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Honors Thesis

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Abstract

This thesis focuses on the three different projects that I worked on during my time in Dr. Yvonne Sun's microbiology lab. The main goal of the first project was to develop an analytical method on UD's campus to analyze SCFAs by using Gas Chromatography. This was achieved by creating defined media that could sustain the growth of *Listeria monocytogenes* (*Listeria*) and developing a successful characterization method on a GC. The main goal of the second project was to determine the effects that SCFA supplementation and age have on *Listeria* infection. This was achieved using *in vitro* methods to assess the effects of SCFAs on LLO production, acetoin production, and cell culture infection and *in vivo* methods to assess the effects of SCFAs and age on mice with *Listeria* infections. The main goal of the third project was to gain a better understanding of the mechanism underlying the regulation of *Listeria*'s LLO production by varying oxygen levels. This was achieved by performing aerobic/anaerobic transition experiments and aerobic/microaerobic/anaerobic experiments.

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Project 1 – The Human Gut Microbiome, Microbial Metabolites, and the Development of Analytical Techniques of SCFA Analysis

Introduction

The Significance of the Human Gut Microbiome on Human Health

The human gut microbiome has a much larger impact on human health than previously known. One example of this involves obesity, which plagues much of our world today. According to the CDC, every state in the U.S. had 20% or more of its adult population classified as obese in 2016. In some states, such as Alabama, the percentage jumped to 35% or more.[1] Obesity is connected with decreased variety of gut bacteria, which in turn is associated with higher levels of systematic inflammation.[2] Because this inflammation plays a role in the development of some cancers, it is likely that the gut microbiota have contributed to a connection between adiposity and gastrointestinal cancers.[3]

The impact that the gut microbiome has on health is further established in our understanding on the gut-brain axis. The gut-brain axis is the signaling pathway between the gastrointestinal tract (gut) and central nervous system. However, despite the recognition and awareness of the gut microbiota as a key factor in human health, whether the relationship is correlative or causal is difficult to establish. Using rodents as experimental hosts, the gut microbiome was shown to play a role in modifying emotional behavior. *Bifidobacterium* and *Lactobacillus* have both been shown to exert positive effects on anxious and depression-like behavior.[4] In human studies, children with autism spectrum disorder (ASD) have reduced protein digestion, which results in higher amounts

of peptides going into circulation and the distal gastrointestinal tract.[5] This could cause dysbiosis, which is a lack of harmony between the beneficial and harmful species in the gut.[5] Dysbiosis could cause changed immune reactions and higher danger of diseases.[6] It has also been suggested that gastrointestinal issues, which could be linked to dysbiosis, may have a causal relationship with ASD because of the frequency that children with ASD have had GI issues.[7]

Alterations in diet, which consequentially alter the gut microbiota, are known to play a role in autoimmune diseases (like multiple sclerosis).[8] For example, high consumption of salt could be a factor in autoimmune disease development because of certain pathogenic cell induction.[9] The human gut microbiota plays a key role in priming the immune system in multiple ways, such as providing immune-stimulatory signals, which may trigger immune responses.[8] Not only does the gut microbiota influence the immune system, but the immune system also controls the composition of the microbiota.[10]

Mechanisms of Microbiome Effect on Human Health

Trimethylamine N-oxide (TMAO) is an example of an intestinal metabolite that impacts human cardiovascular health. TMAO is derived from choline and phosphatidylcholine in one's diet.[11] Choline is first metabolized to trimethylamine (TMA) by the gut microbiota, and TMA is in turn converted to TMAO by FMO3, which is an enzyme in the liver.[12] Interestingly, higher amounts of TMAO have been associated with higher risk of adverse cardiovascular events[13] because this TMA/FMO3/TMAO pathway regulates inflammation and lipid metabolism in many different ways[11] that result

in deposit of fatty materials in the artery linings, or atherosclerosis. TMAO promotes expression of scavenger receptors in macrophages to increase their proinflammatory responses. TMAO also significantly alters cholesterol and sterol metabolism by altering bile acid synthesis and cholesterol transport.[14] Moreover, because phosphocholines are enriched in red meat, subjects that had a combined meat-plant based diet produced more TMAO than those with only a plant based diet following the same ingestion.[14] As a result of this research field, the gut microbiota has demonstrated a direct role in contributing to cardiovascular disease.

Other examples of metabolites that impact health are long chain fatty acids (LCFAs) and short chain fatty acids (SCFAs). The fascinating thing about these molecules is a minor difference in chain length can have a large impact on their health consequences in the human body. LCFAs, which are microbial metabolites found in large quantities in the modern western diet, may be an underlying cause of diseases because they promote differentiation and proliferation of T helper 1 (Th1) and/or T helper 17 (Th17) cells and diminish sequestration of Th1 and Th17 cells in the intestines. SCFAs are metabolites produced by microbial fermentation in the intestines and play multiple roles in benefiting our health.[15] They are able to play a role in the human metabolic regulation, immune homeostasis, and the performance of the nervous system through the activation of G-protein coupled receptors (GPCRs).[16] SCFAs are the ligands of the GPCRs free fatty acid receptors 2 and 3 (FFA2 and FFA3). FFA2 has been found to alter the supply of gut immune cells and inflammatory responses that manage immune homeostasis and protect the epithelial wall of the intestine against tumor development and pathogens, and both FFA2 and FFA3 regulate glucose homeostasis.[17]

What SCFAs Are and How They Are Synthesized

SCFAs are fatty acids consisting of only 2-6 carbons, making them highly difficult to analyze in complex biological samples. They are metabolites produced by microbial fermentation of polysaccharides, starches, and endogenous glycans.[16] Only specific species of bacteria can carry out this fermentation to produce SCFAs, such as *Firmicutes* and *Bacteroidetes*.[16] The most abundant SCFAs produced are acetic acid (C2), propionic acid (C3), and butyric acid (C4), with each usually being produced by a different pathway. The most effective pathway for the production of acetate is the Wood-Ljungdahl pathway. In this pathway, two molecules of carbon dioxide are reduced to form a single molecule of acetate.[18] Propionate is usually produced by a carbon dioxide fixation pathway, while butyrate is typically formed by acetyl-*S* coenzyme A condensation.[19]

Methods to Measure SCFAs (Sample Preparation and GC Instrumentation)

Analysis of SCFAs often relies on a gas chromatograph (GC). However, to detect SCFAs in complex biological samples, sample pretreatment process is required before injection into the GC for cleaner peaks. Three common cleanup treatments include extraction,[20] ultrafiltration,[21,22] and steam distillation.[23] Extraction was performed by suspending fecal samples in water and 50% sulfuric acid. The sample was then homogenized and centrifuged, creating a supernatant. Using the supernatant and ethyl ether, an extraction was performed by shaking and venting. The suspension was then collected and the chosen internal standard (IS) was added before analysis.[20] Ultrafiltration

was performed by homogenizing the fecal sample in sulfuric acid and water. The homogenized sample was then centrifuged. The supernatant was filtered through a microconcentrator and then the sample was acidified and distilled using steam distillation. Acidification and steam distillation steps are used to help with the detection of the wanted compounds.[21] Other literature discussed the use of an ultrafiltration unit.[22] Steam distillation was performed by using a microKjeldahl apparatus.[23]

