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Modulation of *Listeria monocytogenes* Carbon Metabolism by Short Chain Fatty Acids

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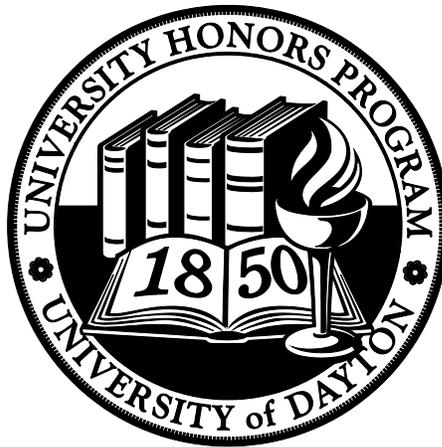
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**Modulation of *Listeria*
monocytogenes Carbon Metabolism
by Short Chain Fatty Acids**



Honors Thesis

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Department: Biology

Advisors: Yvonne Sun, Ph.D.

and Jeremy Erb, Ph.D.

April 2019

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Abstract

Listeria monocytogenes, a bacterial pathogen, is associated with foodborne infections in humans. *Listeria* encounters short chain fatty acids (SCFAs) during its transit through the intestine but its metabolic responses to SCFAs are not fully understood. To determine how *Listeria* metabolism is affected by SCFAs, I performed basic microbiology assays, including monitoring optical density, determining acetoin production, and measuring culture pH levels. I also performed preliminary ^{13}C -NMR assays to provide a more in-depth look into carbon metabolism in SCFA-treated *Listeria*. I found that propionate-supplemented *Listeria* produced significantly more acetoin compared to no supplemented controls. Because acetoin is a product of central carbon metabolism, my result suggests that *Listeria* is capable of changing its carbon metabolism in response to propionate. My preliminary ^{13}C -NMR results have not revealed how carbon metabolism is altered by propionate and are under current investigation. Further investigation will provide more knowledge in the metabolic mechanism associated with *Listeria* responses to SCFAs during intestinal transit.

Dedication

Thank you to the University Honors Program for their support of my research thesis. The UD Dean's Summer Fellowship and Honors Program funded this work. I am grateful for the members of Dr. Sun's Anaerobic Microbiology Lab, especially those whose work and assistance contributed to my thesis: Erica Reinhart and Nathan Wallace. I would also like to thank Dr. Yvonne Sun for her three years of guidance in helping me complete my honors thesis. Her hard work has allowed me to gain invaluable knowledge and practical wisdom from my experiments. Additionally, I would like to thank Dr. Jeremy Erb for ensuring I was properly trained in using the ^{13}C -NMR machine, and for taking the time to help me understand the methodology behind my experiments.



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Introduction

The foodborne Gram-positive bacterial pathogen *Listeria monocytogenes* regularly causes the recall of contaminated food products, costing the U.S. government an estimated 2.8 billion dollars annually¹. A critical component of *L. monocytogenes* is its ability to multiply at dangerous levels even when food is kept at refrigerated temperatures. According to the Center of Disease Control (CDC), *Listeria* is the third leading cause of death from food poisoning in the United States. In healthy individuals, *Listeria* may cause mild gastroenteritis. However, in patients who are immunologically compromised, listeriosis can lead to severe illness, meningitis, stillbirths, and even death². The 21% fatality rate associated with *Listeria* infections in contrast to *Salmonella*'s 0.3% fatality rate, has attracted the attention of the U.S. government in that a “zero tolerance” policy has been implemented, meaning that there is no distinction in recalling foods contaminated at high or low levels³.

Thus far, most *Listeria* research has been conducted under aerobic conditions without taking into account the *in vivo* (physiological) anaerobic conditions of the human gut. Furthermore, there is a lack of knowledge in how fermentation acids produced in the gut affect *Listeria*'s pathogenesis. In order to obtain a better understanding of how to combat *Listeria* infections, a proper simulation of gut chemistry, anaerobic conditions with fermentation acids, is needed.

Recently, the human gut microbiota has become the subject of extensive research, as it is speculated to engage with multiple interactions that affect the host's lifespan, development, immune regulation, and metabolic homeostasis¹. Furthermore, disruption of these microbes has been linked to many serious human diseases: opportunistic infections,

inflammatory diseases, diabetes, malnutrition, and obesity⁴. For instance, *Candida albicans* is usually regulated through its mutualistic relationship with the gut microbiota; however, the ingestion of antibiotics can disrupt the gut environment, and create a set of optimal conditions that allow to fungus to proliferate and become harmful. This in turn leads to the development of thrush or yeast infections, a diagnosis that 75% of women will experience at least once in their lifetime⁵. Among this diagnosis, there are a countless number of other interactions involving the gut microbiota that can influence the health, and overall wellbeing of individuals.

