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Lessening Malaria Parasite Burden with Yogurt

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Lessening Malaria Parasite Burden with Yogurt

Background

While malaria is not a common issue on the minds of health care professionals in the United States, the Center for Disease Control estimates that in 2012, malaria was responsible for over 600,000 deaths and 207 million clinical episodes worldwide. Malaria is a devastating microbial disease which is present in warmer climates, particularly in sub-Saharan Africa and India (2). The prevalence of malaria in these developing areas is coupled with a lack of adequate healthcare which increases the number of people who succumb to the disease. Additionally, it is estimated that 90% of those who die from malaria are ages 5 or younger (2). Clearly, this microbial disease is a systemic health crisis that requires the utmost attention from medical researchers. Several species of the microbes that cause malaria exist within the genus *Plasmodium*, including *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium falciparum*, the last of these being the most deadly (14). The strain of malaria used in this experiment is *Plasmodium yoelii*, which is a non-lethal malaria parasite commonly used in laboratory mice (16).

Laboratory mice are widely used in human disease-related experiments when human testing is not realistic or when experimental trials are still in earlier stages. Mice have similar disease progressions as humans, are small and simple to house, and are easily genetically manipulated. Over 85% of the mouse genome is similar to that of a human, which gives researchers confidence that their experimental results will be relevant and applicable to human diseases (9). Mice are often inbred during experimental testing so that genomes of individuals are nearly identical, which gives researchers more replicates within an experiment. However, it is critical to note that while two mice may have been inbred from the same strain of mouse, housed

in identical conditions, and fed the same diet, no two organisms are ever exactly identical. The strains of mice compared in this experiment were from the Jackson Laboratory (Jax) with locations in Bar Harbor, Maine; Sacramento, California; and Farmington, Connecticut, from the Frederick National Laboratory for Cancer Research (NCI) in Frederick, Maryland, from the Taconic Laboratory with breeding locations in Germantown, New York and Cambridge City, Indiana, and from the Harlan Laboratory with breeding locations in Dublin, Virginia and Frederick Maryland.

C57BL/6 strain of mice from each vendor were kept in identical conditions and fed the same diet, making them as similar as possible. However, the following figure summarizes data that was conducted prior to my entrance in Dr. Schmidt's laboratory and shows that Jackson (Jax), NCI, Harlan (Har), and Taconic (Tac) mice exhibit different levels of parasitemia when exposed to *P. yoelii*:

Figure 1

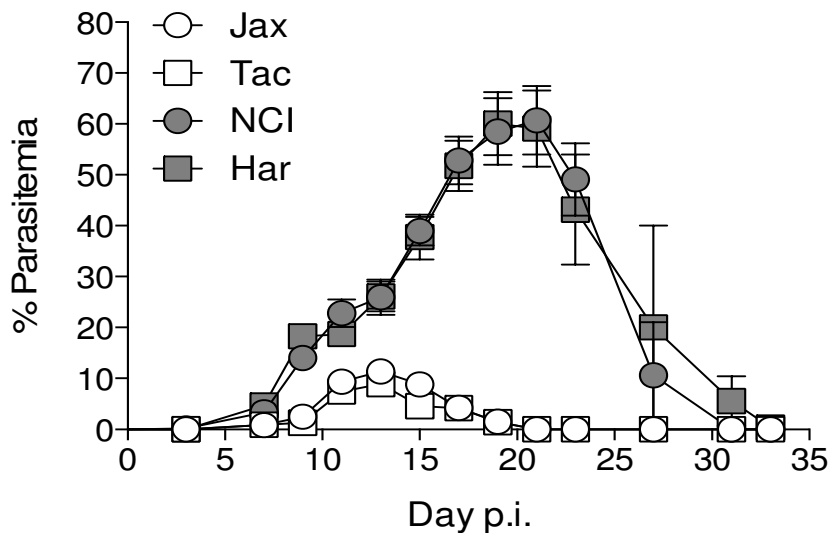


Figure 1: The percentage of parasitemia of mice over time (in days post-infection) from the following laboratories: Jackson (Jax), Taconic (Tac), NCI, and Harlan (Har). The NCI and Harlan mice experienced similar patterns of parasitemia, while Jackson and Taconic mice

experienced similar patterns. Over the course of the study, NCI and Harlan mice experienced a higher parasite burden than Jackson and Taconic mice.

