12-2014

The Effects of Probiotics Supplementation on Health Using Caenorhabditis elegans as a Model System

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THE EFFECTS OF PROBIOTICS SUPPLEMENTATION ON A HEALTH USING CAENORHABDITIS ELEGANS AS A MODEL SYSTEM

A Thesis
Presented to
The Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
Miranda L. Klees
December 2014

Accepted by:
Dr. Min Cao, Committee Chair
Dr. Yuqing Dong
Dr. Thomas A. Hughes
ABSTRACT

The “Western Diet,” prominent among developed nations, often refers to a diet rich in meat proteins and refined sugars. Western society may in part be plagued with obesity and obesity related diseases due to a diet enriched with glucose. With an increasing glycemic index observed in Western society, it comes to no surprise that obesity, diabetes, heart disease, and other obesity related diseases are on the rise. The Centers for Disease Control and Prevention describes obesity as an epidemic, affecting more than 35% of U.S. citizens. Shortened lifespan and increased susceptibility to pathogens are associated with these diseases and linked to increased consumption of foods high in sugar. In order to reverse these observed effects, the healthful benefits probiotics have on immune system stimulation, restoration of shortened lifespan induced by a high glucose diet, and the beneficial effects of probiotics in combination of cranberry extracts in the models system Caenorhabditis elegans were investigated. An exciting benefit of implementing C. elegans in such studies is the innate immunological response and aging pathways homology with humans. By targeting these pathways, C. elegans provides an economical model system that provides application in humans. Here, we investigated the effects probiotics have on high glucose diet in C. elegans. Consistent with previous studies, when C. elegans was supplemented with each probiotic strain tested, lifespan extension was observed. Interestingly, the reversal of glucose induced shortened lifespan and the extension of lifespan in combination of cranberry extracts was strain dependent. The impact that probiotics have on the reversal of detrimental effects associated with a high glucose diet, including protection against known pathogens and
immunological response pathways targeted by probiotics, is currently under investigation.
DEDICATION

I would like to dedicate my work to my family, most importantly my parents. Their love and support through failures and accomplishments have shaped me into the woman I am today. The memory of my mother has been a major driving force for my accomplishments, all the while striving to be the woman she shaped me to be. I would also like to dedicate my work to my brothers and sisters and stepmom: Jacob, Marshall, Tateum, Pyper, and Robyn for all of their support during my journey through graduate school.
ACKNOWLEDGEMENT

I would like to thank my advisor Dr. Min Cao for her invaluable support and guidance in developing this project. I would also like to thank the rest of my committee members Dr. Yuqing Dong and Dr. Thomas Hughes for their support and guidance in all of my decisions and work progress.

I would also like to thank my lab mates for helpful discussions and experimental advice and assistance: Daniel Pederson, Sujay Guha, Ethan Wilson, Joseph Angeloni, Ojas Natarajan, Jessica Dinh, and Xiaxiao Wang. Lastly, I would like to thank my undergraduate research advisor Dr. Joseph Flaherty for all of his support along the way and sparking my interest in research that led to the pursuit of my master’s degree.
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LIST OF ABBREVIATIONS

Bc ........................................................................................................... *Bacillus coagulans*
Bs ........................................................................................................... *Bacillus subtilis*
CBE ........................................................................................................ Cranberry Extracts
CFU ......................................................................................................... Colony Forming Unit
ddH₂O .................................................................................................. Double-Distilled Water
*E. coli* OP50 ........................................................................................ *Escherichia coli* OP50
FUDR ..................................................................................................... 5-Fluoro-2’-deoxyuridine
IL .............................................................................................................. interleukin
LAB ......................................................................................................... Lactic Acid Bacilli/Bacteria
LB ............................................................................................................. Luria-Bertani Broth
Ld ........................................................................................................... *Lactobacillus delbrueckii*
Lf ........................................................................................................... *Lactobacillus fermentum*
Lp ........................................................................................................... *Lactobacillus planatarum*
LPS ......................................................................................................... lipopolysaccharides
MRS ......................................................................................................... Lactobacilli MRS Medium
NGM .................................................................................................... Nematode Growth Medium
NO ......................................................................................................... nitric oxide
p38 MAPK ............................................................................................. p38 mitogen-activated protein kinase
qRT-PCR ............................................................................................. Quantitative Real Time Polymerase Chain Reaction
spp ........................................................................................................... species
subsp ..................................................................................................... sub-species
TNFα ..................................................................................................... tumor necrosis factor alpha
The “Western Diet,” prominent among developed nations, often refers to a diet rich in meat proteins and refined sugars. Western society may in part be plagued with obesity and obesity related diseases due to a diet enriched with glucose. An increasing glycemic index has been observed in western society, and this increase has been linked to obesity, diabetes, and heart disease. The Centers for Disease Control and Prevention describes obesity as an epidemic, affecting more than 35% of U.S. citizens. Though obesity is a complex trait influenced by diet, developmental stage, age, physical activity, and genetics, the recent epidemic is probably reflective of environmental and lifestyle changes, with diet being a major influence. Diet, from a scientific perspective, can be described as the sum of food consumption by an organism. As humans, we have the ability to consciously decide what foods we consume; however, proper nutrition involves consumption of essential vitamins and minerals needed for metabolic processes. Much of our food energy intake is consumed in the forms of carbohydrates, proteins, and fats. The dietary decision of a healthful balance plays a major role on quality of life and health and longevity. Therefore, diet not only affects metabolic activities and overall health, but it also affects lifespan.
II. OBESITY

