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The influence of the Gut Microbiota and Alzheimer's Disease

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Mackenzie Gibbs

Honors Thesis

Spring 2021

The influence of the Gut Microbiota and Alzheimer's Disease

Abstract:

Alzheimer's disease is a neurodegenerative disease that effects the neurons in the brain and can lead to cognitive impairment and death. The main aspects of Alzheimer's research focus on genetic factors contributing to the disease, but the microbiota gut-brain-axis may be playing a potential role in Alzheimer's development. The human body has an abundant microbiota to help our bodies carry out daily needs, but the amount of microbiota present has to be kept in a tolerance range by the immune system. If there is an increase in the amount of gut microbiota present in the human body, inflammation can occur in the CNS and lead to a potential contributor to Alzheimer's development. A climbing assay was performed to study Elav *Drosophila* and AD *Drosophila* locomotor skills. The aim of this study is to survey the diversity of gut microbiota and motor skills in the transgenic Alzheimer's fruit fly (*Drosophila*) model, in comparison with ones in the healthy wild-type *Drosophila*.

Introduction:

Drosophila are ideal disease models that have a short life cycle and contain genes that are also found in humans. Alzheimer's disease is the number one contributor for dementia and the number of cases is increasing. There are two ways in which Alzheimer's develop which include beta-amyloid deposits in the brain and neurofibrillary tangles of the microtubule protein Tau. Mutations in the APP, PS-1, and PS-2 genes are also linked to Alzheimer's disease. Alzheimer's

and other neurodegenerative diseases are the cause of locomotor impairment. A climbing assay is performed to test locomotor impairment in the fruit flies. The human gut plays a major role in the overall health of an individual. If the gut is in dysbiosis, this could result in disease. “Leaky Gut” syndrome occurs when bacteria in the gut passes through the intestine into the blood stream. Once the bacteria are in the bloodstream, lipopolysaccharides (LPS) and proinflammatory cytokines can permeabilize the BBB and enter the brain. This leads to neuroinflammation, amyloid plaques, and tau protein tangles. The brain-gut-microbiota axis reflects the connection between the gut bacteria and the brain. The goal of this experiment is to see if there is an increase in the number of colonies between healthy and AD *Drosophila* and if there is a difference in the type of bacteria present between healthy and AD *Drosophila*.

Experimental Methods:

Fly collection

Collected transgenic Alzheimer’s fruit flies (*Drosophila*) and Elav *Drosophila*. There were five separate trials, 1, 3, 4, 5, 7, each containing # tubes with five *Drosophila* in each tube. Knowledge of which trial contained transgenic Alzheimer *Drosophila* or Elav *Drosophila* was only known by the instructor to remove any bias towards the experiment. Fly food was prepared with 226.8 grams of jazz mix and 1.2 L of water. This mixture was placed into a pot that was sitting on a hot plate. Once the mixture started to boil the hot plate was turned down. After 10 minutes, half an inch of food was poured into vials.

Climbing Assay

Climbing assay was performed for four weeks, every Wednesday in the morning. Five *Drosophila* from each tube was transferred into vials. All vials were marked at the same spot.

After flies were transferred, they were placed on the table for 1 minute to rest. Next, the vials were firmly tapped on a piece of foam and placed upside down on the table. A 10 second timer was started and the number of flies that climbed above the designated line was recorded. After 10 seconds, the flies rested for 1 minute. This process was repeated five times. The number of flies in each vial was written on the outside of the tube. If a fly escaped or died, the new number of flies would be written down.

Gut Dissection

Gut dissection of *Drosophila* was performed on 3/8, 3/10, 3/17, and 3/23. On 3/8, 10 healthy and 10 AD *Drosophila* were obtained from stock cultures. CO₂ anaesthesia was exposed to the flies. Flies were sterilized in ethanol for 30 seconds. Next, sterile PBS was used to rinse the ethanol off the flies. A drop of sterile PBS was placed onto a dissecting dish. Flies were placed onto a dissecting dish under a microscope. The head was removed first, then the entire gut. The guts were then placed in a centrifuge tube that contained 100 uL of sterile PBS. There were two centrifuge tubes, one for healthy *Drosophila* and one for AD *Drosophila*. Guts were homogenized with sterile pestles. 400 uL of sterile PBS was added into centrifuge tube for a total volume of 500 uL. 250 uL of homogenized solution was added to an LB plate and the remaining 250 uL was added to an SDA plate. Mixture was spread onto plates using a sterile spreader. 250 uL of sterile PBS was added to LB and SDA plates for controls. There was a total of six plates. LB plates were in an autoclave for ~72 hours at 37°C. SDA plate was left at room temperature for 4-5 days.