One integral component of the human gut microbiome is the production of short-chain fatty acids (SCFAs). SCFAs are fatty acids containing six or fewer carbons; they are also the product of fermentation of carbohydrates as a result of human bacteria fermentation². The most common SCFAs are acetate, propionate, and butyrate⁶. Several studies have explored the link between diet and the production of SCFAs in our gut, stating that butyrate produced in the colon may inhibit macrophages and contribute to immune responses through the regulation of T lymphocyte production³. Taken together, conditions in the human gut, particularly levels of SCFAs, play an integral role in regulating human health. Thus, in order to understand *Listeria's* biological relevance in the body, experiments must be performed that mimic *in vivo* conditions, as it is unknown how the presence of physiologically relevant fermentation acids interferes with the carbon metabolic pathway.

Therefore, my research objectives include protocols that identify how *Listeria* alters its carbon metabolism in response to different short chain fatty acids (SCFAs) present in the human intestines. It is important to note that *Listeria* can grow by using

glucose as a carbon source under both aerobic and anaerobic conditions, producing acetoin and lactic acid as main byproducts, respectively. Thus, my research objectives are three fold:

- (1) Quantify the production of acetoin in the presence and absence of SCFAs
- (2) Examine Lactate dehydrogenase (LDH) activity in aerobic or anaerobic conditions with or without SCFAs
- (3) Investigate the role of the electron transport chain in the aerobic/anaerobic regulation of LDH activity

Materials and Methods

I. Bacterial Strains and Culture Conditions

Listeria monocytogenes strain 10403S was used in this study. *Listeria* were grown overnight from single colonies in filter-sterilized brain heart infusion (BHI) media at 37 degree Celsius with shaking at 250 rpm. Bacteria were harvested from the overnight cultures in test tubes, each containing 100 microliters of overnight cultures. They were centrifuged for three minutes at 10,000 rpm. The resulting bacterial pellets were washed in 750 microliters of distilled water followed by a second centrifugation. After removing the supernatant, the bacterial pellets were resuspended in solutions listed in Table 1 with each condition performed in triplicates. After 30 minutes of incubation with shaking at 37 degrees Celsius, bacteria were pelleted by centrifugation for three minutes at 10,000 rpm. The resulting supernatant was removed for acetoin analysis while the pellet was used for LDH activity analysis.

	Propionate	Glucose
1	No supplementation	No supplementation
2	25 mM	No supplementation
3	50 mM	No supplementation
4	100 mM	No supplementation
5	25 mM	25 mM
6	No supplementation	25 mM

Table 1: Different experimental conditions used for acetoin and optical density measurements. Results are shown in **FIG 2**.

II. Measurement of Acetoin Concentrations

Acetoin production in the supernatant of overnight *Listeria* cultures was measured through the use of acetoin assay analysis. After the experimental set up, 100 μL of supernatant was placed into a sterile microcentrifuge tube. After that, 50 μL of 5% creatine monohydrate, 100 μL of 1-Naphthol in 95% EtOH, and 100 μL of 40% KOH were added to the experimental conditions. Between each addition, the samples were centrifuged. Ultimately, the samples were incubated for 20 minutes at room temperature. Next, 200 μL of the supernatant was plated into a 96-well flat-bottom plate and the absorbance was read at 560 nm. Based on a standard curve, the concentration of acetoin in the culture supernatant was calculated. Results are shown in (FIG 2, 4).

III. ^{13}C -NMR

Using isotope labeling with ^{13}C NMR, the research objective involves developing appropriate methods for metabolite extraction and identification to follow *Listeria* degradation of ^{13}C -labeled glucose. ^{13}C -glucose was added to follow the formation of ^{13}C metabolites in the central metabolism of *Listeria* grown aerobically or anaerobically with or without the presence of fermentation acids. Because *Listeria* has a sequenced and annotated genome, it is possible to track the possible glucose-derived metabolites.

IV. LDH Cytotoxicity Assay

Listeria cells were incubated overnight in the presence and absence of oxygen. Sonication was used for 30 seconds in 10-second intervals to lyse the cells and subsequently release LDH (Lactate dehydrogenase). Between each interval, the *Listeria*

cells were kept on ice for 30 seconds. The released LDH was transferred to a 96 well plate and an LDH Cytotoxicity Assay was performed. Absorbances were measured at 490 nm and 680nm, and a standard curve was used to analyze the data in Microsoft Excel.

V. M1 and M2 Mixtures

M1 and M2 are physiologically relevant conditions that mimic our human gut⁷. Acetoin and LDH measurements were taken following the inoculation of the cultures in aerobic and anaerobic conditions. Both experiments were normalized using OD measurements and Microsoft excel analysis.

	M1	M2
Acetate	25.5 mM	110 mM
Propionate	2.25 mM	70 mM
Butyrate	2.25 mM	20 mM

Table 2: Concentrations of SCFAs in solution.

