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Establishing the Effect of Ethanol on Listeria Infection

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Abstract

Excessive alcohol consumption is common in the United States, particularly among college campuses. Previous studies have shown that excessive drinking increases the risk of drug dependency, sexual assault and liver damage. Heavy drinking has also proven to impact immune capabilities. Immune cell function and numbers have been shown to be negatively impacted by alcohol treatment. This research project used different cell cultures to model human cells and investigated how exposure to alcohol affects susceptibility to bacterial infection. *Listeria monocytogenes*, a common foodborne bacterium, was used as a model pathogen. A high alcohol preferring mouse model was also used to examine the complex organismal responses to alcohol consumption prior to infections. These experiments were done in order to better understand the effects of alcohol consumption on the function of the human immune system and to help identify strategies to combat negative consequences associated with excessive drinking.

Acknowledgements

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Introduction

Excessive alcohol consumption can prove to be detrimental to health and wellness. When ingested in excess on a regular basis, alcoholic beverages have been known to cause a host of ailments from liver disease to alcoholism. It has been shown that there is an inversely proportional relationship between amount of alcohol consumed and life longevity. According to a paper published *The Lancet*, people who consume 0-100g of alcohol per week can live 3-5 years longer than those who consume more than 200g per week. Alcohol is the fourth leading preventable cause of death in the United States, following smoking tobacco, medical errors and overdoses, and obesity (Wood). Alcohol consumption is particularly common among college campuses as well. According to National Institute on Alcohol Abuse and Alcoholism, about "1 in 4 college students report academic consequences from drinking, including missing class, falling behind in class, doing poorly on exams or papers, and receiving lower grades overall" (College Drinking). Furthermore, alcohol consumption has been known to impair inhibitions, motor function, and critical reasoning—increasing the risk of sexual assault, theft, and unintentional injury such as motor vehicle accidents while intoxicated. Throughout my college career, I have witnessed many of my peers drink excessively every Thursday through Saturday night. The weekly consumption of potentially toxic levels of alcohol motivated me to pursue an honors thesis on the topic.

Chronic alcohol consumption can lead to the development of dependence and addiction by altering serotonin release in the brain. Serotonin is a key compound in feelings of reward and pleasure. According to an article published in *Brain Research*, serotonin metabolism in the brain was significantly reduced during alcohol consumption,

resulting in increased serotonin concentration in the brain. This increased serotonin level in the brain gives an individual a sense of euphoria. After repeated ethanol consumptions paired with serotonin release, the brain's reward pathway is modified so that the individual's happiness and optimism becomes dependent on the consistent consumption of alcohol. (Palaić et. al)

Alcohol travels through the body much like other foods and beverages. Alcohol first begins its journey through the body by consumption of any alcoholic beverage. The ethanol in alcoholic beverages travels to the stomach where approximately half of the ethanol is degraded by stomach enzymes. The other fifty-percent of the alcohol present in the drink remains in the chyme that then travels to the duodenum of the small intestine. In the small intestine, the alcohol diffuses across the epithelial lining to the nearby capillary beds and enters the blood circulation. The alcohol in blood is subsequently filtered from blood inside the liver, where alcohol dehydrogenase breaks down ethanol into acetylaldehyde. From there acetyladehyde travels to individual cells where aldehyde dehydrogenase of the mitochondria convert it in acetate. Acetate is then used as an energy source by the cell. (Cederbaum)

Beliefs about health benefits from drinking held in popular culture are at times unfounded. In popular culture, there are many misconceptions about the health benefits associated with alcohol. In a 1993 study that asked 781 Michigan residents about the reasons they drank alcohol, most reported that environmental stress and social standards were the primary motivations for alcohol consumption (Abbey et. al). This research compliments a later 2008 survey of 34 community members from Woodland and Davis, California showed that health benefits were not among the reasons for buying alcoholic

beverages. Instead, participants reported that taste and brand loyalty were among the strongest reasons for buying alcohol. Despite these primary reasons for alcohol consumption, some participants reported the health benefits of antioxidants present in the wine they drank (Wright et. al). However, according to an article published in *Food Chemistry*, it was reported that during the process of winemaking, many of the strongest antioxidants found in the grapes were lost. Particular types of antioxidants, called anthocyanins, are found mostly in the skin of the grape. During winemaking, the skin is discarded in favor of the juices. These juices form the basis of the wine (Lingua et. al). As stated before, there are clear relationships between health outcomes and alcohol consumption. The results from both survey studies suggest that participants drink for environmental and pleasure-seeking reasons and used perceived health benefits of alcohol as a way of justifying their behavior.