Successful experimental conditions of the GC are vital for successful separation and detection of the wanted compounds. Many various methodologies have been reported and share several common steps. For example, an IS, such as 2-ethyl butyric acid,[24,25] is used for calibration and to adjust for the loss of analyte during analysis in order to obtain accurate results. In other cases, another 6 carbon chemical is used as the IS.[26] A flame ionization detector (FID) is a common detector used for analyses of SCFAs[24,25] because of its sensitivity, uniform response, and linear range.[27] The column used in reported studies vary; some used a fused-silica capillary column,[24,26] and others used a high polarity capillary column.[25] Some columns had a free fatty acid phase (FFAP)[24,25] in order to better analyze SCFAs. The sizes of the columns also varied in internal diameters – 30m x 0.53mm[24,26] and 30m x 0.25mm.[25] The film coating thickness varied from 0.5μm,[24] 0.25μm,[25] and 1μm.[26] Carrier gases are used in GC as the mobile phase, and those used include helium[24,26] and nitrogen.[25] The flow rates of these carrier gases varied greatly – from 1 mL/min,[25] 14.4 mL/min,[24] to 24 mL/min.[26] The detector temperatures were maintained at 240°C[24,25] or 300°C[26] to vaporize the analytes. The injection port temperatures were 200°C[24,26] and 220°C.[25] In each study, the sample volume used was

$1\mu L.$[24–26] Flow rates of hydrogen, air, and nitrogen were 20-30mL/min, 300mL/min, and 20-30mL/min, respectively.[24,25]

What *Listeria monocytogenes* Is and why it Is Important

Listeria monocytogenes (*Listeria*) is a rod-shaped, Gram-positive, facultative anaerobic bacterium,[28] and also a foodborne pathogen that causes listeriosis. Listeriosis cases are generally infrequent in humans, but the mortality rate for those infected is about 20%, making it an extremely dangerous disease. According to data from 2010, *Listeria* infection caused 5,463 deaths, 23,150 illnesses, and172,823 disability-adjusted life-years (which is the measure of the burden of an illness shown by years of life lost).[29] *Listeria* is capable of surviving through food treatment and packaging because it can still propagate in harsh conditions, such as in temperatures of 1.7-45.0°C,[30] in a pH range of 4.7-9.2,[31] and microaerobic or anaerobic environments. *Listeria*, as an enteric pathogen, transits through anaerobic intestinal lumen rich with SCFAs. However, we knew very little about how the bacteria respond to SCFAs or how SCFAs affect *Listeria* pathogenesis. Better understanding of the relationship between SCFAs and *Listeria* may identify novel preventative or therapeutic strategies.

Project Goals

The main goal of this project it to establish an analytical method for SCFA analysis. Although various methods have been established in literature, including Gas Chromatography, Mass Spectrometry, coupled Gas Chromatography-Mass Spectrometry, and High-performance Liquid Chromatography, there are no established methods on the

University of Dayton's campus. Gas Chromatography is the most readily available option in Dr. Erick Vasquez's laboratory and was chosen for this project.

Materials and Methods

Strains

The *Listeria* strain used in this project was the wild-type lab strain 10403s. This strain was streaked onto brain heart infusion (BHI) agar weekly.

Defined Media

Listeria is typically grown in complex medias such as brain heart infusion (BHI). However, accurate detection of SCFAs produced by *Listeria* requires the creation of a defined media. Defined media is able to sustain growth of a specific organism while only consisting of known chemical components. The defined media for this study had to contain no SCFAs as to not interfere with results of SCFA levels in the samples. After a thorough search through the relevant literature, modified Welshimer's broth (MWB)[32] and a defined solid and liquid medium (HTM)[33] were chosen as the baselines for the defined media. MWB was found to support only some growth of the strain 10403s,[34] and was thus altered into HTM. Ammonium sulfate was included in HTM and our defined media because *Listeria* is able to use inorganic nitrogen sources. Therefore, ammonium sulfate can replace glutamine in the defined media.[33] Because we wanted to develop defined media that support robust bacterial growth instead of identifying essential nutritional requirements, we decided to combine the two lists and used higher concentrations whenever possible. Double the concentration of components of MWB and

HTM were used in the defined media, except for thiamine, riboflavin, and biotin, where

50 times, 50 times, and 200,000 times the previous concentrations were used,

respectively. Comparisons of the content in MWB, HTM, and our defined media are

shown in Table 1. The final solution was filter sterilized instead of autoclaved to avoid

degradation of amino acids or caramelization of glucose.

	MWB	HTM	Defined Media
MOPS pH 7.4		100.00mM	200.00mM
KH_2PO_4	48.2mM	4.82mM	96.4mM
Na_2HPO_4	115.5mM	11.55mM	231mM
$MgSO_4$	1.70mM	1.70mM	3.40mM
Ferric citrate	360µM		720µM
Glucose	55.00mM	55.00mM	110mM
Thiamine	2.96µM	2.96µM	148µM
Riboflavin	1.33µM	1.33µM	66.5µM
Biotin	24.00pM	24.00pM	4.8µM
Lipoic acid	0.1mg ml^{-1}		0.2mg ml^{-1}
Histidine	0.1mg ml^{-1}		0.2mg ml^{-1}
Tryptophan	0.1mg ml^{-1}		0.2mg ml^{-1}
Leucine	0.1mg ml^{-1}		0.2mg ml^{-1}
Isoleucine	0.1mg ml^{-1}		0.2mg ml^{-1}
Valine	0.1mg ml^{-1}		0.2mg ml^{-1}
Arginine	0.1mg ml^{-1}		0.2mg ml^{-1}
Cysteine	0.1mg ml^{-1}	0.1mg ml^{-1}	0.2mg ml^{-1}
Methionine	0.1mg ml^{-1}	0.1mg ml^{-1}	0.2mg ml^{-1}
Glutamine	0.6mg ml^{-1}		1.2mg ml^{-1}
$(NH_4)_2SO_4$		0.6mg ml^{-1}	1.2mg ml^{-1}
Agarose		15g liter^{-1}	

Table 1: Content of MWB, HTM, and our defined
media. Empty spots in the table indicate that the
chemical was not used in that media.

Culture Conditions

Listeria strain 10403s was grown overnight in the filter sterilized defined media

described above along with a brain heart infusion (BHI) control. The overnight cultures

were put into either an aerobic or anaerobic environment. The aerobic cultures were placed into a 37°C incubator on a shaker while the anaerobic cultures were placed into an anaerobic chamber and grown statically at 37°C (Type A, Coy Laboratory, Grass Lake, MI, USA). The gas inside the chamber is mainly nitrogen with 2.5% hydrogen. In each environment, there were cultures consisting of 1mL of media. Optical density (OD) of the overnight cultures were read the next day by transferring 200µL of each into a 96-well plate that was read at 600nm using a 96-well plate reader (Synergy4, Biotek, Winooski, VT, USA).

Gas Chromatograph Instrumentation

The process to determine the GC instrumentation was also based on trial, error, and adjustment. In the first trial, a thermal conductivity detector (TCD), water as the SCFA solvent, and an Rtx-1 column were used. This trial yielded poor results and we determined this could have been because a TCD is not a suitable detector for SCFAs. The detector that was then put in was an FID, which has more capabilities for detecting SCFAs. After the second trial, it was realized that the results were unusable because water was used as the SCFA solvent. In order to analyze complex biological samples in the future, the GC method needed to have analyzed standards in the same solvent that would be used for the complex samples. Because extractions of biological samples would take place in diethyl ether, this solvent was used from then on for the SCFAs. The third trial yielded poor results and we determined this was likely because the Rtx-1 column was nonpolar. In order to separately elute the polar SCFAs, a polar column needed to be put into place. This is when we installed the DB-FATWAX UI column, which was

nonpolar and had a FFAP, so it was better equipped to analyze SCFAs. At this point, an FID, diethyl ether as the SCFA solvent, and the DB-FATWAX UI column were used to run a fourth trial. This trial yielded excellent results.