VI. Alterations in Electron Transport Chain Activity

Two different approaches were taken to alter electron transport chain (ETC) activity in *Listeria*. First, mutants lacking the *menB* gene, which encodes a protein involved in the synthesis of menaquinone, were used to determine the consequences of the lack of ETC activity. Second, exogenous fumarate was added to overnight culture as an alternative electron acceptor to stimulate anaerobic ETC activity².

Results

I. Acetoin Production in the Presence of Propionate

Measurement of acetoin concentrations

Listeria central metabolism leads to the production of acetoin under aerobic conditions and lactic acid mainly under anaerobic conditions (**FIG 1**). To determine how propionate alters carbon metabolism, we performed a cell suspension assay where aerobically grown bacteria were harvested, concentrated, and resuspended in fresh media supplemented with propionate and/or glucose. After 30 minutes of incubation, the concentration of acetoin was measured to assess whether propionate alters acetoin production. A total of six different conditions were included (**TABLE 1**). Our results showed that *Listeria* supplemented with propionate produced increased levels of acetoin in a dose-dependent manner. This increase in acetoin production was not a result of stimulating glucose oxidation because the addition of glucose to propionate-containing samples did not result in increased acetoin levels (**FIG 2**). This illustrates that *Listeria* is capable of modifying its central carbon metabolism to produce more acetoin in response to propionate, likely by incorporating propionate into the carbon metabolism (**FIG 1**).

The Citric Acid Cycle: Pyruvate formation from Glucose

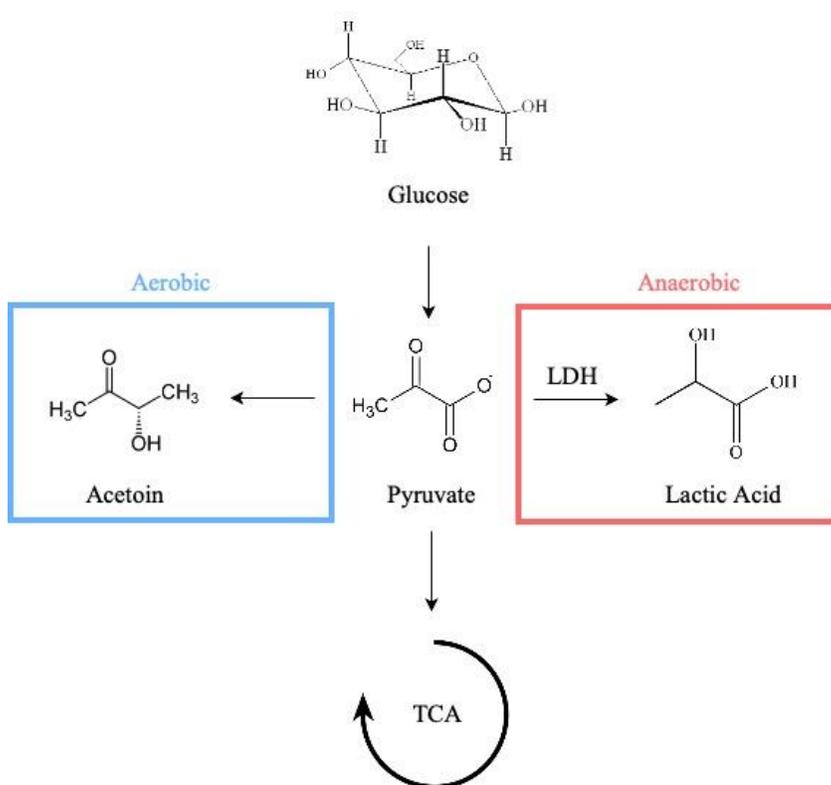


Figure 1. The various fates of glucose under aerobic and anaerobic conditions; LDH denotes Lactate Dehydrogenase, an enzyme that catalyzes the reversible conversion of Pyruvate to Lactic acid. LDH and Acetoin assays were used as a proxy to measure bacterial metabolism anaerobically and aerobically, respectively.

