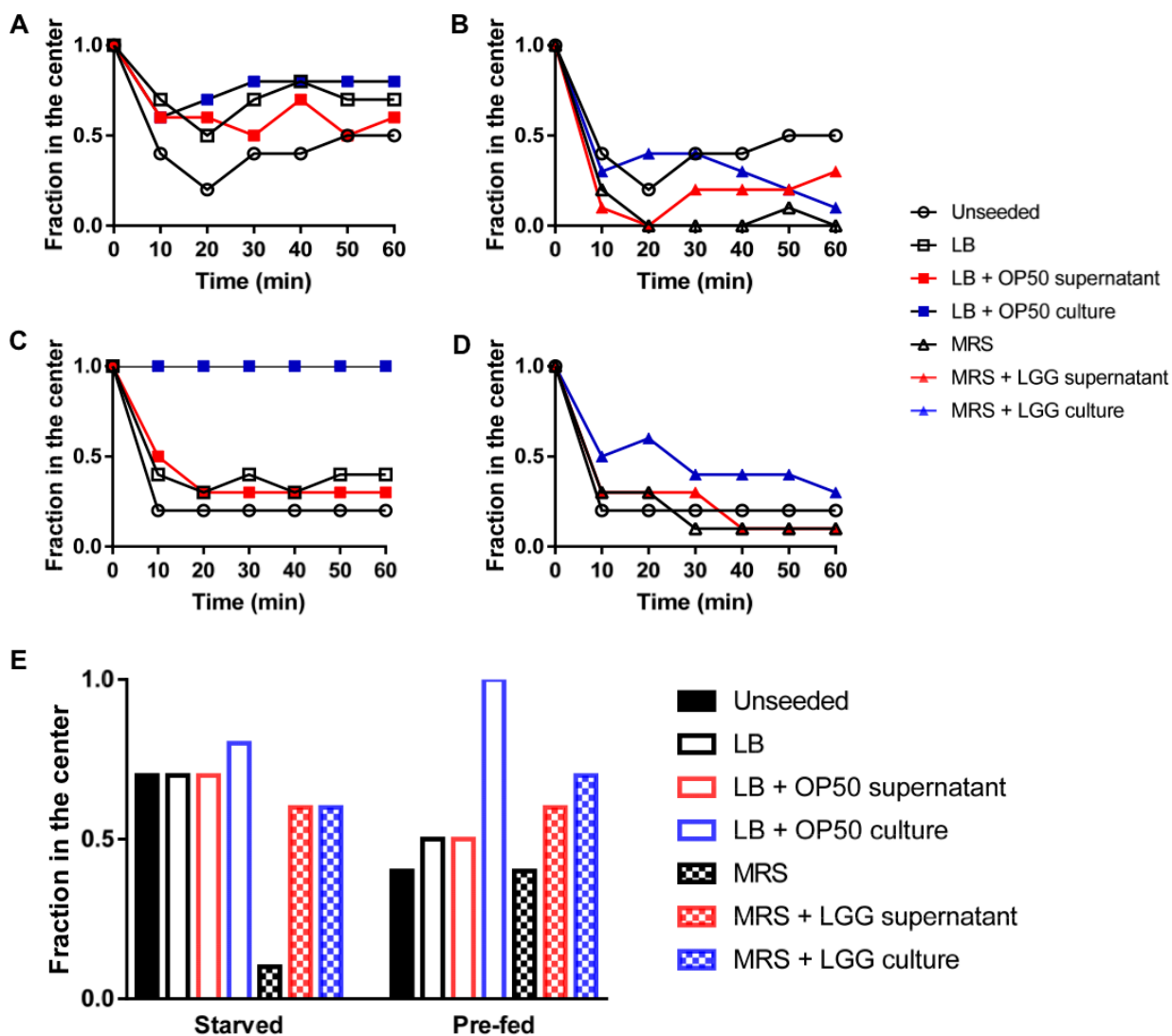


FIG. 2 Aversion to MRS media in *C. elegans* is dependent on prior feeding behaviour. Avoidance assays were performed with ten wild type worms per food source, starved or pre-fed with OP50. Worms were transferred onto plates seeded in the center with one of several food sources, and the fractions of those remaining inside the center were measured at 10 min intervals for 1H for both starved (A-B), and pre-fed worms (C-D). An endpoint count of worms remaining in the center was measured 24H after transfer (E). Data are n=1 plate per food source, with columns representing the fraction of worms remaining in the center of each plate.

worms do not. As our data are derived from a single trial, additional trials would be needed to determine whether these results reflect an avoidance behaviour in *C. elegans* or stochastic variation within the experimental system.