Regular and excessive consumption of alcoholic beverages reduces the effectiveness of the immune system. The immune system prevents pathogens from being able to reproduce and spread in a manner sensitive to changes in their environment. The introduction of ethanol into the body has drastic effects on the function of immune cells, making them less efficient at both detecting pathogens as well as killing them. When cells from spleens, a key immune organ, were extracted from mice and treated with ethanol, they had significantly lower levels of killing *Listeria monocytogenes* and *Borrelia burgdorferi* (Pavia et. al.). In another experiment, lab mice were orally administered 10% (w/v) ethanol in drinking water for 6 weeks before their spleens were harvested and their immune cells were isolated. Overall splenic weight, populations of both CD4+ and CD8+ T cells were all drastically lower in ethanol-treated mice than

those in control mice. The proliferation of CD4+ and CD8+ T cells as well as cytokine release was also decreased upon ethanol treatment. Together, these results showed that ethanol decreased both growth and function of these immune cells.

Drinking alcohol on a regular basis also harms the epithelial barrier. The small intestine is where nutrient absorption begins to take place on a large scale. The cells of the intestinal epithelium must be very tightly bound to prevent any unwanted compounds or pathogens from entering the blood stream. Because of this, cells of the small intestine have what are called tight junctions with neighboring cells. This ensures that the cells are sealed off from larger chemicals and pathogens. However, when a host introduces alcohol into their body, this barrier is compromised. Exposing intestinal cells to ethanol prevents them from forming a strong tight junction, allowing particles to freely pass between them. Researchers have shown that ethanol exposure increases the amount of dye that can pass between tight junction-forming cells. Furthermore, transepithelial resistance, a metric to determine the strength of a tight junction, was decreased significantly from control levels (Wang, et. al). These results show that ethanol prevents the intestinal epithelium from forming proper tight junctions, making them more likely to allow toxic compounds or a pathogen through into the blood stream, allowing it to infect the host body.

Listeria monocytogenes is a foodborne pathogen that was used as a model organism in this honors thesis. *Listeria* is a facultative anaerobic, rod-shaped, gram-positive bacterium. After ingesting contaminated food, *Listeria* can survive passing through the acidic stomach and travel to the duodenum of the small intestine. Once in the small intestine, *Listeria* can pass through the intestinal barrier into the blood stream.

Immune cells rapidly recognize *Listeria* as a pathogen and engulf it into a phagosome. However, *Listeria* produces a toxin, called Listeriolysin O (LLO), which helps it to break free of the phagosome. After it escapes into the cytosol of the immune cell, it quickly begins reproducing. The concentration of *Listeria* becomes so high in the immune cell that it eventually bursts, releasing many bacteria into the surrounding area to be recognized and taken in by other immune cells. *Listeria*'s ability to use immune cells as host rather than others makes it a novel type of pathogen. This ability also proved to be important in studying the effect of ethanol on the function and effectiveness of immune cells.

The main goal of this research project is to establish the effects of ethanol on the immune health of human cells.

Methods

Hemolytic Assay

Hemolytic assays were performed to quantify the activity of the secreted toxin, listeriolysin O, in the bacterial culture supernatant. Because of the ability of LLO to lyse red blood cells, higher levels of lysis are indicative of higher amounts of LLO produced by the bacteria. *Listeria monocytogenes* cultures were prepared by inoculating 1mL of BHI media with 1 bacterial colony from a spread plate. Prior to inoculation, BHI and ethanol were mixed to form concentrations of 0.05% or 0.20% ethanol (v/v). After inoculation, cultures were then placed in either an aerobic or anaerobic incubator for 18 hours. After incubation, cultures were removed from their incubators and were measured for their optical density to quantify their growth. Samples of the cultures were then

placed in a centrifuge to separate the bacterial cells from supernatant, where the toxin of interest is. After centrifuging, each supernatant sample (100µL) was added to the top row of a 96 well plate, along with 5µL of 0.1M DTT, a reducing agent to maximize LLO activity. While incubating at room temperature with DTT, the remaining wells were filled with 100µL hemolytic assay buffer (HAB). After 10 minutes of incubation, 100µL of HAB was added to the supernatant sample, pipetted up and down repeatedly to mix, and 100µL of the mixture was transferred to the next row down to continue to serially dilute the supernatant samples in 1:2 ratio. After dilutions, 100µL of defibrinated sheep blood was added to each well to a final concentration of 1% hematocrit. The plates were placed in an aerobic incubator for 30 minutes at 37°C. After incubation, plates were placed in the centrifuge to separate the intact blood cells from the supernatant. The supernatant was then transferred to a flat-bottom 96-well plate and the optical density was measured to determine the toxin activity of the *Listeria monocytogenes* culture. The activity levels among different cultures were normalized by the corresponding culture optical density.