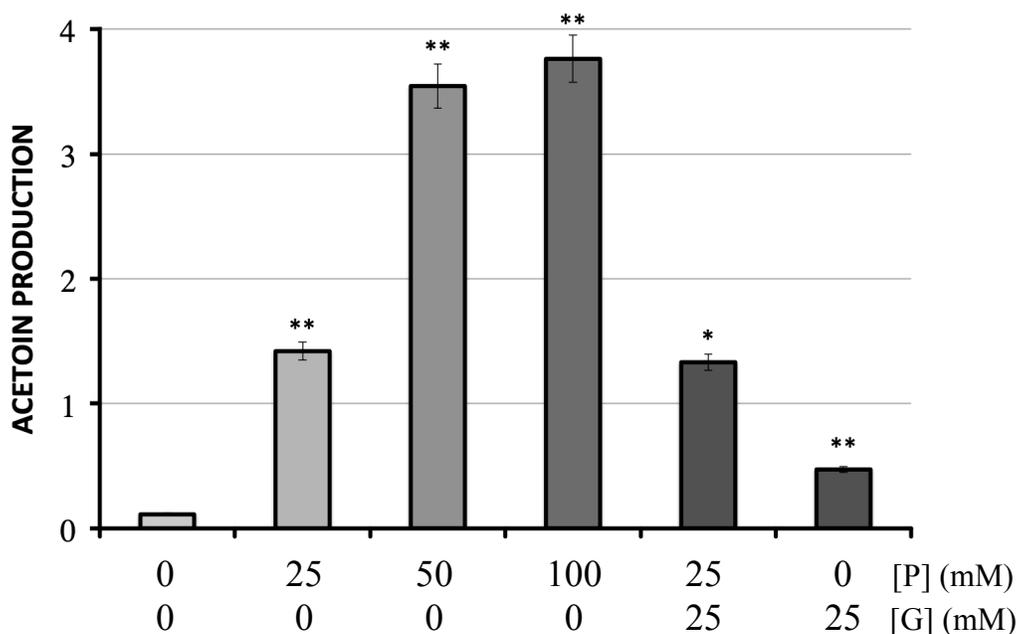


Figure 2. Propionate supplementation results in increased acetoin production. P, propionate; G, glucose; “***”, $0.001 < p < 0.01$; “**”, $0.01 < p < 0.05$.

¹³C- NMR

If propionate is directly used for acetoin production, we predicted that the use of ¹³C-NMR would show ¹³C metabolites consistent with our hypothesis. Using the ¹³Carbon isotope, this experimental method can allow for the identification of any carbon compound, and the tracing of any carbon derived metabolite. In an NMR analysis the number of signals corresponds to the number of carbons, the presence of signal splitting denotes the number of Hydrogen atoms, and the chemical shifts indicate the hybridization of the molecule. Initially our trial involved a standard NMR analysis of the glucose C2 marker shown in (FIG 3). Thus far, preliminary results have not yet revealed how *Listeria's* carbon metabolism is altered by SCFAs and are under current investigation.

Further experimentation would allow us to compare *Listeria* cultures in the presence of SCFAs with or without oxygen.

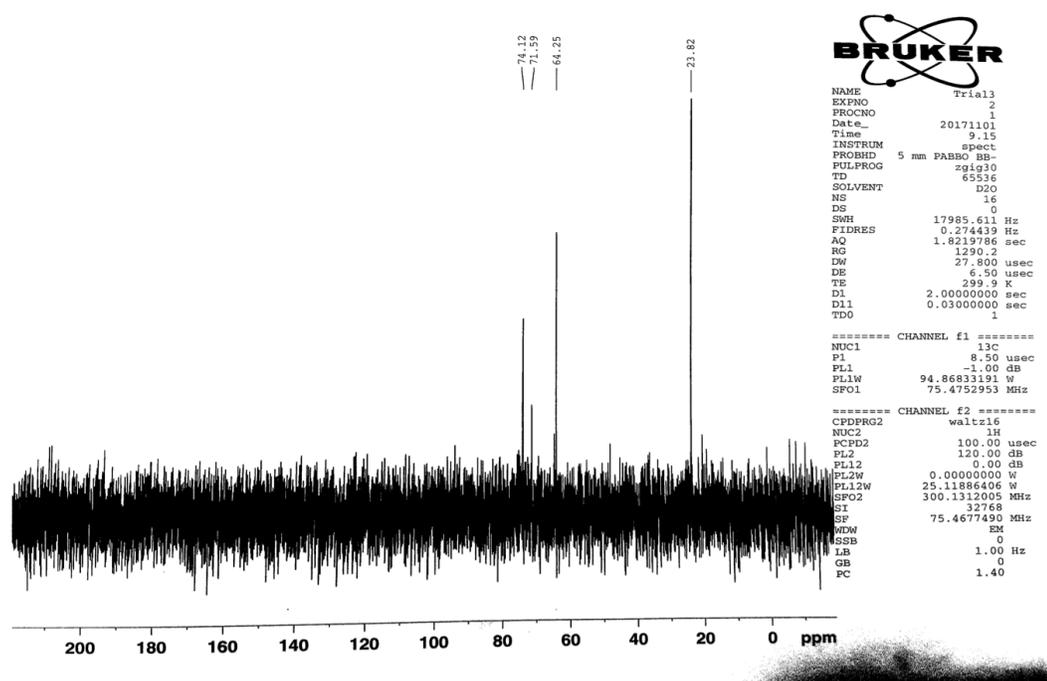


Figure 3. ^{13}C -NMR of glucose stock solution

II. *Listeria's* Metabolism in the Presence of Biologically Relevant SCFAs

Acetoin Measurements

Because the human gut comprises of a mixture of Short Chain Fatty Acids, not simply propionate alone, experiments were designed to better mimic the conditions *Listeria* would encounter *in vivo*. Two mixtures, designated as M1 and M2, were comprised of varying concentrations of propionate, acetate, and butyrate with a greater concentration of all three found in M2 (**TABLE 2**). The results under aerobic conditions indicated that increasing Short Chain Fatty Acid concentration increased acetoin production. Additionally, acetoin production leveled off when supplementation of M2 relative to M1. This observation is not noted under anaerobic conditions, as acetoin production drastically increased with the addition of the M2 mixture. In previous experiments, propionate alone increased acetoin production in a dose dependent manner (**FIG 2**).

