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Influence of External pH and Organic Acids on Internal pH and Acid Anion Accumulation in Listeria monocytogenes and Escherichia coli

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INFLUENCE OF EXTERNAL PH AND ORGANIC ACIDS ON INTERNAL PH AND ACID ANION ACCUMULATION IN *LISTERIA MONOCYTOGENES* *and ESCHERICHIA COLI*

by

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ABSTRACT

Influence of External pH and External Organic Acids on Internal pH and Acid Anion Accumulation in *Listeria monocytogenes* and *Escherichia coli*

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Utah State University, 2021

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Organic acids are widely employed in the food industry to control growth of microbial pathogens including *Listeria monocytogenes* and *Escherichia coli*. There is substantial evidence that intracellular accumulation of acid anions is a major inhibitor to cell growth, and that many bacteria may combat anion accumulation by lowering their intracellular pH (pH_i). In this study, we followed the accumulation of acid anion into the cell pellet and parallel changes in pH_i in two human pathogenic strains of *L. monocytogenes* (N1-227 and R2-499) and in *E. coli* O157:H7 after exposure to sub-bacteriostatic levels of lactic and acetic acids at mildly acidic pH 6.

Log phase bacteria underwent 60 min habituation to treatments including a baseline control of media at pH 7.4, acid control (added HCl to pH 6), or to 4.75 mM added lactic or acetic acids plus their C^{14} tracers. Tritiated water was also included as a tracer. Cells were harvested by centrifugation through a layer of bromododecane, and radioactivity in pellet and supernatant measured using a scintillation counter. Relative accumulation of anion was calculated as the ratio of

C^{14}/H^3 in the pellet divided by the ratio of C^{14}/H^3 in the supernatant. Parallel experiments were performed without tracers, and pH_i measured using a pH-sensitive fluorescent dye. It is noteworthy that our measures of anion accumulation and pH_i were independent rather than being confounded as when employing the common practice of calculating intracellular concentration of anions based on difference in internal and external pH, or conversely calculating pH_i based on intracellular accumulation of acid anion.

The test strains of *Listeria* and *E. coli* accumulated up to 3 times more acetate anion into the cell pellet compared to the supernatant. *Listeria* accumulated less lactate than acetate anion, and *E. coli* did not significantly accumulate lactate. The values for anion accumulation into the pellet were much less than expected for intracellular accumulation based on previous reports and predicted by the measured difference in internal and external pH. Hence, C^{14}-inulin (a sugar excluded from the cell interior) was employed to determine the fractional volume of pellet not available to inulin (i.e., intracellular volume) in comparison to volume of pellet available to water. That fractional volume was calculated as 0.19 for *E. coli*, and it follows that anion accumulation into the intracellular space is minimally 5X greater than measured for the entire pellet. A fractional volume of intracellular space was not measurable for the *L. monocytogenes* strains suggesting it was below the detection limit of our procedure.

Exposure of *E. coli* to acid control (pH 6) or to added organic acids did not induce a change in pH_i from the baseline control (pH 7.4). In contrast, exposure of *Listeria* to the organic acids induced lowered pH_i as compared to baseline in both

strains while the impact of the acid control varied within strain. The acid control induced a lowered pH_i as compared to baseline in only strain R2-499 with exposure to acetic acid inducing an additional lowering of pH_i.

In summary, *Listeria* and *E. coli* were exposed to non-bacteriostatic levels of external organic acids at mildly acidic pH 6. *Listeria* accumulated more acetate than lactate while mounting a defense against anion accumulation that included lowering its pH_i , while *E. coli* accumulated only acetate and apparently made use of combat mechanisms other than lowering pH_i not explored in this study. The methodology employed in these studies was based on independent measures of pH_i and intracellular anion accumulation, and the resulting data brings into question the common, but confounding, practice of using intracellular anion accumulation as a measure of pH_i, and vice versa.

(77 pages)

PUBLIC ABSTRACT

Influence of External pH and External Organic Acids on Internal pH and Acid Anion Accumulation in *Listeria monocytogenes* and *Escherichia coli*

Savannah R. Branson

Listeria monocytogenes and *Escherichia coli* are both among the most common microbial pathogens that cause foodborne illnesses and death. They both are capable of growing over a wide range of conditions. Organic acids are widely employed in the food industry to control growth of these pathogens to help prevent foodborne illnesses. There is substantial evidence that intracellular accumulation of organic acid anions is a major inhibitor to cell growth, and that many bacteria may combat anion accumulation by lowering their intracellular pH (pH_i). In this study, we followed the accumulation of acid anion into the cell pellet and parallel changes in pH_i in two human pathogenic strains of *L. monocytogenes* (N1-227 and R2-499) and in *E. coli* O157:H7 after exposure to sub-bacteriostatic levels of lactic and acetic acids at mildly acidic pH 6.

Cells were exposed to two controls, or to acetic or lactic acid. The accumulation of anions into the cell pellet and pH_i was measured after 60 minutes habituation in these treatments. It is noteworthy that our measures of anion

accumulation and pHi were independent rather than being confounded as when employing the common practice of calculating intracellular concentration of anions based on difference in internal and external pH, or conversely calculating pH_i based on intracellular accumulation of acid anion.

All three bacteria accumulated up to 3 times more acetate anion into the cell pellet compared to their external environment. *L. monocytogenes* accumulated less lactate than acetate anion, and *E. coli* did not significantly accumulate lactate. The values for anion accumulation into the pellet were much less than expected based on previous reports and predicted by the measured difference in internal and external pH. Exposure of *E. coli* to organic acids did not induce a change in pH_i from its baseline pH_i. In contrast, exposure of *L. monocytogenes* N1-227 and R2-499 to the organic acids induced lowered pH_i as compared to baseline in both strains.

In summary, *Listeria* and *E. coli* were exposed to non-bacteriostatic levels of external organic acids at mildly acidic pH 6. *Listeria* accumulated more acetate than lactate while mounting a defense against anion accumulation that included lowering its pH_i, while *E. coli* accumulated only acetate and apparently made use of combat mechanisms other than lowering pH_i not explored in this study. The methodology employed in these studies was based on independent measures of pH_i and intracellular anion accumulation, and the resulting data brings into question the common, but confounding, practice of using intracellular anion accumulation as a measure of pH_i, and vice versa.

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LIST OF ABBREVIATIONS

AC- acetic acid

LA- lactic acid

HCl- hydrochloric acid

BC- baseline control

NaOH- sodium hydroxide

KH_2PO_4 -potassium phosphate

STEC- shiga toxin producing *Escherichia coli*

EHEC- enterohemorrhagic *Escherichia coli*

TSB- tryptic soy broth

TSA- tryptic soy agar

CFSE- 5(6)-carboxyfluorescein diacetate N-succinimidyl ester, a membrane permeant fluorescein-based dye

LT_{50}- the lethal time until 50% population mortality

DPM- disintegrations per minute

CHAPTER I.
LITERATURE REVIEW

Listeria monocytogenes

Listeria monocytogenes is a gram positive, non-spore forming, facultative anaerobic bacteria (20, 72). *L. monocytogenes* is environmentally ubiquitous. It lives saprophytically in the soil and on plants, and it can persist in the intestines of animals. Its survival can be influenced by temperature, the humidity of the soil, and the motility of the bacterium (54). *Listeria* is capable of transitioning into a pathogen when it encounters stressors, such as when it is ingested (23). These stressors include reduced oxygen, bile, endogenous microbiota and low pH (23, 48, 63). Because of its ubiquitous presence, *L. monocytogenes* has the potential to contaminate food processing facilities and food products (40).

L. monocytogenes also has growth and survival properties that make it difficult for the food industry to control its growth. It is psychotropic and can grow at temperatures as low as 0°C and as high as 50°C. It grows within a wide pH range, from 4.6-9.5. It can grow at water activity less than 0.93 and in a concentration of salt up to 10% (46, 44). It is capable of transport and cross contamination through water, air, animals, and humans. It can also survive and persist in biofilms (20, 25, 64). *Listeria* can survive in hostile environments within a food processing facility including equipment, drains, and wet floors in refrigerated rooms of processing facilities (3).

Listeriosis infection

Listeriosis is the infection in humans caused by the consumption of food contaminated with *L. monocytogenes.* In the United States, the incidence of listeriosis is relatively low-only 2.4 cases per million in the population (33). The CDC reported 126 cases of listeriosis in 2018, 121 of which resulted in hospitalization (96%) and 26 resulted in death (21%) (66). Thirteen different serotypes of *L. monocytogenes* have been described, and serotypes 1/2a, 1/2b, and 4b have been associated with most of the foodborne infection cases (22, 72). They cause more than 95% of clinical cases of listeriosis (59).

This thesis in particular studied a 1/2a strain and a 4b strain. There are four evolutionary serovar-related lineages (I-IV) identified for *Listeria*. 4b is in lineage one which is associated with large outbreaks and sporadic human cases. 1/2a is in lineage two which includes the majority of isolates from food and the environment (50). Serotype 4b is responsible for 35-50% of sporadic human cases of listeriosis and for all major foodborne outbreaks of the illness in North America and Europe since the 1980s (59).

Food sources that may harbor *Listeria* include ready to eat deli meats and hot dogs, raw meat and poultry, raw milk, raw vegetables, soft cheeses (particularly those made with non-pasteurized milk), raw and smoked seafood, and ice cream. Potential sources of contamination include food workers, raw materials, and food processing environments. However, the most frequent source of contamination is food contact surfaces (22, 57).

L. monocytogenes can colonize the intestine causing diarrhea and fever in healthy adults, similar to other foodborne illnesses. Because of its similarity to other foodborne illnesses and relative mildness in healthy adults, it often goes undiagnosed. However, listeriosis can be much more dangerous to a certain portion of the population due to its propensity to escape the gut and invade other cells, including the nervous system. Pregnant women, older adults, and people with weakened immune systems are at higher risk of contracting invasive listeriosis (14). A listeriosis infection during pregnancy can result in abortion or early birth. In newborn children the infection can result in conjunctivitis, late-onset meningitis, and pneumonia. An invasive listeriosis infection in the elderly and immunocompromised causes nervous system infection, sepsis, and endocarditis (12).