Caco-2 Colonic Epithelial Cell Infections

This experiment was used to determine the differences in susceptibility to *Listeria* infection between untreated Caco-2 cells and ethanol treated cells. Caco-2 colonic epithelial cells were grown in DMEM media with 10% Fetal Bovine Serum for several days in order to harvest the maximum amount of cells for the infection procedure. Cells were then treated with 0.1% Trypsin to dissociate them from the bottom of the flask. The cells were then placed in the centrifuge along with 10mL of fresh media. After centrifuging, the cell media was poured out and replaced by 10ml of new media. The pellet of cells was then resuspended in the new media. Using a hemocytometer, the

concentration of cells was determined. A total of six million cells were added to a 24-well plate at 1mL per well. The cells were then placed in an aerobic incubator at 37°C for 24 hours to allow for the cells to adhere to the bottom of the well plate. After 24 hours, the Caco-2 cells were treated with media supplemented with 0, 0.05, or 0.2% (v/v) ethanol. After another 24 hours for the cells to acclimate to the new conditions, the cells were then infected with 10^8 *Listeria monocytogenes* colony-forming units (CFU). After 1 hour of incubation with *Listeria*, media infused with gentamicin was added to each well to eliminate the extracellular bacteria. Following another hour of incubation, the cells were then lysed using 0.1% Triton-X and plated onto LB spread plates. The plates were placed in an aerobic incubator at 37°C for at least 48 hours and counted for CFU.

RAW Macrophage Infections

This experiment was used to determine the differences in susceptibility to *Listeria* infection between untreated RAW cells and ethanol treated cells. RAW264.7 macrophage cells were grown in DMEM media with 10% Fetal Bovine Serum for several days. Cells were then gently scraped from the bottom of the plate so they could be placed into a centrifuge tube. The cells were then placed in the centrifuge along with 10mL of fresh media. After centrifuging, the cell media was poured out and replaced by 10ml of new media. The pellet of cells was then resuspended in the new media. Using a hemocytometer, the concentration of cells was determined. A total of six million cells were added to a 24-well plate at 1mL per well. The cells were then placed in an aerobic incubator at 37°C for 24 hours to allow for the cells to adhere to the bottom of the well plate. After 24 hours, the RAW cells were treated with media supplemented with 0, 1, or 2% (v/v) ethanol. After another 24 hours for the cells to acclimate to the new conditions,

the cells were then infected with 10^8 *Listeria monocytogenes* colony-forming units (CFU). After 30 minutes of incubation with *Listeria*, media infused with gentamicin was added to each well to eliminate the extracellular bacteria. Following another hour of incubation, the cells were then lysed using 0.1% Triton-X and plated onto LB spread plates. The plates were placed in an aerobic incubator at 37°C for at least 48 hours and counted for CFU.

HAP2 Mice Infection

This experiment was done in order to help determine animal susceptibility to *Listeria monocytogenes* infection. Lab mice were selected based on their preference to drink alcohol over water. These mice were then crossed to produce High Alcohol Preferring (HAP) mice (Grahame). High alcohol preferring mice were acquired from Dr. Nicholas Grahame and placed in single cages in order to acclimate them to the environment of the vivarium at University of Dayton. For this period of time, all mice received normal water and food. After 12 days, the experimental mice were given water bottles filled with 10% ethanol. At 3, 6, 9, and 12 days following the initial treatment of ethanol, experimental mice were given water as a substitute for 24-hour periods. At 15 days after the initial treatment with ethanol, all mice were infected with 10^8 CFU of *Listeria monocytogenes* provided through inoculated Nutella. At 3 days after infection, all mice were sacrificed in order to harvest the liver and spleen. These organ samples were homogenized and plated at varying dilutions to quantify the number of colony forming units as the bacterial burden in these organs.

Results

Hemolytic Assay

To investigate how ethanol affects *Listeria monocytogenes* pathogenesis, we first analyzed the effect of ethanol on listeriolysin O (LLO) toxin production under aerobic or anaerobic conditions. LLO is a virulence factor that *L. monocytogenes* needs in order to escape the phagosome of macrophages so it can initiate its replication inside the macrophage cytoplasm. The experiment was performed with bacteria grown aerobically or anaerobically to recapitulate the different conditions where *L. monocytogenes* might be exposed to ethanol. For example, ethanol exposure in bloodstream will be aerobic, while ethanol exposure in the large intestines will be anaerobic. Under aerobic conditions, supplementation of 0.05% (v/v) ethanol resulted in a small but significant decrease in LLO production compared to control cultures without ethanol (Figure 1). However, this effect was not observed in the anaerobic cultures. Under anaerobic conditions, supplementation of 0.05% ethanol did not significantly alter LLO production (Figure 1). These results suggest that LLO production by *L. monocytogenes* is sensitive to regulation by ethanol under aerobic but not anaerobic conditions. Therefore, bloodstream alcohol level is likely to impact the outcome of *L. monocytogenes* infections more than the intestinal alcohol levels.

