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Assessing the Effect of Bile and Oxygen Availability on the Redox Status of Listeria monocytogenes Strain F2365

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Assessing the Effect of Bile and Oxygen Availability on the Redox Status of *Listeria monocytogenes* Strain F2365

by

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Abstract

Although bile is a bactericidal agent able to disrupt membrane structure and cellular homeostasis, including the induction of oxidative stress, *Listeria* can tolerate bile and also utilize it as a signal to enhance infection and virulence. Preliminary findings showed that under anaerobic conditions, exposure to bile significantly lowered the amount of oxidative damage present in bile-resistant strain F2365 cells. Similarly, *Listeria* further elicits an adaptive immune response, wherein pre-exposure of the bacterium to stress during food processing or in the host prior to entry into the intestine increases bile tolerance. Based on these previous studies, we hypothesized that bile induces oxidative damage under aerobic, but not anaerobic conditions and that pre-exposure to oxidative stress can improve the oxidative stress response expressed from exposure to bile. The overall goal is to understand the effects of oxygen availability and bile on the redox state of F2365. To do so, oxidative stress marker GSH:GSSG ratio was measured in F2365 under aerobic and anaerobic conditions subsequent to one of four treatments: no treatment, 50 mM H_2O_2 only, 50 mM H_2O_2 followed by 1% bile, and only 1% bile. The results indicate a similar redox state of F2365 under both aerobic and anaerobic conditions. However, exposure to bile induced oxidative stress under aerobic conditions, but not under anaerobic conditions. Additionally, pre-exposure to hydrogen peroxide does not protect F2365 from bile-induced oxidative stress. Further research is needed to determine the normal-state GSH:GSSG ratio in *Listeria* and understand the different mechanisms *Listeria* uses to combat bile under aerobic and anaerobic conditions.

Key Words: *Listeria monocytogenes*, Bile, Oxidative Stress, Anaerobic, GSH:GSSG Ratio, Hydrogen Peroxide

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I would like to express my utmost gratitude to my advisor, Dr. Janet Donaldson, without whom this project would not have been possible. Thank you Doc for giving me the opportunity to work with you and most importantly, for being an impeccable source of knowledge, guidance, support, and inspiration all these years. I couldn't have asked for a better mentor. I would also like to thank all the graduate students I have had the pleasure to work with throughout the years. Thank you Damayanti, Oindrila, and Sally for graciously sharing your knowledge and always being so willing to help. Special thanks to Honors College for their constant assistance and guidance in this journey.

Dedication

To my family.

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List of Abbreviations

ATR	Acid Tolerance Response
bilE	Bile Exclusion Gene
bsh	Bile Salt Hydrolase Gene
CDC	Centers for Disease Control and Prevention
ETEC	Enterotoxigenic *Escherichia coli*
gadB	Glutamate Decarboxylase Beta Gene
GI	Gastrointestinal
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
InlA	Internalin A
InlB	Internalin B
MLN	Mesenteric Lymph Node
PBS	Phosphate Saline Buffer
ROS	Reactive Oxygen Species
SCFAs	Short Chain Fatty Acids
SSA	Salicylic Acid
TSA	Tryptic Soy Agar
VP	Vinylpyridine

Chapter 1: Introduction

Listeria monocytogenes is a rod-shaped, Gram-positive, facultative anaerobe known to cause the food borne illness listeriosis. Although listeriosis is typically asymptomatic in healthy adults, it is generally severe in pregnant women, newborns, older populations, and patients with compromised immunity. Clinical manifestations include meningitis and septicemia, and in the case of pregnant women, complications can result in miscarriage or stillbirth (1). A Centers for Disease Control and Prevention (CDC) survey of listeriosis data from 2009 to 2011 identified 1651 cases nationwide, with a fatality rate of 21% (2). In 2010, approximately 23,150 cases were estimated worldwide, with a mortality rate of approximately 23% (5463 deaths) (3).

Listeria monocytogenes is ubiquitous in the environment, and owing to its ability to resist a variety of stressors such as low pH, low temperature, and high salt concentrations, it easily contaminates food products such as milk and dairy products, various meat products, vegetables, and fruits. Twelve outbreaks were reported from 2009 to 2011; these include five outbreaks from soft cheese made from pasteurized milk, five from different types of cheeses, and two outbreaks linked to raw produce (2). Similarly, inside the mammalian host, including humans, *Listeria* must survive the harsh conditions of the gastrointestinal (GI) tract, such as variations in pH and oxygen availability, presence of bile, and osmolarity, as demonstrated in Figure 1.1 (4). This adaptability observed in *Listeria* is largely due to its ability to sense the conditions of its environment and regulate the expression of genes related to stress response mechanisms for rapid adaptation. Therefore, it is important to understand how this pathogen interacts with these stressors in order to develop treatments targeting those interactions.