Escherichia coli

Escherichia coli is a gram negative, rod-shaped, facultative anaerobic bacterium. Most strains are harmless and coexist with humans and animals in the intestinal tract. However, some strains are pathogenic (31). This research focuses entirely on *E. coli* O157:H7, an enterohemorrhagic (EHEC) strain of *E. coli*. *E. coli* O157:H7 strains are among the most common causes of *E. coli* foodborne illness owing to the Shiga toxins produced by the strain (13). EHEC strains produce Shiga-like toxins (38).

E. coli O157:H7 is a hardy organism. It can survive in food, soil, and water. The pathogen can survive in cattle water troughs contaminated with feces for longer than eight months and persist in the environment of farms for periods of several

years (29). Most human infections are caused by consuming water or food contaminated with bovine feces (1). The pathogen is able to survive and adapt to varying pH, osmolarity, and temperatures in its environment (31).

E. coli O157:H7 infection

E. coli O157:H7 is similar to *Listeria* in that it is tied to fewer cases of foodborne illness than other pathogens like *Salmonella* or *Campylobacter* though it results in higher hospitalization and death rates than those pathogens. Five pathogens are responsible for 90% of food related deaths, and *E. coli* O157:H7 is one of them. The full list, ordered from highest to lowest occurrence, includes *Salmonella* (31%), *Listeria* (28%), *Toxoplasma* (21%), Norwalk-like viruses (7%), *Campylobacter* (5%), and *E. coli* O157:H7 (3%) (37).

Symptoms start three to four days after ingestion of food or liquid that contains the bacteria and subsequent colonization of the intestine (15). Raw meat, raw milk, vegetables, and fruits have been associated with outbreaks. The infectious dose is very low- less than a thousand bacteria can cause illness (58). Symptoms include stomach cramps, diarrhea, bloody diarrhea, and vomiting. Some develop a low-grade fever. Most people feel better within 5-7 days of onset (15). However, around 5-10% of people develop hemolytic uremic syndrome, a life-threatening condition that causes the kidneys to stop working and can result in serious problems. Hemolytic uremic syndrome occurs when toxins from the infection cross the intestine into the bloodstream (67). Most people recover in a few weeks, but for others this condition can cause permanent damage or death (15).

Organic acids

Organic acids are generally recognized as safe (GRAS) by the FDA, and they are widely utilized as acidulants, pH adjusters, flavoring agents, and antioxidants (51, 52, 53), They are also utilized as food preservatives due to their antimicrobial activity. Acetic acid is commonly used to inhibit yeasts and bacteria and its primary food use as a food additive includes include both dairy products and meat product, while lactic acid is primarily used to inhibit bacteria in meat and fermented foods (34).

The USDA requires beef slaughter facilities to have one carcass intervention treatment due to the likelihood of *E. coli* O157:H7 contamination (2). Both acetic and lactic acids are also used to decontaminate meat surfaces of pathogens such as *Salmonella*, *Escherichia coli*, and *Listeria monocytogenes* (4). The USDA does verification testing on ready to eat products based on risk of contamination with *L. monocytogenes*, and formulating products with lactic and/or acetic acids lowers the testing requirements owing to their recognized anti-Listerial activity (68).

Organic acids as antimicrobials

In their protonated form organic acids are charge neutral, and many low molecular weight acids are thus membrane soluble and able to enter the cytoplasm freely by diffusion. *L. monocytogenes* and *E. coli* maintain a pH_i close to neutral, so exposure to these acids results in immediate acid dissociation and the release of protons and anions inside the cell (10) where they may exert antimicrobial activity primarily through two mechanisms. The first is that proton release acidifies the cytoplasm, which can result in the uncoupling of energy production and regulation,

and the second is the intracellular accumulation of dissociated acid anion (34). Accumulation of anion will increase osmolarity of the cell, leading to increased turgor pressure (48). If intracellular proton release exceeds the buffering ability of the cytoplasm or the capability of the proton efflux systems, the pH_i will decrease and cellular functions will be inhibited (5). The hypothesized extent of intracellular anion accumulation has been described by Carpenter and Broadbent (10) to be driven by the difference in external and internal pH, and this is discussed in greater detail in the preliminary studies section of this thesis.

The work of Wang et al. (53) suggests that the antimicrobial effect of lactic acid is the result of morphological and physiological changes in bacterial cells. Their work found that exposure to 0.5% lactic acid (approximately 67mM) for two hours completely inactivated *E. coli*, *Salmonella enteritidis* and *L. monocytogenes*, and that exposure to lactic acid resulted in leakage of proteins from all three pathogens. The content and activity of bacterial proteins were disrupted. Analysis of those proteins confirmed that the lactic acid disrupted the cytoplasmic membrane, and damage to the cytoplasmic membrane was observed in TEM images (53). Similarly, it was found that the fatty acid composition of the membrane of *L. monocytogenes* was altered by exposure to organic acids (61).

There are many ways that *Listeria* may combat the effects of organic acids. *Listeria* may change its membrane composition to adapt (61, 76), lower its pH_i (69) or alter its export systems (76). The extent to which these mechanisms occur in the strains used in this study are not known, especially as it pertains to mildly acidic

environments and levels of external organic acid that are less than bacteriostatic thereby allowing the cell to actively adapt to the imposed stress.

Anion Accumulation

Other studies have looked at measuring the accumulation of organic acid anion when cells are subjected to a weak acid treatment. One study in particular measured anion accumulation into *E. coli* cells to understand the impact of anion accumulation of cellular pools of K^+ and Na^+. To determine acetate accumulation, cells exposed to 8mM of acetate were exposed to C^{14} labeled acetate and H^3 inulin. Inulin is an extracellular marker to help determine the internal cell volume and subsequently internal acetate concentration (42). Inulin is a sugar excluded from the cell interior. The purpose of using inulin is to determine what is "external" to the cell (27). Cells were separated from the supernatant by centrifugation through bromododecane. Internal acetate concentration was calculated from knowledge of the pKa of acetate, the external pH, and the accumulation ratio. A cytoplasmic volume was assumed in the calculation (42). This is the same method used for pH_i determination, except that method utilizes C^{14} labeled benzoic acid (28, 42, 71). The study found that exposure of *E. coli* to 8mM of acetate lowered pH_i from 7.8 to 7 after two minutes of its addition, and cells recovered to a pH_i of 7.4-7.5 after ten minutes (42).

Intracellular pH

Maintaining an optimal intracellular pH (pH_i) is vital for optimal cellular functions, so changes in pH_i can detrimentally affect many of those functions. pH_i can

affect cellular metabolism, intracellular messengers, activation, growth, proliferation, and membrane flow & conductance (41). *L. monocytogenes* is reported to maintain a pH_i = 8 when the external pH ranges from 5 to 9 (8, 45), and invokes several mechanisms to maintain pH_i when the cell is exposed to external acid stress. These mechanisms include systems involving F_0F_1-ATPase (17), arginine and agmatine deiminases (ADI and AgDi, 32), and glutamic acid decarboxylase (GAD, 21). The ADI, AgDI, and GAD systems are involved in resistance to extreme acid conditions. The F_0F_1-ATPase operon is involved in ATR initiation under mild acid stress (7). Habituation of two *Listeria* strains to organic acids significantly increased expression of genes related to the ADI and GAD systems compared to *Listeria* not habituated to organic acids (30), the same two strains are used in the research reported in this thesis.

Similar to *L. monocytogenes*, *E. coli* is reported to maintain a pH_i from 7.2 to 7.8 while growing in medium with pH ranging from 5 to 9 (35). One study found that when external pH was lowered from 7.5 to 5.5, cytoplasmic pH dropped within 20 seconds to a pH ranging from 5.6-6.5. Rapid recovery occurred after 30 seconds to bring pH_i up, and the cells continued to recover slowly over the following five minutes (56). Another study similarly found that *E. coli* cells exposed to M63 growth media at pH 5.5 were able to recover their pH_i to 7.0-7.5 within two minutes of exposure to the lower external pH (35). Weak organic acids in comparison are predicted to have additional antimicrobial activity owing to their internal accumulation (10, 11). Additional studies have found that *E. coli* can survive in extremely acidic environments, pH 2.0 to 2.5 through three different acid resistance

mechanisms. These acid resistance mechanisms are what allow the pathogen to survive passage through stomach acid (11).

Measuring intracellular pH by Fluorescence Staining Technique

Internal pH in bacteria can be followed using a pH-sensitive fluorescence dye (16). One commonly used fluorophore is 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) that is very sensitive to pH excited at its peak wavelength 490 nm but is pH insensitive at 435 nm (47). This property allows for a dual-excitation fluorescent ratio that is sensitive to changes in pH especially around the pKa of fluorescein (6.4). This fluorescent ratio is relatively insensitive to alterations in the focal plane, bleaching, the amount of fluorophore present, cell thickness, and leakage of the dye in that they affect both wavelengths equally (9).

(CFSE) is a membrane permeant fluorescein-based dye. The two acetate chains make the compound permeable to the membrane. Once the dye is inside of the cell, the acetate groups are removed by intracellular esterases. The carboxyfluorescein then exits the cell at a much slower rate, allowing time for CFSE to covalently couple with intracellular amine groups through its succinimidyl moiety. This forms a stable amide bond. They are highly reactive with amines at neutral pH.

Many of the conjugates CFSE makes are short lived molecules or are capable of passing through the plasma membrane. Because of this, a lot of the CFSE taken up by the cells are lost within 24 hours (39). At 37 °C, the intracellular concentrations of CFSE have been observed to diminish by 30-40% within ten minutes after washing (Han and Burgess 2010). As long as enough dye remains in the cell to

produce a measurable signal, pH_i can be measured because it uses a ratio rather than absolute value. There are enough long-living molecules that cells can be measured for their fluorescence for several weeks after labeling if needed (39).

A standard curve needs to be established to measure pH_i. The standard curve relates fluorescence values to pH_i values. pH_i is fixed using ethanol or ionophores (9). A separate curve needs to be created for each strain to account for differences between strains.

Cheng et al. (16) demonstrated that a simple microplate-based fluorescence method could be used to determine the pH_i of *Listeria monocytogenes* cells labeled with CFSE. Those researchers subjected *Listeria* to acid stress and measured the intracellular response over time. They found that at certain pH conditions, organic acids lowered pH_i significantly more than hydrochloric acid. Additionally, the study found that three virulent strains of *L. monocytogenes* were more resistant to the acidic stress than an avirulent strain of *Listeria* tested (16). Another study using this fluorescence method demonstrated that *E. coli* could recover intracellular pH within 2 minutes after exposure to an external medium of 5.5 (35).