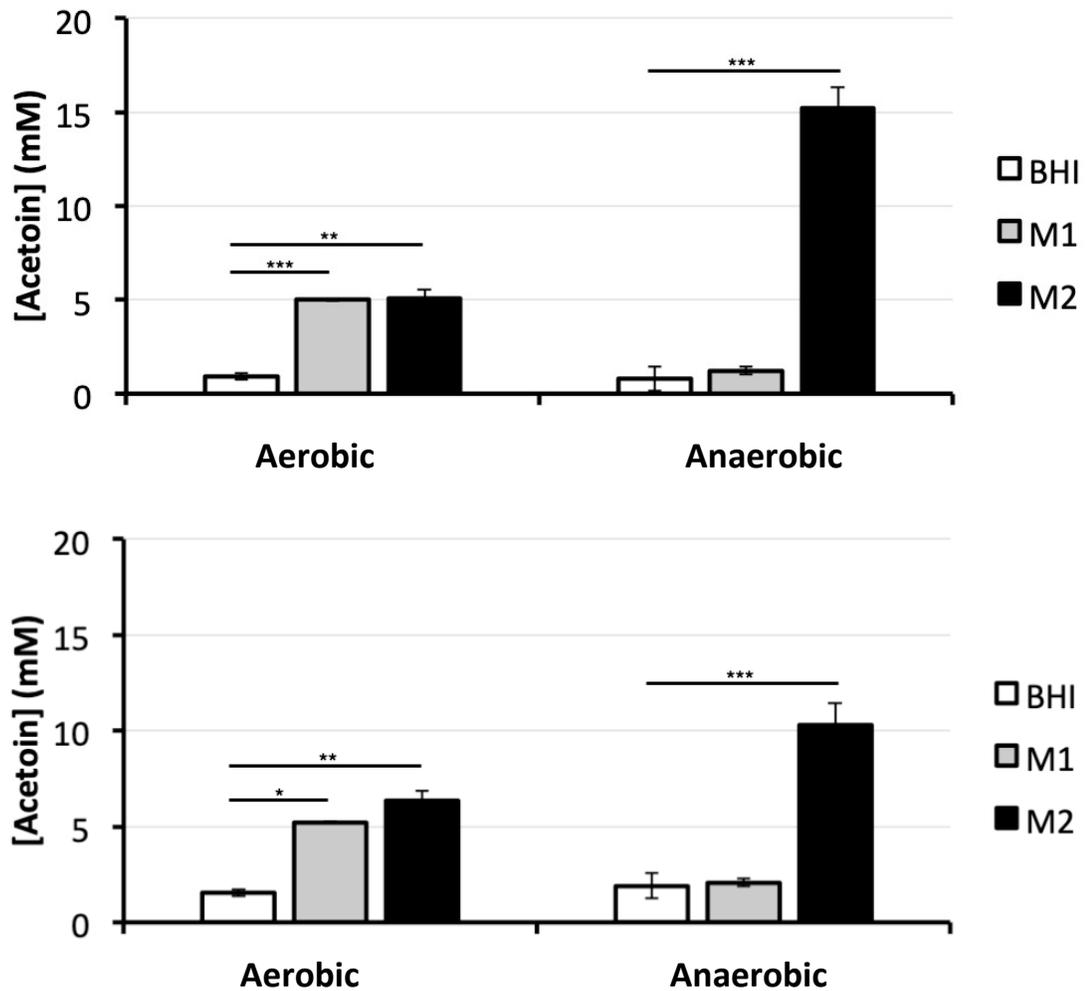


Figure 4. Supplementation of SCFAs increased acetoin production under aerobic and anaerobic conditions, and a larger increase was seen anaerobically. This is consistent across both trials, trial 1 (top) and trial 2 (bottom). “***”, $p < 0.001$ “**”, $0.001 < p < 0.01$; “*”, $0.01 < p < 0.05$.

LDH% Activity

Scientists have often investigated the link between lactate utilization and pathogenicity. Usually, species utilize different mechanisms that regulate LDH activity: genes, enzymes, and kinases⁸. *E. coli* in particular uses an acidic environment to upregulate the genes responsible for adjusting LDH activity⁹. To test whether or not this observation is noted in *Listeria* cultures, an LDH assay was used as a proxy for quantifying LDH activity and OD measurements were taken to normalize the results.