NCI and Harlan laboratory mice exhibit a higher parasite burden when exposed to malarial pathogens than Jackson and Taconic mice. While these mice are all the same strain, their parasitemia levels differ based on where their laboratory is located geographically. In order to identify the reasoning behind this, members of Dr. Schmidt's laboratory sought to determine what component between the vendors of mice was different.

For the remainder of the study for practical purposes, since Jackson and Taconic mice experienced nearly identical parasitemia patterns, as did NCI and Harlan mice, only Jackson and NCI mice were used. Many experimental variables were altered in order to determine the differences between the mice vendors. First, the water and food for the mice were autoclaved to rid both of any bacteria. Second, another strain of mouse, BalbC, from the different vendors was used as opposed to C57BL/6 mice to see if the parasite burden was a result of the strain of mouse. Next, different species of the *Plasmodium* organism were tested. Each of these experiments showed the same result: the parasite burden of Jackson mice was significantly less than that of NCI mice.

Dr. Schmidt's laboratory then began to explore the gut microbiome of the two vendors of mice. Water for all of the mice was inoculated with a cocktail containing broad spectrum antibiotics, which would eliminate much of the composition of the gut microbiome. After treating the mice with *P. yoelii*, the patterns of parasitemia between the mice from the two vendors showed indications of convergence, meaning that the Jackson mice began to experience a higher parasite burden, while NCI mice began to experience a lower parasite burden. This

implies that without the influence of gut microbes, the parasite burdens would be more similar between vendors.

Researchers also used cecal transfers hoping to receive more information on the gut microbiome and its implications in infection progression. The contents of the cecum, the portion of the intestine between the small and large intestine where many gut microbes are present, from NCI mice were transferred to Jackson mice via oral gavage, and vice versa. Just as in the antibiotic experiments, the progression patterns converged and became more similar to the vendor from which the cecum was received. Also, germ-free mice, which have no gut microbes and are essentially “clean slates”, obtained cecal contents, some from Jackson mice and some from NCI mice. The infection progressions then resulted in similar patterns to those from which they received cecal contents, as in those germ-free mice that received contents from the cecum of Jackson mice experienced an increase in parasite burden, while those that received contents from the cecum of NCI mice experienced a decrease. The combination of each of these preliminary tests showed that the parasite burden differences between strains of mice were likely a result of microbial composition differences and not of genetic factors.

Types of immune cells, such as mast cells, phagocytes, basophils, and eosinophils, between each vendor of mice were also analyzed and did not result in any significant differences. This indicates that the two vendors of mice do not possess inherently different innate immune components. If the numbers and types of immune cells were statistically different, it would be clear that this would be the reason behind a difference in infection progression. An analysis confirmed that Jackson mice and NCI mice carry different microbes within their gut that seem to affect their ability to clear infections. The following figure shows the breakdown of the gut microbiome for both NCI and Jackson mice:

Figure 2

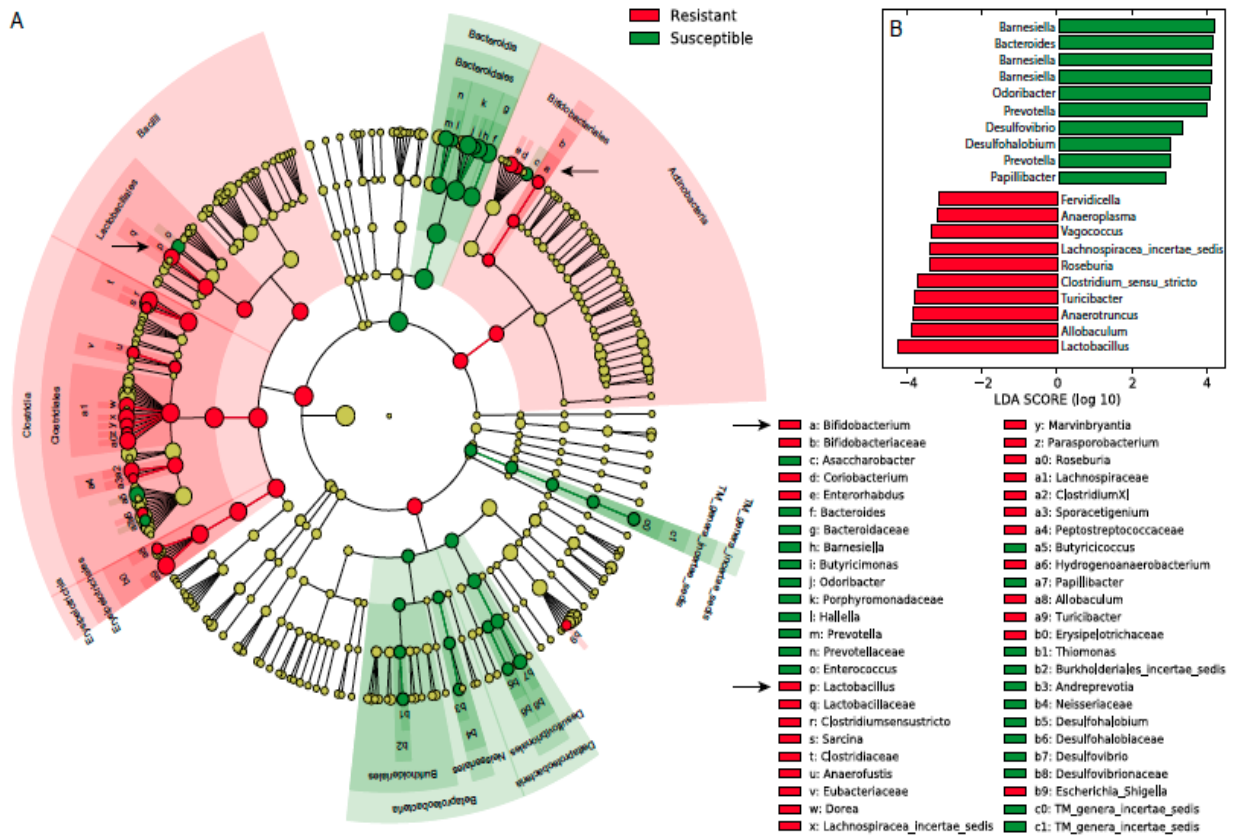


Figure 2: The cecal microbiome from resistant and susceptible mice is different. The (A) portion of the figure is a representation of the microbes found in resistant mice from Jax (red dots), susceptible mice from NCI (green), or both mice (yellow). The (B) portion shows unique microbes found in either resistant (red bars) or susceptible (green bars) mice. Data generated by Nicolas Villarino and Gary LeCleur

This discovery led to the hypothesis that the contents of the gut microbiota could be manipulated so that the parasite burden could be lessened. A 2014 review article displays how this idea has been utilized in previous experiments (1). The review describes how antibodies are produced as a result of microbes present in the gut, which can help protect the host from malaria infection. Dr. Schmidt's lab sought to continue these findings by manipulating the gut microbiome in order to determine its effects on parasite burden.

It is estimated that human microbes outnumber human cells 10 to 1 (17). The human gut is a particularly significant “bacterial hot spot” within the body in terms of containing more, and more diverse, microbes along with the mouth, the skin, and the vaginal tract (7). Gut microbes help with somewhat obvious bodily functions, such as digestion. However, the gut microbiota does not solely maintain homeostasis within the stomach and intestinal tracts of humans. Microbes in the gut engage in what is known as “cross talk” with cells in the mucosal immune system to regulate their production and activity (12). Productive and beneficial gut microflora benefit from a strong and regulated mucosal immune system, and vice versa. Thus, both components seek to help the other to ensure that detrimental pathogens are kept under control. Components such as surface enterocytes, which act as a screen in determining whether components entering the gut should alert the immune system as a potential danger, M-cells, which sample bacteria from the intestinal lumen and present the bacterial antigens to immune cells, and dendritic cells, which stimulate critical T-cell mediated immunity, are all present in the gut (10). This evidence shows that boosting the power and health of gut microbes can, in turn, boost the immune response to bacterial infections.