More detailed information on the GC characterization method is not currently available due to lack of access to research materials during the COVID-19 quarantine.

SCFA Samples and Standards

The SCFA samples were made using the ethyl ether, IS (which consisted of 88% 2-ethylbutyric acid and 12% formic acid), and sodium acetate, sodium propionate, and/or sodium butyrate. The standards were made in the same way, but the concentration of each SCFA varied so that a standard graph could be created.

More detailed information on the creation of the SCFA samples and standards is not currently available due to lack of access to research materials during the COVID-19 quarantine.

Results

Defined Media Supported the Growth of *Listeria*

The process to accurately create the defined media was extensive and initially full of error. It was not realized until after the first few trials that MOPS needed to be adjusted to pH 7.4, as it is naturally more acidic. Other initial errors included a lack of consistency in the amount of substance being transferred. However, by the eleventh trial, accurate defined media was created and gave consistent results when tested, as shown in Figure 1.

In the last 4 trials, especially trials 10 and 11, *Listeria* grew slightly better in the presence of oxygen than it did in the absence of oxygen, which are typically found when *Listeria* is grown in BHI (data not shown).

Figure 1: Consistency of OD (read at 600nm) measurements across 11 trials was used to assess the effectiveness of our defined media to reliably serve as a growth media for *Listeria.* The results show the averages of three replicates.
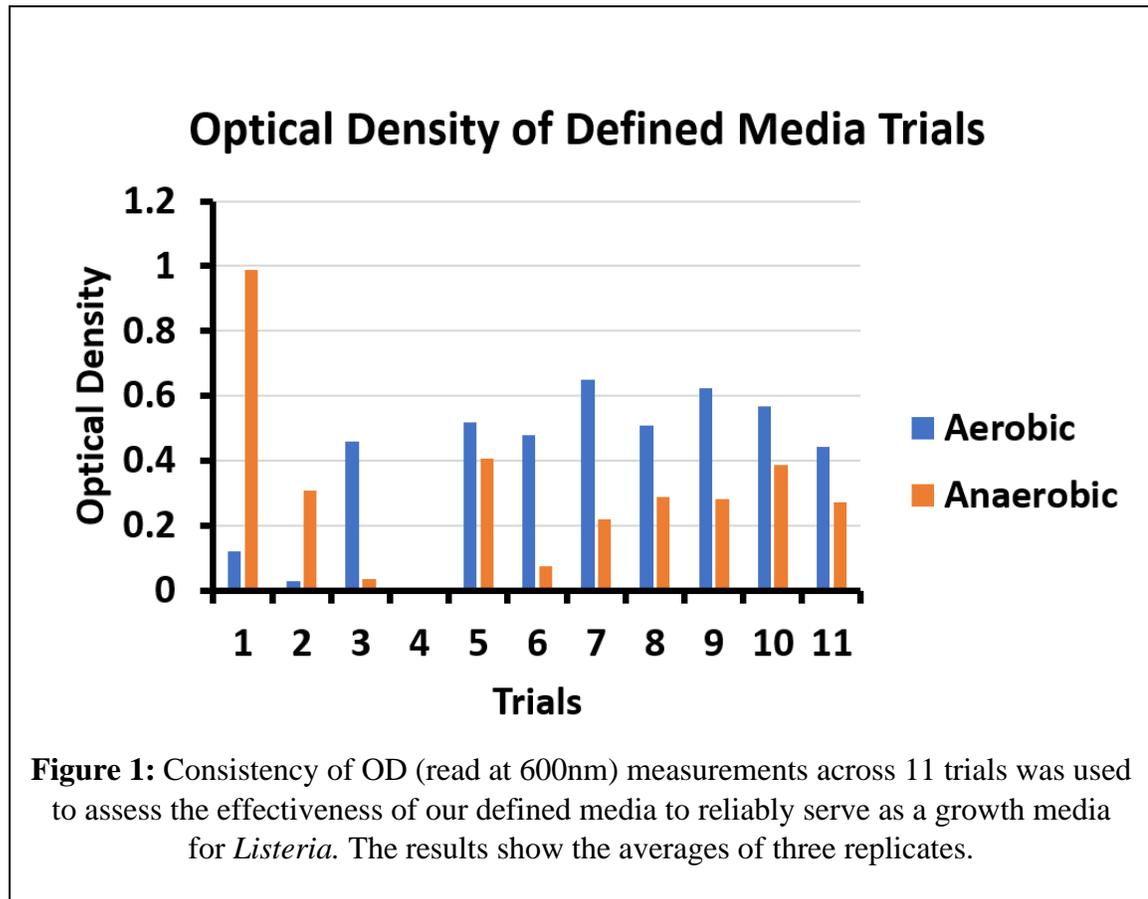
SCFA Characterization Method Established

A clear gas chromatogram was obtained once the GC characterization method had been adjusted through trial and error. An optimal gas chromatogram has clear, sharp, and separate peaks for each compound with minimal noise. This was obtained through our method, as we were able to get results that show the elution of acetate, propionate,

butyrate, and the IS from left to right. Acetate, propionate, and butyrate each had about 10.137, 11.288, and 12.405 minutes of retention time. Acetate eluted before propionate and butyrate because it is the most polar of the SCFAs and a polar column was used in the GC. Once the characterization method was able to accurately detect and elute the SCFAs, standards were successfully analyzed in order to generate equations that could be used to find the concentrations of SCFAs in unknown samples.

Discussion

While creating a viable defined media, a couple of important factors to microbiological research were realized. One of these is that when working with small amounts of chemicals, the slightest variations in transfer and measurement methods can impact the results. Another factor was that it is important to adjust pH in media that bacteria are grown in, because although *Listeria* is able to grow in a wide pH range, it may grow optimally at a certain pH. Once these factors were improved, the defined media appropriately supported *Listeria* growth.

The most important outcomes of this project are that we have 1) developed a defined media that does not contain SCFA and is capable of supporting the growth of *Listeria* and 2) established an analytical method that is capable of separately eluting different SCFAs and giving accurate data that can be used to determine SCFA concentrations in a standard sample. From this method, the standard equations, and the approximate retention times of the SCFAs, we are now able to able to begin analyzing complex biological samples.

Future Directions

In future work on this project, further improvement of the defined media may prove useful. In our defined media, two buffers were used instead of one, which may have impacted the growth of *Listeria*. Now that an accurate analytical method has been established on the GC for the analysis of SCFAs, more complex biological samples can be tested such as the production of SCFAs by *Listeria* and animals. This could be very valuable to our lab as we work to better understand the influence that SCFAs have on *Listeria* infections and human health.