Preliminary Studies

Carpenter and Broadbent (10) proposed that the intracellular accumulation of organic acid anions in mildly acidic environments is due to two factors, the pH gradient between the inside and outside of the cell external concentration of acid anion. Their predictive equation was based on an algebraic manipulation of the Henderson-Hasselbach equation.

1. Protonated organic acid freely transverses the cell membrane:

$[HA]_{out} = H[A]_{in}$

2. Once inside the cell, the protonated acid dissociates according to the Henderson-Hasselbach equation.

   $[A^-]_{in} = \text{antilog}(pH_{in}\text{-}pKa) \times [HA]_{in}$

3. Equation 1 substituted into equation 2:

   $[A^-]_{in} = \text{antilog}(pH_{in}\text{-}pKa) \times [HA]_{out}$

4. Protonated acid outside of the cell is also defined by the Henderson-Hasselbach equation.

   $[A^-]_{in} = \text{antilog}(pH_{in}\text{-}pKa) \times ([A^-]out/\text{antilog}(pH_{out}\text{-}pKa))$

5. Using algebra:

   $[A^-]_{in} = [A^-]_{out} \times \text{antilog}(pH_{in}\text{-}pH_{out})$

6. Protonated acid anions are released equally from acid dissociation.

   $[A^-]_{in} = [H^+]_{released} = [A^-]_{out} \times \text{antilog}(pH_{in}\text{-}pH_{out})$

Because *L. monocytogenes* is reported to maintain a relatively constant pH_i when the external pH ranges from 5 to 9 (8, 45), it was hypothesized that the intracellular accumulation of anions is mainly driven by external pH and external anion concentration (10). *E. coli* can also maintain its pH_i so this should hold true for it as well (35). Accumulation of anion within the cells will increase osmolarity and lead to increased turgor pressure. The cell may combat this by excreting the anion itself or excess H^+ (48).

Other researchers have focused on the external protonated acid driving internalization of acid anion. In these studies researchers control pH and either the

concentration of protonated acid or total acid in the growth media, which leaves external anion concentration to vary depending on the pK_a of the acids involved (80, 81, 82). Carpenter and Broadbent assert in their hypothesis paper that external protonated acid is merely a shuttle, not a driving force for intracellular anion accumulation. Their hypothesis paper indicates that a failure to control external anion concentration has confounded results and likely led to misleading conclusions regarding the antimicrobial action of organic acids (10). The following three studies were designed with this in mind.

Zhang et al. (60) recently tested the inducible resistance of *L. monocytogenes* to acid and bile. Acid and bile are both factors related to the bacteria's survival in the GI tract. Strains were grown in TSB at pH 7.4 (baseline control) or at pH 6.0 containing 0 (HCl control) or 4.75 mM organic acids (L-lactic acid, levulinic acid or acetic acid). Cells were harvested at mid-log phase and then challenged in medium acidified with HCl to pH 3 for one hour. Growth of *Listeria monocytogenes* in TSB at pH 6.0 with organic acids induced more than 1 log survival against the acid challenge test compared to the hydrochloric acid control. The results indicated that the exposure of certain strains of *Listeria monocytogenes* to organic acids under mildly acidic conditions can induce acid resistance, and this may increase the virulence by promoting survival in the GI tract. Past research also suggests that the use of organic acids in the food industry may be increasing virulence of *Listeria* (48, 55). The paper hypothesized that the increase in acid resistance after habituation to the organic acids was linked to intracellular accumulation of organic acids (60).

Zhang et. al (61) also studied changes in the fatty acid composition of *L. monocytogenes* when it was habituated to organic acids. Habituation to organic acids increased five of six branched chained fatty acids (BFCAs) and lowered levels of 15 carbon fatty acids. The study suggested that exposure of *Listeria* to organic acids induced resistance to acid through changes in membrane composition.

The third and most recent study looked at the impact of habituation to acetic and lactic acid on virulence, acid resistance, and bile resistance in *Listeria monocytogenes* (30). Habituation to organic acid resulted in differential expression of genes involved with acid and bile resistance genes as well as genes for virulence, membrane transport, cell motility, quorum sensing and carbohydrate and amino acid metabolism. The study supported the hypothesis that habituation to organic acid can induce *in situ* virulence in *Listeria monocytogenes* and gave a comprehensive review of the mechanisms it uses to adapt to organic acids. Li (30) also determined the in vivo virulence of *L. monocytogenes* using the *Galleria mellonella* wax worm model. Virulence was determined by injecting cells into *G. mellonella* larvae. After injection, both the survival of *G. mellonella* and the *L. monocytogenes* growth kinetics in the larvae were evaluated. The lethal time until 50% population mortality (LT_{50}) was used as a measurement for virulence. When *Listeria* was habituated to organic acid, the LT_{50} significantly decreased.

CHAPTER II.

ESCHERICHIA COLI AND *LISTERIA MONOCYTOGENES* ACCUMULATE ACID ANION AND ALTER pH_I IN RESPONSE TO ORGANIC ACIDS

Abstract

Organic acids are widely employed in the food industry to control growth of microbial pathogens including *Listeria monocytogenes* and *Escherichia coli*. There is substantial evidence that intracellular accumulation of acid anions is a major inhibitor to cell growth, and that many bacteria may combat anion accumulation by lowering their intracellular pH (pH_i). In this study, we followed the accumulation of acid anion into the cell pellet and parallel changes in pH_i in two human pathogenic strains of *L. monocytogenes* (N1-227 and R2-499) and in *E. coli* O157:H7 after exposure to sub-bacteriostatic levels of lactic and acetic acids at mildly acidic pH 6.

Log phase bacteria underwent 60 min habituation to treatments including a baseline control of media at pH 7.4, acid control (added HCl to pH 6), or to 4.75 mM added lactic or acetic acids plus their C^{14} tracers. Tritiated water was also included as a tracer. Cells were harvested by centrifugation through a layer of bromododecane, and radioactivity in pellet and supernatant measured using a scintillation counter. Relative accumulation of anion was calculated as the ratio of C^{14}/H^3 in the pellet divided by the ratio of C^{14}/H^3 in the supernatant. Parallel experiments were performed without tracers, and pH_i measured using a pH-sensitive fluorescent dye. It is noteworthy that our measures of anion accumulation and pH_i were independent rather than being confounded as when employing the common practice of calculating intracellular concentration of anions based on

difference in internal and external pH, or conversely calculating pH_i based on intracellular accumulation of acid anion.

The test strains of *Listeria* and *E. coli* accumulated up to 3 times more acetate anion into the cell pellet compared to the supernatant. *Listeria* accumulated less lactate than acetate anion, and *E. coli* did not significantly accumulate lactate. The values for anion accumulation into the pellet were much less than expected for intracellular accumulation based on previous reports and predicted by the measured difference in internal and external pH. Hence, C^{14}-inulin (a sugar excluded from the cell interior) was employed to determine the fractional volume of pellet not available to inulin (i.e., intracellular volume) in comparison to volume of pellet available to water. That fractional volume was calculated as 0.19 for *E. coli*, and it follows that anion accumulation into the intracellular space is minimally 5X greater than measured for the entire pellet. A fractional volume of intracellular space was not measurable for the *L. monocytogenes* strains suggesting it was below the detection limit of our procedure.

Exposure of *E. coli* to acid control (pH 6) or to added organic acids did not induce a change in pH_i from the baseline control (pH 7.4). In contrast, exposure of *Listeria* to the organic acids induced lowered pH_i as compared to baseline in both strains while the impact of the acid control varied within strain. The acid control induced a lowered pH_i as compared to baseline in only strain R2-499 with exposure to acetic acid inducing an additional lowering of pH_i.

In summary, *Listeria* and *E. coli* were exposed to non-bacteriostatic levels of external organic acids at mildly acidic pH 6. *Listeria* accumulated more acetate than

lactate while mounting a defense against anion accumulation that included lowering its pH_i , while *E. coli* accumulated only acetate and apparently made use of combat mechanisms other than lowering pH_i not explored in this study. The methodology employed in these studies was based on independent measures of pH_i and intracellular anion accumulation, and the resulting data brings into question the common, but confounding, practice of using intracellular anion accumulation as a measure of pH_i, and vice versa.

Introduction

Listeria monocytogenes and *Escherichia coli* are pathogens of concern for foodborne illness, and each is among the top five causes of food-related death (37). Both pathogens are hardy organisms that can survive in many different types of environments, including food production facilities (29, 40). The food industry often uses organic acids in the environment and as food additives to limit growth of these, and other, pathogens. For example, acetic and lactic acids are used to decontaminate meat surfaces of *E. coli* and *L. monocytogenes* (4) and as antilisterial additive in meat products (18).

It has been hypothesized that a primary mechanism by which organic acids exert antimicrobial activity is their intracellular accumulation of acid anions (34), and that such accumulation is driven by the difference in external and internal pH (10). It is understood that some bacteria employ a lowering of their internal pH as a primary mechanism to combat accumulation of organic acid anions (69, 70). Other researchers have focused on the external protonated acid driving internalization of

acid anion (80, 81, 82). Carpenter and Broadbent assert in their hypothesis paper that external protonated acid is merely a shuttle, not a driving force for intracellular anion accumulation. Their hypothesis paper indicates that a failure to control external anion concentration has confounded results and likely led to misleading conclusions regarding the antimicrobial action of organic acids (10).

Hence, this study explored the extent to which mid-log phase cells of two human pathogenic strains of *L. monocytogenes* and *E. coli* accumulate acid anions and respond with altered pH_i when exposed to less-than-bactericidal levels of organic acids at mildly acidic pH. External concentration of organic acids was set at 4.75mM for both lactic and acetic acid as done in previous studies with the two *Listeria* strains.

