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Bile-induced damage in Listeria monocytogenes

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BILE-INDUCED DAMAGE IN *LISTERIA MONOCYTOGENES*

By

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Listeria monocytogenes is an enteric pathogen that can replicate within bile, yet this capability differs between strains. This project analyzed whether the pathogenic potential of the strain affects the ability to resist bile. We tested this hypothesis by examining the effect of bile on the morphology of a virulent strain (EGD-e) and an avirulent strain (HCC23) under aerobic and anaerobic conditions. Our data showed that exposure to bile greatly impacted the growth of HCC23. Additionally, scanning electron microscopy and transmission electron microscopy analyses indicated that bile affects the cell envelope of EGD-e and HCC23 differently. Our results suggest that differences exist in the ability of EGD-e and HCC23 to survive and replicate in the presence of bile. We propose that the virulence capability of *L. monocytogenes* directly correlates to its ability to resist the detergent properties of bile.

DEDICATION

This thesis is dedicated to my family and friends who have supported me throughout this process. I would like to especially thank my parents, Marie and Larry Merritt, for their love and continuous support. Without these individuals this work would not have been possible.

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INTRODUCTION

Introduction

The digestive system typically plays a vital role against combating potentially pathogenic microorganisms before food-borne infections are established. The digestive system combats these microbes through producing several bactericidal agents along the tract. Some of these bactericidal agents are gastric secretions, hydrochloric acid, and bile. These agents all have distinct roles in ensuring infections do not arise, but depending on the conditions, they are not always effective in eliminating pathogens.

Gastric secretions and hydrochloric acid together lower the pH of the stomach to approximately 3.0. This acidic environment destroys a majority of bacteria that initially enter the stomach. The importance of this acidic environment is evident in studies with patients with the disease hypochlorhydria where production of less gastric juice results in an increase in the number of bacteria that survive within the stomach. Since the bactericidal property of the stomach is weak in these patients, potentially pathogenic microbes can then migrate to the small intestine to establish disease. This is evident by the fact that hypochlorhydria patients are more prone to infections by *Helicobacter pylori* and *Salmonella* (31, 53).

Bile is another bactericidal agent that is found in the digestive system. Bile is produced in the liver and stored in the gallbladder (37). The circulation of bile through the digestive system is a part of the enterohepatic circulation, which is activated with the intake of food. During this process the production of cholecystokinin triggers contraction of the gallbladder. This contraction leads to the release of bile into the intestines where it will eventually circulate back to the liver via the bloodstream (44). Bile comes into contact with ingested bacteria in the gastrointestinal tract during the enterohepatic circulation and inhibits the colonization of bacteria within the small intestine (21). The small intestine, which contains a very high amount of bile acids, typically harbors very few bacteria. If less bile is secreted, such is observed in patients with cirrhosis of the liver, bacterial overgrowth is observed in the small intestine, (13, 49) suggesting that bile has bactericidal properties in addition to aiding the digestion of fatty acids.

The composition of bile plays a role in its ability to exert its bactericidal effects. Bile is composed of a multitude of components, such as proteins, ions, pigments, cholesterol, and various bile salts. The bile salts are initially produced in unconjugated forms, such as cholate (CA), chenodeoxycholate (CDCA), deoxycholate (DOCA), lithocholate (LCA), and ursodeoxycholate (UDCA) (20). Further metabolism in the liver results in the formation of "conjugated" bile salts through the attachment of either a glycine or taurine to the side chain of these various bile salts. These conjugated bile salts can then pass into the gallbladder and continue through the enterohepatic circulation (44) (Fig. 1). Since the composition of bile, especially in regards to the type of bile salts present, may change as it passes through the gastrointestinal tract,

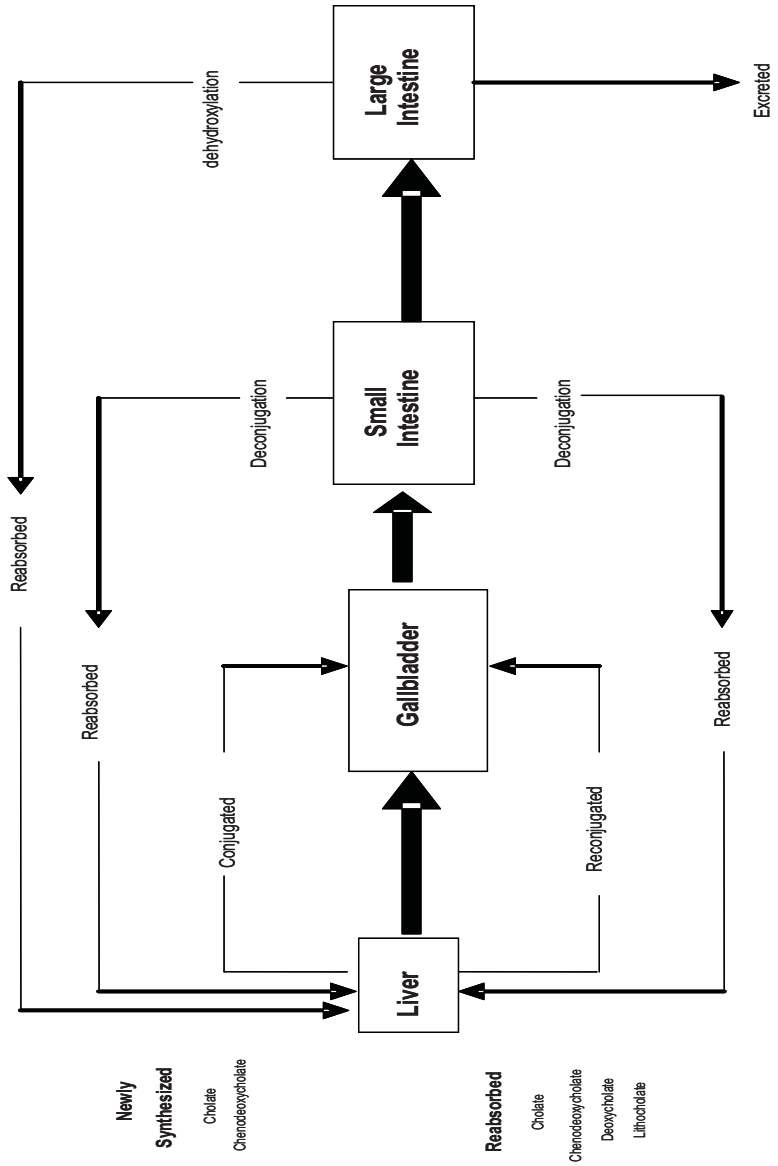

Figure 1.1 A diagram illustrating bile salt synthesis, processing, and cycling through the human gastrointestinal system.

understanding the differences in the antimicrobial properties of both conjugated and unconjugated forms of bile salts is of great importance in combating against resistant pathogenic bacteria. As a result many studies elucidating the role of bile salts in bacterial virulence have been conducted on bile mixtures that contain both conjugated and

unconjugated forms of salts, such as bile from ox gallbladder (oxgall) (14, 25, 55), bovine bile (38) and human bile (2).

Even though the gastric microbial barriers of the stomach and small intestine decrease the chance of colonization by pathogenic bacteria, they do not provide protection against bacteria that have adapted to survive within these extremely harsh conditions. The enteric bacteria are one class of bacteria that have mechanisms that allow them to survive and proliferate within the human gut. Several of these bacteria invade the gallbladder, including *Listeria monocytogenes, Salmonella enterica*, intestinal colonizer Enteroaggregative *Escherichia coli*, and feces present *Bacillus cereus* (12, 19, 22, 28). It is possible that the ability of these microbes to be able to survive in the presence of large quantities of bile salts is directly related to their ability to establish invasive infections.

In recent years there has been much work dedicated to understanding the role of bile salts in the resistance of these enteric bacteria. It has been speculated that the pathogenic potential of an enteric bacterium is directly related to its ability grow in the presence of bile. However, to determine if this hypothesis is true, the mechanisms by which bacteria are able to grow in bile environments need to be determined. To date, the mechanisms by which bile induces cell death are poorly understood; it has not been determined whether cell death results from damage at the membrane and/or DNA level. It is possible that the antimicrobial effect of bile salts elicit various mechanisms of resistance including the activation of several different stress response genes involved in membrane synthesis and protection and also in DNA repair (6, 28, 41).

Determining the effect that bile salts have on the integrity of the bacterial membrane has been mainly investigated through molecular analyses studying the

regulation of membrane proteins in the presence of bile (46). The upregulation of genes encoding outer membrane proteins, efflux pumps, and cell membrane biosynthesis enzymes are good indicators of the effect of bile and its interaction with the bacterial cell membrane (33, 45, 46). The membrane damaging capability of bile is exhibited with mutations in tol-pal genes that are essential for preserving the outer membrane of gram-negative bacteria such as *E. coli* and *Erwinia chrysanthemi* (15, 43) . These findings corroborate that the membrane and various components of the membrane are important for bacterial resistance to bile salts. The membrane components of efflux pumps in various pathogenic and commensal bacteria such as *E. coli, V. cholera* and *C. jejuni* expel bile salts from the interior after they have breached the cell membrane (10, 29, 54).

In addition to looking at the upregulation of genes involved in cell membrane synthesis, studies have also investigated the interaction of bile and the bacterial cell membrane by analyzing the composition of the membrane grown in the presence of bile. Bile alters the fatty acid composition as well as the ratio of membrane proteins to phospholipids, resulting in an altered cell surface structure in bacteria such as *Bifidobacterium animalis* and *Lactobacillus reuteri* (47, 52). Visual confirmation of cell surface deformities induced by bile can be accomplished by scanning electron microscopy or transmission electron microscopy (7, 8, 47). The integrity of the membrane of enteric bacteria in the presence of bile plays an important role in allowing for survival in the human digestive system.

The effect of bile on the integrity of the membrane has been reviewed in detail by others (3). Therefore, this review will focus on the mechanisms recently discovered that allow for protection and continued proliferation in bile environments of the Gram-

negative enteric pathogenic bacteria *Escherichia coli* and *Salmonella enterica* (Table 1) and the Gram-positive enteric pathogenic bacteria *Listeria monocytogenes* and *Bacillus cereus* (Table 2). The aim of this review is to aid in establishing a cohesive link between the effects of bile on bacteria to determine a common mechanism of resistance as it relates to protection of the DNA and the cell membrane among the enterics.

Bile Induced Damage in Gram Negative Bacteria

Escherichia coli.