Microbes present in the gut in humans can be a result of several factors. A person’s diet can affect his or her microbial composition, particularly if he or she consumes the same types of food over an extended period of time or a lifetime (4). For example, a person living in the United States or the United Kingdom would likely consume very different types of food than a person living in Jamaica or Liberia. Taking antibiotics can also eliminate regulatory gut microbes and change routine functioning within the host (8). Another one of the most significant contributions to the makeup of the gut microbiome is that which occurs from a mother to a child, referred to as maternal transfer. Microbes are taken from the mother’s enteric tract, part of the gastrointestinal

system, as well as from the skin on the breast of the mother, and are transferred orally to a newborn (6). The types of microbes transferred to the child are not constant within one mother for the child's entire time breastfeeding; they are altered based on the age of the infant. For example, newborns require more bacterial diversity, which is present in colostrum, than older infants to establish a gut microbiome (6). This is one reason why infants have weaker immune systems than fully grown adults. Maternal transfer is one of the largest contributing factors that determines the gut microbiome that an offspring will possess.

While the microbial composition figure for these mice is incredibly complex, the size of the bars corresponding to *Lactobacilli* and *Bifidobacteria* microbial strains show that these two types of bacteria were present in significant amounts within the microbiota of the mice from both vendors and were shown to be resistant to *P. yoelii*. *Lactobacilli*, similar to the word "lactose", are unsurprisingly associated with dairy products, as are *Bifidobacteria*. This led to the further question: Could increasing the amount of *Lactobacilli* and *Bifidobacteria* within the gut microbiota boost immune response to the malaria parasite? Previous experiments have associated an increased amount of *Lactobacillus* in the gut as showing defenses to other health issues, such as arthritis (15). Thus, Dr. Schmidt's laboratory decided to administer yogurt to Jackson mice and NCI mice in the hopes of lessening the parasite burden of the mice from each vendor, which is the experiment in which I participated and to which I contributed. This brings the experiment full circle with the formation of the hypothesis that administering yogurt to both Jackson mice and NCI mice will lessen the parasite burden of a *P. yoelli* infection compared to when yogurt is not administered to the mice.

Methods

Throughout the experimental procedure, all mice were kept in identical conditions. There were 4 mice per cage and each mouse was fed a diet of NIH-31 Modified Open Formula Mouse/Rat Irradiated feed (5). While in preliminary experiments other vendors of mice were used, for this particular analysis, only Jackson and NCI mice were used.

Experimental Categories of Mice	
Jax + yogurt (Experimental)	NCI + yogurt (Experimental)
Jax no yogurt (Control)	NCI no yogurt (Control)

There were 7-9 total replicates for each experimental category (approximately 32 mice total).

Experimental mice were administered yogurt via an oral gavage which ensured that each mouse ingested the yogurt. Mice do not have gag reflexes so all yogurt that was ingested was digested.

The yogurt was created using a starter culture containing *Lactobacillus bulgaricus*, *L. acidophilus*, *L. lactis*, *Bifidobacterias lactis*, and *Streptococcus thermophilus*. The culture was enriched with probiotic powder containing *L. acidophilus*, *L. rhamnosus*, *L. salivarius*, *L. plantarum*, *L. casei*, *L. lactis*, *B. breve*, *B. infantis*, *B. longum*, *B. bifidum*, and *B. lactic*. These cultures were obtained from Custom Probiotics, Inc. in Glendale, California.

After infecting each mouse with *P. yoelii*, data was collected from both experimental and control mice from both vendors every other day beginning on Day 5 of the infection until Day 29. Measurements are not taken before Day 5 because the parasite has not had time to cause

notable damage and are not taken after Day 29 because the infection is cleared after 30 days in this non-lethal strain of *Plasmodium*. Taking measurements each day is not necessary to analyze differences over time in the percentage of infected cells.