Our results show that there was no change in LDH activity under aerobic conditions; this observation was consistent with our hypothesis, as LDH is normally activated under anaerobic conditions. Anaerobically, the more concentrated SCFA mixture (M2) downregulated LDH activity, and the results were consistent across both trials (**FIG 5**).

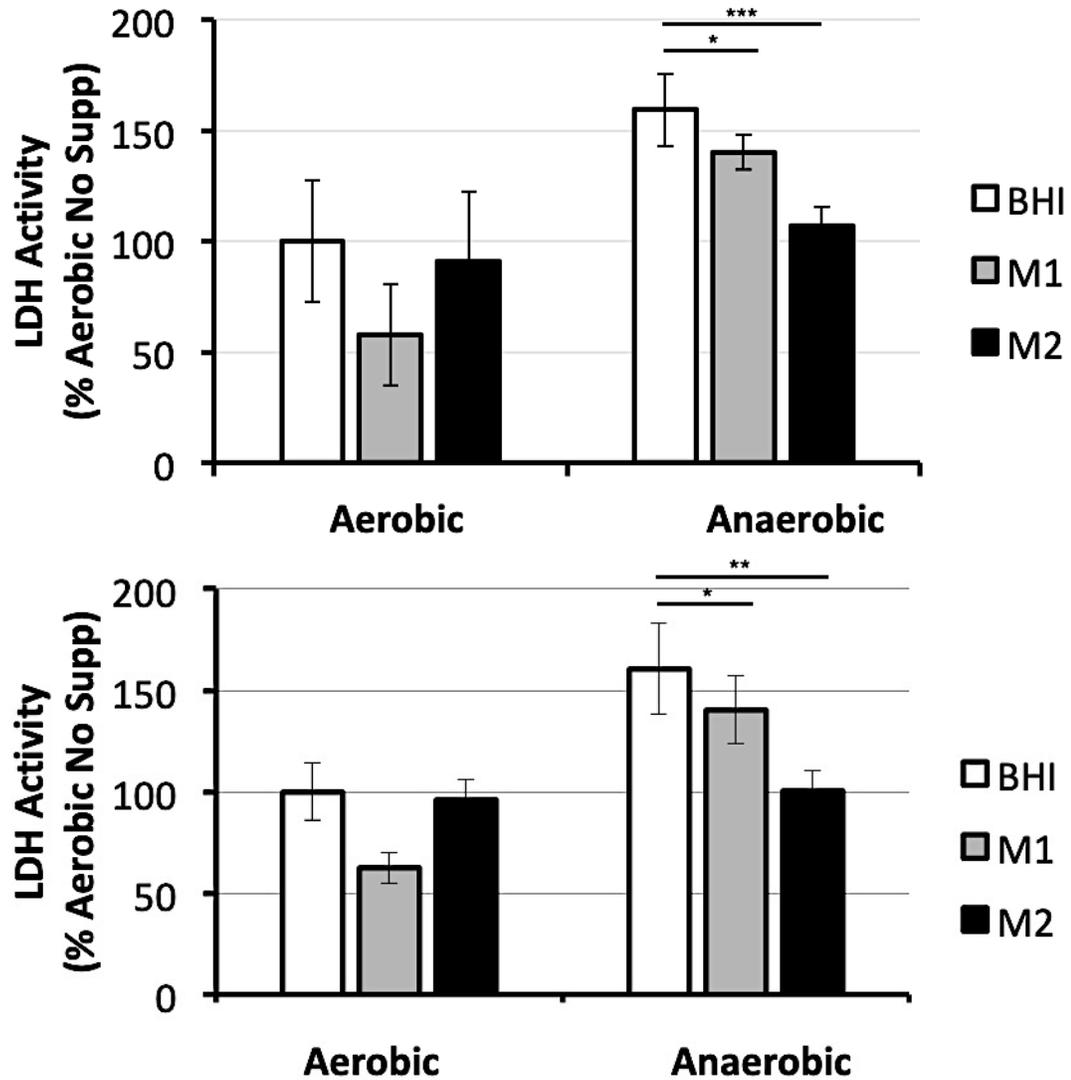


Figure 5. LDH activity decreased with increasing concentrations of SCFA mixtures under anaerobic but not aerobic conditions. This is consistent across both trials, trial 1 (top) and trial 2 (bottom). “***”, $0.001 < p < 0.01$; “*”, $0.01 < p < 0.05$.

pH Measurements

Anaerobic conditions lowered the pH overall across all trials. Furthermore, the pH decreased with decreased concentrations of SCFAs.