DISCUSSION

C. elegans has been widely used as a model to test for beneficial bacteria that can expand longevity and improve host defense against pathogenic bacteria (9-13). Different strains of *Lactobacilli* have been found to prolong *C. elegans* lifespan through several different mechanisms (9-13). One of the key regulators in the longevity mechanism is the transcription factor DAF-16, which is involved in the insulin transduction pathway in *C. elegans* (11, 17, 22). LGG is one of the most well studied *Lactobacilli* bacteria. However, the effect and underlying mechanisms of LGG on *C. elegans* aging have not been extensively studied. In this study, we hypothesized that LGG would increase the longevity of *C. elegans* in a DAF-16 dependent manner. We monitored the lifespan of WT *C. elegans* (N2) and the *daf-16* knockout mutant (GR1307) while feeding them with LGG, or its normal food source *E. coli* OP50. Our results have shown that the probiotic LGG does not increase the lifespan of *C. elegans*. By tracking the aversion behaviour of *C. elegans* on different media seeded onto NGM over time, we have also shown preliminary evidence that the media for growing food source bacteria may affect lifespan assay results.

Our results showed that LGG has no significant effect on *C. elegans* longevity (Fig. 1). The median lifespan of WT worms fed on LGG was four days shorter than those fed on OP50 (Table 1). As our data are derived from only two trials, statistical analysis cannot be performed to determine whether this is significant or not. This was observed in both WT and *daf-16* mutant, suggesting that the decrease is independent of the DAF-16 pathway. Interestingly, many censored worms were observed to be desiccated on the walls of the Petri dish in the WT and *daf-16* mutant + LGG treatments. These worms are counted as censored, but not dead, because their death is not due to aging but other reasons which drives the worms to move away from the seeded areas. An experimental parameter that may lead to the significant censorship may be the bacterial lawn preparation. As observed in the avoidance assay, worms pre-fed on OP50 may behave differently when they are transferred onto LB media versus MRS media, and when they were on OP50 supernatant versus LGG supernatant (Fig. 2B, 2D). We eliminated the possibility of pH change in the culture as no pH difference was observed between the LB and MRS media and the incubated bacteria

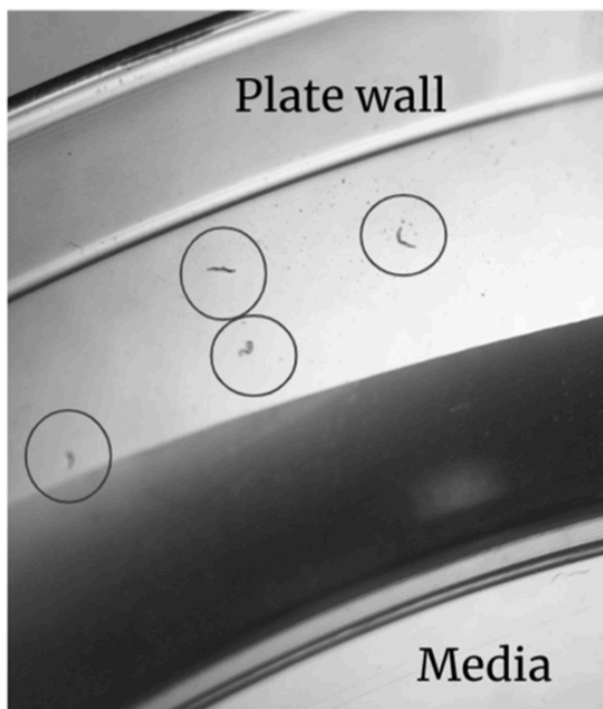


FIG. 3 Aversion to MRS media in starved worms results in censorship. Desiccated worms that had crawled up the sides of the plate were noted during our aversion assay. Worms that had been starved for two days prior to the assay, then transferred to a center seeded with MRS media alone, crawled up the side of the plate where they dried up (circled).

