ANTIMICROBIAL ACTION OF THE PEPSIN HYDROLYSATE OF LACTOFERRIN (LFH) ON *ESCHERICHIA COLI* O157:H7

by

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ABSTRACT OF THE DISSERTATION

ANTIMICROBIAL ACTION OF THE PEPSIN HYDROLYSATE OF LACTOFERRIN (LFH) ON *ESCHERICHIA COLI* O157:H7

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Karl Matthews, Ph. D.

Foodborne illnesses are a significant problem and a major public health concern in the United States and throughout the world. The control of microbial pathogens in foods is a significant concern and numerous methods have been employed to control or prevent the growth of pathogenic microorganisms in food, including the use of synthetic and natural antimicrobial agents. There exist a plethora of literature on "natural" antimicrobial compounds (*e.g.* nisin, lactoferrin) and their possible use in food systems to eliminate or control the growth of pathogenic microorganisms. The actual antimicrobial mechanism of action for some antimicrobials has been extensively studied and well documented but for other potential natural biopreservatives, such as lactoferrin, the actual mechanism of action is not well defined. Lactoferrin is a 78 kilo Dalton cationic iron-binding antimicrobial glycoprotein produced in many mammalian secretions, including milk, tears, saliva, and serum. Previous research has focused on iron starvation and cell membrane damage. However, treatment with pepsin yields a peptide fragment, termed

lactoferricin that lacks the iron binding sites and is still antimicrobial. It has also been hypothesized that the peptide, due to its small size, in comparison to the whole molecule, might be able to penetrate the outer membrane or that the smaller size of lactoferricin may facilitate its access to microbial cell surface components. The peptide and pepsin hydrolysate have been shown to depolarize the outer membrane of *E. coli*, however, this is likely not the mechanism of action. The data presented in this study demonstrate that the pepsin hydrolysate of lactoferrin (LfH) exerts its antimicrobial action on the inner membrane of *E. coli* O157:H7 by forming pores in the membrane. This membrane damage results in a loss of energy and ion balance (potassium ion (K^+) efflux and decreases in intracellular ATP concentrations coupled with increases in extracellular ATP concentrations) leading to a collapse of membrane potential ($\Delta\Psi$) and a loss of cell viability.

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I would also like to thank all my friends in our labs as their assistance, even with the smallest of tasks helped me in many ways to accomplish this research.

And last but by no means last, I thank my wife, Kim, and our children, Christopher and Alexis, who have endured missing a husband and father on many a day or night so that I could complete this daunting task. Their love and support never wavered.

DEDICATION

To the memory of my father, Dr. Wade Thomas Murdock, MD and my father in law Frank Antonacci. You were both taken from us too soon but your love and support will always be with me.

TABLE OF CONTENTS

LISTS OF TABLES

LIST OF ILLUSTRATIONS

Chapter 1

Chapter 3

Chapter 4

Chapter 5

Chapter 6

LIST OF ABBREVIATIONS

CDW	Cell dry weight
$\Delta\Psi$	Delta Psi, transmembrane potential
Di-S-C3-(5)	Fluorescent probe 3,3' –dipropyltyiadicarbocyanine iodide
DMSO	Dimethylsulfoxyde
EDTA	Ethylenediaminetetraacetic acid
GRAS	Generally Recognized as Safe
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IU	International Units
Lf	Lactoferrin, the parent protein
Lfcin	Lactoferricin, the purified peptides from lactoferrin
LfH	Pepsin Hydrolysate of Lactoferrin
LPS	Lipopolysaccharide(s)
MIC	Minimum Inhibitory Concentration
MLC	Minimum Lethal Concentration
OD_{xxx}	Optical density at a specific wave length "xxx"
PYG	Peptone Yeast Glucose (1% peptone, 0.025% yeast extract, 1% glucose)
TSA	Tryptic Soy Agar
TSB	Trypticase Soy Broth
UHT	Ultra-high temperature, a pasteurization process 145^{o}F (62.8^{o}C) for 30 min or 163^{o}F (72.8^{o}C) for 15 s

CHAPTER 1

I. INTRODUCTION

A. Background and Significance

Foodborne illnesses are a significant problem throughout the world today. However, according to health officials, the likelihood of significant decreases in the occurrence of foodborne illnesses is remote. Despite progress improving the quality and safety of foods, the Council for Agricultural Science and Technology (CAST) states that any food product can be contaminated. Furthermore, new species of pathogenic microorganisms (*i.e.* emerging pathogens) are frequently isolated and known pathogenic microorganisms continue to adapt and evolve, often increasing in virulence. For example, in the early 1990s the U.S. Public Health Service identified *Escherichia coli* O157:H7, *Salmonella* species, *Listeria monocytogenes* and *Campylobacter jejuni* as the four most serious foodborne pathogens, all of which (excluding *Salmonella* species) were not recognized as sources of foodborne diseases twenty years ago. Additionally, microorganisms, such as *Salmonella* DT104 and *Staphylococcus aureus*, are developing resistance to many known antimicrobial compounds, thus increasing their potential to cause disease (Hall, 1997).

Today's challenge is to develop effective means of controlling pathogenic microorganisms while they employ sophisticated mechanisms of mutations and adaptations to withstand stresses and avoid control measures. To further complicate this challenge is the desire of the industry and the public to develop "natural" antimicrobial compounds, those compounds found in nature that have a pharmacological or biological activity. Currently there is a vast amount of literature on natural antimicrobial

compounds (*e.g.* nisin, lactoferrin) and their possible use in food systems to control the growth of pathogenic microorganisms. Nisin is considered GRAS and is one example of a compound used in some food products as a natural biopreservative. Nisin produced by *Lactococcus lactis* spp. *Lactis*, is the only bacteriocin with FDA-approved GRAS status for use in products such as pasteurized processed cheese and salad dressings (Anonymous 2000, Cleveland, *et al.* 2001). The mechanism of action for nisin has been extensively studied and well documented (Winkowski, *et al.*, 1996, Chatterjee, *et al.*, 2005, Breukink, 2006). Another potential example of a natural biopreservative is lactoferrin (Lf), which has been demonstrated, in an activated form, to be useful in food products (Naidu, 2002). However, unlike nisin the actual mechanism of action for lactoferrin is not yet completely understood. In the present work, LfH was studied, due to the likelihood of greater acceptance by industry and government regulatory agencies due to the GRAS status of Lf (Sofos, *et al*. 1998).

Many studies report that Lf possesses antimicrobial activity towards a large number of Gram-negative and Gram-positive foodborne pathogens; therefore, there is considerable potential for widespread use of Lf as a biopreservative in food products (Ellison, *et al.*, 1988, Ellison and Giehl, 1991, Bellamy, *et al.*, 1992, Chapple, *et al.*, 1998). Lf, a member of the transferrin protein family, is a cationic iron-binding glycoprotein that is present in most biological systems (Bellamy, *et al.*, 1993). It consists of a single chain with a molecular weight of seventy-eight kilo Daltons (78kDa) and contains two lobes with four domains (two amino and two carboxyl termini) that allow it to reversibly bind two iron ions (Figure 1) (Odell, *et al.*, 1996, Ye, *et al.*, 2000). The biological properties of Lf include regulation of adsorption of iron and metals as well as

varying degrees of antimicrobial activity. In fact, Lf is considered one of the major antimicrobial components in mammalian exocrine secretion (Naidu, *et al.*1993). Additionally, Lf has been shown to act synergistically with other proteins and biological antimicrobial constituents, such as lysozyme and immunoglobulin (Chapple, *et al.*, 1998, Ye, *et al.*, 2000).

The study of the antimicrobial activity of Lf becomes more complex since its peptide fragments, termed lactoferricin (Lfcin), possess antimicrobial activity as well. These fragments are composed of two chains, which correspond to a region of the surface helix near the N-terminus of the whole molecule. The length of the peptide fragments appears to vary slightly depending on the source, either bovine or human. However, one characteristic that remains constant is the lack of the iron ion binding sites on the peptide fragment (Hoek, *et al.*, 1996). It can also be hypothesized that the peptide, due to its small size (approximately one twenty-fifth the size of the whole molecule) might be able to penetrate the outer membrane or that the smaller size of lactoferricin may facilitate its access to microbial cell surface components (Yamauchi, *et al.*, 1993). In conclusion, Lfcin has been shown to exert a more potent antimicrobial effect than its parent molecule Lf (Table 1) (Bellamy, *et al.*, 1993, Yamauchi, *et al.*, 1993, Lawyer, *et al.*, 1996, Odell, *et al.*, 1996, Vorland, *et al.*, 1999(a), Vorland, *et al.*, 1999(b)).

The initial hypothesis proposed was that the antimicrobial effect of Lf on microorganisms was due to its ability to bind iron ions, therefore, depriving the cell of iron, which is one of the most important inorganic compounds required for survival (Yamauchi, *et al.*, 1993). Some of the earliest work done with Lf supported this hypothesis (Stuart, et al., 1984). However, additional studies with the non-iron-binding

peptide, Lfcin proved this partially correct (Ellison and Geihl, 1991, Erdei, *et al.*, 1994, Chapple, *et al.*, 1998).

Further research suggested that the antibacterial activity may be due to a destabilization or disruption of the outer membrane due to release of lipopolysaccharide (LPS) or potentially membrane porin binding may be involved (Yamauchi, *et al.*, 1993, Erdei, *et al.*, 1994, Hancock and Chapple, 1999, Vorland, *et al.*, 1999(a), Vorland, *et al.*, 1999(b)). Yamauchi and associates (1993) demonstrated as much as a 58% release of LPS in the presence of lactoferrin (compared to less than 5% for controls) and the associated viability studies demonstrated a >90% decrease in bacterial population. On the other hand, Sallmann and associates demonstrated increased conductance in the ion channels associated with porin OmpC, OmpF and PhoE mutants (Sallmann, *et al.*, 1999). While the latter two mechanisms appear to demonstrate effective control, additional work with both porin mutants and outer membrane LPS removal is necessary to clarify the actual mechanism of Lf and Lfcin.

II. EVALUATION OF THE POTENTIAL MECHANISMS

A. Elimination of Iron Starvation as Potential Antimicrobial Mechanism

Initially the proposed hypothesis for the antimicrobial effect of Lf on microorganisms was its ability to bind iron ions present in the environment; thereby, depriving microorganisms of iron, which is one of the most important inorganic compounds required for survival (Yamauchi, *et al.*, 1993). While there are some supporting data for this hypothesis and proposed mechanism, there exists significant

research data pointing out the weaknesses of this hypothesis. Most of the conflicting research focused on the non-iron-binding peptides, Lfcin, and iron-super-saturated systems.

Early research performed at the University of Alaska indicated that while the bactericidal effects of Lf were reduced significantly or eliminated in the presence of divalent cations (*e.g.* Fe^{+}), there was still a bacteriostatic effect produced in the presence of iron-super-saturated molecules (systems) (Stuart, *et al.*, 1984). Additional experiments with increasing concentrations of Lf, to remove any available iron in the system, demonstrated a consistent increase in the delay associated with microbial growth (bacteriostatic effect). This change from approximately 5.7 hours to 8.7 hours indicated that the mechanism, which caused inhibition or death, must be more complex than one of a simple nutrient deprivation (Stuart, *et al.*, 1984). Additionally, Bellamy and associates reported that while apo-lactoferrin (iron free lactoferrin) would cause a rapid loss of bacteria viability this effect could not be reproduced using an iron-deficient medium alone (Bellamy, *et al.*, 1992). This work supported the theory that simple nutrient deprivation was not the sole cause of the antimicrobial effect.

To further strengthen the hypothesis that simple nutrient depletion was not the cause for the antimicrobial effect of Lf, work was done with the peptide fragments of Lf, termed Lfcin that are generated upon proteolytic pepsin digestion. Bellamy and associates reported that Lfcin was devoid of both tyrosine (Tyr) and histidine (His) residues, which are essential for the iron binding function of Lf (Figure 2) (Bellamy, *et al.*, 1992 Bellamy, *et al.*, 1993). In the tertiary folding of the native Lf molecule there is a close association of the negatively charged histidine and tyrosine amino acids, which

specifically aid in the binding of iron atoms. In Lf, an iron atom is coordinated to the amino acids ^{61}Asp, ^{92}Tyr, ^{192}Tyr and ^{253}His in the N terminal lobe and another atom is coordinated to the corresponding amino acids ^{396}Asp, ^{433}Tyr, ^{525}Tyr and ^{595}His in the C terminal lobe (Ward, *et al*., 1996). These two areas form the clefts for the binding of the positively charged iron ion (Figure 1).

With the absence of these essential putative iron-binding sites and relative clustering of basic amino acids, a net positive charge is produced suggesting a cause for the antimicrobial activity of lactoferricin. This type of asymmetric clustering of basic residues has been reported in various types of antimicrobial peptides including magainins and defensins, which have a high binding affinity for negatively charged parts of microbial membranes. Lfcin peptide fragments contain the primary residues (20 - 37), which are required for high affinity binding to LPS (Kang, *et al.,* 1996, Chapple, *et al.*, 1998, Chapple, *et al.*, 2004, Farnaud, *et al.*, 2004). It is also reasonable to hypothesize that the peptide, due to its small size (approximately 3 to 5 kDa versus approximately 78 kDa) might be able to penetrate the outer membrane, come into contact with and cause damage to the inner membrane.

Furthermore, the smaller size of Lfcin may facilitate its access to microbial cell surface components (Ye, *et al.*, 2000). In addition, the inability of Lfcin to bind iron eliminates conformation changes that occur in the molecule upon iron binding, which ultimately might cause a decrease in activity. Therefore, based on the early research presented above, the "iron starvation" hypothesis was dismissed as the primary mechanism and the focus turned towards the molecular interactions that potentially occur with Lf and Lfcin.

B. Other Potential Mechanisms for Lactoferrin and Lactoferricin Activity

Lf and Lfcin, like any cationic molecule, have high affinities for binding to LPS in Gram-negative bacterial cell walls as well as lipotechoic acids in Gram-positive bacteria (Hoek, *et al.* 1996, Hancock and Chapple, 1999). After binding, Lfcin is thought to release LPS thus compromising the membrane permeability barrier of the microorganism. Figure 3 indicates this general mechanism. Lfcin is hypothesized to bind to and release the LPS, which will cause a potential loss in membrane stability. Additionally, there is the potential for cationic molecules, such as Lfcin, to cross the outer membrane to the inner membrane where additional damage may occur.

During the formation of the outer membrane, cationic ions, such as Ca^{2+}, are incorporated into the membrane to stabilize the anionic charge of the LPS core. By binding to the outer membrane in place of these stabilizing cations, Lfcin and Lf can destabilize cellular membranes and cause release of LPS from the outer membrane. Studies using ^{3}H labeled lipopolysaccharide demonstrated a significant release of LPS from the outer membrane of selected foodborne pathogens upon exposure to these compounds (Table 2) (Ellison, *et al.*, 1988, Ellison and Geihl, 1991, Yamauchi, *et al.*, 1993). It was assumed that this release could cause the outer membrane to become more permeable to these molecules as well as other potentially microbial lethal compounds.

Scanning transmission electron microscopy demonstrated Lfcin and Lf caused the release of LPS resulting in destabilization of the outer membrane and appeared as separation in the outer membrane, potentially characterizing the bactericidal mechanism.

The research showed that, for example, upon exposure of *Escherichia coli* to Lfcin, cytoplasmic clumping or coagulation was observed along with "blistering" of the outer membrane.

In the electron micrographs in Figure 4, it can be observed that the control, in standard buffered medium, (Figure 4a) displays no separation of the outer membrane. While in Figures 4b and 4d, exposure to Lfcin at half of the minimum inhibitory concentration resulted in significant separation typically at the ends of the cells or at the junction of dividing cells. The collapse of the membrane integrity adversely affects the membrane potential, causing cellular distortion and ultimately cell lysis (Chapple, *et al.*, 1998).

Similarly, other reports indicate that Lf and Lfcin may have specific surface binding receptors. Bellamy and associates performed a series of dose-dependent binding studies using ^{14}C labeled Lfcin and *Escherichia coli* to support this hypothesis (Figure 5) (Bellamy, *et al.*, 1992, Bellamy, *et al.*, 1993).

In their research, the authors demonstrated that the amount of labeled Lfcin bound was dependent on the amount added to the sample. Additionally, the viability studies indicated the maximum bactericidal effect at the optimum pH for lactoferrin-cell binding activity. These data suggest that Lf cell binding is a prerequisite for antimicrobial activity (Bellamy, *et al.*, 1993). In this research, a two to three log loss of viability was observed and more peptide molecules were bound (greater than 10^6 per cell) than would be expected for binding to a specific protein receptor. Therefore, it is reasonable to conclude that the receptors are likely to be highly repeated surface components.

In an effort to determine if there were more specific, repeated cell surface components researchers began to focus on the porin protein components of the cell membrane. To allow nutrients to pass through the outer membrane and reach the periplasmic space, the outer membrane contains water-filled pores that consist of trimers of the proteins called porins. Each porin has the capacity to form a channel, which constitutes a major permeability barrier against nutrients as well as antimicrobial compounds in the outer membrane of Gram-negative bacteria. The major porins in Gram-negative foodborne pathogens, such as *Escherichia coli*, are OmpC, OmpF and PhoE. Both OmpF and OmpC are similar in their function to act as a diffusion channel for relatively small charged molecules. By comparison, PhoE is an anion-selective diffusion channel (Erdei, *et al.*, 1994, Naidu, *et al.*, 1993, Sallmann, *et al.*, 1999).

While some reports indicate that Lf and Lfcin bind to the porins in the outer membrane, there is some debate as to which porins are the primary binding sites for Lf and Lfcin. Some research indicates that OmpF and OmpC are the primary binding sites while other work demonstrates that OmpC and PhoE are the major targets (Erdei, *et al.*, 1994, Sallmann, *et al.*, 1999). However, in both studies the results support the proposal that the antibacterial activity of Lf and Lfcin may depend on their ability (partially or completely) to bind to the porins and alter membrane conductance or stability (Erdei, *et al.*, 1994, Sallmann, *et al.*, 1999).

The binding of Lf to specific OmpF and OmpC porin phenotypes was investigated using strains of *Escherichia coli* genetically modified to express specific porins as well as wild type cells and ^{125}I-labeled Lf (Erdei, *et al.*, 1994). The lack of either OmpF or OmpC did not considerably alter the Lf binding as compared to the parent strains, while

strains that lacked both OmpF and OmpC (*e.g.* $PhoE^+$ mutants) demonstrated significantly less binding (Table 3). Furthermore, the mutants deficient in all three porins demonstrated negligible binding capability for Lfcin (Erdei, *et al.,* 1994). Subsequent analysis of the outer membranes using urea-SDS-PAGE and Western blot analysis demonstrated strong binding associations with OmpC and OmpF mutants while weak (if any) binding interaction was observed with the PhoE mutants (Erdei, *et al.*, 1994, Naidu, *et al.*, 1993).

Further evaluation of the actual binding of Lf to the porins demonstrated that porin binding interactions vary slightly, which might lead to the destabilization of the membrane by LPS removal, or changes in porin ion channel conductance (Erdei, *et al.*, 1994, Chapple, *et al.*, 1998). Figure 6 shows the differing binding characteristics between lactoferrin and porins OmpC and PhoE, while OmpF is not pictured since it was determined in simultaneous experiments that lactoferrin did not bind appreciably (molar binding ratios of 1.9 : 1.8 : 0.1 respectively) (Sallmann, *et al.*, 1999). Lf appears to have a greater binding affinity for OmpC than PhoE. Based on this research, Lf impedes the ion fluxes through OmpC but not PhoE. However, the weaker binding of Lf to PhoE (instead of OmpC) could allow for more protein interactions with other cell surface components such as LPS (Sallmann, *et al.*, 1999).