Escherichia coli is an enteric pathogen and has been extensively studied as a model organism for the effect of bile on gram-negative bacteria. An initial study in 1991 by Kandell and Bernstein investigated whether bile salts could directly induce damage to the DNA of *E. coli* using a modified SOS chromotest (23). In the presence of chenodeoxycholic acid and sodium deoxycholate, *E. coli* had increased expression of the gene *sulA*. *sulA* is part of the SOS response system of bacteria and acts

Table 1.1

Bile Response-Associated Genes Induced in Gram-Negative Bacteria

Genes/operons	Induction	Bile Salt	Organism/Strain	Reference
SOS response				
sulA	+	NaDC	*E.coli* JL1705	(23)
sulA	+	NaCDC	E.*coli* JL1705	(23)
umuDC	-	NaGC	*E.coli* K12	(6)
umuDC	+	NaUDC	*E.coli* K12	(6)
umuDC	-	NaCDC	*E.coli* K12	(6)
umuDC	-	NaDC	*E.coli* K12	(6)
dinD	+	NaGC	*E.coli* K12	(6)
dinD	+	NaUDC	*E.coli* K12	(6)
dinD	+	NaCDC	*E.coli* K12	(6)
dinD	+	NaDC	*E.coli* K12	(6)
uvrB	-	NaDC	*S.typhimurium* SV5142	(41)
uvrB	-	Ox bile	*S.typhimurium* SV5142	(41)
dinB	+	NaDC	*S.typhimurium* DA7974	(41)
dinB	+	Ox bile	*S.typhimurium* DA7974	(41)
umuDC	-	NaDC	*S.typhimurium* SV5144	(41)
umuDC	-	Ox bile	*S.typhimurium* SV5144	(41)
Oxidative Genes				
micF	+	NaGC	*E.coli* K12	(6)
micF	+	NaUDC	*E.coli* K12	(6)
micF	+	NaCDC	*E.coli* K12	(6)
micF	+	NaDC	*E.coli* K12	(6)
zwf	+	NaGC	*E.coli* K12	(6)
zwf	-	NaUDC	*E.coli* K12	(6)
zwf	+	NaCDC	*E.coli* K12	(6)
zwf	+	NaDC	*E.coli* K12	(6)
soi28	-	NaGC	*E.coli* K12	(6)
soi28	-	NaUDC	*E.coli* K12	(6)
soi28	+	NaCDC	*E.coli* K12	(6)
soi28	+	NaDC	*E.coli* K12	(6)
katG	+	NaGC	*E.coli* K12	(6)
katG	-	NaUDC	*E.coli* K12	(6)

(Table 1 continued)

katG	-	NaCDC	*E.coli* K12	(6)
katG	-	NaDC	*E.coli* K12	(6)
dps	+	NaDC	*S. typhimurium* SV5158	(41)
katG	+	NaDC	*S. typhimurium* SV5157	(41)
fumC	+	NaDC	*S. typhimurium* SV5154	(41)
Stress Response				
clpB	-	NaGC	*E.coli* K12	(6)
clpB	+	NaUDC	*E.coli* K12	(6)
clpB	+	NaCDC	*E.coli* K12	(6)
clpB	+	NaDC	*E.coli* K12	(6)
uspA	+	NaGC	*E.coli* K12	(6)
uspA	-	NaUDC	*E.coli* K12	(6)
uspA	-	NaCDC	*E.coli* K12	(6)
uspA	+	NaDC	*E.coli* K12	(6)
dps	+	NaDC	*S.typhimurium* SV5158	(41)
Base-excision Repair				
alkA	-	NaDC	*S.typhimurium* SV5110	(41)
alkA	-	Ox bile	*S.typhimurium* SV5110	(41)
tagA	-	NaDC	*S.typhimurium* SV5111, SV5112	(41)
tagA	-	Ox bile	*S.typhimurium* SV5111, SV5112	(41)
mutM	-	NaDC	*S.typhimurium* SV5213	(41)
mutM	-	Ox bile	*S.typhimurium* SV5213	(41)
mutY	-	NaDC	*S.typhimurium* SV5167	(41)
mutY	-	Ox bile	*S.typhimurium* SV5167	(41)
nei	-	NaDC	*S.typhimurium* SV4992	(41)

(Table 1 continued)

nei	-	Ox bile	*S.typhimurium* SV4992	(41)
nth	-	NaDC	*S.typhimurium* SV4994	(41)
nth	-	Ox bile	*S.typhimurium* SV4994	(41)
Mismatch repair				
mutH	-	NaDC	*S.typhimurium* SV4802	(40)
mutL	-	NaDC	*S.typhimurium* SV4721	(40)
mutS	-	NaDC	*S.typhimurium* SV4858	(40)
DNA Repair				
impB	+	NaDC	*E.coli* 60 A	(22)
nfo	+	NaGC	*E.coli* K12	(6)
nfo	+	NaUDC	*E.coli* K12	(6)
nfo	+	NaCDC	*E.coli* K12	(6)
nfo	+	NaDC	*E.coli* K12	(6)
ada	-	NaGC	*E.coli* K12	(6)
ada	+	NaUDC	*E.coli* K12	(6)
ada	-	NaCDC	*E.coli* K12	(6)
ada	-	NaDC	*E.coli* K12	(6)
recA	+	NaGC	*E.coli* K12	(6)
recA	-	NaUDC	*E.coli* K12	(6)
recA	+	NaCDC	*E.coli* K12	(6)
recA	-	NaDC	*E.coli* K12	(6)
recA	+	NaDC	*S.typhimurium* SV4933	(41)
recA	+	NaC	*S.typhimurium* SV4851	(41)
recA	+	NaCDC	*S.typhimurium* SV4851	(41)
recA	+	NaGC	*S.typhimurium* SV4851	(41)
recA	+	NaTC	*S. typhimurium* SV4851	(41)

(Table 1 continued)

recA	+	NaGCDC	*S.typhimurium* SV4851	(41)
recA	+	NaDC	*S.typhimurium* SV4869	(41)
recA	+	Ox bile	*S.typhimurium* SV4869	(41)
recB	+	NaDC	*S.typhimurium* SV4844	(41)
recB	+	Ox bile	*S.typhimurium* SV4844	(41)
recC	+	NaDC	*S.typhimurium* SV5082	(41)
recC	+	Ox bile	*S.typhimurium* SV5082	(41)
recD	-	NaDC	*S.typhimurium* SV5166	(41)
recD	-	Ox bile	*S.typhimurium* SV5166	(41)
recF	-	NaDC	*S.typhimurium* SV5080	(41)
recF	-	Ox bile	*S.typhimurium* SV5080	(41)
recJ	-	NaDC	*S.typhimurium* SV5076	(41)
recJ	-	Ox bile	*S.typhimurium* SV5076	(41)
ada	-	NaDC	*S.typhimurium* SV5111	(41)
ada	-	Ox bile	*S.typhimurium* SV5111	(41)
ogt	-	NaDC	*S.typhimurium* SV5141	(41)
ogt	-	Ox bile	*S.typhimurium* SV5141	(41)
nfo	+	NaDC	*S.typhimurium* SV5159	(41)
dinB	+	NaDC	*S.typhimurium* DA7974	(41)
dinB	+	Ox bile	*S.typhimurium* DA7974	(41)

(Table 1 continued)

Transcriptional regulation				
merR	-	NaGC	*E.coli* K12	(6)
merR	+	NaUDC	*E.coli* K12	(6)
merR	+	NaCDC	*E.coli* K12	(6)
merR	-	NaDC	*E.coli* K12	(6)
ada	-	NaGC	*E.coli* K12	(6)
ada	+	NaUDC	*E.coli* K12	(6)
ada	-	NaCDC	*E.coli* K12	(6)
ada	-	NaDC	*E.coli* K12	(6)
marR	+	NaDC	*S. typhimurium* JSG782	(42)
marRAB	+	NaDC	*S. typhimurium* JSG782	(42)
marRAB	-	NaTC	*S. typhimurium* JSG782	(42)
marRAB	-	NaGC	*S. typhimurium* JSG782	(42)
marRAB	-	NaGCDC	*S. typhimurium* JSG782	(42)
acrAB	+	NaC	*S. typhimurium* JSG782	(42)
Cell Wall				
osmY	+	NaGC	*E.coli* K12	(6)
osmY	+	NaUDC	*E.coli* K12	(6)
osmY	+	NaCDC	*E.coli* K12	(6)
osmY	+	NaDC	*E.coli* K12	(6)

Table 1.2

Bile Response-Associated Genes Induced in Gram-Positive Bacteria

Genes/operons	Induction	Bile Salt	Organism/Strain	Reference
Motlility				
motA	+	NaC:NaDC	*B.cereus* 14579	(28)
cheY	+	NaC:NaDC	*B.cereus* 14579	(28)
fliS	-	NaC:NaDC	*B.cereus* 14579	(28)
fliF	-	NaC:NaDC	*B.cereus* 14579	(28)
fliG	-	NaC:NaDC	*B.cereus* 14579	(28)
flagellum-specific ATP synthase	-	NaC:NaDC	*B.cereus* 14579	(28)
flgE	-	NaC:NaDC	*B.cereus* 14579	(28)
flagellin (BC1657)	-	NaC:NaDC	*B.cereus* 14579	(28)
flagellin (BC1658)	+	NaC:NaDC	*B.cereus* 14579	(28)
flagellin (BC1659)	+	NaC:NaDC	*B.cereus* 14579	(28)
Transportation				
ABC transporter permease	+	NaC:NaDC	*B.cereus* 14579	(28)
Di-or tripeptide Transporter	+	NaC:NaDC	*B.cereus* 14579	(28)
Lincomycin Resistance	+	NaC:NaDC	*B.cereus* 14579	(28)
Na+ driven multidrug efflux pump	+	NaC:NaDC	*B.cereus* 14579	(28)
Transporter, Drug/Metabolite Exporter	+	NaC:NaDC	*B.cereus* 14579	(28)
Multidrug resistance protein B (BC4000)	+	NaC:NaDC	*B.cereus* 14579	(28)
Multidrug resistance protein B (BC4568)	+	NaC:NaDC	*B.cereus* 14579	(28)
Multidrug resistance protein A	+	NaC:NaDC	*B.cereus* 14579	(28)
Multidrug resistance protein B (BC4707)	+	NaC:NaDC	*B.cereus* 14579	(28)
Bacitracin transport permease protein BCRB	+	NaC:NaDC	*B.cereus* 14579	(28)
gadE (lmo0448)	+	oxgall	*L.monocytogenes* LO28	(4)
yxiO (lmo1417)	+	oxgall	*L.monocytogenes* LO28	(4)

(Table 2 continued)

Transcription Regulation				
RNA polymerase sigma factor	+	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator ctsR	+	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator, GntR family (BC4603)	-	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator, GntR family (BC1302)	+	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator, LytR family	+	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator, MarR family	+	NaC:NaDC	*B.cereus* 14579	(28)
tcdA-E operon negative regulator	+	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator, TetR family (BC3160)	+	NaC:NaDC	*B.cereus* 14579	(28)
Transcriptional regulator, TetR family (BC1814)	+	NaC:NaDC	*B.cereus* 14579	(28)
Bm3R1	+	NaC:NaDC	*B.cereus* 14579	(28)
plcR	+	NaC:NaDC	*B.cereus* 14579	(28)
hrcA	+	NaC:NaDC	*B.cereus* 14579	(28)
Two-component response regulator	+	NaC:NaDC	*B.cereus* 14579	(28)
zurR	+	oxgall	*L.monocytogenes* LO28	(4)
Stress Response				
groES	+	NaC:NaDC	*B.cereus* 14579	(28)
groEL	+	NaC:NaDC	*B.cereus* 14579	(28)
clpP	+	NaC:NaDC	*B.cereus* 14579	(28)
clpB	+	NaC:NaDC	*B.cereus* 14579	(28)
cspD	+	NaC:NaDC	*B.cereus* 14579	(28)
hsp20	+	NaC:NaDC	*B.cereus* 14579	(28)
sodA1	+	NaC:NaDC	*B.cereus* 14579	(28)
terD	+	NaC:NaDC	*B.cereus* 14579	(28)
thioredoxin	+	NaC:NaDC	*B.cereus* 14579	(28)

(Table 2 continued)

thioredoxin reductase	+	NaC:NaDC	*B.cereus* 14579	(28)
Peptide methionine sulfoxide Reductase	+	NaC:NaDC	*B.cereus* 14579	(28)
DNA Repair				
Site-specific recombinase	+	NaC:NaDC	*B.cereus* 14579	(28)
mutS	-	NaC:NaDC	*B.cereus* 14579	(28)
Superfamily I DNA and RNA helicases	+	NaC:NaDC	*B.cereus* 14579	(28)
Cytosine-specific methyltransferase	-	NaC:NaDC	*B.cereus* 14579	(28)
Type I restriction-modification system restriction subunit (BC4456)	+	NaC:NaDC	*B.cereus* 14579	(28)
Type I restriction-modification system methylation subunit (BC4459)	+	NaC:NaDC	*B.cereus* 14579	(28)
Carbamoyl-phosphate synthase small chain	-	NaC:NaDC	*B.cereus* 14579	(28)
pyrK	-	NaC:NaDC	*B.cereus* 14579	(28)
carA	+	NaC:NaDC	*B.cereus* 14579	(28)
uvrA	+	porcine bile	*L.monocytogenes* LMB472	(26)
Virulence				
Hemolysin BL lytic component L1	+	NaC:NaDC	*B.cereus* 14579	(28)
Internalin	-	NaC:NaDC	*B.cereus* 14579	(28)
Collagenase	-	NaC:NaDC	*B.cereus* 14579	(28)
(continued)				
Perfringolysin O	+	NaC:NaDC	*B.cereus* 14579	(28)
Sphingomyelin Phosphodiesterase	+	NaC:NaDC	*B.cereus* 14579	(28)
capA (lmo0516)	+	oxgall	*L. monocytogenes* LO28	(4)
Cell Wall				
Peptidoglycan N-acetylglucosamine deacetylase	-	NaC:NaDC	*B.cereus* 14579	(28)