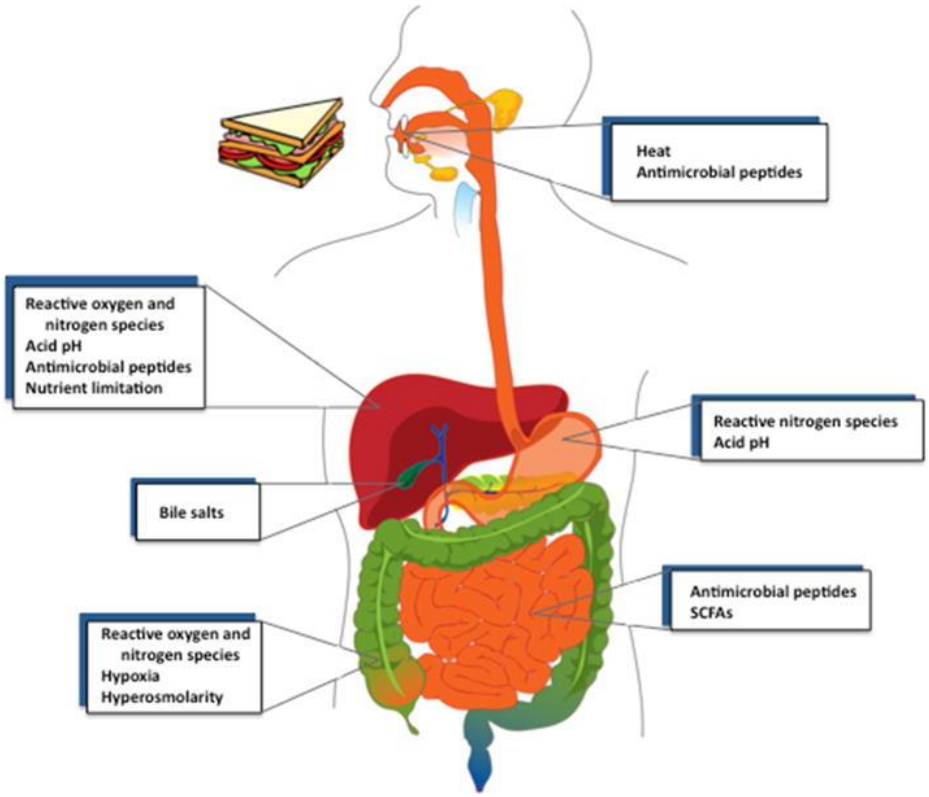

Figure 1.1. Stressors commonly encountered by foodborne enteric bacteria within the gastrointestinal tract. *Upon ingestion of contaminated food, foodborne bacteria must resist the acidic conditions, bile salts, reactive oxygen species and nitrogen species, changes in oxygen availability, short chain fatty acids (SCFAs), and many other stressors (4).*

Bile and variation in oxygen availability are two common stressors encountered by *L. monocytogenes* within the GI tract. Although bile is a bactericidal agent that causes damage to the membrane and DNA of enterics (5), *Listeria* is not only able to tolerate bile, but also can utilize bile as a signal to enhance infection and virulence (6, 7, 8). However, there is a scarcity of literature on the impact of bile under physiologically

relevant anaerobic conditions. Similarly, the effect of bile on the redox state of *L. monocytogenes* under anaerobic conditions is yet to be deciphered. Therefore, the aim of this study is to decipher the effects of bile and oxygen availability on the redox status of *L. monocytogenes*. Preliminary findings showed that under anaerobic conditions, exposure to bile significantly lowered the amount of oxidative damage present in bile-resistant strain F2365 cells (9). Similarly, *Listeria* further elicits the adaptive immune response, wherein pre-exposure to stress during food processing or in the host prior to entry into the intestine, increases bile tolerance (10). Based on these previous studies, this project tests the hypothesis that bile induces oxidative damage under aerobic, but not anaerobic conditions and that pre-exposure to oxidative stress can improve the oxidative stress response expressed from exposure to bile. The rationale for this project is that understanding the impact of bile under physiolgically relevant conditions will help us better understand the bile resistant mechanisms utilized by *L. monocytogenes*. Additionally, identification of the environmental and host factors that contribute to *Listeria*'s virulence and proliferation can help to identify therapeutic targets for listeriosis prevention and treatment.

Chapter 2: Literature Review

2.1. The intracellular lifecycle of *Listeria monocytogenes*

Inside the GI tract, *L. monocytogenes* invades the intestinal epithelial cells and macrophages and replicates intracellularly within these cells, which allows for the pathogen to avoid the host immune system. This internalization into the mammalian cells is accomplished by inducing phagocytosis largely through the action of two surface-expressed proteins, InlA and InlB (internalins A and B), on different host cell surface receptors (11, 12, 13). This is followed by several events in succession: lysis of the phagocytic vacuole by listeriolysin O and phospholipase C enzymes, replication inside the cytoplasm, recruitment of actin to the bacterial surface for actin-based motility using pseudopods, and internalization of *Listeria* by neighboring cells (13). Inside the neighboring cell, the bacterium is housed inside a two-membrane phagosome, whose lysis then infects the cytoplasm and re-initiates the entire cycle (13). This mechanism allows *Listeria* to disseminate throughout the host, avoiding detection by the immune cells.