A porin conductance study performed by Sallmann and associates demonstrated up to a 25 pA current fluctuation in the porins. This fluctuation was determined to be due to the rapid opening and closing of the porin channels. Raising the concentration of Lf significantly increased the frequency of the current fluctuations and in some instances, a blocking effect, a complete interference with porin channel function, was observed. These

results were primarily observed in OmpC mutants while little to no effect was observed with the PhoE or OmpF mutants. These data suggest that OmpC and not OmpF or PhoE is primarily affected by the antimicrobial activity of lactoferrin. Additionally, PhoE binds lactoferrin weakly, which may lead to potentially other antimicrobial activity (Sallmann, *et al.*, 1999).

III. TABLES AND ILLUSTRATIONS

Table 1: Minimum Inhibitory Concentrations for lactoferrin and lactoferricin for several typical food-borne pathogenic microorganisms. Data derived from Bellamy *et al.*, 1992, Bellamy, *et al.*, 1993, Hoek, *et al.*, 1996, and Hancock and Chapple, 1999.

	Minimum Inhibitory Concentration (μg/ml)	
Organism	Lactoferrin	Lactoferricin
Escherichia coli O111	500 – 3000	6 – 500
Staphylococcus aureus NCTC 6571	2000 – 8000	14 – 500
Listeria monocytogenes NCTC 7973	1000 – 2000	15 – 69
Bacillus cereus, ATCC 6633	Not determined	29 – 172
Salmonella species (Salford)	1000 - 4000	72 – 172

Table 2: Release of ^{3}H labeled lipopolysaccharide from selected foodborne pathogenic microorganisms by lactoferrin and lactoferricin. Data compiled from Yamauchi, *et al.*, 1993.

	Percent ^{3}H lipopolysaccharide released		
Microorganism	Control	Lactoferrin (2000μg/ml)	Lactoferricin (100μg/ml)
Escherichia coli CL99 1-2	2.0 ± 1.3	8.5 ± 2.1	26.6 ± 3.4
Salmonella montevideo SL 5222	3.4 ± 1.9	26.4 ± 2.0	39.8 ± 10.6
Salmonella typhimurium SL696	4.5 ± 2.6	22.8 ± 6.8	45.5 ± 1.8

Table 3: Percentage of the total amount of ^{125}I-labeled lactoferrin bound to the cell outer membrane porins of specific OmpF, OmpC and PhoE mutant and wild type strains of *Escherichia coli*. Adapted from Erdei, *et al.*, 1994.

Porin phenotype			% ^{125}I-lactoferrin bound
OmpF	OmpC	PhoE	
+	+	-	25.2
+	+	-	26.0
-	-	+	13.3
+	-	-	20.8
+	-	-	31.0
-	+	-	25.8
-	+	-	30.9
-	-	-	4.2

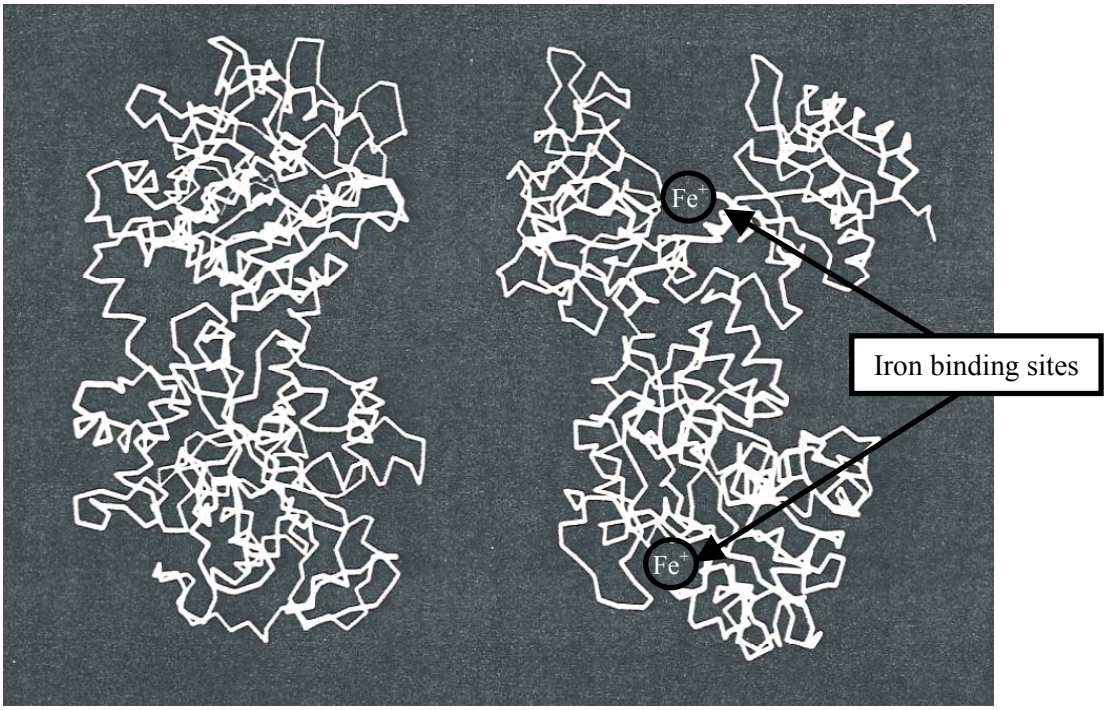

Figure 1: 3-D structure of the typical lactoferrin molecule (right structure is 90^{o} rotation), N-lobes are upper. The ferric ion binding sites are indicated by the globes & arrows. Adapted from Odell, *et al.*,1996.

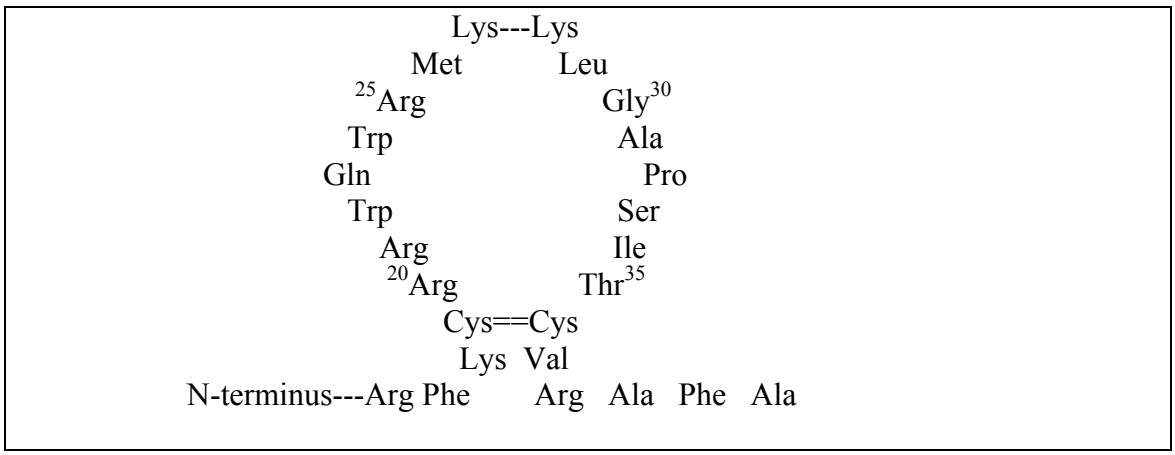

Figure 2: The primary sequence of Lfcin as identified by N-terminal sequencing and mass spectroscopy. The numbers indicate the amino acid residue sequence position from the N terminus. The putative iron binding sites, which form the two binding domains, are located at positions 92, 192, 253, 433, 525 and 595. Adapted from Bellamy, *et al.*, 1992, Bellamy, *et al.*, 1993, and Hoek, *et al.*, 1997.

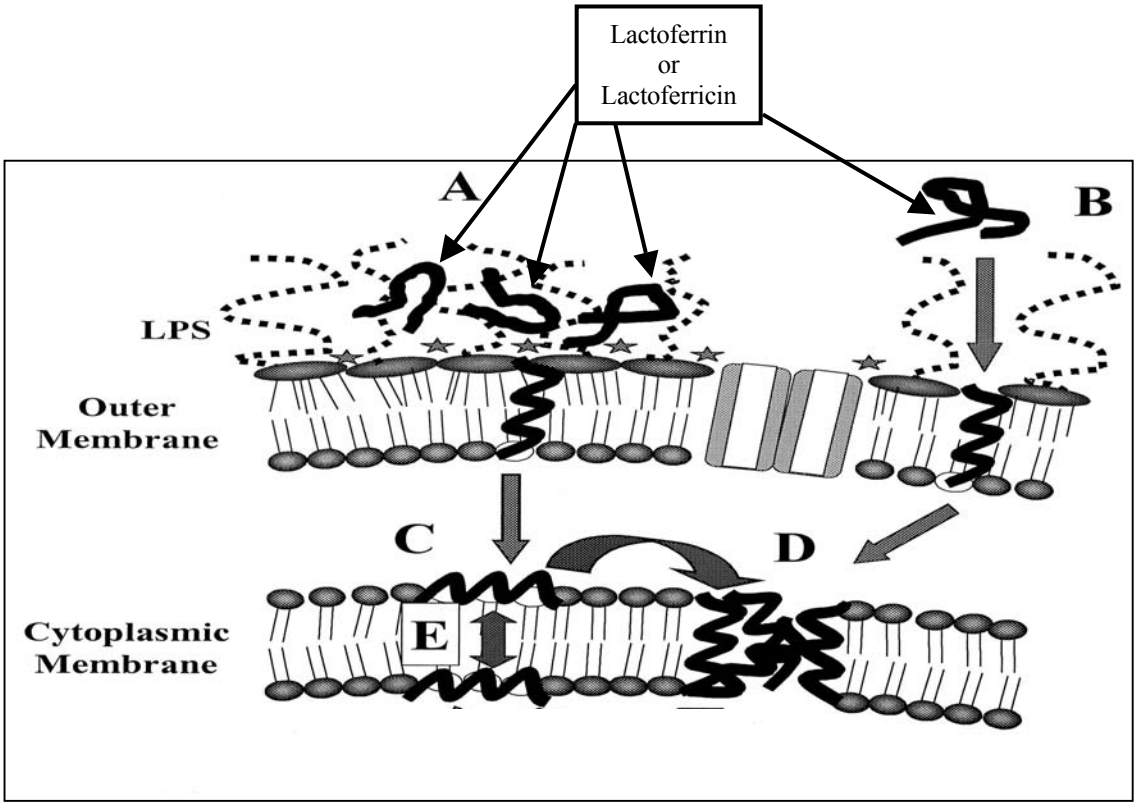

Figure 3: Proposed mechanism of action of the cationic molecules lactoferrin or lactoferricin with the cell membrane of Gram-negative bacteria. These cationic molecules are thought to bind to the lipopolysaccharide (LPS) and cross the barrier. Figure adapted from Hancock and Chapple, 1999.

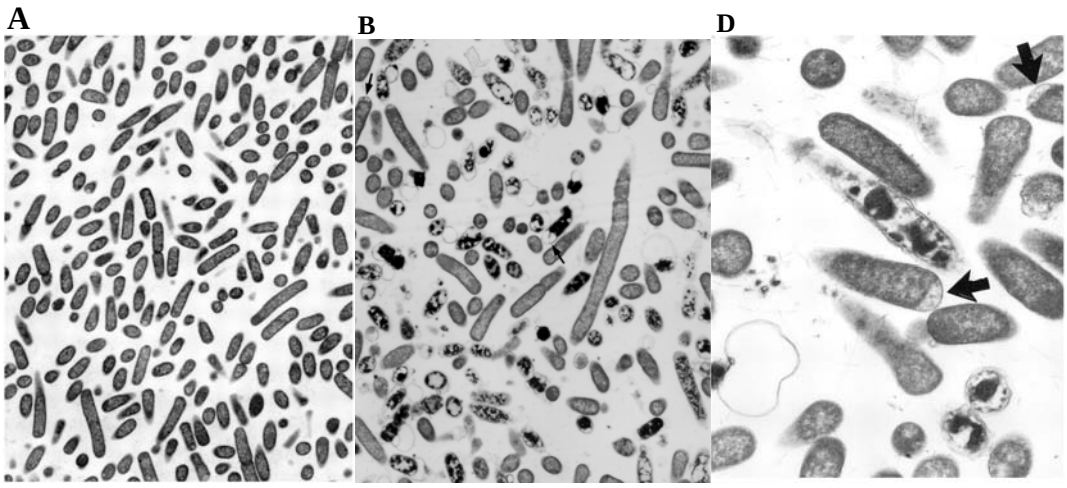

Figure 4. Electron micrographs of *Escherichia coli* O111. (a) Control culture under normal growth conditions (magnification, 3,600 ×). (b) Culture after incubation in the presence of half MIC value of lactoferricin peptide (magnification 7,200 ×). (d) Culture after incubation in the presence of half MIC value of lactoferricin peptide (magnification 13,500). Arrows in (b) and (d) indicate the separation of the outer membrane. Adapted from Chapple, 1998.

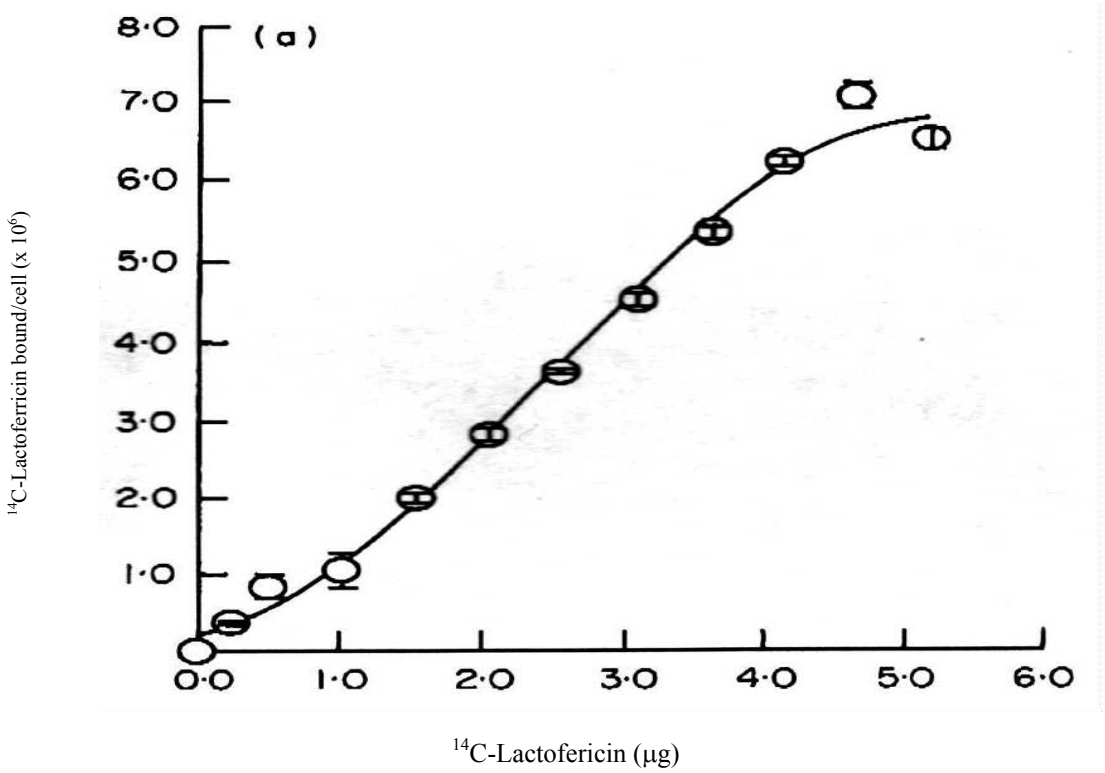

Figure 5: Binding of ^{14}C labeled Lactoferricin at various peptide concentrations (μg/ml) to *Escherichia coli* O111 at pH 6.0. Bellamy, *et al.*, 1993.

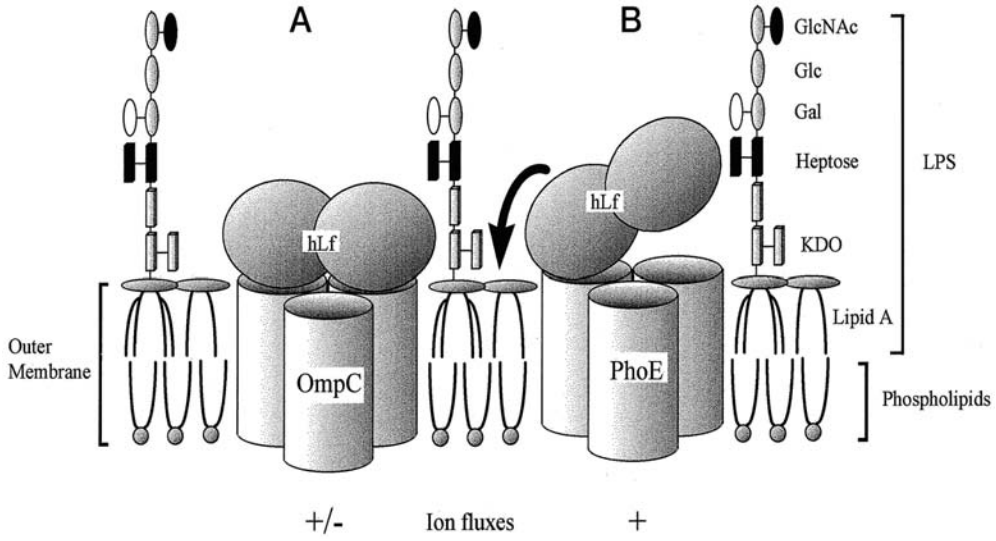

Figure 6: Schematic representation of lactoferrin-porin binding. Section "A" demonstrates the close interaction (tight binding) that occurs between OmpC and lactoferrin while section "B" illustrates the weaker interactions between lactoferrin and PhoE. Adapted from Sallmann, *et al.*, 1999.

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CHAPTER 2

I. HYPOTHESIS

Previously, there were two well-accepted hypotheses for the antimicrobial mechanism of lactoferrin (Lf), the hydrolysate (LfH) and lactoferricin (Lfcin) on foodborne pathogens that have supporting data. These two hypotheses propose that Lf, LfH or Lfcin act on the outer membrane causing a loss of viability by either (1) the destabilization or disruption of the outer microbial membrane due to release of lipopolysaccharide or (2) interference with porin ion channels. While there are data to support both of these theories, bacteria can survive in cell wall-less forms (***e.g.*** L-form, spheroplasts). Therefore, we hypothesize that Lf, LfH or Lfcin exerts their antimicrobial action on the inner membrane of *E. coli* O157:H7 causing a loss of energy and ion balance materials (K^+ and ATP) leading to a collapse of the membrane potential ($\Delta\Psi$) and a loss of cell viability.

II. STATEMENT

The pepsin hydrolysate of Lactoferrin (LfH) acts on the inner membrane of *E. coli* O157:H7 causing disruption in the membrane stability, which ultimately leads to cell death.

III. OBJECTIVES

Chapter 4:

A. Determine the antimicrobial activity of lactoferrin (Lf) and the hydrolysate (LfH) in a model broth system.

B. Evaluate the antimicrobial activity of lactoferrin (Lf) and the hydrolysate (LfH) in a model food system (suitability for use in foods).

Chapter 5:

C. Evaluate the potential for synergistic effects of lactoferrin (Lf) and the hydrolysate (LfH) with nisin of *E. coli* O157:H7 and *Listeria monocytogenes*.

Chapter 6:

D. Evaluate the interaction of the hydrolysate (LfH) with the inner membrane of *E. coli* O157:H7 through potassium efflux (K^+), ATP changes and membrane potential ($\Delta\Psi$).