On days of data collection, blood samples were taken from the tail of each mouse by clipping the tip of the tail, squeezing a small drop of blood onto a microscope slide, and smearing the blood in an even layer onto the slide using another slide. The blood on the tip of the tail clotted typically within one minute, and the mouse was returned to the cage. The blood smears were left to air dry for approximately 15 minutes. The blood was then fixed onto the slide by soaking the slide in a methanol solution for one minute. The slides were then immersed in a Giemsa stain for one hour. The stained slides were left out to air dry approximately overnight. Two smears were fixed onto one slide to conserve resources. As an undergraduate researcher, I acquired the blood samples from the mice and performed the fixing and staining of slides, first under supervision and then independently.

After the slides had been stained and dried, they were counted. The slides were placed under a microscope and were viewed under the 1000x lens. The lens contained a grid with which to count cells. After viewing the slide under the microscope, the grid was positioned onto the blood smear which well-represented the entire sample. A “well-placed” grid involved selecting an area of the smear that was not so congested that counting was impossible but not so sparse that the results of the count could vary immensely based on the experimenter doing the counting. It was also critical to choose an area with a parasitemia level that was representative of the entire smear and not an outlier. Each cell present within the grid was counted and recorded. Then, the number of parasitized cells within the grid were also counted and recorded.

Figure 3

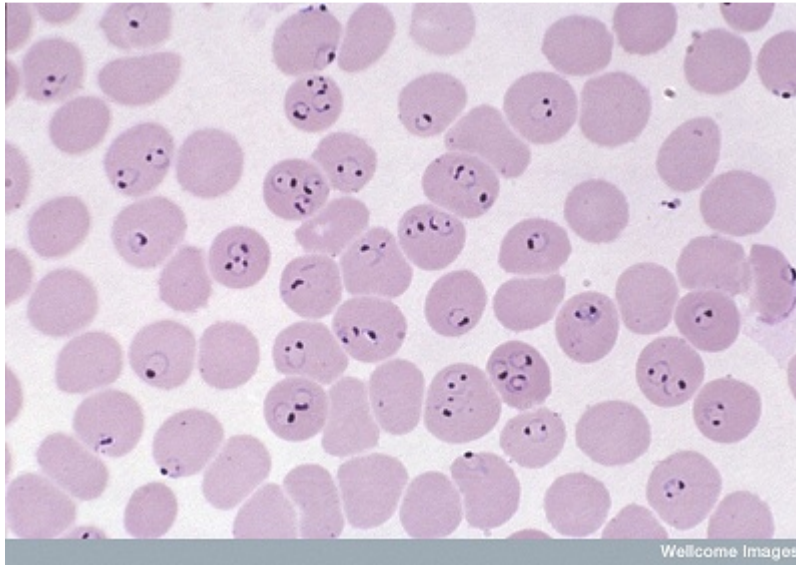


Figure 3: Image of red blood cells infected with Plasmodium
Source for image: <http://www.southsudanmedicaljournal.com> (Magnified 1000X)

The image above displays one that would be typically viewed during a malaria infection progression under a microscope. The parasitized cells are those which seem to have holes or dark marks within them.

The slide was then moved to another representative portion of the blood sample, and the same procedure was repeated. For each mouse each day of data collection, this process was repeated 5 times. Each count was entered into an Excel spreadsheet, where the average number of parasitized cells in the grid per mouse per day was compared with the average number of total cells in the grid per mouse per day. Using these averages, the overall percent of parasitemia was calculated for each mouse each data collection day. At the end of data collection (Day 29), the average parasitemia for each day was calculated for the Jackson control mice, the Jackson experimental mice, the NCI control mice, and the NCI experimental mice (yielding 13 data points over time for each of the 4 experimental categories). In addition to obtaining the blood

samples and preparing the slides for data collection, I also had a substantial part in counting the cells on the slides to obtain the parasitemia percentages, as well as inputting the data into Excel.

This was my largest contribution to the experimental procedure.

