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Article

The Effects of Altered Microbiome on Caenorhabditis elegans Egg Laying Behavior

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SUMMARY

Studies suggest the importance of the gut microbiome in human health and disease. However, the human microbiome is complex, and simpler models are needed to better understand the interaction between gut microbiota and the body. We developed a simple microbiome model system using the worm *Caenorhabditis elegans* (*C. elegans*). We hypothesized that if wild-type *C. elegans* feeds on bacteria other than lab standard *E. coli* OP50, there will be a change in its gut microbiome, thus influencing egg retention behavior. Compared with the control, the worms fed *Comamonas* bacteria retained more eggs than the worms fed *Bacillus* ($p < 0.001$). Therefore, when wild-type *C. elegans* is grown on varying bacteria, a change in gut microbiome may explain the differences noted in egg retention behavior. The *C. elegans* model created in this study is a simple representation of the more complex human-microbiota interaction occurring in our bodies. An interesting application of this model is finding out to what extent host response to various medications is affected by the microbiome and whether this can be used to guide a personalized approach to treating diseases.

INTRODUCTION

Caenorhabditis elegans (*C. elegans*) is a small and clear roundworm with a short lifespan. In nature, it is found in soil and rotting fruits and vegetables where it feeds on bacteria (1). Due to its simplicity, fast development, and sharing 40% genetic similarity with humans, *C. elegans* has served as a simple model system for various diseases found in humans (1). The human gut microbiome, a collection of microorganisms that live in our intestines, has in recent years been a focus of much research due to its potential role in our health, development, and tendency to get ill. Microbes in a healthy human adult are estimated to outnumber human cells by a ratio of 10:1, and the total number of genes in the microbiome exceeds the number of genes in the human genome by a factor of at least 200 (2). It is possible that gut microbiota can also influence the response to medications such as cancer therapies, and this may be the next frontier to be explored in humans when developing more effective cancer treatments (3). The human microbiome and its interaction with the host is very complex and much more research is needed in this arena (4, 5).

C. elegans and its simple bacterial diet provides a useful model to study host-bacteria interactions in a more controlled fashion. The type of bacteria eaten by the worm may change its rate of growth, as well as the ability to resist environmental stressors such as osmotic stress, and high or low temperatures (6). The microbes ingested by this worm appear to lead to a variety of effects that are beneficial to the worm apart from their role as mere food (7). It appears that live, metabolically active bacteria are needed to achieve the positive health benefits for the worm. Fluorescently tagged bacteria that were eaten by the worm have been visualized colonizing the gut and staying in the intestine to form the worm's microbiome (8). Most experiments with *C. elegans* do not take into consideration that a diverse worm microbiome may impact worm responses to experimental stimuli. More needs to be known about the effect of worm microbiota on its health and behavior.

C. elegans are self-fertilizing hermaphroditic worms. During its life cycle, *C. elegans* passes from egg stage through four larval stages before reaching adulthood, all within two to four days. One worm can, on average, produce 300 offspring over a 3-day period (4-10 eggs laid per hour). At any point in time, usually 10-15 fertilized eggs are retained within the uterus for several hours before being laid. The number of eggs in the uterus is a function of both the rate of egg production and the rate of egg laying. An intact motor and neural circuit is necessary for the egg laying process to be successful (8).

The egg laying behavior of *C. elegans* is influenced by a variety of factors including environmental stressors such as overcrowding, availability of food, temperature, and availability of sperm. If the environment is not favorable for egg laying—for example, there is not enough food or the food is harmful—the worms retain the eggs longer until the environment becomes more favorable (1). Egg retention (also called the egg in worm assay) was chosen for this study because it is a relatively easy assay to observe in the lab and results in a large sample size over a short period of time.

The purpose of this study was to better understand the host-microbiome interaction by developing a simple microbiome model system to determine the impact of different gut microbiota on *C. elegans* behavior. We hypothesized that if wild-type *C. elegans* is allowed to grow on bacteria other than the standard strain of *E coli* (OP50) normally used in labs, there would be a change in the worm's gut microbiome that could affect egg retention behavior. While altering the *C. elegans* gut microbiome by allowing it to feed and develop on three different strains of bacteria, we observed worms' egg retention capacity. Our study showed that, indeed, egg retention of the worms differed according to the bacteria they were raised on and presumably incorporated in their gut.