(Table 2 continued)

UDP-N-acetylglucosamine 4-epimerase	-	NaC:NaDC	*B.cereus* 14579	(28)
UDP-bacillosamine synthetase	-	NaC:NaDC	*B.cereus* 14579	(28)
Beta-1,3-N-acetylglucosaminyltransferase	-	NaC:NaDC	*B.cereus* 14579	(28)
Cell wall endopeptidase, family M23/M37	+	NaC:NaDC	*B.cereus* 14579	(28)
S-layer homology domain / putative murein endopeptidase	+	NaC:NaDC	*B.cereus* 14579	(28)
Fatty Acid Biosynthesis and Degradation				
Fatty acid desaturase	+	NaC:NaDC	*B.cereus* 14579	(28)
Lysophospholipase L2	+	NaC:NaDC	*B.cereus* 14579	(28)
CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	+	NaC:NaDC	*B.cereus* 14579	(28)
Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta	-	NaC:NaDC	*B.cereus* 14579	(28)
Metabolism				
crr	+	NaC:NaDC	*B.cereus* 14579	(28)
(R,R)-butanediol dehydrogenase	+	NaC:NaDC	*B.cereus* 14579	(28)
Pyruvate kinase	+	NaC:NaDC	*B.cereus* 14579	(28)
pfkA	-	NaC:NaDC	*B.cereus* 14579	(28)
(continued from page 12)				
Fructose-1,6-bisphosphatase	+	NaC:NaDC	*B.cereus* 14597	(28)
NADH-dependent butanol dehydrogenase A	+	NaC:NaDC	*B.cereus* 14579	(28)
Flavodoxin	+	NaC:NaDC	*B.cereus* 14579	(28)
Protein Synthesis				
rpsS	-	NaC:NaDC	*B.cereus* 14579	(28)
rplK2	+	NaC:NaDC	*B.cereus* 14579	(28)
infA	-	NaC:NaDC	*B.cereus* 14579	(28)
fmt	-	NaC:NaDC	*B.cereus* 14579	(28)

(Table 2 continued)

pheS	-	NaC:NaDC	*B.cereus* 14579	(28)
miaA	+	NaC:NaDC	*B.cereus* 14579	(28)
Other				
thiocillin	+	NaC:NaDC	*B.cereus* 14579	(28)
thiocillin	+	NaC:NaDC	*B.cereus* 14579	(28)
lytB	+	oxgall	*L.monocytogenes* LO28	
bsh (lmo2067)	+	oxgall	*L.monocytogenes* LO28	(5)
pva (lmo0446)	+	oxgall	*L.monocytogenes* LO28	(5)
Bile acid 7-alpha dehydratase	+	oxgall	*L.monocytogenes* LO28	(5)

to halt cell division through inhibiting the formation of the FtsZ ring, which is a critical step in the early stages of cell division. This result indicates that the SOS response is induced in the presence of bile salts, and the activation of the SOS response is essential for the survival of the bacterium in the presence of bile. The authors compared their results to those of SOS-deficient cells in the presence of mitomycin c, a known inducer of the SOS response. Both studies produced similar results, supporting the theory that bile salts induce DNA damage *in vivo* in bacteria and that exposure to bile activates the SOS response.

Expanding upon these results, Berstein *et al.* (1999) investigated the ability of various bile salts to induce stress response genes to gain a general understanding of the effect of bile on the repair and stress response of *E. coli* (6). They tested the effect that the bile salts sodium deoxycholate, sodium chenodeoxycholate, sodium ursodeoxycholate, and sodium glycocholate had on 13 specific *E. coli* stress-response

genes (*osmY*, *recA*, *umuDC*, *micF*, *clpB*, *dinD*, *zwf*, *soi28*, *nfo, katG*, *uspA*, *merR, ada*). Using a similar technique as the Kandell and Bernstein study, the promoters of each gene were fused with a *lacZ* reporter gene allowing for detection of activity by measuring the level of β-galactosidase. The results of the study indicated that three promoters, *dinD*, *micF* and *osmY*, were significantly activated by all four bile salts. *dinD* is well known for being induced in the presence of DNA damage (24, 30, 36, 56), but its function remains unknown. The increase in expression of *dinD* indicated that the SOS response is a possible mechanism elicited in response to bile salts. *osmY* encodes for a periplasmic protein of unknown function commonly involved in osmotic stress, and *micF* is a negative regulator for the outer membrane porin protein OmpF (34). The increased transcription levels of *osmY* and *micF* genes are also indicators of oxidative damage (11, 34). Together, these results suggest that bile salts induce DNA damage through oxidative stress.

Recently a study on enteroaggregative *E. coli* showed that bile salts may induce error-prone DNA repair in strains containing an *imp*-positive locus (22). Error prone repair involves the polymerases Pol IV, encoded by *dinB*, and Pol V, encoded by *umuDC*. This mechanism allows for cells to continue to replicate in the presence of DNA damage, although it also leads to an increase in spontaneous mutations (17). In the study, the expression pattern of the repressed LexA gene *impB,* which is involved in error-prone DNA repair and a known homologue of *umuC*, was analyzed following treatment with either UV irradiation or bile salts. Following treatment, the SOS response gene *lexA* was derepressed and the *impB* gene was upregulated. Thus, it was proposed that SOS was induced to allow for repair of the damaged genome and continued survival. In support of

this hypothesis, treatment of the *impB* mutants of *E. coli* with 1% sodium deoxycholate significantly decreased the survival rate. This study provided further evidence that bile salts are damaging not only the membranes of bacterial cells, but also the DNA.

Salmonella. *Salmonella typhimurium* is an important enteric pathogen and is associated with diseases such as gastroenteritis. Additionally, it is a chronic colonizer of the gastrointestinal system (35). A recent study investigated the role of the drug resistance operon *marRAB* in conferring bile resistance to *S. typhimurium* (42). The *marRAB* operon, a regulator of multiple antibiotic resistance, consists of a repressor (*marR*) and a positive transcriptional regulator (*marA*) of antibiotic resistance genes, such as the efflux genes *acrA* and *acrB* (51). Using microarray analyses, β-galactosidase activity assays, gel electrophoretic mobility shift assays, and bile resistance assays, they demonstrated that the *marRAB* operon is activated in the presence of bile. This work suggested that resistance to bile and to antibiotics is interconnected to the survival of *S. typhimurium* in a host. A model was proposed in which bile salts enter the bacterium and then the bile salts bind with MarR, resulting in increased transcription of the *mar* operon. This regulation would in turn affect unknown genes involved in surviving the host's environment. The *acrAB* efflux pump, which was also found to be necessary for bile resistance, is transcribed in tandem to allow for the excretion of bile salts from inside the bacterium. Based on their model, activation of *marRAB*, which has been shown to possibly induce a decreased level of the transcription of the porin protein OmpF (1), would reduce influx into the cell, while *acrAB* would promote the efflux of bile salts out of the cell, thus creating a mechanism for resisting the damaging effect of bile salts.

The DNA damaging effect of bile and the bacterial response mechanisms utilized during exposure to bile has also been analyzed in *S. enterica* using the bile sensitive DNA adenine methyltransferase (Dam) mutant SV4392 (40). Using a random insertion to desensitize the strain to bile, they discovered that the mismatch repair proteins MutH, MutL and MutS confer bile sensitivity to *dam* mutants. RecA, a recombination protein, is a well known indicator of SOS response and is important to repair processes in bacteria (39). A β-galactosidase activity assay demonstrated that the SOS response was induced in the presence of sodium deoxycholate and ox bile but only when a functional RecA protein was present. Inversions were detected in three alleles: *hisC3072* (a +1 frameshift), *hisG46* (a nucleotide substitution causing a missense mutation), and *leuA414* (a nucleotide substitution resulting in an amber codon). This work provided evidence that bile increases the frequency of nucleotide substitutions, frameshifts and chromosomal rearrangements, further supporting the idea that bile is a DNA damaging agent and possibly produces double strand DNA breaks.

Another study by Prieto *et al*. provided evidence for the SOS response and use of homologous recombination as repair mechanisms in the presence of bile (41). This study showed that RecA, RecBCD and PolV are required for survival in the presence of bile (41). The RecBCD pathway is a recombination repair process activated in the presence of double stranded breaks and has been shown to be essential to *S. enterica*'s virulence (9). To determine whether bile induces oxidizing or alkylating DNA damage, various assays were performed using strains deficient in genes involved in oxidative repair or alkylation damage repair. Bile was found to act more as an oxidizing agent rather than an alkylating agent based on the minimal inhibitory concentrations against mutants deficient in specific

repair pathways. Those same data also indicated a role for base excision repair in the presence of bile-induced damage. The investigators proposed a model for DNA repair in response to bile-induced damage: initial lesions produced by bile are repaired by Dam-directed mismatch repair and by base excision repair, which in turn induce the SOS response and possibly impair DNA replication. This, in turn, would then require DinB and RecBCD to repair the damaged DNA and aid in restarting replication. This study was essential in supporting the theory that bile salts act as DNA damaging agents and that the role of DNA repair in virulent bacteria allows for survival and proliferation within the host digestive system.

Bile Induced Damage In Gram Positive Bacteria

Bacillus cereus

Bacillus cereus is a common cause of food-borne acquired infections, making it an important bacterium to study in relation to its interaction with the host's gastrointestinal tract. However, the pathogenesis of this bacterium is not fully understood, especially in regards to its ability to colonize the human intestine. There are two proposed methods of infection: 1) infections are mediated by the production of a toxin and 2) infections are mediated by the production of spores and subsequent release of a toxin (50). In both cases either the cells or the endospores must resist the presence of bile salts to establish the infection. A study conducted on 40 strains of *B. cereus* in the presence of bile showed that low levels of bile salts had a significant effect on the survival. The study found 100 genes were upregulated and 133 genes were downregulated (28). Genes

involved in general stress response, such as efflux pumps and transcriptional regulators (including MarR) were upregulated. Several genes associated with cell motility, cell wall and membrane synthesis, and DNA replication, recombination and repair were downregulated in the presence of bile. However, bile inducing oxidative damage was supported by the upregulation of genes involved in oxidative protection (superoxide dismutase and thioredoxins) and several chaperon-encoding genes. The motility genes *motA* and *cheY* were also upregulated, possibly indicating the cell's response to the bile salts. Additionally, the strains were only able to grow in the presence of low concentrations of bile salts (sodium cholate:sodium deoxycholate, 1:1). The upregulation of genes encoding efflux pumps and other membrane components, as well as transcriptional regulators and chaperones, provides support that membrane and DNA protection mechanisms are utilized for the survival of *B. cereus* in the presence of bile.

This same study also tested the possibility that spore-production is essential for the pathogenesis of *B. cereus* (28). Spores were grown in the presence of a bile salt mixture. The authors found that spores were able to tolerate high levels of bile, indicating that the spores are much more resistant to bile damage. This result suggests that *B. cereus* endospore formation could be a preferred mechanism of establishing an enteric infection through its ability to resist bile.

Listeria monocytogenes

Listeria monocytogenes is a food-borne pathogen that is responsible for nearly 28% of food related deaths each year (32). This gram positive bacterium can grow in similar environments as the gram negative *Salmonella enterica*, including that of the

gallbladder (19). Additionally, both *L. monocytogenes* and *S. enterica* respond to stress similarly and have similar virulence systems (18). As a result of these similarities, the influence of bile on the host-microbe interaction is becoming an important area of research, as this mechanism is poorly understood. Several genes have been identified in *L. monocytogenes* to be important for bile resistance, including genes involved in the preservation of the cell envelope and in stress response (4). Recently, it was found that *L. monocytogenes* contains certain genes required for bile resistance and these genes are regulated by the main virulence regulator *prfA* (5, 16). These genes are the *btlB* and *bsh* genes and are involved in detoxifying bile salts that have been conjugated with either glycine or taurine (5). Another important discovery was the identification of a novel bile exclusion system, also under the regulation of *prfA,* that allows the bacterium to survive in high concentrations of bile salts (48). Additionally, it was found that the nucleotide excision repair protein UvrA is important for survival in bile (26). The deletion of *uvrA* resulted in a significant impairment on the growth of *L. monocytogenes* in as little as 0.3% bile salts.