Results

Figure 4

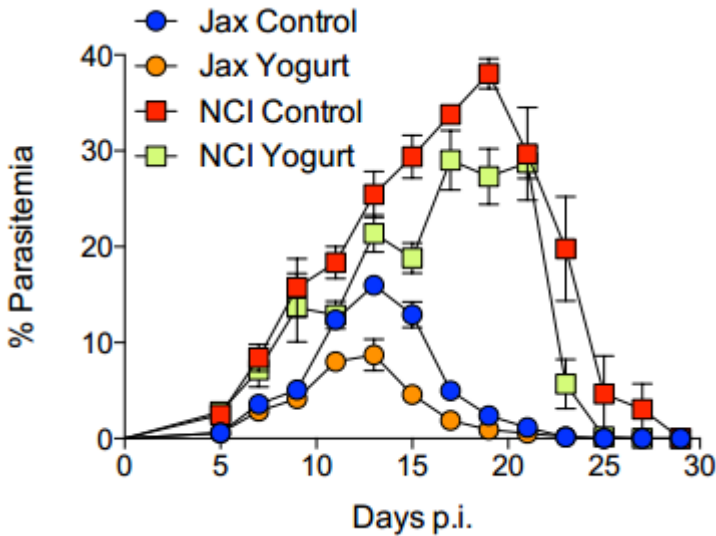


Figure 4: The percent parasitemia of the Jackson (Jax) control mice, the Jackson mice that received yogurt, the NCI control mice, and the NCI mice that received yogurt over time, in days post-infection. The numbers were obtained using the averages calculated each day for each experimental category in an Excel spreadsheet created during data collection. The overall parasite burden was higher in both categories of NCI mice compared to Jackson mice. The experimental (received yogurt) mice from both Jackson and NCI vendors expressed a pattern of lower parasitemia over time than the control mice.

Figure 5

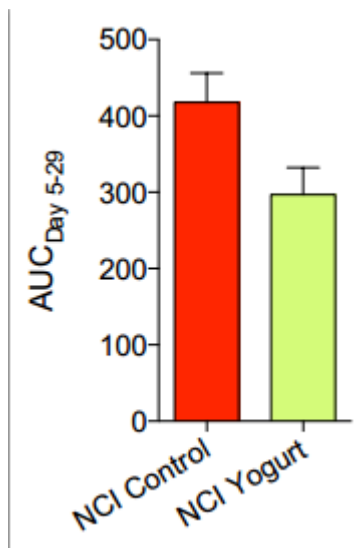


Figure 5 shows the Area Under the Curve for NCI mice that received yogurt as well as for NCI control mice that did not receive yogurt over the time of data collection (every other day from Day 5-Day 29). The Area Under the Curve is obtained using a summation of trapezoids of the infection progression curve, which is indicative of the level of parasitemia. The NCI mice that received yogurt had significantly less parasite burden than the control NCI mice ($p=.0418$).

Figure 6

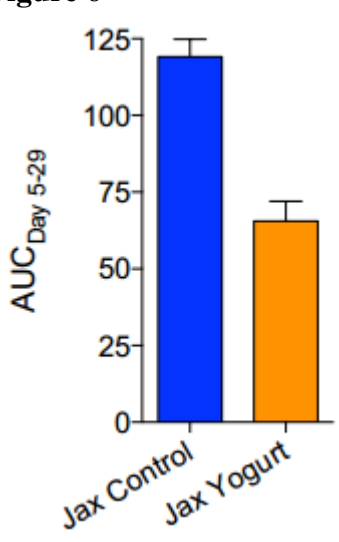


Figure 6 shows the Area Under the Curve for Jackson (Jax) mice that received yogurt as well as for Jackson control mice that did not receive yogurt over the time of data collection (every other day from Day 5-Day 29). The Area Under the Curve is obtained using a summation of trapezoids of the infection progression curve, which is indicative of the level of parasitemia. The Jax mice that received yogurt had significantly less parasite burden than the control Jax mice ($p<.0001$).

The AUC for both types of Jackson mice was less than the AUC for both types of NCI mice (Figure 4).