As previously mentioned, obesity is a complex characteristic influenced by many factors including but not limited to diet, lifestyle, and genetics. The phenotype is responsible for 6-10% of national healthcare costs in the United States; in addition to, a lifetime of medical costs that are $10,000 greater in obese patients in comparison to non-obese. Researchers argue that the rise in obesity is the result of an increase in sedentary lifestyles \(^2,^3\) while others argue that the culprit is over-consumption and gluttony \(^4,^5\). In the 1960s, the average American weighed 168 pounds; today, that average has increased nearly 15 pounds. Since the 1970s, obesity rates have doubled according to the Centers for Disease Control and Prevention. Medications, cosmetic surgeries, and diet may be methods for short-term treatment of obesity; however, long-term treatment involves a lifelong commitment to healthy diet habits and increased physical activity. Still, most people who previously suffered from obesity have relapses within 5 years of surgeries or other methods of weight loss.

Obesity is characterized by excess body fat accumulation as a result of increased energy intake compared to expenditure to the point that it becomes detrimental to health. When more calories are consumed than expended, the resulting phenotype is weight gain. Increased body fat proportionally increases the risk for chronic diseases such as diabetes, heart disease, and cancer. Not only are these chronic diseases associated with obesity, but bacterial diversity shifts are also observed in these patients \(^6,^7\). Other side effects of obesity include immune system failures and increased sensitivity to pathogens.
III. OBESITY & THE MICROBIOME

The gut is home to a vast array of microorganisms that aid in digestive health, immune stimulation, and provide a competitive environment to prevent colonization of many harmful pathogens. With the alteration of diet associated with Western society, the microbial community is severely impacted within the gut. The human body and gastrointestinal tract are colonized at birth, and continues to shift based on factors, such as breastfeeding, diet, and environmental exposure, until the microbial community becomes stabilized around 3 years of age. The human gut is largely comprised of Actinobacteria, Firmicutes, and Bacteroidetes. The functions of the gut microbiota obtained throughout our lifespan are essential to the metabolism of xenobiotic compounds, amino acids, and carbohydrates. The balance of bacteria within the gut can be rapidly altered by dietary shifts. For example, when mice were fed a high-fat diet, the gut microbial composition shifts were observed in 24 hours. When altered gut microbiota, resultant of a high-fat diet, were transferred to germ-free mice, the subjects experienced increased adiposity, consistent with the suggestion that adiposity and obesity are linked to microbiota. Specifically, a reduction of Bacteroidetes was observed in obese subjects; however, not all studies consistently observed this reduction. When obesity is induced via diet the resulted mice experienced an increase of *Eubacterium dolichum*, a member of the Firmicutes phyla and a decrease in *Bifodobacterium*, a member of the Actinobacteria phyla. Energy restriction and increased physical activity have also been linked to changes in the gut microbiota. These microorganisms provide necessary enzymes involved in utilization of non-
digestible carbohydrates, involved in cholesterol reduction, important for biosynthesis of vitamins K and B\textsuperscript{21,22}, and involved in deconjugation and dehydroxylation of bile acids which, necessary in solubilizing and absorbing lipids\textsuperscript{23}. Factors other than diet also played a crucial role in microbiota, including but not limited to, gut microbiota shifts in obese subjects which were dependent on age\textsuperscript{24}.

IV. OBESITY & IMMUNE RESPONSE

A high-fat diet is associated with chronic low-grade inflammation and increased susceptibility to infection\textsuperscript{25}. In obese patients, the innate immune system responds by increased macrophage infiltration in adipose tissue and production of inflammatory adipokines\textsuperscript{26}. Increased plasma lipopolysaccharides (LPS) levels were observed with obesity\textsuperscript{17,27}, activating the synthesis of inflammatory cytokines TNF\textalpha. Chronic activation of the innate immune system results in increased risk for development of obesity; dietary compounds may in part be responsible\textsuperscript{26}.

V. CAENORHABDITIS ELEGANS AS A MODEL SYSTEM

\textit{C. elegans} is a favorable model system for studying aging and immune response for many reasons: 1) ease of maintenance, 2) homological pathways, including aging and immunity, with humans, 3) transparent, 4) economically favorable, and 5) relatively short lifespan. \textit{C. elegans} offers a faster, efficient analysis of human related diseases while allowing us to infer evolutionary history of immune components to study conserved traits and obtain information about probable functions. Similar to humans, many pathogens
have the ability to colonize the digestive tract of *C. elegans*, giving rise to an ideal model to study pathogenesis. However, many pathogens may exhibit other modes of infection in the worm, adhering to the cuticle or producing lethal toxins \(^{28, 29}\). We are able to determine the pathology of such pathogens by determination of a decrease in lifespan.