culture (data not shown). In our lifespan assay experiment, the bacterial lawns on NGM were prepared by plating 100 μ L of the bacteria overnight culture on NGM medium and dried overnight before use. Therefore, the MRS media used for LGG overnight culture could be contributing to the aversion event. One way to potentially resolve this issue would be to adopt the method used by Zanni *et al.*, which is to wash the bacteria in M9 buffer before plating to remove any residual media (23).

Our observation does not agree with the increase in lifespan observed by Zanni *et al.* and Guantarios *et al.* (23, 24). Zanni *et al.* reported a maximum lifespan of 18 days for WT worms fed with OP50 and a 26-day lifespan for those fed on LGG (23). While we observed a similar WT lifespan as Zanni *et al.* when fed with LGG, our data showed a much longer WT lifespan than theirs when fed on OP50 (23). The difference in experimental set up such as incubation temperature, bacteria lawn preparation and animal condition prior to experiment may have led to the difference in the trend observed. First, the incubation temperature in our assay was 20 °C whereas Zanni *et al.* used 16 °C (23). However, it has been previously reported that lower temperature enhances the longevity of *C. elegans* (25). Therefore, it is likely that other experimental parameters may contribute to this difference. One thing that is worthwhile to note is the difference in bacterial lawn preparation in the two studies. Zanni *et al.* prepared the lawns by resuspending 10 mg bacteria in M9 buffer for plating (23). As mentioned before, we suspect that this difference in plate preparation may have an impact on worm lifespan. By washing off the LB media in the OP50 culture, a metabolite within the media that is favored by *C. elegans* may be removed and consequently lead to a shorter lifespan of worms. Therefore, further lifespan assay should be repeated using plates prepared by the buffer-washing method to eliminate any possible impact from the bacterial culture media.

Preliminary results from the avoidance showed that both starved and pre-fed worms adopt similar trends in media preference after overnight plating, except for on the MRS media lawn (Fig 2E). This suggests that the starved and pre-fed worms would behave similarly when adapting to fresh food source. The aversion against MRS media is greater in starved worms than the pre-fed worms. The reason for this is unclear and no previous studies has reported similar observation. As we only performed the avoidance assay with 10 animals per treatment and only one replicate was done, the results from the experiment may be influenced by the small sample size. We believe more replication and larger sample size is necessary and may provide more convincing results. Based on our observations, animals who left the center have a clear preference for the food environment and would either return to the center or stay as far away as possible from it by moving up the petri dish wall.

Conclusions We monitored the effect of LGG diet on the longevity of *C. elegans* in both WT and *daf-16* mutant to investigate possible underlying mechanism. In contrast to our hypothesis, results from our experiments showed that LGG does not increase the lifespan of *C. elegans*. The non-significant decrease in lifespan of LGG-fed worms is not dependent on the DAF-16 pathway. We also designed an avoidance assay where we monitor the movement of starved and OP50 pre-fed worms after they are transferred onto plates with different conditions. Preliminary results from the assay indicated that starved animals may be averse to the MRS media, and may therefore impact results from the lifespan assay.

Future Directions First, the avoidance assay should be repeated with more worms as the assay was only performed once with 10 animals in this paper due to time constraints. In addition, as previously mentioned, we recommend further repeating the lifespan assay done on bacterial lawn that has been washed to remove residual culture media and use animals that are previously exposed to the same conditions. Also, the sample size of the survival assay is low compared to those in other studies due to large amount of censorship so increasing the sample size is necessary. Experiments may also include the *L. rhamnosus* CNCM I-3690, which has shown to increase *C. elegans* longevity by Grompone *et al.* as a positive control if the strain is available (11).