III. Alterations in Electron Transport Chain Activity

As mentioned previously, LDH is an enzyme that is most often utilized under anaerobic conditions. The main deviations in aerobic respiration and anaerobic fermentation are seen in the aerobic use of the electron transport chain (ETC). For instance, under aerobic conditions, oxygen is available as the final electron acceptor, which ultimately powers ATP-Synthase through the use of proton motor force. Anaerobically, an alternate electron acceptor is used, such as NO_3 , as observed with the bacterium *E. coli*¹⁰. In order to better understand the mechanism *Listeria* uses in switching from aerobic to anaerobic respiration, $\Delta menB$ strains were used, as they cannot synthesize menaquinone, an important component of the Electron Transport Chain (ETC).

Menaquinones are known to contribute in the switch between aerobic respiration and anaerobic lactic acid fermentation¹¹. Thus, $\Delta menB$ mutants were used in conjunction with fumarate supplementation to alter ETC activity in the presence and absence of oxygen. The results showed that WT *Listeria* increased LDH activity under anaerobic conditions, and $\Delta menB$ mutants exhibited no change in LDH activity independent of oxidative conditions, this indicates that the absence of menaquinones inhibits anaerobic LDH activity in *L. monocytogenes*.

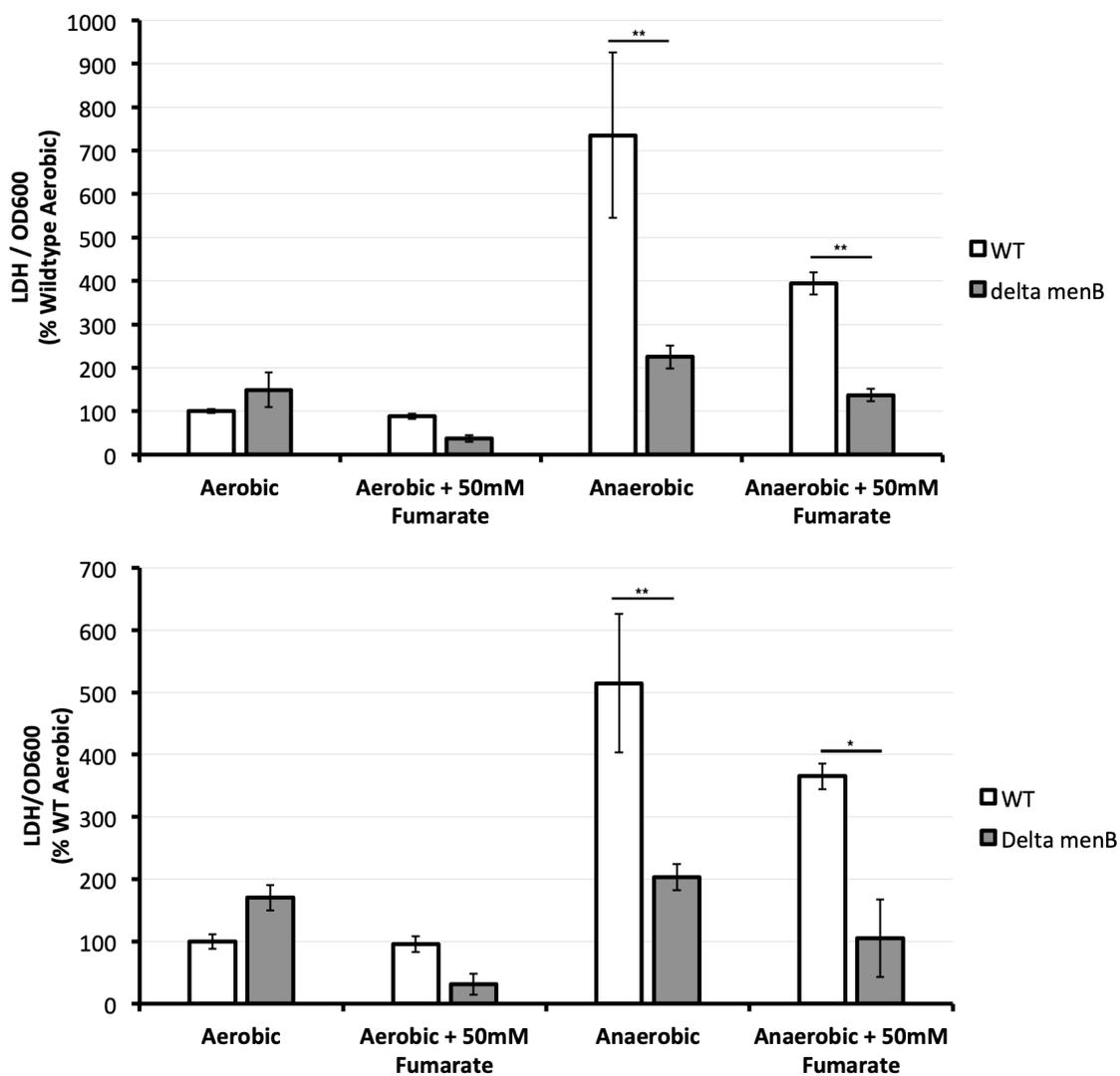


Figure 6. WT LDH activity is higher in anaerobic than aerobic conditions. The addition of fumarate as an alternative electron acceptor did not change aerobic LDH activity but decreased anaerobic LDH activity. $\Delta menB$ mutant exhibited a significant decreased LDH activity under anaerobic conditions compared to WT. This is consistent across trials, trial 1 (top) and trial 2 (bottom). “***”, $0.001 < p < 0.01$; “*”, $0.01 < p < 0.05$.