Though much less complex than the mammalian immune response, *C. elegans* immune defenses similarly consists of an innate immune response in which it is solely reliant. In its natural habit, the soil-dwelling nematode feeds on bacteria and has frequent encounters with pathogens. Most pathogens infect the worm via ingestion and then establish in the intestine where the majority of the damage occurs. In response to invasive pathogens the worms have developed defenses that include a behavioral response, physical barriers, and physiological response. As the first line of defense, behavioral responses must be employed to reduce the number pathogen encounters. However, exposure is inevitable and the worm must elicit physiological response to prevent colonization of the gut during the process of feeding. The p38 mitogen-activated protein kinase (MAPK) pathway and the insulin-like receptor pathway

![Figure 1-1. *C. elegans* life cycle at 25°C (artwork from http://www.scq.ubc.ca/genetic-studies-of-aging-and-longevity-in-model-organisms/).]
are two pathways involved in immune response, longevity, and mammalian homology. These two pathways interact and respond to a vast array of stress conditions. The p38 MAPK pathway is activated in response to pathogens such as Pseudomonas aeruginosa and Salmonella enterica, as well as in response to other pathogens and other stressors such as acute dehydration. The insulin-like receptor pathway is also important to the response to P. aeruginosa, Enterococcus faecalis, and Staphylococcus aureus. This pathway also has complex interactions with other regulatory elements such as the heat shock factor-1 (HSF-1) and the superoxide dismutase-3 (SOD-3).

*C. elegans* possesses a complex and interactive innate immunological response that has been extensively studied. The advantages of using this experimental system as a means to study application in humans is evident through the extensive study of these evolutionarily conserved pathways.

VI. PROBIOTICS SUPPLEMENTATION

A probiotic can be defined as any microorganism believed to provide health benefits upon consumption. The most common types of probiotics found in our diet are *Bifidobacterium* and lactic acid bacteria. Several strains of lactic acid bacteria, mainly *Lactobacillus* spp. have been shown to induce longevity as well as alleviating the effects of infection caused by *Vibrio parahaemolyticus, Salmonella typhimurium, Salmonella enteritidis, Staphylococcus aureus*, and *Enterococcus faecalis*. Bifidobacteria have the ability to increase lifespan in *C. elegans* via the p38 MAPK pathway, a homologous innate immunological pathway with humans. However, the
pathway in which each probiotic targets may be dependent on the strain of probiotic and the infectious strain of bacteria. Kim et al. suggested that *Lactobacillus acidophilus* had minimal effects on Gram-negative bacteria *Pseudomonas aeruginosa* and *Salmonella enterica* Typhimurium. On the contrary, *L. acidophilus* provided protection from *Enterococcus faecalis* and *Staphylococcus aureus* infection while targeting the p38 MAPK pathway suggested during Gram-positive immune response in *C. elegans*\(^7\). The aforementioned probiotics and the probiotics utilized in this study are often consumed in yogurts, fermented foods and dietary supplements. More interestingly, previous studies suggest that probiotics have the ability to enhance the immune response in not only *C. elegans* but also in a higher organism model system, mice\(^{37, 40-42}\).

**VII. CRANBERRY & APPLICATION**

The probiotics targeted in this study are often consumed in yogurts, fermented foods, and dietary supplements. Though probiotic supplementation is an emerging area of research, little is known about the benefits they may have on healthspan and lifespan, particularly when supplemented with nutraceuticals. Previous studies suggest that probiotics have the ability to enhance the immune response in not only *C. elegans* but also in mice\(^{37, 40-42}\). Numerous studies have also linked nutraceuticals to longevity and stress resistance\(^{43, 44}\). Still, little is known of the benefits of synergistic supplementation. For years, the North American cranberry (*Vaccinium macrocarpon*) has been ingested to alleviate the effects of a urinary tract infection by preventing adherence of bacteria to host’s cells\(^{45}\). However, there is no
evidence suggesting that cranberry is actually effective in treating or even preventing a urinary tract infection. Preliminary data suggests components in cranberry may prevent plaque formation on teeth, kill *Helicobacter pylori* (the causative agent of stomach cancer and ulcers), and even prevent tumor development. Research suggests that cranberry provides many nutritional and healthful benefits, owing to its antioxidant, antimicrobial, anti-apoptotic, anti-aging, anticancer, anti-inflammatory, and enhancement of endothelial functions. Recently published research from our lab suggests that cranberry, when studied in the soil dwelling nematode model organism *C. elegans*, effectively extends lifespan and promotes healthy aging while elevating resistance to certain stressors, including bacterial infection.