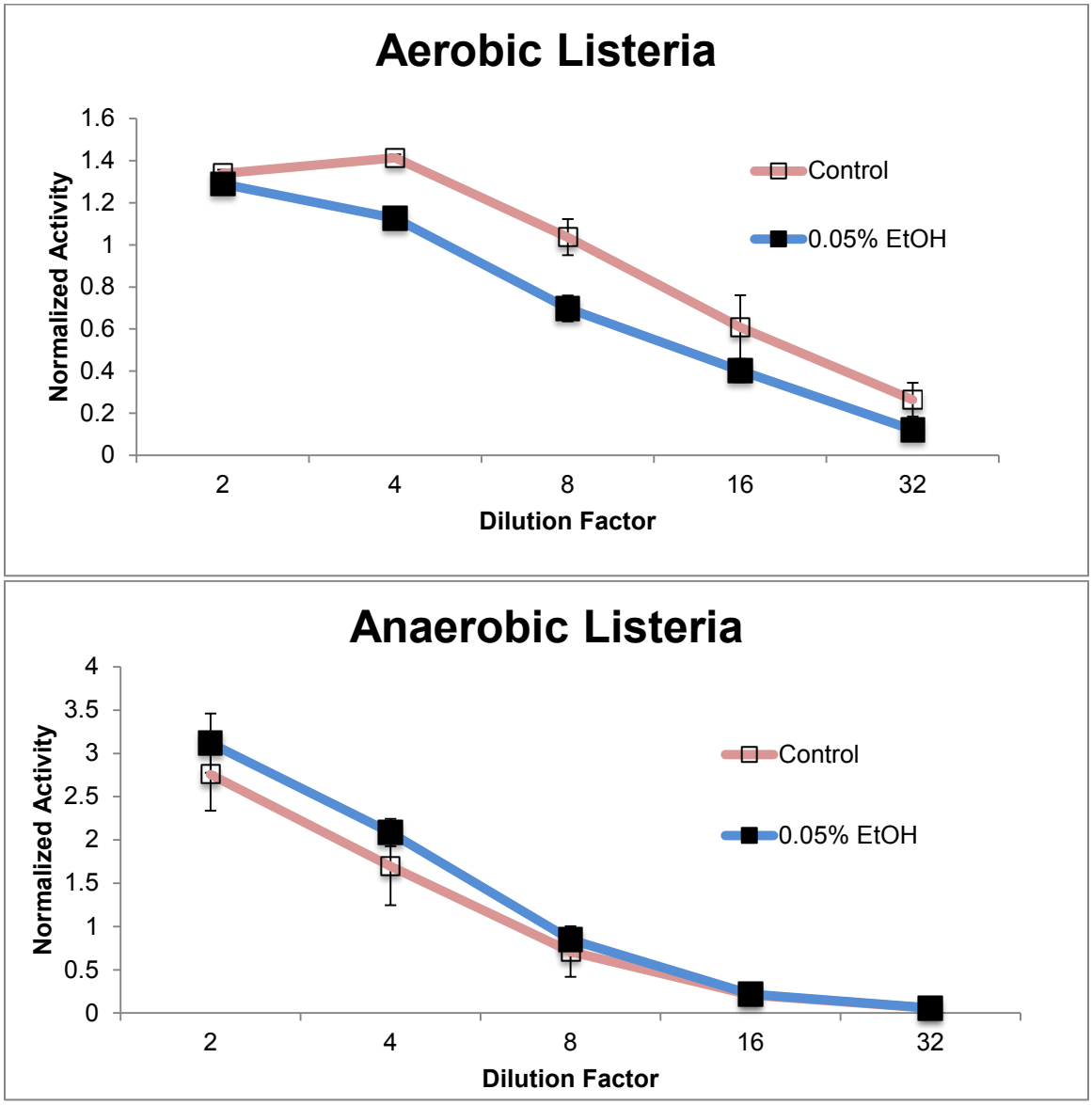

Figure 1: Aerobically grown *L. monocytogenes* (top) was grown with 0.05% ethanol for 18 hours. A hemolytic assay was performed to quantify Listeriolysin O toxin production. Ethanol significantly decreases toxin production in aerobically grown *L. monocytogenes*. Anaerobically grown *L. monocytogenes* (bottom) was grown with 0.05% ethanol for 18 hours. A hemolytic assay was performed to quantify Listeriolysin O toxin production. Ethanol does not significantly alter toxin production.