CHAPTER 3

I. COMPREHENSIVE MATERIALS AND METHODS

A. Bacterial Strains

Escherichia coli O157:H7 (ATCC 43895), *Salmonella enterica* serovar Stanley (ATCC 7308), *Staphylococcus aureus* (ATCC 13565) and *Listeria monocytogenes* (ATCC 19111) were used. In preparation for all experiments a fresh culture was prepared from a frozen stock culture vial, checked for purity and transferred to the appropriate medium for the assay (as defined in each protocol) and incubated at 35°C ± 2°C. All cultures were checked for typical characteristics of the microorganism. This was performed using a series of tests including Gram-staining, latex agglutination to determination of *O* and *H* antigens (*e.g.* Wellcolex® *E. coli* O157:H7 or RIM™ *E. coli* O157:H7 Latex Test, Remel Inc., Lenexa, KS, USA) and other typical rapid methods (*e.g.* BBL™ Enterotube™ II, BD Diagnostic Systems, Franklin Lakes, NJ, USA). Any atypical cultures were discarded and new cultures initiated prior to performing any experiments.

In general, Peptone Yeast Glucose broth (1% peptone, 0.025% yeast extract, 1% glucose (PYG)) was the growth medium for all experiments. All cultures were streaked plated for purity on Tryptic Soy Agar (TSA, Becton Dickinson, USA), and stocks were maintained in a 50:50 mixture of glycerol and 1% peptone broth at -20°C. All working cultures were prepared by transferring a loop of culture from the frozen stock onto a fresh

plate of TSA streak plating for isolation and incubating at 35°C ± 2°C for 16-24 h. An isolated colony was transferred to a container of fresh PYG medium of sufficient volume for the individual study and incubated at 35°C ± 2°C, under agitation, for 8-12 h for log phase cells and 16-24 h for stationary phase cells. Prior to use, all cultures were diluted into fresh PYG medium or the appropriate buffer as defined in each protocol.

B. Lactoferrin and Lactoferrin Pepsin Hydrolysate preparation

Lf obtained from Immu-Cell Corporation (Portland, ME) as a lyophilized powder was stored at -20°C until needed for individual studies. Lf was re-suspended at the appropriate concentrations needed for each individual study in sterile water (Fisher Scientific Water LC-MS Ultra Pure Chromosolv® or equivalent), PYG broth or the appropriate buffer as defined in each study. A sample of Lf was subjected to Tris-HCl SDS PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) analysis (7.5% or 10% tris polyacrylamide gel) to determine purity using BIORAD pre-stained broad range molecular weight markers. Figure 1, SDS PAGE for Lf, indicates purity with one major band at approximately 78 kilo Daltons (kDa). All experiments were performed using freshly prepared Lf.

LfH was prepared using the methods described by Bellamy, *et al.* (1991) and the resulting lyophilized powder was stored in sealed containers at – 20°C until re-suspended for use in all experiments. Briefly, Lf was dissolved in sterile water (milli Q, Millipore, Bedford, MA) at a concentration of 5%. The pH was adjusted to 3.0 ± 0.1 using sterile 1 N HCl. Porcine pepsin (Sigma P6887, MW approximately 35kDa) was added to the

solution to a final concentration of 3.0% weight to weight of substrate to pepsin. The mixture was incubated for 4 h at 37°C under agitation. Following the incubation the reaction was terminated (*e.g.* inactivate the pepsin) in a preheated 80°C water bath for 15 minutes measuring the temperature internally and under agitation to ensure even heat distribution to terminate the reaction completely. The mixture was subsequently cooled in an ice bath to room temperature (15 – 25°C). After cooling, the pH of the solution was adjusted to 7.0 $\pm$ 0.1 using sterile 1 N NaOH. The solids were removed by centrifugation at approximately 15,000 × *g* for 30 minutes at 5°C. Additionally, to ensure removal of the heat-inactivated pepsin the supernatant was passed through a 30,000 MW cut off filter (Pall Macrosep Omega, Amicon Ultra or equivalent). The resulting supernatant was lyophilized and stored at - 20°C until re-suspended for each study as described in Figure 2.

A sample of LfH was subjected to Tris-Tricine SDS PAGE analysis (16.5% tris-tricine polyacrylamide gel) to determine purity using pre-stained kaleidoscope polypeptide molecular weight markers. Figure 3, SDS gel for LfH, indicates there are three primary bands at approximately 9kDa, 5 kDa and 3.5 kDa. Previous literature reports that the peptide fragments suspected to express antimicrobial activity are approximately 2.6 to 5.8 kDa (Dionysius and Milne, 1997, Bellamy, *et al.*, 1992, Hoek, *et al.*, 1997). These data indicate the hydrolysate contains fragments within the range suspected to contain antimicrobial activity.

The antimicrobial activity of Lf and LfH was confirmed using studies in PYG and foods.

C. Minimum Inhibitory and Minimum Lethal Concentration Assays measured in PYG

Antimicrobial assays were designed using a modification of the methods described by Bellamy, *et al.* (1992), Shin, *et al.* (1998) and the Clinical and Laboratory Standards Institute (2003) for Lf and LfH.

Stock solutions of Lf and LfH were prepared in PYG broth at appropriate concentrations (*e.g.* 16,000 μg/ml (16 mg/ml)) and filter sterilized using 0.45 μm and 0.2 μm pore size cellulose acetate membrane filters in sequence (Pall Corporation Acrodisc ®, Nalgene or equivalent). The solutions were all prepared fresh or prepared and stored at -20^{o}C until needed.

All minimum inhibitory concentration (MIC) and minimum lethal concentrations (MLC) were determined using a temperature controlled 96-well plate reader (MRX, Dynex Tech Inc) with software (Revelation, version 4.06) measuring optical density (OD) at 630nm. All assays were performed in 96-well "U" (round) bottom sterile polystyrene non-treated plates (Costar®, Falcon® or Becton Dickinson or equivalent). All lids were removed before the assay was initiated and replaced with sterile sealing film (Axygen Scientific or equivalent) to eliminate potential moisture build up on the inside of the lid during the assay that could impact the unit's ability to accurately read the OD. PYG was added to all wells (100μl), excluding the highest concentration to be assayed. Two hundred (200) μl of PYG containing two-times the needed maximum concentration (*e.g.* 16,000 μg/ml) of Lf or LfH were added to the first well in each row or column depending on the orientation of the assay (assay series). All assay series were

subsequently 1:2 serially diluted and subsequently inoculated with 10µl of logarithmic growth phase cells from the appropriate culture to achieve approximately 10^5 colony forming units (CFU) per ml. Following the addition of the culture, all wells were mixed using an 8 or 12 channel pipette to ensure consistent mixing (5 repeats) in all wells and minimize the potential for contamination. The microtiter plate was inserted into the plate reader and incubated at 35°C ± 2°C for approximately 24 h. The OD_{630} was determined for each well every 15 to 30 minutes following two 10 s mixing cycles (shakes) (PC Assay File: MIC_CM_OD_630.asy). The MIC was considered the lowest concentration of the Lf or LfH that showed no increase in OD. MLCs were determined by transferring 10µl from each well to a new 96-well plate containing 100 µl of sterile PYG (without Lf or LfH) in all wells. The microtitre plate was then re-incubated using the same assay as described for the MIC. The MLC was considered as the lowest concentration where growth was observed.

D. Assay for Lf and LfH antimicrobial activity in Ultra-high temperature (UHT) milk with or without EDTA

Ultra-high temperature milk purchased from a local supermarket, used prior to labeled expiration and was re-pasteurized to reduce potential contamination from sampling following standard USDA procedures. The milk was placed in a sterile container and subsequently pasteurized by heating to 145°F (62.8°C) for 30 min or 163°F (72.8°C) for 15 s (USDA.gov). All repasteurizations were performed in a pre-heated circulating water bath using an alcohol sanitized thermometer to measure internal

temperature of the milk. Following re-pasteurization the milk was evenly distributed to sterile conical centrifuge tubes. Lf, LfH and EDTA including combinations of Lf / EDTA and LfH / EDTA were added to the samples to bring the final volume to 2.0 ml per sample at final concentrations of 8000, 4000, 2000, 1000, 500, 250 and 125 μg/ml (Lf or LfH) with and without EDTA at a final concentration of 10 mM (mg/ml). All tests were performed in duplicate and repeated in triplicate. The resulting dilution series was inoculated with 100 μl of logarithmic growth phase cells from the appropriate culture to achieve approximately 10^5 CFU per ml. Following the addition of the culture all tubes were subsequently mixed using a Vortex Genie for 15 s ensure consistent distribution of the cells. Samples were incubated at 2 to 8°C for up to 14 d or 35°C $\pm$ 2°C for 24 h under agitation. At time points during the incubation period, 100 μl aliquots were removed from each series and drop plated on TSA. All plates were incubated at 35°C $\pm$ 2°C for 18-24 h.

E. Assay Lf and LfH antimicrobial activity in Ultra-high temperature (UHT) milk with pH neutralization or pH acidification.

As described above, UHT milk was re-pasteurized following standard USDA procedures (*e.g.* by heating to 145°F (62.8°C) for 30 min or 163°F (72.8°C) for 15 s). Following re-pasteurization the milk (initial pH approximately 6.5) was evenly distributed to sterile conical centrifuge tubes and the pH adjusted to either 4.0 $\pm$ 0.1 or 7.0 $\pm$ 0.1 using sterile 1N NaOH or 1N HCl and a third sample was not pH adjusted to act as a control. Lf or LfH were added to the samples to bring the final volume to 2.0 ml per

sample at final concentrations of 32,000, 16,000, 8000, 4000, 2000, 1000, 500, 250 and 125 μg/ml. All assays were performed in duplicate and repeated in triplicate. The resulting dilution series was inoculated with 100 μl of logarithmic growth phase cells from the appropriate culture to achieve approximately 10^5 CFU per ml. Following the addition of the culture all tubes were subsequently mixed using a Vortex Genie for 15 s to ensure consistent distribution of the cells. Samples were incubated at 2 to 8°C, representing normal storage conditions, for up to 14 d or 35°C $\pm$ 2°C, representing an abuse condition, for 24 h under agitation. At time points during the incubation period, 100 μl aliquots were removed from each series and plated on TSA. All plates were incubated at 35°C $\pm$ 2°C for 18-24 h.

F. Assay for antimicrobial activity in liquid egg product

Frozen liquid egg product (egg beaters ®) was obtained from a local food store and used prior to labeled expiration. The product was thawed in a 30°C water bath to ensure proper thawing and provide a reduced period of time for potential contamination and growth. The thawed liquid egg was mixed well using a Vortex Genie for 15 s and transferred to sterile conical centrifuge tubes. Lf or LfH were added to the samples to bring the final volume to 4.0 ml per sample at final concentrations of 32,000, 16,000, 8000, 4000, 2000, 1000, 500, 250 and 125 μg/ml. The ranges tested were 32,000 to 2,000 for *S. aureus* and *Sal. Stanley*, 2000 to 125 for *L. monocytogenes* and 8000 to 500 for *E. coli* O157:H7. All tests were performed in duplicate and repeated in triplicate. The resulting dilution series was inoculated with 100 μl of logarithmic growth phase cells

from the appropriate culture to achieve approximately 10^5 CFU per ml. Following the addition of the culture, all tubes were subsequently mixed using a Vortex Genie for 15 s to ensure consistent distribution of the cells. Samples were incubated at 2 to 8°C, representing normal storage conditions, for up to 14 d or 35°C ± 2°C, representing an abuse condition, for 24 h under agitation. At time points during the incubation period, 100 µl aliquots were removed from each series and plated on TSA. All plates were incubated at 35°C ± 2°C for 18-24 h.

G. Development of spheroplast cells from *E. coli* O157:H7

The methods used were modified from *Bacterial Membranes* by Kaback (1975), Birdsell and Cota-Robles (1967) and Gumpert (1996). The process utilizes Penicillin G (Sigma P-8721), ethylenediaminetetraacetic acid (EDTA) (Sigma ED2P) and lysozyme (Sigma L-6876) to partially or completely remove the outer membrane from the cells to generate a spheroplast. Photo micrographs were obtained throughout the process to evaluate and document the effectiveness of the procedure. From a frozen culture, a plate of TSA was streak inoculated and incubated for 18-24 h at 35°C ± 2°C. Following incubation, an isolated colony was Gram stain to ensure purity and have a baseline photo for the cellular morphology changes. Nutrient broth was inoculated from the TSA plate and incubated for 18-24 h at 35°C ± 2°C under agitation. Subsequently 100 µl of this culture was transferred to 10 ml of Penn Assay Medium (Difco Antibiotic Medium 3 #0243-17) and incubated for 18-24 h at 35°C ± 2°C under agitation. Following the incubation period, a photograph (100× magnification under oil immersion) was taken of a

representative (5 µl placed on a microscope slide) sample from the culture. To initiate the transformation of the wild-type cells to spheroplasts, filter sterilized Penicillin G (1000 IU/ml), lysozyme (200 mg/ml) and EDTA (0.05% wt/v) were added to the medium and the culture incubated for an additional 18-24 h at 35°C ± 2°C. At 2 h and 24 h of incubation, samples were removed from each broth culture and photographs taken at 100× magnification under oil immersion. The sample was then separated into two equal parts. One (1) ml was transferred to fresh Penn Assay and incubated for 18-24 h at 35°C ± 2°C to examine for reversion to wild-type cells. The remaining portion of the sample was treated a second time with Penicillin G (1000 IU/ml), lysozyme (200 mg/ml) and EDTA (0.05% wt/v) and re-incubated for an additional 18-24 h at 35°C ± 2°C. At 2 h and 24 h of incubation, samples were removed and photographs taken at 100 × magnification under oil immersion. The sample was then transferred to fresh Penn Assay medium and re-incubated for an additional 18-24 h at 35°C ± 2°C under agitation to examine for reversion to wild type cells. Photographs were taken at 100 × under oil immersion.

To determine the effect of Lf and LfH on the *E.* coli O157:H7 spheroplasts, antimicrobial assays/MIC studies were performed as described above.

H. Determination of the leakage of cellular constituents (*e.g.* Potassium (K+)) in LfH treated *E. coli* O157:H7.

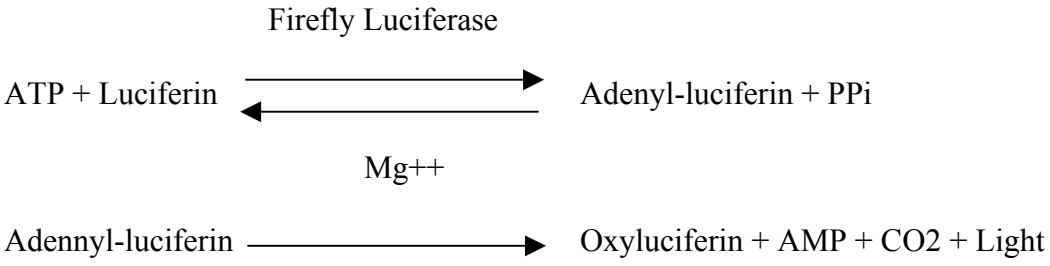
The determination of K^+ leakage from *E. coli* O157:H7 was measured with a potassium ion electrode model number 6250 (Jenco Electronics, LTD., San Diego, CA)

and a Jenco Microprocessor ph/Mv/Ion/Temp Meter model number 6219 (Jenco Electronics, LTD., San Diego, CA). To evaluate the effect of LfH against *E. coli* O157:H7, the ion potential response generated using the Jenco potassium ion electrode was converted to percentages based on a baseline (untreated cells) and a maximum efflux (permeabilized cells) (Orlov, *et al.*, 2002, Ohmizo, *et al.*, 2004).

E. coli O157:H7 cells were grown to an OD_{600} of approximately 1.0 in 50 ml of sterile PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 minutes at 5°C. The cells were washed twice with cold 10mM tris acetate, 100mM NaCl, pH 7.4 + 0.1 and re-suspended in the same buffer to an OD_{600} of approximately 30 (approximately 100 × concentration) and retained on ice until used for each experiment. All experiments were completed with 30 minutes of cell washing. The concentrated cells were diluted 1:10 prior to use in each experiment in fresh 10mM Tris Acetate 100mM NaCl, pH 7.4 $\pm$ 0.1. The potassium efflux was measured over time in untreated cells, permeabilized cells and cells treated with 4000 and 8000 μg/ml LfH. In each experiment, the efflux was monitored and recorded approximately every 30 s, excluding the total efflux from permeabilized cells, which was recorded as an initial reading and monitored for any increase over the same time period. Prior to use in each experiment, the probe was calibrated with standard solutions containing 1 mM (Fluka 60142, 1M in H_2O), 0.1 mM and 0.01 mM KCl, and 5M NaCl ionic strength adjuster buffer (Jenco K00IS01 or equivalent).

I. Determination of the intra- and extra-cellular and total ATP levels of LfH treated *E.* coli O157:H7.

The levels of total, intra- and extra-cellular ATP were determined using bioluminescent quantification of ATP. This procedure uses the enzyme luciferase to oxidize luciferin to adenyl-luciferin in the presence of limiting ATP (McEntire, *et al.*, 2004, Sigma Product Technical Bulletin, ref JWM 09/04, Bonnett, *et al.*, 2006) as the amount of ATP in the sample is proportional to the amount of light emitted and detected by a spectrophotometer (*e.g.* luminometer) (reference equations below).



Adenosine 5'-Triphosphate (ATP) Bioluminescent Assay Kits (Sigma FL-AA) were purchased and used in conjunction with a luminometer (Luminoskan TL Plus luminometer, Labsystems Oy, Helsinki, Finland) to evaluate samples for the presence of extracellular and total ATP presence. Intracellular ATP was determined by a calculation of the difference in the total and extracellular ATP concentrations.

A standard calibration curve of the moles ATP per assay versus the relative light intensity was generated for each experiment using re-suspended ATP standard provided in the Sigma kit (FL-AAS) at a 1:10 dilution in the ATP Assay Mix Dilution Buffer (FL-

AAB). To generate the standard curve, the ATP standard was then serially diluted to obtain a 10^0 to 10^{-8} dilution series. Equal amounts of this dilution series were added with ATP Assay Mix (FL-AAM, enzyme/substrate preparation containing luciferase, luciferin, MgSO4, DTT, EDTA, BSA and tricine buffer salt). Immediately following the combination, the samples were placed in the luminometer and measurements taken. All samples were tested in duplicate and all assays were repeated in triplicate to obtain results for each dilution of the standard.

E. coli O157:H7 cells were grown to mid log phase in PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 min at 5°C. The pellet was re-suspended and washed twice in equal volumes of 50mM HEPES (Fisher Scientific BP-299, 1M HEPES). A final resuspension in one half the original HEPES buffer volume was retained on ice for all studies. For energization, cells were re-suspended in one half of their original volume in 50 μM HEPES with 0.2% glucose and allowed to remain at room temperature for 20 minutes and then used for time dependent assays. All cultures were used within 30 minutes.

To determine background levels of ATP and potential interference within the system samples were prepared with 5000 μg/ml and 10,000 μg/ml LfH in HEPES buffer (*e.g.* antimicrobial buffer mix). Antimicrobial buffer mix was diluted 1:10 in fresh buffer and combined with an equal portion of ATP Assay Mix (enzyme/substrate preparation) in a round cuvette. Measurements were taken immediately. All samples were tested in duplicate and all assays were repeated in triplicate. To determine extracellular levels of ATP, 100 μl of the cell suspension was diluted 1:10 in fresh buffer and combined with an equal portion of ATP Assay Mix in a round cuvette. Measurements were taken

immediately. All duplicate samples were tested in triplicate. To determine total levels of ATP (intracellular plus extracellular ATP), 100 µl of the cell suspension was diluted 1:10 in fresh buffer, centrifuged at 10,000 × g for 30 seconds. The resulting pellet was mixed with 10 µl dimethylsulfoxyde (DMSO) for permeabilization. Following 5 minutes of permeabilization at room temperature, 990 µl fresh buffer was added to the pellet (final volume 1000 µl). Equal portions of the permeabilized cell suspension and ATP Assay Mix were combined in round cuvettes. Measurements were taken immediately. All samples were tested in duplicate and all assays were repeated in triplicate. Time dependent changes in intracellular and extracellular ATP levels were determined as described above following exposure of the cell suspension to 5000 µg/ml and 10,000 µg/ml LfH.