**VIII. SIGNIFICANCE**

Previous research suggests that probiotics such as *Lactobacillus* isolates can control and alleviate the effects of pathogenic infections; however, much still needs to be studied. *C. elegans* provides an effective model of study of pathogenesis with application in humans. Another exciting benefit of implementing *C. elegans* in such studies is the homology in humans in the innate immunological response pathways and aging pathways. The optimal combination of probiotics that successfully stimulates the immune system has yet to be studied. If we are able to determine a favorable combination of probiotics that have the ability to reverse the effects of a high glucose diet while promoting healthy aging and aiding in immune stimulation, we may be able to further provide insight into the effects that a high-glucose diet has on overall health and how to
reverse such detrimental effects. Studies can then be carried out in a higher organism such as mice to further confirm implication in supplementing a particular combination of probiotics in our diet.
CHAPTER TWO
MATERIALS & METHODS

I. BACTERIAL STRAINS AND WORM STRAINS

*Lactobacillus fermentum* (ATCC 9338), *L. planatarum* (ATCC 8014), *L. delbrueckii* (ATCC 7830), and *B. coagulans* were all acquired from VWR. *B. subtilis* CU1065 was a lab strain originally acquired from Cornell University. *E. coli* OP50 was acquired from lab stocks. *Lactobacillus* strains were cultured anaerobically in 5% CO₂ on MRS agar at 37°C for 48 hours. *Bacillus* spp. were cultured aerobically on LB agar at 37°C overnight. *E. coli* OP50 was cultured on LB+streptomycin (100μg/mL) at 37°C overnight.

*Bacillus* spp. and *E. coli* OP50 overnight cultures for lifespan assays were inoculated in LB broth and incubated at 37°C shaking at 150 rpm. *Lactobacillus* strains were inoculated in MRS broth and incubated at 37°C stationary in 5% CO₂.

N2 worms were acquired from the Caenorhabditis Genetics Center (CGC). Worms were maintained on nematode growth medium (NGM) seeded with *E. coli* OP50 at 25°C, and transferred to fresh plates every two days.

II. CBE PREPARATION

The cranberry extracts, HI-PAC BL DMAC 5, used in this study were obtained from DECAS cranberry products, Inc. and were received in the powdered form. For working stock solutions, CBE was dissolved in ddH₂O at a concentration of 5% and final concentration used was 0.2% (2mg/mL).
III. PREPARATION OF MEDIA

NGM for maintenance was prepared via standard protocol in 60mm plates. *E. coli* OP50 was dropped on the center of the plates the night prior to transfer of worms. NGM+glucose plates were prepared via standard protocol with the addition of 2% D-glucose final concentration in 60mm plates. Bacterial strains were dropped onto the center of the plates 2 hours prior to worm transfer.

NGM-FUDR 35mm plates were used for lifespan assays. Five times concentrated bacterial cultures were dropped in 100μL aliquots onto the center of plates 2 hours prior to worm transfer. D-glucose was added with a final concentration of 2% to NGM-FUDR plates prior to pouring and 5mL aliquots were added to each plate. For CBE testing, CBE was dissolved in water and dropped and spread confluent onto 5mL NGM-FUDR the night prior to start of assay.

IV. GROWTH CURVE

A single colony of each strain was inoculated from a freshly streaked plate into 5 mL MRS for *Lactobacillus* strains, LB for *Bacillus* strains, and LB+strep (100μg/mL) for *E. coli* OP50. *Lactobacillus* spp. were grown statically overnight at 37°C in 5% CO₂. *E. coli* OP50 and *Bacillus* strains were grown under aerobic conditions overnight at 37°C. On the next day, the overnight cultures were transferred into sterile 15 mL centrifuge tube and centrifuged at 4000 rpm for 10 min at room temperature. Cell pellets were resuspended in 10 mL NGM liquid medium. Aliquots of 50 μl were plated from the following dilutions $10^5$ and $10^6$ onto designated agar plates. This was the CFU for T=0.
Resuspended cultures were transferred in 3 mL aliquots into each glass test tube, and then desired concentrations of glucose were added to corresponding tubes. Tubes were incubated under aerobic conditions at 37°C. *Lactobacillus* tubes were kept at 37°C CO₂ incubator. At each time point (T=2, 4, 12, 24, 48 hr), serial dilutions were performed and dilutions to be plated were estimated based on cultural density. Aliquots of 50 μL were plated on corresponding plates. CFU/mL were calculated the next day using the standard plate count method.

V. LIFESPAN ASSAYS

Lifespan assays were carried out at 25°C. Worms were synchronized by transferring 20 gravid worms to 60mm NGM plates seeded with *E. coli* OP50 2 days prior to the start of assays. Worms were allowed to lay eggs for 5 hours, and then parent worms were removed from plates, leaving only synchronized eggs. Worms were then incubated until L4 stage. Overnight cultures of *Bacillus* spp. and *E. coli* OP50 were concentrated by centrifugation and removing 80% of supernatant. Cultures were then resuspended in remaining medium. *Lactobacillus* overnight cultures were concentrated via centrifugation and removing 100% of supernatant. Cultures were resuspended in 1 mL of M9 buffer. Aliquots of 100μL were dropped on the center of NGM-FUDR 35mm plates with and without 2% D-glucose and allowed to dry for 2 hours. Worms in L4 stage were transferred to assay plates; 30 worms per plate. Plates were subsequently checked daily and dead worms were counted and recorded. Day of transfer was defined as zero. Statistical analysis was carried out through SPSS software under Kaplan-Maier lifespan
analysis. P-values were determined via log rank test, and P<0.05 was accepted as statistically significant.
CHAPTER THREE