The ability of *L. monocytogenes* to survive in the presence of bile was also found to be influenced by the growth atmosphere (aerobic or anaerobic), the growth phase (stationary or exponential), and strain specificity (27). Four different strains isolated from food, environment, or clinical settings were subjected to both acid and bile and various atmospheric conditions, including air, 100% nitrogen, 40% carbon dioxide: 60% nitrogen, and 100% carbon dioxide. The acidic and bile environments were utilized to model the environment that ingested bacteria would encounter within the human digestive system. In all environments tested, stationary cells were much more resistant

than exponential cells. In general the bile salt environment proved to be more difficult for the strains to resist. It was found that only the stationary bacterial cells grown in air and 100% nitrogen survived after being exposed to the bile salt environment. These results suggest that atmospheric conditions and strain specificity of *L. monocytogenes* determine the microbe's ability to resist bile. Despite the possibility of being strain specific, these studies indicate that the pathogenic potential of *L. monocytogenes* is related to its ability to resist bile and possibly activate repair systems in the presence of bile.

Concluding Remarks

Bile is an important antimicrobial component of the human digestive system. The ways in which bacteria, both gram negative and gram positive, cope with its toxic effect differ in the exact mechanism, but a general theme can be determined. These bacterial models show that resistance is not exclusive to just overcoming damage to the membrane or the DNA, but rather is a result of a combination of repair mechanisms. One mechanism several enteric bacteria posses is that of efflux pumps to remove bile salts out of the cell, thus preventing potential damage to the membrane. If the membrane is compromised by bile salts, then the toxic effects could be conveyed onto the DNA, leading to extensive damage in the form of reactive oxygen species. This would lead to a cessation of replication and eventually cell death. Many recent studies, as outlined above, have focused upon determining the role that DNA repair has in the virulence capability of enterics. While the level of resistance seems to vary, the ability of the bacterium to breach certain areas of the host digestive system is contingent on its ability to resist damage induced by bile salts. Bile has repeatedly been found to be an oxidative agent

with the ability to induce the SOS response in several bacteria. The identification of this mechanism of damage as well as the bacteria's resistance and repair could aid in understanding its interaction with similar bactericidal agents and provide a better understanding of the role of the host response in the enteric infection process. While bile does induce both DNA damage and membrane damage, the interaction between the two types of damage is still not greatly understood in any bacteria. In particular, research pertaining to the connection of the pathogenic potential of a bacterium to its ability to resist bile is still in its infancy. The relationships between the virulence capabilities of these various pathogenic bacteria that are able to survive within the human digestive tract and the expression of resistance genes and repair mechanisms need to be further analyzed.

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CHAPTER II

EFFECT OF BILE ON THE MEMBRANE INTEGRITY OF VIRULENT AND AVIRULENT STRAINS OF *LISTERIA MONOCYTOGENES*

Introduction

Listeria monocytogenes is a gram-positive bacterial pathogen and is the causative agent of the food-borne illness listeriosis. *L. monocytogenes* is responsible for nearly 28% of reported food-related deaths each year in the United States (23). This bacterium is able to proliferate in a wide range of environments, including temperatures ranging from -0.4 to 50°C, stressful environments encountered within food processing plants, and high salt and acidic environments encountered during infections (4, 8, 9, 11). These characteristics make this microbe a very dangerous source of food contamination. It is because of the morbidity associated with this microbe that the FDA issued a "zero-tolerance policy" for the presence of *Listeria* in ready-to-eat (RTE) food products in 1989, which led to a drastic reduction in the incidence of *Listeria* contaminated RTE-products. However, there are still many reported cases of *Listeria* contamination and listeriosis annually (5), indicating that it is essential to understand the pathogenesis associated with this microbe.

The establishment of listeriosis infection is dependent upon the ability of *L. monocytogenes* to survive the acidic and bile environments encountered in the gastrointestinal tract and to invade and replicate within the epithelial cells lining the

intestinal tract. Resistance to bile is considered a major virulence determinant for enterics, is evident by the fact that the numerous commensal organisms found throughout the gut are tolerant to bile. Bile acts as a detergent and has been linked to the degradation of phospholipids and fatty acids found within the membranes of the gram-positive enteric bacteria *Bifidobacterium animalis* and *Lactobacillus reuteri* (30, 33). Bacteria have evolved several different mechanisms of resistance against bile to survive. One mechanism utilized by several bacteria is a two component regulatory system that acts to detect the presence of bile salts and upregulate response mechanisms after receiving a signal via transmembrane sensing domains of histidine protein kinases (21, 25, 28, 31). Another mechanism is through the use of bile-specific efflux pumps that remove the bile salts that have crossed into the cytoplasm. These bile transporters and pumps have been identified not only in gram-negative bacteria such as *Escherichia coli* and *Salmonella enterica* (24, 34) but also gram-positive bacteria such as *Bifodobacterium longum and Lactococcus lactis* (12, 22, 35). *L. monocytogenes* has several genes involved in bile tolerance, including the *bilE* operon that excludes bile from the cytoplasm and *bsh*, which encodes a bile salt hydrolysis enzyme involved in converting bile to a less toxic form (2, 6, 32). These genes have been shown to be essential for resistance to bile during the hepatic and intestinal stages of listeriosis (6). In addition, the virulence regulator *prfA* regulates expression of these bile tolerance genes (2, 6, 32), indicating the necessity for bile resistance in the pathogenesis of *L. monocytogenes*.

Bile is mostly composed of bile salts, cholesterol, and phospholipids. The detergent activity of bile is primarily attributed to the bile salt component (13). Two forms of bile salts are found within the digestive tract: unconjugated and conjugated.

Unconjugated bile salts are initially synthesized through the enzymatic conversion of cholesterol, expelled from hepatocytes, and then are converted to conjugated forms following the addition of either a glycine or taurine (16). Unconjugated forms of bile salts are the most toxic against microbes, yet are not present in the GI tract (17). Conjugated forms are the predominant types of bile salts encountered by bacteria in the GI tract. Through a process known as enterohepatic circulation, bile salts undergo structural alterations enabling their travel from the liver to the gallbladder, where they are stored until the intake of food triggers the release of bile into the small intestine (16, 29). From here the bile salts are reabsorbed and returned back to the liver (29). As a result of this circulation through the gastrointestinal tract, the concentration and composition of the bile salts undergo several changes due to conjugation, deconjugation and dehydroxylation, which in turn causes altered cytotoxicity against microbes (16).

L. monocytogenes is highly resistant to bile (1, 2). However, it has not been analyzed whether this capability is directly related to pathogenicity. To determine if bile resistance is related to the virulence capability of *L. monocytogenes*, we examined the effect that conjugated bile salts and a mixture containing unconjugated and conjugated bile salts have on the survival and maintenance of membrane integrity for the virulent strain EGD-e (serovar 1/2a) and the naturally isolated avirulent strain HCC23 (serovar 4a) (7) under both aerobic and anaerobic conditions. We found that bile salts affected the membrane of both EGD-e and HCC23, but this effect was much more severe in the avirulent strain. These results suggest that the pathogenic potential of *L. monocytogenes* is related to bile tolerance, but bile tolerance cannot be used as a sole indicator for

virulence capability. We present these data and suggest a model for how bile can act as a bactericidal agent against *L. monocytogenes*.

Materials and Methods

Bacterial strains, bile salts, and growth conditions

The *L. monocytogenes* strains EGD-e (serovar 1/2a) and HCC23 (serovar 4a) were grown in brain heart infusion (BHI) media at 37°C. For growth in the presence of bile salts, 1 ml of BHI was supplemented with 0 mg (0%), 100 mg (10%) or 200 mg (20%) of either bile from bovine or ovine (oxgall), sodium glycodeoxycholate (GDCA), or sodium taurodeoxycholate hydrate (TDCA) (Sigma Aldrich). All experimental methods were performed with cultures grown either aerobically or anaerobically. Anaerobic conditions were achieved by placing Wheaton serum bottles containing 1 ml of bile-infused BHI media in a vinyl anaerobic chamber for two days (Type B, Coy Laboratory Products INC.), after which bottles were capped with aluminum seals. Syringes were used to inoculate cultures and remove samples. Anaerobic conditions for BHI plates were achieved by incubating the plates in a BBL Gas Pak System. Anaerotest strips were used to verify anaerobic conditions.

Growth analysis in the presence of bile salts

Fresh, overnight cultures of EGD-e or HCC23 were diluted 1:100 in 2 ml of bile-infused BHI medium containing 0%, 10%, or 20% oxgall, GDCA, or TDCA and were grown at 37°C in a shaking incubator under aerobic or anaerobic conditions. For each

time point, 2 µl of culture were used for OD_{600} measurements with a Nanodrop ND-1000. Pathlengths for the Nanodrop readings were adjusted 10 fold in accordance with the manufacturer. Three independent experiments were completed and averaged for each bile salt under both anaerobic and aerobic conditions. Additionally, cultures grown in 0%, 10% or 20% of oxgall, GDCA or TDCA for 6 hr at 37°C were plated onto BHI agar and incubated overnight at 37°C. Analyses of growth on agar plates were performed for three, independent experiments under both aerobic and anaerobic conditions.

Scanning electron microscopy (SEM)

Fresh overnight cultures of EGD or HCC23 were diluted 1:100 in 2 ml of BHI media infused with either 0% or 20% oxgall. After a 6 hr shaking incubation at 37°C, cells were centrifuged at 8,000 *g* for 3 min (Eppendorf Centrifuge 5415R). The resulting bacterial cell pellets were fixed in 2.5% (v/v) glutaraldehyde in 0.1M cacoldylate buffer, washed in 0.1M cacoldylate, post-fixed in 1% (v/v) osmium tetraoxide in 0.1M cacoldylate buffer, re-washed in distilled water, dehydrated in an ethanol series, and dried in an hexamethyldisilazane (HMDS) series. Samples were sputter coated with gold-palladium (Polaron SEM coating system) prior to observations with a field emission scanning electron microscope (JEOL JSM-6500F). Samples were prepared from three independent experiments of 0% and 20% oxgall under both aerobic and anaerobic conditions. The length and width of 20 individual cells from each independent experiment were collected for analysis using the JEOL-PC-SEM 6500 software provided with the microscope. A mean average was calculated for cell length and cell width for data collected from the control cells (0% oxgall) and treated cells (20% oxgall). The

mean average from the control cells was compared to the mean average from the treated cells within a strain using a student *t*-test. A p-value < 0.05 indicated that the parameter measured (cell length or cell width) had significantly changed during exposure to oxgall.

Transmission electron microscopy (TEM)

Sample preparation for TEM observations were performed as indicated above for the SEM with the following exceptions. After the dehydration step, the cells were treated in a stepwise resin/ethanol series and embedded into resin at 68-70°C overnight. The cells were then sectioned using an ultramicrotome (Reichert-Jung Ultracut E) and viewed under a transmission electron microscope (JEOL JEM-100CXII). The width of the cell wall, cell membrane and cell envelope for 20 individual cells from each independent experiment was collected for analysis. Microscopic analyses were performed on three independent experiments for cells grown under aerobic or anaerobic conditions in the presence of either 0% or 20% oxgall. A mean average was calculated for the cell wall, cell membrane, and cell envelope thickness for control cells (0% oxgall) and treated cells (20% oxgall). The mean average from the control cells was compared to the mean average from the treated cells within a strain using a student *t*-test. A p-value < 0.05 indicated that the parameter measured (cell membrane, wall, or envelop thickness) had significantly changed during exposure to oxgall.