2.2. Stressors commonly encountered by *L. monocytogenes*

Once ingested, *L. monocytogenes* first encounters the acidic condition of the stomach. Although low pH acts as an important initial host defense system to infections, several virulent strains of *L. monocytogenes* contain different regulatory systems to manage the acidic stress. One such system is the acid tolerance response (ATR), wherein bacteria exposed to mildly acidic stress acquire advanced acid resistance and maintain pH homeostasis (14). This phenomenon has been shown in a previous study, where

preexposure to mildly acidic conditions enhanced the survival of *L. monocytogenes* upon subsequent exposure to lethal acid (15). In another study, the intraintestinal population levels of *L. monocytogenes* and its translocation rate to the mesenteric lymph node (MLN) increased post acid-adaptation in a murine model of intragastric infection (16). This is particularly important in food processing as the ATR system was also shown to provide cross-protection against other stress, including food-related stresses like heat and osmotic stress (17).

Bile is another prominent stressor that *L. monocytogenes* encounters within the intestines. After production in the liver from cholesterol, bile is stored in the gall bladder and released into the duodenum following the intake of food (5, 18, 19). Table 2.1 summarizes the components of bile (20, 21, 22). Because bile is a digestive secretion able to disrupt bacterial membrane structure, affect membrane proteins, and disturb cellular homeostasis, an enteric pathogen's ability to resist bile is crucial to its ability to survive and colonize the GI tract (5). Interestingly, despite the high concentration of bile in the gall bladder, *L. monocytogenes* is able to grow extracellularly in the lumen of the gall bladder in animals, revealing the gall bladder lumen as a niche for this pathogen (23). The bacteria replicating in the gall bladder can serve as a reservoir of infection, as they can be effectively expelled from the organ, move into the intestinal tract and cause reinfection (24).

Table 2.1 Bile composition within gall bladder and liver (21, 22, 25)

Constituent	Gall Bladder Bile %	Liver Bile %
Water	89.0	98.0
Solids	11.0	2.0
Inorganic salts	0.8	0.75
Bile salts/acids	6.0	0.9
Mucin and pigments	3.0	0.4
Cholesterol	0.38	0.06

Two genes, bile salt hydrolase (*bsh*) and bile exclusion (*bilE*), are key genes involved in bile resistance (26, 27). The bile salt hydrolase, which catalyzes the deconjugation of bile acids, contributes to the survivability of *L. monocytogene*s in the intestinal lumen and liver, thereby acting as a virulence factor (26). Similarly, functional inactivation of *bilE* has been shown to significantly reduce the bacterium's resistance to lethal and physiological concentrations of bile, suggesting a virulence role (27). Both *bsh* and *bilE* are regulated by transcriptional activators *prfA* and Sigma B (σ^B) (26, 27). Although low oxygen tension increases *bsh* activity, the *bsh* gene transcription and production level of the PrfA regulatory protein are not altered by hypoxia, which suggests that there is likely a post-transcriptional mechanism: a *prfA*-independent and oxygen-dependent regulation of *bsh* activity (26). Similar to acid tolerance, a pathogen's ability to resist bile inside the host can be altered by its pre-exposure to stress during food processing or in the host before entry into the intestine (10). These phenomena of adaptive resistance (stress hardening) and cross-adaptation might exist because many stresses cause activation of the same set of stress response proteins (28). For example, when *L. monocytogenes* was pre-exposed to acid (pH 5.5), heat (42^{o}C), salt (5% NaCl), or SDS (0.01%); bile tolerance was increased (10).

L. monocytogenes is a facultative anaerobe and encounters varying oxygen availability inside the GI tract as well as during food processing. Several studies have shown that oxygen availability either during food processing or inside the host influences the pathogen's ability to survive and respond to stressors inside the host. For example, *L. monocytogenes* grown under oxygen-restricted conditions is approximately 100 times more invasive in Caco-2 (human epithelial colorectal adenocarcinoma) cells and is significantly more invasive in guinea pigs (29). Similarly, transcriptional analysis of *L. monocytogenes* under hypoxic conditions revealed 161 differentially expressed genes; internalins (A & B), which are important for GI invasion and survival, are upregulated under hypoxic conditions (30). Another study conducted to determine if oxygen availability influenced bile resistance found a significant increase in bile resistance under anaerobic conditions; however, this phenomenon occured in a strain-dependent manner (31).

2.3. Stress response mechanisms of *L. monocytogenes*

Listeria's ability to resist stressors is largely attributed to two regulatory factors. One of them is Sigma B (σ^B), an alternative sigma factor characterized in several Gram-positive bacteria including *Bacillus subtilis* (32) and *Staphylococcus aureus* (33). The alternate sigma factor σ^B has been recognized as a general stress-responsive sigma factor (34), with σ^B-dependent genes including both stress response {glutamate decarboxylase beta *gadB* (acid response) , *ctc* (osmotolerance), glutathione reductase gene *lmo1433* (oxidative stress)} and virulence genes (*inlA*, *inlB*, and *bsh*) (35). Sigma factor σ^B is also implicated in the transcriptional activation of the second regulatory protein, PrfA (36). It was observed that *L. monocytogenes* virulence and invasion are governed by a complex

σ^B-PrfA regulatory network (36). The PrfA regulon largely consists of virulence genes, appearing to be switched "on" after the bacteria enter the host cell, while σ^B-dependent mechanisms leads to the expression of stress response genes induced under environmental and host stress (36). An elaborate system in *L. monocytogenes* allows for cross-talk between the regulatory circuits of σ^B and PrfA, ensuring optimal expression of stress-related functions and virulence genes in the host environment, as shown in Figure 2.2 (25, 37, 38). Because the σ^B-PrfA network regulates the global stress response, one of its potential roles is to prime *Listeria* preexposed to mild or sub-lethal stress to develop resistance to subsequent exposure of the same or a different stressor, resulting in a robust and persisting strain in a given environment (39, 40).