Future researchers may also improve the design of the avoidance assay. A limitation to the assay is that it cannot distinguish between the movement of *C. elegans* that are averse to the treatment or are foraging for other food by traveling further. As Shtonda and Avery

demonstrated, worms may exhibit dietary choice and hunt for higher quality food that can be easily eaten (21). Some of the animals may be moving outside the circle for better foods but eventually crawl up to the side of the walls and die. To take this into account, further experiments where an additional ring of the same treatment can be added in the plate. While animals hunting for higher quality food would move across the ring to search for other food, those averse to the treatment would stay in between the ring and the center and not crawl up to the walls. It is recommended to perform the assay in a bigger petri dish as the distance between the two treatment may also impact the way *C. elegans* behave. Future research may also focus on determining whether LGG is able to colonize the intestine of *C. elegans* by feeding them with fluorescent bacteria and monitoring the propagation of those bacteria throughout the *C. elegans* lifespan (26).

ACKNOWLEDGEMENTS

We would like to thank the University of British Columbia (UBC), Department of Microbiology and Immunology for funding this project. We would also like to thank Dr. David Oliver and Mihai Cirstea for their guidance and support throughout the term. We would like to thank Dr. Brett Finlay and Dr. Don Moerman for sourcing the materials and strains used in this project, with a special thanks to Mark Edgley for his expertise and training he provided in *C. elegans* husbandry. We would like to acknowledge teams 1a (Joshua Bowman, Robin Couput, Anthony Pookpun, Kendrew Wong) and 3V (Samantha Feng, Tina Liao, Catherine Wang, Aya Zakaria) for their collaborations, and the UBC Wesbrook Building media room staff for their support.

CONTRIBUTIONS

All authors were equally involved in all steps of this project from start to finish, from designing, planning and conducting experiments, to writing the final paper. Major contributions MC: executing experiments in the lab, and writing introduction and abstract of the paper. RDR: executing experiments in the lab, troubleshooting and organizing data, and writing methods and results of the paper. LL: assisting in laboratory experiments and writing discussion and future directions of the paper. CT: gathering foundational information for the project, statistical analysis and figure formatting on Graphpad, and writing results of the final paper.

REFERENCES

1. **O'Reilly LP, Luke CJ, Perlmutter DH, Silverman GA, Pak SC.** 2014. *C. elegans* in high-throughput drug discovery. *Advanced Drug Delivery Reviews.* 69-70:247-253.
2. **Kaletta T, Hengartner MO.** 2006. Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov.* 5:387-398.
3. **Riddle DL, Blumenthal T, Meyer BJ, Priess JR.** 1997. *The Biological Model.* Cold Spring Harbor Laboratory Press.
4. **Kim W, Hendricks GL, Lee K, Mylonakis E.** 2017. An update on the use of *C. elegans* for preclinical drug discovery: screening and identifying anti-infective drugs. *Expert Opin Drug Discov.* 12:625-633.
5. **Doré J, Simrén M, Buttler L, Guarner F.** 2013. Hot topics in gut microbiota. *United European Gastroenterology Journal.* 1:311-318.
6. **Guinane CM, Cotter PD.** 2013. Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. *Therapeutic Advances in Gastroenterology.* 6:295-308.
7. **Valdes AM, Walter J, Segal E, Spector TD.** 2018. Role of the gut microbiota in nutrition and health. *Bmj.* 361:k2179.
8. **Clark LC, Hodgkin J.** 2014. Commensals, probiotics and pathogens in the *Caenorhabditis elegans* model. *Cell. Microbiol.* 16:27-38.
9. **Ikeda T, Yasui C, Hoshino K, Arikawa K, Nishikawa Y.** 2007. Influence of lactic acid bacteria on longevity of *Caenorhabditis elegans* and host defense against *salmonella enterica* serovar enteritidis. *Appl. Environ. Microbiol.* 73:6404-6409.
10. **Park MR, Ryu S, Maburutse BE, Oh NS, Kim SH, Oh S, Jeong S, Jeong D, Oh S, Kim Y.** 2018. Probiotic *Lactobacillus fermentum* strain JDFM216 stimulates the longevity and immune response of *Caenorhabditis elegans* through a nuclear hormone receptor. *Sci Rep.* 8:7441.
11. **Grompone G, Martorell P, Llopis S, González N, Genovés S, Mulet AP, Fernández- Calero T, Tiscornia I, Bollati-Fogolin M, Chambaud I, Foligné B, Montserrat A, Ramón D.** 2012. Anti-inflammatory *Lactobacillus rhamnosus* CNCM I-3690 strain protects against oxidative stress and increases lifespan in *Caenorhabditis elegans*. *PLoS ONE.* 7:e52493.