Results

Analysis of EGD-e and HCC23 growth in the presence of bile salts

The ability of virulent and avirulent strains of *L. monocytogenes* to grow in the presence of 0%, 10% or 20% oxgall, GDCA or TDCA was investigated under both aerobic and anaerobic conditions. Under aerobic conditions, growth was impaired for both EGD-e and HCC23 in the presence of oxgall, TDCA, and GDCA (Fig. 2.1). The growth of HCC23 in the presence of bile salts was much more impaired than that observed for EGD-e. The data show that increasing the concentration of bile salts exaggerates the growth deficiency exhibited by HCC23 (Fig. 2.1A). However, the avirulent strain did appear to maintain some viability through spectrophometric analysis. To confirm that the HCC23 cells were still viable following exposure to bile salts, cells were plated after 6 hr of incubation in the presence of oxgall, TDCA, or GDCA. Our results confirmed that HCC23 was viable in the presence of high concentrations of bile salts (data not shown). HCC23 growth never recovered in the presence of bile, yet bile was not 100% lethal to this avirulent strain. The growth of EGD-e in the presence of bile salts was impaired as compared to the control cells (Fig. 2.1B). However, in contrast to HCC23 bile-exposed cells, growth of EGD-e increased following an extended lag period (Fig. 2.1B). Under

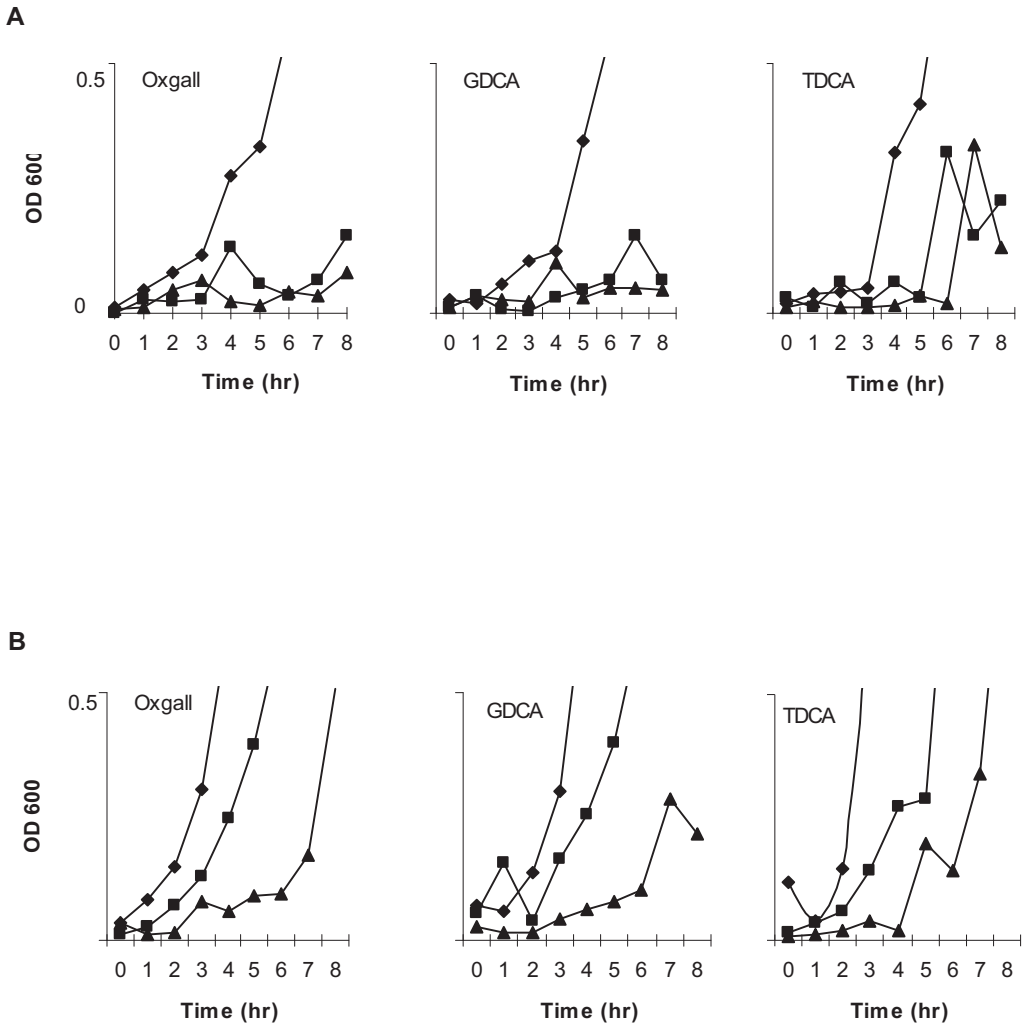

Figure 2.1 Growth Response Curves. HCC23 (A) and EGD-e (B) grown under aerobic conditions in 0% (♦), 10% (■) and 20% (▲) oxgall, GDCA or TDCA. The averages of three independent experiments are graphed.

normal growth conditions, EGD-e exhibited a 1 hr lag phase. In the presence of 10% oxgall, TDCA, or GDCA this growth period was extended to 2 hr. In the presence of 20% oxgall, TDCA, or GDCA this growth period was extended to 5 or 6 hr depending on the bile salt present. The effect of the bile salt was similar regardless of whether conjugated GDCA or TDCA or oxgall containing a mixture of conjugated and unconjugated bile salts was used.

To determine if similar growth patterns were observed in conditions found within the human digestive system, we analyzed the ability of EGD-e and HCC23 to grow in the presence of bile salts under anaerobic conditions. In general, the growth of both strains was less prolific under anaerobic conditions. However, similar growth patterns were observed for both strains in the presence of oxgall, TDCA, and GDCA under anaerobic conditions (Fig. 2.2) as compared to aerobic conditions (Fig. 2.1). Both strains exhibited a decrease in growth with an increase in the concentration of bile salt. The concentration of HCC23 in the presence of 20% bile salt remained low until a slight increase by 8 hr of incubation (Fig. 2.2A). However, further analysis indicated that this increase does not continue at 12 hr and 24 hr of incubation (data not shown). EGD-e showed similar growth patterns in 10% bile salts as observed under aerobic conditions. However, the increase of bile salts to 20% had a slightly greater inhibitory affect on the growth of EGD-e under anaerobic conditions. These results were also further confirmed by plating cells following a 6 hr incubation in the presence of 20% bile salts on BHI agar. While the plates exhibited viable cell growth of both strains in 20% bile, there were fewer colonies

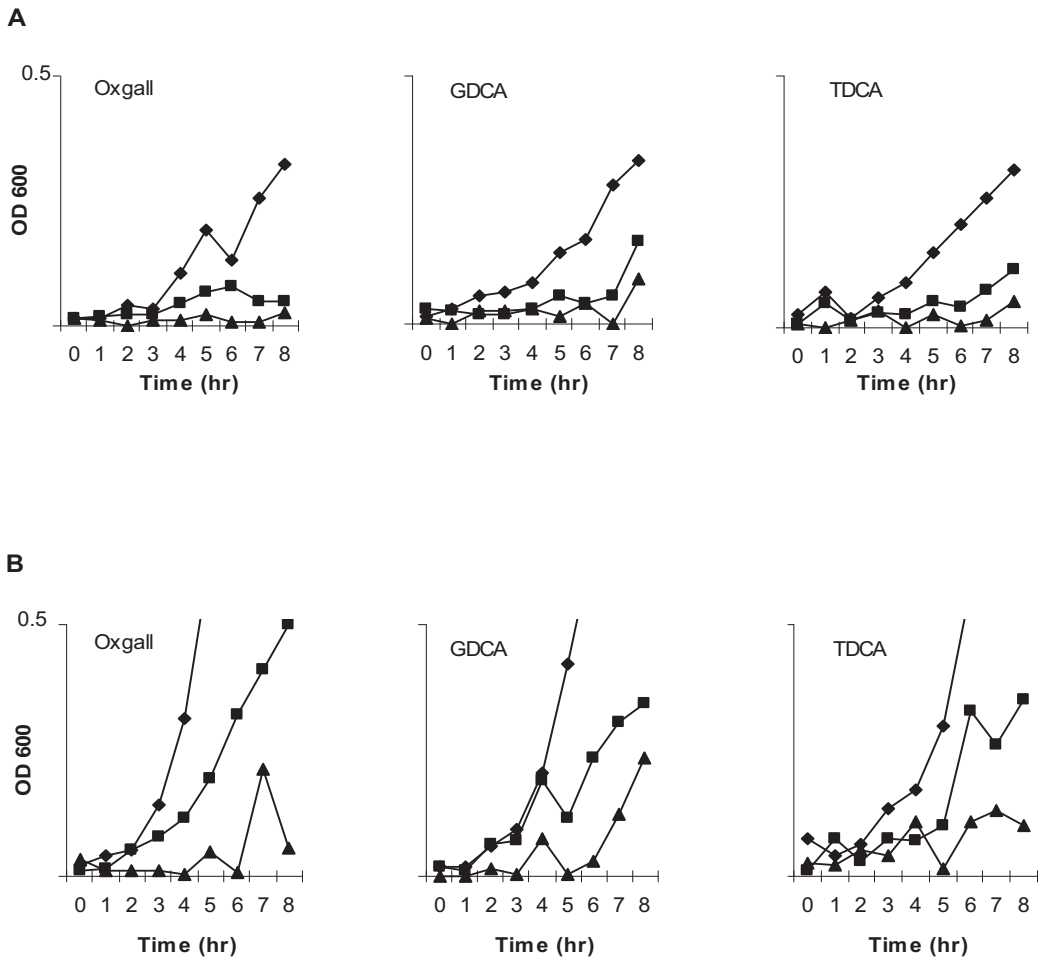

Figure 2.2 Growth Response Curves. HCC23 (A) and EGD-e (B) grown under anaerobic conditions in 0% (♦), 10% (■) and 20% (▲) oxgall, GDCA or TDCA. The averages of three independent experiments are graphed.

of the treated HCC23 present compared to its control than the treated EGD-e cells compared to its control. Similar to what was observed under aerobic conditions, the effect of the bile salt was not specific to certain bile salts.

Bile salts induce morphological changes in both virulent and avirulent strains of L. monocytogenes

To investigate the effect that bile salts have on the cell surface of HCC23 and EGD-e, samples exposed to bile salts were examined using a scanning electron microscope. Since similar growth patterns were observed for oxgall, TDCA, and GDCA treated cells, only oxgall was used for morphological analyses. Changes in cell length and width were examined for three independent experiments for untreated and bile-exposed (treated) EGD-e and HCC23 cells under aerobic or anaerobic conditions. Nearly 78% of oxgall treated HCC23 cells exhibited visible surface deformities, such as indentations, that indicated a loss of rigidity to the surface (Fig. 2.3A). These distortions were not present in the control treatment of HCC23, signifying that these alterations to the membrane were due to the presence of bile salts. EGD-e cells showed less deterioration of the surface of the membrane when exposed to 20% oxgall. Only 22% of the bile treated EGD-e cells exhibited minimal damaged to the cell surface.

The length and width of control and treated cells were measured to determine if oxgall alters the shape of the *L. monocytogenes*. Oxgall did not significantly alter the cell length of either EGD-e or HCC23 (Table 2.1). The cell width of both strains was significantly altered by the presence of bile salt. The cell width of HCC23 decreased in the presence of 20% oxgall. Interestingly, the cell width of EGD-e increased in the presence of oxgall. This correlates with SEM micrographs showing indentations throughout the surface of the cell wall in HCC23, while the rigidity and structure of the EGD-e cell membrane remained intact with little visible change (Fig. 2.3).

To determine if similar deformities occur under anaerobic conditions, EGD-e and HCC23 exposed to 20% oxgall under anaerobic conditions were observed with the SEM. As seen under aerobic conditions, the presence of bile salt induced damage to the surface of HCC23 cells (Fig. 2.3B). Under anaerobic conditions nearly 73% of HCC23 cells exhibited damage to the cell surface, while only 27% of EGD-e cells exhibited minimal damage to the cell walls. In anaerobic conditions growth in the presence of oxgall only significantly changed the cell morphology for HCC23 (Table 2.1). HCC23 exhibited a significant decrease in the cell length and cell width (Table 2.1).