The results in Figures 4, 5, and 6 indicate that our hypothesis was correct: administering yogurt containing *Lactobacillus* and *Bifidobacteria* species lessened the parasite burden for both Jackson mice and NCI mice. The results from both vendors were statistically significant using a Student's T-Test (Figures 5 and 6). Area Under the Curve was used for these experiments because it gives an indication of the level of parasitemia throughout the course of the infection, rather than at one specific time point. Expectedly, NCI mice experienced a higher parasite burden throughout the infection, both the experimental mice and the control mice, than the Jackson mice. For mice from both vendors, ingesting yogurt laden with microbes that often colonize the gut decreased the severity of the *P. yoelii* infection. The results of this experiment not only have implications in the scientific exploration of the effect of the gut microbiota on immune response but also possess rising possibilities in humanitarian realms.

Discussion

The gut microbiota is an area of increasing interest in medical research because of experiments such as this because they demonstrate a method to manipulate the immune system without medicine, without genetic alteration, and without invasive procedures. A fairly obvious next step from this experiment is attempting to recreate the same results in a human. While mice have similar immune patterns to humans, they are also very different organisms. Human testing is difficult in this experiment because it would require researchers to work with individuals as soon as they had been infected with the pathogen and take their blood at regular intervals. Also, infection progresses at a different rate in humans than it does in mice, which could require very drawn out laboratory visits. The optimism in this experiment with regards to human testing is

that it does not require the administration of anything that could be harmful to humans; it is simply a yogurt. A more broad continuation could be monitoring the effect of gut microbe administration as treatment for other diseases besides *Plasmodium* malaria infections. If the gut microbiota clearly regulates the parasite burden in response to malaria, it is likely that it will do so in response to other bacterial pathogens, as well. One example of this was shown in a 2014 study, which showed that the gut microbiome regulated antibody production in response to the influenza vaccine (11).

The methodology behind how the gut microbiome regulates immunity or parasite burden to a disease can be understood more thoroughly with a grasp of the GALT (gut-associated lymphoid tissues). GALT are tissues that line intestinal mucosal surfaces and are a subset of the MALT (mucosal-associated lymphoid tissues) which are present in other mucosal surfaces of the body, such as the tonsils. These are the areas of the gut in which pathogens are sampled and create immune responses (3). These tissues contain high numbers of immune cells that help clear infections. However, GALT cells are adaptive and must be stimulated by commensal flora in order to function properly (13). Thus, boosting the number and variety of commensal flora in the gut, such as through the administration of probiotic yogurt, creates a propensity for stimulated GALT cells. This will result in a stronger immune response to any infection. Boosting the virility GALT will ultimately boost overall health.

Ever-increasing knowledge of the gut microbiome can lead to more specific studies in terms of the type of microbes present within the intestines. In this experiment, *Lactobacilli* and *Bifidobacteria* species were used to create larger numbers and more diversity within the microbiome of the mice. However, other species of commensal bacteria could have even stronger and more beneficial effects on the human gut microbiome. Once the most effective

species in aiding the immune system and stimulating GALT cells are realized through human testing, diets, vitamin supplements, and probiotic treatments could be administered in order to create overall better health and immunity within humans. There are two truly optimistic and hopeful outcomes of an experiment such as this: the gut microbiome has a significant effect on immune response to pathogen infections, and the gut microbiome is not expensive or difficult to alter.

The true tragedy of the malaria disease is that it is not only complex and lethal, but it also affects, predominantly, an area of the world in which adequate health care is difficult by which to come. Costly trial vaccines and extensive antibiotic treatments are not readily available to those in suffering countries and are difficult to deliver from countries that are able to provide aid. However, none of these complex treatments were used in this experiment: only yogurt. If this experiment is taken to the next level in human testing, the implications would be significant in terms of opportunities to help with the malaria epidemic for countries with more health care resources. Education in terms of immune-boosting diet could be given to suffering nations, and vitamins and probiotic supplements could be donated. This is the main reason why I chose this experiment to devote my undergraduate research experience. The implications in terms of science and overall understanding of human health are significant, but, also, too many doors are opened in terms of humanitarian opportunities to ignore or not pursue further. Lessening the blow of *Plasmodium* infections in suffering nations could be as simple as eating yogurt.

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