ANTIMICROBIAL EFFICACY OF LIPOSOME ENCAPSULATED NISIN AND NISIN'S INHIBITION AGAINST *Listeria monocytogenes* IN FLUID MILK AT DIFFERENT STORAGE TEMPERATURES

A Thesis

by

SHANNON ELISE SCHMIDT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2009

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	T. Matthew Taylor
Committee Members,	Glenn Holub
	Joseph Sturino
Intercollegiate Faculty Chair,	Jimmy T. Keeton

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ABSTRACT

Antimicrobial Efficacy of Liposome Encapsulated Nisin and Nisin's Inhibition Against *Listeria monocytogenes* in Fluid Milk at Different Storage Temperatures. (August 2009)

Shannon Elise Schmidt, B.S., Texas A&M University

Chair of Committee: Dr. Matthew Taylor

Nisin is a naturally occurring food antimicrobial that inhibits many Gram-positive pathogens, including *Listeria monocytogenes*, a bacterial pathogen responsible for ~500 deaths in the U.S. annually. Factors known to counteract the nisin activity in a food matrix include: antimicrobial interaction with food components, insolubility, protease inactivation, and target cell-driven envelope modifications. Encapsulating nisin in liposomes can help protect nisin functionality by regulating its introduction to the external environment. The objectives of this study were to determine the encapsulation efficiency (%EE) of nisin within liposomes as a function of encapsulation method and the capacity of liposomal nisin to inhibit *L. monocytogenes* in fluid milk.

Phosphatidylcholine (PC) and phosphotidyl-DL-glycerol (PG) were used to prepare three lipid molar formulations: PC, PC/PG 7:3, and PC/PG 6:4 (mol.%). Liposomes were formulated to entrap the self-quenching fluorophore calcein and nisin. Unencapsulated analyte was removed via size-exclusion chromatography, and percent EE was determined. To determine antilisterial activity of liposomes, fluid milk samples containing *L. monocytogenes* (4 $\log_{10}$ CFU/mL) in combination with liposomal or

unencapsulated nisin at 50 IU/mL were mixed and aerobically stored at 5°C and 20°C. Surviving *L. monocytogenes* were enumerated via plating on a non-selective microbiological medium after 0, 1, 3, 6, 12, 24, 48, and 72 hours of incubation.

Encapsulation of nisin via extrusion resulted in a mean EE% of 84.20%, 77.33% and 80.78% for PC, PC/PG 7:3, and PC/PG 6:4 liposomes, respectively. Freeze-thaw cycling formed liposomes without detectable fluorophore entrapment. *L. monocytogenes* populations grew to 5 $\log_{10}$ CFU/mL after 72 hours at 5°C and 8 $\log_{10}$ CFU/mL at 20˚C after 48 hours. Unencapsulated nisin exerted statistically greater inhibition of *Listeria* in skim milk compared to liposomal nisin, regardless of incubation temperature. No statistically significant differences in *Listeria* populations exposed to free or encapsulated nisin in whole milk were observed at either incubation temperature. Results indicate storage temperature and presence of milk fat exert greater influence then nisin delivery (free vs. encapsulated) over *Listeria* inhibition. Further research is needed to confirm these findings and develop more effective means of liposome entrapment of nisin for the inhibition of foodborne bacterial pathogens.

DEDICATION

To my family

Your love has carried me through it all.

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I would like to thank my committee chair, Dr. Matthew Taylor, and my committee members, Dr. Glenn Holub and Dr. Joseph Sturino, for their support, understanding, and guidance throughout these several years. You all have been a wonderful team to work with, and I appreciate everything you have done for me.

Thank you also to my lab mates, colleagues, friends, and the department faculty and staff for all of your support and encouragement. You have made my time at Texas A&M University an enjoyable and memorable experience. I appreciate all of your help inside and outside of the laboratory.

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TABLE OF CONTENTS

Page

ABSTRACT iii

DEDICATION v

ACKNOWLEDGEMENTS vi

TABLE OF CONTENTS vii

LIST OF FIGURES x

LIST OF TABLES xi

CHAPTER

I INTRODUCTION: LISTERIA MONOCYTOGENES 1

- Classification 1
- Growth Requirements 3
- Environment 5
- Incidence 8
- Cost of Illness 8
- Foodborne Outbreaks 9
- Listeriosis 11
- Pathogenicity 13
- Intracellular Invasion 13

II LIPOSOMES 15

- Liposome Properties 16
- Vesicle Preparation 19
- Reverse Phase Evaporation 20
- Detergent Depletion 21
- Freeze-Drying Rehydration 22
- Freeze-Thaw Cycling 23
- Sonication 23
- High-Pressure Homogenization and Microfluidizers 24
- Extrusion 25

CHAPTER Page

Liposomes in Drug Delivery 27
Liposomes in Gene Therapy 29
Liposomes in Cosmetics 30
Liposomes in Food Industry 31

III NISIN 37

Chemical and Physical Properties 38
Stability and Solubility 39
Antimicrobial Spectrum 40
Mode of Action 42
Factors Affecting Nisin Action 43
Nisin Resistance 45

IV MATERIALS AND METHODS 47

Culture Resuscitation and Maintenance 47
Antimicrobial Preparation 47
Fluorescence Probe Preparation 48
Liposome Preparation and Encapsulation via Extrusion 48
Pathogen Survivor Assay 50
Analysis of Data and Experimental Design 51

V RESULTS 52

Liposomal Encapsulation Efficiency of Nisin 52
Survival of *L. monocytogenes* in Skim and Whole Milk from Liposome-Encapsulated and Free (Unencapsulated) Nisin at 5°C 53
Survival of *L. monocytogenes* in Skim and Whole Milk from Liposome-Encapsulated and Free (Unencapsulated) Nisin at 20°C 56

VI DISCUSSION 60

VII CONCLUSION 66

REFERENCES 68

Page

VITA……... 80

LIST OF FIGURES

Page

Figure 1 Liposome with Bilayer in Aqueous Solution. 16

Figure 2 LiposoFast™ Mini-Extruder. 26

Figure 3 Structure of Nisin A. 39

Figure 4 Encapsulation Efficiency of Liposomes Formed via Extrusion. 52

Figure 5 Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin in 2 mM PC Liposomes at 5°C. 54

Figure 6 Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin in 2 mM PC/PG 7:3 Liposomes at 5°C. 55

Figure 7 Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC/PG 6:4 Liposomes at 5°C. 56

Figure 8 Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC Liposomes at 20°C. 57

Figure 9 Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC/PG 7:3 Liposomes at 20°C. 58

Figure 10 Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC/PG 6:4 Liposomes at 20°C. 59

LIST OF TABLES

Page

Table 1 Size and Lamellae-Dependent Classification of Liposomes....................19

Table 2 Common Preparation Techniques for Different Types of Liposomes 27

Table 3 Various Encapsulated Food Ingredients..32

CHAPTER I

INTRODUCTION: LISTERIA MONOCYTOGENES

The first reported case of human listeriosis occurred when a soldier in World War II developed meningitis. Since then, it has emerged as a major foodborne disease. Its surfacing resulted from several factors including medical progress, more immunocompromised individuals, changes in food processing methods, food preparation and handling, and food consumption habits (Rocourt and Cossart 1997). *Listeria* is considered a public health concern due to its severity, high case-fatality rate, and long incubation time that averages 31 days and ranges from 11-70 days (Rocourt and Cossart 1997; Ryser and Marth 1999; Lorber 2007). There are several differences with *Listeria* compared to other foodborne pathogens such as a high mortality rate (20%-30%), its ability to cause spontaneous abortion, and its status as an intracellular pathogen (Bell and Kyriakides 2005; Jay and others 2005; FDA 2007; Painter and Slutsker 2007).

Classification

This pathogen belongs to the genus *Listeria* within the *Clostridium* sub-branch. *Listeria's* phylogenic position is partially dependent on its low G + C DNA content (36%-42%) (Rocourt and Cossart 1997; Ryser and Marth 1999; Jay and others 2005). *Listeria* has a low G + C DNA content because it contains fewer G and C DNA bases than A and T bases as compared to other bacteria. This is one of many features used to classify bacterial genomes, specifically Gram-positive bacteria. There are six species of *Listeria*, and only Listeria monocytogenes and Listeria ivanovii are pathogenic. However,

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This thesis follows the style of the Journal of Food Science.

L. monocytogenes is the only species considered a public health concern because *L. ivanovii* is primarily associated with animal disease. According to its numerical taxonomy, *Listeria* is closely related to lactic acid bacteria. Teichoic acids are found in the cell wall of Gram-positive bacteria and extend to the surface of the peptidoglycan layer. They are either covalently bonded to N-acetylmuramic acid of the peptidoglycan layer or linked to a D-alanine. Lipoteichoic acids are a combination of lipids and teichoic acids. The lipoteichoic acids present in most Gram-positive bacteria assist in *Listeria's* virulence mechanisms (Ryser and Marth 1999). The lipoteichoic acids are polyphosphoglycerol substituted with a D-alanyl (D-Ala) ester or glycosyl residue (Abachin and others 2002). Studies have shown that mutants of Gram-positive bacteria deficient in D-Ala esters of lipoteichoic acids have an increased cell surface electronegativity (Abachin and others 2002). This allows for more efficient binding of cationic compounds and more susceptibility to cationic, pore-forming, antimicrobials.

The genus *Listeria* possesses several characteristics that distinguish it from other bacterial genera. This group contains regular, short rods that range from 1-2 μm in length (Holt and others 1994). The rods are found in single, short chains, and are arranged in V and Y forms, or in palisades (Holt and others 1994; Ryser 1999). Cells are Gram positive, do not produce spores, and are not encapsulated. When grown at 20-25°C, *Listeria* can be motile by peritrichous flagella and are weakly mobile around 37°C (Holt and others 1994; Ryser and Marth 1999). They also move in a tumbling motion at

lower temperatures ~20°C by twisting and wriggling. Their speed will increase until they quickly move in different directions.

Growth Requirements

Listeria is considered a psychrotroph by some and grows optimally from 30-37°C, but it can also grow below 10°C (Holt and others 1994; Rocourt and Cossart 1997). The microbe's generation time slows to 30-40 hours at 4°C in fluid skim milk (Ryser and Marth 1999). Therefore, refrigeration is not sufficient to assure the safety of a food (Madigan and Martinko 2006). The bacterium's virulence is increased at lower temperatures rather than higher, which can increase the bacterium's ability to cause disease. *Listeria* requires at least four B vitamins (biotin, riboflavin, thiamine, and thioctic acid) and five amino acids (cysteine, glutamine, isoleucine, leucine, and valine) for sufficient growth (Jay and others 2005). *Listeria* spp. typically grow best around pH neutrality; however, they can grow at a pH range of 5.6-9.6 (Ryser and Marth 1999). *Listeria* can acquire enhanced resistance to acid stress due to growth phase-dependent acid resistance (AR) and adaptive acid tolerance response (ATR) (Gahan and others 1996; Ferreira and others 2003). Adenosine triphosphate (ATP) presence enables enhanced resistance to lethal acid exposure and results from exposure of bacterial cells to mild acidic conditions over a period of time. Acid adaption may induce cross protection against heat, ethanol, oxidation, osmotic stress, and some antimicrobials (Ferreira and others 2003). ATR enhances *Listeria's* ability to survive host challenges like exposure to gastric fluid, bile, competitive intestinal flora, and organic acids found in the small intestine (Ferreira and others 2003). After subjecting Listeria to non-lethal

acidic pH (4.8) for several hours its capacity to resist subsequent lethal acid stress increased (Phan-Thanh and others 2000). The amount of acidity *Listeria* can survive depends on the strain and kind of acid present. Its acid resistance is also growth-phase dependent. In the stationary phase *Listeria* has a natural acid tolerance to a certain extent. *Listeria's* metabolism will decrease in an acidic medium, physiological processes will slow down, and fewer proteins will be synthesized (Phan-Thanh 2002). However, *Listeria* synthesizes a number of indispensible proteins that help it resist acidity (Phan-Thanh 2002). In another study Listeria were subjected to acid and osmotic shock treatments after the beginning of growth and results showed shorter lag phases and longer generation times (Cheroutre-Vialette and others 1998). As incubation temperature is raised above refrigeration, *Listeria* generation time will decrease (Bell and Kyriakides 2005). *Listeria* can grow in some low-pH foods, including fermented products depending on water activity (a_w), food matrix, temperature, and other intrinsic factors (Lado and Yousef 2007). Experiments have shown the pathogen able to survive 1-4 days in orange juice (pH 3.6) stored at 4°C and more than a year in cheddar cheese (pH 5.1) stored at 13°C and 6°C (Lado and Yousef 2007). The Pathogen Modeling Program (PMP) from the USDA showed D-values to decrease with pH in a log-linear fashion (USDA 2003; Lado and Yousef 2007). *Listeria* becomes more sensitive as acidity (pH <4.5) increases along with increase in temperature. Studies have shown that *Listeria* can alter its morphology during stressful environmental conditions. When the pH is greater than 9.0, *Listeria* will become filamentous or elongated chains will form that are 2X greater in length (Efstathios and others 2007). Once the stress is removed,

the cells will return to normal morphology and rapidly subdivide. They will grow optimally with an a_w around 0.97 but have the ability to multiply at an a_w as low as 0.90 (Ryser and Marth 1999).

Listeria are facultatively anaerobic or microaerophilic. Cells are catalase-positive and Cytochrome oxidase negative (Holt and others 1994; Delves-Broughton and others 1996; Ryser and Marth 1999). The growth rate will increase in the presence of fermentable sugars, specifically glucose, due to the presence of glucose oxidase through the Embden-Meyerhof anaerobic glycolytic pathway, which yields pyruvate and lactate (Benedict 1990; Ryser and Marth 1999; Jay and others 2005). *Listeria* ferments lactic acid from glucose as the major metabolic end product. The pathogen is also capable of fermenting rhamnose but is unable to utilize xylose. *Listeria* completes the citric acid cycle and produces acetate and lactate with small amounts of isovaleric, isobutyric, and isohydroxy acids (Patchett and others 1991). *Listeria* is able to hydrolyze esculin into glucose and esculetin to form a black-colored complex with ferric iron (III) ions, a characteristic that has been exploited by microbiologists to identify the microbe in specific media like PALCAM, Fraser broth, and modified Oxford's medium (MOX) (Van Netten and others 1989; Hammer and others 1990; Jay and others 2005; Gorski 2008).