Caco-2 Cell Infections

To better understand whether the different LLO production levels in response to ethanol can alter *L. monocytogenes*-host interactions, different cell-culture infection experiments were performed. The first cell type I used as a host model were Caco-2 cells, which are human colonic epithelial cells that represent the first cell layer *L. monocytogenes* has to invade prior to disseminating to other organs. First, I treated the Caco-2 cells with 0.05% ethanol prior to infections and removed ethanol during infections. At 1-hour post infection, there was a significant increase in the number of intracellular *L. monocytogenes* in cells pretreated with ethanol compared to that in cells without ethanol pretreatment. At 2 hours post infection, there were no significant differences in the number of intracellular *L. monocytogenes* between control and pretreated Caco-2 cells (Figure 2). These observations suggest that the effects of ethanol only *L. monocytogenes*-Caco-2 interactions are likely limited to the early stage of infection.

Next, I included the ethanol treatment both prior to infections and during infections to determine the effect of long-term ethanol exposure only *L. monocytogenes*-host interactions. At 1-hour post infection, a decreasing trend in the amount of intracellular *L. monocytogenes* was observed with the increasing ethanol concentrations during infections. However, this trend was not observed at 2 hours post infection (Figure 2). These results further confirm that the effects of ethanol on *L. monocytogenes* infection are likely restricted to the initial adherence and entry.