RESULTS

I. EXPERIMENTAL DESIGN

Previous studies showed that 2% glucose significantly reduced lifespan in *C. elegans*\(^{51}\). Consistent with this studied, 2% D-glucose significantly decreased the lifespan of N2 worms; therefore, 2% D-glucose was used to represent a high glucose diet. Similarly, previous studies from our lab determined 2mg/mL was the ideal concentration of CBE supplementation to extend lifespan and modulate the immune response\(^ {44, 50}\). Based on previous studies, a series of experiments were employed to determine the effects that probiotics had on reversing the effects of a high glucose diet in *C. elegans* and the effects probiotics have on lifespan in combination with CBE (Figure 3-1).

![Diagram of the experimental design of lifespan assays carried out at 25°C with different diets.](image)

**Figure 3-1.** Diagram of the experimental design of lifespan assays carried out at 25°C with different diets.
II. EFFECTS OF 2% D-GLUCOSE AND 2mg/mL CBE ON BACTERIAL GROWTH

Glucose induced shortened lifespan was first observed in *C. elegans* (Table 3-2). Dietary restriction is a well-documented cause of lifespan extension in the worm model. To determine if 2mg/mL CBE and/or 2% D-glucose would affect bacterial growth, growth curves using the standard plate count method were performed. Each strain was treated with either 2% D-glucose or 2mg/mL CBE for up to 72 hours, growth was not significantly inhibited (Figure 3-2 A-F).
Figure 3-2. Growth curves of each probiotic with and without 2% D-glucose and with and without 2mg/mL CBE in NGM broth. A) E. coli OP50 B) B. subtilis C) B. coagulans D) L. fermentum E) L. planatarum F) L. delbrueckii.
III. EFFECT OF PROBIOTICS ON LIFESPAN

Previous studies suggested that probiotic strains significantly extend lifespan in *C. elegans*; however, the observable extension has also been suggested to be dose dependent\(^{39-42, 52}\). The five strains tested in this study all extended lifespan when supplemented during L4 stage of worms (Figure 3-3, Table 3-1). *B. subtilis* fed worms had one day lifespan extension compared to the control. *B. coagulans* fed worms had 4 days lifespan extension compared to the control, *L. fermentum* had 3 days lifespan extension, *L. planatarum* had 1.5 days extension, and *L. delbrueckii* had 2 days lifespan extension.

![Figure 3-3](image-url)

**Figure 3-3.** Probiotic strains extended lifespan of N2 worms compared to *E. coli* OP50 at 25°C. *L. planatarum* exhibited greatest lifespan extension with mean lifespan of 16.042 days.
Table 3-1. Effect of probiotics on lifespan of N2 worms at 25°C

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean±SE (Days)</th>
<th>N</th>
<th>P-value</th>
<th>% Increase of Lifespan</th>
</tr>
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<tbody>
<tr>
<td>E. coli OP50</td>
<td>13.442±0.244</td>
<td>95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>17.261±0.336</td>
<td>92</td>
<td>&lt;0.05</td>
<td>28.41%</td>
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<tr>
<td>L. fermentum</td>
<td>16.663±0.213</td>
<td>92</td>
<td>&lt;0.05</td>
<td>28.45%</td>
</tr>
<tr>
<td>L. planatarum</td>
<td>15.085±0.249</td>
<td>82</td>
<td>&lt;0.05</td>
<td>12.22%</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>15.480±0.307</td>
<td>75</td>
<td>&lt;0.05</td>
<td>15.16%</td>
</tr>
<tr>
<td>E. coli OP50</td>
<td>12.737±0.459</td>
<td>99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>14.468±0.369</td>
<td>109</td>
<td>&lt;0.05</td>
<td>13.6%</td>
</tr>
</tbody>
</table>

Probiotic strains extended lifespan compared to E. coli OP50, consistent with previous research. P-value <0.05 considered significant. Data shown are representative of one round of lifespan assays.
IV. EFFECT OF D-GLUCOSE ON LIFESPAN

Consistent with previous studies, when glucose was supplemented at a concentration of 2% into the worm diet, lifespan was significantly reduced. *C. elegans’* lifespan was significantly reduced 15.7% as compared to the control strain without glucose (Figure 3-4, Table 3-2).