On 3/10, 9 healthy and 9 AD *Drosophila* were obtained from stock cultures. Flies were sterilized in ethanol for 30 seconds. Ethanol was removed using a pipette and sterile PBS was rinsed over the flies. The flies were placed on a dissecting dish on top of a drop of sterile PBS.

The dissected guts were placed into centrifuge tube containing 100 uL of sterile PBS. The rest of the procedure was the same as 3/8 except no extra sterile PBS was added to the centrifuge tubes.

On 3/17, the procedure was the same as 3/10 with one exception. The total volume of sterile PBS that was mixed with the guts in the centrifuge tubes was 250 uL. On 3/23, the procedure was identical to 3/17 except a total of 36 *Drosophila* were dissected.

Luria broth (LB) is a nutrient-rich media used to grow bacteria. LB plates were made from LB agar mix and purified water. The mix was autoclaved and poured onto petri dishes. Sabouraud Dextrose Agar (SDA) is used for cultivation of yeasts/fungus. SDA plates were made from SDA mix and purified water. The mix was also autoclaved and poured onto petri dishes. Dissecting dishes, pestles, forceps, and spreaders were all sterilized in ethanol. Pipette tips and centrifuge tubes were autoclaved for sterilization.

DNA Extraction

Three separate SDA plates, containing fungal growth, was used for DNA extraction. A sample of bacterial fungus was removed with sterile forceps and placed into a centrifuge tube containing 360 uL of ATL buffer. 40 uL of proteinase K was added into the centrifuge tube. The tube was then hand homogenized with vortex and pestle and then incubated at 56°C for 20 minutes. 400 uL of AL buffer was added after incubation. The tube was placed in a centrifuge machine to collect supernatant and then placed in new Eppendorf tube. The tube was centrifuged at 10,000 rpm for 1 minute. 400 uL of ethanol was added and solution was placed into DNeasy mini spin column. The tube was centrifuged again at 8,000 rpm for 1 minute and flow through was discarded. DNeasy tube was placed into a new collection tube and 400 uL of AW1 was added. The tube was centrifuged again at 8,000 rpm for 1 minute. Collection tube was discarded and DNeasy spin tube was placed into a new collection tube. 500 AW2 was added into tube and

then centrifuged at 14,000 rpm for 3 minutes to dry membrane. DNeasy tube was placed into 1.5 mL centrifuge tube and 200 uL of AE buffer was added. The tube was incubated at room temperature for 1 minute and then centrifuged at 8,000 rpm for one minute. Run-off was collected for PCR experiment in 1.5 mL tube. The DNA extraction experiment was performed two different times due to negative results during the first trial.

PCR

Polymerase chain reaction (PCR) is used to produce copies of a DNA sequence. The three fungal primers were used by adding 10 uL of master mix, 3 uL of H₂O, 1 uL of forward primer and 1 uL of reverse primer. After this was added into a PCR tube, 5 uL of DNA was added. For the first PCR trial, cycle 1 was set to 94°C for 3 minutes, the next 35 cycles were set to 94°C for 1 minute, 55°C for 30 seconds and then 72°C for 1 minute. The last cycle was set to 72°C for 5 minutes and then to 10°C to cool. For trial 2, cycle 1 was set to 94°C for 2 minutes, the next 35 cycles were set to 94°C for 1 minute, 50°C for 45 seconds, and 72°C for 1 minute. The last cycle was set to 72°C for 5 minutes and 10°C to cool.