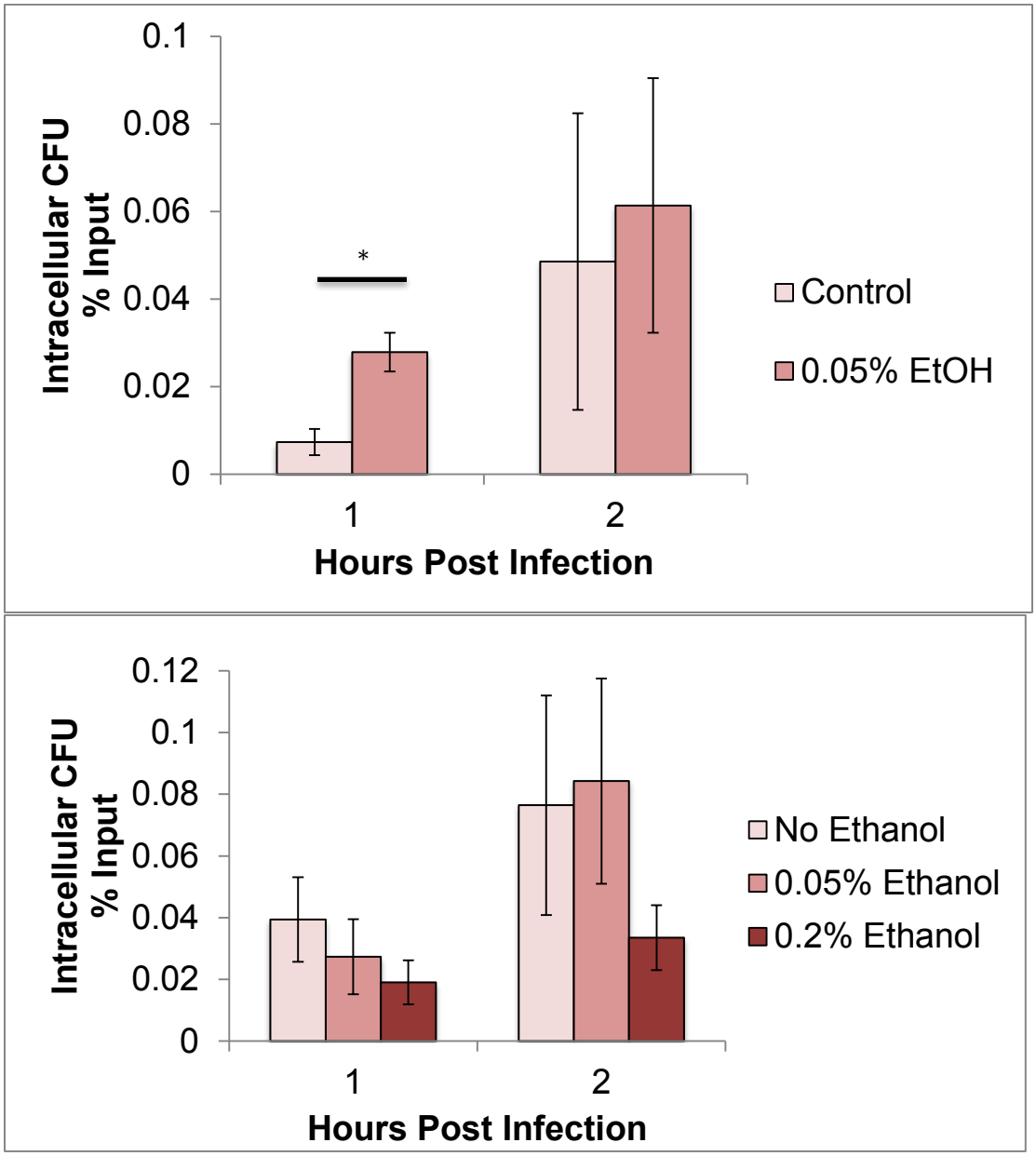

Figure 2: Caco-2 cells were infected with 10^8 CFU of *L. monocytogenes* for 1 hour before being lysed and plated for intracellular bacterial burden at 1 and 2 hours post-infection. When ethanol was not present during the infection procedure (top), intracellular CFU significantly increased for ethanol-pretreated cells. When ethanol was present (bottom), intracellular CFU decreased for ethanol-pretreated cells.

RAW Cell Infections

To investigate the effects of ethanol on immune cells, we used RAW264.7 macrophages and assayed for intracellular bacterial burden after pre-treatment with ethanol. This cell line is used to represent immune cells that phagocytose *Listeria monocytogenes* and contribute to the killing of *L. monocytogenes*. First, we treated RAW264.7 cells with 1% or 2% ethanol for 24 hours prior to infection and removed the ethanol during infections. At 1-hour post infection, there was a significant increase in intracellular *L. monocytogenes* in RAW264.7 cells pretreated with ethanol compared to those without ethanol treatment (Figure 3). This trend was observed at 2 hours and 4 hours post infection. When we analyzed the rate of clearance of *L. monocytogenes* by the RAW264.7 cells between 1 and 2 hours post infection, we saw that ethanol-treated macrophages were more efficient at removing *L. monocytogenes* than macrophages without the ethanol treatment (Figure 3). From these results, we concluded that, compared to untreated controls, ethanol-treated macrophages are more susceptible to infections but are better at killing the intracellular *L. monocytogenes*.