The approach to this research was unique. Here intracellular pH (pH_i) and intracellular anion accumulation are measured in two separate but parallel experiments. In past studies, pH_i and intracellular anion accumulation have been measured together (28, 42). pH_i in this research was measured separately to remove confounding factors. Past studies used a similar protocol to measure intracellular acid anion accumulation with C^{14}-labeled acetate and inulin, but there were differences in the approach to handling the data and developing the final dependent variables (28, 42). In the prior work, the cell pellet was dried and the internal acetate concentration was calculated utilizing a previously established relationship between dry cell weight and internal cell volume. pH_i was subsequently calculated as predicted by the Henderson-Hasselbach equation (28, 42). This research instead used accumulation ratios of C^{14}/H^3 in the pellet and supernatant to

determine a relative accumulation value in the pellet in comparison to the external environment rather than actual internal concentration in mM, and also employed independent means of measuring pH_i.

Objectives

This study explored how *L. monocytogenes* and *E. coli* respond to organic acids in mildly acidic pH environments. The research is based on the hypothesis that intracellular accumulation of acid anions by bacteria in slightly acidic environments is driven by two predictive factors, external anion concentration and external acidity (10). Here, we describe the extent to which mid-log phase cells of two human pathogenic strains of *L. monocytogenes* and *E. coli* accumulate acid anions and respond with altered pH_i when exposed to less-than-bacteriostatic levels of organic acids at mildly acidic pH. This was chosen so that cells are exposed to environments where they are under stress but are given the opportunity to adapt. It is useful to understand a situation like this because it may happen in a food industry setting- where acid may accumulate in drain for example. This thesis had two objectives employing 2 strains of *L. monocytogenes* and 1 strain of *E. coli*. The same two *Listeria* strains have been studied multiple times in the past decade by this lab and this research continues to study the phenomena of these two strains (30, 60, 61). The strain of *E. coli* was used as an outside control because it is gram negative and has been studied by other researchers (42).

1. Determine accumulation of acid anion into the cell pellets after 1 hour exposure to 4.75mM lactic or acetic acid at mildly acidic pH 6.
    a. Determine concentration of inulin associated with the cell pellets after 1 hour exposure to inulin.
2. Determine pH_i after 1 hour exposure to a baseline control (pH 7.4), an acid control (pH 6), 4.75mM lactic acid (pH 6), and 4.75mM acetic acid (pH 6).

Materials and Methods

Bacterial strains and growth conditions

Two different strains of *L. monocytogenes* were used (Table 2-1). These two strains have previously been shown to mount an inducible resistance to both acid and bile in response to organic acids (60) and represent the two serotypes that are most commonly virulent in humans. The two strains have also been studied extensively under the same conditions set forward in this research (30, 60, 61). R2-499 is serotype 1/2a and N1-227 is 4b. One strain of *E. coli* O157:H7 was selected for this study as well as an outside control.

Table 2-1. Strains selected for this study

Bacteria	Strain	Source
L. monocytogenes	FSL R2-499	Human isolate associated with US outbreak linked to sliced turkey (60)
L. monocytogenes	FLS N1-227	Food isolate associated with US outbreak (60)
E. coli O157:H7	H1730	Human isolate associated with a lettuce outbreak (62)

Original cultures were stored as frozen stock cultures at -80^{o}C in tryptic soy broth (TSB, pH=7.4; Becton, Dickinson and Company, Sparks, MD) supplemented with 20% v/v glycerol. Prior to use, cultures were propagated on tryptic soy agar

(TSA; Becton, Dickinson and Company, Sparks, MD) plates and incubated at 37°C for 24 hours. A single colony was transferred from the TSA plate into TSB and incubated overnight at 37°C with shaking (220 rpm).

Overnight working cultures were harvested by centrifugation (2500 x g for 10 min; Sorvall RT1, Thermo Scientific, Germany) then diluted to an optical density at 600nm (OD_{600}) of 0.03 in TSB. A 1% inoculum of cells was transferred into 10mL TSB (pH 7.4) and incubated at 37°C with shaking (220rpm) into mid-log phase, 3 hours for *E. coli* and 4 hours for *Listeria*. Cells were individually collected by centrifugation and used.

Experimental overview and statistical analysis

Objective 1

Each bacterial strain was grown to mid log phase in standard media and conditions, then cells were harvested and resuspended in habituation media for one hour. Habituation media included tryptic soy broth (TSB) at pH 6 containing 4.75mM lactic or acetic acid, each spiked with the respective isotope-labeled organic acid (Table 2-2). Spike was present for the entire hour cells were grown in habituation treatment. Cells were separated by centrifugation, and C^{14} labeled organic acid and H^3 water was measured in both the pellet and supernatant. The ratio of C^{14}:H^3 in each pellet and supernatant is reflective of relative concentration. This relative concentration in the pellet compared to relative concentration in the supernatant allowed for calculation of an accumulation factor.

Table 2-2. Objective 1 habituation treatments

Treatment Name	Media	pH	Organic acid concentration	Tritiated water	C^{14} Isotope Spike
Lactic Acid (LA)	TSB	6	4.75mM lactic acid	10 uCi	1 uCi lactic acid
Acetic Acid (AC)	TSB	6	4.75mM acetic acid	10 uCi	1 uCi acetic acid

Acid anion accumulation values were calculated as:

$$\frac{(C^{14} \text{ pellet counts}/H^{3} \text{ pellet counts})}{(C^{14} \text{ supernatant counts}/H^{3} \text{ supernatant counts})}$$

Statistical analysis was done using a t-test with single treatment of habituation media that included two levels, lactic and acetic acid. The dependent measurement was the calculated accumulation factor. The treatment means for anion accumulation were deemed significant if the 95% CI was greater than 1. Significant differences in means of the treatment levels were identified by $P<0.05$. All data reported is a mean of four independent experiments, with three replications per experiment.

Objective 1A

Each bacterial strain was grown to mid log phase, then cells were resuspended in habituation media. Cells were habituated for one hour in tryptic soy broth (TSB) at pH 6 containing C^{14} labeled inulin. Habituation media was spiked with isotopes (Table 2-3). Spike was present for the entire hour cells were grown in

habituation treatment. Cells were separated by centrifugation, and C^{14} labeled inulin and H^3 water was measured in both the pellet and supernatant.

Table 2-3. Objective 1A habituation treatments

Treatment Name	Media	pH	Organic acid concentration	Tritiated water	C^{14} Isotope Spike
Inulin control (IC)	TSB	6	0 mM	10 uCi	1 uCi inulin

This approach did not employ calculations of absolute concentration. The ratio of C^{14}:H^3 in each pellet and supernatant is reflective of relative concentration. This relative concentration in the pellet compared to relative concentration in the supernatant was used to calculate the internal cell space as fractional volume of the pellet. Fractional internal volume was determined by comparing the average C^{14}/H^3 values of the pellet to the supernatant. If their means were not significantly different ($P<0.05$), we concluded there was no measurable fractional internal volume. The fractional internal volume found in the pellet was calculated as 1 – [(the fraction of system total volume available to inulin in pellet) / (the fraction of total system volume available to ^3H in pellet)]. More specifically, this was calculated from:

$$1- \frac{(C^{14}\text{ pellet counts}/ (C^{14}\text{ counts pellet}+ C^{14}\text{ counts supernatant})}{(H^3\text{ pellet counts}/H^3\text{ counts pellet}+ H^3\text{ counts supernatant})}$$

Data was analyzed using a t-test at $P<0.05$ to evaluate if the supernatant had significantly higher relative concentration of inulin than found in the pellet, and if significant the internal cell space was calculated as a fraction of the total pellet volume available to water. All data reported is a mean of four independent experiments, with three replications per experiment.

Objective 2

Each bacterial strain was grown to mid log phase, then cells were resuspended in one of four habituation media (Table 2-4). All treatments besides the baseline control were adjusted to pH 6 with hydrochloric acid, an anion-free acid. The two other acid treatments contained 4.75 mM added L-lactic acid (pKa 3.88) or acetic acid (pKa 4.76). After an hour in habituation media pH_i was determined using a pH sensitive fluorescent dye, and employing a standard curve previously established for each bacterial strain.

Table 2-4. Objective 2 habituation treatments

Treatment	pH	mM organic acid
Baseline control (BC)	7.4	N/A
Acid control (HCL)	6	N/A
Lactic Acid (LA)	6	4.75 lactic acid
Acetic Acid (AC)	6	4.75 acetic acid

Statistical analysis was done using one-way analysis of variance (ANOVA) with single treatment of habituation media that included four levels; BC, HCL, LA and AC (Table 2-4). Dependent measurement was the measured pH_i of cells. Differences in means of the treatment levels were identified by Tukey's test at $P<0.05$. All data reported is a mean of four independent experiments, with three replications per experiment.

Accumulation of organic acid

Accumulation of organic acid inside the cells were determined using isotopes. C^{14} labeled lactic and acetic acid (acetic acid, sodium salt -[1-^{14}C] and lactic acid, sodium salt, L-[^{14}C(U)], Perkin Elmer, Waltham, MA) were used as tracers to measure the amount of organic acid anion incorporated into the cell. The extremely small spike of additional C^{14} labeled organic acid added to the lactic and acetic acid treatments was determined to not impact the total concentration of the treatment (4.75mM for both, Table 2-2). Tritiated water (Water, [^{3}H], Perkin Elmer, Waltham, MA) was also used in each treatment. Tritiated water was used because it was assumed that it would equilibrate between the interior and the exterior of the cell, resulting in uniform distribution. The C^{14}/H^3 ratio provides a means to assess the relative acid anion concentration in both the cell pellet and supernatant. C^{14}-labeled inulin (Inulin- carboxyl, [carboxyl-^{14}C], Perkin Elmer, Waltham, MA) was also employed in an effort to determine the intracellular fraction of the pellet volume. Inulin is a water-soluble carbohydrate that is impermeable to the cell membrane (43).

For this work, 10mL of mid log cells grown in TSB were collected by centrifugation and resuspended in TSB broth adjusted to deliver one of the three individual treatment levels employed for objective 1 and 1A. (IC, LA, or AC, Table 2-2 and 2-3). Cells were incubated for one hour at 37°C with shaking (220 rpm). After incubation 0.5mL bromododecane (1-Bromododecane, Sigma Aldrich, St. Louis, MO) was added to the centrifuge tube. Cells were then centrifuged. Bromododecane created a layer between the supernatant and pellet after centrifugation allowing for easy decanting of the supernatant and stripping cells of external water (36).