Discussion

Listeria encounters a wide range of intestinal conditions in the human gut, notably SCFAs and anoxic conditions. In order to gain a better understanding of *Listeria*'s carbon metabolism, the experiments performed intended to mimic the conditions of the human gut.

Our experiments demonstrated that *L. monocytogenes* cultures supplemented with propionate alone increased acetoin production in a dose dependent manner (**FIG 2**), while *Listeria* supplemented with M2 showed a smaller increase in acetoin production in comparison to M1 (**FIG 4**). This observation could indicate enzymatic saturation in acetoin production, a phenomenon seen in other experiments performed with *E. coli*⁹. In other words, the increase in SCFA concentration could have lead to the occupation of all the binding sites available for the enzymes that catalyze the production of acetoin from pyruvate, thus limiting its production. Alternatively, the concentrated SCFA mixture could contain allosteric inhibitors such as acetate and butyrate, which also limit the production of acetoin through manipulation of the enzyme at the allosteric site. In order to test this hypothesis, future experimentation would be needed to examine acetoin production in the presence butyrate and acetate alone.

It is important to note that these explanations do not take into account the large increase in acetoin production seen anaerobically with *Listeria* supplemented with M2. This observation could point to *Listeria*'s ability to switch acetoin-producing enzymes in the presence and absence of oxygen. Thus, there could be two different points of saturation in the enzymes responsible for acetoin production. Furthermore, under

anaerobic conditions SCFAs could upregulate the specific genes responsible for producing acetoin, so the observed effect could be the product of generic manipulation. Evidence of *Listeria's* ability to genetically regulate acetoin production has been cited in alternative experiments as well¹².

As mentioned previously, *E. coli* bacteria have the ability to upregulate LDH activity under anaerobic conditions, as the lower pH activates genes responsible for upregulating the enzyme⁹. The results shown in (FIG 5) are consistent with the pH measurements observed, where the pH increased with increasing SCFA concentration. The inhibitory effects of SCFAs on LDH activity in acidic conditions could be explained with Le Chatelier's principle. In the anaerobic reaction denoted in (FIG 1), the increased proton concentration could drive the reaction to favor lactic acid production, as the differences in structure between Lactic Acid and Pyruvate involve the addition of a proton; therefore, an acidic environment could facilitate the reaction. Because the conversion of Pyruvate to Lactic acid is reversible, this would give reason as to why the reaction was driven in the direction that produces Lactic acid, and ultimately lowered the pH.

Alternatively, perturbations in LDH activity in the presence of increased SCFA concentration could point to saturation in the enzyme responsible in the regulation of LDH activity. If all the active sites in the enzyme are occupied, this could limit or level off enzymatic activity. In order to better understand this observation, different concentrations of SCFAs would have to be tested in order to determine whether or not a saturation point exists. Furthermore, the SCFAs may bind to alternative sites on the enzyme other than the active site, and limit its activity. This is known as allosteric

inhibition, and could explain why a decrease in LDH activity is observed with increased SCFA concentrations (**FIG 5**).

Future work in this area is needed to confirm my preliminary findings. The use of carbon tracking in ^{13}C -NMR, could narrow down the pathways involved in *Listeria*'s glucose metabolism. Additionally, experimental conditions consisting of acetate, propionate, and butyrate alone would be needed for further comparison in acetoin production. Taken together, the results thus far indicate that *Listeria monocytogenes* is capable of modifying its carbon metabolism in acidic conditions, in the presence and absence of oxygen, and in the presence and absence of SCFAs.

Listeria monocytogenes is an intracellular pathogen that is capable of replicating efficiently in the cytosol of many eukaryotic cell types, and because of this it can easily spread to neighboring cells in dangerous levels¹³. It is important to note that there is still little information concerning the metabolic capacities and the metabolic adaptation processes that enable these bacteria to efficiently replicate in the cytosol of their host cells. This research can help bridge the gaps in knowledge around the metabolic capabilities of *L. monocytogenes*. Moreover, this research contains medical implications as a better understanding of *Listeria*'s carbon metabolism can help pharmaceuticals develop more effective antibiotics in response to the changing composition of the bacteria *in vivo*.

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