12. **Nakagawa H, Shiozaki T, Kobatake E, Hosoya T, Moriya T, Sakai F, Taru H, Miyazaki T.** 2016. Effects and mechanisms of pro-longevity induced by *Lactobacillus gasseri* SBT2055 in *Caenorhabditis elegans*. *Aging Cell*. 15:227-236.
13. **Sharma K, Pooranachithra M, Balamurugan K, Goel G.** 2018. Multivariate analysis of increase in life span of *Caenorhabditis elegans* through intestinal colonization by indigenous probiotic strains. *Probiotics Antimicrob Proteins*. <https://doi.org/10.1007/s12602-018-9420-0>
14. **Vanderhoof JA, Whitney DB, Antonson DL, Hanner TL, Lupo JV, Young RJ.** 1999. *Lactobacillus GG* in the prevention of antibiotic-associated diarrhea in children. *The Journal of Pediatrics*. 135:564-568.
15. **World Health Organization.** 2002. Guidelines for the Evaluation of Probiotics in Food. https://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf
16. **Yan F, Liu L, Cao H, Moore DJ, Washington MK, Wang B, Peek RM, Acra SA, Polk DB.** 2017. Neonatal colonization of mice with LGG promotes intestinal development and decreases susceptibility to colitis in adulthood. *Mucosal Immunol*. 10:117-127.
17. **Sun X, Chen W, Wang Y.** 2017. DAF-16/FOXO Transcription Factor in Aging and Longevity. *Frontiers in Pharmacology*. 8:548.
18. **Caenorhabditis Genetics Center.** n.d. Strain information: GR1307. <https://cgc.umn.edu/strain/GR1307>
19. **Amrit FRG, Ratnappan R, Keith SA, Ghazi A.** 2014. The *C. elegans* lifespan assay toolkit. *Methods*. 68:465-475.
20. **Calahorra F, Alejandro E, Ruiz-Rubio M.** 2009. Osmotic avoidance in *Caenorhabditis elegans*: synaptic function of two genes, orthologues of human NRXN1 and NLGN1, as candidates for autism. *Journal of Visualized Experiments*.
21. **Shtonda BB, Avery L.** 2006. Dietary choice behavior in *Caenorhabditis elegans*. *Journal of Experimental Biology*. 209:89-102.
22. **Zhao L, Zhao Y, Liu R, Zheng X, Zhang M, Guo H, Zhang H, Ren F.** 2017. The Transcription factor DAF-16 is essential for increased longevity in *C. elegans* exposed to *Bifidobacterium longum* BB68. *Scientific Reports*. 7:7408-7.
23. **Zanni E, Laudenzi C, Schifano E, Palleschi C, Perozzi G, Uccelletti D, Devirgiliis C.** 2015. Impact of a complex food microbiota on energy metabolism in the model organism *Caenorhabditis elegans*. <https://doi.org/10.1155/2015/621709>
24. **Guantario B, Zinno P, Schifano E, Roselli M, Perozzi G, Palleschi C, Uccelletti D, Devirgiliis C.** 2018. *In vitro* and *in vivo* selection of potentially probiotic Lactobacilli from Nocellara del Belice Table Olives. *Frontiers in Microbiology*. 9:595.
25. **Lee S, Kenyon C.** 2009. Regulation of the longevity response to temperature by thermosensory neurons in *Caenorhabditis elegans*. *Current Biology*. 19:715-722.
26. **Aballay A, Yorgey P, Ausubel FM.** 2000. *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Current Biology*. 10:1539-1542.