The supernatant was decanted and diluted so that disintegrations per minute (DPM) would be similar to that of the pellet. 1mL of the diluted supernatant was added to 18mL liquid scintillation cocktail (Emulsifier Scintillator Plus, Perkin Elmer, Waltham, MA). The entire cell pellet was cut out of the centrifuge tube using guillotine dog nail clippers. The entire cell pellet was added to 18mL liquid scintillation cocktail along with 1mL TSB. Liquid volume in both the supernatant and pellet samples were identical (1mL) to ensure similar counting efficiency. Counts were measured using a scintillation counter (Beckman LS 6500, Beckman Coulter, Brea, CA).

Measurement of pH_i

The pH_i was determined using 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE, Sigma Aldrich, St. Louis, MO), a membrane permeant fluorescein-based dye that at 492nm is sensitive to pH (47). The first step was to determine a calibration curve that would relate fluorescent values to pH_i values. A

10-uM solution of CFSE was prepared from a concentrated stock solution in DMSO by dilution in a 10mM KH_2PO_4 buffer for use as the dye.

To determine how acid treatments impacted pH_i we first established a calibration curve to relate cell fluorescent values to pH_i. 10mL of mid log cells grown in TSB were collected by centrifugation and resuspended in 10mL CFSE staining solution (10uM) and incubated at 37°C for 30 minutes. The cells were collected by centrifugation then suspended in 10mL KH_2PO_4 buffer (pH 6.0) supplemented with 10mM glucose (to energize the cells) and incubated at 37°C for another 30 minutes to remove unbound dye. To permeabilize cells and equilibrate intracellular and external pH for the calibration curve, the stained cells were then placed in ethanol (63%, v/v) for 30 minutes at 37°C. The dye is bound to metabolic items of the cell so after exposure to ethanol it remains bound. The bacterial cells were harvested by centrifugation and suspended in 10mL TSB broth medium whose pH was adjusted with HCl or NaOH to range from 5.0 to 8.0, in 0.5 increments.

Fluorescence was measured using a microplate fluorometric reader (Synergy H1, BioTek, Winooski, VT). The fluorescent $ratio_{492/435}$ was obtained by dividing fluorescence at 492 nm by that at 435 nm. These wavelengths are pH sensitive and pH insensitive, respectively (47). Undyed cells in TSB were used as a blank to correct for any naturally occurring fluorescence. The calibration curve was plotted by polynomial fitting between $ratio_{492/435}$ and the pH_i of the equilibrated cells corresponding to the broth pH (5.0 to 8.0), respectively.

It was necessary to establish a different calibration curve for each strain because of the nature of the intracellular dye binding to molecules within the cell that could vary by strain.

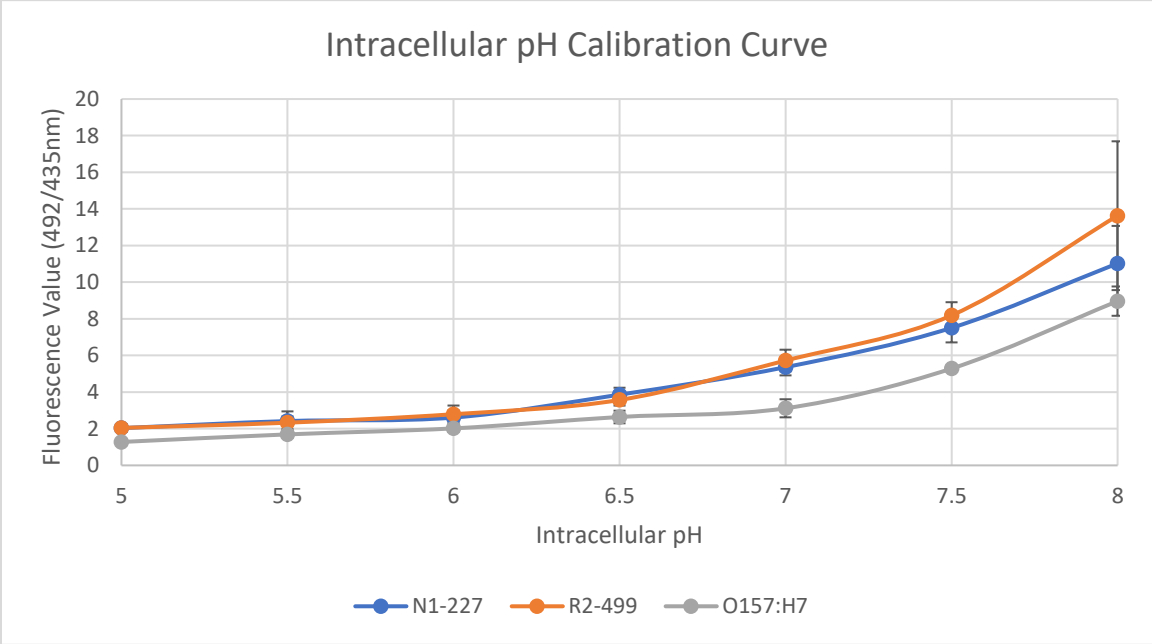

Figure 2-1. pH_i calibration curves for *L. monocytogenes* and *E. coli* at mid log phase. Error bars represent standard error of the mean.

The standard error is relatively small until the higher pH values, similar to other work using the same method (16). After the curve was established, polynomial fitting was used to establish an equation for each strain relating fluorescence values (492/435nm) to pH_i. See appendix B for individual strain calibration curves and equations. In each equation, y and x represent the fluorescence value and pH_i, respectively. To calculate for pH_i the fluorescence value (y) is used in the polynomial equation to solve for x. The R^2 for each equation is >0.99 which demonstrates the regression lines very closely fit the data.

The last step is the conversion of measured absorbance values to pH_i using the standard curve for each corresponding strain. Mid log cells were dyed in 10um CFSE then washed and energized in in KH_2PO_4 buffer (pH 6.0) supplemented with 10mM glucose as described above. The cells were then collected by centrifugation and suspended in TSB broth adjusted to deliver one of the four individual treatments (BC, HCl, LA, or AC). The cells were incubated at 37°C with shaking in the microplate fluorometric reader for 60 minutes then fluorescence values at 492 and 435nm were measured. pH_i was calculated from the mean value plotted against the pH calibration curve.

Results and Discussion

Acid anion accumulation

Results from acid accumulation studies are presented in figure 2-2. Across all three species, acetate anion accumulated into the cell pellet to a significantly greater extent than lactic acid. Levels of lactate in *Listeria* N1-227 and R2-499 cell pellets were 1.54 and 1.66 times higher, respectively, than supernatant levels. No significant accumulation of lactate was detected in the *E. coli* pellet. In contrast, the concentration of acetate in the cell pellet was more than two-fold greater than in the supernatant for all three bacteria. *L. monocytogenes* N1-227, R2-499, and *E. coli* O157:H7 accumulated acetate anion 2.28, 2.41, and 2.64 times more than the supernatant respectively.

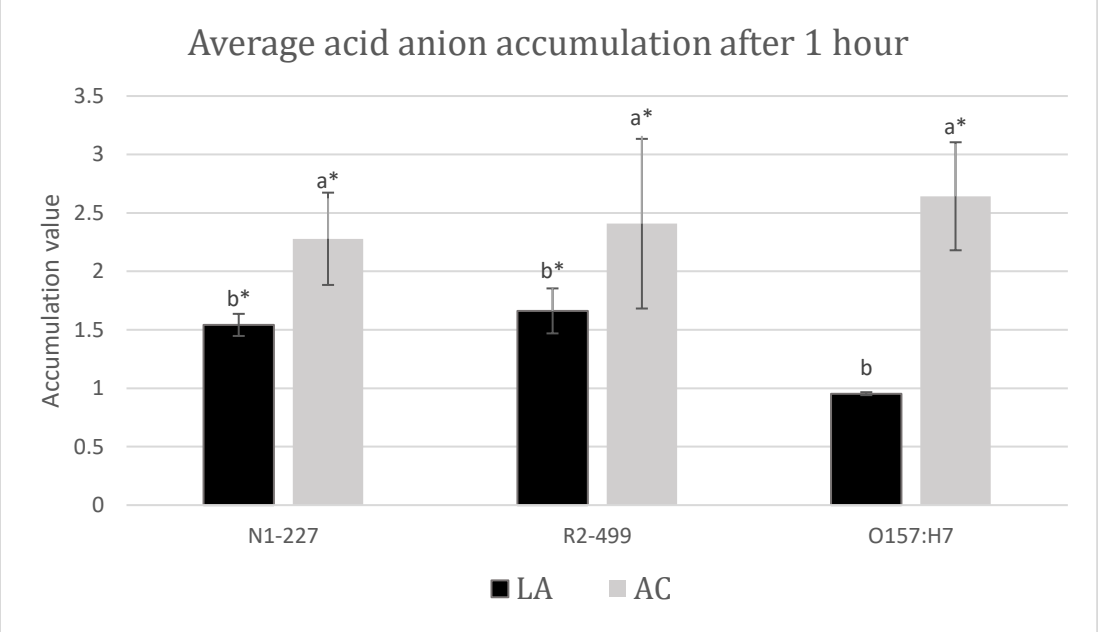

Figure 2-2. Acid anion accumulation into pellets of *L. monocytogenes* N1-227, *L. monocytogenes* R2-499, and *E. coli* O157:H7 following one hour in habituation media. Values represent treatment means and include bars of the standard error. Treatment details: LA, lactic acid treatment (TSB adjusted to pH 6.0 with HCl

containing 4.75mM lactic acid); AC, acetic acid treatment (TSB adjusted to pH 6.0 with HCl containing 4.75mM acetic acid). Superscripts identify significant differences within strains ($P<0.05$). Asterisk signifies accumulation was significantly greater than 1 ($CI<95\%$)

Prior studies report that acetic acid inhibits growth of L. *monocytogenes* more than lactic acid in terms of total acid added as weight by volume (19, 24, 49), and the results presented here suggest that this may be due to the greater accumulation of acetate. Roe. et al reported that at pH 6, growth in 8mM acetate resulted in an internal pool of 240mM acetate anion in *E. coli* (42). Results here are much lower in comparison. Thus, C^{14} inulin was employed as a control to try and assess how much of the pellet volume was truly intracellular. The fractional volume of the pellet not available to inulin as compared to water was calculated as representative of the relative intracellular space. A fractional volume of 0.19 was identified for *E. coli*. Because *E. coli* had a measurable internal volume, an additional accumulation factor was estimated based on fractional internal volume. Total intracellular accumulation of anion was determined by dividing 1 by 0.19. The fractional volume of the *E. coli* pellet then suggests that accumulation of anion is possibly 5-fold greater than measured in the pellet. Both *L. monocytogenes* strains did not have a fractional volume significantly different from 0 suggesting it was below the detection limit of our procedure. We hypothesize that this is because it would require numerous repetitions to detect a significant fractional internal volume so close to zero. The smaller the fractional internal volume, the larger the total effect on the pellet. So while the methodology employed here was able to identify significant accumulation of organic acids into the pellet, our results with

inulin suggest that the pellet values underestimate intracellular concentrations of anion, and the true extent of anion accumulation is likely much higher than measured.