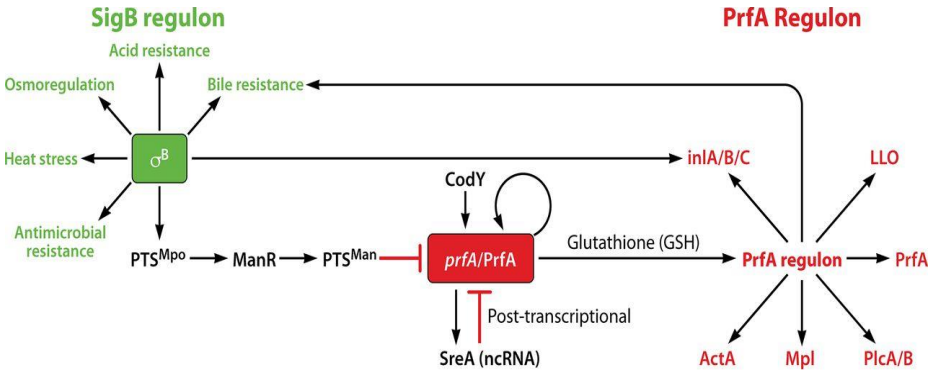

Figure 2.2 Cross-talk between the regulatory circuits controlled by σ^B and PrfA regulons. *The σ^B and PrfA regulons control various mechanisms required for survival within the mammalian GI tract, including genes required for internalization into eukaryotic cells (38).*

2.4. Overview of oxidative stress generation

As mentioned above, food borne pathogens like *L. monocytogenes* are commonly stressed during food processing. For example, in cheese manufacturing processes, the

pathogens that are present in raw milk are exposed to a variety of stresses, including hydrogen peroxide (40). Oxidizing agents, such as hydrogen peroxide (H_2O_2), ozone, and chlorine, are popular biocidal agents used in many food, medical, and environmental applications (41). However, the use of such agents exposes the pathogens to sub-lethal concentrations of oxidative stress, which can cause stress hardening and increase the pathogens' resistance to lethal concentrations of same or different stressor (10). Inside the GI tract, bile salts induce oxidative stress in bacteria, as evidenced by the upregulation of genes that are related to oxidative stress (42). For example, in *Campylobacter jejuni*, the bile salt sodium deoxycholate elevates reactive oxygen species (ROS), ultimately resulting in DNA damage; the bacterium adapts to deoxycholate to by producing enzymes that mitigate ROS accumulation (20). In *Escherichia coli*, bile salts cause widespread protein unfolding, triggering protein aggregation *in vitro* and *in vivo*. *In vivo*, bile salts also cause disulfide or oxidative stress by reducing the reduced glutathione to oxidized glutathione ratio (GSH:GSSG) (43).

Oxidative stress is the imbalance between reactive oxygen species generation and elimination (44). Reactive oxygen species are highly reactive free radicals capable of causing damage to proteins, lipids, and nucleotides, and thereby negatively affecting bacterial cells (45). Glutathione (L-gamma-glutamyl-L-cysteinylglycine), a low molecular weight intracellular tripeptide with a thiol group, exists predominantly as reduced glutathione (GSH) (46). GSH is an antioxidant and is one of the most important scavengers of ROS; therefore its ratio to oxidized glutathione (GSSG) is used as a marker of oxidative stress (46). The accumulation of GSSG occurs when cells are exposed to

increased levels of oxidative stress, therefore, a decreased level of the GSH:GSSG ratio is an indicator of oxidative stress (46).

2.5. Project rationale and hypothesis

Enteric pathogens, including *L. monocytogenes*, have evolved to utilize bile as a signal to enhance infection and virulence (6). In enterotoxigenic *Escherichia coli* (ETEC), components of bile, such as the bile salt sodium deoxycholate and the glycoconjugated primary bile acid sodium glycocholate, induce the expression of colonization factors (surface proteins that adhere the bacterium to the intestinal epithelium) (7). Another study on ETEC found that bile salts induce the expression of many evirulence factors like heat-stable and heat-labile enterotoxins (8). Similarly, a study to understand the impact of bile on *L. monocytogenes* under physiologically relevant anaerobic conditions revealed that under anaerobic conditions, exposure to bile significantly lowered the amount of oxidative damage present in bile-resistant *L. monocytogenes* strain F2365 cells (9).

Taking into account these previous studies, this study tests the hypothesis that bile induces oxidative damage under aerobic, but not anaerobic conditions and that pre-exposure to oxidative stress can improve the oxidative stress response expressed from from exposure to bile. Although the impact of bile under aerobic conditions has been well studied, there is a scarcity of literature on its effects under physiologically relevant anaerobic conditions. Similarly, the effect of bile on the redox state of *L. monocytogenes* under anaerobic conditions is yet to be deciphered. Through this study, the effects of bile and anaerobic conditions on the redox status of *L. monocytogenes* are evaluated.