All ATP data were converted to dry weight of cells as follows. *E. coli* O157:H7 was incubated for approximately 8 hours in 20 ml PYG under agitation and centrifuged at approximately 7,000 x *g* for 10 minutes at 5^{o}C. The resulting pellet was removed and washed twice in 50 mM HEPES. The pellet was re-suspended in 2 ml of sterile saline and mixed well. Six aluminum weigh dishes were pre-weighed using an analytical balance (Denver Instruments Company, model TR-64). A volume of 0.5 ml of cell suspension was placed into each of four aluminum dishes and 0.5 ml sterile saline was placed into the remaining two aluminum dishes. The dishes were placed into drying oven at approximately 105^{o}C (VWR Model 1305U) and allowed to dry overnight. After drying, the aluminum dishes were each reweighed immediately following removal from the drying oven. The average weight difference of the saline containing aluminum dishes was removed from the weight of each individual aluminum dish containing cells. The

dry weight of cells was then converted to cell dry weight (CDW) and used for normalization of all ATP values.

J. Determination of the transmembrane potential in LfH treated *E. coli* O157:H7

Relative determination of the transmembrane potential ($\Delta\Psi$) in pepsin hydrolysate treated *E. coli* O157:H7 was determined using the fluorescent probe 3,3' – dipropyltyiadicarbocyanine iodide (Di-S-C3-(5)) as described by Bruno, *et al.* (1992) and Herranz, *et al.* (2001) with modifications as described by Sims, *et al.* (1974), Schuldiner and Kaback (1975), and Katsu, *et al.* (1984). A Spex Industries Fluorolog spectrofluorometer model F1T11 with excitation and emission wavelengths of 643nm and 666nm respectively with a 10nm slit width and a 700 s assay duration with reading every 0.1 second will be used for all assays.

E. coli O157:H7 cells were grown to mid log phase in 50 ml PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 min at 5^{o}C. The pellet was re-suspended and washed twice in equal volumes of 50mM Potassium HEPES (K-HEPES) buffer (Fisher Scientific BP-299, 1M HEPES) pH 7.0 $\pm$ 0.1 with a final resuspension in one one-hundredth (1/100) the original K-HEPES buffer volume in buffer containing 100 mM KPO_4 (Sigma Chemical Co.), 20% Glucose (Sigma Chemical Co.), 5 mM HEPES and 1mM K-EDTA (Sigma Chemical Co) and retained on ice prior to all studies. All cultures were used within 30 minutes.

Briefly 100 mM $KHPO_4$ was prepared using a mixture of 500 mM K_2HPO_4 (mono basic) and 500 mM KH_2PO_4 (di basic). The pH of each solution was determined. The basic solution was added to the acidic solution until the pH reached 7.0 $\pm$ 0.1. The resulting solution was diluted with sterile water to reach a final concentration of 100mM.

Assays were conducted as described previously using the process outlined below. A volume of 1980 µl 50 mM K-HEPES-1 mM EDTA and 20 µl concentrated cells in the same buffer was added to a cuvette and placed in the spectrofluorometer. The fluorescence measurements were initiated followed by the addition of 10 µl Di-S-C3-(5) (stock 2mM, final concentration 10 µM) with 1mM K-EDTA and 10 µl of the cell concentrate in buffer with quick mixing with the pipette. After the stabilization of the fluorescence, 5 µl nigericin (stock 5mM, final concentration 12.5 µM) with 1 mM K-EDTA was added with quick mixing using the pipette. Following this either buffer with 1 mM K-EDTA or LfH in buffer with 1 mM K-EDTA (5000 µg/ml and 10,000 µg/ml) was added to the cuvette. In all assays 10 µl valinomycin (stock 2 mM, final concentration 10 µM) with 1 mM K-EDTA was added to deplete any residual $\Delta\Psi$.

K. Viability Assays in LfH treated *E. coli* O157:H7

The viability of *E. coli* O157:H7 cells was determined following treatment with the MIC and 2 x MIC levels of LfH according to the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Kit # L13153, Invitrogen Molecular Probes, Eugene, Oregon) product protocol. All samples were visualized using an Olympus BH2-RFCA fluorescence microscope fitted with a Pixera camera as outlined below (Invitrogen Molecular Probes

product insert MP 07007, Li, 2002). The LIVE/DEAD® BacLight™ Bacterial Viability assay would demonstrate damage to the membrane caused by LfH. The LIVE/DEAD® BacLight™ Bacterial Viability assay utilizes a mixture of two dyes, SYTO®-9 a membrane-permeable green-fluorescent nucleic acid stain and propidium iodide a membrane-impermeable red-fluorescent nucleic acid stain to differentiate between viable and non-viable cells. The dyes differ in their spectral characteristics and ability to penetrate intact membranes of viable cells. SYTO®-9 labels all cells as it can penetrate both intact and damaged membranes and under fluorescence microscopy, the cells will appear green. Propidium iodide cannot penetrate intact membranes and, therefore, will only stain cells with damaged membranes. When the two stains are present in a cell, the propidium iodide stain will quench the fluorescence of the SYTO®-9 and the cells will appear red under fluorescence microscopy. In cases where the cell membrane is only slightly damaged (non-lethal damage) the propidium iodide is not able to penetrate the membrane (due to size of the molecule) and thus the cell will appear viable (green).

E. coli O157:H7 cells were grown to mid log phase in PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 min at 5°C. The pellet was re-suspended and washed twice in equal volumes of sterile water (Fisher Scientific Water LC-MS Ultra Pure Chromosolv®) with a final resuspension in one-half the original volume of sterile water and retained on ice prior to all studies. All cultures were used within two hours.

To determine viable cells within a control (negative control = viable) equal portions of the 2x stock solution of SYTO®-9/propidium iodide and the negative control culture were mixed in a microfuge tube and incubated at room temperature in the dark for 15 min. Following incubation, 5 µl of the mixture was placed on three separate

microscope slides and covered with a cover slip. Each sample was then observed under fluorescent microscopy and five random fields (ensuring only to select fields with similar appearing cell quantities on a given slide) were counted from each slide. All assays were repeated in triplicate.

To determine non-viable cells within a control (positive control = non-viable) the final sterile water resuspension of the cells, following washes, was replaced with filter sterilized 70% isopropyl alcohol (Fisher Scientific, 2-Propanol HPLC grade) and allowed to incubate at room temperature for one hour. Following the isopropyl alcohol incubation equal portions of the 2x stock solution of SYTO®-9/propidium iodide and the positive control culture were mixed in a microfuge tube and incubated at room temperature in the dark for 15 min. Following incubation, 5 µl of the mixture was placed on three separate microscope slides and covered with a cover slip. Each sample was then observed under fluorescent microscopy and five random fields (ensuring only to select fields with similar appearing cell quantities on a given slide) were counted from each slide. All assays were repeated in triplicate.

To determine the effect of LfH on *E. coli* O157:H7, the cells were mixed with MIC and 2x MIC levels of LfH and allowed to incubate at room temperature for one hour. Following the incubation equal portions of the 2x stock solution of SYTO®-9/propidium iodide and the treated culture were mixed in a microfuge tube and incubated at room temperature in the dark for 15 min. Following incubation, 5 µl of the mixture was placed on three separate microscope slides and covered with a cover slip. Each sample was then observed under fluorescent microscopy and five random fields (ensuring

only to select fields with similar appearing cell quantities on a given slide) were counted from each slide. All assays were repeated in triplicate.

II. ILLUSTRATIONS

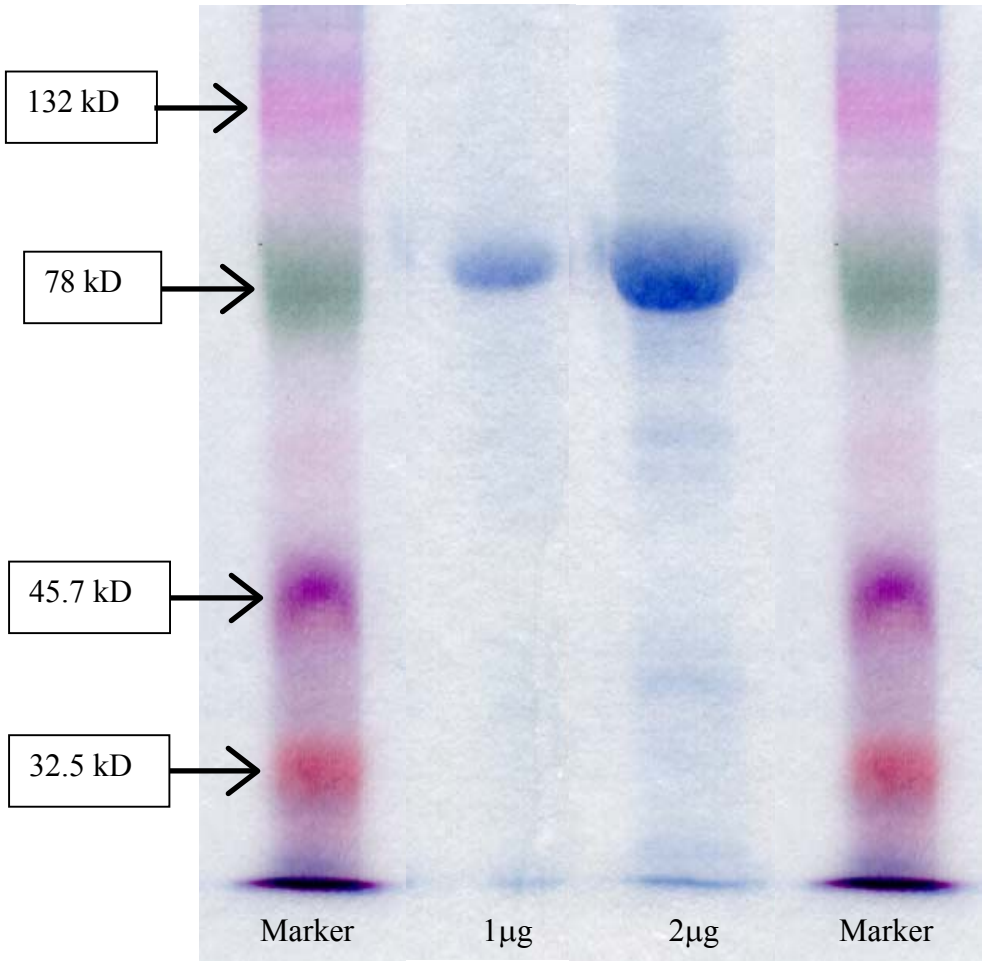
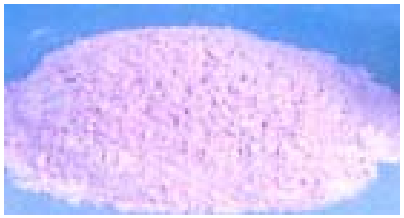


Figure 1: Purity of bovine lactoferrin (Lf) on a 20% sodium dodecyl sulphate gel before hydrolysis with pepsin. Molecular weights of kaledioscope markers are indicated on the left side of the gel.



Suspend at 5% in water and adjust to pH 3.0

Add 3% Pepsin wt/wt substrate

Incubate at 37°C for 4 hours

Terminate peptic reaction by heat (80°C) for 15 minutes

Cool to 5°C and adjust pH to 7.0

Centrifuge at 15,000 *g* for 30 minutes and collect supernatant followed by 30,000 MW filtration

Freeze Dry

Lactoferricin / Hydrolysate

Figure 2: Schematic diagram for the preparation of Lactoferrin Hydrolysate (LfH) from Lactoferrin (Lf)

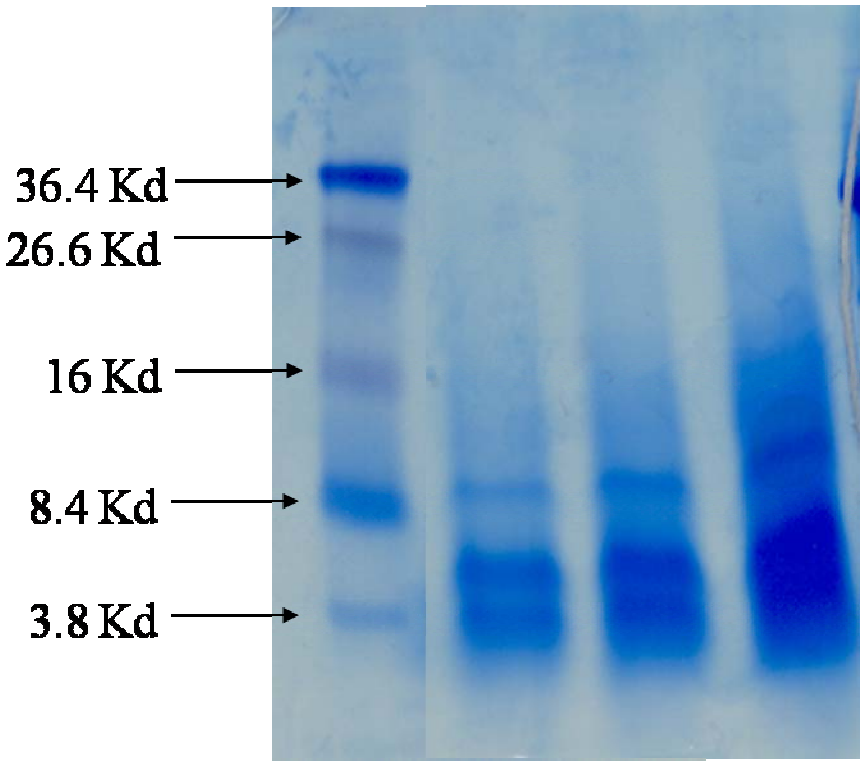


Figure 3: Purity of bovine lactoferrin hydrolysate (LfH) on a 20% sodium dodecyl sulfate gel following hydrolysis with pepsin. Molecular weights of kaledioscope markers are indicated on the left side of the gel.

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CHAPTER 4

The following data were published in: Journal of Applied Microbiology Volume 93 Issue 5 Page 850 - 856 - November 2002

Murdock, C. A. and Karl Matthews. *Antibacterial Activity of Pepsin-Digested Lactoferrin on Foodborne Pathogens in Buffered Broth Systems and UHT Milk with and without EDTA.* J Applied Microbiology, 93: 850-856, 2002.

Objectives:

A. Lf and LfH possess antimicrobial activity towards *E. coli* O157:H7 and *Listeria monocytogenes* in a model broth system.

B. Lf and LfH exert bacteristatic or bactericidal effects on *E.* coli O157:H7 and *Listeria monocytogenes* in UHT milk.

JOURNAL OF APPLIED MICROBIOLOGY

Journal of Applied Microbiology Volume 93 Issue 5 Page 850 - 856 - November 2002

Running head: Activity of lactoferrin peptides on foodborne pathogens

Title: Antibacterial Activity of Pepsin-Digested Lactoferrin on Foodborne Pathogens in Buffered Broth Systems and UHT Milk with EDTA

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I. ABSTRACT:

Aims: To evaluate the antimicrobial activity in peptone yeast extract glucose (PYG) broth and ultra-high temperature (UHT) milk of bovine lactoferrin pepsin hydrolysate (LfH) against the foodborne pathogens *Salmonella* Stanley, *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus.*

Methods and Results: The LfH was suspended in PYG and the minimum inhibitory concentration for each pathogen determined. The LfH was also suspended in UHT milk adjusted to pH 4.0 $\pm$ 0.1 or 7.0 $\pm$ 0.1, samples incubated at 4 or 35^{o}C and the change in bacterial cell population determined. Experiments in UHT milk were conducted using *L. monocytogenes* and *E. coli* O157:H7. At pH 4 $\pm$ 0.1, LfH reduced the population of *E. coli* O157:H7 and *L. monocytogenes* by approx. 2 log; however, only *E. coli* O157:H7 was inhibited in samples adjusted to pH 7 $\pm$ 0.1. The addition of EDTA (10 mg ml^{-1}) to UHT milk supplemented with LfH did not markedly influence the growth of *E. coli* O157:H7 or *L. monocytogenes.*

Conclusions: The results suggest that, under low pH and refrigeration conditions, LfH can limit the growth or reduce the population of pathogenic bacteria in a dairy product.

Significance and Impact of the Study: Natural preservatives that are active against Gram-negative and Gram-positive bacteria are desirable to the food industry. This study demonstrates that LfH is effective in a complex food system. Moreover, the LfH used was not purified, making its use by industry more attractive.

II. INTRODUCTION

Foodborne illnesses associated with *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella enterica* serovar Enteritidis and *Listeria monocytogenes* present a major public health concern in the US and throughout the world (Hall 1997). A number of methods have been employed to control or prevent the growth of these pathogens in food, including the use of synthetic and natural antimicrobial agents (Payne, *et al.* 1994; Branen and Davidson 2000). There is a wealth of literature on 'chemical' antimicrobial compounds and the use of these compounds in food systems to eliminate or control the growth of pathogenic micro-organisms. Alternatively, two natural compounds that have received considerable attention are nisin and lactoferrin (Lf). The mechanism of action of nisin has been elucidated (Winkowski, *et al.* 1996; Montville and Chen 1998), but the mode of action of Lf is not yet known.

Lactoferrin, a member of the transferrin protein family, is a cationic iron-binding glycoprotein that is found in many exocrine secretions, including milk, tears, saliva and serum, and is putatively one of the most powerful antimicrobial agents in milk (Bellamy, *et al.* 1993; Chantaysakorn and Richter 2000). Lactoferrin is a single-chained molecule with a molecular weight of approx. 78 kDa, composed of two lobes with four domains that facilitate the reversible binding of two iron ions (Odell, *et al.* 1996; Ye, *et al.* 2000). Lactoferrin exerts antimicrobial activity against a variety of Gram-negative and Gram-positive bacteria (Ellison and Giehl 1991; Payne, *et al.*; Dionysius and Milne 1997; Branen and Davidson 2000). The antimicrobial activity of the whole molecule and peptide fragments of the whole molecule are of interest for the control of pathogens in

food. The peptide, lactoferricin B, is composed of two chains, corresponding to a region of the surface helix near the N-terminus of the whole molecule. Depending on the source of Lf, bovine or human, the lengths of the peptide fragments from pepsin digestion may vary slightly, but are generally composed of 25 amino acid residues (Hoek, *et al.* 1997; Hwang, *et al.* 1998). The mechanism of action of the purified lactoferricin B peptide that has the greatest antimicrobial activity has not been clearly elucidated but it is believed to act by damaging the outer cell wall and destabilizing the cytoplasmic membrane (Yamauchi, *et al.* 1993; Sallmann, *et al.* 1999; Vorland, *et al.* 1999).

Studies investigating the antimicrobial activity of Lf or pepsin digest of Lf in food systems are limited. Payne, *et al.* (1990) reported the bacteristatic effect of Lf against *L. monocytogenes* in ultra-high temperature (UHT) pasteurized milk. Further studies by Payne, *et al.* (1994) demonstrated that Lf alone or in combination with EDTA or lysozyme in UHT milk had little practical effect against *E. coli* O157:H7, *Pseudomonas fluorescens*, *Salm.* Typhimurium or *L. monocytogenes*. However, Lf peptides were reported to exhibit antibacterial activity against *E. coli*, *Ps. fluorescens*, *L. monocytogenes* and *Bacillus cereus* in peptone yeast extract glucose (PYG) medium at concentrations 4–80 times lower than the native molecule (Dionysius and Milne 1997). Similarly, Shin, *et al.* (1998) demonstrated the enhanced activity of the pepsin digest of Lf and lactoferricin against *E. coli* O157:H7 in Bactopeptone broth. Others have reported a reduction of approx. 0·7 and 2 log CFU *E. coli* O157:H7 in 1% peptone solution containing 50 or 100 μg ml^{-1} lactoferricin B, respectively (Venkitanarayanan, *et al.* 1999).