Gel electrophoresis

Gel electrophoresis was used to separate the DNA fragments produced from PCR. 0.8 grams of agarose was added to 100 uL of 1 X TAE in an Erlenmeyer flask. The mixture was microwaved for 1-minute full power, taken out and mixed up, and reheated again. After the mixture was fully dissolved, it was cooled to 60°C and poured into a gel tray with a well comb. The agar was cooled in the tray for ~30 minutes or until it solidified. The well comb was gently removed from the agarose. Samples were loaded into the wells. A DNA marker was added into the first well. The DNA marker contained 1 kb ladder 3 uL + 2 uL of Syber green dye, 3 uL 6X sample buffer, and 12 uL of H₂O. There was a total of 3 samples, 1', 2', and 3', produced from

PCR. Each sample contained 5 uL DNA (PCR) + 2 uL Syber green dye, 3 uL 6X sample buffer, and 10 uL of water. The gel was run at 100V for 30 minutes. The gel was carefully removed from the gel box and taken to a digital gel photography system. The results of the first trial were negative. The whole procedure was performed again on 4/2/21, starting with DNA extraction. A higher amount of fungal growth was used in the second trial which gave better results.

Results

Climbing Assay

The data from each week was entered into an excel spreadsheet. The average and standard error of each trial was recorded and put into a bar graph. The average for week one was 50.4, week two was 53.5, and week three was 50.5. The overall average was 50.5 and the standard error was 4.436054.

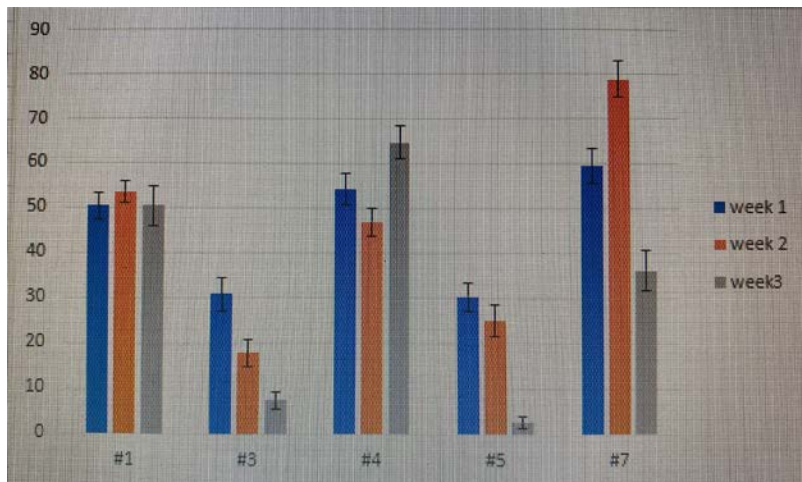


Figure 1. Data from climbing assay

Gut Dissection

The bacterial results from the 3/10 dissection showed 10 colonies on the AD LB plate, 6 colonies on the healthy LB plate and 0 on the LB control plate. The bacterial results from the 3/17 dissection showed 7 colonies on the AD LB plates, 1 colony on the healthy LB plate and 0 on the LB control plate. The colonies on the healthy plate were almost double in size compared to the AD plate.