Chapter 3: Methodology

3.1. Bacterial strain and culture conditions

The strain of *Listeria monocytogenes* used in this study was F2365 (serovar 4b). F2365 cells were stored as a frozen stock in 20% glycerol at -80^{o}C. The cells were routinely grown on Tryptic Soy Agar (TSA) prior to being cultured in Tryptic Soy Broth (TSB) at 37^{o}C. Anaerobic studies were conducted inside a Coy anaerobic chamber supplied with a gas mix of 95% N_2 / 5% H_2. Before initiating experiments under anaerobic conditions, freshly autoclaved media were placed in the chamber for 3 days prior to usage.

3.2. Sample preparation

Cultures were grown overnight at 37^{o}C in a shaker incubator and were then inoculated at a 1:100 ratio in TSB and grown to mid-logarithmic phase (OD_{600}= 0.3-0.5) under aerobic and anaerobic conditions. After the cells reached the mid-logarithmic phase, the cultures were split into four separate 10 mL aliquots and centrifuged at approximately 9000 x *g* for 5 minutes. One aliquot was collected at this time as the 'non-treated' cells. The remaining three aliquots were subjected to the following treatments: TSB supplemented with 50 mM H_2O_2 for 30 minutes; TSB supplemented with 50 mM H_2O_2 for 30 minutes, followed by resuspension in TSB supplemented with 1% porcine bile for 1 hour; TSB supplemented with 1% bile for 1 hour. To collect and store the samples for further use, the cells were washed three times with Phosphate Buffer Saline (PBS) and pellets were stored at -80^{o}C. A minimum of three independent replicates was performed.

3.3. Glutathione colorimetric detection assay

A glutathione colorimetric detection kit (Invitrogen, Catalog # EIAGSHC) was used to determine the GSH:GSSG ratio according to the manufacturer's instructions. Briefly, pellets were first washed in ice-cold PBS, followed by resuspension in ice-cold 5% salicylic acid (SSA) (1 g of aqueous 5-sulfo-salicylic acid dehydrate in 20 mL water). Cells were then lysed using 0.1 mm Zirconia beads in a homogenizer (Fisher Bead Mill 24 Homogenizer). After lysis, samples were incubated at 4^{o}C, followed by centrifugation at approximately 22,000 x *g* for 10 minutes at 4^{o}C. The resulting supernatant was separated into two aliquots: one for the measurement of total GSH (total GSH includes both GSH and GSSG) and one for the measurement of oxidized glutathione GSSG. For GSSG measurements, the samples were treated with 2-vinylpyridine (2VP) solution (1 µl for every 50 µl of sample) for 1 hour at room temperature. The 2VP solution was prepared by adding 27 µL of 2-vinylpyridine to 98 µL of ethanol. The 2VP treatment blocks any free GSH in the sample, thereby allowing for the measurement of GSSG. The samples (2VP treated and untreated) were then diluted by adding 4 volumes of the kit's assay buffer, bringing the SSA concentration to 1% with the total dilution of 1:5.

Standard dilutions were prepared as follows: for total GSH, 25 µl of the kit's Oxidized Glutathione Standard was added to 475 µl of sample diluent (prepared by diluting 5% SSA with Assay Buffer in the ratio 1:5, pH adjusted to >6) for a final concentration of 25 µM total GSH. Two-fold serial dilutions were made in sample diluent (12.5, 6.25, 3.125, 1.56, 0.791, and 0 µM). For the GSSG standard, 1 µL of 2VP solution was added to 50 µl of Oxidized Glutathione Standard and incubated for 1 hour at room temperature. Then 25 µL of this treated standard was added to 475 µL sample diluent

(final concentration of 12.5 μM GSSG). Again, serial dilutions were conducted in sample diluent (6.25, 3.125, 1.56, 0.781, 0.391, and 0 μM GSSG).

A colorimetric detection reagent was prepared by mixing Detection Reagent Concentrate and Assay Buffer in the ratio of 1:9. Similarly, the reaction mixture was prepared by mixing NADPH Concentrate, Glutathione Reductase Concentrate, and Assay Buffer supplied in the kit in the ratio of 1:1:8. To perform the assay, 50 μl of the standards or samples were added to the wells of a half area 96-well plate in duplicate. This was followed by the addition of 25 μl of colorimetric detection reagent and 25 μl of reaction mixture. The contents were mixed well. The plate was incubated for 20 minutes at room temperature before reading the absorbance at 405 nm. Finally, standard curves were generated for both GSSG and total GSH. The GSSG and total GSH concentration for the samples was calculated from the standard curve and adjusted for dilution. Free GSH was determined by subtracting the GSSG concentration from the total GSH and the GSH:GSSG ratio was calculated by dividing the free GSH concentration by GSSG concentration of each sample.

Chapter 4: Results

4.1. Extent of oxidative stress induced in F2365 was similar in both aerobic and anaerobic conditions.

The GSH:GSSG ratios in the F2365 cells grown under aerobic and anaerobic conditions were first compared. The GSH:GSSG ratios in untreated cells under aerobic and anaerobic conditions were analyzed via a two-tailed T-test (Figure 4.1). No significant difference is observed between the two treatment groups ($p > 0.05$).