Project 2 – Effects of SCFA and Age on *Listeria* Pathogenesis

Introduction

Background on Listeriosis

Listeria is able to effectively infect the body by evading immune detection in a host in a variety of different ways. One way is by cell-to-cell infection, which allows *Listeria* to avoid detection outside of cells while continuing to spread. Another way that *Listeria* evades immune detection is by targeting host T cells. By targeting host T cells during *Listeria* infection, inflammation and innate immunity are repressed.[35] *Listeria* produces the toxin LLO which induces apoptosis of T cells.[36–38] *Listeria* can evade innate immune detection by altering components of its cell wall in order to avoid binding with pattern recognition receptors. One way that *Listeria* does this is by deacetylating the *N*-acetylglucosamine residues in its peptidoglycan, thus decreasing its sensitivity to lysozyme.[39] Another way that *Listeria* evades immune detection is by modifying host

gene expression. An example of this is alterations to histones H3 and H4 during early infection, which correlates with decreased transcription of host immunity genes.[40]

The elderly population is characterized by compromised immune systems, lending to even higher susceptibility to infection by *Listeria*. A study has shown the impact of *Listeria* on the elderly, as the risk of infection of *Listeria* increases with age in adults ≥65 years old.[41] Although the average annual rate of infection for *Listeria* is lower than that of *Salmonella* and *Campylobacter*, the percentage of patients hospitalized was higher in patients with *Listeria*. *Salmonella* and *Listeria* were shown as the leading causes of death in the study, but percentage of deaths was increased 10-fold in patients with *Listeria*.[41] Because studies have suggested that T cell response impacts human susceptibility to listeriosis,[42,43] further studies have been conducted examining factors such as T cell response in aging murine models of listeriosis. A study found that aging mice usually have an increased baseline of Treg responses, and when infected by *Listeria*, this effect is only exacerbated. This causes a decrease in pro-inflammatory responses during infection, which leads to decreased pathogen elimination from systemic organs. The study also found that T cell expression of CD39 and CD73 is augmented in aging mice.[44] In humans, increased expression of CD39 has been found to possibly impair formation of vaccine-specific memory T cells.[45]

Listeria-associated Infective Endocarditis

In some patients, *Listeria* infections are able to travel to the heart and cause infective endocarditis. This is a serious form of infection, as patients with endocarditis due to *Listeria* have been found to have a 41% mortality rate. This mortality rate is twice

as high as that of endocarditis associated with other related pathogens, and notably 10 times higher than that of Streptococcus-associated endocarditis.[46] *Listeria*-associated endocarditis tends to cause severe symptoms, as 50% of patients are found to have acute congestive heart failure.[47] Because of the severity of this type of infection, research has been done recently to determine whether certain *Listeria* isolates have enhanced capability to colonize heart tissue. A study by Alonzo et al. (2011) found this increased capability of myocardial invasion in a cardiac case isolate (07PF0776). In this study, 10-times as many bacteria were recovered from heart tissue in mice infected by the 07PF0776 strain than the 10403s strain that is typically studied.[48]

Varying SCFA Concentrations Influences Pathogenesis

As stated previously, SCFAs are primarily the result of microbial fermentation in the gut. SCFA concentrations vary at different locations of the gut, with two notably distinct locations being the distal ileum and the colon. The distal ileum contains about 25.5mM acetate, 2.25mM propionate, and 2.25mM butyrate, while the colon contains about 110mM acetate, 70mM propionate, and 20mM butyrate.[49] It is thought that SCFA may signal for bacterial invasion of the ileum while lowering invasion of the colon. An example of findings consistent with this mechanism come from a Lawhon et al. (2002) study that investigated the role SCFAs and the sensor kinase BarA had in *Salmonella* invasion. It was found that invasion gene expression is restored in *barA* mutants grown in SCFA solutions mimicking ileal conditions. However, this restoration does not take place in *barA* mutants grown in SCFA solutions mimicking colonial conditions, and. However, colonic SCFA conditions do not have this restorative effect in *barA* mutants and reduce

invasion gene expression in the wild-type strain.[49] These results may explain why

Salmonella tend to invade the distal ileum and show the importance of examining the

impact of ileal and colonic SCFA conditions on the pathogenesis of *Listeria*.

Project Goals

The main goal of this project is to determine the effects of SCFA and age on

Listeria infection. This is done partially through *in vitro* methods by assessing the effects

of SCFAs on LLO production, acetoin production, and cell culture infection. Oral

infection of mice is also used to give an *in vivo* approach to assessing the effects of

SCFAs and age on *Listeria* infection.

Materials and Methods

Strains

The *Listeria* strains used include the wild-type lab strain 10403s and a clinically

isolated cardiotropic strain 07PF0776 (provided by Dr, Nancy Freitag at The University

of Illinois College of Medicine). These strains were streaked onto brain heart infusion

(BHI) agar weekly.

M_{hi} and M_{lo} SCFA Supplementation

Two SCFA mixtures that were mentioned in a previous study[49] are used in this

study as a way to study the effects of relevant SCFA concentrations in the body. The

colonic conditions have a higher concentration of SCFAs and the ileal condition have a

lower concentration of SCFAs, so they are respectively labelled Mixture$_{hi}$ (M_{hi}) and

Mixture$_{lo}$ (M$_{lo}$) in this study. M$_{hi}$ consists of 110mM acetate, 70mM propionate, and 20mM butyrate, while M$_{lo}$ consists of 25.5mM acetate, 2.25mM propionate, and 2.25mM butyrate.

Acetoin Assay

A modified Voges-Proskauer test was used to quantify the acetoin production in *Listeria* cultures. 100µL of acetoin standard or culture supernatant was combined with 70µL of 0.5% creatine monohydrate (C3630-100G, Sigma, St. Louis, MO, USA), 100µL of 5% alpha-Naphthol (N1000-10G, Sigma) in 95% ethanol, and 100µL of 40% KOH in water. The mixture was vortexed then incubated at room temperature for 15 minutes. The absorbance was measured at 560nm in a flat-bottomed 96-well plate.

Hemolytic Assay

OD of overnight cultures and timepoint cultures were measured at 600nm. 500µL of cultures were centrifuged to obtain a bacterial pellet separate from supernatant. 100µL of supernatant and 5µL of 0.1 M dithiothreitol were incubated at room temperature for 15 minutes in a round-bottom 96-well plate. A 1:2 serial dilution of these samples was performed with 100µL of hemolytic assay buffer. The hemolytic assay buffer consisted of 125 mM sodium chloride (Ameresco #0241-5KG, Framingham, MA, USA) and 35 mM sodium phosphate dibasic (Fisher 7558-79-4) adjusted to pH 5.5. Hemolytic assay buffer was used to prepare defibrinated sheep blood at 2% hematocrit. 100µL of the prepared blood was added to each well, and the samples were incubated at 37°C for 30 minutes. Intact blood cells were separated from the rest of the sample by centrifuging the

plates at 2000 rpm for 5 minutes. 120µL of the supernatant was transferred to a flat-bottom 96-well plate and the absorbance was measured at 541nm using a microplate reader.

Cell Culture Infection

RAW264.7 macrophages were cultured at 37°C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Corning 10-013-CV, Corning, NY, USA) containing 10% Fetal Bovine Serum (Corning 35-010-CV) and penicillin/streptomycin (BioWhittaker 17-603E, Rockland, ME, USA). The macrophages were seeded $6x10^6$ cells per plate in a 24-well plate and incubated at 37°C overnight. *Listeria* cultures were grown overnight in their respective oxygen (aerobic and anaerobic) and SCFA conditions (no supplement, M_{hi}, and M_{lo}). The cultures were centrifuged for 3 minutes at 10,000rpm, washed, and resuspended in Phosphate-buffered Saline (PBS). Culture OD was used to calculate the volume of bacterial culture suspension needed to achieve a multiplicity of infection of 10. At 30 minutes post infection of the macrophages, the wells were washed twice with Dulbecco's PBS (DPBS) (World Wide Life Sciences, 61211088). DMEM with 10µg/mL gentamicin was supplemented into the wells to remove extracellular *Listeria*. Macrophages were lysed using 0.1% Triton-X at 2, 4, and 8 hours post infection. The lysate was then plated on LB plates, which were incubated at 37°C for 2 days. Colonies were counted by an aCOLyte 3 plate reader (Synbiosis) and then intracellular colony forming units(CFUs)/well was calculated.