Environment

L. monocytogenes is widely distributed throughout the environment. Its natural habitat consists of soil, water, and plant material (particularly those undergoing decay), animal feces, sewage, and silage (Ryser and Marth 1999; Jay and others 2005; Madigan

and Martinko 2006). Although widely distributed, the numbers of organisms in most environmental habitats are very low. The bacteria can survive longer under adverse environmental conditions than many other non-sporulating bacteria (Ryser and Marth 1999). *Listeria* spp. have the ability to colonize, multiply, and persist on processing equipment making it a particular threat to the industry (Rocourt and Cossart 1997; Ryser and Marth 1999). Specifically, *Listeria* can attach to stainless steel, glass, wood, porcelain, iron, plastic, propylene, rubber, and paper eventually forming a biofilm (Lado and Yousef 2007). Attachment and biofilm formation occurs in the following sequence: cell deposition on the surface through hydrophilic interactions and presence of flagella, cell adhesion to the surface through hydrophilic interactions and presence of fibrils, surface colonization, biofilm formation, and biofilm development through growth and presence of capillary water channels (Lado and Yousef 2007).

Animals such as sheep, goat, and cattle are common reservoirs; therefore, foods of animal origin are usually associated with *L. monocytogenes* (Rocourt and Cossart 1997; Jay and others 2005; CIDRAP 2008). Commonly contaminated foods are uncooked meats and vegetables or unpasteurized milk. Some of the highest risk foods are ready-to-eat (RTE), which are stored under refrigeration for long periods of time and not required to be fully reheated prior to consumption (Ryser and Marth 1999; Jay and others 2005; Madigan and Martinko 2006). Research has shown these foods to be cross-contaminated immediately post-processing with *L. monocytogenes* at greater than 100 CFU/g (Rocourt and Cossart 1997). In a survey of the prevalence of *L. monocytogenes* in RTE foods in the United States, smoked seafood, deli salads, and luncheon meats were found to harbor

the pathogen at rates of 4.31%, 2.36%, and 0.89%, respectively (Burnett and others 2005). Previous experiments have shown that RTE turkey breast supported higher *Listeria* growth rates on growth curves than cured ham and cold-smoked salmon due to presence of salts of lactate or diacetate (Burnett and others 2005). The organism has also been found in raw milk, soft cheeses, fresh and frozen meat, poultry, seafood, and on fruits and vegetables (Jay and others 2005). Dairy, particularly milk, was the first and most often studied food product for *L. monocytogenes* (Rocourt and Cossart 1997; Jay and others 2005). Soft cheeses are of greatest concern because of the frequency of *Listeria* (2-10%) and the bacterial load (10^1-10^7 CFU/g) within the product due to their ability to grow in high salt, slightly acidic conditions, and in the presence of lactic acid bacteria (LAB) starter cultures (Rocourt and Cossart 1997; Cataldo and others 2007). The accidental use of raw milk in soft cheese due to inadequate pasteurization or improper use of raw milk for making cheese at home has created many problems with *Listeria* growth in soft cheeses. *Listeria* grows well in this matrix because soft cheeses are commonly treated with brine in the production process. This leads to increased salt concentrations that may inhibit competing organisms (Linnan and others 1988). The process temperature and short ripening times also drive *Listeria* transmission. Soft cheeses do not undergo a heat treatment (40˚C-50˚C) like hard cheeses, and soft cheeses usually do not go through a ripening process so they never develop a strongly acidic pH to reduce bacterial growth. However, the United States requires non-ripened cheeses (aged less than 60 days) to use pasteurized milk for their production (FDA 1998). Ripened cheeses (aged more than 60 days) are not required to use pasteurized milk for

their production due to low water activity and pH content (FDA 1998). Many *Listeria* outbreaks have been documented with the primary vehicles being foods listed above.

Incidence

In the United States, approximately 2,500 people develop listeriosis each year; of these, ~500 people die (CDC 2008). The incidence of listeriosis has continued to decline in recent years; from 1989-1993 the incidence declined from 7.4 to 4.4 million cases partially due to regulatory agencies enacting the zero-tolerance policy for processed meats (USDA 1993; Ryser and Marth 1999). In 1996 the incidence per 100,000 persons was 0.46 compared to an incidence rate of 0.27 in 2007 (CIDRAP 2008). However, as of 2007, the national health objective of 0.24 incidence set for 2010 had not been met (CIDRAP 2008). The overall case-fatality rate of systemic or invasive listeriosis is 20%-30% for epidemic and sporadic cases, and the mortality rate is higher (38%-40%) for the immunocompromised, elderly, pregnant women, and people with central nervous system (CNS) problems (Rocourt and Cossart 1997; CDC 2008). People with weakened CNS, cancer, or those using immunosuppressive medication following organ transplantation are at an increased risk of developing bacterial meningitis known as inflammation of the membranes and cerebrospinal fluid surrounding the brain and spinal cord.

Cost of Illness

Listeriosis is a costly disease due to its severity, incidence of residual symptoms, and high case-fatality rate (Rocourt and Cossart 1997). Estimates for total costs of listeriosis cases in 2000 approximated $2.3 billion (Crutchfield and Roberts 2000). This amount was due to 2,493 cases, 2,298 hospitalizations, and 499 deaths (CDC 1999;

Crutchfield and Roberts 2000). In 1993, the estimated total costs of listeriosis were ~$264 million. This total cost was determined from several subcategories. This includes acute illness medical costs of $61.7-$64.8 million. There were three case categories present for acute illness medical costs. Maternal hospitalized cases cost $3.1 million, newborn/fetus hospitalized cases cost $14.3-$17.4 million, and other adult hospitalized cases cost $44.3 million (USDA 1993). Medical and special education costs from chronic listeriosis, which was only present in newborn/fetal cases, cost $7.2 million (USDA 1993). Productivity losses from acute listeriosis cost $125.8-$154.4 million, while productivity losses due to chronic listeriosis cost $38.0 million per year (USDA 1993). The estimated total cost for listeriosis has lowered some from 1993-2000, but it is still considered a costly disease.

Foodborne Outbreaks

The first *Listeria* confirmed foodborne outbreak occurred during 1981 in Nova Scotia, Canada, over a six-month period (Rocourt and Cossart 1997; Ryser and Marth 1999). There were 41 patients affected, and 37 were pregnancy-associated cases. The vehicle for *Listeria* transmission was found to be coleslaw. It is believed the cabbage used to make coleslaw was fertilized with sheep's manure, which caused the cross-contamination. During 1983 in Boston, Massachusetts 49 cases were confirmed over a two-month period with a case fatality rate of 29% (Rocourt and Cossart 1997; Ryser and Marth 1999). The illness was strongly associated with drinking a specific brand of pasteurized whole or 2 percent milk (Fleming and others 1985). The milk associated with disease came from a group of farms on which listeriosis in dairy cows was known

to have occurred at the time of the outbreak. Multiple serotypes of *L. monocytogenes* were isolated from raw milk obtained from these farms after the outbreak (Fleming and others 1985). At the plant where the milk was processed, inspections revealed no evidence of improper pasteurization. However, it is possible some of the *Listeria* cells survived the pasteurization process. The largest North American outbreak occurred during 1985 in California. There were 142 cases over an eight-month period with 93 pregnant cases and 49 non-pregnant cases (Linnan and others 1988; Rocourt and Cossart 1997; Ryser and Marth 1999). The case fatality rates were 32% and 37%. The source of contamination was determined to be Mexican Queso Fresco soft cheese due to inadequate pasteurization of the raw milk used to produce this cheese (Linnan and others 1988). This outbreak helped determine *Listeria* has longer incubation periods (11-30 days) than most foodborne pathogens (Ryser and Marth 1999). A four-year outbreak occurred during 1983-1987 in Switzerland affecting 122 cases due to soft cheese contamination. In 1989-1990 the United Kingdom suffered 300 cases from pate´(Rocourt and Cossart 1997). France had an outbreak in 1992 with 278 confirmed cases due to pork tongue cross-contamination. The contamination probably occurred during distribution (Rocourt and Cossart 1997). During 1997 in Italy, 1,566 children contracted febrile gastroenteritis from tuna and corn salad; 292 were hospitalized and 87% of stool cultures were positive for *L. monocytogenes* (CIDRAP 2008). “Cleugh's Frozen Foods' [recalled] frozen strawberries sold to Jamba Juice locations in Arizona, Nevada, and Southern California during 2006 that was due to *Listeria* contamination”. It

created a scare to consumers that reduced confidence in the safety of the product (FDA 2006).

Listeriosis

Listeriosis is an illness caused by the infectious bacterium *Listeria monocytogenes*. It is characterized by a sudden onset of fever, sever headache, vomiting, and other influenza-type symptoms (USDA 1993). Roughly 85%-95% of all listeriosis cases are attributed to food (USDA 1993). Listeriosis has caused premature death in fetuses, newborns, and some adults. Susceptible populations include neonates, the elderly, pregnant women, immunocompromised (people with predisposed disease leading to T-cell mediated immunity), and AIDS victims (Rocourt and Cossart 1997; Jay and others 2005; Madigan and Martinko 2006). Adults with listeriosis most frequently contract sepsis, meningitis, or meningo-encephalitis. Central nervous system symptoms may include fever, malaise, ataxia, seizures, and altered mental status (Painter and Slutsker 2007). Spontaneous abortion of a pregnancy is strongly associated with a decease in T-cell mediated immunity which is responsible for resistance to *Listeria* (Lorber 1990). Most cases of listeriosis during pregnancy occur in otherwise healthy women who show nonspecific symptoms that appear as a mild illness. During pregnancy, the form of listeriosis is bacteremia and virtually never meningitis even though meningitis is the most common form of listeriosis in other at risk groups. A quarter of patients have a bacteremic form of listeriosis showing fever, fatigue, myalgia, malaise and isolation of *L. monocytogenes* from blood cultures without evident foci of origin or metastatic infection (Lorber 1990; Ryser and Marth 1999). Most patients with

bacteremia have underlying conditions like hematologic malignancy or immunosuppression. In clinical cases of listeriosis, 30% occur in people younger than 3 weeks old and people older than 40 years of age (USDA 1993).

Infected pregnant women can transmit the illness to their newborns/fetuses before or during delivery by transplacental transmission (Painter and Slutsker 2007). Infants infected *in utero* may be aborted early on, stillborn, or are born with early onset neonatal septicemia (Lorber 1990; USDA 1993; Jay and others 2005). Infants infected at or shortly after birth can develop late onset neonatal meningitis and will age and develop chronic neurological complications. Babies infected shortly after birth will typically be premature with a low birth weight. They can develop respiratory issue including pneumonia and granulomatosis infantiseptica (Lorber 1990). Late onset neonatal infection is manifested as meningitis in the second to fourth week after birth.

The infective dose of *L. monocytogenes* depends on many factors. These include the immunological status of the host, exposure to particular foods, and the virulence factors of the organism. Data indicates the amount of *L. monocytogenes* in contaminated food responsible for epidemic and sporadic foodborne cases is more than 100 CFU/g (Rocourt and Cossart 1997). However, the infective dose of *L. monocytogenes* is unknown but is believed to vary with the strain and susceptibility of the victim as stated earlier. Fewer than 1,000 total organisms may cause disease in cases associated with the consumption of raw and pasteurized milk (FDA 2007).

Pathogenicity

L. monocytogenes is one of the most invasive bacteria known because it can infect many different cells (macrophages, fibroblasts, hepatocytes, and epithelial cells). If *L. monocytogenes* is contracted orally, it will cross the intestinal barrier at the site of entry at epithelial cells or M cells in Peyer's Patches (Rocourt and Cossart 1997; Madigan and Martinko 2006). Bacteria are then internalized by surrounding gastrointestinal (GI) cells to escape macrophage attack where they will survive and replicate. They are then transported by blood to regional lymph nodes. Upon reaching the liver and spleen, most *Listeria* are killed quickly (Rocourt and Cossart 1997). During the early phases of the illness, hepatocytes are the target for neutrophils and eventually for mononuclear phagocytes (Rocourt and Cossart 1997; Jay and others 2005; Madigan and Martinko 2006). Further spreading and infection may occur depending on the level of T-cell response. Therefore, infection is not localized at the site of entry and can involve many cell types and tissues (Rocourt and Cossart 1997).

Intracellular Invasion

Listeria spread directly from cell-to-cell to shelter themselves from host defenses. Soon after entry into the target cell, the bacteria are internalized in membrane-bound vacuoles. These vacuoles are lysed in less than thirty minutes, and the bacteria are released into the cytoplasm where they will begin to replicate (Rocourt and Cossart 1997; Ryser and Marth 1999; Jay and others 2005). The protein toxin Listeriolysin O (LLO) belongs to a family of thio-activated, cholesterol-dependent, pore-forming toxins (CDTX) responsible for this mechanism (Kuhn and Goebel 2007). LLO will oligomerize

in the target cell membrane to form stable pores due to its affinity for cholesterol within the cell's membrane. Once inside the cytosol, a surface protein ActA helps form actin tails that will cover the bacteria and rearrange into a polar tail (Rocourt and Cossart 1997; Ryser and Marth 1999; Jay and others 2005). This tail will propel the organism toward the cytoplasmic membrane. Upon reaching the membrane, the bacteria push out to form a protrusion called a filopodium that has a bacterium at its tip (Jay and others 2005). A neighboring cell then internalizes the protrusion through phagocytosis making a two-membrane-bound vacuole. The new vacuole is lysed and the invasion process is repeated. Specific virulence genes that include LLO and the two bacterial phospholipases, phosphatidylinositol-specific phospholipase C (PIPLC) and the broad-range phospholipase C (PCPLC), aid this mechanism (Rocourt and Cossart 1997; Jay and others 2005). This is a very effective invasion method that allows *Listeria* to occupy many cells and organelles within the host.