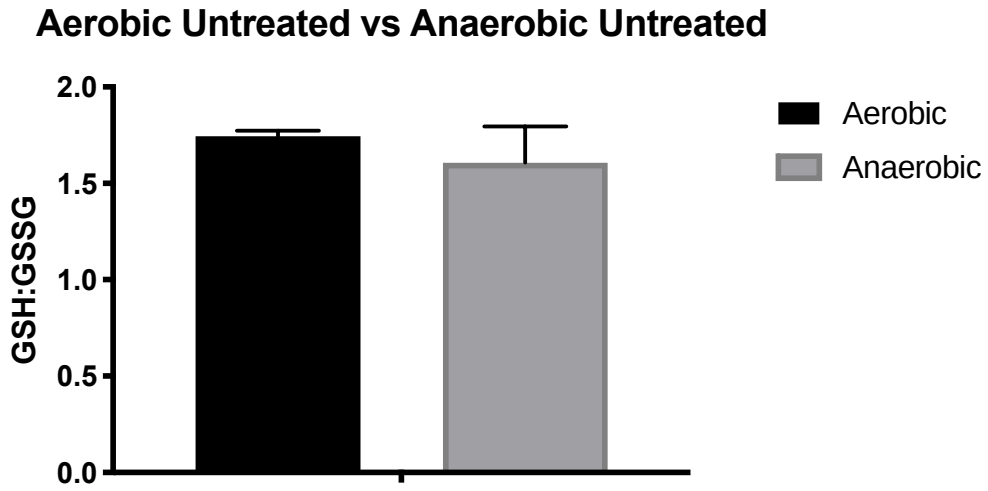

Figure 4.1 GSH:GSSG ratio in F2365 cultured under either aerobic or anaerobic conditions. *Data represent the mean of three independent replicates. Error bars represent standard deviation of triplicate, independent sample measurements. No significant difference was found between the two treatment groups ($p > 0.05$).*

4.2. Bile induces oxidative stress under aerobic, but not anaerobic, conditions.

It was previously hypothesized that bile induced oxidative damage to F2365 under aerobic conditions and reductive damage under anaerobic conditions. Therefore, to determine if bile induced different types of damage in *L. monocytogenes,* cells were treated with bile under both aerobic (Figure 4.2) and anaerobic (Figure 4.3) conditions and the GSH:GSSG ratio was analyzed. Alterations in the ratio of GSH:GSSG can indicate oxidative damage being present in the cell (i.e., increase in the amount of GSSG in comparison to GSH). Treatment with H_2O_2 was used as a control for oxidative stress. Statistical significance between the treatment conditions was determined with One-way ANOVA. As expected, there is a significant decrease in the GSH:GSSG ratios between untreated cells and H_2O_2-treated cells as well as between untreated cells and bile-treated cells ($p < 0.001$), indicating that both hydrogen peroxide and bile induce oxidative stress in F2365 under aerobic conditions. Under anaerobic conditions, however, the difference in the GSH:GSSG ratio was only significant between untreated cells and H_2O_2-treated cells ($p < 0.05$), indicating bile did not induce oxidative damage under anaerobic conditions.

4.3. Pre-exposure to H_2O_2 does not protect F2365 against bile-induced oxidative stress.

To determine if pre-exposure to an oxidizing agent could protect *L. monocytogenes* F2365 from oxidative damage induced by bile, cells were pretreated with H_2O_2 prior to treatment with bile. This was tested under both aerobic (Figure 4.2) and anaerobic (Figure 4.3) conditions. Statistical significance between the treatment conditions was determined with One-way ANOVA. There was no significant difference

in the GSH:GSSG ratios between the H_2O_2-treated cells and the cells treated with bile post exposure to H_2O_2 under both aerobic and anaerobic conditions ($p > 0.05$). Under aerobic conditions, cells treated with bile post exposure to H_2O_2 continued to show oxidative stress; the GSH:GSSG ratio was significantly lower ($p < 0.001$) compared to that in the untreated cells (Figure 4.2). However, this was not the case under anaerobic conditions, as no statistically significant difference was observed between untreated cells and cells treated with H_2O_2 followed by bile ($p > 0.05$).

Under aerobic conditions, the GSH:GSSG ratio was significantly lower for cells pre-treated with H_2O_2 prior to exposure to bile, as compared to the cells only treated with bile ($p < 0.01$). However, under anaerobic conditions, there was no significant difference in the GSH:GSSG ratios between the conditions ($p > 0.05$).