Bile salts induce damage at the cell membrane in both virulent and avirulent strains of *L. monocytogenes*

To further investigate the effect that bile salts have on the cell wall of EGD-e and HCC23, cells exposed to 20% oxgall under aerobic and anerobic conditions were examined using the TEM. Cells were analyzed for alterations in the thickness of the layers that make up this natural bacterial barrier of defense. Visible differences in the nucleoid and the cytoplasm of control cells and the treated cells also contributed to the analysis of the TEM micrographs (Fig. 2.4A). Comparing the nucleoids of the HCC23 control cells to the bile treated cells revealed that in the presence of bile

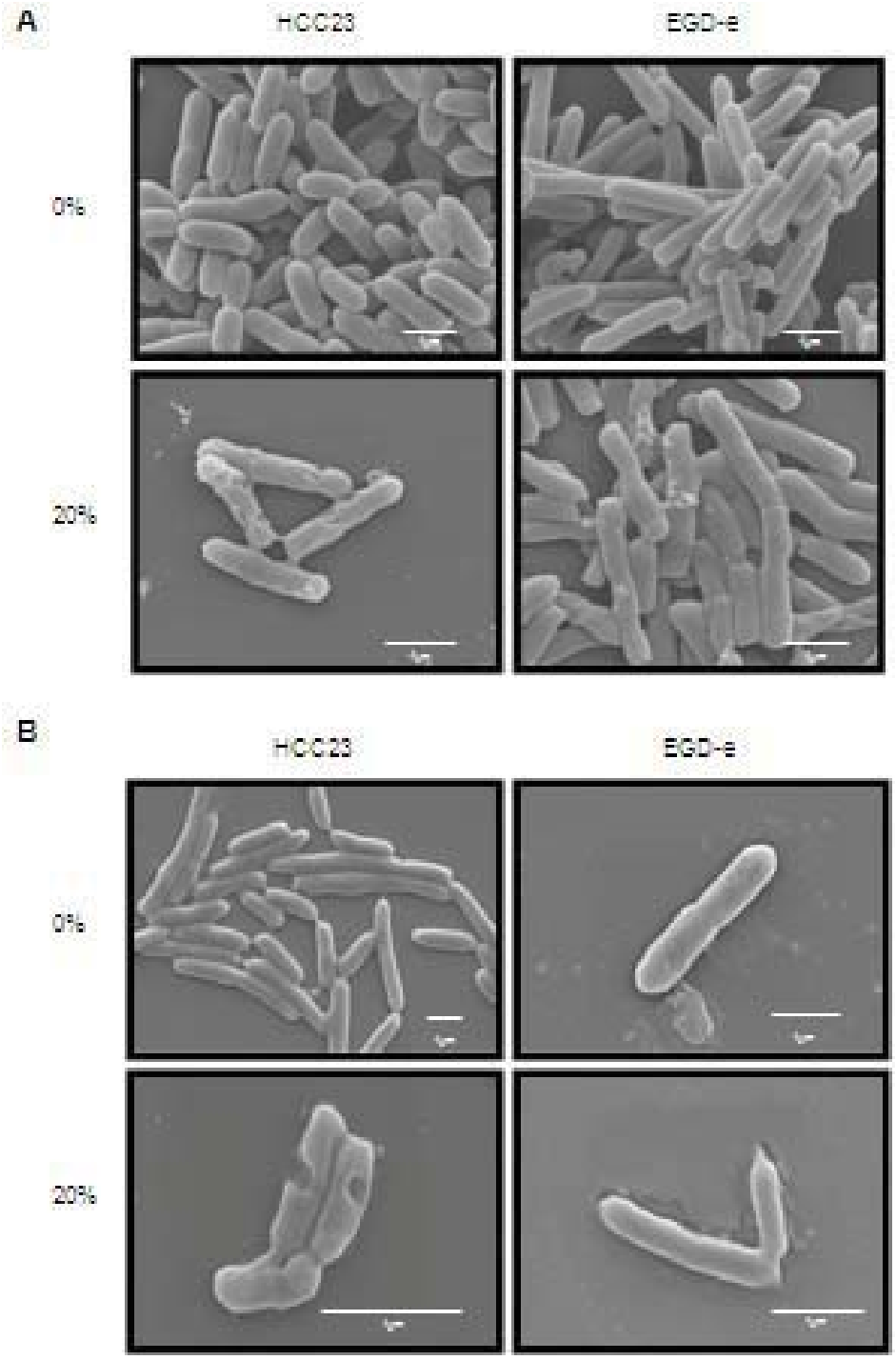

Figure 2.3 Morphological changes in aerobically grown (A) and anaerobically grown (B) HCC23 and EGD-e cells after 6 hours in 0% or 20% oxgall as investigated by scanning electron microscopy.

Table 2.1

Average cell length and width of HCC23 and EGD-e cells in the presense of 0% and 20% oxgall under aeroibic and ananerobic conditions. *depicts significant changes ($p<0.05$) in treated cells as compared to untreated cells

Strain, % oxgall	Average (μm)			
	Cell Length		Cell Width	
aeroibic	aerobic	anaerobic	aerobic	anaerobic
HCC23, 0%	1.329	1.518	0.519	0.503
HCC23, 20%	1.340	1.406*	0.375*	0.429*
EGD-e, 0%	1.347	1.450	0.376	0.529
EGD-e, 20%	1.376	1.376	0.512*	0.508

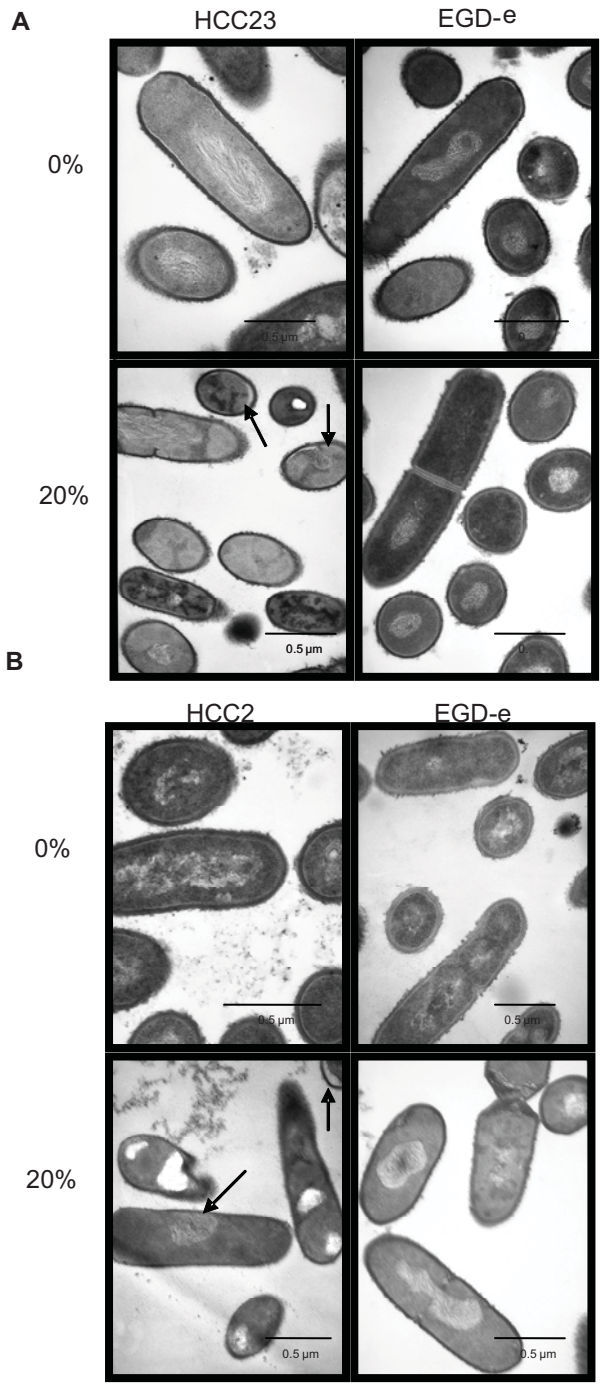

Figure 2.4 Morphological changes in aerobically grown (A) and anaerobically grown (B) HCC23 and EGD-e cells after 6 hours in 0% or 20% oxgall were investigated by transmission electron microscopy. Arrows indicate deformities including damaged nucleoid and membrane dissociating from the cytoplasm. Magnification of 40,000x for all photos.

nearly 28% of the cells exhibited fragmentation of the DNA (Fig. 2.4A). Less than 10% of the bile salt exposed EGD-e cells exhibited this same nucleoid alteration. Yet in both strains, approximately 8% of the cells exhibited areas where the membrane was dissociated from the cytoplasm but had an intact membrane and nucleoid (Fig. 2.4).

Comparing the thickness of the cell wall, membrane, and envelope between the control cells and treated cells of both strains revealed that bile had a significant effect on both strains. Oxgall treated HCC23 and EGD-e cells grown under aerobic conditions showed a significant decrease in the average cell wall and cell envelope thickness when compared to their respective control cells (Table 2.2). EGD-e cells also had a significant decrease in the thickness of the cell membrane. Under aerobic conditions, the presence of a high concentration of oxgall caused a decrease in the cell wall possibly contributing to the significant decrease in the thickness of the cell envelope.

The TEM micrographs of EGD-e and HCC23 grown under anaerobic conditions in the presence of 20% oxgall also indicated that the nucleoid and cytoplasm may be affected by the presence of bile (Fig. 2.4B). Nearly 54% of the HCC23 bile-treated cells exhibited damage to the nucleoid compared to only 9% of EGD-e cells. Cells exhibiting dissociation of the membrane from the cytoplasm under aerobic conditions were seen in 7% of HCC23 cells and only 2% of EGD-e cells.

Using anaerobic conditions and high concentration of bile salt to model the gallbladder of the human digestive system (27), alterations in the thickness of the cell envelope of EGD-e and HCC23 cells were analyzed to determine if membrane damage

Table 2.2

Average thickness of the cell wall, membrane and envelope of HCC23 and EGD-e cells grown in 0% or 20% oxgall under aerobic and anaerobic conditions. * depicts
significant changes ($p<0.05$) in treated cells as compared to untreated cell.

Strain, % oxgall	Average Thickness (nm)					
	Cell Wall		Cell Membrane		Cell Envelope	
	aerobic	anaerobic	aerobic	anaerobic	aerobic	anaerobic
HCC23, 0%	20.31	25.57	5.02	3.72	25.02	29.07
HCC23, 20%	15.57*	13.15*	5.58	3.32*	21.13*	16.25*
EGD-e, 0%	17.79	25.87	8.54	3.50	27.32	29.47
EGD-e, 20%	15.91*	20.17*	5.21*	5.74*	20.91*	26.06*

could potentially occur *in vivo*. Oxgall induced significant changes to the cell wall, membrane and envelope of both strains (Table 2.2). The average thickness of the HCC23 cell wall, membrane and envelope significantly decreased when grown in the presence of bile under anaerobic conditions. While the cell wall and cell envelope of the EGD-e decreased, the cell membrane thickness increased when grown in the presence of oxgall. Bile had a significant effect on the thickness of the cell wall, cell membrane and cell envelope of both strains, yet the micrographs suggest that the changes to the HCC23 cell membrane may also allow damage to the nucleoid and cytoplasm contained inside the cell.

HCC23 may exhibit intracellular accumulation of bile salts

TEM micrograph examination indicated that HCC23 had patterns of intracellular darkening following 6 hr of exposure to oxgall. To determine if this cytoplasmic defect was due to an influx of bile salts, we examined HCC23 cells at 3 hr and 6 hr in the presence of 0% or 20% oxgall under anaerobic conditions. TEM micrographs indicated that a visible darkening occurred within the cytoplasm of these cells. After 3 hr of bile exposure, approximately 13% of the treated HCC23 cells exhibited the phenotype of darkening in the cytoplasm. By 6 hr 20% of HCC23 cells exhibited this phenotype. HCC23 cells that were not exposed to oxgall did not have any areas of intracellular darkening within the cytoplasm. Additionally, no areas of cytoplasmic darkening were observed within EGD-e cells in the presence or absence of bile salts (Fig. 2.5).