CHAPTER II

LIPOSOMES

Since the first observation of phospholipid vesicles (liposomes), liposome-derived technologies have become one of the cornerstones of bio-nanotechnology (Bangham 1972; Jesorka and Ormar 2008). Liposomes are small vesicles formed from amphiphilic lipids suspended in an aqueous environment that enclose an aqueous core. Theses vesicles can incorporate many functional components within their interior making them very versatile. Material may be entrapped in the lipid bilayer or in the aqueous phase, depending on its inherent lipophilicity/hydrophilicity (Skeie 1994). Liposomes can be made entirely from naturally occurring substances and can be therefore nontoxic, biodegradable and non immunogenic (Lasic 1995). Due to liposome versatility and ability to act as targeted release-on-demand carrier systems for water and oil-soluble compounds, they have been used in a number of industrial applications including drug delivery, gene therapy, cosmetics, ecological preservation, and food processing (Lasic 1995; Laye and others 2008). Liposomes are predominantly composed of phospholipids that spontaneously form bilayers when polar solvents, such as water, are mixed with dried lipid. Polar head groups from phospholipids will orientate towards the polar, aqueous environment, while hydrophobic tails tend to cluster together to minimize their contact with water, forming a lipid bilayer as seen in figure 1 (Wiggins 1990; Kim and Baianu 1991).

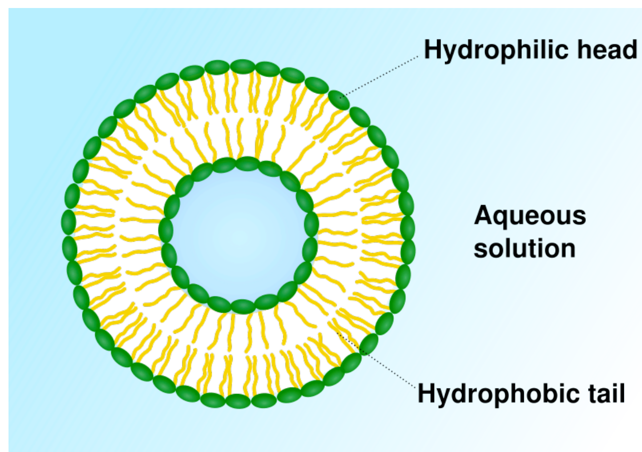

Figure 1-Liposome with Bilayer in Aqueous Solution.
Phospholipid head groups represented by green spheres and hydrophobic tails represented by yellow lines (Wikimedia 2009).

However, these vesicles can be formed from many lipid species and classes creating different polymorphic phases such as the bilayer or hexagonal (H_{II}) organization (Hope and others 1985). Different methods of preparation will assist in determining the size of the vesicle making large or small unilamellar or multilamellar vesicles among other possibilities.

Liposome Properties

The primary phospholipids used for the production of liposomes include the zwitterion phosphatidylcholine (PC) and the anionic phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS), and phosphatidylethanolamine (PE) (Jesorka and Ormar 2008). Each of these lipids may possess a different combination of fatty acid chains in the hydrophobic region of the molecule giving different degrees of saturation. Each phospholipid is unique and has complex phase transition profile. The gel to liquid crystalline phase transition temperature (T_M) is defined as the temperature

required to induce a change in the lipid physical state from the ordered gel phase in which hydrocarbon chains are fully extended and closely packed to the disordered liquid crystalline phase where the hydrocarbon chains are randomly oriented and fluid (Taylor and others 2005; Voet and others 2006; Avanti 2009). There are several factors that influence the phase transition temperature such as acyl chain length, headgroup charge, headgroup species, and degree of unsaturation. Usually, the longer hydrocarbon chain lengths mean there will be higher phase transition temperatures (Voet and others 2006). Introducing a double bond puts a kink in the chain requiring lower temperatures to induce an ordered packing arrangement (Avanti 2009). Sometimes cholesterol is added to a system to improve stability. Cholesterol is the major sterol component in most mammalian membranes, and is not homogeneously distributed among different organelles. One of the specific physical features of cholesterol is the planar steroid ring, a conformationally rigid structure, which governs much of the interactions of cholesterol in a lipid bilayer (Raffy and Teissie 1999). Cholesterol lowers membrane permeability at elevated temperatures and modulates membrane-protein interactions thus imparting better stability (Samad and others 2007). Cholesterol decreases membrane fluidity thus creating rigidity because its steroid ring system interferes with the motions of fatty acid side chains (Voet and others 2006). When cholesterol is present in large amounts, it acts as a permeability barrier for the membrane by introducing conformational ordering of the lipid chain (Raffy and Teissie 1999). Specifically, it alters the freedom of formation of carbon molecules in the acyl chain. It increases its mechanical stiffness while keeping the membrane fluid.

Liposomes are most frequently classified by their size, and number of bilayers (lamellae). Liposomes can vary widely in size from <30nm referred to as small unilamellar vesicles (SUVs) to 20-100nm referred to as large unilamellar vesicles (LUVs) to >100nm called giant unilamellar vesicles (GUVs) (Kim and Baianu 1991; Taylor and others 2005; Samad and others 2007; Taylor and others 2007). These are vesicles with only a single bilayer membrane; however, liposomes with more than a single bilayer membrane are called multilamellar vesicles (MLVs) (Lasic 1995). Larger vesicles will have a better chance at trapping a higher volume, however sizes >300 nm will scatter light making them visible to the naked eye showing a cloudy appearance (Taylor and others 2005). Table 1 shows a summary of the different liposome classifications. These various sizes are a result of the many different preparations and methodologies used today.

Table 1- Size and Lamellae-Dependent Classification of Liposomes[a]

Vesicle Type	Abbreviation	Diameter Size	Number of Lipid Bilayer
Small Unilamellar vesicle	SUV	20-100 nm	One
Large Unilamellar vesicle	LUV	>100 nm	One
Giant Unilamellar vesicle	GUV	>1 micro meter	One
Oligolamellar vesicle	OLV	0.1-1 micro meter	~ 5
Multilamellar vesicle	MLV	>0.5 nm	5-25

[a]Vesicle types with their abbreviation, size, and number of lipid layers (Kim and Baianu 1991; Lasic 1995; Taylor and others 2005; Samad and others 2007).

Vesicle Preparation

Multilamellar vesicles were first prepared using a simple film-hydration technique (Bangham and others 1965). The lipid solution is initially dried either via evaporation, spray drying, or lyophilization to produce a thin film. The sample is then hydrated with an aqueous solution and mechanically agitated. Vesicles spontaneously form when the film is exposed to an excess volume of aqueous buffer and agitated (Lasch and others 2003). This produces vesicles that are heterogeneous in size, a major disadvantage, as well as, possible sample degradation occurring (Kim and Baianu 1991; Jesorka and Ormar 2008). They have large diameters, multiple internal compartments, a low entrapment volume, and are inconsistent from batch to batch (Mui and Hope 2007). The main advantage of MLVs is that their lipids are not subjected to harsh treatments

like exposure to organic solvents or high-intensity ultrasound. MLVs can be transformed into unilamellar vesicles through various mechanical processing methods.

LUVs are thought as the most useful liposome because they are more homogeneous than MLVs and have higher encapsulation efficiency than SUVs (Kim and Baianu 1991; Mui and Hope 2007; Samad and others 2007; Jesorka and Ormar 2008). This is due, in part, to their sufficiently large radius and single bilayer. Several common methods of preparation that are considered non-mechanical methods are reverse phase evaporation, detergent dialysis, and freeze-thaw.

SUVs consist of a single lipid bilayer and have a relatively homogeneous size distribution. Encapsulation efficiency for SUVs is around 1-2% of the original enzyme preparation (Skeie 1994). SUVs will spontaneously fuse when they drop below the phase transition temperature of the lipid forming the vesicle (Avanti 2009). There are several methods that produce SUVs including sonication, high-pressure homogenization, and extrusion all of which are considered mechanical methods.

Reverse Phase Evaporation

In reverse-phase evaporation (REV) the lipid mixture and aqueous solution to be encapsulated are dispersed in an organic solvent. The system is subjected to homogenization forming an emulsion. The emulsion structure is similar to inverted micelles (hydrophilic headgroups interacting with aqueous phase while hydrophobic fatty acid tails interact with organic solvent) (Lasch and others 2003; Taylor and others 2005; Samad and others 2007). The solvent is removed by evaporation creating a gel-like state. The gel-like state collapses and some inverted micelles disintegrate resulting

in excess phospholipid that helps to form a complete bilayer around the remaining micelles (Winterhalter and Lasic 1993). This produces heterogeneous vesicles (100-1000 nm) with high entrapment efficiencies (up to 65% of aqueous phase can be trapped within vesicles) (Lasch and others 2003). However, the material to be encapsulated is exposed to organic solvent that may lead to protein denaturation and complete removal of the solvent is almost impossible (Skeie 1994; Lasch and others 2003; Taylor and others 2005; Mui and Hope 2007).

Detergent Depletion

This method is used for the removal of small molecular weight material from liposome dispersions that escaped entrapment and for the complete removal of detergents from mixed detergent lipid micelles to produce homogenous liposomes (Lasch and others 2003; Taylor and others 2005). Detergents are a class of molecules that disrupt or form hydrophobic and hydrophilic interactions among molecules in biological samples. Common detergents used include sodium cholate, alkyl(thio)glucosides, and alkyloxypolyethylenes (Lasch and others 2003). Sodium cholate is a water-soluble ionic detergent and is one of the least denaturing of ionic detergents. At concentrations > 9.5 mM, sodium cholate forms small micelles around (900-1,200 Da) that allows easy removal by dialysis or gel filtration, if needed (Pierce 2006). Cholate and deoxycholate produce the most homogenous liposome populations (Lasch and others 2003). Alkyl(thio)glucosides and alkyloxypoly-ethylenes are nonionic detergents meaning they lack a charged group. At high concentrations nonionic detergents solubilize biological membranes by forming mixed micelles of detergent,

phospholipid, and integral membrane proteins. At low concentrations they may bind to the hydrophobic regions of most membrane proteins, making them soluble in aqueous solution; however, they do not form mixed micelles (Lodish and others 2000). Micelles are formed when more surfactant than lipid is present. As surfactant molecules are removed from the aqueous phase using dialysis, surfactant molecules present in micelles will be removed (Taylor and others 2005). This creates mixed surfactant-containing liposomes and further dialysis is required to completely remove the surfactant to produce surfactant-free liposomes. Other methods used to deplete the detergent include dilution, gel-filtration, and adsorption (Lasch and others 2003). The detergent used determines the size distribution of vesicles formed. Detergent depletion is a very flexible method because it allows the preparation of a large variety of liposomes and proteoliposomes. It is a mild treatment so even sensitive proteins and encapsulated materials can survive while physical (sonication, extrusion) and chemical treatments (organic solvents) can induce loss of functionality. However, this method is time consuming and small amounts of surfactant can stay in the system.

Freeze-Drying Rehydration

Freeze-dried rehydration vesicles (FRVs) are formed from preformed vesicles to refine and improve their properties instead of simply producing them (Lasch and others 2003). These vesicles are formed from preexisting liposomes that have been subjected to dehydration-rehydration cycles. In this method, lipids are hydrated with the aqueous mixture containing the material to be entrapped after which they are freeze-dried, leading to a dispersion of solid lipids in a finely subdivided form (Samad and others

2007). Rehydration above the gel-liquid crystalline phase transition temperature will cause the membranes to fuse and reseal to produce MLVs (Skeie 1994; Taylor and others 2005). These vesicles are much larger than the initial liposomes and high encapsulation efficiencies can be achieved (up to 45%) (Lasch and others 2003; Taylor and others 2005). To further improve encapsulation efficiencies, liposome may be subjected to multiple freeze-thaw cycles above their phase transition.

Freeze-Thaw Cycling

Freeze-thawing involves submerging a sample under water and in several different temperatures with large gradients. During freezing, solutes are expelled from the ice phase and the material to be encapsulated is concentrated in the residual fluid (Burger and others 2002; de Kroon and others 2005; Laye and others 2008). Small aggregates of neutral species begin to form followed by co-aggregation of the positively charged encapsulate species (Burger and others 2002). Electrostatic interaction of the positively charged solution and negatively charged lipids results in vesicle formation (Laye and others 2008).

Sonication

Sonication applies sound (ultrasound) to agitate particulates. This method is among the first mechanical treatments of amphiphilic lipids (Mui and Hope 2007). Ultrasonication generates alternating low-pressure and high-pressure waves in liquids, leading to the formation and violent collapse of small vacuum vesicles. This event is called cavitation and it creates extreme pressure and temperature gradients in these vesicles along with powerful shear-forces. In these conditions, large liposomes

spontaneously form when mixed with aqueous solutions. High-shear forces will eventually lyse large liposomes to form smaller vesicles (Taylor and others 2005). There are two approaches to sonication. One is immersing a metal probe directly into a mixture of liposomes (Kim and Baianu 1991; Mui and Hope 2007; Samad and others 2007; Jesorka and Ormar 2008). This method is usually used for small volumes and requires high energy (Samad and others 2007). The second approach involves the mixture held in a glass vial and placed in a bath sonicator and is useful for large volumes. Unlike the probe, this method can be carried out in a closed container under nitrogen and cannot be contaminated with the metal from the probe tip (Kim and Baianu 1991). However, bath sonicators are preferred because they maintain a uniform energy distribution resulting in homogeneous liposomes. The mechanical agitation of sonication may create problems like enzyme activity reduction and foaming, which loses lipids.

High-Pressure Homogenization and Microfluidizers

The main advantages of homogenized liposomes are their single bilayer membrane and small and homogeneous vesicle size (Lasch and others 2003). The processes work mostly under mild conditions, are cost-effective and may be scaled up easily (Bachmann and others 1993). Several types of homogenizers are available including the French pressure cell, Ultra-Turrax® high-shear mixers, and microfluidizers. Gap and interaction-chamber machines give high levels of energy dissipation and small particles where high-shear mixers give lower energy use and larger particle sizes (Lasch and others 2003).

The Microfluidizer is a high-pressure homogenizer that can quickly produce a

large volume of liposomes in a continuous and reproducible manner without using sonication, detergents, solvents, or alcohols (Thompson and Singh 2006). Microfluidization is based upon the interaction between two fluid streams at high velocities. The aqueous buffer solution of pressurized in continuous flow, and split into two streams that are forced together at high velocities (>500 m/s) causing large phospholipid bilayer sheets to break into smaller pieces (Kim and Baianu 1991; Thompson and Singh 2006). Microfluidizers usually create smaller particle sizes than other homogenizes, which improves the macroscopic appearance and physical stability (Barnadas-Rodriguez and Sabes 2001).