The antimicrobial activity of Lf peptides in foods may individually or collectively be influenced by water activity, pH, food components (lipid, protein and carbohydrate) or cations (Mg2+ and Ca2+) (Bellamy, *et al.* 1993). The objective of this study was to determine the activity of a pepsin digest of bovine Lf against selected foodborne pathogens using a model broth system and a representative food system. In the present study, the lactoferrin hydrolysate (LfH) was studied, due to the likelihood of greater acceptance by industry and government regulatory agencies (Sofos, *et al*. 1998).

III. MATERIALS AND METHODS

A. Bacteria

Escherichia coli O157:H7 ATCC 43895, *Salm. enterica* serovar Stanley ATCC 7308, *Staph. aureus* strains ATCC 13565 and ATCC 10832 and *L. monocytogenes* ATCC 19111 were used. All cultures were streaked for purity on tryptic soy agar (TSA), cultured in PYG broth (1% peptone, 0·025% yeast extract and 1% glucose) and stocks maintained in a 50:50 glycerol : peptone broth at −20^{o}C. Working cultures were obtained by transferring a loop of stock culture to PYG agar, incubating overnight and transferring an isolated colony to 10 ml of PYG broth. Cultures were incubated in PYG broth at 35^{o}C for 16–20 h with shaking (160 rev min^{-1}). Before using in assays, cultures were diluted 1:100 in fresh PYG broth, except for *L. monocytogenes*, which was diluted 1:10.

B. Lactoferrin and lactoferrin hydrolysate

Lactoferrin (Immu Cell Corporation, Portland, ME, USA) was stored at −20°C. An Lf sample was subjected to sodium dodecyl sulfate (SDS)-PAGE analysis (20% polyacrylamide gel) to determine purity. The method of Bellamy, *et al*. (1992) was used to obtain an LfH. In brief, bovine Lf was dissolved in sterile water (milli Q; Millipore, Bedford, MA, USA) to a concentration of 5.0% and the pH adjusted to 3.0 $\pm$ 0.1 using filter-sterilized 1 N HCl. Porcine pepsin (Sigma) was added to achieve a final concentration of 3.0% (weight/weight of substrate). The mixture was incubated at 37°C for 4 h with shaking at 150 rev min^{-1}. Following incubation, the reaction was terminated by incubation in a preheated 80°C water bath for 15 min followed by cooling at 5°C for 15 min. After cooling, the solution was adjusted to pH 7.0 $\pm$ 0.1 using filter-sterilized 1 N NaOH. Insoluble solids were removed by centrifugation at 15,000 × g for 30 min. Additionally, to ensure removal of the heat inactivated pepsin the supernatant was passed through a 30,000 MW cut off filter (Pall Macrosep Omega, Amicon Ultra or equivalent). The resulting supernatant fluid was lyophilized and stored at − 20°C until needed. Hydrolysate samples were separated by loading onto a 20% SDS-PAGE gel to confirm complete hydrolysis. A premade low molecular weight kaleidoscope marker was used to determine the molecular weight of the resultant protein hydrolysate. The Lf and LfH stock solutions (8000 μg ml^{-1}) were prepared in sterile distilled water, filter sterilized (0.45 μm followed by 0.22 μm pore size cellulose acetate) and stored at − 20°C until needed.

C. Assays for antimicrobial activity in peptone yeast extract glucose

The antimicrobial activity of Lf and LfH was determined using a modification of the methods described by Bellamy, *et al*. (1992) and Shin, *et al*. (1998). Stock solutions of Lf and LfH were prepared in PYG broth at a concentration of 16,000 μg ml^{-1}. The resulting mixture was filter sterilized (0.45 μm followed by 0.22 μm pore size cellulose acetate) and stored at − 20°C until needed. Minimum inhibitory concentrations (MIC) were determined using a temperature-controlled 96-well plate reader (MRX; Dynex Tech Inc., Chantilly, VA, USA) with software (Revelation) measuring optical density (O.D.) at 630 nm. Peptone yeast extract glucose was added to all wells and an equivalent volume of Lf or LfH added to the first well and serially diluted (1:2 dilutions). The concentrations of Lf or LfH tested were 16,000, 8000, 4000, 2000, 1000, 500, 250 and 125 μg ml^{-1}. Logarithmic growth phase cell cultures of *L. monocytogenes*, *E. coli* O157:H7, *Staph. aureus* (ATCC 13565 and ATCC 10832) or *Salm.* Stanley were added to corresponding wells to achieve 10^5−10^6 CFU $well^{-1}$. The microtitre plate was incubated at 35°C for 24 h and the $O.D._{630}$ of each well read every half hour following two repeats of shaking for 5 s. The MIC was considered the lowest concentration of the Lf or LfH that showed no increase in O.D. Minimum lethal concentrations (MLC) were determined by transferring 10 μl from all wells to a new 96-well plate containing 100 μl of fresh PYG in each well without Lf or LfH. The microtitre plate was incubated at 35°C for 24 h and the $O.D._{630}$ of each well read every half hour following two repeats of shaking for 5 s.

D. Assays for antimicrobial activity in ultra-high temperature (UHT) milk and pH-neutralized or acidified UHT milk

Ultra-high temperature milk was added to sterile conical centrifuge tubes at varying volumes and Lf, LfH or a combination of Lf or LfH with EDTA added to bring the final volume to 2 ml. Ultra-high temperature milk was acidified to pH 4.0 $\pm$ 0.1 using 1N HCl and neutralized to pH 7.0 $\pm$ 0.1 using 1 N NaOH. Neutralized or acidified milk was added to sterile conical centrifuge tubes at varying volumes and Lf or LfH and EDTA added to bring the final volume to 2 ml. The concentrations of Lf and LfH tested were 8000, 4000, 2000, 1000, 500, 250 and 125 μg ml^{-1} in combination with 10 mg ml^{-1} EDTA. Logarithmic growth phase cells of either *L. monocytogenes* or *E. coli* O157:H7 (to achieve 10^5–10^6 CFU ml^{-1}) were added to appropriate tubes. Samples were incubated in duplicate at 4°C for 7 d and at 35°C for 1 d with shaking at 160 rev min^{-1}. After incubation, 100-μl aliquots were spread plated onto TSA, incubated at 35°C for 18 h and colonies enumerated.

IV. RESULTS AND DISCUSSION

The results of the present study suggest that the peptide hydrolysate of Lf has greater antimicrobial activity than the whole molecule against *E. coli* O157:H7, *Staph. aureus*, *Salm*. Stanley and *L. monocytogenes*. Previous studies have investigated the activity of Lf in UHT milk and tryptic soy broth (TSB) or the activity of LfH hydrolysate in PYG or other bacterial growth media (Payne, *et al*. 1990; Payne, *et al*. 1994; Shin, *et*

al. 1998; Branen and Davidson 2000). In this study, we used UHT milk as a surrogate food to investigate the activity of LfH under varying pH, temperature and in the presence of cations and food components (lipid, carbohydrate and protein). Determining the activity of LfH in a representative food matrix is essential since activity in bacterial growth media probably does not represent activity in a food.

The relative purity of Lf and LfH was determined prior to conducting experiments (Fig. 1). A single major band of approx. 78 kDa was present, suggesting that the preparation was essentially free of other substances that may influence activity of the Lf (Fig. 1a). Digestion of Lf with pepsin yielded a peptide with three primary bands at approximately 9kDa, 5 kDa and 3.5 kDa (Fig. 1b). In this study, similar to previous studies, the pepsin digest was used rather than the isolated peptides (Shin, *et al*. 1998; Branen and Davidson 2000).

Antimicrobial activity of Lf or LfH suspended in PYG broth was demonstrated against *E. coli* O157:H7, *Staph. aureus*, *Salm.* Stanley and *L. monocytogenes.* For each pathogen tested, the LfH was more active against the cells than the whole molecule (Table 1). The slight variability between the results reported here and those of other studies is probably associated with strain variation, method of culturing or variation in the purification methods for Lf and LfH (Ellison and Giehl 1991; Dionysius and Milne 1997; Shin, *et al*. 1998; Branen and Davidson 2000). The PYG broth was evaluated based on previous studies suggesting that other non-divalent cation-limiting media can reduce the effect of or completely eliminate the inhibitory activity of Lf (Dionysius and Milne 1997; Branen and Davidson 2000). Of the four pathogens evaluated, the reduction in MLC was

greatest for *E. coli* O157:H7 and *L. monocytogenes* (Table 1). Therefore, all subsequent studies were conducted using *E. coli* O157:H7 and *L. monocytogenes.*

The addition of EDTA to UHT milk containing Lf or LfH had no effect on the survival of *E. coli* O157:H7 or *L. monocytogenes* (Table 2). Unexpectedly, a > 2·5 log decrease in *E. coli* O157:H7 was noted in samples containing 4000 μg ml^{-1} Lf only, the highest concentration tested. However, in the presence of 10 mg ml^{-1} EDTA and 4000 μg ml^{-1} Lf only a 0.5 log decline occurred in the population of *E. coli* O157:H7. In contrast, Payne, *et al*. (1994) reported an approx. 4.0 log reduction in *E. coli* O157:H7 at a concentration of 1·0 mg ml^{-1} of EDTA; however, the survival of *L. monocytogenes* was not affected at the same concentration of EDTA. Payne, *et al*. (1994) also demonstrated that the combination of Lf and lysozyme was less effective than that of EDTA and lysozyme. This was reported to be due to the potential for iron saturation of the Lf molecule, which is reported to reduce the antimicrobial activity of Lf (Hoek, *et al*. 1996; Dionysius and Milne 1997). Moreover, Lf saturated with other divalent cations has a reduced antimicrobial effect on foodborne pathogens (Payne, *et al*. 1990; Payne, *et al*. 1994). We also conducted studies to determine the efficacy of Lf and LfH against *E. coli* O157:H7 or *L. monocytogenes* in liquid whole eggs; little reduction in the cell population occurred (data not shown). These results are not remarkable since it is likely that the Lf molecule is saturated with iron or other divalent cations in eggs.

Foods (*e.g.* milk) may encounter extremes of temperature during storage, transport and handling. The pH can vary considerably between products, including milk products, e.g. regular compared with buttermilk. In the present study, the activity of Lf and LfH at 4 and 35°C, pH 4.0 $\pm$ 0.1 and 7.0 $\pm$ 0.1 and against *E. coli* O157:H7 (Tables 3

and 4) and *L. monocytogenes* (Tables 5 and 6) was investigated. For *E. coli* O157:H7, the control (no Lf or LfH) decreased only slightly (≈0.5 log) during the 168 h (7 d) incubation period at 4°C in acidified milk (Table 3). The presence of Lf or LfH did not result in further marked inhibition of the growth of *E. coli* O157:H7. At 35°C, regardless of the concentration of Lf or LfH, the population of bacteria increased from 2 to 4 log CFU ml^{-1} in 24 h. In UHT milk adjusted to pH 7.0 $\pm$ 0.1 and held at 4°C the population of bacteria increased slightly, except at LfH concentrations of 4000 and 8000 μg ml^{-1} (Table 4). For samples incubated at 35°C for 24 h there was an approx. 5 log increase in the *E. coli* O157:H7 population in media containing Lf; however, in samples containing 2000, 4000 or 8000 μg ml^{-1} LfH only, a 3 log increase occurred in the bacterial population. The data for *L. monocytogenes* were similar to those for *E. coli* O157:H7 in that Lf and LfH did not greatly influence growth of the pathogen. Indeed, at pH 4.0 $\pm$ 0.1 the LfH suppressed the growth of *L. monocytogenes* but at pH 7.0 $\pm$ 0.1 there was no effect (Tables 5 and 6). At 35°C a several log increase in the population of *E. coli* O157:H7 (Table 3) and *L. monocytogenes* (Table 5) occurred in UHT milk adjusted to pH 4.0 $\pm$ 0.1. Limited growth of these pathogens in nutrient-rich medium at low pH is not striking based on the growth characteristics of each microbe (Anon. 1996).

The results of the present study extend the scope of knowledge on activity of the pepsin hydrolysate of Lf in food systems against foodborne pathogens. Branen and Davidson (2000) reported that LfH was active in PYG but was only marginally effective in the more complex TSB medium. Therefore, the likelihood that LfH would be active in a complex food system was questionable particularly since studies have demonstrated that the whole Lf molecule was not effective in controlling or suppressing the growth of

foodborne pathogens in milk (Payne, *et al*. 1990; Payne, *et al*. 1994). However, our findings demonstrate suppression of *E. coli* O157:H7 in UHT milk containing 2000 μg ml^{-1} LfH (Table 2) and an approx. 2 log decrease in the population of *E. coli* O157:H7 occurred during a 7-d incubation period (Table 3). Previous studies have demonstrated that, although LfH is effective against a range of foodborne pathogens in simple nutrient media, no practical meaningful reduction in the population of a target pathogen occurred in ground beef, chicken skin extract or carrot juice (Venkitanarayanan *et al*. 1999; Chantaysakorn and Richter 2000). However, the results presented here suggest that, under low pH and refrigeration conditions, LfH can limit the growth or reduce the population of pathogenic bacteria contaminating a dairy product. This may also hold true for activity against a range of spoilage bacteria.

Based on our results and those of others, Lf probably has an extremely limited potential as an antimicrobial agent in food. However, as the understanding of the behavior of LfH in complex media and food systems advances, the potential exists for the use of LfH as a food preservative based on the results of this study. The influence of cations on the activity of LfH should continue to be explored, but not to the exclusion of the impact of temperature, pH and osmolarity. Gaps in knowledge also exist in the understanding of the mechanism of action of LfH, the elucidation of which would facilitate the application of LfH as a food preservative.

V. ACKNOWLEDGEMENTS

The authors thank John Rejman and the Immu Cell Corporation for providing Lf. The work described in this paper was funded in part by the New Jersey Agricultural Experiment Station.

VI. TABLES AND ILLUSTRATIONS

Table 1. Antimicrobial activity of Lactoferrin (Lf) and lactoferrin hydrolysate (LfH) in peptone yeast extract glucose broth towards common foodborne pathogens.

Organism	Lf		LfH	
	MIC (μg/ml)	MLC (μg/ml)	MIC (μg/ml)	MLC (μg/ml)
E. coli O157:H7	4000	8000	2000	4000
S. stanley	4000	4000	4000	8000
S. aureus	8000	>8000	4000	8000
L. monocytogenes	2000	4000	1000	1000

[a]MIC, minimal inhibitory concentration; MLC, minimal lethal concentration

Table 2. Antimicrobial activity of lactoferrin (Lf), lactoferrin hydrolysate (LfH) and EDTA towards *Escherichia coli* O157:H7 and *L. monocytogenes* in ultra-high temperature milk at 37°C.

Concentration	Log_{10} CFU/ml			
	E. coli O157:H7		*L. monocytogenes*	
	0 mg/ml EDTA	10 mg/ml EDTA	0 mg/ml EDTA	10 mg/ml EDTA
Lf μg/ml				
0	9.18[a]	9.06	5.97	6.48
250	9.13	9.11	6.39	7.03
500	9.18	9.04	6.79	6.60
1000	8.44	9.17	6.69	6.60
2000	8.11	9.18	6.59	6.34
4000	6.61	8.50	6.97	6.39
LfH μg/ml				
0	8.77	8.77	6.58	6.82
125	8.39	9.08	7.08	6.50
250	8.44	8.91	7.22	6.61
500	8.45	8.70	7.26	7.09
1000	8.48	9.18	7.58	7.08
2000	8.57	9.08	7.62	7.52
4000	8.44	8.90	7.73	7.86

[a] Minimum and maximum S.D. 0.04 and 0.13 respectively for any result

Table 3. Antimicrobial activity of lactoferrin (Lf) and lactoferrin hydrolysate (LfH) against *Escherichia coli* O157:H7 in acidified (pH 4.0 ± 0.1) ultra-high temperature milk at 4°C and 35°C

Temp. (°C)	Time (h)	Log_{10} CFU/ml						
		Concentration of Lf (μg/ml)						
		0	250	500	1000	2000	4000	8000
4	0	3.82[a]	3.82	3.82	3.82	3.82	3.82	3.82
	24	3.34	3.45	3.32	3.57	3.37	3.45	3.73
	72	3.37	3.03	3.44	3.45	3.22	3.26	3.18
	120	3.49	3.18	3.00	3.10	2.82	3.03	3.07
	168	3.00	2.40	3.12	0.00[b]	2.52	2.52	2.92
35	0	4.03	4.03	4.03	4.03	4.03	4.03	4.03
	24	6.29	7.67	6.98	7.78	7.14	8.40	7.75
		Concentration of LfH (μg/ml)						
		0	250	500	1000	2000	4000	8000
4	0	3.82	3.82	3.82	3.82	3.82	3.82	3.82
	24	3.39	3.43	3.35	3.51	3.06	2.08	2.00
	72	3.37	3.28	3.38	3.30	3.00	2.13	2.30
	120	3.50	3.25	3.11	3.19	2.40	2.03	2.20
	168	3.18	2.33	3.08	3.00	2.00	2.00	2.00
35	0	4.03	4.03	4.03	4.03	4.03	4.03	4.03
	24	6.28	6.75	6.01	6.18	6.17	6.41	5.75

[a] Minimum and maximum S.D. 0.01 and 0.16 respectively for any result
[b] No growth occurred, therefore, data point not included in S.D. calculation

Table 4. Antimicrobial activity of lactoferrin (Lf) and lactoferrin hydrolysate (LfH) toward *E. coli* O157:H7 in neutralized (pH 7.0 ± 0.1) ultra-high temperature milk at 4°C and 35°C

Temp. (°C)	Time (h)	Log_{10} CFU/ml						
		Concentration of Lf (μg/ml)						
		0	250	500	1000	2000	4000	8000
4	0	3.82[a]	3.82	3.82	3.82	3.82	3.82	3.82
	24	3.52	4.03	3.54	3.88	3.85	3.68	3.68
	72	3.57	3.93	4.10	3.86	4.10	3.51	3.83
	120	3.89	4.09	4.18	3.94	4.28	3.10	3.81
	168	4.16	3.73	4.39	3.90	3.80	3.60	3.66
35	0	4.03	4.03	4.03	4.03	4.03	4.03	4.03
	24	8.83	9.38	9.38	9.36	9.45	9.41	9.31
		Concentration of LfH (μg/ml)						
		0	250	500	1000	2000	4000	8000
4	0	3.82	3.82	3.82	3.82	3.82	3.82	3.82
	24	3.49	3.94	3.82	3.75	3.85	2.95	2.40
	72	3.60	3.96	3.93	3.90	3.90	3.00	2.56
	120	3.99	3.90	3.95	3.99	3.83	2.94	2.99
	168	4.23	4.18	4.10	4.00	3.90	2.18	2.00
35	0	4.03	4.03	4.03	4.03	4.03	4.03	4.03
	24	8.84	9.00	9.89	8.82	7.41	7.19	7.30

[a] Minimum and maximum S.D. 0.02 and 0.19 respectively for any result

Table 5. Antimicrobial activity of lactoferrin (Lf) and lactoferrin hydrolysate (LfH) against *L. monocytogenes* in acidified (pH 4.0 ± 0.1) ultra-high temperature milk at 4°C and 35°C

Temp. (°C)	Time (h)	Log_{10} CFU/ml						
		Concentration of Lf (μg/ml)						
		0	125	250	500	1000	2000	4000
4	0	3.53[a]	3.53	3.53	3.53	3.53	3.53	3.53
	24	3.74	3.57	3.35	3.59	3.81	3.71	3.61
	72	3.08	3.72	3.60	3.63	3.56	4.05	3.94
	120	3.43	3.48	3.56	3.12	3.41	3.53	3.53
	168	3.55	3.41	3.56	3.28	3.47	3.44	3.26
35	0	3.55	3.55	3.55	3.55	3.55	3.55	3.55
	24	8.21	8.21	6.29	7.77	7.20	7.73	6.61
		Concentration of LfH (μg/ml)						
		0	125	250	500	1000	2000	4000
4	0	3.53	3.53	3.53	3.53	3.53	3.53	3.53
	24	3.35	3.50	3.20	3.00	2.70	2.26	2.40
	72	3.21	3.24	3.41	2.85	2.45	2.50	2.57
	120	3.50	3.41	3.10	2.80	2.25	2.00	2.24
	168	3.20	3.40	3.00	2.98	2.10	2.00	2.00
35	0	3.55	3.55	3.55	3.55	3.55	3.55	3.55
	24	7.51	7.00	6.00	5.95	5.25	5.50	5.00