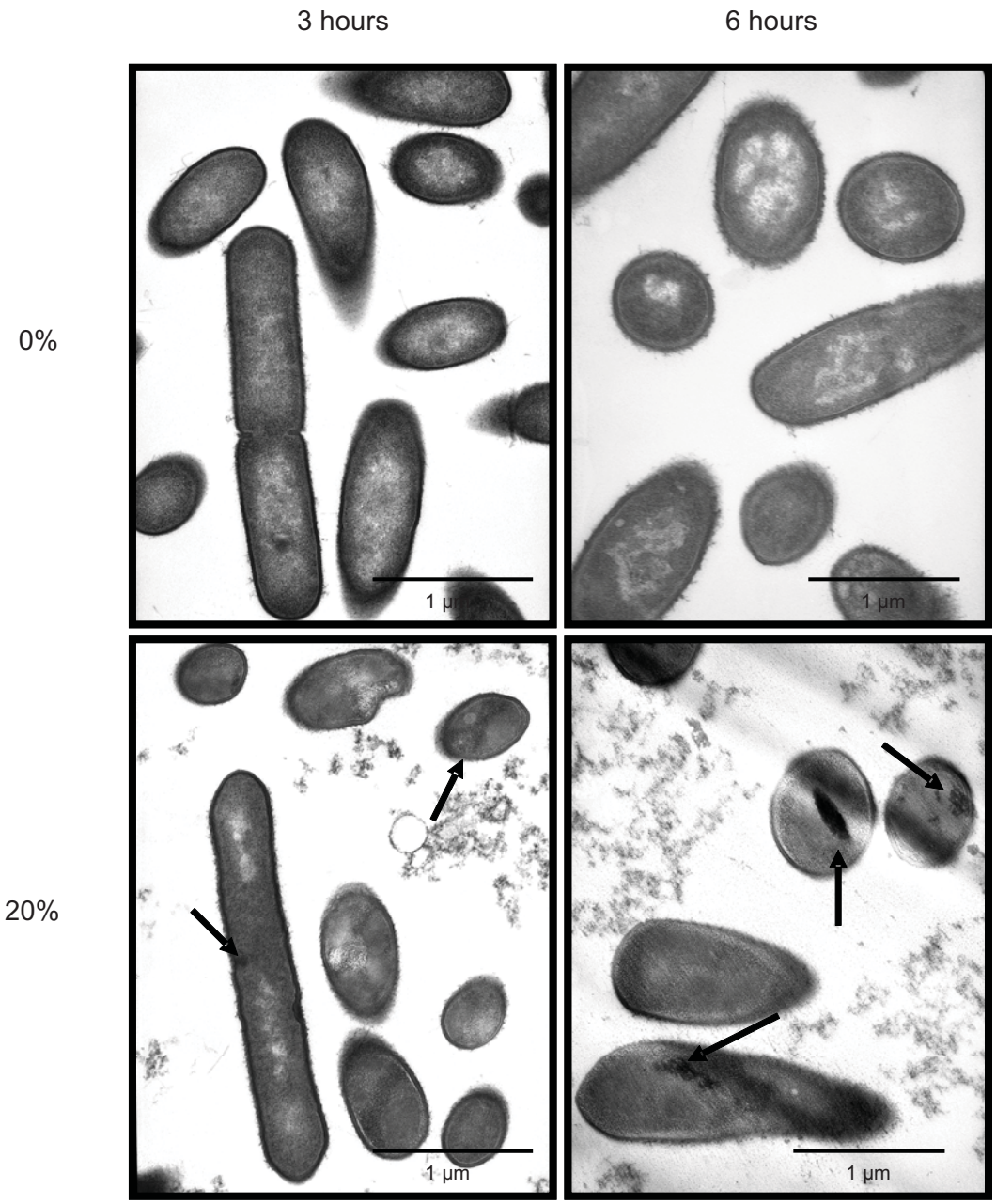

Figure 2.5 TEM images of HCC23 anaerobically grown cells in the presence of 0% or 20% oxgall at 3 and 6 hrs exhibiting an increase darkening within the cytoplasm. Arrows indicate darkened cytoplasm. Magnification: 27,000x for 3 hr photos and 40,000x for 6hr photos.

Discussion

L. monocytogenes acts as a foodborne enteric pathogen. It must be able to invade and survive within the human digestive system to establish infections. The gastric juices that make up this hostile environment serve as a natural barrier to combat the bacteria as it travels through the liver, gallbladder, and the intestinal tract. Throughout this environment, bacteria are exposed to various concentrations of bile ranging from 15% or higher (13). Bile and gastric enzymes function to break down the lipid constituent of food. Bile also acts as a bactericidal agent against bacteria that possibly enter the digestive system through ingestion of contaminated food products. Bile resistance has been proposed to be an indicator for bacterial pathogenicity, yet this has not been well characterized for *L. monocytogenes*. To establish an infection this pathogenic microorganism must be able to resist the detergent effect of bile and must also be able to repair any damage induced by this bactericidal agent. In the current study our purpose was to look at the effect that bile has on the bacterial membrane and to determine whether that effect differed based on the virulence capability of the strain.

Bile salts are the damaging component of bile. Their presence in various concentrations throughout the body makes them a constant source of damage to the bacteria (27). With the recent finding that *L. monocytogenes* is able to replicate extracellularly within the gallbladder (14, 15) where bile salt concentrations can be 15% or higher, it has been suggested that resistance to high concentrations of bile salts is essential for the pathogenesis of this pathogen. To investigate whether virulence is related to the ability to resist bile, we determined the ability of the naturally isolated avirulent strain HCC23 and the virulent strain EGD-e to grow in the presence of high

concentrations of bile salts. We found that both strains were able to grow in the presence of conjugated and non-conjugated forms of bile salts. However, the growth of HCC23 was severely impaired in the presence of all forms of bile salts tested.

However, the virulent strain exhibited the capability to adapt to the environment and continue to proliferate in the presence of bile salts. For all conditions tested, EGD-e had an extended lag period followed by a rapid increase in cell growth. This result suggests that these cells are able to adapt to this stressful environment, repair any damage that might have been introduced by the bile salts, and resume replication.

Utilizing scanning electron microscopy, we were able to visually observe the effect bile salts have on the cell surface of virulent and avirulent strains of *L. monocytogenes*. Exposure to oxgall resulted in very little damage to the EGD-e cell surface as compared to the untreated EGD-e cells. HCC23 exhibited more damage to the cell surface, as was indicated by indentations observed on the cell surface. To quantify these changes in the cell surface, the length and width of HCC23 and EGD-e cells were measured. Under aerobic conditions, the cell width of both strains was significantly altered; HCC23 had a decrease in cell width and EGD had an increase in cell width. Under anaerobic conditions, the length and width of only the HCC23 cells were significantly decreased when grown in the presence of bile salts. It is possible that the HCC23 cells became shorter and less wide as cytoplasmic material is lost through the compromised membrane. In the gram-positive bacteria *Lactobacilli* and *Bifidobacteria,* it has been shown that bile salts dissipate the transmembrane electron potential (20). This disrupts the membrane integrity and allows the leakage of protons, potassium ions, and

other cellular components out of the cell (20). The fact that EGD-e cells are expanding in cell length could indicate a mechanism utilized to keep the membrane intact.

Analysis of the TEM images resulted in several conclusions concerning the effect of bile on HCC23 and EGD-e cells. The cell wall, membrane, and envelope of both strains were significantly affected by the presence of bile. This decrease in the thickness of the layers of the membrane is probably due to the detergent effect of bile salts. The damaged nucleoid, dissociation of the membrane from the cytoplasm, and dark accumulation within the cell occurred in the presence of bile salts in the avirulent strain. This supports the conclusion drawn from the SEM data that bile salts affect the individual layers of the cell envelope thus making it more permeable. The space within the cell where the membrane seems to be dissociating from the cytoplasm could also be due to the loss of intracellular material, which would also correlate to the overall decrease in cell width of HCC23.

We believe the alterations occurring at the membrane level allow the bile salts to affect the intracytoplasmic components of *L. monocytogenes* and this effect is exaggerated in the avirulent strain HCC23. The increase in intracellular darkening occurs over time in the cytoplasm of HCC23 cells and we believe could indicate intracellular accumulation of bile salts. Because our SEM data indicates that the membranes of the HCC23 cells are being significantly affected in the presence of bile salts it is no surprise that the intracellular darkening was specific to the HCC23 cells. The damage occurring at the membrane would allow a flux of material, such as the bile salts, into the cell. In this case bile salts that were able to breach the cell wall and accumulate within the cytoplasm could then exert DNA damage and lead to cell death. This idea is supported by the fact

that more HCC23 cells exhibited a damaged nucleoid than the EGD-e cells. This damage to the nucleoid would render the cell non-functional, which would explain the decrease in growth, especially in HCC23 cells, in increasing concentration of bile salts.

From these data and literature supporting the detergent properties of bile salts on lipids (10) , we propose the following model for the effect of bile on the viability of *L. monocytogenes* (Figure 2.6). Bile salts act on the phospholipids and fatty acids of the membrane to disrupt the proton and potassium ion pumps, thus altering the transmembrane electron gradient and allowing for the influx of bile salts into the cell and efflux of cellular components out of the cell. Bile salts move into the cytoplasm and are targeted to the nucleoid. The bile salts then induce DNA damage, most likely through reactive oxygen species. If damage is too profound for repair, the cell will cease to replicate and will eventually undergo cell death. DNA damage induced by bile salts is well described in gram-negative bacteria (3, 18, 26) but remains in its infancy in gram-positive bacteria, though a recent study did find the induction of the nucleotide excision repair gene *urvA* in L. *monocytogenes* cells in presence of bile salts (19). The thickness of

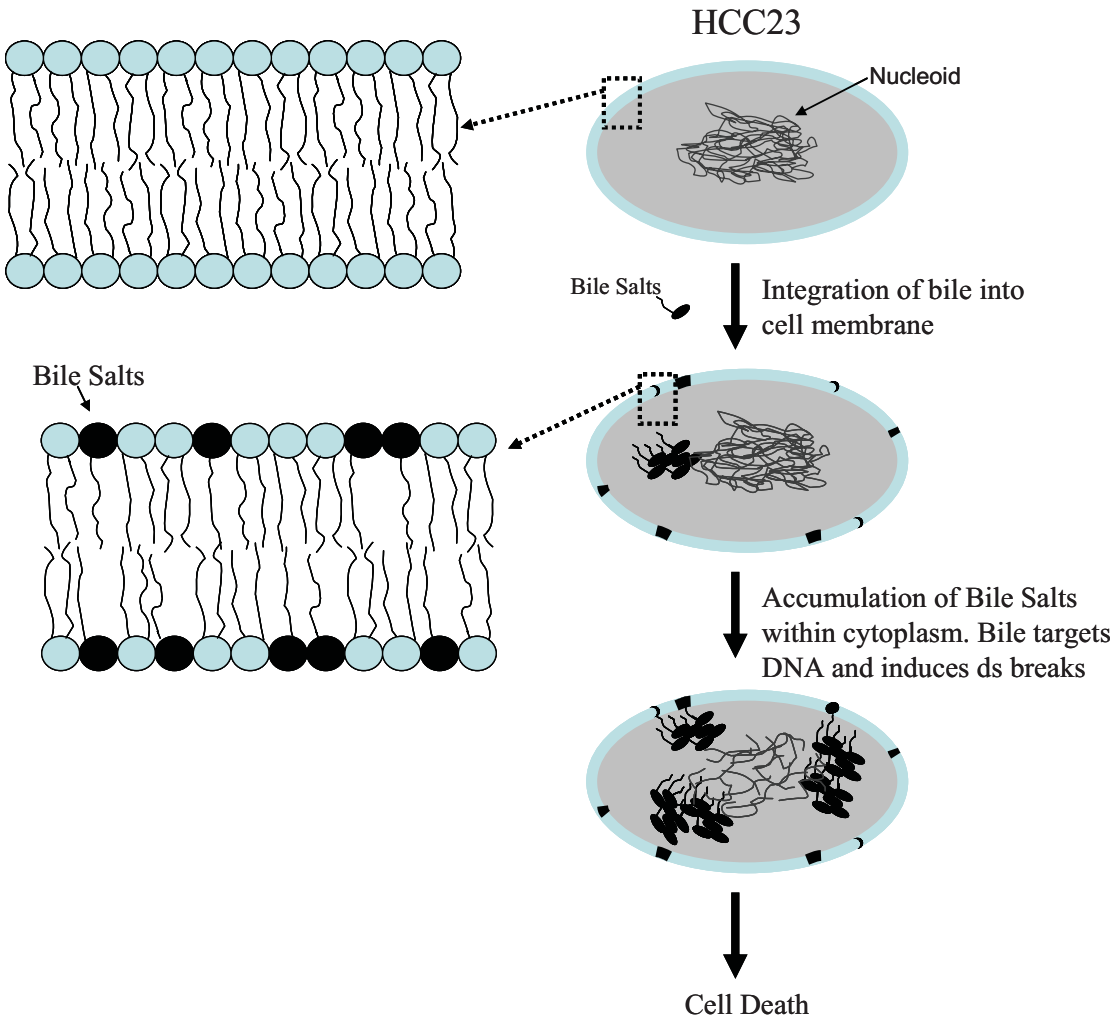

Figure 2.6 A model for bile induced damage in *L. monocytogenes* HCC23 cells. Bile salts damage the structure of the membrane resulting in the loss of the electron chemical gradient, the influx of bile salts into the cell and the efflux of intracellular components. Finally the bile salts accumulate inside the cell and act on the nucleoid of the cell to damage the DNA. If the damage reaches the point beyond repair the cell will undergo cell death.