Extrusion

Extrusion involves forcing a sample of large liposomes (LUVs) through cylindrical pores of filters uniform in size resulting in a homogenous population of smaller vesicles (SUVs) with vesicle sizes correlating to the size of filters used (Mayer and others 1986; Taylor and others 2005). By forcing large liposomes through smaller pores, they are sheared and resealed rapidly, consequently entrapping the targeted substance. The large vesicles are subjected to shear-induced tensions in the bilayer that makes the membrane unstable and smaller vesicles are produced as a result (Mui and Hope 2007). Liposomes are usually subjected to multiple passes through membranes (MacDonald and others 1991). It is important for this method to be performed within solutions containing the material to be encapsulated because anything trapped before will leak out during extrusion and then be resealed within the new smaller liposomes (Taylor and others 2005; Jesorka and Ormar 2008). Completion of extrusion at

temperatures above the main gel-liquid crystalline phase transition temperature is required because lipids in the gel-liquid state cannot be effectively extruded at low pressures (MacDonald and others 1991). This is most likely due to higher viscosities and decreased deformability. Hand held extruders have shown on average processing pressures ranging from 200-300 lb/inch^2 proving significant pressure can be generated for benchtop procedures (MacDonald and others 1991). An example of a hand held extruder is the LiposoFast™ Mini-Extruder produced by Avestin Inc. (Ottawa, Ontario, Canada) shown in Figure 2.

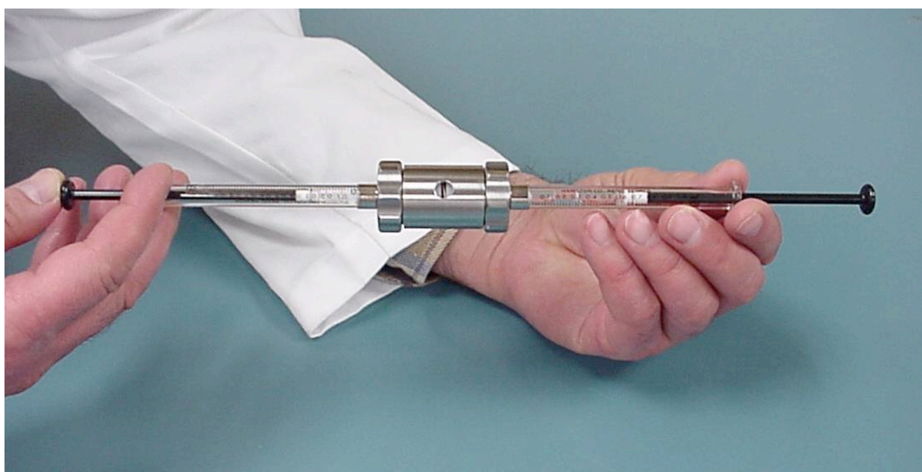

Figure 2-LiposoFast™ Mini-Extruder.
Hand held benchtop extruder produced by Avestin Inc. The extruder uses two 1mL syringes that are luer lock at each end of the extruder. The sample is passed back and forth through membranes with specific pore sizes. The sample is sheared as it is passed through the pores, and the new vesicle size correlates to the size of filters used (Avestin 2009).

Producing liposomes through extrusion offers several advantages such as the absence of residual organic solvent or detergents, the process works well with many types of lipids, and it produces liposomes homogeneous in size distribution and with high encapsulation efficiencies (Hope and others 1985; Mayer and others 1986).

Subjecting liposomes to several freeze-thaw cycles before extrusion may also allow for larger inter-lamellar spacing and much larger trapped volumes (Hope and others 1985; Mayer and others 1986; Mui and Hope 2007). Table 2 gives an overview of liposome preparation techniques and their resulting liposome classification.

Table 2: Common Preparation Techniques for Different Types of Liposomes[a]

Multilamellar preparation	Unilamellar preparation		
MLV	SUV	LUV	GUV
Thin-film dehydration (evaporation-dried, spray-dried, or lyophilized lipid material)	High-energy sonic fragmentation Extrusion High-pressure homogenization Solvent injection	Freeze-thaw cycling Swelling in non-electrolytes De-/rehydration Detergent dialysis Reverse evaporation	De-/rehydration Electroformation Solid-film hydration Detergent dialysis

[a]Various liposome preparation techniques and liposome classification outcome (Bangham and others 1965; Kim and Baianu 1991; Skeie 1994; Burger and others 2002; Lasch and others 2003; Taylor and others 2005; Mui and Hope 2007; Samad and others 2007; Jesorka and Ormar 2008; Laye and others 2008).

Liposomes in Drug Delivery

One of the first applications applied to liposomes was encapsulating drugs and therapeutics for localized delivery (Gregoriadis 1976b; Gregoriadis 1976a; Papahadjopoulos 1978). Liposomes are very effective in this area because they can enclose many different classes of substances such as antibacterial, antiviral, and anticancer drugs, as well as hormones, enzymes, nucleotides, steroids, and

bronchodilators. The aim of liposomal drug delivery is to achieve a high localization of active compounds at disease sites such as tumors, or inflammations (Ranade 1989; Jesorka and Ormar 2008). Localization is the controlled release when a change in the liposome structure or in its reactivity promotes the release of encapsulated material. Controlled release helps to ensure release of the target-drug through a specific mechanism at the target site. There are two main controlled-release mechanisms. One is based on the development of an affinity reaction between the target and the liposome. The second is a triggered-release and involves incorporating an environmentally-responsive (pH, temperature change, or light irradiation) molecule into the liposome that will cause structural changes in the bilayer membrane (Jesorka and Ormar 2008). Alteration of liposome surface charge has also been shown to enhance drug entrapment and release (Ranade 1989). Several liposomal drug delivery applications include:

- Enhancing drug solubilization for Amphotericin-B, Minoxidil, Paclitaxels, and Cyclosporins
- Protection of sensitive drug molecules like Cytosine arabinosa, DNA, RNA, Anti-sense oligo-nucleotides, and Ribozymes
- Enhancing intracellular uptake of anticancer, anti viral and antimicrobial drugs
- Altering pharmacokinetic and bio-distribution for prolonged or sustained release of drugs with short circulatory half lives (Samad and others 2007)

Liposomal aerosols have been used to treat respiratory disorders for sustained release, prevention of local irritation, reduced toxicity, and improved stability. These aerosols can treat diseases of the eye including dry eyes, keratitis, corneal transplant

rejection, uveitis, endopthelmitis, and proliferative vitro retinopathy by being used as a vector for genetic transfection and monoclonal antibody directed vehicles (Samad and others 2007). Liposomes can act as anti-infective agents by removing pathogens such as protozoa, bacteria, and fungus that reside in the liver and spleen. The polyene antibiotic, Amphotericin B, used to treat fungal infection because of renal toxicity has been encapsulated at normal doses for the treatment of these infections. Small and stable liposomes can target different tumors since they can circulate longer and pass through vessel walls into surrounding tissues through enhanced vascular permeability improving anticancer therapy (Gabizon 1992). Several formulations of liposomal drug formulations are Doxil, EVACT™, DaunoXome, VincaXome, and Mikasom (NeXstar Pharmaceuticals, Inc., Boulder, CO).

Liposomes in Gene Therapy

The aim of gene therapy is to deliver DNA, RNA, or antisense sequences to cells in order to alleviate symptoms or prevent diseases (Lasic and Templeton 1996; Templeton and Lasic 1999). Some major applications are gene replacement, addition of genes for production of natural toxins, sensitizing cells to other treatments, and over-expression of highly immunogenic genes for immune self-attack. Diseases that could benefit from gene therapy by restoration of mutated genes and enhancement of the body's response include cystic fibrosis, hemophilia, sickle cell anemia, immune system deficiencies, transmissible viral diseases (HIV, hepatitis), neurological diseases (Parkinson's disease), and Alzheimer's (Lasic and Templeton 1996). It was determined that cationic liposomes can electrostatically interact with anionic DNA, RNA, and

proteins, and complex into small stable particles that maintain increased transfection efficiencies (Templeton and Lasic 1999; Jesorka and Ormar 2008). Using liposomes for gene therapy has many advantages including:

1. Unlimited size of nucleic acids that can be delivered
2. Low cost and ease of producing liposome complexes that deliver therapeutics on a large scale
3. Ability to target liposome complexes by colloidal or surface properties
4. Lack of immunogenicity

(Templeton and Lasic 1999)

In order to target liposomes to a specific cite, ligands should be added by ionic interactions or by covalent attachments. Ligands are signal-triggering molecules that bind to a site on a target protein. Typically polyethylene glycol-conjugated (PEGylated) lipids are used to prepare noninteracting liposomes; however, normal liposomes can become sterically stable by incubation with PEG-lipid micelles making them interact with DNA.

Liposomes in Cosmetics

In 1963, the use of lipid vesicles as systemic and topical drug delivery systems began attracting attention. The liposome bilayer structure contains phospholipids or sphingolipids which resembles natural membranes making this system applicable to the cosmetic industry (Arnaud 1995). Liposomes, depending on composition, can alter cell membrane fluidity and fuse with cells to deliver active drugs and ingredients to the target site (Betz and others 2005). Some experts believe liposomes do not penetrate as

intact vesicles or permeate the skim, but deform into fragments (Jesorka and Ormar 2008). Liposomes offer advantages because the lipids used are well hydrated and can reduce the dryness of the skin, which is a primary cause for its ageing. The vesicles within creams, ointments, and other solutions will deliver drugs in a concentration dependent manner across the stratum corneum to the epidermis and dermis (Betz and others 2005). Liposome cosmetics range from pastes (creams, gels, and ointments) to formulations containing various extracts, moisturizers, antibiotics, and recombinant proteins for wound or sunburn healing (Lasic 1995). Due to their high and long lasting moisture content, most products are anti-ageing creams. Other products include sunscreens, perfumes, hair conditioners, and aftershaves. It should be noted that simple liposome formations do not allow penetration into the lower epidermal layers of the skin and therefore, are of minor value as transdermal drug delivery systems (Geho 1995; Jesorka and Ormar 2008).

Liposomes in Food Industry

Food ingredients are encapsulated in liposomes for the same reason many other compounds are, and this is to improve stability of the ingredients by protecting them from metal ions, pH, free radicals, or enzymatic degradation (Reineccius 1995; Gibbs and others 1999). Liposomes work well for the food industry because they are natural, biodegradable, non-toxic, and versatile systems for both water and oil soluble components. Main applications within the field include altering the texture of food components, encapsulation of ingredients, additives, and antimicrobials, controlled release of flavors, and increasing the bioavailability of nutritional components like

vitamins and minerals (Reza Mozafari and others 2008). Table 3 shows various food ingredients that have been encapsulated over the years.

Table 3-Various Encapsulated Food Ingredients[a]

Ingredients	Specific Compounds	Applications	References
Favoring agents (oils, spices, and seasonings	Citrus oil, peppermint, tumeric, paprika	Flavors are delicate and usually volatile, encapsulating them creates protection from evaporation, chemical reactions, oxidation, or migration within food.	(Cheetham 1999; Barbosa-Canovas and others 2005)
Sweeteners	Aspartic acid	Encapsulating sweeteners reduces their hygroscopicity, improves their flowability, and prolongs their sweetness perception	(Schobel and Yang 1989)
Acids	Ascorbic acid, fumaric acid, lactic acid, malic acid	For flavor modifiers, preservatives, and processing aids for dough conditioners, and cured meat processing	(Barbosa-Canovas and others 2005)
Lipids	Omega-3 fatty acids, carotenoids, polysterols	Encapsulation helps stabilize fatty acids from rancidity, and autoxidation, which increases shelf-life. Used in infant formulas, bread mixes, fish oil capsules	(Hoch 1997; Schrooyen and others 2001)
Enzymes	Chymosin (milk coagulant enzyme)	Enzyme encapsulation in liposomes provides good distribution in the curd and prevents interaction with milk proteins at the vat stage allowing shortened coagulation times. Enzymes can maintain viability by avoiding exposure to ions, protons, and free radicals.	(Picon and others 1994; Barbosa-Canovas and others 2005)

Table 3 Continued

Ingredients	**Specific Compounds**	**Applications**	**References**
Microorganisms	Lactobacillus, Bifidobacterium,	Encapsulation helps in segregating the bacterial cell from the adverse environment of the product thus potentially reducing cell loss. Encapsulation helps increase the survival and delivery of microorganisms. This is used within the dairy industry to protect probiotics within products.	(Sultana and others 2000; Annan and others 2007)
Antioxidants	Flavonoids, Cyclodextrins,	Encapsulation enhances solubility, dissolution rate, membrane permeability and bioavailability drugs. Improves stability to air and light. Flavonoids have been added to broccoli and other vegetables.	(Calabro and others 2004; Duncan 2006)
Preservatives	Salt	Controls water absorption, rancidity, and yeast growth in meat products, pretzels, and yeast dough	(Shahidi and Han 1993; Barbosa-Canovas and others 2005)
Vitamins and Minerals	Vitamins C, A, D, K, beta-carotene, B group Iron, Calcium,	Encapsulation provides better stability, extends shelf-life, protects from oxidation. Used in dried milk, juices, orange oil, fluid milk.	(Schrooyen and others 2001; Duncan 2006)
Fragrances		Encapsulation helps control fragrance evaporation, interactions with other components, oxidation and chemical degradation in air fresheners, perfumes, etc.	(van Soest 2007)

[a]Ingredients that have been encapsulated for the food industry.

One of the first studied liposome applications in food products was for reducing the ripening time and enhancing flavor development in cheese production (Law and

King 1985; Alkhalaf and others 1988; Kirby 1990; Law and King 1991; Lasic 1995). Due to the longevity of ripening, it is a substantial cost of producing cheeses. Therefore, reducing the time required is of great benefit to the industry. Incorporating enzymes into cheese has shown to reduce production times; however, addition of free enzymes in milk causes premature proteolysis. These enzymes are soluble in water and are inactivated during curd formation resulting in unfavorable curd consistency and low yields (Arnaud 1995; Reineccius 1995; Gibbs and others 1999; Reza Mozafari and others 2008). Experiments in a Saint-Paulin cheese demonstrated up to 60% of liposomal-entrapped enzyme was retained whereas only 20% of free enzyme was retained. Within fifteen days of ripening, the liposome treated cheese had achieved an equivalent level of proteolysis as the non treated cheese at 45 days (Arnaud 1995).