[a] Minimum and maximum S.D. 0.03 and 0.20 respectively for any result

Table 6. Antimicrobial activity of lactoferrin (Lf) and lactoferrin hydrolysate (LfH) toward *L. monocytogenes* in neutralized (pH 7.0 $\pm$ 0.1) ultra-high temperature milk at 4°C and 35°C

Temp. (°C)	Time (h)	Log_{10} CFU/ml						
		Concentration of Lf (μg/ml)						
		0	125	250	500	1000	2000	4000
4	0	3.53[a]	3.53	3.53	3.53	3.53	3.53	3.53
	24	3.92	3.99	3.82	3.85	3.86	3.97	3.50
	72	5.40	5.98	5.57	5.44	5.62	5.50	5.55
	120	6.33	6.66	6.70	6.76	6.80	6.75	6.79
	168	7.51	7.52	7.52	7.56	7.56	7.56	7.58
35	0	3.55	3.55	3.55	3.55	3.55	3.55	3.55
	24	9.67	9.55	9.60	9.52	9.45	9.45	9.61
		Concentration of LfH (μg/ml)						
		0	125	250	500	1000	2000	4000
4	0	3.53	3.53	3.53	3.53	3.53	3.53	3.53
	24	3.75	3.20	3.48	3.51	3.50	3.20	3.50
	72	5.50	5.50	5.28	4.90	5.24	5.00	5.20
	120	5.90	6.12	6.24	6.35	6.50	5.86	6.00
	168	7.00	7.00	6.80	7.50	6.80	6.50	6.20
35	0	3.55	3.55	3.55	3.55	3.55	3.55	3.55
	24	8.60	8.25	8.00	7.80	6.90	7.00	7.50

[a] Minimum and maximum S.D. 0.02 and 0.23 respectively for any result

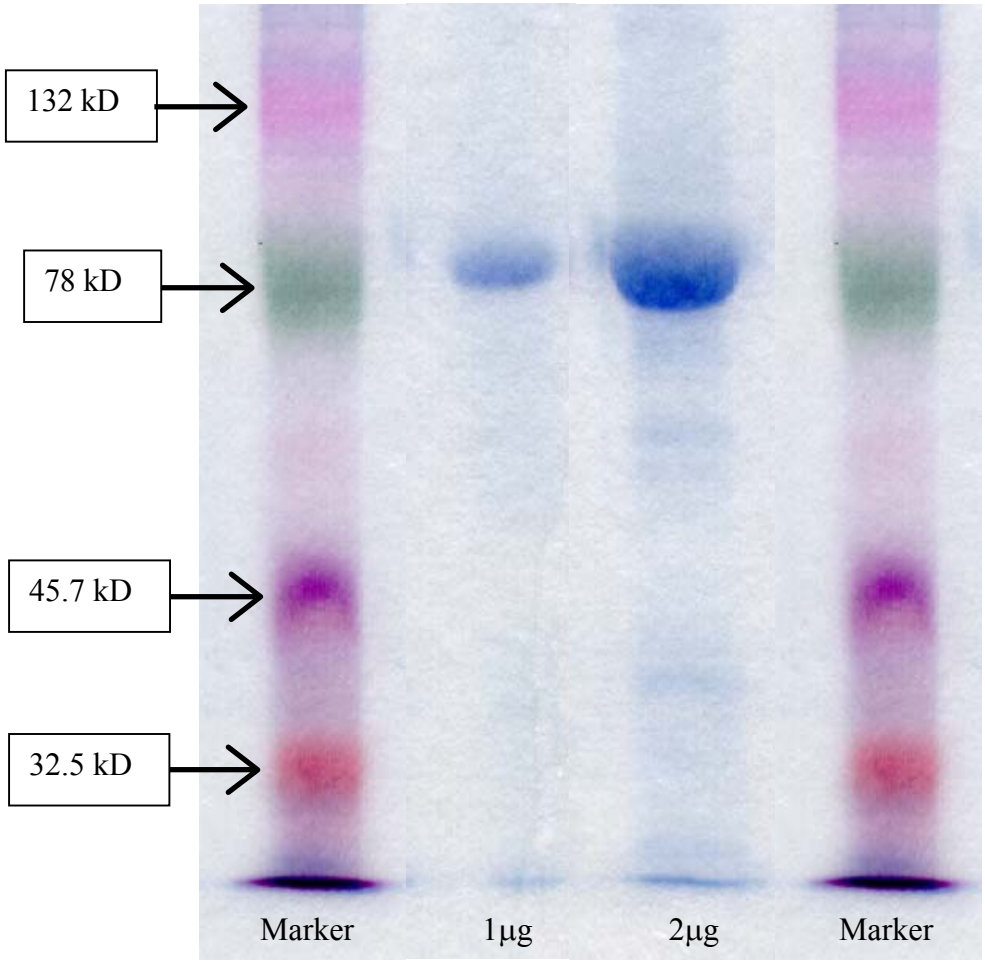

Figure 1a: Purity of bovine lactoferrin (Lf) on a 20% sodium dodecyl sulphate gel before hydrolysis with pepsin. Molecular weights of kaledioscope markers are indicated on the left side of the gel.

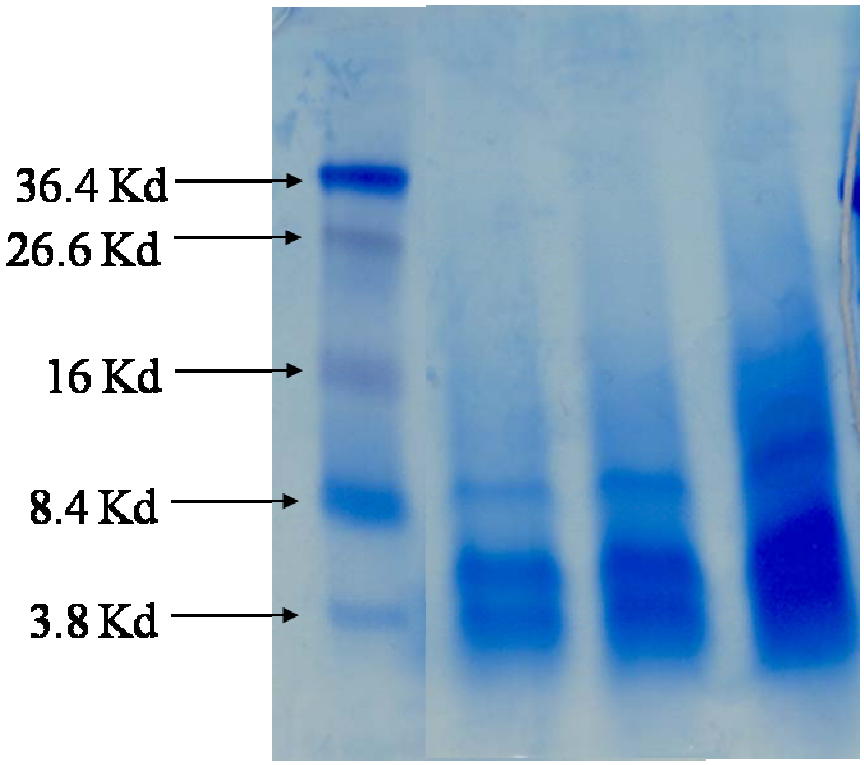

Figure 1b: Purity of bovine lactoferrin hydrolysate (LfH) on a 20% sodium dodecyl sulfate gel following hydrolysis with pepsin. Molecular weights of kaledioscope markers are indicated on the left side of the gel.

VII. BIBLIOGRAPHY

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CHAPTER 5

The following data were published in: Letters of Applied Microbiology, Volume 44, Pages 255 – 261 – 2007.

Murdock, C. A., Jennifer Cleveland, Karl Matthews and Michael Chikindas. *The Synergistic Effect of Nisin and Lactoferrin and the Inhibition of Listeria monocytogenes and Escherichia coli O157:H7*.

Objectives:

A. The antimicrobial activities of Lf and nisin act synergistically towards *E. coli* O157:H7 and *Listeria monocytogenes*.

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The synergistic effect of nisin and lactoferrin on the inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7

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Running head: Antimicrobial synergy of nisin and lactoferrin

Key words: Lactoferrin, nisin, antibacterial peptides, foodborne pathogens

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I. ABSTRACT

Aims: The goal of this study was to determine whether nisin and lactoferrin would act synergistically to inhibit the growth of *Listeria monocytogenes* and *Escherichia coli* O157:H7.

Methods and Results: Lactoferrin and nisin separately or in combination were suspended in peptone yeast glucose broth and following inoculation with *L. monocytogenes* or *E. coli* O157:H7 growth inhibition of each pathogen was determined. At 1000 μg ml $^{-1}$ lactoferrin *L. monocytogenes* was effectively inhibited. However, *E. coli* O157:H7 initially was inhibited and then grew to cell density similar to the control. A combination of 500 μg ml $^{-1}$ lactoferrin and 250 IU ml $^{-1}$ nisin effectively inhibited the growth of *E. coli* O157:H7, whereas, 250 μg ml $^{-1}$ lactoferrin and 10 IU ml $^{-1}$ nisin were inhibitory to *L. monocytogenes.*

Conclusions: The results suggest that lactoferrin and nisin act synergistically to inhibit the growth of *L. monocytogenes* and *E. coli* O157:H7.

Significance and Impact of the Study: Natural preservatives that are active against Gram-positive and Gram-negative pathogens are desirable to the food industry and consumers. This study demonstrates that lactoferrin and nisin work synergistically reducing the levels required independently inhibiting growth of two major foodborne pathogens. Previous reported results indicated a low level of antimicrobial activity; however, this work was not performed in low divalent cation concentration media. It has been suggested that non-divalent cation limiting medium such as Trypticase Soy Broth (TSB), can reduce or completely eliminate the inhibitory activity. Further knowledge of

these interactions can increase the understanding of the antimicrobial activity of lactoferrin. This should make the use of these compounds by industry more attractive.

II. INTRODUCTION

Foodborne illnesses are a significant problem and a major public health concern (Swaminathan, *et al*. 2005). The control of microbial pathogens in foods is a significant concern and numerous methods have been employed to control or prevent the growth of pathogenic microorganisms in food, including the use of synthetic and natural antimicrobial agents (Payne, *et al*. 1994). There is a growing consumer demand for "natural" methods of controlling microorganisms that may be associated with food. Nisin and lactoferrin are examples of compounds that due to their origin can be used in food products as "natural" biopreservatives.

Bacteriocins are ribosomally-synthesized peptides that mostly kill closely-related bacteria (Klaenhammer 1993). Nisin is produced by *Lactococcus lactis* spp. *lactis* and is the only bacteriocin with FDA-approved GRAS status for use in products such as pasteurized processed cheese and salad dressings (Anonymous 2000, Cleveland, *et al.* 2001). Nisin's antimicrobial mechanism has been extensively studied and well documented (Winkowski, *et al.* 1996). Nisin first binds to the cell membrane through ionic interactions of the C terminus and then forms pores in the membrane by the penetration of the hydrophobic N terminus (Breukink, *et al*. 1998). This results in disruption of the proton motive force and leakage of cellular materials (Okereke and Montville 1992, Bruno, *et al.* 1994, Breukink, *et al.* 1998). The effectiveness of nisin against Gram-negative cells is generally low due to the inability of nisin to penetrate the

cell wall, which prevents access to the inner membrane. However, when used in combination with chelators such as EDTA, nisin is also effective against Gram-negative bacteria (Stevens, *et al*. 1991, Branen and Davidson. 2004).

Lactoferrin, a 78 kDa anionic iron-binding antimicrobial glycoprotein, is also a natural biopreservative that is found in many mammalian secretions, including milk, tears, saliva, and serum (Odell, *et al* 1996, Ye, *et al.* 2000). It is also thought to be one of the most powerful antimicrobial agents in milk (Bellamy, *et al*. 1993, Chantaysakorn and Richter 2000). The antibacterial mechanism of bovine lactoferrin has been studied but has yet to be clearly defined. Lactoferrin was originally thought to inhibit cell growth by limiting the amount of iron in the environment. Further studies showed that it may act as a more general chelator, releasing lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria (Ellison and Giehl 1991). These peptides can interact with the binding sites on the LPS, causing distortion in the outer membrane integrity and ultimately forming pores or "blebs" (Chapple, *et al.* 2004). Lactoferrin has been also shown to exert antimicrobial activity against other pathogenic microorganisms such as *E. coli* and *L. monocytogenes* (Payne, *et al.* 1990, Ellison and Giehl 1991, Payne, *et al.* 1994, Dionysius and Milne 1997). Treatment with pepsin yields antibacterial peptides with varying lengths (typical 25 residues), lack the iron binding sites and correspond to a region of the surface helix near the N-terminus (Hoek, *et al.* 1997, Hwang, *et al.* 1998, Vorland, *et al.* 1999a, Vorland, *et al.* 1999b). This peptide has been shown to depolarize the *E. coli* membrane (Ellison and Giehl 1991, Ulvatne, *et al.* 2001), although the mechanism of action has not been clearly defined.

Synergy of lactoferrin and lactoferricin (the peptide) with antibiotics (Sanchez and Watts 1999, Vorland, *et al.* 1999a), antifungal drugs (Kuipers, *et al.* 1999) and lysozyme (Ellison, 1994) has been demonstrated. Synergy with other compounds such as chelators (EDTA) or physical conditions (high pressure) offers a type of a "multiple hurdle approach" which combines different synergistically-acting preservation methods to inhibit the growth of microorganisms (Branen and Davidson 2000, Masschalck, *et al.* 2001, Murdock and Matthews 2002, Branen and Davidson 2004). The lack of activity by nisin against Gram-negative organisms is due to the nisin's inability to penetrate the cell wall. However, in the presence of lactoferrin that has the ability to permeabilize the Gram-negative cell wall, nisin could then have access to the inner membrane thus providing a novel system for control of Gram-negative pathogens, such as *E. coli* O157:H7 (Ellison 1994, Branen and Davidson 2004). These previous reported results indicated a low level of antimicrobial activity; however, this work was not performed in low divalent cation concentration media such as Peptone Yeast Extract Glucose (PYG) Broth, *e.g.* media in which the addition, directly or indirectly, of divalent cations such as Fe^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+} is limited. It has been suggested that other non-divalent cation limiting medium such as Trypticase Soy Broth (TSB), can reduce or completely eliminate the inhibitory activity (Bellamy *et al.*, 1992, Jones, *et al.*, 1994). Additionally, since the binding site for the divalent cations is localized to the area of the lactoferrin molecule that is associated with the antimicrobial activity it would be reasonable to suggest that this could impair the molecules ability to permeabilize the Gram-negative cell wall (Yamauchi, *et al.* 1993, Dionysius and Milne, 1997, Branen and Davidson, 2000).

The objective of this study was to determine the synergistic activity of bovine lactoferrin and nisin against *E. coli* O157:H7 and *L. monocytogenes* in a low or divalent cation limiting model broth system. Using this approach we can determine if the cell wall permeabilizing activity of lactoferrin can allow nisin to be exposed to the inner membrane and thus cause cell damage and ultimately cell death.

III. MATHERIALS AND METHODS

A. Bacteria

Escherichia coli O157:H7 ATCC 43895 and *Listeria monocytogenes* Scott A ATCC 19111 were used. All cultures were streaked for purity on Tryptic Soy Agar, cultured in Peptone Yeast Extract Glucose (PYG) Broth (1% Peptone, 0.025% yeast extract and 1% glucose) and stocks were maintained in a 50:50 Glycerol : Peptone Broth at –20^{o}C. Working cultures were prepared by transferring a loop of stock culture to fresh PYG agar, incubating at 37^{o}C for 16 h and transferring an isolated colony to 10 ml of PYG broth. Cultures were incubated in PYG broth at 35^{o}C for 16 to 20 h with shaking at 160 rpm. Cultures were diluted 1:100 in fresh PYG broth before use in the 96 well plate assay.

B. Antimicrobials

Bovine lactoferrin (approximately 15% saturated, range 12-20%) was obtained from Immu Cell Corporation (Portland, ME, USA) and stored at –20^{o}C until used for assays. A lactoferrin sample was subjected to sodium dodecyl sulphate (SDS)-PAGE

analysis using a Kaleidoscope™ polypeptide standard (Bio-Rad, CA, USA) to determine the purity and molecular weight of the protein (data not shown). Bovine lactoferrin was added to the sterile PYG medium and the solution was filter sterilized by passing through a 0.45 μm cellulose acetate filter and then through a 0.22 μm cellulose acetate filter. A stock solution was stored at -20^{o}C for up to 90 days. Nisin preparation (Novasin 2.5%, gift from Rhodia Inc. Cranbury, NJ.) was autoclaved in a 0.02 mol HCl and 0.75% NaCl solution (nisin diluent), adjusted with 1mol HCl to pH 3.0 $\pm$ 0.1 (stock concentration of $1x10^5$ IU $m1^{-1}$).

C. Microtitre Plate Assay

A temperature controlled Dynex 96 well plate reader MRX with Revelation software was used to monitor optical density. Lactoferrin (100 μl) diluted in PYG was added to wells to achieve a final concentration of 0- 5000 μg $m1^{-1}$. Nisin (100 μl) was added to a final concentration of 0-1000 IU $m1^{-1}$. Wells used to evaluate the effects of the two antimicrobials combined contained a 50:50 mixture of each of the two prepared stock solutions. *L. monocytogenes* or *E. coli* O157:H7 was diluted in fresh PYG to achieve 10^5-10^6 CFU $m1^{-1}$ and 100 μl added to wells. The microtiter plate was incubated at 37°C for 26 h with OD630 read every half hour immediately following a 5 s shake cycle. All assays were performed in triplicate and repeated twice.

D. Fractional Inhibitory Concentration (FIC)

Synergy was determined using the Fractional Inhibitory Concentration (FIC) index. This index is calculated by utilizing the minimum inhibitor concentrations (MICs) of the antimicrobial compounds alone and the respective MICs when the compounds are combined. The formula is $([A]/MIC_A) + ([B]/MIC_B)$, where MIC_A and MIC_B are the MICs of the compounds alone and [A] and [B] are the MICs of the compounds when used together. This calculation is interpreted as synergy (<0.5), partial synergy (>0.5 but <0.75), additive effect (>0.75 but <1.0), indifference (>1.0 but <4.0) and antagonism (>4.0) (Berenbaum, 1981).

IV. RESULTS AND DISCUSSION

The results of this study indicate that nisin used in combination with lactoferrin can be an effective approach to control *E. coli* O157:H7 and *L. monocytogenes*. This study as well as previous studies indicated nisin had a limited effect on the growth of *E. coli* O157:H7 whereas *L. monocytogenes* was inhibited in its presence (Cleveland *et al.*, 2001). In most previous studies a typical medium (*e.g.* TSB) was used, which contains high levels of divalent cations. However, in this study we used PYG medium, which contains a very low level of divalent cations. In the present study *L. monocytogenes* was inhibited by 25 IU $m1^{-1}$ nisin in PYG. This is more than 10 times lower than the inhibitory concentration in Tryptic Soy Broth, a typical medium with high levels of divalent cations, *e.g.* Fe^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} and some salts (Bellamy *et al.*, 1992, Ellison 1994, Jones, *et al.*, 1994, Branen and Davidson 2004). The growth conditions in PYG medium may have made the organism more sensitive to nisin. The PYG medium was

used based on previous studies suggesting that other non-divalent cation-limiting media, *e.g.* Tryptic Soy Broth (TSB), can reduce or completely eliminate the inhibitory activity of lactoferrin (Bellamy *et al.*, 1992, Jones, *et al.*, 1994). Furthermore, divalent cations may bind to the area of the lactoferrin molecule associated with the antimicrobial activity, thus impairing the lactoferrin molecule's ability to permeabilize the Gram-negative cell wall. Ultimately, the permeabilization of the Gram-negative cell wall would decrease the possibility for nisin to be exposed to the inner membrane and cause cell damage or death. (Yamauchi, *et al.* 1993, Dionysius and Milne, 1997, Branen and Davidson, 2000).