One possible explanation for not finding a significant fractional internal volume in *Listeria* may be related to past observations seen in our research. As reported by others, we have found that the OD_{600} for these *Listeria* strains did not correlate with actual viable cell numbers (60, 79). The reason for this discrepancy is unknown, but we have hypothesized that it could be due to cell clumping, and that may play a factor in these results as well. Another explanation is that carryover of inulin may be different in Gram negative versus Gram-positive cells, possibly explaining the difference in measured fractional internal volume between *Listeria* and *E. coli*. Gram negative bacteria, like *E. coli*, have a periplasm that serves as a multipurpose compartment separate from the cytoplasm (73). The periplasm can have a different pH than the cytoplasm (32). This periplasm may play a role in the difference seen in fractional internal volume.

pH_i values

Results from pH_i studies are presented in Figure 2-3. Under these conditions, most of the organic acids would be in the undissociated anion form in the cells because their pKa < pHi (10). Acetic acid exposure resulted in a significantly lower ($P<0.05$) pH_i compared to exposure to the baseline control in both *Listeria* species (Figure 2-3). In N1-227 cells, the acid control and lactic acid were intermediate (not significantly different) from the baseline control or acetic acid. In contrast, all three acid treatments resulted in a significantly lower pH_i in R2-499 cells in comparison to

the baseline control. Exposure to the acetic acid treatment resulted in a significantly lower pH_i than cells in the acid control in R2-499, while the pH_i in cells given the lactic acid treatment was intermediate between that of cells from the acid control or acetic acid treatment. Both *Listeria* strains lowered pH_i in response to acetic acid. Lowering pH_i is a mechanism known to combat external acidity and intracellular anion accumulation (69, 70).

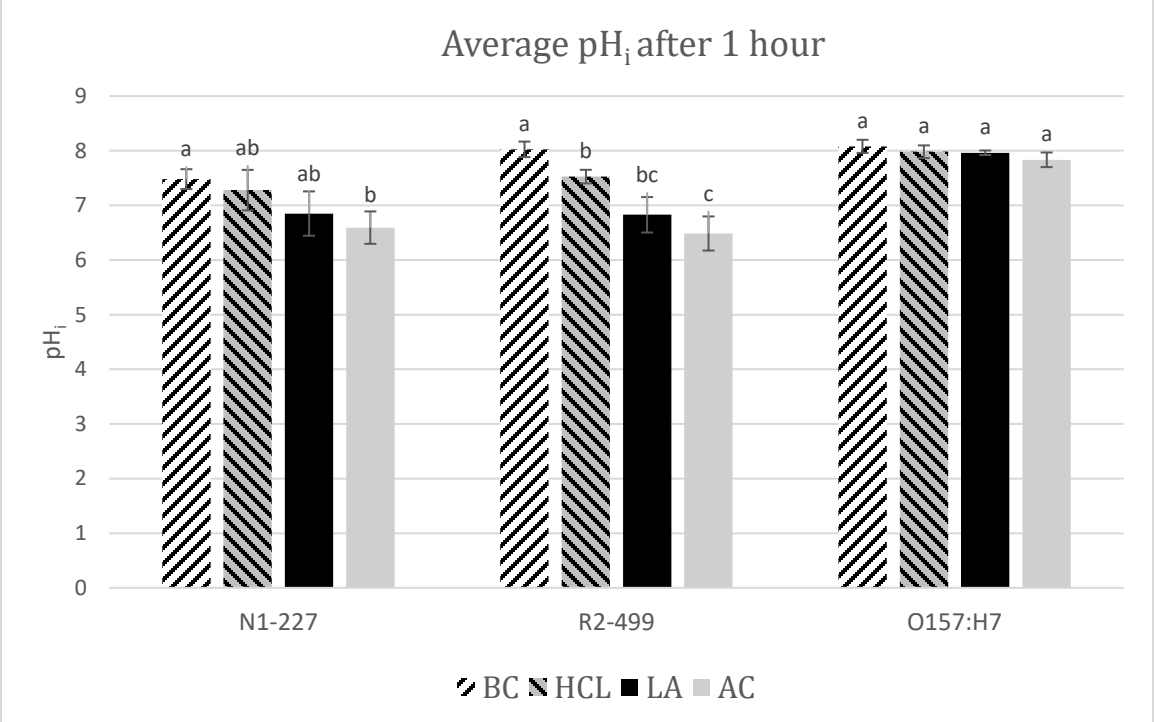

Figure 2-3. pH_i values of *L. monocytogenes* N1-227, *L. monocytogenes* R2-499, and *E. coli* O157:H7 after one hour in habituation treatment media. Error bars represent standard error of the mean. Treatment details: BC, baseline control (TSB pH 7.4); HCl, pH control (TSB adjusted to pH 6.0 with HCl); LA, lactic acid treatment (TSB adjusted to pH 6.0 with HCl containing 4.75mM lactic acid); AC, acetic acid treatment (TSB adjusted to pH 6.0 with HCl containing 4.75mM acetic acid). Superscripts identify significant differences within strains.

Only strain R2-499 lowered pH_i to combat just external acidity. It has not previously been reported that *Listeria* would lower its pH_i in response to just

external acidity (8, 45, 78). This finding has been reported in lactic acid bacteria (78). N1-227 lowered pH_i significantly from the baseline control when exposed to the added stress of external acid, and only acetic acid. The cells may have alternative methods to combat lactate accumulation such as export mechanisms. Both lactate and acetate are part of normal metabolism in *L. monocytogenes* (74, 77).

E. coli O157:H7 pH_i did not significantly change in response to any of the four treatments (Figure 2-3). This finding is in agreement with past studies that reported *E. coli* pH_i rapidly drops after exposure to acid before recovering within 5 minutes (35). Based on the pH_i, *E. coli* would be expected to accumulate acid anions to a much greater extent than both *Listeria* strains. In contrast, this research found that *E. coli* accumulated acetate at a level that was comparable to both *Listeria* strains, and accumulated even less lactate than either *Listeria* strain. *E. coli* may largely rely on other means to combat the accumulation of anion rather than lowering pH_i. For example, the cells may change their membrane composition or upregulate export mechanisms (75, 76).

Conclusions & Further Research

This study used two parallel experiments to investigate how *L. monocytogenes* and *E. coli* O157:H7 respond to organic acids in mildly acidic pH environments. The two *Listeria* strains, exposed to the same conditions, has been studied previously. This research builds on a long history with these two strains, while *E. coli* was used as an outside "control" and has been studied by other researchers. Results from this study increases the understanding of the organic acid response in these species and builds on the knowledge of the physiology of these

two *Listeria* species. Understanding their physiology and how they combat exposure to organic acids may impact how they are applied and used in the food industry as antimicrobials.

Our results suggest the potential of these strains surviving organic acid exposure through various mechanisms, including lowering pH_i to combat intracellular anion accumulation. Results indicate acetate and lactate anion were accumulated into cells significantly more than their environment in both strains of *Listeria*, while only acetate anion was significantly accumulated in *E. coli.* Results from the inulin control indicate that intracellular anion accumulation is likely much higher than measured.

We also found that organic acid exposure induces a significant drop in *Listeria* pH_i, but not in *E. coli* O157:H7 ($P<0.05$, ANOVA). These strains of *E. coli* and *Listeria* may have different mechanisms to combat intracellular acid anion accumulation. *Listeria* may lower pH_i as a mechanism accumulation. *E. coli* may largely rely on other means to combat the accumulation of anion rather than lowering pH_i. It is hypothesized that these two strains of *Listeria* may have specific mechanisms to handle lactate but not acetate, and may be more effective at combating lactate than acetate. Additional research needs to be done at different external pH and external acid concentrations to truly understand their physiological response to the organic acids, and more strains would need to be studied to make a broader conclusion.

It is also worthwhile to note that these results bring into question the risks of using intracellular anion accumulation as a measure of pH_i as done in previous

studies (28, 42). The methodology employed in these studies was based on independent measures of pH_i and intracellular anion accumulation, and the results found here brings into question the common, but confounding, practice of using intracellular anion accumulation as a measure of pH_i, and vice versa. The risks of doing so include differences in how various species may handle acid exposure and accumulation of anions.

Future research should track changes in intracellular anion accumulation and pH_i over time, and in response to external pH, to better understand their interaction. All of the experiments done here were at mildly acidic pH 6, excluding the baseline control. It would be interesting to see how time and external pH effect these variables, including inulin accumulation into the pellet. In this study we did not find that *E. coli* employed lowering its pH_i. However, it could also be that *E. coli* lowers pH_i initially to provide a general protection from anion accumulation into the cells until it can ramp up other specific systems such as export systems or nonspecific systems such as membrane changes to combat anion accumulation (75, 76). This study may have missed any initial lowering of pH_i because the cells had 60 minutes to adjust. Additional research should also be done to better define intracellular volume utilizing inulin. Finally, our lab has previously studied transcriptomic data of the two *Listeria* strains exposed to these conditions. It would be useful to reexamine that transcriptomic data in connection with these results.