Many vitamins are unstable to processing and react to many environmental stimuli. Encapsulation has shown to be beneficial by increasing their stability and protecting them from the environment. Ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E) are susceptible to oxidation and both are stabilized after encapsulation. Ascorbic acid showed a 50% survival after refrigerated storage for 50 days compared to the free control, which was fully degraded after 20 days (Arnaud 1995; Reineccius 1995). Vitamin E is more effective at preventing lipid oxidation when in liposomes versus in free form because it is kept from dissolving in the oil phase.

Other ingredients that have benefitted from encapsulation are acidulants because it increases the shelf life of their flavors and prevents loss of color due to controlled release. Lactic and citric acids enhance flavors and reduce production times in cured

meats like pepperoni, hard salami, and summer sausages (Gibbs and others 1999). Sodium bicarbonate can be encapsulated to prevent it from reacting with acid and water, providing uniformity. Sodium chloride is encapsulated to increase flow ability and reduce clumping and caking. Sweeteners can be degraded by temperature and moisture, therefore, encapsulating them allows for slow release during chewing and flavor retention (Gibbs and others 1999).

To prevent spoilage in various cheeses, Thapon and Brule (1986) encapsulated the antimicrobials lysozyme and nisin. One important reason for this is the addition of an antibiotic directly to the cheese curd would kill its starter culture (Reza Mozafari and others 2008). Antilisterial effects of pediocin AcH were seen upon encapsulation of bacteriocin in beef tallow and muscle slurries (Degnan and Luchansky 1992). Encapsulation of nisin has many advantages such as reducing or prohibiting nisin's affinity to non-target components, lengthening the time of its preservative effects, decreasing the risk of resistant strains developing, and providing a means of targeting the bacteria (Reza Mozafari and others 2008). Benech and others (2002b) tested encapsulated nisin Z in liposomes composed of phosphatidylcholine (PC) and unencapsulated nisin in a cheese-milk solution at a final concentration of 300 IU/g cheese. They found that *Listeria innocua* counts were reduced by 1.5-3.0 logs within 6 months (Benech and others 2002a). Laridi and others (2003) tested several commercially prepared proliposomes encapsulated with nisin against the effects of fatty-acid composition, pH, cholesterol, and nisin Z content on the encapsulation efficiency (EE) of the liposomes. Hydrogenated PC liposomes, lower cholesterol concentration, lower pH

(3.6) allowed for higher EE while higher percentages of unsaturated fatty acids did not influence the EE (Laridi and others 2003).

CHAPTER III

NISIN

Nisin is an antimicrobial polypeptide or bacteriocin that was discovered in 1928 by researchers during cheese production when a 'lactic streptococci' inhibited the growth of *Lactobacillus* cheese starter cultures (Rogers and Whittier 1928). Nisin was further characterized by the National Institute for Research in Dairying and given the name nisin (Whitehead 1933; Mattick and Hirsch 1947; Delves-Broughton and others 1996). Its name is derived as 'Group **N** *Streptococcus* **I**nhibitory **S**ubstance' (Hurst 1981). The suffix '-**in**' was commonly used for antibiotics; however, nisin is considered a bacteriocin because it lacks the key attributes that make antibiotics unsuitable for food usage. Several attributes include: nisin not persisting in the body or environment, and it is not associated with development of bacterial resistance to nisin itself or to any medically important antibiotic (Thomas and others 2000).

The first commercial extract of nisin called Nisaplin® was produced in 1957 by Aplin & Barrett, Ltd. (Norman Hansen 1993; Delves-Broughton and others 1996; Thomas and Delves-Broughton 2005). Nisaplin® has a standard potency of 1 million International Units per gram and contains ~2.5% nisin A along with salts and milk solids from milk fermentation by *Lactococcus lactis* subspecies *lactis* (Thomas and Delves-Broughton 2005). The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Committee on Food Additives approved nisin for use in food in 1969. Nisin is currently approved as a food preservative in over 50 countries. It was given generally recognized as safe (GRAS) status in the United Stated in 1988 by the

Food and Drug Administration (FDA) (FDA/HHS 1988; CFSAN 2008). Nisin contains several characteristics that make is suitable for food preservation. It is non-toxic to humans, is produced by a bacterium not known to exert pathogenesis against humans, is not used for clinical therapies, and is digested quickly.

Chemical and Physical Properties

Nisin is an antimicrobial polypeptide that is produced during milk fermentation by the organism *Lactococcus lactic* subspecies *lactis*. It is a bacteriocin meaning it has the ability to kill or inhibit other bacteria. It is classified as a Class Ia lantibiotic due to its structure. Lantibiotics are a family of membrane active peptides that contain unusual amino acids and lanthionine rings. These are specifically thioether amino acids lanthionine and B-methyl lanthionine and also modified amino acids like dehydrated serine and threonine (Klaenhammer 1993; Montville and Chen 1998; Thomas and others 2000; Wiedemann and others 2001). It is considered a Group A lantibiotic because it has a linear structure rather than a circular structure like Group B lantibiotics. Nisin is comprised of 34 amino acid residues and has a molecular mass of 3510 Daltons. It has 5 internal ring structures (Rings A-E) formed by disulfide bridges that are contributed by lanthionine and B-methyl lanthionine as seen in figure 3 (Gross and Morell 1971; Klaenhammer 1993). Ring A is formed by lanthionine and rings B-E by four B-methyl lanthionine residues (Thomas and others 2000; Cheigh and Pyun 2005; Thomas and Delves-Broughton 2005). The B-methyl lanthionine bridge is between residues 8 and 11 while the lanthionine bridge is between residues 3 and 5 or 3 and 7 (Gross and Morell 1971).

Figure 3-Structure of Nisin A.
Nisin is comprised of 34 amino acid residues and contains 5 internal ring structures formed by disulfide bridges (Gross and Morell 1971).

There are two forms of nisin, which are nisin A and nisin Z. They differ at position 27 where nisin A has a histidine and nisin Z has an asparagine residue (Cheigh and Pyun 2005). Nisin carries a poly-cationic charge due to three lysine residues and one histidine residue (nisin Z) or two histidine residues (nisin A). It is an amphiphilic molecule because it is hydrophobic at the N-terminus and hydrophilic at the C-terminus (Thomas and others 2000).

Stability and Solubility

It is important to understand the stability and solubility of nisin when using it as a food preservative. Most notable is that its stability and solubility drastically increase as the pH is lowered and become less stable and soluble as pH is raised to neutral and alkaline levels (Hurst 1981; Delves-Broughton 1990; Liu and Hansen 1990; Rollema and others 1995). It is optimally stable around pH 3-3.5 (Thomas and Delves-Broughton

2005). It is so stable that at pH 2 it can withstand autoclaving temperatures (115°C to 121°C) without inactivation, but 40% of its activity is lost at pH 5 and more than 90% is lost at pH 6.8 (Hurst 1981; Rollema and others 1995; Delves-Broughton and others 1996). Pasteurization temperatures are less damaging to nisin. During standard processed cheese manufacturing, as least 80% activity will be retained (Delves-Broughton and others 1996). It is not known whether nisin instability at high pH is a consequence of denaturation, chemical modification, or configuration changes. At alkaline pH the reactivity of unsaturated amino acids will undergo a variety of additional reactions that may contribute to this instability (Rollema and others 1995). The dehydro residues become susceptible to modification by nucleophiles that are present at high pH like hydroxide ions, deprotonated amines, and deprotonated hydroxyl groups (Liu and Hansen 1990; Thomas and others 2000; Thomas and Delves-Broughton 2005). Reactions with these nucleophiles can be intermolecular or intramolecular and possibly cause cross-linking that will form large aggregates. Buffer concentration can also affect nisin solubility because nisin is inversely and linearly proportional to phosphate buffer concentration. Certain food components have been shown to protect nisin during heat processing compared to buffer solutions (Liu and Hansen 1990; Delves-Broughton and others 1996)

Antimicrobial Spectrum

Nisin shows antimicrobial activity against many Gram-positive bacteria but little to no activity against Gram-negative bacteria. Nisin's antimicrobial spectrum is in a concentration dependent; the cytoplasmic membrane is the primary site of porulation in

vegetative cells. Nisin will bind to the membrane, insert and orient itself, create a pore upon binding to peptidoglycan precursor molecule named Lipid II, and destroy the transmembrane potential causing rapid efflux of ions, amino acids, and ATP out of the cell resulting in death. Nisin is usually sporostatic rather then sporicidal. However, spores are sensitive to nisin and sensitivity increases with increasing heat damage and acidic conditions (Fowler and Gasson 1991). Nisin's activity against spores does not affect the germination process. Instead, nisin prevents post-germination swelling and later spore outgrowth (Morris and others 1984; Thomas and others 2000). The mode of action will be covered in greater detail in the following paragraphs.

The outer membrane of Gram-negative bacteria does not allow nisin to reach the cell membrane without altering the permeability of the outer membrane. It has been shown that chelators remove divalent cations like Ca^{2+} and Mg^{2+} ions from the outer membrane, destabilizing it and releasing phospholipid and lipoproteins (Delves-Broughton 1993; Boziaris and Adams 1999). Chelators are compounds that can sequester metal ions and form stable metal complexes. Examples of chelators include ethylenediamine tetraacetic acid (EDTA), some acid salts (e.g. citrate), and pyrophosphate. Other agents can remove lipids and phospholipids from Gram-negative cell walls making them more sensitive to nisin like citric acid, lactates, and polyphosphates. Sub-lethal heating, freezing, hydrostatic pressure, or organic acids that can sensitize the cell wall and expose the membrane to nisin will increase its antimicrobial activity towards Gram-negative bacteria (Ganzle and others 1999; Thomas and Delves-Broughton 2005).

Mode of Action

Nisin acts upon vegetative cells at the outer membrane by binding and inserting itself into the membrane, forming pores, and destroying the proton motive force. Due to the cationic and amphiphilic nature of nisin, it has a strong affinity for anionic lipids in many membranes. Nisin attaches to the cytoplasmic membrane through electrostatic interaction of the negatively charged lipids present in the membrane to the positively charged C-terminus region of nisin (El-Jastimi and Lafleur 1997; Breukink and Kruijff 1999; Bonev and others 2000). There is no significant binding of nisin to vesicles with zwitterionic lipids showing the affinity of this peptide is greatly dependent on the lipid charge (El-Jastimi and Lafleur 1997). It has been determined that nisin needs an energized membrane to dissipate the membrane potential in the process of causing cell leakage (Abee 1995; Breukink and Kruijff 1999). Binding of nisin induces a change in the secondary structure that involves conformational reorganization. It specifically promotes the formation of beta-turns (El-Jastimi and Lafleur 1997; Breukink and Kruijff 1999).

Once nisin binds to the membrane, its amphiphilic properties allow it to insert into the membrane at the lipid phase. This is done with the help of lipid II, the bactoprenol-bound peptidoglycan precursor for cell wall synthesis (Wiedemann and others 2001). Lipid II is considered a key element during synthesis of bacteria cell walls. The undecaprenyl tail of lipid II is used as a carrier that transports the peptidoglycan subunit from the cytoplasm to the extracellular domain (Hsu and others 2002). The N-terminal region of nisin first recognizes the lipid II headgroup, and a tight nisin/lipid II

complex is formed. The amphiphilic nature of nisin along with lipid II allows it to insert into bilayers in a perpendicular orientation on the membrane interface (Hsu and others 2002; Hasper and others 2004). This complex is also anchored onto the membrane by the undecaprenyl tail of lipid II. Subsequent conformational rearrangements take place due to aggregation of the nisin/lipid II complexes(Wiedemann and others 2001; Hasper and others 2004). Specifically, large chemical shift perturbations were found for the first two rings (A and B) (Lubelski and others 2008).

Multiple molecules of nisin and lipid II are assembled at the interface of the bilayer forming pores via an intermediate state called the prepore complex B (Hasper and others 2004). The pores produced are uniform in size and are very stable with increased lifetimes (6 seconds) compared to pores in the absence of lipid II (milliseconds). The final pore complex formed consists of 8 nisin and 4 lipid II molecules (Hasper and others 2004). Upon pore formation, the C-terminus translocates across the membrane (Breukink and Kruijff 1999). When the C-terminal of nisin is inserted into the membrane, the pore-forming process is complete. The presence of pores causes inhibition of amino acid uptake, and small metabolites, ions (K^+), cellular ATP, or solutes begin to flow out of the bacterial cell resulting in cell death (Abee 1995; Thomas and Delves-Broughton 2005).

Factors Affecting Nisin Action

Several factors in foods are known to counteract the action of nisin. Bacteriocin activity in food matrices may be affected by changes in the solubility and charge of bacteriocins, binding and interaction of bacteriocins to food components, inactivation by

proteases, and target cell envelope changes that occur in response to environment factors (Ganzle and others 1999). Heat treatments with higher temperatures and longer processes result in a greater nisin loss due to structural changes in the antimicrobial. In foods that are non-heat treated or are minimally processed, nisin can be degraded during storage by proteolytic enzymes developed from microbial, plant, or animal origins (Delves-Broughton and others 1996; Abee and Delves-Broughton 2003). Nisin is shown to work better in liquid and homogenous foods because it is more evenly distributed throughout the matrix. Phospholipids in meat are suggested to cause binding of nisin, making it unavailable (Delves-Broughton and others 1996). Some food additives such as sodium metabisulfite (antioxidant, bleaching, and antimicrobial agent) and titanium dioxide (whitener) have been shown to act as antagonists and degrade nisin (Abee and Delves-Broughton 2003; Thomas and Delves-Broughton 2005). Divalent and trivalent cations (Ca^{2+}, Mg^{2+}, or Gd^{3+}) reduce efficiency of nisin Z against *L. monocytogenes* because they interact with certain negatively charged phospholipid headgroups (Gupta 1968; Thomas and Delves-Broughton 2005). Divalent cations like Mg^{2+} bind to anionic phospholipids resulting in an enhanced rigidity of the cytoplasmic membrane and a reduced affinity of nisin to the cytoplasmic membrane (Ganzle and others 1999).