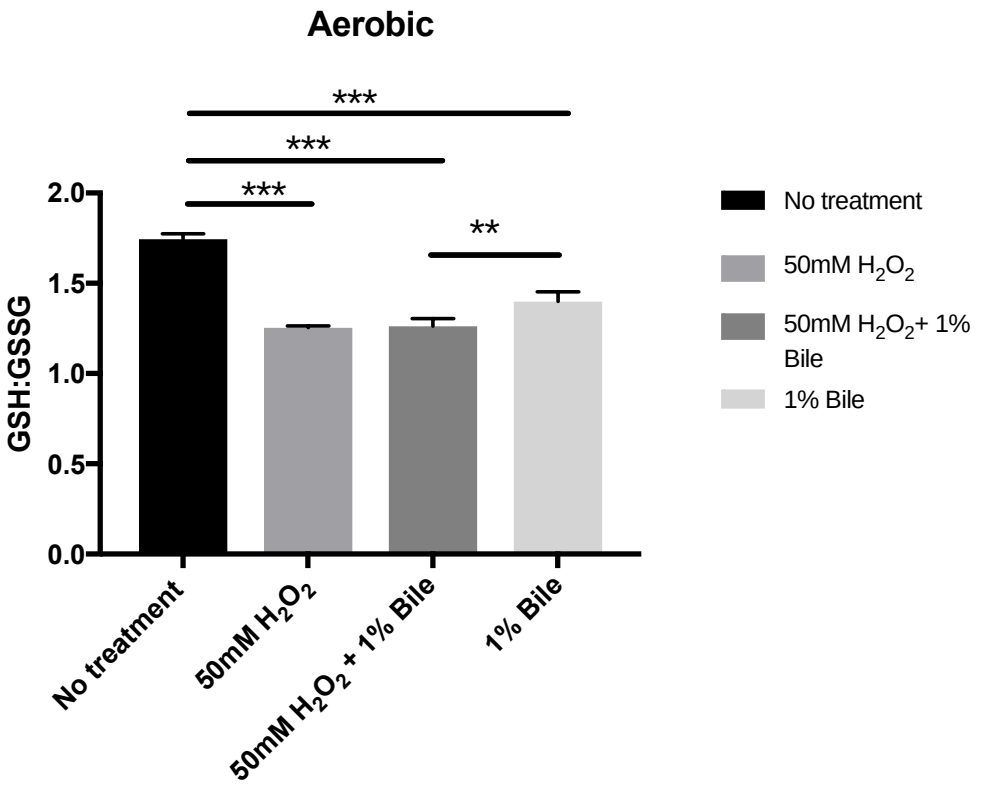

Figure 4.2 GSH:GSSG ratio in F2365 cells under aerobic condition. *Cells were exposed to one of four different treatments- no treatment, treatment with 50mM H_2O_2 for 30 min, treatment with 1% bile for 1 h after treatment with 50mm H_2O_2 for 30 min, and treatment with only 1% bile for 1 h. Each graph represents the mean of three independent replicates. Error bars represent standard deviation of the three replicates. Statistical significance between the treatment conditions was determined with One-way ANOVA. *** indicates $p < 0.001$ and ** indicates $p < 0.01$.*

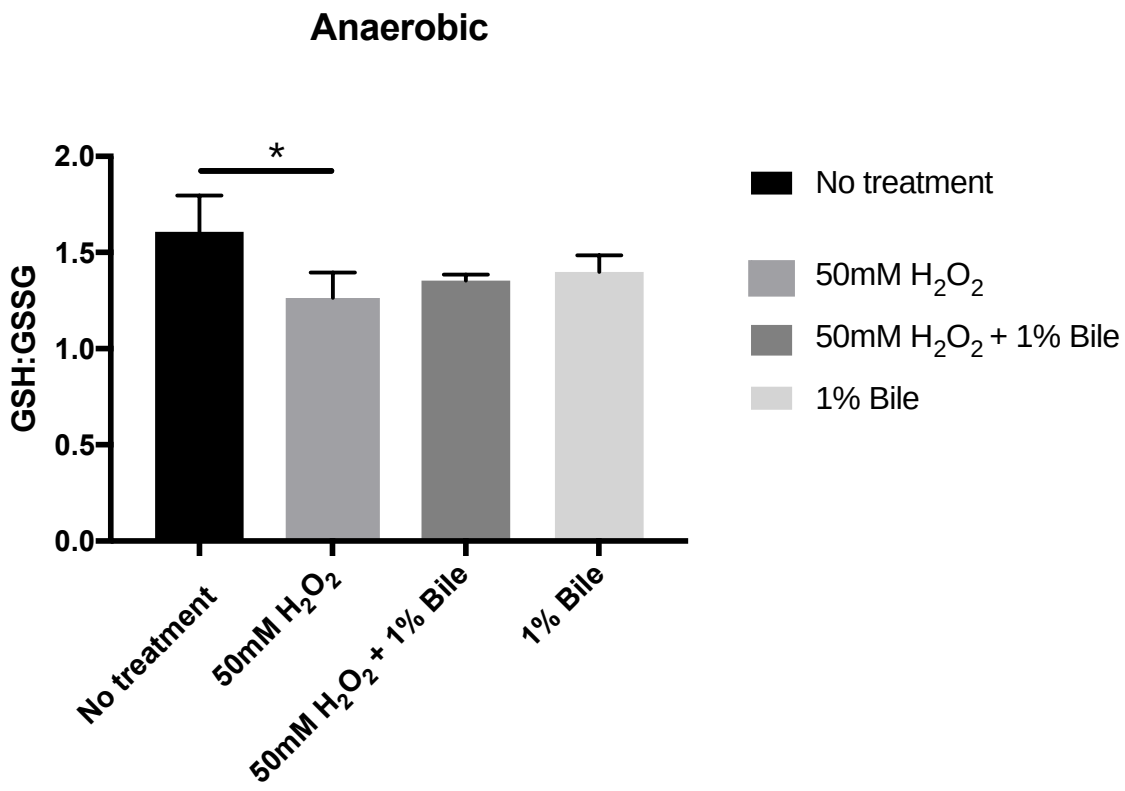

Figure 4.3 GSH:GSSG ratio in F2365 cells under anaerobic conditions. *Cells were treated with one of four different treatments- no treatment, treatment with 50mM H_2O_2 for 30 min, treatment with 1% bile for 1 h after treatment with 50mm H_2O_2 for 30 min, and treatment with only 1% bile for 1 h. Each graph represents the mean of three independent replicates. Error bars represent standard deviation of the three replicates. Statistical significance between the treatment conditions was determined with One-way ANOVA. * indicates $p < 0.05$.*