Mouse Infections

For the old cohort of mice, 3 month old female BALB/c mice were purchased from Jackson laboratory and aged in our vivarium until they were 16-18 months old. The young female BALB/c cohort of mice were purchased at 2 months of age and acclimated in our vivarium for 1 week. While in our vivarium, mice were aged/acclimated 4 to a cage on a 12-hour light and 12-hour dark schedule. Once the experiment began, mice were moved to individual cages and given designated SCFAs through drinking water. These SCFA treatments were the no supplement control, 110mM acetate, 70mM propionate, 20mM butyrate, M_{hi}, M_{lo}, and 200 mM NaCl. The drinking water was replaced biweekly for the 5 weeks of the experiment. In order to find the amount of water consumption for each mouse, before and after weights of the water bottles were recorded during water replacement. The mice were weighed weekly to observe changes from SCFA treatment and were familiarized with Nutella (the mode of infection) through feeding.

After 28 days of SCFA treatment, the mice were starved for 24 hours before infection. On the day of infection, the mice were each given 10^8 bacterial CFUs of either the strain 10403s or 07PF0776 inside Nutella. 4 to 5 days post infection the mice were euthanized by CO_2 asphyxiation and their organs (heart, liver, spleen, gallbladder, colon, and fecal contents of colon) were extracted. The organs were sonicated in 1% tergitol NP-7 by a 60 sonic dismembrator. The organ homogenates were diluted and liver, spleen, gallbladder, and heart samples were plated onto LB agar plates while ileum, colon, and colonic content samples were plated on Oxford agar plates. The plates were incubated at

37°C for 2 days and the colonies were counted using an aCOLyte 3 plate reader
(Synbiosis).

Results

SCFA Supplementation Affects LLO Production of Strains 10403s and 07PF0776

These experiments were performed mainly by Dr. Erica Rinehart, while I assisted
her in some of the hemolytic assays. Data for these experimental results have been
recently published,[50] but will be described here. There are multiple important effects that
SCFA supplementation has on the LLO production of *Listeria* strains 10403s and
07PF0776, as noted by looking at hemolysis. M_{lo} supplementation leads to an increase of
LLO production while M_{hi} supplementation leads to a decrease of LLO production in
strain 10403s under aerobic conditions when compared to the no supplement control.
However, this is not seen in the 07PF0776 strain under aerobic conditions; M_{lo}
supplementation leads to a decrease of LLO production while M_{hi} supplementation leads
to no significant difference in LLO production. Under anaerobic conditions, there is no
production of LLO in the 10403s strain while there is in the 07PF0776 strain. SCFA
supplementation affects the 07PF0776 strain similarly under anaerobic conditions as it
did under aerobic conditions, however one important difference is that there is a
significant increase in LLO production when supplemented with M_{hi} that was not present
under aerobic conditions. Overall, there is clearly an oxygen concentration, strain, and
SCFA dependent difference on *Listeria*'s production of LLO.

SCFA Supplementation Affects Carbon Metabolism of Strains 10403s and 07PF0776

There are multiple important effects that SCFA supplementation has on the carbon metabolism of *Listeria* strains 10403s and 07PF0776, as noted by looking at acetoin production. Under aerobic condition, M_{lo} supplementation leads to a significant increase of acetoin production in the 07PF0776 strain, but not in the 10403s strain, while M_{hi} supplementation leads to a significant increase of acetoin production in both strains. There was also a significant increase of acetoin production in both strains under aerobic conditions from M_{hi} supplementation when compared to M_{lo}. Acetoin production occurred in both strains under anaerobic conditions, but SCFA supplementation did not significantly affect it.
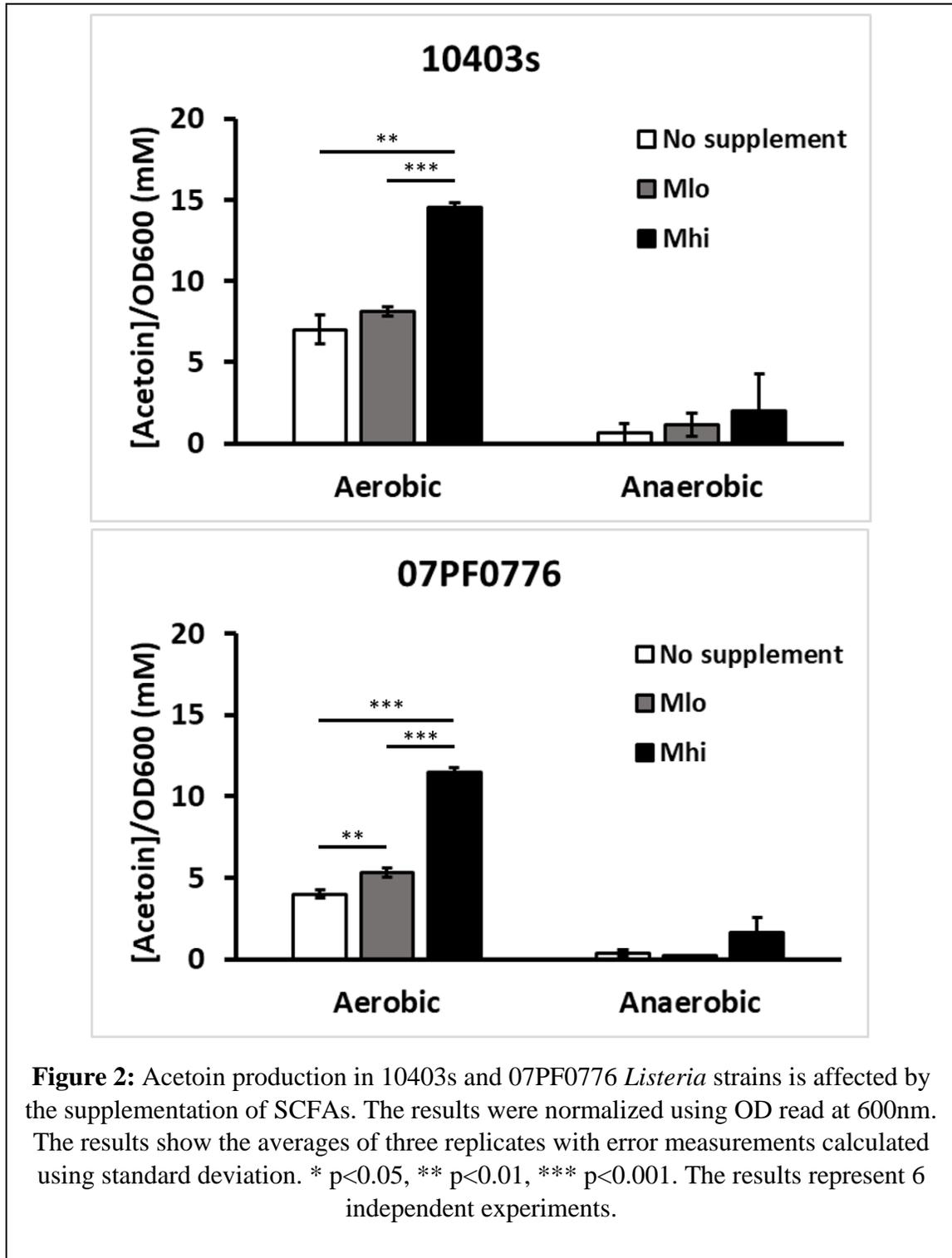
Figure 2: Acetoin production in 10403s and 07PF0776 *Listeria* strains is affected by the supplementation of SCFAs. The results were normalized using OD read at 600nm. The results show the averages of three replicates with error measurements calculated using standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The results represent 6 independent experiments.