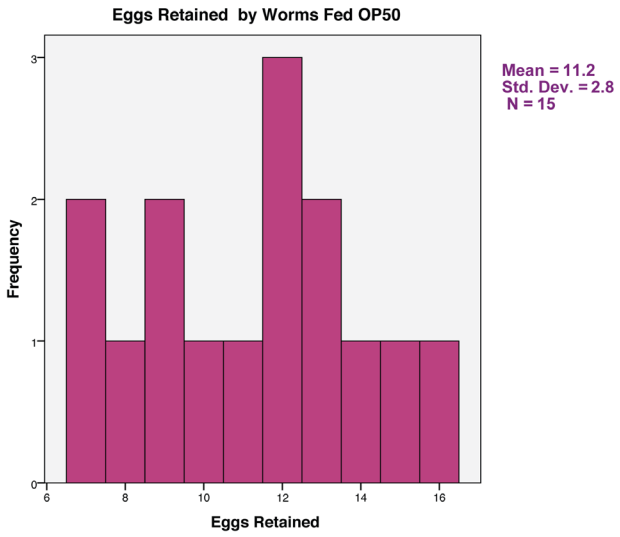

Figure 1. Number of eggs retained by worms fed OP50, using the egg retention assay. The *y*-axis indicates the number of worms that retained eggs at each specified count. The mean number of eggs retained by worms fed control *E. coli* was 11.2 ± 2.8 eggs.

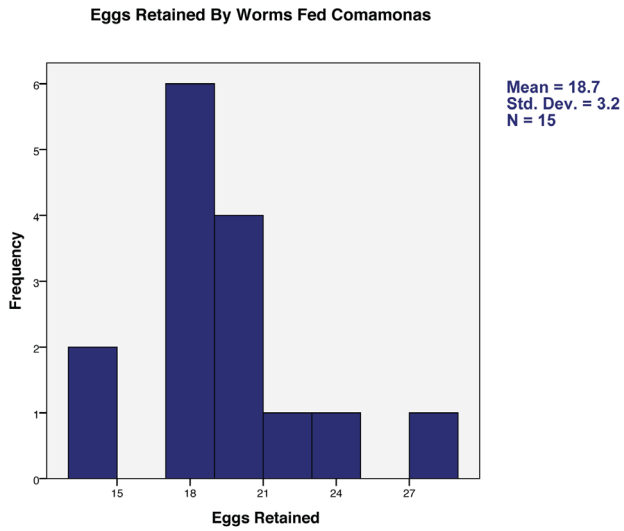

Figure 2. Number of eggs retained by worms fed *Comamonas* sp., using the egg retention assay. The *y*-axis indicates the number of worms that retained eggs at each specified count. The mean number of eggs retained by worms fed *Comamonas* sp. was 18.7 ± 3.2 eggs.

RESULTS

The bacteria chosen in this experiment have been shown to alter the gut microbiome and are non-toxic to *C. elegans* worms (9). We chose *E. coli* OP50 as the control bacteria since *C. elegans* worms are traditionally fed this bacteria in the lab. The two experimental bacteria used in this study are found on rotting fruits and vegetables in nature where they are eaten by *C. elegans*; thus they were good choices for further exploration (9). The experimental bacteria are different enough from *E. coli* OP50 that they likely would have an effect on the microbiome and egg retention behavior, but not so different as to be lethal to the worms (9). It was important to select bacteria that would not be lethal to *C. elegans*. If the chosen bacteria were toxic food sources, then any changes noted in egg retention may have been due to their lethality rather than the potential impact of the bacteria on their microbiome.

E. Coli OP50	*Comamonas*	*Bacillus*
15	14	11
9	18	5
14	27	10
11	21	11
12	19	8
12	18	7
8	18	6
7	19	9
13	19	2
16	17	6
7	19	10
13	18	14
9	14	13
10	17	6
12	23	9

Table 1. Number of eggs retained by C. elegans grown on three different bacterial strains

Summary Statistics	*E. coli* OP50 N = 15	*Comamonas* N = 15	*Bacillus* N = 15
Mean ± SD	11.2 ± 2.8	18.7 ± 3.2	8.5 ± 3.2
Mode	12	18	6
Median	12	18	9
Min-Max	7-16	14-27	2-14

Table 2. Summary statistics for egg retention by worms grown on three different bacterial strains.

The constant variables in this study included the amount of agar in the Petri dish, the age of the worm, the temperature at which the worms were maintained, the amount of time exposed to the type of bacterial lawn), and the strain of *C elegans* (wild-type). The independent variable was the type of bacterial lawn the worms were allowed to feed on (*E. coli* OP-50, *Bacillus megaterium*, or *Comamonas* sp., DA1877) The dependent variable studied was the egg retention of adult worms. The control group consisted of worms that were fed and raised on the standard laboratory *E. coli* OP50. Fifteen worms were studied per bacterial strain. Worms were allowed to grow on their respective bacterial strains from egg stage until adulthood.