Figure 2. AD LB plate bacterial colonies

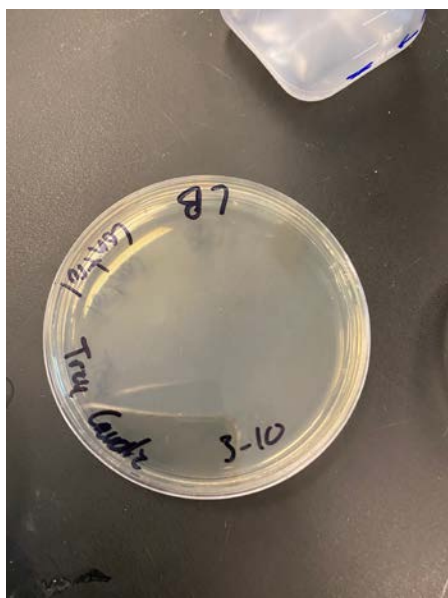


Figure 3. Control LB plate



Figure 4. Healthy LB plate bacterial colonies

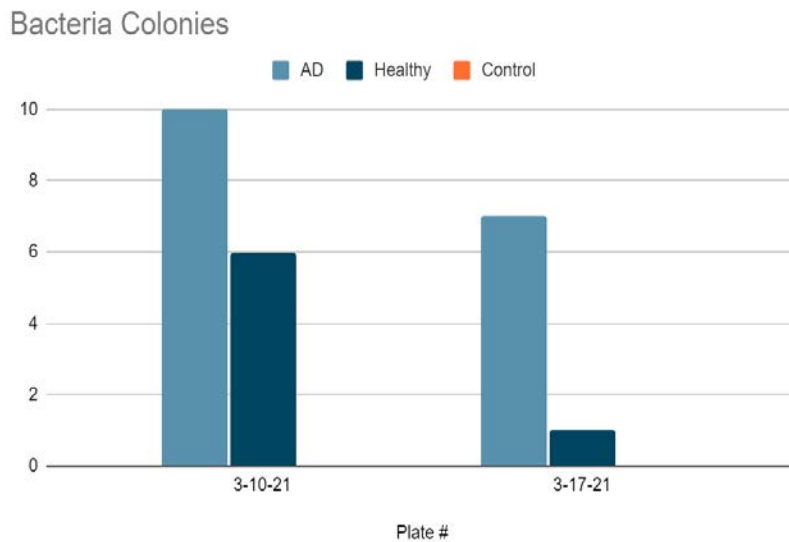


Figure 5. Bacterial colonies from 3/10 and 3/17 plates.

Results from the SDA plates revealed 3 different fungi. Figure 6 shows the first fungus that was found in the healthy *Drosophila*, which was *Didymella*. *Cladosporium* was found in healthy *Drosophila* as shown in Figure 7. Figure 8 shows the fungus *Penicillium citrinum* which was found in AD *Drosophila*.



Figure 6. *Didymella* fungus found on healthy *Drosophila* SDA plates.



Figure 7. *Cladosporium* fungus found on healthy *Drosophila* SDA plates.

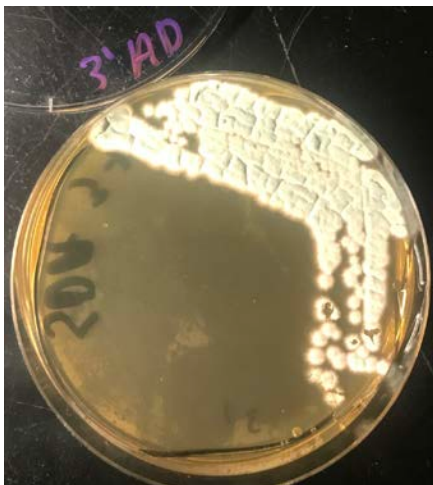


Figure 8. *Penicillium citrinum* fungus found on AD *Drosophila* SDA plates.

DNA extraction, PCR, Gel electrophoresis

The first DNA extraction trial gave no results on the gel electrophoresis image. In figure 9, there are no bands in wells 2, 3, and 4. Lane 1 contained the kb ladder. During the second DNA extraction, a higher amount of fungal substance was used, and the PCR settings were altered. In figure 9, there are bands present in wells, 6, 7, 8. Lane 5 contained the second kb ladder. The three bands shown are DNA fragments extracted from fungi. The DNA fragments in lane 6, 7, and 8 were sent off to be sequenced. The results were analyzed using the Blast program. Lane 6 is the fungus *Didymella*, lane 7 is *Cladosporium* and lane 8 is *Penicillium citrinum*.



Figure 9. Gel electrophoresis image

Discussion

The climbing assay was performed to compare the locomotor skills of *Elav Drosophila* to *AD Drosophila*. The bacterial results from the gut dissection on 3/10 showed that the healthy *Drosophila* bacterial colonies were bigger than the *AD Drosophila* bacterial colonies. One cause of this could be that the healthy bacteria replicated faster. Both trials from 3/10 and

3/17 revealed that there are a higher number of bacterial colonies grown on AD *Drosophila* LB plates compared to healthy *Drosophila* LB plates. Didymella fungus, that was found on the healthy *Drosophila* SDA plate, is found in plants. So far there is no connection between Didymella and Alzheimer's disease. One study was found that connects Didymella and an increase in pulmonary infection. The second fungus found in healthy *Drosophila* was Cladosporium. Studies have showed this fungus being present in both healthy and AD *Drosophila* but there is an increase in the amount found in AD *Drosophila*. The third fungus, found in AD *Drosophila*, was Penicillium citrinum. There are studies that have found a link between this fungus and pneumonia patients. There is a correlation between pneumonia and Alzheimer's patients. Scientists are currently studying the function of an anti-Alzheimer's drug by using this fungus. A future direction from this study is to look at how the immune system is affected with the progression of Alzheimer's. Another future direction is to sequence the DNA from the bacteria colonies and see what species are present in both healthy and AD *Drosophila*.