SCFA Supplementation Affects Strains 10403s and 07PF0776 Infection of Cell Cultures

These experiments were performed mainly by Dr. Erica Rinehart, while I assisted her in some of the seeding, infecting, plating, and colony counting. Data for these experimental results have recently been published,[50] but will be described here. There are multiple important effects that pre-exposure of *Listeria* strains to SCFA has on cell culture infections, as noted by looking at infections of RAW264.7 macrophages. Under aerobic conditions, SCFA treatment significantly affected intracellular growth of the 10403s strain, with M_{lo} increasing growth and M_{hi} decreasing growth, but did not significantly affect intracellular growth of the 07PF0776 strain. Under anaerobic conditions, M_{lo} significantly decreased intracellular growth of the 10403s strain and M_{hi} had no significant effect. However, there again is no significant effect of SCFA treatment on the intracellular growth of the 07PF0776 strain grown anaerobically.

Another important result that came from this experiment is that it shows a difference of intracellular growth between the strains. At 8 hours post infection under both aerobic and aerobic conditions, the 07PF0776 strain has less intracellular survival than the 10403s strain.

Age of Mice and SCFA Supplementation of Strains 10403s and 07PF0776 Affects Infection of BALB/c Mice

These experiments were performed mainly by Dr. Erica Rinehart, while I assisted her in some of the sonicating, diluting, and plating of the organs and colony counting. Data for these experimental results are included in her doctoral thesis but will be described here. There are multiple important effects that pre-exposure of female BALB/c

mice to SCFA has on infection with *Listeria* strains. There is also an important impact that age has on the infection of these mice. In both young and old mice, infection with the 07PF0776 strain leads to a higher bacterial load in the spleen than does infection with the 10403s strain. In the old mice infected with the 07PF0776 strain, SCFA treatment resulted in increased bacterial burden, with there being a significantly higher bacterial burden from treatment with M_{hi} than M_{lo}. Also, in old mice, infection with the 07PF0776 strain leads to a higher bacterial load in the gallbladder than does infection with the 10403s strain. Of the mice that were treated with acetate, young mice had a higher bacterial load in the heart than old mice. And, lastly, old animals seemed to have lower bacterial burden in the heart when treated by propionate, butyrate, and M_{lo}, but this difference was only significant with M_{lo}.

Discussion

Overall, these findings are highly relevant and have an important impact on what is known today about *Listeria* infections. These studies have shown that there is clearly an oxygen concentration, strain, and SCFA treatment dependent difference on *Listeria*'s infection of macrophages and production of LLO and acetoin. Because LLO plays multiple roles in infection and pathogenesis, it is important to know that its production is altered by SCFA levels found in the gut, and that the 10403s and 07PF0776 strains respond differently in LLO production to alteration by different SCFA levels. Because SCFA supplementation altered acetoin levels, it is likely that SCFA affect *Listeria*'s carbon metabolism. The macrophage infections showed that there are notable differences in the 10403s and 07PF0776 strains, both in terms of normal infection abilities and

impact of SCFA pre-exposure. Also, there seems to be a difference between the effects of SCFA on aerobic and anaerobic cultures, although the significance of this was not tested. The mice infections led to multiple relevant findings. Firstly, some mouse organs seem to be holding higher bacterial burden when infected by the 07PF0776 strain than the 10403s strain, such as the spleen and gallbladder. Secondly, different SCFA treatments have different affects in different areas of mice. Increasing SCFA concentrations may have caused a higher 07PF0776 bacterial burden in the spleen of old mice, acetate treatment may have increased susceptibility of young mice to infections of the heart, and propionate, butyrate, and M_{lo} treatments may be able to protect old mice from infections reaching the heart.

Future Directions

An example of a future direction of this project is that the mice experiments need to be repeated. One reason for this is that this experiment has only taken place one time in our lab. Another reason is because the n values of our experiment could be increased in order to provide more ability to find significance in results.

Project 3 – Effects of Oxygen Levels on *Listeria* Pathogenesis

Introduction

Oxygen Concentration Varies in the Intestines

Humans and most mammals are obligate aerobes, meaning that they need oxygen to complete metabolic processes. Although these organisms need oxygen to survive in the broad sense, some areas in the body exist where oxygen concentration is low (microaerobic) or absent (anaerobic). Oxygen concentrations vary throughout different regions of the gastrointestinal (GI) tract and tend to decrease from the proximal to the distal regions.[51] Differences in oxygen concentration in the GI tract impact the processes that occur throughout it, such as bacterial growth. For example, it has been shown that gut microbiota segregate radially in response to the oxygen gradient and there is an increase in growth of oxygen-tolerant organisms on the mucosal surface due to its oxygen abundance.[52] Along with changes to the growth of these symbiotic bacteria comes altered growth to pathogenic bacteria as well.

Enteric Pathogens are Facultative Anaerobes

Two examples of enteric pathogens that adjust components of pathogenesis due to oxygen concentration are *Shigella flexneri* and *Listeria*. *Shigella* is a Gram-negative enteropathogen that causes shigellosis, or bacillary dysentery.[53] It enters host cells by delivering invasion plasmid antigen (Ipa) effectors through a type three secretion system (T3SS). *Shigella* contains the *fnr* gene which gives rise to the transcriptional activator FNR, which increases the transcription of genes necessary for anaerobic pathways of pathogenesis. It has been shown that anaerobic conditions lead to reduced Ipa secretion

and T3SS needle elongation in *Shigella*, likely regulated by FNR, to ready the pathogen for infection.[54]

Listeria Produces Multiple Virulence Factors

Listeria infection typically begins with the ingestion of contaminated foods. The bacterium then travels into the stomach and through the intestines. *Listeria* likely translocates into the host through different mechanisms, with one study suggesting that *Listeria* invades the intestinal epithelium.[55] Two internalin proteins, InlA and InlB, which are located on the *Listeria* membrane, are crucial to *Listeria* crossing the intestinal and placental barriers. InlA works by binding to the transmembrane protein E-cadherin on the intestinal epithelium, causing cytoskeletal rearrangements that induce internalization.[56,57] As an intracellular pathogen, *Listeria* can spread from cell to cell and can disseminate into peripheral organs very rapidly.[58]

There are a few processes that varying oxygen levels are known to affect in *Listeria*. It has been found that compared to aerobically grown *Listeria*, anaerobically grown *Listeria* was more efficient in entering host cells but less successful in intracellular growth.[59] This difference could contribute to the explanation of how pre-exposure of *Listeria* to anaerobic conditions can alter pathogenesis as well as the microbial community composition after infections.[60] Moreover, the expression of LLO, a protein produced by *Listeria* that forms pores in the phagosomal membrane to assist the bacterial escape from phagosomes, is under strong regulation by oxygen concentration present during growth. LLO protein is more abundant in aerobically grown *Listeria* compared to

that in anaerobically grown *Listeria*.[59] However, the underlying cause of the anaerobic suppression of LLO production is not fully understood.