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APPENDICES

Appendix A: Anova Data- Measurement of pH_i

Table A-1. ANOVA for measurement of pH_i in *L. monocytogenes* R2-499 with single treatment of habituation media that included four levels; BC, HCL, LA and AC.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
R2-499 BC	8	64.2	8.025	0.1619
R2-499 HCl	8	60.21	7.5263	0.1239
R2-499 LA	8	54.62	6.8275	0.8399
R2-499 AC	8	51.88	6.485	0.7806

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	11.488	3	3.8294	8.0353	0.0005	2.9467
Within Groups	13.344	28	0.4766			
Total	24.832	31				

Table A-2. ANOVA for measurement of pH_i in *L. monocytogenes* N1-227 with single treatment of habituation media that included four levels; BC, HCL, LA and AC.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
N1-227 BC	8	59.84	7.48	0.2645
N1-227 HCl	8	58.23	7.2788	1.1004
N1-227 LA	8	54.8	6.85	1.3141
N1-227 AC	8	52.74	6.5925	0.7028

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.8923	3	1.2974	1.5346	0.2274	2.9467
Within Groups	23.672	28	0.8454			
Total	27.564	31				

Table A-3. ANOVA for measurement of pH_i in *E. coli* O157:H7 with single treatment of habituation media that included four levels; BC, HCL, LA and AC.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
O157: H7 BC	8	64.66	8.0825	0.1101
O157:H7HCl	8	63.85	7.9813	0.1079
O157:H7 LA	8	63.7	7.9625	0.0142
O157:H7AC	8	62.67	7.8338	0.1433

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.2504	3	0.0835	0.8892	0.4588	2.9467
Within Groups	2.6286	28	0.0939			
Total	2.879	31				

Appendix B: Additional Data from Measurement of pH_i

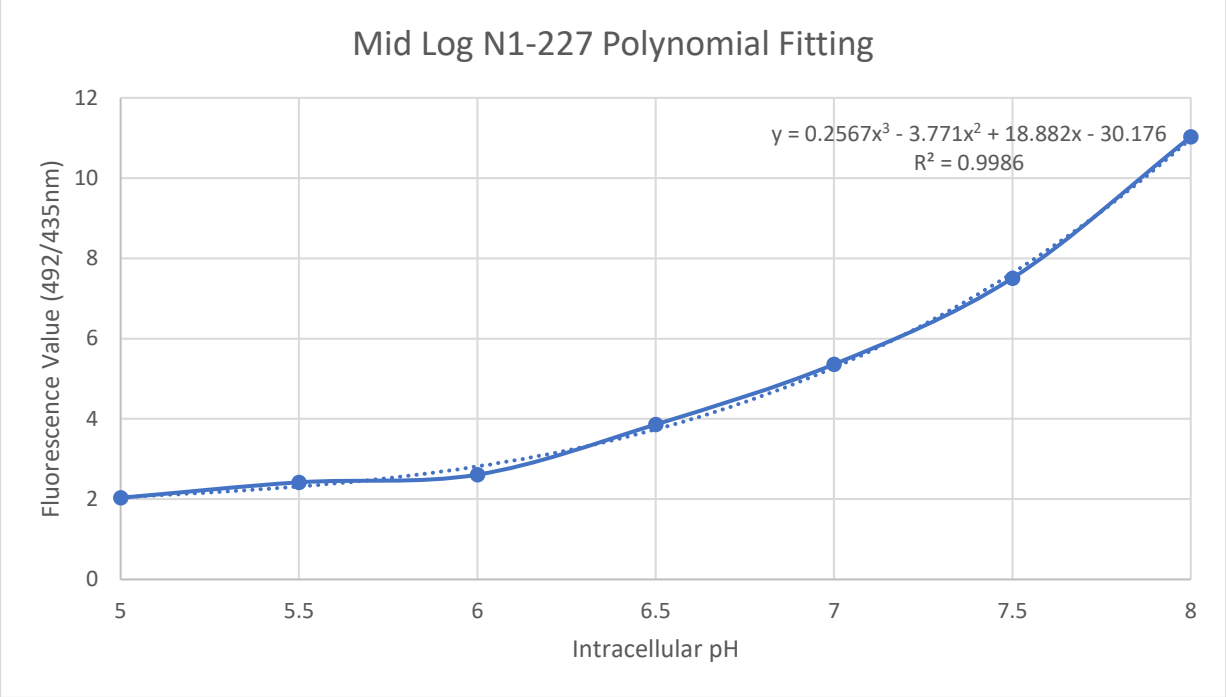

Figure B-1. Polynomial fitting for strain *L. monocytogenes* N1-227 at mid log. Dotted line is the fitted regression line.

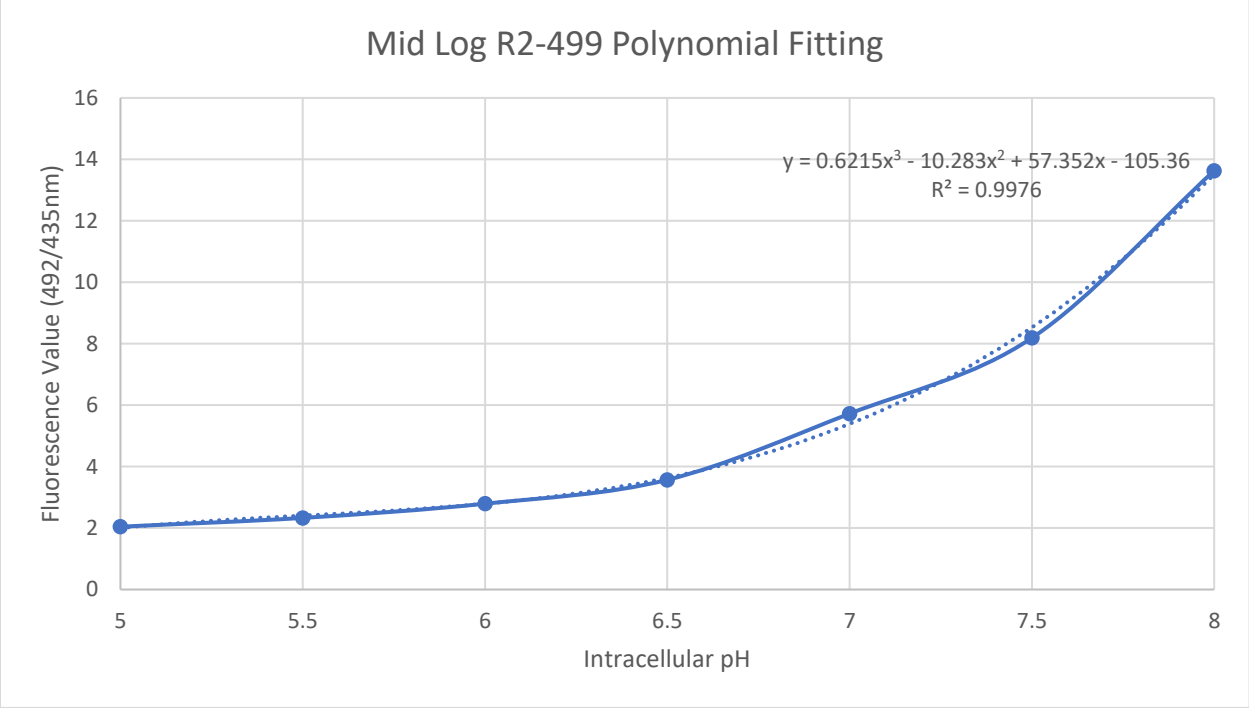

Figure B-2. Polynomial fitting for strain *L. monocytogenes* R2-499 at mid log. Dotted line is the fitted regression line.

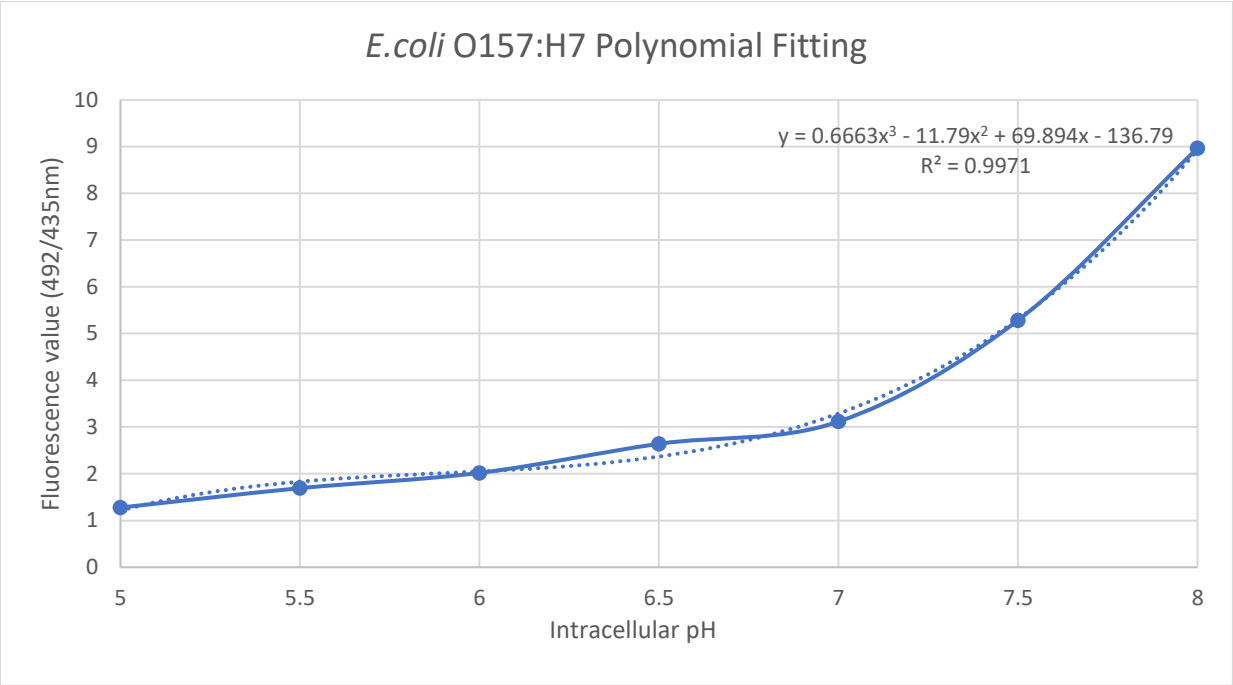

Figure B-3. Polynomial fitting for strain *E. coli* O157:H7 at mid log. Dotted line is the fitted regression line.

The pH_i values of the *L. monocytogenes* strains were initially monitored every ten minutes over the course of one hour to see how they changed over time (Figures B-4 and B-5). All data is an average of triplicate wells, and the experiment was run four times.