![Graph showing lifespan reduction](image)

**Figure 3-4.** Glucose significantly reduced lifespan of N2 worms under normal lab conditions at 25°C. Lifespan was reduced by 2 days, P<0.05. Data shown are representative of one round of lifespan assays.
V. PROBIOTICS REVERSAL EFFECTS OF GLUCOSE INDUCED SHORTENED LIFESPAN

As previously mentioned, each probiotic strain extended lifespan, and glucose was determined to reduce lifespan. When probiotics were supplemented with glucose, reversal of the glucose induced shortened lifespan was dependent on the strain of probiotic. Though lifespan was not fully restored, three of the five strains exhibited some level of lifespan restoration. The control strain exhibited a 15.7% decrease in lifespan. *B. subtilis* only exhibited a 5.3% decrease, *B. coagulans* had an 11.39% decrease, *L. fermentum* had a 16.1% decrease, and *L. delbrueckii* had an 8.7% decrease. *L. planatarum* exhibited the most dramatic decrease of 64.6% reduction of lifespan (Figure 3-5, Table 3-2).
Figure 3-5. Lifespan curves of N2 worms fed on each probiotic with or without 2% glucose supplemented into diet at 25°C. A) Control E. coli OP50 B) B. subtilis fed worms C) B. coagulans fed worms D) L. fermentum fed worms E) L. planatarum fed worms F) L. delbrueckii fed worms.
Table 3-2. Lifespan of N2 worms on high glucose diet and probiotics at 25°C

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean±SE (Days)</th>
<th>N</th>
<th>P-value</th>
<th>% Decrease of Lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli OP50</td>
<td>13.442±0.225</td>
<td>95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>OP50+2% glucose</td>
<td>11.333±0.207</td>
<td>69</td>
<td>&lt;0.05</td>
<td>15.70%</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>13.477±0.202</td>
<td>86</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bs+2% glucose</td>
<td>12.759±0.138</td>
<td>87</td>
<td>&lt;0.05</td>
<td>5.30%</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>17.261±0.221</td>
<td>92</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Bc+2% glucose</td>
<td>15.301±0.142</td>
<td>73</td>
<td>&lt;0.05</td>
<td>11.39%</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>16.663±0.262</td>
<td>92</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lf+2% glucose</td>
<td>13.986±0.240</td>
<td>73</td>
<td>&lt;0.05</td>
<td>16.10%</td>
</tr>
<tr>
<td>L. planatarum</td>
<td>15.085±0.273</td>
<td>82</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lp+2% glucose</td>
<td>5.342±0.130</td>
<td>73</td>
<td>&lt;0.05</td>
<td>64.60%</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>15.48±0.256</td>
<td>75</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ld+2% glucose</td>
<td>14.135±0.318</td>
<td>74</td>
<td>&lt;0.05</td>
<td>8.70%</td>
</tr>
</tbody>
</table>

N2 lifespan was shortened with all strains when supplemented with glucose. Lifespan restoration was observed in *Lactobacillus delbrueckii*, *Bacillus subtilis*, and *Bacillus coagulans*. Worms fed *Lactobacillus planatarum* exhibited an extreme decrease in lifespan when supplemented with 2% D-glucose. Data shown are representative of one round of lifespan assays.
VI. EFFECTS OF PROBIOTICS IN COMBINATION WITH CBE

Previous studies in our lab have determined that the optimum concentration of cranberry supplementation was 2mg/mL. At this concentration C. elegans’ lifespan is extended significantly without affecting bacterial growth of different strains such as Vibrio cholerae and E. coli OP50. When CBE was supplemented at the time of exposure to probiotics, C. elegans lifespan was extended (Figure 3-6 A-E). We can also conclude that when C. elegans is fed probiotics in succession with CBE supplementation, lifespan was significantly extended in comparison without CBE treatment. L. planatarum only exhibited bacterial extension effect, and lifespan was not further extended when 2mg/mL CBE was supplemented into the diet (Figure 3-6 A-E).
Table 3-3. Lifespan of N2 worms fed on probiotics and CBE diet at 25°C

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean±SE (Days)</th>
<th>N</th>
<th>P-value</th>
<th>% Increase of Lifespan</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli OP50</td>
<td>12.737±0.459</td>
<td>99</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>14.468±0.369</td>
<td>109</td>
<td>&lt;0.05</td>
<td>13.6%</td>
</tr>
<tr>
<td>B.s.+2mg/mL CBE</td>
<td>15.359±0.106</td>
<td>64</td>
<td>&lt;0.05</td>
<td>20.6%</td>
</tr>
<tr>
<td>B. coagulans</td>
<td>15.295±0.181</td>
<td>88</td>
<td>&lt;0.05</td>
<td>20.1%</td>
</tr>
<tr>
<td>B.c.+2mg/mL CBE</td>
<td>16.136±0.130</td>
<td>81</td>
<td>&lt;0.05</td>
<td>26.7%</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>13.44±0.511</td>
<td>90</td>
<td>&lt;0.05</td>
<td>5.5%</td>
</tr>
<tr>
<td>L.f.+2mg/mL CBE</td>
<td>15.575±0.320</td>
<td>87</td>
<td>&lt;0.05</td>
<td>22.3%</td>
</tr>
<tr>
<td>L. planatarum</td>
<td>14.9±0.194</td>
<td>100</td>
<td>&lt;0.05</td>
<td>17%</td>
</tr>
<tr>
<td>L.p.+2mg/mL CBE</td>
<td>14.693±0.300</td>
<td>75</td>
<td>&lt;0.05</td>
<td>15.4%</td>
</tr>
<tr>
<td>L. delbrueckii</td>
<td>14.364±0.459</td>
<td>66</td>
<td>&lt;0.05</td>
<td>12.8%</td>
</tr>
<tr>
<td>L.d.+2mg/mL CBE</td>
<td>15.742±0.304</td>
<td>66</td>
<td>&lt;0.05</td>
<td>23.6%</td>
</tr>
</tbody>
</table>