the cell envelope layers in EGD-e cells were significantly affected, but the overall shape of the cell was not altered. This suggests that EGD-e has a mechanism for excluding the bile salts from the cell. Recently, a bile exclusion system (BilE) in *L. monocytogenes* was characterized for its ability to prohibit bile salts from entering the cell (32). *bilE*

expression was also shown to be regulated by the main virulence regulator PrfA, which indicates that the exclusion of bile is related to virulence. Yet, even in this work, radiolabelled bile salt was still shown to accumulate within the cell whether *bilE* was present or mutated, thus indicating that other mechanisms may be in place to export the bile once it has penetrated the membrane. This would account for the few damaged nucleoids observed and the absence of significant changes to the overall cell shape for EGD-e. Interestingly, HCC23 (NCBI Reference Sequence NC_011660) contains the *bilE* operon and shows 91% sequence similarity to the *bilE gene* in EGD-e (NCBI Reference Sequence NC_003210). It also shows 97% similarity to the *bsh* gene of EGD-e (lmo2067) and 99% similarity to the proposed bile salt dehydrolase, *btlB* gene (lmo0754). HCC23 lacks *pva* (lmo0446), which is the only other gene identified for bile resistance in *L.monocytogenes*. This indicates that there may be other genes in EGD-e that are yet to be characterized for their involvement with tolerance and adaptation to bile.

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CHAPTER III

CONCLUSION

Bile represents an important natural mechanism of defense in the human gastrointestinal system. Enteric pathogens must have the ability to survive the stressful conditions created by the gastric juices and bile. It is this resistance to bile that enables some bacteria to invade and establish infections in the many organs that make up the human digestive system. The fact that bacteria have evolved mechanisms to sense the bile salts, as seen in *Lactobacilli* and *Bifidobacteria,* as well as utilize antibiotic resistance efflux pumps to remove bile salts, indicates that bile provides a barrier to the survival of bacteria (3, 7).

The effect of bile on the membrane and DNA of several different gram-negative and gram-positive pathogenic bacteria was investigated in a literature review in chapter 1 of this thesis. The aim of this review was to illustrate how bile induces damage on enteric bacteria and illustrate that bacteria utilize several different mechanisms to cope with the presence of bile and its resulting damage. Bile has been shown to upregulate several genes involved in efflux pumps, membrane biosynthesis, as well as DNA repair and general stress response (2, 4-6). This work leads to the idea that the ability and mechanisms of the bacteria to overcome the presence of bile may be specific to each strain.

To further investigate the influence of bile on bacteria and how it relates to pathogenicity, a virulent strain (EGD-e) and a naturally occurring avirulent strain (HCC23) of *L. monocytogenes* were grown under aerobic and anaerobic conditions in the presence of various concentrations of bile salts. The growth of each strain was assessed to ensure that growth was possible under high concentrations of bile salts that mimic those encountered throughout the digestive tract (1). Comparing the growth of EGD-e and HCC23 in media containing 0%, 10%, or 20% bile from bovine (oxgall), sodium glycodeoxycholate (GDCA), and sodium taurodeoxycholate (TDCA) indicated that increasing the concentrations of bile salts decreases the viability of *L. monocytogenes*. In HCC23, the presence of 20% bile salts greatly inhibited the growth of the strain but did not eliminate the organism, regardless of whether the cells were grown under aerobic or anaerobic conditions. EGD-e cells showed a decrease in growth in 20% bile salts, and this effect was exacerbated under anaerobic conditions that mimic those found in the human digestive system.

Micrographs from scanning electron microscopy and transmission electron microscopy of both strains in 20% oxgall under aerobic and anaerobic conditions gave visible confirmation of bile induced damage on the membrane of the cells; this effect was exaggerated in HCC23. Measuring the length and width of the cells along with the thickness of the cell wall, cell membrane and cell envelope indicated that bile alters the cell membrane of both avirulent and virulent strains of *L. monocytogenes*. The HCC23 cells became slightly shorter and thinner and the layers of the cell envelope significantly decreased when exposed to bile. EGD-e cells also experienced a significant decrease in most of the layers comprising its cell envelope, indicating that bile also has an effect on

its cell structure. Another important discovery from the TEM images was the darkening that occurred within the nucleoid and cytoplasm in bile exposed HCC23 cells over time. We believe this indicates that the nucleoid is damaged and that the darkening is actually the accumulation of the bile salts within the cytoplasm of the cell.

These data and the supporting literature lead us to propose a model in which bile salts induce damage to the membrane of the cells allowing for changes in the electron chemical gradient and the permeation of the membrane for the flux of bile salts into the cell and cellular components out of the cell. Once the bile salts have accumulated in the cytoplasm, the DNA is damaged. As a result this may cause an increase in expression of several genes involved in the general stress and repair, such as those discussed in several bacteria in Chapter 1. If excessive damage occurs, the cell will lose its membrane integrity, the cell will experience extensive genomic damage, leading to a cessation of replication and ultimately cell death.

The importance of this work lies in its ability to give insight to the host-microbe interaction of *L. monocytogenes* and several other pathogenic bacteria within the human digestive system. This physiological study indicates a need for further research into the DNA damaging capabilities of bile salts on *L. monocytogenes* and the repair mechanisms involved in this and other enteric pathogens allowing them to establish infection within the human gastrointestinal tract. The resistance to bile is a useful indicator pathogenicity of a bacterial strain.

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APPENDIX A

ATTEMPTED METHODS: PULSE FIELD GEL ELECTROPHORESIS

Pulse Field Gel Electrophoresis

The ability of bile to induce double-stranded breaks into the DNA of *Listeria monocytogenes* was investigated using pulse field gel electrophoresis. This method has previously been utilized by others to examine the effect of DNA damaging agents on bacterial and mammalian cells (2, 3, 5). The single-stranded, double-stranded or intact DNA will migrate through the agarose gel differently once a charge is administered. The intact DNA should migrate slowly while the damaged DNA will migrate through the gel more quickly, allowing for separation and determination of the amount of DNA damaged by bile salts.

Materials and Methods

The strains EGD-e and HCC23 were subjected to a 6 hr exposure to 0%, 10%, or 20% of oxgall at 37°C under anaerobic and aerobic conditions. Following the exposure, the cell concentration was adjusted to $1x10^8$ cells/ml and integrated into plugs that fill the wells of a 1% agarose gel (Pulsed Field Certified Agarose Bio-Rad #162-0137) in 0.5X Tris-Borate-EDTA. The plugs were made using the Bio-Rad CHEF Bacterial Genomic DNA Plug kit (#170-3592). The gel was run in 0.5X TBE buffer for 40 hours at 160 V (6-7 V/cm) using a ramp from 3 ms forward/1 ms back to 180 ms forward/60s backward (2) on a CHEF Mapper XA System (Bio-Rad #170-3670). The gel was then stained in ethidium bromide and photographed using a BioRad gel documentation workstation.

Results and Discussion

The assay was attempted in order to assess the effect that bile salts have on the genomic integrity of EGD-e and HCC23. Previous studies in gram-negative bacteria such as *E. coli* and *Salmonella enterica* have indicated that bile salts induce damage on the DNA of enteric bacteria (1, 4). We were not able to achieve separation of the bands from the plugs or the standard marker after several attempts. After contacting BioRad representatives, changing the running buffer, and changing the running program, we were not able to resolve the problems with the assay. The problem may be the running conditions program and settings since the standard marker did not properly migrate.

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APPENDIX B

ATTEMPTED METHODS: COMET ASSAY

Comet Assay

The comet assay, also known as single cell gel electrophoresis, is a way to visualize double and single-stranded breaks within the DNA. This assay involves the electrophoresis of lysed cells embedded in agarose. If DNA is damaged, the DNA will migrate from the intact DNA, forming a "comet" tail to appear from the intact DNA. This occurs because when a charge is applied, the intact DNA will migrate slower as it is confined to the nucleoid and damaged DNA will migrate away from the intact nucleoid. This can then be visualized by staining the cells and then viewing the cells with a fluorescent or confocal microscope. The purpose was to use the CometAssay assay kit (Trevigen) to determine whether bile salts induce DNA damage and to assess whether the amount of damage differs between the virulent strain EGD-e and the avirulent strain HCC23 of *Listeria monocytogenes*.

Materials and Methods

Overnight cultures of HCC23 and EGD-e cells were diluted 1:100 into fresh BHI media supplemented with either 0% or 10% oxgall. Cultures were then grown at 37°C for 4 hr and samples were extracted at 1, 2, 3, and 4 hr. Cell concentrations were determined using Beer's law in order to determine a 1:10 ratio of low melting agarose to cells. 1 x10^7 CFU/ml were centrifuged for 10 min at 13 rpm, after which cells were washed in cold PBS (MP tablets, 2810305). Using the CometAssay kit (Trevigen)(#4250-050-K), the cells were lysed and placed in an alkaline solution made of NaOH and 200 mM EDTA. After a short 20 min electrophoresis at 25 V, the cells were dried, stained with the nucleic

acid stain SYBR Green I, and visualized under a confocal laser scanning microscope (Axiovert 200 M Inverted Research microscope).

Results and Discussion

The data collected from the comet assay did not provide conclusive results. However, it was still difficult to examine the DNA due to magnification restraints of the microscope. Some "comet tails" appeared to occur, yet once magnified the quality of the photo and the appearance of a distinct tail were lost. This assay may be better served under a higher powered confocal or higher powered fluorescent scope. The prospect of the cells exhibiting the tails indicated that DNA damage was occurring but could be better determined through an alternative assay.

APPENDIX C

ATTEMPTED METHODS: LIVE/DEAD ASSAY

Live/Dead Assay

The LIVE/DEAD *Bac*Light Bacterial Viability Kit (Invitrogen, L70012) is a way to assess viability of bacterial cells based on whether the cell membrane is intact or compromised. This kit utilizes two nucleic acid stains: SYTO 9 and propidium iodide. SYTO 9 stain will label all the bacteria in a population, but propidium iodide will only penetrate through damaged membranes and will cause a reduction in the SYTO 9 stain. When the cells are examined using a fluorescent or confocal microscope, the SYTO 9 will excite/emit at 480/500 nm and propidium iodide will excite/emit at 490/635 nm. Based on these dyes the viability of cells and the degree that bile salts are compromising the membranes can be determined for the avirulent HCC23 strain and EGD-e strain of *Listeria monocytogenes*.

Materials and Methods

Overnight cultures of EGD-e or HCC23 were diluted 1:100 into BHI media containing either 0% or 10% oxgall. The cultures were grown at 37°C for 6 hr and samples were extracted at 1, 2, 3, 4, 5, and 6 hr post inoculation. At each time point, 1 ml of cells were centrifuged at 8,000 *g*, washed in PBS, then resuspended in 1 ml of cold PBS. The component A (SYTO 9 dye, 3.34 mM in DMSO) and component B (propidium iodide, 20 mM in DMSO) were mixed in a 1:1 ratio and 3 µl of this dye mixture were added to every 1 ml of sample. This stained bacterial suspension was incubated at room temperature in the dark for 15 min. 5 µl of the stained bacterial sample was transferred to a glass microscope slide and viewed under a fluorescent microscope.

Results and Discussion

We were able to visually examine both treated and untreated HCC23 or EGD-e cells. However, it was difficult to determine if the membrane was intact or compromised. Cells contained both SYTO 9 and Propidium iodide stains. This was also observed under control conditions. This might be due to too much stain or poor filters on the fluorescent scope. This assay may be better served using a flow cytometer to give a percentage of live bacterial cells versus those cells with damaged or compromised membranes.