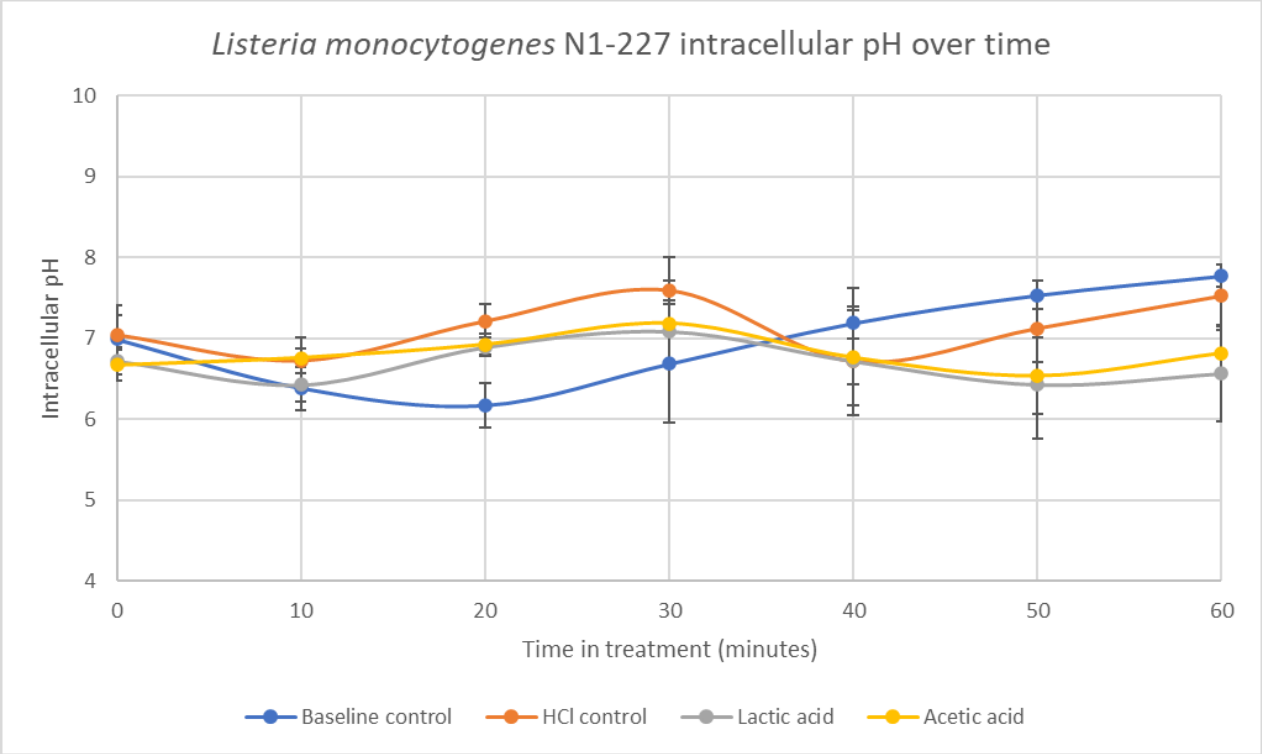

Figure B-4. Preliminary results for *L. monocytogenes* N1-227 from tracking pH_i over time. Error bars represent standard error of the mean.

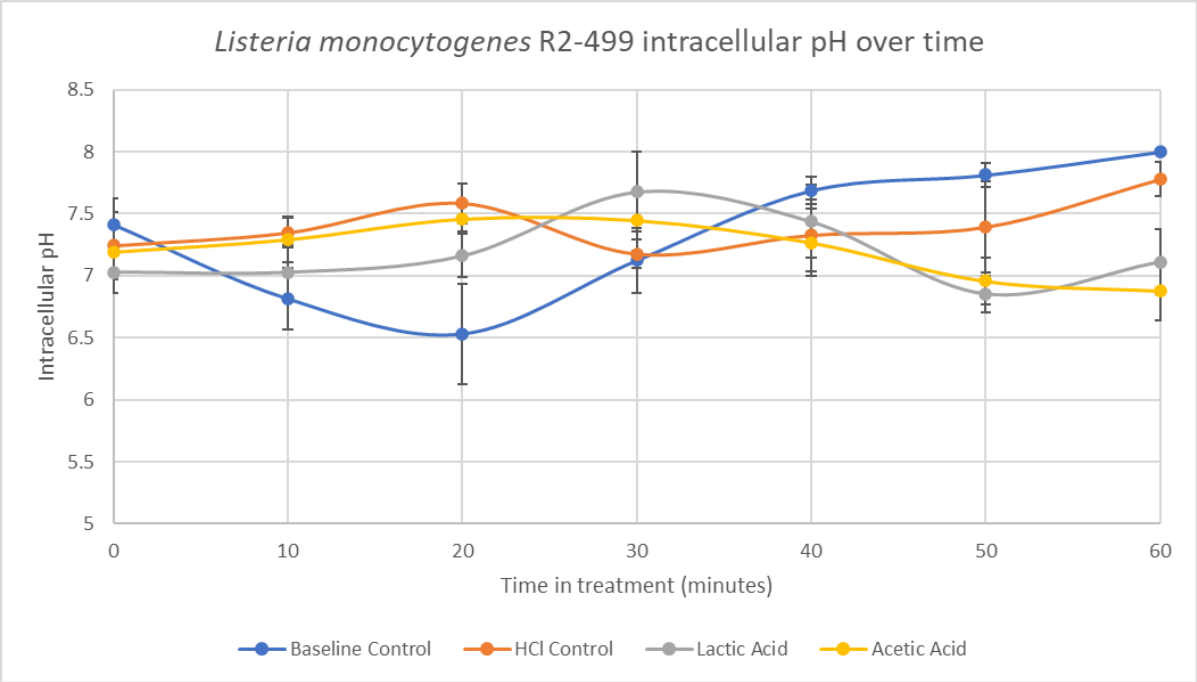

Figure B-5. Preliminary results for *L. monocytogenes* R2-499 from tracking pH_i over time. Error bars represent standard error of the mean.

Results shown in Figures B-4 and B-5 indicate that the cells need time to equilibrate and stabilize pH_i while adapting to the habituation treatments. This is especially noticeable in the baseline control, which drops to a lower pH by twenty minutes consistently in both strains before pH_i rises again. We could not determine if the pH_i is actually changing early on or if it is an artifact of the dye stabilizing relative to other adaptions of cellular metabolism. The cells appear to stabilize by 40-60 minutes, as indicated by the graph and standard error bars. Based on these findings, we decided pH_i measurements in future experiments should be collected after one hour in habituation media.

Appendix C: T-Tests from Accumulation of Organic Acid & Inulin

Table C-1. T-test for accumulation of organic acid in *L. monocytogenes* R2-499 with single treatment of habituation media that included two levels, lactic and acetic acid

t-Test: Two-Sample Assuming Equal Variances
L. monocytogenes R2-499

	Lactic Acid	Acetic Acid
Mean	1.6617234	2.4078
Variance	0.1923734	0.7257
Observations	18	18
Pooled Variance	0.4590387	
Hypothesized Mean Difference	0	
df	34	
t Stat	-3.303734	
P(T<=t) one-tail	0.0011267	
t Critical one-tail	1.6909243	
P(T<=t) two-tail	0.0022534	
t Critical two-tail	2.0322445	

Table C-2. T-test for accumulation of organic acid in *L. monocytogenes* N1-227 with single treatment of habituation media that included two levels, lactic and acetic acid

t-Test: Two-Sample Assuming Equal Variances
L. monocytogenes N1-227

	Lactic acid	Acetic acid
Mean	1.542025829	2.27860572
Variance	0.094581064	0.39473445
Observations	18	18
Pooled Variance	0.244657756	
Hypothesized Mean Difference	0	
df	34	
t Stat	-4.467469739	
P(T<=t) one-tail	4.16345E-05	
t Critical one-tail	1.690924255	
P(T<=t) two-tail	8.3269E-05	
t Critical two-tail	2.032244509	

Table C-3. T-test for accumulation of organic acid in *Escherichia coli O157:H7* with single treatment of habituation media that included two levels, lactic and acetic acid

t-Test: Two-Sample Assuming Equal Variances
E. coli O157:H7

	Lactic acid	Acetic acid
Mean	0.95369419	2.642461
Variance	0.01250465	0.462054
Observations	18	18
Pooled Variance	0.23727928	
Hypothesized Mean Difference	0	
df	34	
t Stat	-10.400662	
P(T<=t) one-tail	2.1126E-12	
t Critical one-tail	1.69092426	
P(T<=t) two-tail	4.2252E-12	
t Critical two-tail	2.03224451	

Table C-4. T-test from inulin control comparing *L. monocytogenes* N1-227 supernatant & pellet

t-Test: Two-Sample Assuming Equal Variances
L. monocytogenes N1-227

	SUP	*PEL*
Mean	0.228696	0.269221
Variance	0.001249	0.010339
Observations	18	18
Pooled Variance	0.005794	
Hypothesized Mean Difference	0	
df	34	
t Stat	-1.59716	
P(T<=t) one-tail	0.059742	
t Critical one-tail	1.690924	
P(T<=t) two-tail	0.119485	
t Critical two-tail	2.032245	

Table C-5. T-test from inulin control comparing *L. monocytogenes* R2-499 supernatant & pellet

t-Test: Two-Sample Assuming Equal Variances
L. monocytogenes R2-499

	SUP	*PEL*
Mean	0.222636	0.269221
Variance	0.000954	0.010339
Observations	18	18
Pooled Variance	0.005646	
Hypothesized Mean Difference	0	
df	34	
t Stat	-1.85988	
P(T<=t) one-tail	0.035786	
t Critical one-tail	1.690924	
P(T<=t) two-tail	0.071572	
t Critical two-tail	2.032245	

Table C-6. T test from inulin control comparing *E. coli* O157:H7 supernatant & pellet

t-Test: Two-Sample Assuming Equal Variances
E. coli O157:H7

	SUP	*PEL*
Mean	0.171279356	0.132260669
Variance	0.000134141	0.000799938
Observations	18	18
Pooled Variance	0.00046704	
Hypothesized Mean Difference	0	
df	34	
t Stat	5.41647947	
P(T<=t) one-tail	2.47093E-06	
t Critical one-tail	1.690924255	
P(T<=t) two-tail	4.94E-06	
t Critical two-tail	2.032244509	