Listeria Produces Different Metabolites in Response to Oxygen Availability

Listeria growth under aerobic or anaerobic conditions can be easily distinguished by the metabolites that are produced. For example, under aerobic, but not anaerobic, conditions, *Listeria* produces acetylmethylcarbinol or acetoin.[61] As a result, the production of acetoin has been proposed as an indicator for *Listeria* contamination in food products.[62] In contrast, under anaerobic conditions, the production of lactic acid dominates, contributing to the more pronounced acidification of culture media, compared to growth under aerobic conditions.

Project Goals

One thing our research lab is dedicated to is gaining knowledge of how *Listeria* responds to the presence or absence of oxygen. Although it has been established that LLO production is higher in *Listeria* grown aerobically than anaerobically, the reason for this is not fully understood. The main goal of this project is to better understand the mechanism underlying the regulation of LLO production by varying oxygen levels, and more specifically to consider the possibility that there is a post-transcriptional inhibition of LLO in *Listeria* grown anaerobically.

Materials and Methods

Strains

The *Listeria* strains used include the wild-type lab strain 10403s and an inducible LLO (iLLO) strain.[63] The iLLO strain is induced by addition of IPTG and allows for the control of the level of *hly* transcription to assess post-transcriptional regulation. These strains were streaked onto brain heart infusion (BHI) agar weekly.

Culture Conditions

Aerobic/Anaerobic Transition Experiments

Listeria strain 10403s was grown overnight in the filter sterilized defined media described above. The overnight cultures were put into either an aerobic or anaerobic environment. The aerobic cultures were placed into a 37°C incubator on a shaker while the anaerobic cultures were placed into an anaerobic chamber and grown statically at 37°C (Type A, Coy Laboratory, Grass Lake, MI, USA). The gas inside the chamber consists mostly of nitrogen with 2.5% hydrogen. 50mL conical tubes with 10mL and 15mL of inoculated BHI were placed into the anaerobic and anaerobic environments, respectively. Optical density (OD) of the overnight cultures were read the next day by transferring 200μL of each into a 96-well plate that was read at 600nm using a plate reader (Synergy4, Biotek, Winooski, VT, USA).

The bacteria grown aerobically was concentrated into 1mL of BHI. 100μL of this concentrated solution was used to inoculate 6 tubes of 3 mL BHI each. 3 of these were then placed into the aerobic environment and 3 were placed in the anaerobic

environment. The same was done to the anaerobic overnight cultures. After this, hemolytic assays were performed at 2 and 4 hours of incubation.

Aerobic/Microaerobic/Anaerobic Experiments

The iLLO strain was grown overnight in the filter sterilized defined media described above. The overnight cultures were put into either an aerobic, anaerobic, or microaerobic environment. The aerobic cultures were placed into a 37°C incubator on a shaker while the anaerobic cultures were placed into an anaerobic chamber and grown statically at 37°C (Type A, Coy Laboratory, Grass Lake, MI, USA). The gas inside the chamber consists mostly of nitrogen with 2.5% hydrogen. The microaerobic cultures were placed into a small chamber along with a gas-pak that works to rid the air of oxygen. The small chamber was then placed inside the 37°C incubator. It was placed at a tilt to ensure adequate surface area of the cultures for gas exchange. 3 tubes consisting of 3mL of culture with 1mM IPTG were each placed in the aerobic, anaerobic, and microaerobic conditions. Optical density (OD) of the overnight cultures were read the next day by transferring 200µL of each into a 96-well plate that was read at 600nm using a plate reader (Synergy4, Biotek, Winooski, VT, USA) and then a hemolytic assay was performed.
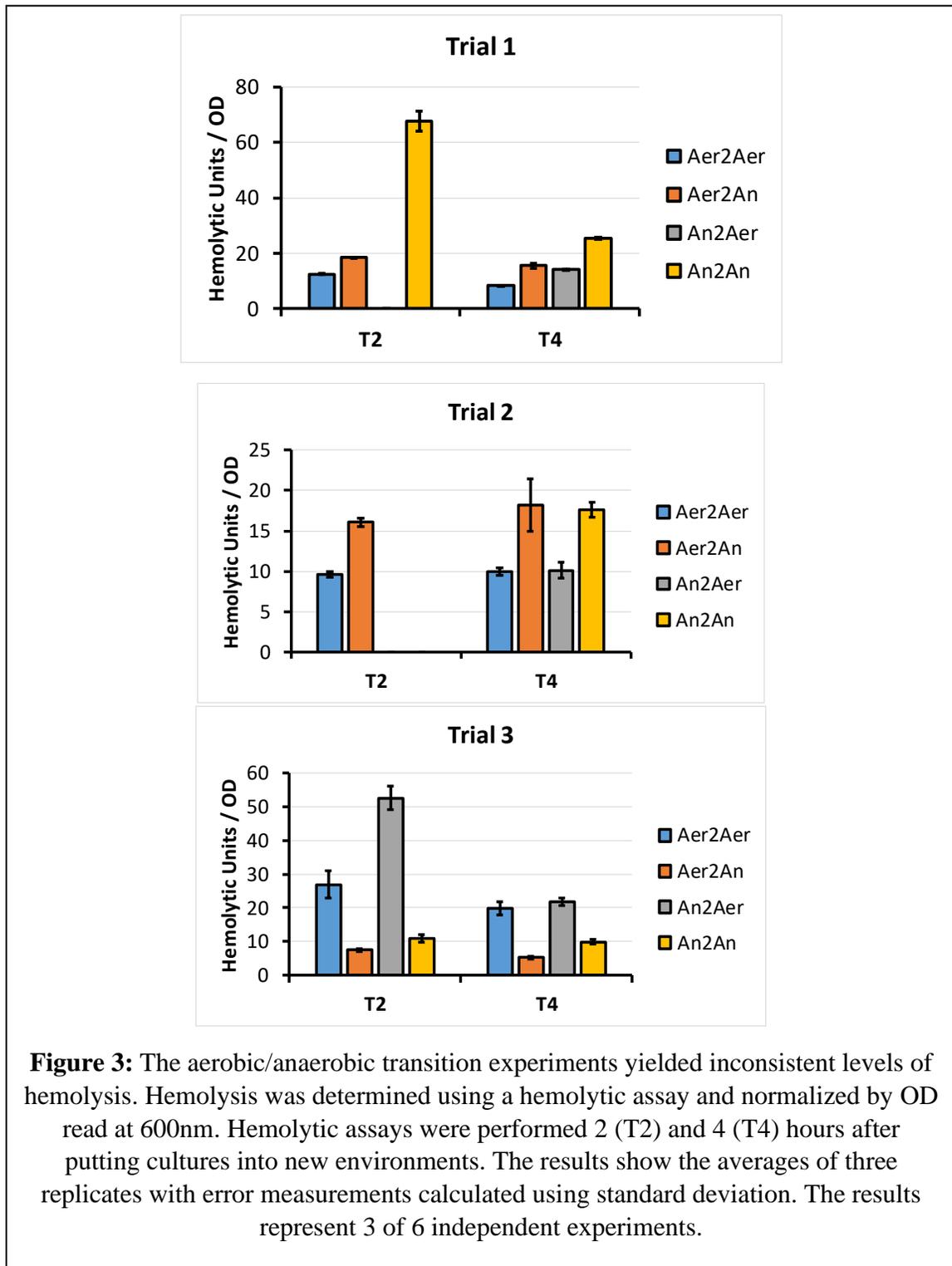
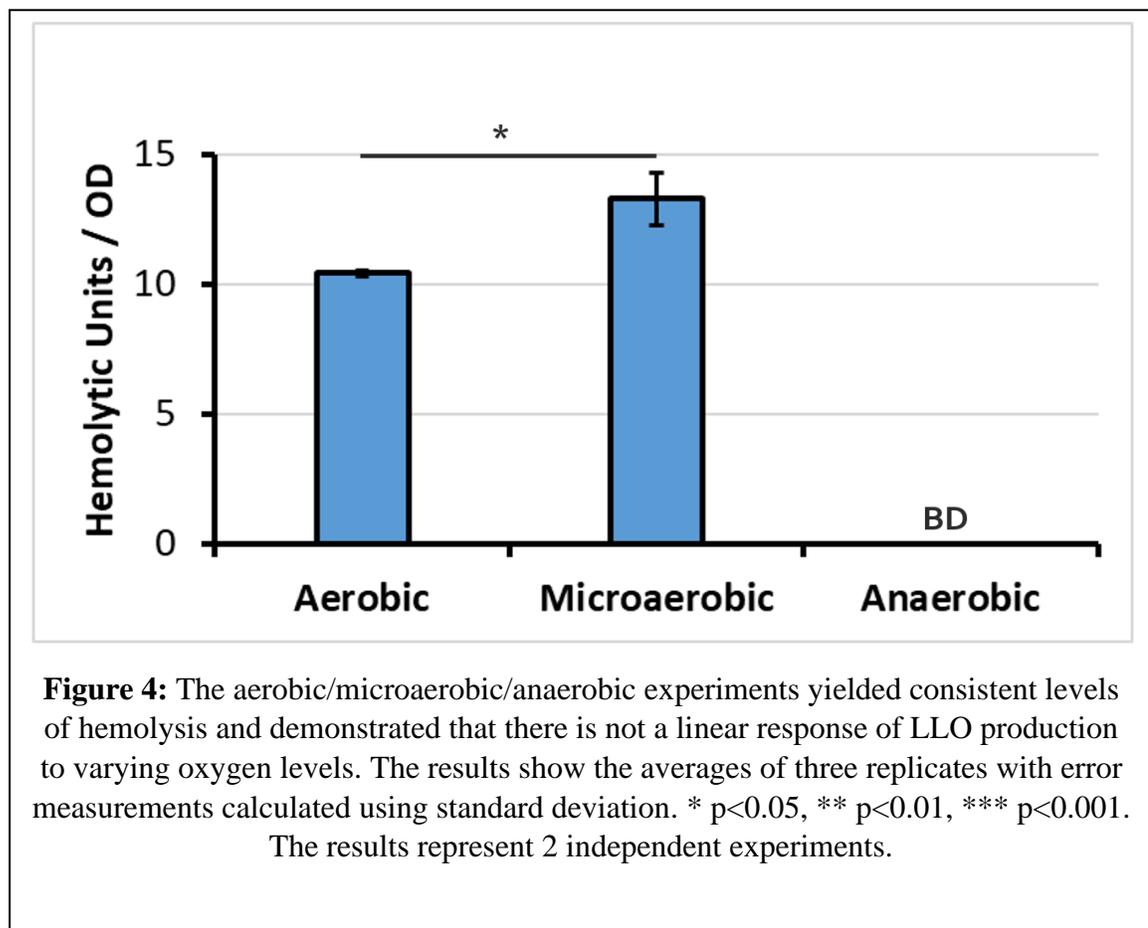Hemolytic Assay