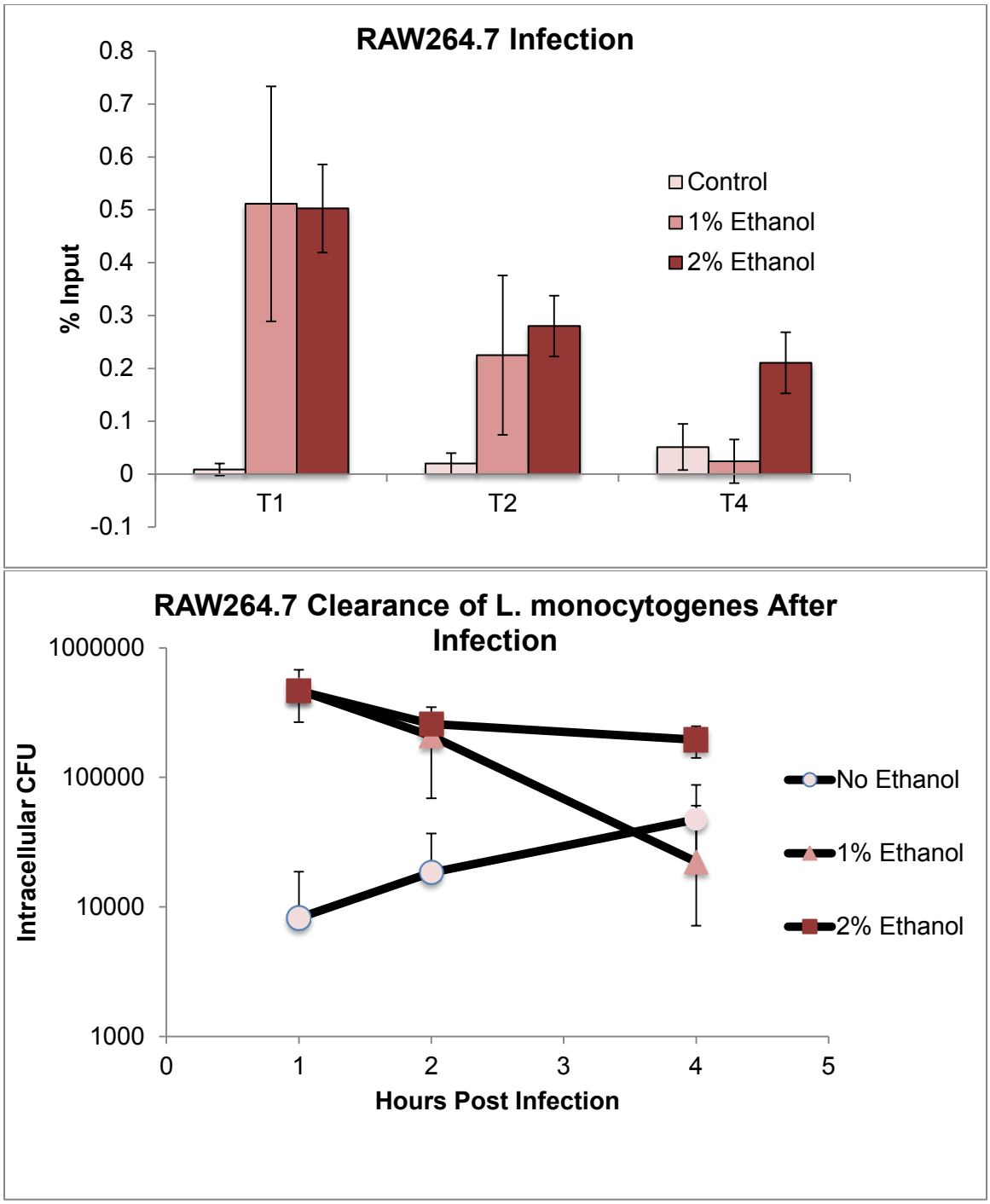

Figure 3: RAW264.7 macrophages were infected with 10^8 CFU of *L. monocytogenes* for 30 minutes before gentimicin treatment of extracellular environment. Cells were then lysed at 1,2, and 4 hours post-infection and plated in order to quantify intracellular bacterial burden (top). Data was also analyzed in order to show clearance rate of *L. monocytogenes* over the same time intervals (bottom). Ethanol-pretreated cells had increased bacterial burden compared to controls. Ethanol-pretreated cells also had increased bacterial clearance of *L. monocytogenes.*

HAP2 Mice Infection

Finally, we wanted to determine whether our observations from cell culture experiments have in vivo relevance. To investigate the in vivo effects of ethanol on *L. monocytogenes* infections, we used High Alcohol Preferring (HAP) mice as an animal model of infection. The HAP mice are bred by choosing individual mice that prefer to drink ethanol more than their peers. These mice are bred over several generations in order to produce the High Alcohol Preferring mice that we used in our experiments. During normal alcohol treatment of 10% v/v ethanol, they maintain a blood alcohol level around 0.08%, which is the legal limit of intoxication in the United States. However, these mice are able to break down ethanol very efficiently and without many of the consequences that human heavy-drinkers suffer. Chronic excessive alcohol consumption is often coupled with liver damage. HAP mice do not exhibit these symptoms, making them adequate models to examine the effects of ethanol on immune health. While this animal model has been used to study the behavior of alcohol intoxication, to our knowledge, this study represents the first time this animal model was used for infectious disease research. Out of the 39 total mice, 20 were subjected to a 2-week treatment with 10% ethanol. The remaining 19 mice received water for the duration of the experiment. The mice were then orally infected with a single dose of *L. monocytogenes*. After analyzing the results we found a significant increase in bacterial burden in the spleens of ethanol-drinking females versus their male counterparts. However, no significant difference was observed between bacterial burden in livers and spleens of ethanol-treated versus untreated animals. When infection was plotted based on the average daily ethanol or water consumption, no significant difference was found either. (Figure 4)

Figure 4: High Alcohol Preferring (HAP) mice were infected with 10^8 CFU of *L. monocytogenes*. After 3 days, mice were sacrificed and spleens and livers were harvested, diluted, and plated for CFU of *L. monocytogenes*. Using a single-tailed T-test, we found there was a significant difference in the impact of ethanol between male and female mice. Besides this finding, there were no other significant correlations.

Discussion

Hemolytic assay

When *L. monocytogenes* was grown in cultures with varying concentrations of ethanol, only in aerobic conditions did the *L. monocytogenes* produce less toxin with ethanol present. However, no significant difference in toxin production was found in the anaerobic cultures. Although this is an interesting finding, the environment of the intestinal epithelium is most often anaerobic. Furthermore, the results obtained mean that any increase or decrease in Caco-2 or HAP2 mice susceptibility must be attributed to the host, not *L. monocytogenes.* Thus, the results attained from the hemolytic assay suggested that in the environment of the intestinal epithelium, *L. monocytogenes* is not affected by the presence of alcohol.