Listeria monocytogenes was inhibited by 1000 μg $m1^{-1}$ lactoferrin, however, up to 5000 μg $m1^{-1}$ lactoferrin was not inhibitory to *E. coli* O157:H7 under the assay conditions (Fig. 1 and 2). Previous studies by several laboratories have indicated that concentrations from 4000 to 6000 μg $m1^{-1}$ are inhibitory to *E. coli* O157:H7 under similar assay conditions (Ellison and Giehl, 1991; Dionysius and Milne, 1997; Shin, *et al*. 1998; Branen and Davidson 2000, Murdock and Matthews, 2002). Furthermore, additional studies have indicated that both *L. monocytogenes* and *E. coli* are inhibited by lactoferrin at concentrations that may vary slightly depending on assay conditions but are similar to those observed in these studies (Branen and Davidson, 2000). Finally, studies performed in other laboratories indicate that both *L. monocytogenes* and *E. coli* have varying degrees of inhibition dependent on the media and other study parameters (*e.g*. pH, temperature, etc.) (Murdock and Matthews, 2001, Branen and Davidson, 2004).

In these studies synergistic inhibition of *L. monocytogenes* was observed when as little as 10 IU $m1^{-1}$ nisin was used in combination with 250 μg $m1^{-1}$ lactoferrin (Fig. 4) and 250 IU $m1^{-1}$ nisin and 500 μg $m1^{-1}$ lactoferrin inhibited growth of *E. coli* O157:H7

(Fig 3). The FICs for each compound was calculated to be 0.35 (Table 1). However, as indicated previously, neither antimicrobial was able to inhibit the growth of either bacterium at these concentrations alone. These data support the hypothesis that lactoferrin and nisin can synergistically function to inhibit the growth of pathogenic bacteria. Additional studies performed indicate that a combination of 1000 IU ml^{-1} nisin and 5000 μg 1^{-1} lactoferrin did not inhibit *Salmonella* Stanley (data not shown), suggesting that the synergistic effect demonstrated against *L. monocytogenes* and *E. coli* O157:H7 is not simply the result of chelation of ions by lactoferrin and suggests the variance in activity may be due to differences in the outer membrane or LPS structure (Branen and Davidson, 2004).

Previous studies in Tryptic Soy Broth and food systems have indicated a slight synergistic effect on *L. monocytogenes* (but not *E. coli*) when lactoferrin and nisin are combined. The presence of divalent cations in these systems may influence the activity of the lactoferrin against Gram-negative bacteria such as *E. coli* O157:H7 (Branen and Davidson, 2004). In this current study, with a medium that has low concentration of divalent cations, a noticeable difference was observed. These data support the hypothesis that divalent cations in the medium may alter the effectiveness of lactoferrin, thus altering its ability to have any significant antimicrobial effect.

The results of the present study suggest that nisin used in combination with lactoferrin is an effective approach to control the growth of *E. coli* O157:H7 and *L. monocytogenes*. Previously, chelators such as EDTA have been used to permeabilize the outer membrane of Gram-negative cells to nisin. Based on results of the present study the iron binding function of lactoferrin was not the sole factor involved in nisin's ability to

act on *E. coli* O157:H7 in the presence of lactoferrin since *S.* Stanley was not similarly affected by the compounds when used in combination. Future research will be directed toward elucidating why *E. coli*, but not *Salmonella* Stanley, responds to a combination of nisin and lactoferrin. *L. monocytogenes* was inhibited by much lower concentrations of each antimicrobial compared to *E. coli* O157:H7; whether the mechanism of action is the same as that for *E. coli* O157:H7 can be determined.

V. ACKNOWLEDGEMENTS

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VI. TABLES AND ILLUSTRATIONS

Table 1. MIC and FIC calculations for Lactoferrin and Nisin.

Organism	Antimicrobial	MIC[1] Alone	MIC[2] Combined	FIC Index[3]
L. monocytogenes	Lf (μg/ml)	1000	250	0.35
	Novasin (IU/ml)	100	10	
E. coli O157:H7	Lf (μg/ml)	5000	500	0.35
	Novasin (IU/ml)	1000	250	

[1] Minimum Inhibitory Concentration of each antimicrobial
[2] Minimum Inhibitory Concentration when the two antimicrobials are combined
[3] Fractional Inhibitory Concentration Index (<0.5 indicates synergy)

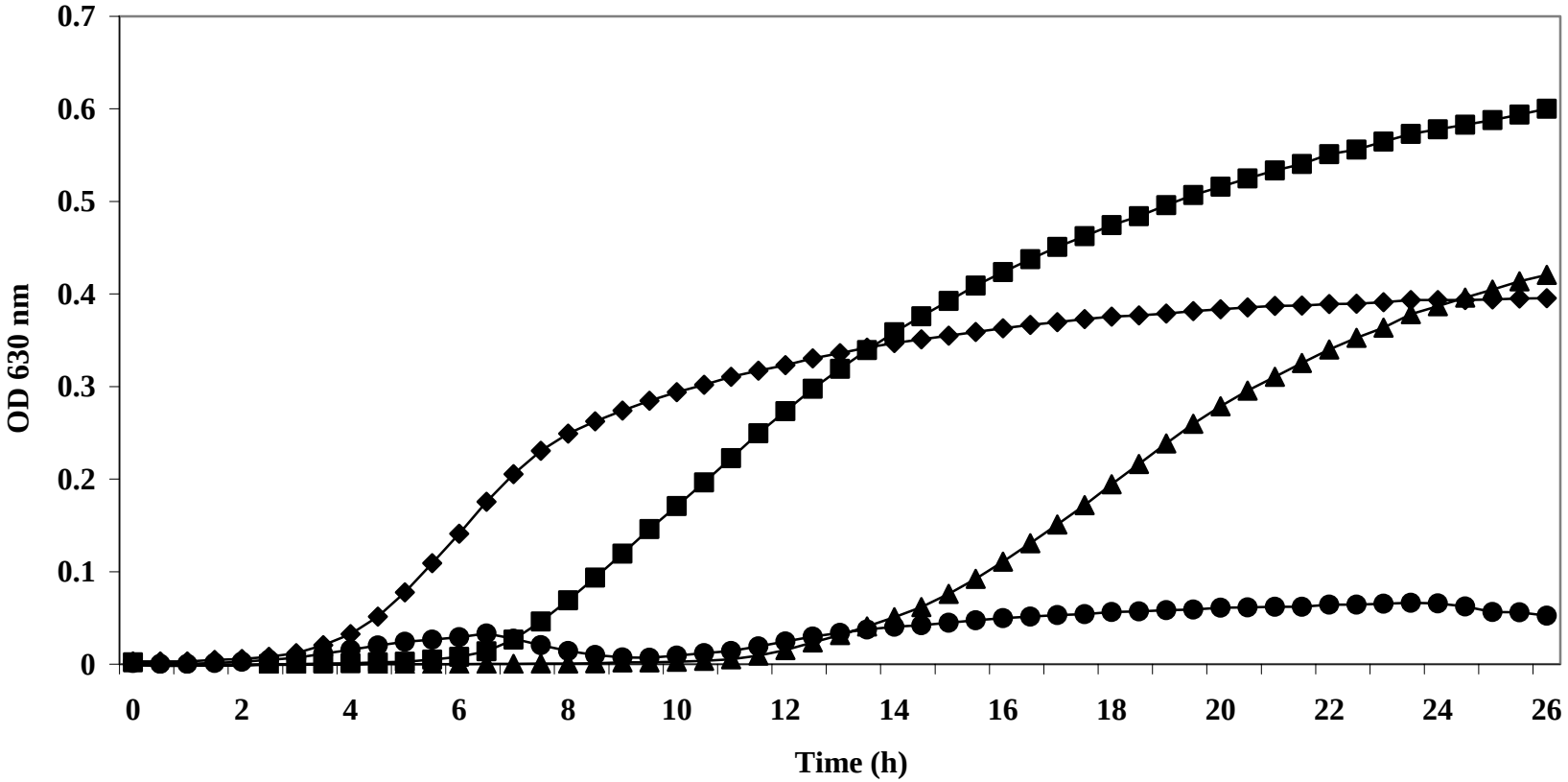

Figure 1. Lactoferrin inhibits growth of *L. monocytogenes* Scott A in PYG broth at 37^{o}C in a concentration-dependant manner. Control, (♦); 250 μg ml^{-1} Lactoferrin, (■) ; 500 μg ml^{-1} Lactoferrin, (▲); and 1000 μg ml^{-1} Lactoferrin, (●).

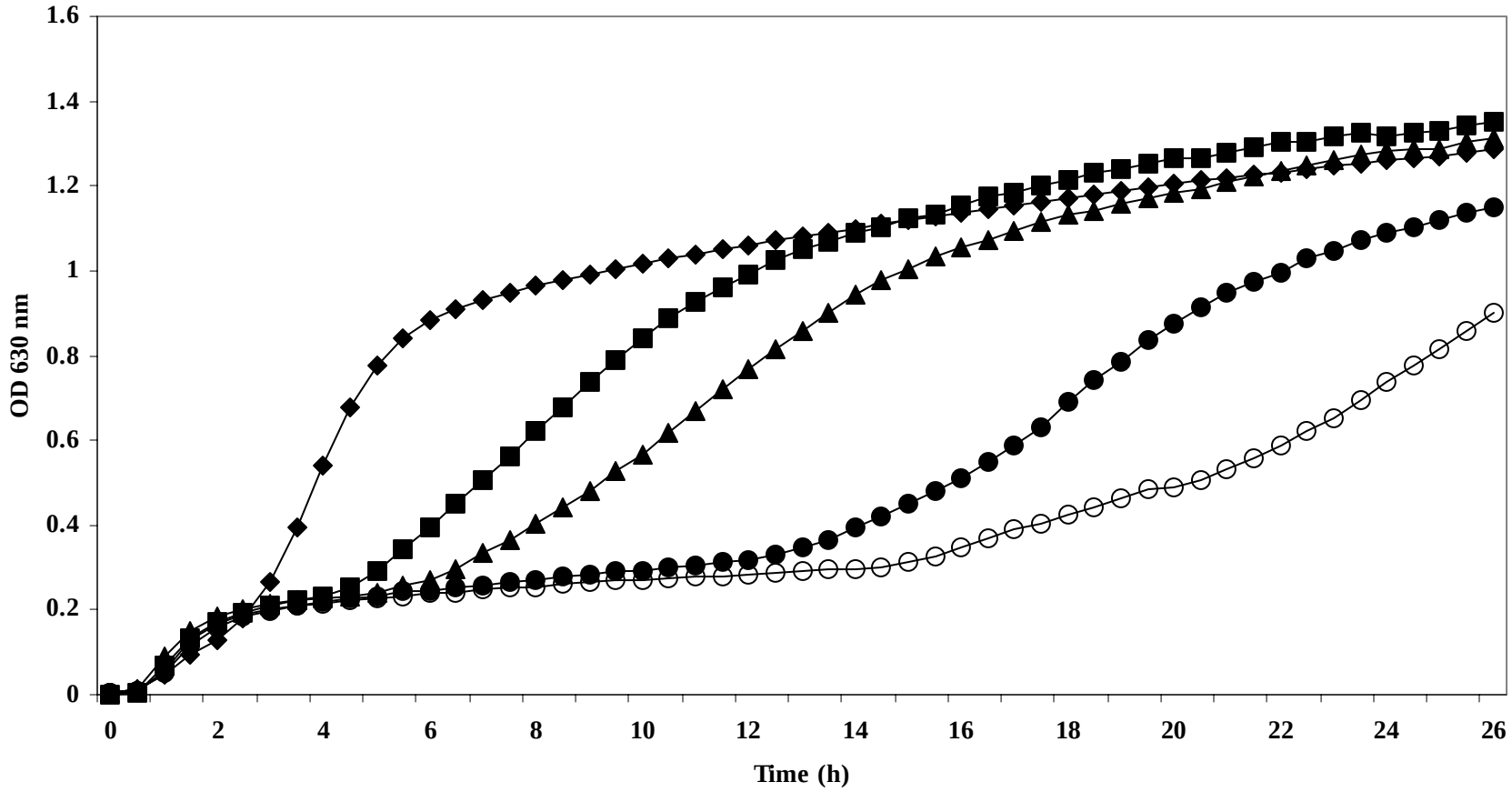

Figure 2. Lactoferrin inhibits growth of *E. coli* O157:H7 in PYG broth at 37°C in a concentration-dependant manner. Control, (♦); 250 μg ml^{-1} Lactoferrin, (■) ; 500 μg ml^{-1} Lactoferrin, (▲); 1000 μg ml^{-1} Lactoferrin, (●); and 2500 μg ml^{-1} Lactoferrin, (○).

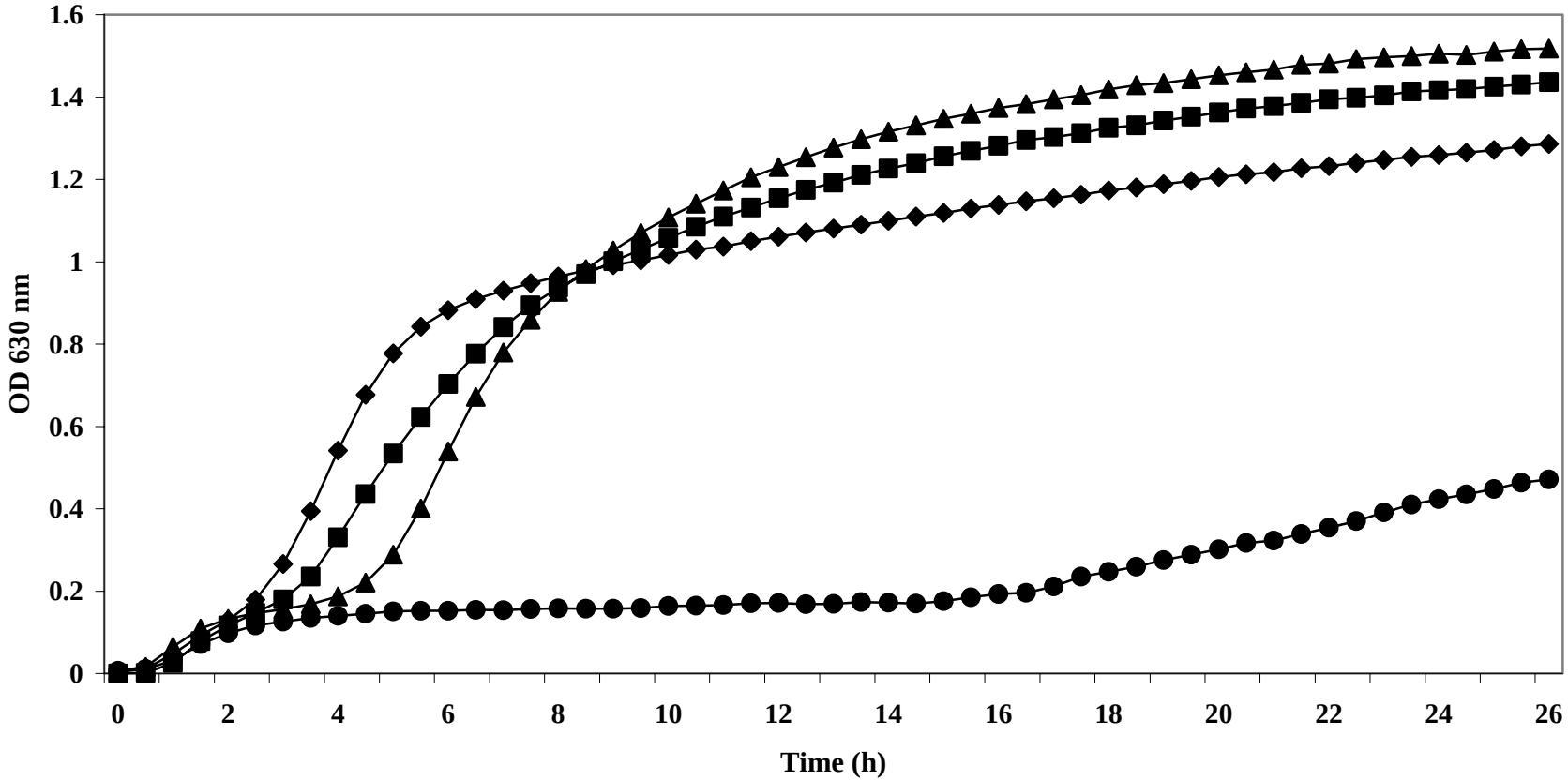

Figure 3. Nisin and lactoferrin act synergistically against *E. coli* O157:H7 in PYG broth at 37°C. Control, (♦); 500 μg ml^{-1} Lactoferrin, (■) ; 250 IU ml^{-1} Nisin, (▲); 500 μg ml^{-1} Lactoferrin + 250 IU ml^{-1} Nisin, (●).

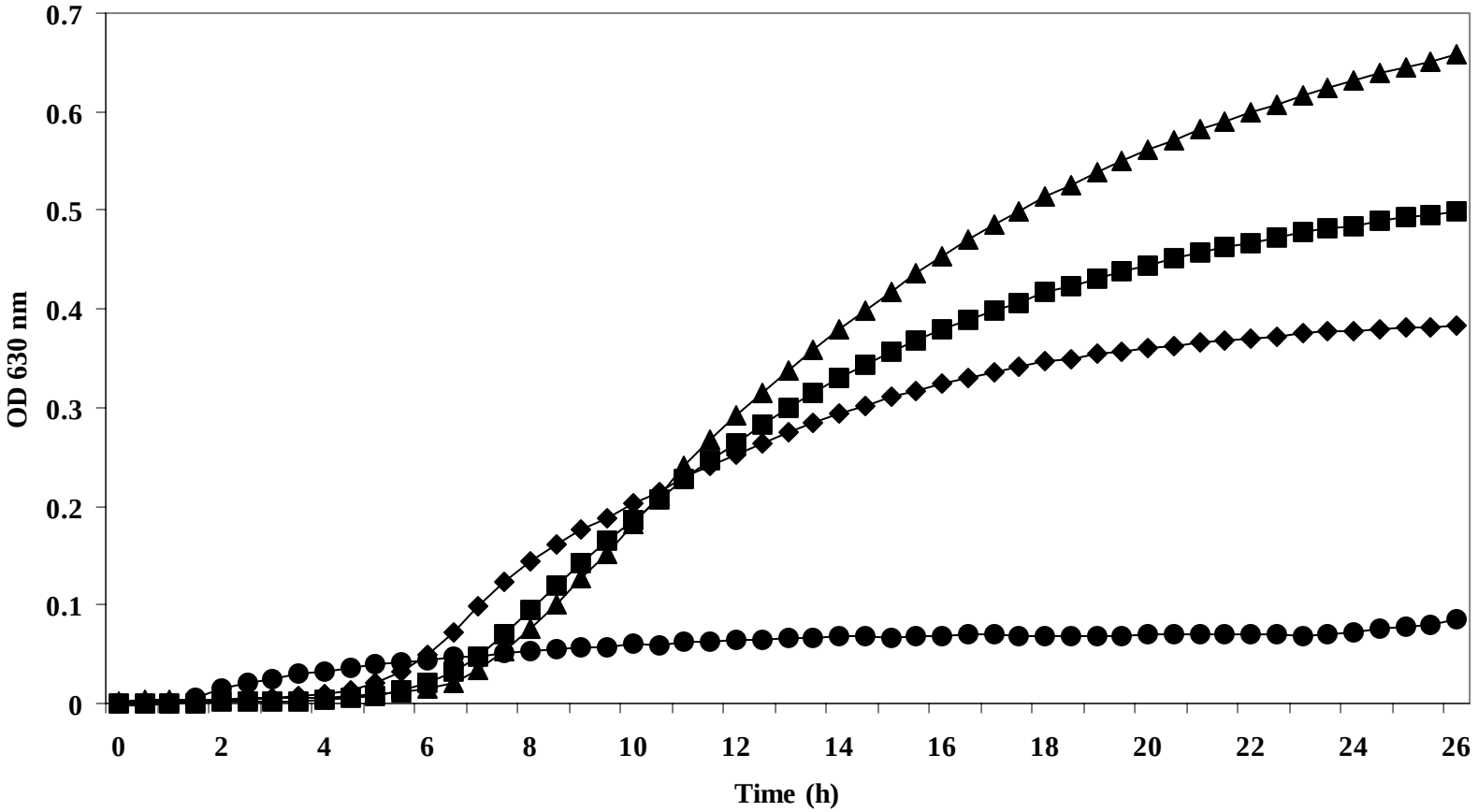

Figure 4. Nisin and lactoferrin act synergistically against *L. monocytogenes* Scott A in PYG broth at 37^{o}C. Control, (♦); 250 μg ml^{-1} Lactoferrin, (■) ; 10 IU ml^{-1} Nisin, (▲); 250 μg ml^{-1} Lactoferrin + 10 IU ml^{-1} Nisin, (●).