Table 1 shows the number of eggs retained by bacterial strain for each worm trial. We also calculated statistics

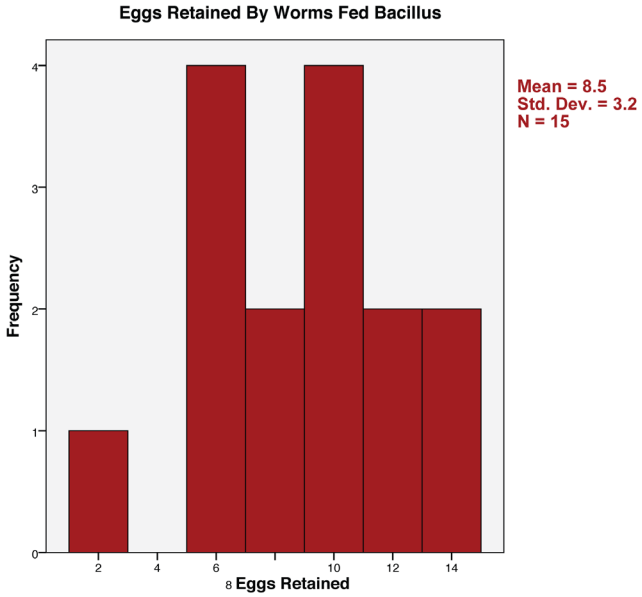

Figure 3. Number of eggs retained by worms fed *Bacillus*, using the egg retention assay. The *y*-axis indicates the number of worms that retained eggs at each specified count. The mean number of eggs retained by worms fed *Bacillus* was 18.7 ± 3.2 eggs.

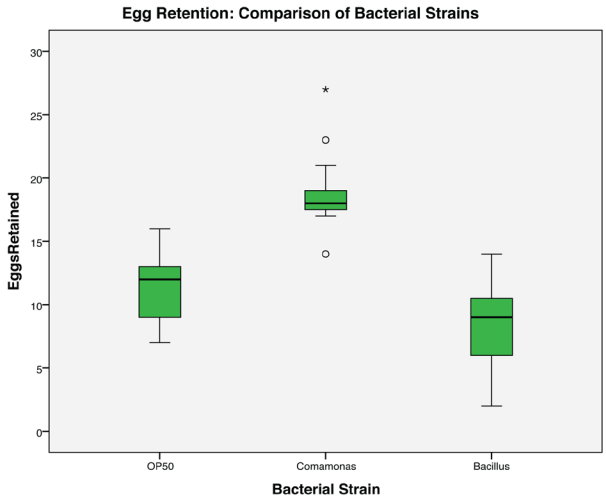

Figure 4. Comparison of eggs retained by worms grown on three different bacterial strains. *E. coli* OP50 was the control bacteria. Experimental bacteria were compared to *E. coli* OP50. A three-way comparison was made using one-way ANOVA. There was a statistically significant difference in the mean number of eggs retained when comparing *Comamonas* and *Bacillus* to *E. coli* OP50 (p <0.001). Worms that grew on *Comamonas*, sp, retained more eggs than the control, and those grown on Bacillus retained less eggs than the control.

including the mean, median, mode, and standard deviation of egg retention for each bacteria strain (**Table 2**). The mean number of eggs retained by worms fed control *E. coli* was 11.2 ± 2.8 (**Figure 1**), the mean eggs retained by worms fed *Comamonas* sp. was 18.7 ± 3.2 (**Figure 2**), and the mean eggs retained by worms fed *Bacillus megaterium* was 8.5 ± 3.2 eggs (**Figure 3**). Worms grown on *Comamonas* sp retained more eggs and worms grown on *Bacillus megaterium* retained fewer eggs than *E. coli* OP50, both statistically significant differences as measured by *t*-test (p < 0.001; **Figure 4**).

DISCUSSION

The study demonstrated that when *C. elegans* worms were allowed to feed and develop on different bacterial cultures, there was a change in egg retention behavior. In previous research on *C. elegans*, fluorescently tagged bacteria eaten by the worm were visualized within the transparent body as colonizing the gut (8). There are far more bacterial cells in *C. elegans* then worm somatic cells, a relationship similar to what is seen in humans. In addition, worms that are fed dead bacteria or nutrients that contains no bacteria have shorter lifespans (8). Together, these findings suggest that bacteria are more than just a food source for the worm and indeed need to be metabolically active for the worm to thrive, indicating an important interaction between the worm host and its bacteria.