Chapter 5: Discussion

Bile is a bactericidal factor produced by the mammalian host and is encountered by *L. monocytogenes* inside the small intestine and gall bladder. One of the ways bile affects bacteria is by inducing oxidative stress, which is the imbalance between reactive oxygen species generation and elimination (42, 43, 44). Reactive oxygen species are highly reactive free radicals capable of causing damage to proteins, lipids, and nucleotides, and thereby adversely affecting bacterial cells (45). One of the most important scavengers of ROS is reduced glutathione (GSH), therefore its ratio to oxidized glutathione (GSSG) is used as a marker of oxidative stress (46). Enteric pathogens, including *L. monocytogenes*, have evolved to utilize bile as a signal to enhance infection and virulence (6, 7, 8). Preliminary results have shown that in the strain F2365, exposure to bile significantly lowered the amount of oxidative damage present in the cells under anaerobic conditions (9). Based on these previous results, this study tested the hypothesis that bile induces oxidative damage under aerobic, but not anaerobic conditions and that pre-exposure to oxidative stress can improve the oxidative stress produced from exposure to bile. Through this study, we aimed to decipher if bile affects the redox state of *L. monocytogenes* differently under aerobic and anaerobic conditions.

To determine if there is a difference in the redox state of F2365 cells grown under aerobic and anaerobic conditions, the GSH:GSSG ratios in the F2365 cells grown under aerobic and anaerobic conditions were compared. There is no significant difference in the GSH:GSSG ratios between the cells grown under the two conditions. In *E. coli*, in normal redox state, 99.5% glutathione exists as GSH and 0.17-0.33% exists as GSSG, with a

GSH:GSSG ratio of 300-600 (47). Compared to that ratio, the ratio observed in F2365 is very low (about 1.7), which might indicate oxidative stress in both aerobic and anaerobic conditions. However, because a normal GSH:GSSG ratio for *L. monocytogenes* is unknown, such an assumption cannot be made. It is interesting that the GSH:GSSG ratios between the aerobic and anaerobic conditions do not indicate any difference. Further research is required to investigate the GSH:GSSG ratio in *L. monocytogenes* at various oxygen concentrations to determine the impact of oxygen on the oxidative stress state of the cell.

The GSH:GSSG ratios were measured in cells subjected to one of four treatments: no treatment, 50 mM H_2O_2 only, 50 mM H_2O_2 followed by 1% bile, and 1% bile only. As expected, there is a significant decrease in the ratios between untreated and H_2O_2-exposed cells, as well as between untreated cells and bile-exposed cells, suggesting both hydrogen peroxide and bile induce oxidative stress in aerobic conditions. In anaerobic conditions, however, only H_2O_2 induced oxidative stress. There is no statistical difference in the GSH:GSSG ratio between the untreated cells and the bile treated cells, which indicates that bile does not induce oxidative stress under anaerobic conditions. This could explain the increase in bile resistance in F2365 under anaerobic conditions (31). This indicates that bile-resistant strain F2365 responds to bile differently based on oxygen availability. This is important as *Listeria* encounters varying levels of oxygen inside the gastrointestinal tract.

There is no significant difference in GSH:GSSG ratios between the cells treated with H_2O_2 only and the cells treated with bile after exposure to H_2O_2. This is applicable to both aerobic and anaerobic conditions. However, under aerobic conditions, treatment

with bile continued to maintain oxidative stress in the cells, which was not the case in anaerobic conditions. Anaerobically, an oxidative stress post bile exposure was not observed. This also indicates a different effect of bile between aerobic and anaerobic conditions.

This trend was also observed for a comparison of bile-treated cells with bile-treated F2365 pre-exposed to H_2O_2. Contrary to the hypothesis that pre-exposure to hydrogen peroxide prior to bile exposure protects F2365 from bile-induced oxidative stress, it was seen that pre-exposure to hydrogen peroxide induced more stress than bile alone. However, this was only true for aerobic conditions. Under anaerobic conditions, neither bile alone nor pre-exposure to H_2O_2 induced oxidative stress. Taken together, these results could be interpreted to support that either bile-resistant characteristic of F2365 is due to its ability to resist bile-induced oxidative stress under anaerobic conditions, or under anaerobic conditions bile lacks the ability to induce oxidative stress in F2365. Further research is needed to understand bile's mechanism of action under aerobic and anaerobic conditions, and to determine whether the similar GSH:GSSG ratios observed under both aerobic and anaerobic conditions indicate oxidative or reductive stress. Future studies should include analyzing the production of reactive oxygen species directly and monitoring the expression levels of different stress response genes under these conditions.

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