Several factors in dairy products limit the activity of nisin, such as its adsorption to fat and the surface of protein globules, a heterogeneous distribution in dairy matrices, and the inhibition of non-resistant starter cultures (Sobrino-Lopez and Martin-Belloso 2008). Due to its hydrophobic nature, nisin will bind to fat in foods which interferes with uniform distribution making nisin unavailable for bacterial inhibition (Delves-Broughton

and others 1996). Several studies have shown that activity of nisin decreases as the milk fat concentration increases (Jung and others 1992; Bhatti and others 2004; Sobrino-Lopez and Martin-Belloso 2008). Bhatti and others (2004) determined phospholipids in 2% or higher fat market milk bound a larger portion of nisin making it unavailable to react with the cell membrane of *L. monocytogenes*. Skim milk was not affected to the extent of the higher fat content milk so similar nisin concentrations were sufficient to disrupt the listerial cell membrane (Bhatti and others 2004). It is possible to use emulsifiers, which function to reduce surface tension effects that are elicited between polar and nonpolar molecules, to prevent binding of nisin to milk fat globules. The surfactant Tween 80® has the ability to displace proteins or peptides such as nisin from milk fat globules and restoring or retaining its activity in milk (Jung and others 1992; Bhatti and others 2004; Sobrino-Lopez and Martin-Belloso 2008).

Nisin Resistance

It is also possible for bacteria to develop resistance against nisin, because they respond to acid, osmotic, and thermal stresses in the environment (Bonnet and others 2006). These responses increase their resistance and virulence. Many Gram-positive bacteria have been shown to be resistant to nisin because they have the ability to synthesize the enzyme nisinase that can inactivate nisin. Another resistance mechanism involves adaptation of cells by subjecting them to sub-lethal concentrations of nisin over a period of time. Several studies have shown *L. monocytogenes* mutants can easily be produced in the laboratory by exposure to high concentrations of nisin (Harris and others 1989; Ming and Daeschel 1993; Mazzotta and Montville 1997). These adaptations were

shown to develop from changes in the cell envelope specifically in the cell membrane and peptidoglycan. Researchers have shown nisin resistant *L. monocytogenes* possessed lipids with higher phase transition temperatures, higher percentage of straight-chain fatty acids, and lower percentage of branched-chain fatty acids (Ming and Daeschel 1993; Crandall and Montville 1998; Li and others 2002; Abee and Delves-Broughton 2003) This resulted in decreased fluidity of the membrane, which decreased the efficiency of nisin pore formation in the resistant mutant. Listeriae are known to resist lethal acid after exposure to mild acidic conditions. This response is referred to as the acid tolerance response (ATR). Bonnet and Montville (2006) proved ATR-induced *L. monocytoge*nes cells survived for 30 days at 4°C in the presence of nisin unlike the control. It is possible ATR protects *L. monocytogenes* because these resistant mutants have more rigid membranes due to changes in the proportion of fatty acids.

CHAPTER IV

MATERIALS AND METHODS

Culture Resuscitation and Maintenance

Listeria monocytogenes strain Scott A (LM SA) was obtained from Texas A&M University Department of Animal Science Food Microbiology Laboratory culture collection and kept on tryptic soy agar (TSA) slants (Becton Dickinson, Sparks, MD) at 4°C. Revival was completed by transferring LM SA three times in tryptic soy broth plus 0.6% yeast extract (TSB-YE) (Becton-Dickinson) at 35°C for 24 hr for full recovery of the cells. The first day LM SA was co-transferred from the slant into Fraser broth (Becton Dickinson) and on PALCAM medium (Becton Dickinson) to confirm correct phenotype (esculin hydrolysis). The second day LM SA was transferred from Fraser broth into TSB-YE and streaked onto TSA plus 0.6% yeast extract (TSA-YE). The third day LM SA was transferred a third time into TBS-YE and streaked onto TSA-YE. All broths and plates were incubated aerobically at 35°C for 24 hours per transfer.

Antimicrobial Preparation

Nisin (CAS# 1414-45-5) (2.5% nisin; 1 million IU/g) from Sigma-Aldrich Co. (St. Louis, MO) was solubilized in 0.02M HCl (EM Science, Gibbstown, NJ) at a concentration of 0.1 g/10 mL (10,000 IU/mL). The stock was submerged in boiling water for four minutes, cooled, and stored at 4°C overnight. Each day a new working stock was prepared at a concentration of 50 IU/mL using 0.1X phosphate buffer saline PBS (0.017M KH_2PO_4, 0.05M Na_2HPO_4) (Mallinckrodt Chemicals, Phillipsburg, NJ), and (1.5M NaCl) (Sigma-Aldrich Co., St. Louis, MO) adjusted to pH 7.4.

Fluorescence Probe Preparation

Calcein (CAS# 1461-15-0) (Sigma-Aldrich Co.) was solubilized in 1.0 M NaOH (Fisher Scientific, Fair Lawn, NJ) to a concentration of 50 mM and adjusted to pH ~ 9.0. The calcein was stored at room temperature in an amber vial to keep from reacting with light.

Liposome Preparation and Encapsulation via Extrusion

Egg-sourced L-α-phosphatidylcholine (PC) and L-α-phosphatidyl-DL-glycerol (PG), obtained solubilized in chloroform from Avanti Polar Lipids (Alabaster, AL), was used as major components of liposome formulations. Three different lipid molar combinations were prepared to a concentration of 60 mM: PC, PC/PG 70:30 (mol. fraction), and PC/PG 60:40 (mol. fraction). Each lipid formulation was prepared and solvent was evaporated under nitrogen gas. Any traces of chloroform were removed using a vacuum desiccator for 15 minutes and then refrigerated at 4°C inside the oxygen free environment overnight to prevent lipid oxidation. Each lipid combination was rehydrated using 0.5 mL of phosphate buffer saline (PBS) containing 50 mM of the fluorescent probe calcein (Sigma-Aldrich St. Louis, MO) with 50 IU/mL nisin (Sigma-Aldrich, St. Louis, MO) the day after desiccation. Tubes were agitated to remove lipid from the test tube walls and allow vesicle formation. All tubes were then subjected for four cycles of freeze-thawing. Liposomes were immersed in 0°C water, tepid water, and 60°C water for 11 seconds each in sequential order. Tubes were vortexed for 8-10 seconds following each cycle. Each formulation was then passed 7 times each through sandwiches of 400nm-200nm-400nm and 100nm-50nm-100nm polycarbonate

membranes (Avestin, Ottawa, Canada) using a LiposoFast™ Basic Extruder (Avestin) to produce liposomes homogenous in size. This procedure was done while the extruder was immersed in 60°C water to keep lipids in a liquid-crystalline phase to facilitate extrusion. Keeping the lipids above their transition temperature allows for a greater occupied volume due to the highly mobile state of the molecules (Voet and others 2006). Filter sterilization of each combination was completed to prevent cross-contamination using Acrodisc Tuffryn-HT low protein binding 0.2 μm pore diameter syringe filters (Pall Corporation, Ann Arbor, MI). After extrusion, the unencapsulated material was removed by size-exclusion chromatography (SEC). The sample was passed over a Bio-Gel P-6 DG Desalting Gel column (Bio-Rad Laboratories, Hercules, CA) while using 0.1X PBS as the system's buffer. The sample was then plated on 24-well, flat bottom, black upper, lidded microplates (Genetix, Boston, MA) at concentrations of 60mM and 30mM. Twenty micro liters of 10% Triton X-100 (Sigma-Aldrich, St. Louis, MO) were added to activate the release of calcein. After the sample was plated, the fluorescence strength was measured using an Infinite M200 microplate reader (Tecan, Durham, NC). The plate reader was programmed to read excitation and emission wavelengths of 495 and 515nm, respectively. Fluorescence readings were taken before and after the addition of Triton X-100 to estimate the amount of encapsulated calcein within the system. The encapsulation efficiency (EE) percentage was determined using the formula:

$$EE\ (\%) = (1 - F/F_t) \times 100$$

where F is the fluorescence strength before to the addition of Triton X-100, and F_t is the fluorescence strength after the addition of Triton X-100 (Were and others 2003).

Pathogen Survivor Assay

To determine the antilisterial activity of unencapsulated and encapsulated nisin within skim and whole milk for extrusion encapsulation method and each liposome formulation (PC, PC/PG 7:3, and PC/PG 6:4), pathogen survivor assays were conducted. Borden® Ultra-high-temperature (UHT) processed fat free skim milk and whole milk (Diversified Foods, Metairie, LA) were used as the experimental food matrix. In a test tube with milk (skim or whole), *L. monocytogenes* was added to a final inoculum concentration of 4 $\log_{10}$ CFU/mL along with liposomes containing encapsulated or free nisin at 50 IU/mL that were diluted to a final concentration of 2mM. The ratio of milk:*L. monocytogenes*:liposomes was always kept at 8:1:1 to make sure concentrations of each component was consistent between treatments and replications. This was conducted with *L. monocytogenes* Scott A for extruded liposomes and each liposome formulation. There was a positive control that only contained *L. monocytogenes* and no liposomes as well as an unencapsulated nisin control which did not contain any liposomes and encapsulated nisin within liposomes. Tubes were then vortexed for ~ 8 seconds and incubated at 5°C or at 20°C to model refrigerated storage and temperature abuse, respectively. Samples stored at 20°C were removed from the incubator at time intervals of 0, 1, 3, 6, 12, 24, and 48 hr. Samples stored under refrigeration were sampled at the above time points and again after 72 hr of incubation. After samples were removed from the incubator, they were serially diluted using 0.1% peptone water (Becton Dickinson) and pour plated on TSA-YE medium. Once solidified, plates were inverted and incubated aerobically at 35°C for 48 hours prior to survivor enumeration.

Analysis of Data and Experimental Design

Encapsulation efficiency, as a function of liposome formulation and entrapment method, was carried out using a 3x2 factorial: 3 liposome formulations x 2 encapsulation methods. Microbiological testing (i.e. pathogen survival) assays were carried out using a complete factorial design with balanced means: 3 lipid formulations x 3 treatments (encapsulated, free, control) x 2 milk types (skim, whole) x 2 replicates. Sampling times were specific to each experimental temperature and were treated as repeated measures; experimental temperature was removed from the statistical model as no comparisons between samples incubated at 5°C or 20°C were made. For 5°C-incubated samples, 8 population assays were taken at 0, 1, 3, 6, 12, 24, 48, and 72 hr; samples incubated at 20°C were assayed at 0, 1, 3, 6, 12, 24, and 48 hr. Overall, the 5°C experimental design consisted of 287 degrees of freedom, while the 20°C experiment consisted of 251 degrees of freedom. Statistical analysis of significant differences between sample means was carried out via 2-way analysis of variance (ANOVA) and Tukey's mean separation tests with $\alpha = 0.05$. All statistical analyses were carried out using SPSS 16.0.1 (SPSS Inc., Chicago, IL).

CHAPTER V

RESULTS

Liposomal Encapsulation Efficiency of Nisin

Liposomes were encapsulated with nisin and calcein to determine the encapsulation efficiency percentage (EE) of extrusion based on particular lipid formulations. The PC formulation showed a mean EE of 84.20%, while PC/PG 7:3 had 77.33% and PC/PG 6:4 had 80.78% (Fig. 4). None of the lipid formulations for extrusion showed significant differences in the mean EE as determined experimentally.

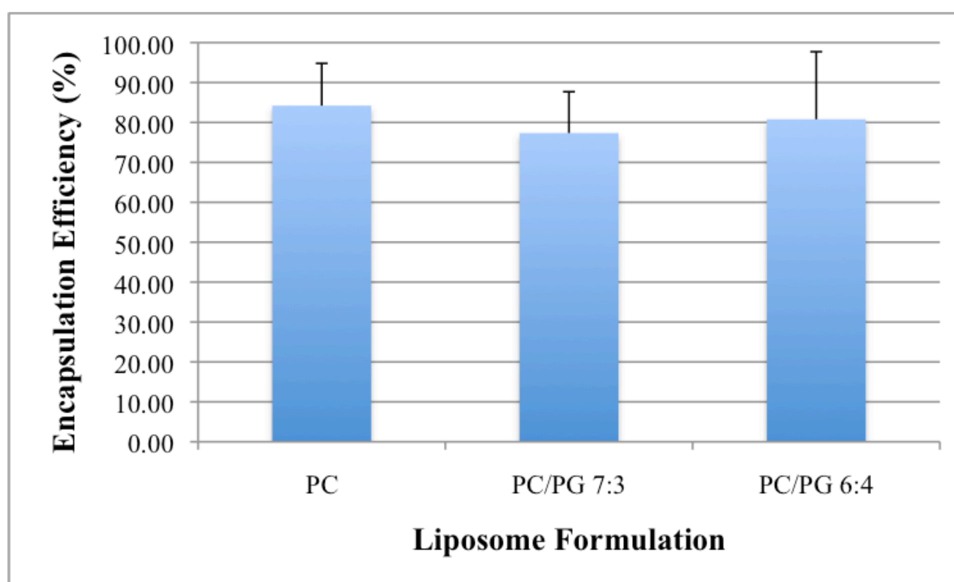

Figure 4-Encapsulation Efficiency of Liposomes Formed via Extrusion.
Bars represent means of triplicate replications with error bars indicating one standard deviation from the mean. Calcein (50 mM) was excited at 490 nm and emission determined at 515 nm.

Encapsulation efficiency of nisin was compared against extrusion using freeze-thaw. The freeze-thaw method produced a non-detectable EE of nisin (Data not shown).

Survival of *L. monocytogenes* in Skim and Whole Milk from Liposome-Encapsulated and Free (Unencapsulated) Nisin at 5°C

The survival of *Listeria monocytogenes* Scott A at 5°C was determined in the presence of encapsulated and free nisin in UHT skim and whole fluid milk. Initial *Listeria* counts for all lipid formulations began with 4.5 $\log_{10}$ CFU/mL.