Usually 10-15 eggs are retained in the worm at any given point in time. In our study, worms that were raised on *E. coli* OP50 fell into this range. However, worms that grew up on *Comamonas*, sp. retained significantly more eggs than the expected, and worms that grew up on *Bacillus* retained fewer. The worms were raised on the bacteria since they were eggs, thus controlling the length of exposure to the bacteria and making it highly likely that the only gut microbiome present was what they ate (*i.e.* the bacteria on the plate). The environmental stressors were kept constant for all worms to prevent bias in egg retention capacity.

Although this experiment shows that changes in bacterial exposure, and in all likelihood worm microbiome, impacts worm behavior, the study was not designed to analyze the worm microbiome or determine whether these differences lead to changes in offspring viability and longevity. It is possible that the experimental bacteria cause differences in egg production rate or egg laying rate, either of which could lead to the differences seen in egg retention. The exact mechanism by which the microbiome affects worm egg retention requires further study.

The host-microbiome model created in this study is a simple representation of the more complex human-microbiota interaction occurring in our bodies. Observing the impact of different bacteria on host behavior, as accomplished in this study, is the first step in understanding the pathways and genes used by bacterial cells in influencing host cells. Eventually studies are needed to find ways to change these pathways to control or treat disease. These studies need to first occur in animal models before they can be applied to humans. In humans, the gut microbiome may play a role in diseases such as asthma, inflammatory bowel disease, obesity, diabetes, and even anxiety (2). Gut microbiota may also play a role in changing individual responses to treatment. For example, some human studies have shown that individual variations in response to certain lipid-lowering drugs may

be due to the role that person's gut microbiome plays in the metabolism of the drug (4). Interestingly, early studies on the *C. elegans* microbiome suggest that different species of bacteria in *C. elegans* impact its response to cancer drugs, with some cancer drugs being more effective than others depending on the type of bacteria that resides in the worm's gut (3). An interesting application of this host-microbiome model is finding out to what extent host response to various medications is affected by the microbiome and whether this can be used to guide a personalized and more precise approach to treating disease in humans. In the future, it may be possible to alter the gut microbiota to enhance response to therapies or boost the body's own immune system.

When *C. elegans* feeds and grows on different bacteria, there is likely a change in its gut microbiome. We observed differences in egg retention behavior depending on what bacteria the worms were allowed to feed and grow on. Sophisticated gene sequencing analyses are needed to better outline host-microbiome interactions and determine the mechanism underlying our observations noted in this study.

MATERIALS AND METHODS

Worm Maintenance

3 cm nematode growth media (NGM) plates for worm growth were prepared by pouring NGM onto sterile petri dishes and allowing them to cool and set over 24 hours. *Escherichia coli* OP50 and the other two experimental bacteria (*Bacillus megaterium* and *Comamonas* sp.) were cultured and allowed to grow overnight at 37°C. A sterile pipette was used to seed each NGM plate with 100µl of bacterial culture and the bacteria were allowed to grow on the plates for 24 hours. Worms were placed on the plates and allowed to develop and lay eggs.

Age Synchronization

Ten young, fertile adult worms were picked using a worm pick and transferred to NGM plates freshly seeded with *E. coli* OP50 or experimental bacteria. The worms were allowed to lay eggs for 60–90 min. The parents were removed and the plates were incubated at 20 °C for 48 h to obtain L4 larvae.

Egg Retention (Egg in Worm Assay)

A 20% bleach solution was prepared, then a 10 µl drop of bleach solution was added to fifteen distinct locations on a 96 well plate. Fifteen age-synchronized adult worms from each experimental and bacterial lawn were picked, washed with M9 Buffer, and placed on a clean agar plate containing no bacteria. Then the worms were transferred into each bleach drop, one worm per well. The worm cuticle was allowed to dissolve for 10 min or until the worm burst open, expelling the eggs. The eggs were then counted under a dissecting microscope and the results were recorded. [Protocol adopted from Gardner, *et. al.* (10)]

Data Analysis and Statistics

Statistical software, SPSS version 12 was used to compare the egg retention of experimental bacterial strains with control, using the student *t*-test. A three-way comparison was made using one-way ANOVA. For all comparisons, p-value < 0.05 was considered statistically significant.

Safety Measures and Risk Assessment

All standard lab safety measures were followed, including the use of protective eyewear and safety aprons, and gloves. Sterile techniques were used when handling the bacteria. All bacteria used were BSL-1 bacteria. There were no other risks associated with this study.

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