Caco-2 Epithelial Cells

Under the Caco-2 infection procedure with *L. monocytogenes*, two sub-experiments were performed—with ethanol present during the infection process in one experiment but absent in the other experiment. These two experimental setups showed different results. In the experiment where ethanol was not present during infection, the results illustrated that prior treatment with ethanol made Caco-2 cells more susceptible to *L. monocytogenes* infection. Thus, Caco-2 cells are compromised by ethanol, increasing their susceptibility to *L. monocytogenes* infection. One possible mechanism by this increase is the compromisation of tight junctions. During the 24 hours before treatment with ethanol, Caco-2 cells are placed in wells with media where they are to form a monolayer of cells held together by tight junctions. Tight junctions are barriers between

epithelial cells that eliminate space between cells, helping to form a cell layer that prevents unwanted molecules and pathogens from passing through. Based on the results, it is likely that ethanol compromises these barriers. These results mirror a study explained in the *Molecular Medicine Reports Journal* where Caco-2 cells were subjected to 1, 2.5, 5, 7.5 and 10% ethanol for 4 h. The researchers found that ethanol pre-treatment lower transepithelial resistance and higher permeability between the tight junctions were recorded. There was also higher expression of Claudin-1 and lower expression of ZO-1 genes, which are key in the maintenance and creation of tight junctions. (Wang)

In the experiment where ethanol was present during the infection, a decreasing trend of susceptibility was displayed at 1-hour post infection. In these ethanol treatment conditions, either the Caco-2 cells are more resistant to infection or the infectivity of *L. monocytogenes* was compromised. These results agree with the data attained from the hemolytic assay. Under aerobic conditions, toxin production of *L. monocytogenes* is reduced, thus lessening *L. monocytogenes* burden on the host.

RAW264.7 Macrophages

When RAW264.7 macrophages were pretreated with ethanol and subsequently infected with *L. monocytogenes*, the intracellular bacterial burden is increased significantly. However, the clearance rate of pre-treated RAW264.7 macrophages was significantly increased compared to ethanol-free controls. These results suggest that under ethanol pre-treatment, RAW264.7 macrophages are more susceptible to infection but can kill intracellular pathogens more efficiently. Another potential mechanism for these results is that when the macrophages are pre-treated with ethanol, they absorb it to maintain intracellular concentration of ethanol that remains long after the ethanol is

removed from the media. This concentration of ethanol may impact *L. monocytogenes* pathogenesis due to it being an intracellular pathogen. Based on previous experiments, the production of LLO toxin is relatively unaffected by the presence of ethanol. However, the presence of ethanol within the RAW264.7 cells may also affect *L. monocytogenes* in ways that have not been examined in this research project. Some potential mechanisms that could be altered by ethanol include the ability of *L. monocytogenes* to escape the macrophage phagosome, grow within the macrophages, or polymerize actin in order to escape the macrophage. Further research must be done in order to determine the cause of increased infection burden coupled with increased pathogen clearance.

HAP2 Mice

When HAP2 mice were orally infected with *L. monocytogenes*, it was shown that there was no significant difference in bacterial burden between ethanol supplemented mice and control mice. However, we observed that these mice are particularly had higher *L. monocytogenes* burden. When infected with 10^8 CFU, average infection rates were around 10^4 CFU per organ. One explanation of this is that because these mice are bred to prefer ethanol rather than water their tolerance may have made them more susceptible to infection. Furthermore, when plates were counted for CFU of *L. monocytogenes*, there were many contaminations present, signaling that these mice were not only susceptible to *L. monocytogenes* infection, but to other pathogens as well. Although there were no significant differences in infection of *L. monocytogenes* found between the HAP2 mice tested, they seem to be more vulnerable to infections than other laboratory mice typically used for as host models of infections. These results are similar to a study published in *Alcoholism: Clinical and Experimental Research*, where researchers tested the *L.*

monocytogenes burden and clearance rates of mice spleens and livers as well as histology of these organs after ethanol ingestion. Colony counts of the spleen and liver were significantly higher for ethanol-treated mice. Ethanol treated cells had better clearance rates of Listeria than their non-ethanol treated counterparts. There was also greater liver inflammation and damage during infection. Also, ethanol-treated mice had much higher mortality when infected with *L. monocytogenes* (Saad et. al). These results, coupled with mine, show that ethanol compromises and damages primary immune organs when followed by *L. monocytogenes* infection

Conclusion

Chronic and excessive alcohol consumption has been shown to cause problems for college students through lower grades as well as increased incidences of sexual assault and unintentional injury. In my thesis project, I wanted to focus on the biological effects of alcohol consumption. This was done by the use of *L. monocytogenes*, Caco-2 colonic epithelial cells, RAW264.7 macrophages, and HAP2 High alcohol preferring mice. The results I obtained showed that ethanol exposure can impact *L. monocytogenes*-host interactions and that toxin production by *L. monocytogenes* in response to ethanol exposure is regulated by the presence or absence of oxygen. More experiments are needed to determine the mechanisms behind the responses to ethanol.

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