Described in Project 2

Results

Aerobic/Anaerobic Transition Experiments are Currently Inconclusive

6 trials of the aerobic/anaerobic transition experiments were conducted, with 3 being shown in Figure 3, and the results were inconclusive because of a wide variety of responses. Although it was expected that there would be a higher amount of LLO produced by 4 hours (T4) than 2 hours (T2) this occasionally was not the case. These results also do not provide enough evidence to draw conclusions between the environment of the cultures. It is important to note that these results are likely not due to experimenter error throughout the trials, as the error measurements were typically very small.

Figure 3: The aerobic/anaerobic transition experiments yielded inconsistent levels of hemolysis. Hemolysis was determined using a hemolytic assay and normalized by OD read at 600nm. Hemolytic assays were performed 2 (T2) and 4 (T4) hours after putting cultures into new environments. The results show the averages of three replicates with error measurements calculated using standard deviation. The results represent 3 of 6 independent experiments.

Aerobic/Microaerobic/Anaerobic Experiments Give More Insight into Regulation of LLO

2 trials were conducted of the aerobic/microaerobic/anaerobic experiments, with 1 representative trial being shown in Figure 4. Lysis, which represents the presence of LLO, is below detection (BD) in anaerobic samples and higher in aerobic samples. Interestingly, though, microaerobic samples produced significantly more LLO than aerobic samples.

Figure 4: The aerobic/microaerobic/anaerobic experiments yielded consistent levels of hemolysis and demonstrated that there is not a linear response of LLO production to varying oxygen levels. The results show the averages of three replicates with error measurements calculated using standard deviation. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The results represent 2 independent experiments.

Discussion

Because the results of the 6 aerobic/anaerobic transition experiments were inconclusive, they did not help us to understand the regulation of LLO production by

varying oxygen concentrations. However, this form of experiment is still very useful, as it offers an important look at the difference between *hly* transcript and LLO produce in aerobic as compared to anaerobic environments. In order to hopefully use these experiments in the future for more conclusive results, they should be improved in the ways discussed in the future directions.

The aerobic/microaerobic/anaerobic experiments did not prove our initial thought that there may be a post-transcriptional inhibition of LLO in *Listeria* grown anaerobically. This is because these experiments showed that there is not a linear response of LLO production to varying oxygen levels. There may be some type of post-transcriptional modification taking place, but it is not linearly regulated by oxygen levels. More research and duplications of these experiments need to be done in order to better determine what is causing a low amount of LLO production in anaerobic cultures and a high amount in microaerobic cultures.

Future Directions

There are multiple improvements that can be made to the aerobic/anaerobic transition experiments that could possibly lead to better results. One improvement is that the bacteria from the overnight cultures should be washed before switching environments the next day. Without doing this, LLO that was previously synthesized likely skewed the results somewhat. Another improvement that could be made is for the bacteria from the overnight cultures to be normalized before switching environments. This may be a better option than normalizing by OD. For the aerobic/microaerobic/anaerobic experiments, qPCR should additionally be used in order to determine that IPTG is accurately

functioning to produce the same amount of *hly* transcript in all of the varying oxygen environments.

Appendix

PowerPoint and Poster Presentations

1. Berry Summer Thesis Institute Symposium, August 2018

2. Honors Student Symposium 2019, March 2019

3. American Society for Microbiology (ASM) Microbe 2019, June 2019

4. Honors Student Symposium 2020, March 2020

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