Listeria grew to a final cell density of 5.0 $\log_{10}$ CFU/mL after 72 hours at 5°C in the presence of PC liposomes without nisin (positive control) (Fig. 5). Skim milk with PC encapsulated liposomes had a final cell count of 0.90 $\log_{10}$ CFU/mL and free nisin reduced *Listeria* counts to < 1.0 $\log_{10}$ CFU/mL after 72 hour of 5°C incubation (Fig. 5). Both encapsulated and free nisin within skim milk caused a reduction in cell density significantly different from the positive control across all lipid formulations (i.e. PC, PC/PG 7:3, and PC/PG 6:4). PC liposomes with encapsulated and free nisin in whole milk at 5°C created a bacteriostatic effect and held *Listeria* population counts to 4.0 $\log_{10}$ CFU/mL after 72 hours (Fig. 5).

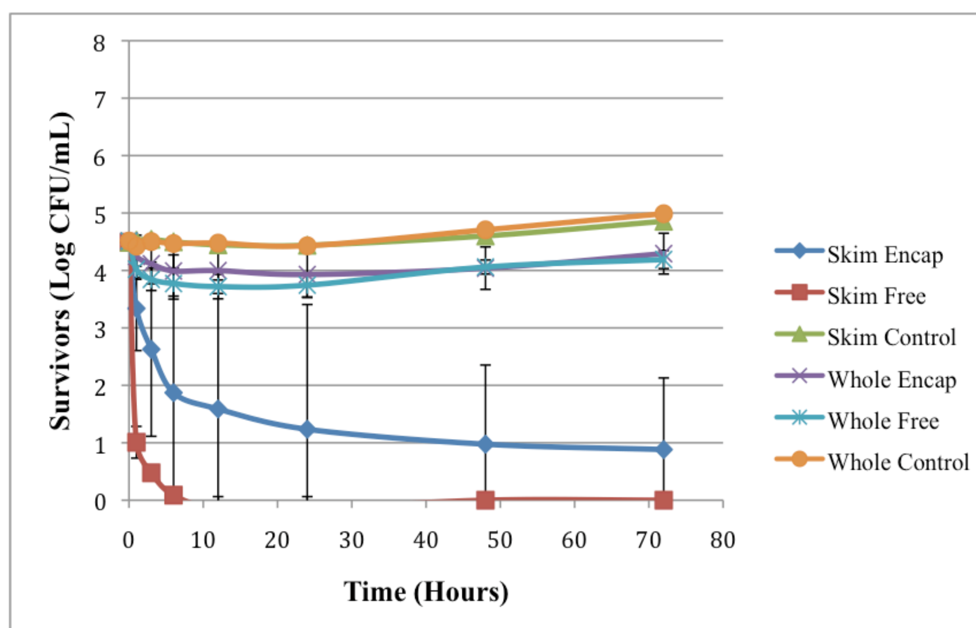

Figure 5-Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin in 2 mM PC Liposomes at 5°C.
Symbols represent means of duplicate replications with error bars indicating one standard deviation from the mean.

The positive controls (i.e. skim and whole control) allowed *Listeria* to grow to a final cell density of 5.5 $\log_{10}$ CFU/mL after 72 hours of incubation (Fig. 6). Liposomes produced with PC/PG 7:3 lipids at 5°C in skim milk (i.e. encapsulated and free) reduced *Listeria* counts nearly 4 logs with < 1.0 $\log_{10}$ CFU/mL remaining after 72 hours incubation (Fig. 6). PC/PG 7:3 liposomes with encapsulated and free nisin in whole milk resulted in slight reductions after 24 hours (Fig. 6). However, there was a complete recovery of the pathogen after 72 hours incubation with populations equivalent to 4.5 $\log_{10}$ CFU/mL density that was 0.5 $\log_{10}$ CFU/mL higher than the initial density (Fig. 6).

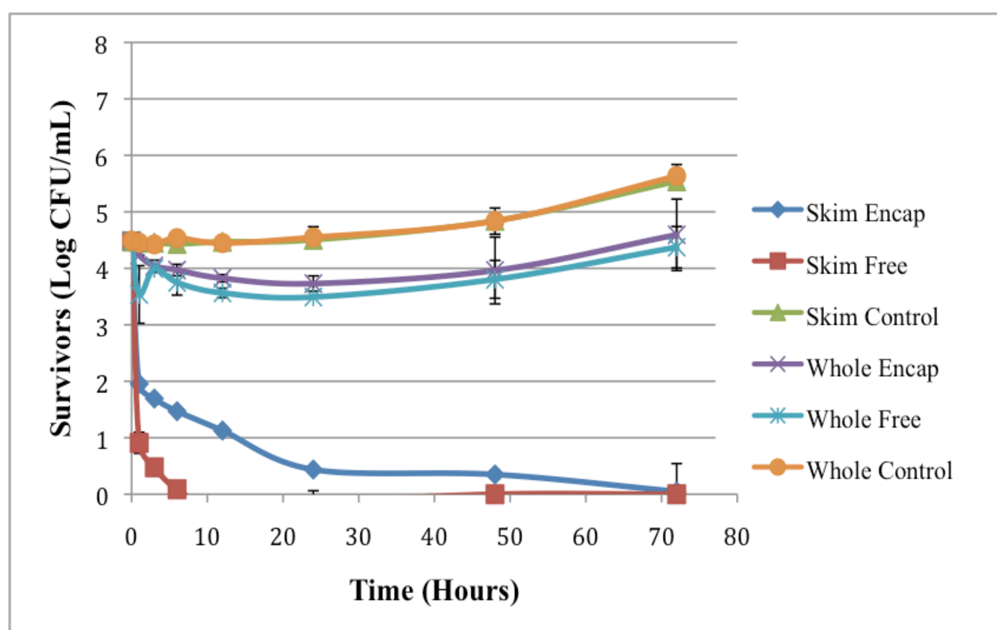

Figure 6-Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin in 2 mM PC/PG 7:3 Liposomes at 5°C.
Symbols represent means of duplicate replications with error bars indicating one standard deviation from the mean.

Skim and whole milk positive controls had a final *Listeria* cell density of 5.0 $\log_{10}$ CFU/mL after incubation in 5°C for 72 hours (Fig. 7). PC/PG 6:4-entrappped and free nisin in whole milk resulted in a decreased cell density following experiment completion (3.7 $\log_{10}$ CFU/mL) (Fig. 7). Encapsulated nisin within skim milk resulted in reduced counts of ~0.70 $\log_{10}$ CFU/mL and free nisin lower the *Listeria* population to <1.0 $\log_{10}$ CFU/mL within 72 hours (Fig. 7).

Statistical analysis confirmed lipid combinations (i.e. PC, PC/PG 7:3, and PC/PG 6:4) were not significantly different from each other at 5°C with respect to the populations of *L. monocytogenes* following experiment completion.

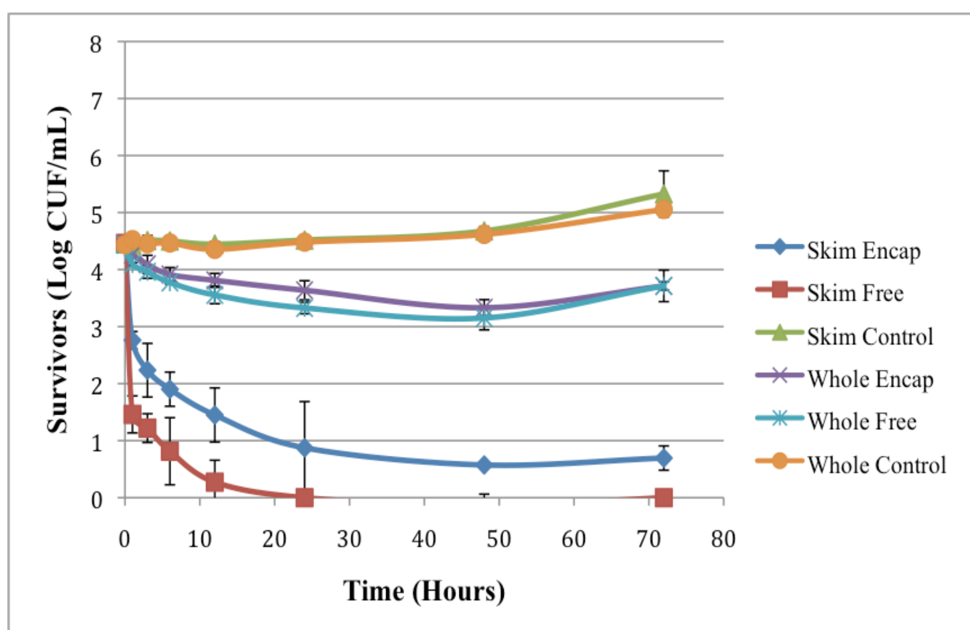

Figure 7-Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC/PG 6:4 Liposomes at 5°C.
Symbols represent means of duplicate replications with error bars indicating one standard deviation from the mean.

Survival of *L. monocytogenes* in Skim and Whole Milk from Liposome-Encapsulated and Free (Unencapsulated) Nisin at 20°C

The survival of *Listeria monocytogenes* Scott A in temperature-abused skim and whole milk was determined for liposomes with encapsulated and free nisin. Initially, all milk samples were inoculated to *Listeria* cell densities of 4.5 $\log_{10}$ CFU/mL.

Listeria positive controls grew to a final cell density of 8.0 $\log_{10}$ CFU/mL after 48 hours of incubation at 20°C for PC liposomes (Fig.8). Both encapsulated and free nisin in whole milk had *Listeria* populations reduced during the first 3 hours, but complete recovery of the pathogen occurred following 48 hours of incubation (Fig. 8). Liposomes with encapsulated nisin in skim milk had final *Listeria* cell counts of 7.3 $\log_{10}$ CFU/mL and free nisin had 5.0 $\log_{10}$ CFU/mL after 20°C incubation for 48 hours.

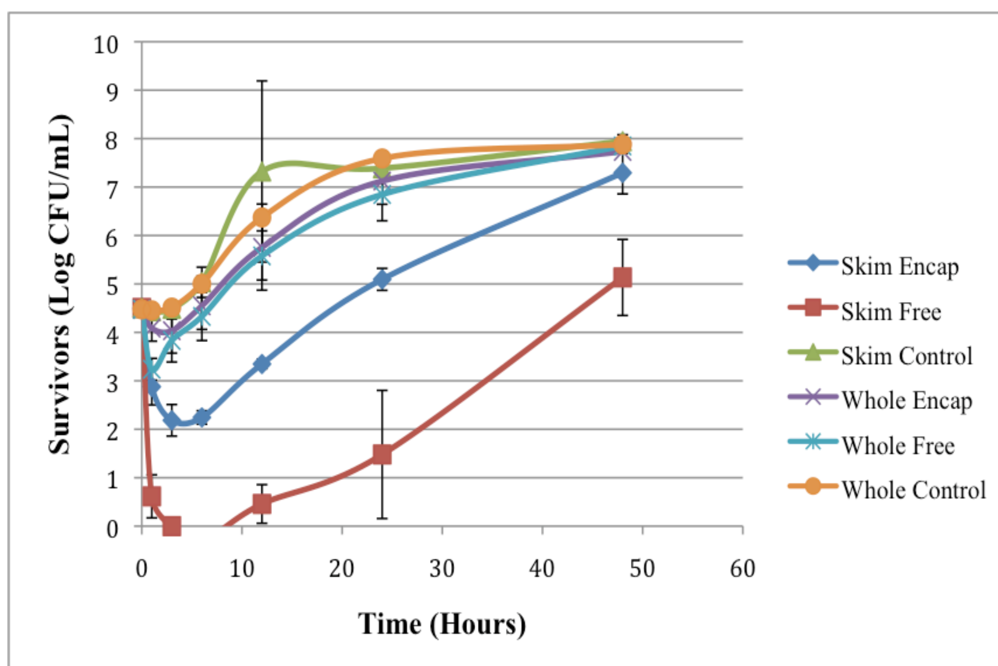

Figure 8-Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC Liposomes at 20°C.
Symbols represent means of duplicate replications with error bars indicating one standard deviation from the mean.

Positive controls for skim and whole milk used during PC/PG 7:3 liposome testing showed final cell densities of 8.0 $\log_{10}$ CFU/mL from 48 hours of incubation (Fig. 9). Encapsulated and free nisin were able to reduce *Listeria* populations for 6 hours; however, populations recovered and maintained exponential growth for 48 hours (Fig. 9). Liposomes with encapsulated nisin in skim milk allowed pathogen growth to final cell counts of 7.0 $\log_{10}$ CFU/mL whereas, free nisin allowed final cell counts of 2.4 $\log_{10}$ CFU/mL following 48 hours of incubation (Fig. 9). Encapsulated and free nisin in whole milk reduced *Listeria* growth for the first 3 hours of incubation, but full recovery of the pathogen occurred after 48 hours incubation at 20°C. Liposomes with

encapsulated nisin had a final *Listeria* count of ~7.5 $\log_{10}$ CFU/mL while free nisin had 7.8 $\log_{10}$ CFU/mL (Fig. 9).

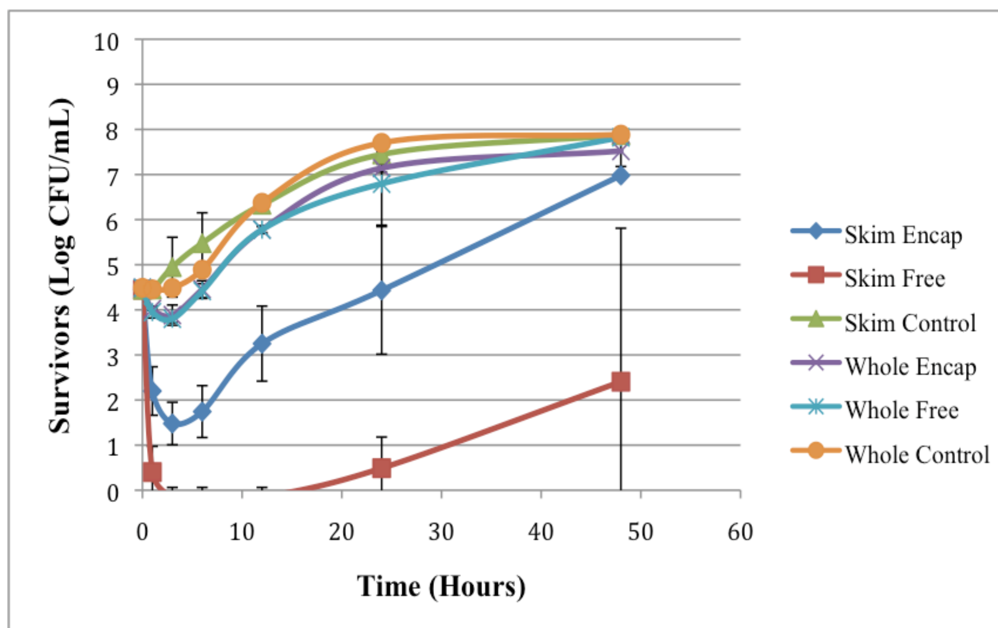

Figure 9-Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC/PG 7:3 Liposomes at 20°C.
Symbols represent means of duplicate replications with error bars indicating one standard deviation from the mean.