N2 lifespan was increased with all strains when supplemented with CBE except L. planatarum. Lifespan extension was observed in *Lactobacillus delbrueckii, Lactobacillus fermentum, Bacillus subtilis*, and *Bacillus coagulans*. Data shown are representative of one round of lifespan assays.
Figure 3-6. N2 lifespan curves carried out at 25°C with and without 2mg/mL CBE and probiotics. A) *B. subtilis* B) *B. coagulans* C) *L. fermentum* D) *L. planatarum* E) *L. delbrueckii*. 
CHAPTER FOUR

DISCUSSION

Over a century ago, a Russian scientist by the name of Élie Metchnikoff first hypothesized that probiotics could beneficially modify the gut flora by replacing harmful pathogens. Recently, manufacturers’ marketing of foods containing beneficial probiotics has fueled the renewed interest in probiotics research. However, the consumption of probiotics as a means to provide health benefits can be traced back to the Greeks and Romans through the consumption of fermented food products such as cheeses and other dairy products.

The modern hypothesis, adapted from Metchnikoff’s findings, stating that probiotics and microbiota have major influences on human health, has further evolved with the recent increase in consumer awareness. Consumers and researchers have expanded the research interests to target the use of oral pill supplementation of probiotics into diet and some have even experimented with fecal transplantations as a means to combat digestive disorders. Of particular importance are the LAB strains. Lactobacillus is a genus of Gram-positive facultative anaerobes or microaerophilic rod-shaped bacteria. Their production of lactic acid from the conversion of lactose and other sugars, create an acidic environment, prohibiting the growth of some harmful pathogens. The lower pH also provides an environment for a balanced ecosystem in the gut. Metabolism and metabolite production by Lactobacillus spp. are not only species dependent but also dependent on the strain and/or subspecies.
In combination with previous research of beneficial supplementation of probiotics as a means to extend lifespan in *C. elegans* and research suggesting a high glucose diet shortens lifespan in *C. elegans*, the reversal effects of probiotics supplementation on a high glucose diet were studied. Consistent with previous research, the probiotic strains used in this study significantly extended lifespan when supplemented individually (Figure 2-3).

*Lactobacillus planatarum* are heterofermentative bacteria producing D-lactic acid, L-lactic acid, and acetic acid through the Embden-Meyerhoff pathway and are also known to produce hydrogen peroxide to eliminate other competitive bacteria. They are found in fermented plant matter such as pickles and kimchi and the most common bacteria used in silage. Supplementation of *L. planatarum* may provide anti-inflammatory effects via reduction of IL-1β, IL-6, and C-reactive protein (CRP), immune response components involved in inflammation, while increasing IL-10. *L. planatarum* has the ability to extend lifespan in *C. elegans* (Figure 3-3, Table 3-1); however, when glucose is supplemented into *C. elegans* diet along with *L. planatarum*, the shortened lifespan induced by glucose is worsened dramatically (Figure 3-5E, Table 3-2). The exhibited shortened lifespan could be a result of a multitude of reasons ranging from metabolites produced by the bacteria to protective compound produced by the bacteria such as hydrogen peroxide. Similarly with glucose, *L. planatarum* fed worms had a slight reduction in lifespan when supplemented with CBE (Figure 3-6D). *L. planatarum* fed worms exhibited a 17% increase in lifespan; however, when supplemented with CBE, there was only a 15.4% increase in lifespan (Table 2-3).
Lactobacillus delbrueckii are homofermentative bacteria that produce lactic acid. They are comprised of four subspecies separated by their metabolism. L. delbrueckii is commonly found in yogurts and cheeses. Worms fed on L. delbrueckii exhibited extension in lifespan (Table 3-1), and when supplemented with a high glucose diet, the probiotic restored lifespan compared to the control. The control exhibited a 15.7% decrease in lifespan when fed on a high glucose diet; however, when the worms were fed a high glucose diet with L. delbrueckii, lifespan was only reduced 8.7%. The mechanism for restorative properties has yet to be determined. L. delbrueckii subsp. bulgaricus produces nitric oxide (NO). NO has previously been shown to extend lifespan in C. elegans. NO is responsible for mucus generation, motility, and water and electrolyte transport. More interestingly, when L. delbrueckii lifespan extension properties were further enhanced when supplemented with CBE, with an increase from 12.8% lifespan extension to 23.6% lifespan extension compared to the control (Table 3-3, Figure 3-6E).