Listeria grew to final cell densities in skim and whole positive controls at 20°C during PC/PG 6:4 testing to 8.0 $\log_{10}$ CFU/mL (Fig. 10). Liposome encapsulated nisin and free nisin in skim milk were able to reduce pathogen counts during the first 3 hours points, but after 48 hours of incubation there was full recovery. Encapsulated nisin had *Listeria* counts of 6.8 $\log_{10}$ CFU/mL and free nisin had 6.4 $\log_{10}$ CFU/mL after 48 hours (Fig. 10). Within whole milk encapsulated and free nisin allowed pathogen growth after 48 hours to 7.7 $\log_{10}$ CFU/mL (Fig. 10).

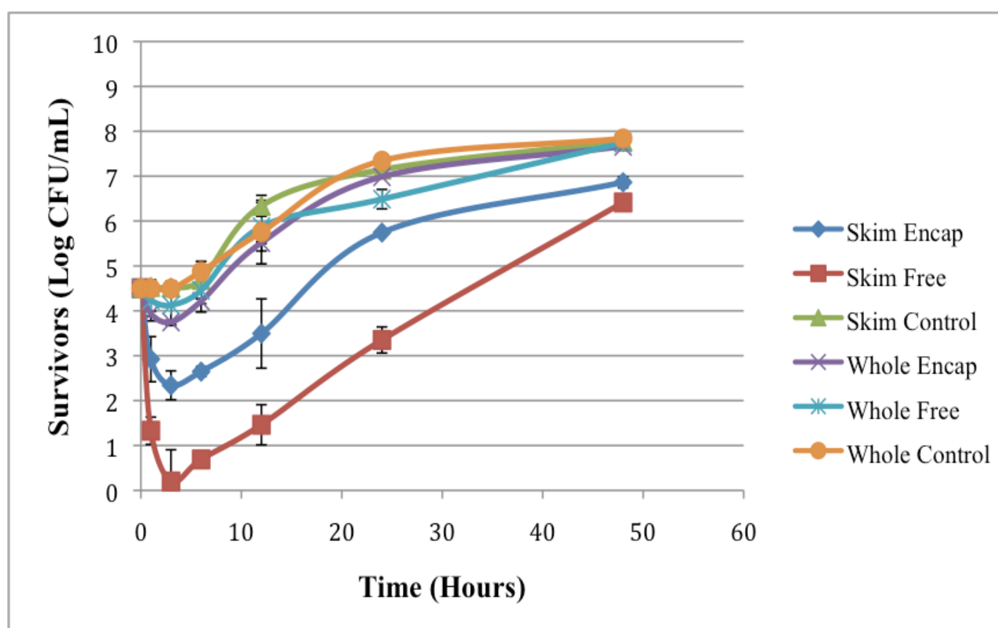

Figure 10-Survivors of *L. monocytogenes* in Skim and Whole Milk in Presence of 50 IU/mL Encapsulated and Free Nisin within PC/PG 6:4 Liposomes at 20°C.
Symbols represent means of duplicate replications with error bars indicating one standard deviation from the mean.

Statistical analysis showed significant differences between the log means of pathogen populations across lipid formulations which was not observed during pathogen survivor assays completed at 5°C. Unencapsulated nisin within skim milk was the only formulation for which lower cell counts were consistently maintained as compared with other treatments. Liposome formulations applied to skim milk consistently lowered *Listeria* cell densities at least 1.0 $\log_{10}$ CFU/mL more than liposomes applied to whole milk, with significant statistical differences resulting in *L. monocytogenes* populations between the milks (i.e. skim and whole). However, after 48 hours most cell populations had increased to 8.0 $\log_{10}$ CFU/mL. Therefore, exposure of *L. monocytogenes* to liposomal and free nisin did not ultimately inhibit growth of the pathogen.

CHAPTER VI

DISCUSSION

It was determined that liposome production by the extrusion method allowed for the highest encapsulation efficiency (EE) of nisin. Therefore, it was used for liposome production during the antimicrobial assay. All lipid formulations used exhibited EE% >50% indicating nisin was successfully entrapped. There were slight differences seen in the EE between the three formulations, although analysis of sample means revealed no statistically significant differences ($p<0.05$). PC had the highest entrapment percentage while PC/PG 6:4 had a higher entrapment percentage than PC/PG 7:3. These results support previously reported experiments where researchers found PC entrapped a larger content of nisin than PC/PG formulations (Were and others 2004). However, because similar amounts of nisin were entrapped in all lipid combinations it is possible PC/PG formulations released the bacteriocin more quickly than PC alone due to differences in stability. Adding cholesterol to PC/PG formulations may help increase the membrane stability and allow for slower controlled release of nisin. Cholesterol can be used to strengthen liposome membranes because it lowers membrane permeability at elevated temperatures and modulates membrane-protein interactions (Samad and others 2007). It decreases membrane fluidity and creates rigidity from its steroid ring system interfering with the motions of fatty acid side chains (Voet and others 2006).

PC/PG 6:4 was the only lipid formulation that maintained a lower cell density of *L. monocytogenes* than the initial cell count (4.5 $\log_{10}$ CFU/mL) at 5°C (Fig. 4) even though statistical analysis confirmed lipid combinations were not significantly different

from each other. It was also found that at 20°C the lipid type created statistically different mean log counts. It is possible that liposomal charge has an influence on nisin's interaction with bacteria. According to the chemical structure of phospholipids, PC liposomes have a net charge of zero while PG-containing liposome have an overall negative charge. *Listeria* also has a negative charge so there may have been electrostatic repulsion between the PC/PG liposomes and *Listeria's* cell surface that prevented direct contact between the liposomes and the pathogen (Were and others 2004). It is possible that because direct contact could not occur nisin had to travel within the food matrix until it came in contact with the pathogen. The external environment of the food matrix may have created interactions that altered nisin's activity spectrum thus creating statistically different means between lipid combinations.

Results for 5°C and 20°C indicate an initial reduction or bacteriostatic effect in *Listeria* populations within the first 3 hours, although evidence of pathogen growth was observed after 3 hr incubation at 20°C and after 48 hr incubation at 5°C, similar to previously reported experiments and expected microbiological growth patterns of *L. monocytogenes* (Figures 4-7) (Song and Richard 1997; Schillinger and others 1998; Vignolo and others 2000). Schillinger and others (1998) tested the bactericidal effect of nisin (10-500 IU/mL) against *L. monocytogenes* Scott A and showed that even at the highest antimicrobial concentration (500 IU/mL) *Listeria* numbers did not remain at a low level but increased to 10^4 CFU/mL within 24 hours and to 10^8 CFU/mL after 48 hours. Most likely nisin was destroyed during encapsulation or incubation or it simply did not destroy all the *Listeria* cells in the sample. Nonspecific binding of nisin to

Listeria cells, nisin bound to dead cells, or sub lethal amounts of nisin bound to *Listeria* are all possible scenarios for pathogen repopulation (Degnan and others 1993). However, it is possible that the survivor *Listeria* cells adapted and became resistant to nisin over a period of time. Song and Richard (1997) reported *Listeria* survivors displayed increased resistance towards the bacteriocin they were in contact with. Hurst (1981) reported in earlier works that resistant cells were easily obtained by 'training' specific bacteria to grow in the presence of increasing concentrations of nisin.

There are consistent differences in *Listeria* counts between skim and whole milk samples. Therefore, any variable tested with different milk fat percentages will have statistically different mean log values. Skim milk (<0.5% milk fat) maintained more lower *Listeria* counts whereas whole milk, with higher milk fat content, did not. The presence of milk fat has been shown to reduce the antimicrobial effects of nisin due to its interaction and adsorption to fat and the surface of protein globules (Thomas and Delves-Broughton 2005; Sobrino-Lopez and Martin-Belloso 2008). Nisin's amphipathic nature allows it to bind to fat in foods which interferes with uniform distribution making nisin unavailable for bacterial inhibition (Delves-Broughton and others 1996). Many studies have shown the antimicrobial activity of nisin is reduced as the milk fat concentration is increased (Jung and others 1992; Bhatti and others 2004; Sobrino-Lopez and Martin-Belloso 2008). Bhatti and others (2004) have shown nisin is unavailable to react with *Listeria's* cell membrane in 2% or higher milk due to present phospholipids binding a large portion of the bacteriocin. Skim milk was not affected as significantly because less milk fat was present; therefore, similar nisin concentrations were sufficient

to disrupt the listerial cell membrane (Bhatti and others 2004). However, at 5˚C whole milk was able to maintain a 1.0 log_{10} CFU/mL lower *Listeria* population than positive controls due to reduced levels of *Listeria* growth in refrigerated temperatures. Even at the highest milk fat content, nisin has an effect on *Listeria* counts despite its interaction with fat globules present. This is important for the industry because a reduction of 1 log can mean increased shelf life and safety of the milk and an increase of 1 log has the potential to contaminate and create many foodborne illness cases.

There are several differences seen in *Listeria* population counts between the encapsulated and free nisin treatments that need to be addressed. The 5˚C and 20˚C treatments showed free nisin maintained lower *Listeria* counts than encapsulated nisin within skim milk, and both free and encapsulated *Listeria* counts were very similar within whole milk. In the case of whole milk, nisin probably interacted and became bound with the higher amount of milk fat present making it unavailable to act upon and reduce the *Listeria* population. The results show free nisin had better inhibition of the pathogen than encapsulated nisin does not agree with previous studies (Benech and others 2002a; Benech and others 2002b; Were and others 2004). However, several key differences exist between this experiment and previous research findings. There were higher amounts of nisin used within previous studies such as 250-300 IU/mL, compared to 50 IU/mL used for this experiment (Benech and others 2002b). That study showed a correlation of increasing the concentration of nisin with increased entrapment efficiency that ultimately led to an increased inhibition of *Listeria* (Benech and others 2002b). Other studies have used broth like Tryptic Soy Broth, beef tallow and beef muscle

slurries, or cheddar cheese for the growth medium, while fluid milk was used for this study (Degnan and Luchansky 1992; Degnan and others 1993; Benech and others 2002a; Benech and others 2002b). These matrices are very different from one another, and it is possible these differences attributed to the difference in results. Nisin stability is highly influenced by cheese components and storage conditions, which can affect nisin activity (Benech and others 2002a; Benech and others 2002b). During cheese ripening, nisin peptides may be degraded by proteolytic enzymes or nisinase from lactic acid bacteria. Nisin may diffuse to the fat phase of the cheese matrix and contribute to a decline in nisin activity (Benech and others 2002b). Cheddar cheese also possesses many factors that help suppress the growth of *Listeria* including lower water activity, slightly acidic pH, solid matrix, etc. The fluid milk matrix used for this study was an ideal environment for *Listeria* to flourish. Results confirmed nisin could not compete with *Listeria* in an ideal environment where whole milk was temperature abused for 72 hours.

Different methods have been used in previous studies to enumerate bacterial growth. Several studies have used spectrophotometers to measure the optical density (i.e. turbidity) of experimental samples (Were and others 2004; Taylor and others 2006). Spectrophotometers measure the sample cell density as a function of light scattering; light scattering techniques are rapid and nondestructive to the cells, but do not measure cell numbers or CFU. This can be problematic because it gives a rough estimate of cell populations and not an actual count. This study used CFU/mL counts from plated *Listeria* on growth media to enumerate the pathogen. These counts, and counts from

studies using optical density measurements, may be different due to their methods of measuring cells present.

All of these differences between previous studies and the present study are possible explanations to the differences seen with free and encapsulated nisin treatments. It is possible that little EE% of nisin was achieved within the liposomes and with less nisin present *Listeria* counts were not reduced as well. Another possible scenario is nisin was only attached to the surface of the liposomes. The small amount of nisin that was surface attached could not have eliminated as much *Listeria* as free nisin.

CHAPTER VII

CONCLUSION

This study compared encapsulation efficiency percentages (EE%) between extrusion and freeze-thaw methods. It was determined that extrusion allowed higher encapsulation percentages of nisin (i.e. 84.20%, 77.33%, 80.78%) whereas freeze-thaw had undetectable fluorophore entrapment. Therefore, extrusion was chosen for liposome production during pathogen survivor assay testing.

Liposomes were produced using three lipid molar formulations: PC, PC/PG 7:3, and PC/PG 6:4 (mol. fraction) with encapsulated and free nisin within skim and whole milk. *L. monocytogenes* was used to determine how well each formulation could suppress pathogen growth. It was determined that lipid molar formulations did create statistically different log means. Free nisin inhibited *Listeria* growth more significantly at 20°C than encapsulated nisin; however, differences were not as significant in 5°C. Milk fat percentages (i.e. skim and whole milk) created statistically significant difference at both incubation temperatures. Skim milk was able to reduce and maintain lower *Listeria* counts than whole milk due to smaller amounts of milk fat present that kept nisin from interacting with present fat globules. Nisin was able to reduce the *Listeria* population by ~1.0 $\log_{10}$ CFU/mL at refrigerated temperature showing the bacteriocin can improve the safety of milk products and overall safety of food manufactures within the industry.

Further research is needed to improve EE using crude and inexpensive lipids so the industry can use these methods on an industrial scale. Liposome design should be

studied in the future to improve methods that allow for faster production with less variability in encapsulation efficacy. Liposome stability over periods of time (i.e. minutes, hours, and days) should be determined by testing fluorescence signal strength at normal and abused temperatures and within many different lipids. This will help determine how long antimicrobials like nisin are released from liposomes and how long they can convey safety measures within a product.

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