L. fermentum are heterofermentative bacteria. Although L. fermentum may be responsible for dental caries, it can also be utilized as a probiotic. The probiotic is commonly found in sourdough breads and other fermented plant products. Some strains have the ability to metabolize cholesterol. Though this could be a possible cause of lifespan extension in C. elegans (Table 3-1), the mechanism of lifespan extension has yet to be studied. Though worms fed L. fermentum exhibited lifespan extension, when supplemented with a high glucose diet, the reduction of lifespan was nearly the same as the control worms fed on E. coli OP50 (Figure 3-5D, Table 3-2). On the other hand, when the probiotic was supplemented with CBE, lifespan was further extended from 5.5% to
Interestingly, this combination may provide insight into the combination of probiotics and fruit extracts commonly supplemented together in the diet.

*B. subtilis* are spore forming obligate aerobes, commonly found in the human gut. Because of the spore forming abilities that allow the bacteria to survive under harsh conditions, the bacteria have become targets for probiotic supplementation in pasteurized foods and beverages. *B. subtilis* have the ability to extend lifespan in *C. elegans* through nitric oxide production\textsuperscript{55}. Here, *B. subtilis* had the ability to reverse the effects of a high glucose diet in *C. elegans*, though not fully. *B. subtilis* fed worms exhibited only a 5.3% decrease in lifespan when supplemented with glucose; whereas, the control strain had a 15.7% decrease in lifespan. More interestingly, when worms were fed *B. subtilis* in combination with CBE, their lifespan was further increased from 13.6% to 20.6%.

*B. coagulans* are spore forming facultative anaerobes, and are used as a probiotic in livestock feed. *B. coagulans* have the ability to extend lifespan in *C. elegans* (Table 3-1), and have some restorative properties when supplemented with a high glucose diet. In worms fed *B. coagulans* and a high glucose diet, lifespan was only decrease 11.39% as compared to the 15.7% decrease in lifespan exhibited in the control. Lifespan in worms was also further extended when supplement in combination with CBE, extending lifespan from 20.1% to 26.7%.

In this study, we observed the ability of probiotics to reverse the effects of a high glucose diet in *C. elegans*. Owing to *C. elegans*’ pathway homology in humans, our findings can provide insight into the possible reversal effects of probiotic supplementation in humans. As previously mentioned, obesity and other diseases
associated with a high glucose diet have become a major issue in developed nations. The insulin-like signaling pathway involved in aging and innate immune response in *C. elegans* is among the pathways of homology in humans.

Insulin, a hormone involved in regulating metabolism and absorption of glucose in mammals, and the homolog IGF-1 have been shown to regulate lifespan in many organisms and is involved in other cellular processes such as stress response\(^{57-59}\). The FOXO transcription factor DAF-16 in the insulin/IGF-1 signaling pathway and decreased activity of heat shock transcription factor HSF-1 reduced the rate of tissue aging, resulting in increased lifespan\(^{60, 61}\). The DAF-16 and HSF-1 transcription factor may be inhibited by a high-glucose diet. A downstream gene encoding a glycerol channel *aqp-1* was also involved in lifespan reduction induced by a high glucose diet\(^{51}\). In mammals, aquaporin glycerol channels are repressed by insulin\(^{62, 63}\).

Our findings may also have implications in prevention and/or cure of obesity related diseases such as type 2 diabetes, tumor formation and heart disease. Studies could be further carried out in a higher organism such as mice. Because of the pathway homology including insulin/IGF-1-mediated signaling (IIS) pathway, p38 kinase pathway, and other immunological/stress response pathways between *C. elegans* and humans, these findings may be of high human health importance. In addition these findings may lead to relatively inexpensive and highly accessible prevention or treatment for infection.
FUTURE DIRECTIONS

Through execution of these objectives, we were able to provide more insight into the effects probiotics have on overall health with possible application in humans. In addition, our findings may provide insight into future studies such as probiotics’ effect on the microbiome. Based on our preliminary data that 1) each probiotic strain extends lifespan significantly compared to the control, and 2) with the exception of *L. planatarum* and *L. fermentum*, each strain restores lifespan when exposed to a high glucose diet, though not fully, we propose to test the possible pathway involvement in this observed protection. Because of the pathway homology including insulin/IGF-1-mediated signaling (IIS) pathway, p38 kinase pathway, and other immunological/stress response pathways between *C. elegans* and humans, we intend to further test the pathways involved in the alleviated effects of a high glucose diet triggered by probiotic supplementation. The transcription factor of the IIS pathway DAF-16 is required for longevity in *C. elegans*. The DAF-16 target *sod-3* encodes a superoxide dismutase involved in stress response. And the downstream gene of DAF-16, *aqp-1*, which encodes for a protein in the aquaporin glycerol channel, may be involved in glucose-induced decreased lifespan in *C. elegans*. Similarly in mammals, aquaporin glycerol channels are repressed by insulin. We can test these pathways via qRT-PCR. If these genes are involved in the regulation of restored lifespan, we would expect to see increased expression in *daf-16, sod-3*, and *aqp-1* in worms fed probiotics compared to *E. coli* OP50 and increased expression in worms fed probiotics in the presence of
glucose. Finally, we would expect to see down-regulation of these genes when worms are fed our control strain *E. coli* OP50 with glucose.

Successful determination as to which pathways are involved in probiotic mediated restoration of lifespan will provide insight into the mechanism with application in humans and potentially lead to further studies in a higher organism.
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