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CHAPTER 6

The following data will be submitted to the Journal, Probiotics and Antimicrobial Proteins for peer reviewed publication.

Murdock, C. A. and Karl R. Matthews. *The Pepsin Hydrolysate of bovine Lactoferrin (LfH) interacts with the Cytoplasmic Membrane and causes a Collapse of the Membrane Potential in E. coli O157:H7*

Objectives:

A. Determine whether LfH causes a loss of intracellular ATP coupled with an increase in extracellular ATP in *E. coli* O157:H7.

B. Determine whether LfH causes a rapid potassium efflux (K^+) in *E. coli* O157:H7.

C. Determine whether LfH causes a complete dissipation of the membrane potential ($\Delta\Psi$) in *E. coli* O157:H7.

D. Determine whether LfH causes a significant loss in viability of *E. coli* O157:H7 as determined by nucleic acid stain.

I. ABSTRACT

Aims: In the present study the ability bovine LfH to disrupt the cytoplasmic membrane of *Escherichia coli* O157:H7 was investigated.

Methods and Results: Lactoferrin (Lf) and LfH antimicrobial activities were compared against *E. coli* O157:H7 and *E. coli* O157:H7 spheroplasts. The effect of LfH on the cytoplasmic membrane of *E. coli* O157:H7 cells was determined by evaluating potassium efflux (K^+), changes in ATP concentrations, and membrane potential ($\Delta\Psi$). LfH produced a rapid efflux of potassium ions, a significant decrease in intracellular levels of ATP coupled with a substantial increase in extracellular ATP levels and a complete dissipation of the $\Delta\Psi$. Viability assays demonstrated a significant increase in non-viable cells in the presence of LfH versus the controls.

Conclusions: The results suggest that LfH causes a collapse of the membrane integrity by pore formation in the inner membrane leading to the death of the cell.

Significance and Impact of the Study: Results of this study suggest that the mechanism of action of LfH on *E. coli* O157:H7 involves an interference with the inner membrane integrity leading to the death of the cell.

II. INTRODUCTION

Foodborne illnesses associated with *E. coli* O157:H7 present a major public health concern in the US and throughout the world (Hall, 1997, Mead, *et al*., 1999, Rangle, *et al.*, 2005). According to the Center for Disease Control, *E. coli* O157:H7 accounts for approximately 73,000 cases of foodborne illnesses and nearly 60 deaths per year. Additionally, this same report indicated that over the ten-year study these numbers are increasing. Finally, data also indicate that over 50% of these cases were transmitted by foods (Mead, *et al*., 1999, Rangle, *et al.*, 2005).

E. coli O157:H7 was first recognized as a pathogen in 1982 following an outbreak associated with ground beef contamination. Since this onset *E. coli* O157:H7 has been linked to contamination of ground beef, produce (*e.g.* lettuce, apples, melons, sprouts) and dairy products (*e.g.* milk, butter, cheese, ice cream) (Rangle, *et al.*, 2005). *E. coli* O157:H7 has several characteristics that distinguish it from other strains of *E. coli*, most importantly are the production of Shiga toxins and a low infectious dose for susceptible populations (Neill, 1989, Tilden, *et al* 1996, Teunis, *et al.*, 2004). Infections caused by *E. coli* O157:H7 can lead to a debilitating or deadly disease know as hemolytic uremic syndrome (HUS) characterized by hemolytic anemia, thrombocytopenia and renal injury (including failure in severe cases) (Riley, *et al.* 1983, Neill, 1989, Su, *et al.* 1995).

The control of microbial pathogens in foods is a significant concern and numerous methods have been employed to prevent the growth of pathogenic microorganisms in food, including the use of synthetic and natural antimicrobial agents such as Lf, lysozyme, EDTA, bacteriocins, monolaurin, lactic acid, hydrogen peroxide (Ellison and Giehl, 1991, Holzapfel, *et al.*, 1994, Payne *et al*., 1994, Venkitanarayanan, *et al.,* 1999,

Branen and Davidson 2000, Cleveland, *et al.*, 2001, Pellegrini, A. 2003). Additionally, these studies and others have demonstrated the potential for synergistic effects by combining some of these antimicrobial compounds. There is a substantial amount of data published on many of these antimicrobial compounds and for some the mechanism of action has been detailed (*e.g.* nisin) (Winkowski, *et al*., 1996; Montville and Chen, 1998, Sablon, *et al.*, 2000, Chatterjee, *et al.*, 2005, Breukink, 2006, Drider, *et al.*, 2006, Willey, *et al.*, 2007). However, while Lf has received considerable attention over the past several years the mechanism of action has not been completely elucidated.

Lf, a member of the transferrin protein family, is a cationic iron-binding glycoprotein that is found in many exocrine secretions, including milk, tears, saliva and serum, and is potentially an extremely powerful and useful antimicrobial agent from milk (Bellamy *et al*., 1992, Bellamy *et al*., 1993; Chantaysakorn and Richter 2000). Lf is a single-chained molecule with a molecular weight of approximately 78 kDa, composed of two lobes with four domains that facilitate the reversible binding of two iron (Fe^{+}) ions (or other divalent cations) between the inner faces of the inter domain clefts (Odell, *et al*., 1996; Farnaud, 2003). Lf exerts antimicrobial activity against a variety of Gram-negative and Gram-positive bacteria (Payne, *et al.*, 1990, Ellison and Giehl, 1991, Dionysius, *et al.*, 1993, Payne, *et al.*, 1994, Shin, *et al.*, 1998, Yu, *et al.*, 1999, Chantaysakorn and Richter, 2000, Naidu, 2000, Branen and Davidson, 2003, Al-Nabulsi and Holley, 2005, Al-Nabulsi and Holley, 2006). However, when Lf binds divalent cations (*e.g.* Fe^{+}, Ca^{+}, Mg^{+}) it loses much if not all of its antimicrobial activity, therefore, its activity would most likely be reduced in foods (Bellamy, *et al*., 1993, Shimazaki, 1998, Naidu, 2000). Studies investigating the antimicrobial activity of Lf in food systems do not demonstrate

the same activity. Payne *et al*. (1994) and Murdock and Matthews (2002) demonstrated that Lf alone or in combination with EDTA or lysozyme in UHT milk had little practical effect against *E. coli* O157:H7, *Pseudomonas fluorescens*, *Salm. typhimurium* or *L. monocytogenes*.

Treatment with pepsin yields antibacterial peptides composed of two chains with varying lengths, typical 25 residues, corresponding to a region of the surface helix near the N-terminus (Hoek, *et al.* 1997, Hwang, *et al.* 1998), that are termed lactoferricin (Lfcin) when purified. Additionally, the peptides lose the ability to bind divalent cations, which have been demonstrated to deplete the antimicrobial activity of the parent molecule, Lf (Bellamy, *et al*., 1993, Shimazaki, 1998, Naidu, 2000).

Lfcin were reported to exhibit antibacterial activity against *E. coli*, *P. fluorescens*, *L. monocytogenes* and *Bacillus cereus* in peptone yeast extract glucose (PYG) medium at concentrations 4–80 times lower than the native molecule (Dionysius, 1997). Similarly, Shin *et al*. (1998) demonstrated the enhanced activity of LfH and Lfcin against *E. coli* O157:H7 in peptone broth. Others have reported a reduction of approximately 0·7 and 2 log CFU *E. coli* O157:H7 in 1% peptone solution containing 50 or 100 μg/ml Lfcin B, respectively (Venkitanarayanan, *et al*. 1999). Additionally, LfH was found to inhibit the growth of *E. coli* in carrot juice, ground beef and UHT milk (Venkitanarayanan, *et al*. 1999, Chantaysakorn and Richter, 2000, Murdock and Matthews, 2002).

The mechanism of action of Lfcin peptide, which has the greatest antimicrobial activity, has not been clearly elucidated. Lfcin was originally thought to inhibit cell growth by limiting the amount of iron in the environment; however, further studies demonstrated that it might act as a more general chelator, releasing lipopolysaccharide

(LPS) from the outer membrane of Gram-negative bacteria (Ellison and Giehl, 1991). These peptides can also interact with the binding sites on the LPS, causing distortion in the outer membrane integrity and ultimately forming pores or "blebs" (Chapple, *et al.* 2004). Other research indicated that Lf and LfH might act by damaging the outer cell wall and destabilizing the cytoplasmic membrane (Yamauchi, *et al*. 1993; Sallmann, *et al*. 1999; Vorland, *et al*. 1999).

Data from our laboratory indicates LfH has similar activity against *E. coli* O157:H7 spheroplasts as it does against the cell wall containing *E. coli*. The objective of this study was to determine the activity of bovine LfH against the cytoplasmic membrane of *E. coli* O157:H7 as the potential mechanism of cell death. In the present study, LfH was studied, due to the likelihood of greater acceptance by industry and government regulatory agencies due to the GRAS status of lactoferrin (Sofos, *et al*. 1998).

III. MATHERIALS AND METHODS

A. Bacteria

E. coli O157:H7 ATCC 43895 was streaked for purity on tryptic soy agar (TSA, Becton Dickinson, USA), cultured in peptone yeast extract glucose (PYG) broth (1% peptone, 0·025% yeast extract and 1% glucose) and stocks were maintained in a 50:50 glycerol : peptone broth at -20^{o}C. Working cultures were obtained by transferring a loop of frozen stock culture to TSA agar, incubating overnight at 35^{o}C $\pm$ 2^{o}C and transferring an isolated colony to PYG broth of sufficient volume for the experiment. Cultures were

incubated in PYG broth at 35°C ± 2°C under agitation for 8-12 hours for log phase cells and 16-24 hours for stationary phase cells. Before using in susceptibility assays, cultures were diluted 1:100 in fresh PYG broth and in all other experiments cultures were diluted as outlined in the methods sections below.

B. Preparation of Lactoferrin and lactoferrin hydrolysate

Bovine Lf obtained from Immu Cell Corporation (Portland, ME, USA) was stored at -20°C until needed for individual studies. Lactoferrin was re-suspended at the appropriate concentrations needed for each individual study in sterile water (Fisher Scientific Water LC-MS Ultra Pure Chromosolv® or equivalent), PYG broth or the appropriate buffer as indicated and the solution was filter sterilized by passing through 0.45 μm and 0.22 μm pore size cellulose acetate filters (Pall Corporation Acrodisc ®, Nalgene or equivalent) in sequence. All experiments were performed using freshly prepared lactoferrin. Purity and molecular weight was determined by sodium dodecyl sulphate (SDS)-PAGE analysis using the manufacturers protocol (7.5% tris polyacrylamide gel, BIORAD Precast Ready Gel and broad range molecular weight markers) (data not shown) (Murdock and Matthews, 2002).

LfH was prepared using the methods described by Bellamy, *et al.* (1991) and the resulting lyophilized powder was stored in sealed containers at – 20°C until re-suspended for use in individual experiments. LfH was re-suspended and processed as described above for Lf. Briefly, to prepare LfH, Lf was dissolved in sterile water (Fisher Scientific Water LC-MS Ultra Pure Chromosolv® or equivalent) at a concentration of 5%. The pH

was adjusted to 3.0 ± 0.1 using sterile 1 N HCl. Porcine pepsin (Sigma P6887) was added to the solution to a final concentration of 3.0% weight to weight of substrate to pepsin. The mixture was incubated for 4 hours at 37°C under agitation. Following incubation the reaction was terminated (e.g. inactivate the pepsin) by placing the solution in a preheated 80°C water bath for 15 minutes measuring the temperature internally and under agitation to ensure even heat distribution to terminate the reaction completely. The mixture was subsequently cooled in an ice bath to room temperature. After cooling, the pH of the solution was adjusted to 7.0 ± 0.1 using sterile 1 N NaOH. The solids were removed by centrifugation at approximately 15,000 × g for 30 min. Additionally, to ensure removal of the heat inactivated pepsin the supernatant was passed through a 30,000 MW cut off filter (Pall Macrosep Omega, Amicon Ultra or equivalent). The resulting supernatant was lyophilized and stored at -20°C until re-suspended for each individual study. Purity and molecular weight was determined by Tris-Tricine SDS-PAGE analysis using the manufacturers protocol (16.5% tris-tricine polyacrylamide gel, BIORAD Precast Ready Gel and kaleidoscope polypeptide molecular weight markers) (data not shown) (Murdock and Matthews, 2002).

C. Lf and LfH Sensitivity Assay

The antimicrobial activity of Lf and LfH was determined using a modification of the methods described by Bellamy *et al*. (1992) and Shin *et al*. (1998). Stock solutions of Lf and LfH were prepared as described above. Minimum inhibitory concentrations (MIC) were determined using a temperature-controlled Dynex 96-well plate reader (MRX; Dynex Tech Inc., Chantilly, VA, USA) with software (Revelation) measuring

optical density (OD) at 630 nm. PYG was added to all wells and an equivalent volume of Lf or LfH re-suspended in PYG added to the first well and serially diluted (1:2 dilutions). The concentrations of Lf or LfH tested were 8000, 4000, 2000, 1000, 500, 250, 125 and 62.5 µg/ml. Logarithmic growth phase cell cultures of *E. coli* O157:H7 and the *E. coli* O157:H7 spheroplasts were added to corresponding wells to achieve 10^4–10^5 CFU well/ml. The microtiter plate was incubated at 35°C ± 2°C for 18 - 24 h and the OD_{630} of each well read every 15 minutes following two repeats of shaking for 5 s. The MIC was considered the lowest concentration of the Lf or LfH that showed no increase in OD. All triplicate samples were tested in duplicate.

D. Development of spheroplast cells from E. coli O157:H7

The methods used were modified from *Bacterial Membranes* by Kaback (1975), Birdsell and Cota-Robles (1967) and Gumpert, *et al.* (1996). The process utilizes Penicillin G (Sigma P-8721), Ethylenediaminetetraacetic acid (EDTA) (Sigma ED2P) and lysozyme (Sigma L-6876) to partially/completely remove the outer membrane from the cells to generate a spheroplast. Photo micrographs were obtained throughout the process to evaluate and document the effectiveness of the procedure. From a frozen culture, a plate of TSA was streak inoculated and incubated for 18-24 hours at 35°C ± 2°C. Following incubation, a Gram stain was performed to check for purity and have a baseline photo for the cellular morphology changes. Nutrient broth was inoculated with a colony picked from the TSA plate and incubated for 18-24 h at 35°C ± 2°C under agitation. Subsequently 100 µl of this culture was transferred to 10 ml of Penn Assay Medium (Difco Antibiotic Medium 3 #0243-17) and incubated for 18-24 h at 35°C ± 2°C

under agitation. Following the incubation period, a photograph (100 × magnification under oil immersion) was taken of a representative sample from the culture. To initiate transformation of the wild type cells to spheroplasts, Penicillin G (1000 IU/ml), lysozyme (200 mg/ml) and EDTA (0.05% wt/v) were added to the medium and the culture re-incubated for an additional 18-24 h at 35°C ± 2°C under agitation. At 2 and 24 h of incubation, samples were removed from each broth culture and photographs taken at 100 × magnification under oil immersion. The sample was then separated into two parts, and 1 ml was transferred to fresh Penn Assay and incubated for 18-24 h at 35°C ± 2°C under agitation to examine for reversion to wild type cells. The remaining portion of the sample was treated a second time with filter sterilized Penicillin G (1000 IU/ml), lysozyme (200 mg/ml) and EDTA (0.05% wt/v) and incubated for an additional 18-24 h at 35°C ± 2°C under agitation. At 2 and 24 hours of incubation, samples were removed and photographs taken at 100 × magnification under oil immersion. The sample was then transferred to fresh Penn Assay medium and re-incubated for an additional 18-24 h at 35°C ± 2°C under agitation to examine for revision to wild type cells. Photographs were taken at 100 × magnification under oil immersion.

To determine the effect of Lf and LfH on the *E. coli* O157:H7 spheroplasts, antimicrobial assays/MIC studies were performed as described above.

E. Potassium efflux (K+) assay

The determination of K^+ leakage from *E. coli* O157:H7 was measured with a potassium ion electrode model number 6250 (Jenco Electronics, LTD., San Diego, CA) and a Jenco Microprocessor pH/Mv/Ion/Temp Meter model number 6219 (Jenco Electronics, LTD., San Diego, CA). To evaluate the effect of LfH against *E. coli* O157:H7 the ion potential response generated using the potassium ion electrode was converted to a percentage based on a baseline (untreated cells) and a maximum efflux (permeabilized cells) (Orlov, *et al.* 2002, Ohmizo, *et al.,* 2004).

E. coli O157:H7 cells were grown to an OD_{600} of approximately 1.0 in 50 ml of sterile PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 min at 5°C. The cells were washed twice with cold 10mM Tris Acetate, 100mM NaCl, pH 7.4 $\pm$ 0.1 buffer and re-suspended in the same buffer to an OD_{600} of approximately 30 (approximately 100 × concentration) and retained on ice until used for each experiment. All cultures were used within 30 min. The concentrated cells were diluted 1:10 in fresh 10mM Tris Acetate 100mM NaCl, pH 7.4 $\pm$ 0.1 buffer prior to use in each experiment. The potassium efflux was measured over time for untreated cells, permeabilized and treated (exposed to 4000 and 8000 μg/ml LfH) cells. In each experiment, the efflux was monitored and recorded approximately every 30 seconds, excluding the total efflux from permeabilized cells, which was recorded as an initial reading and monitored for any increase over the same time period. Prior to use in each experiment, the probe was calibrated with standard solutions containing 1 mM (Fluka 60142, 1M in H_2O), 0.1 mM and 0.01 mM KCl, and 5M NaCl ionic strength adjuster buffer (Jenco K00IS01 or equivalent).

F. ATP assay

The levels of total, intra- and extra-cellular ATP were determined using bioluminescent quantification of ATP. This procedure uses the enzyme luciferase to oxidize luciferin to adenyl-luciferin in the presence of limiting ATP (McEntire, *et al.,* 2004, Bonnett, *et al.*, 2006, Sigma Product Technical Bulletin, ref JWM 09/04) as the amount of ATP in the sample is proportional to the amount of light emitted and detected by a spectrophotometer (*e.g.* luminometer). Adenosine 5'-Triphosphate (ATP) Bioluminescent Assay Kits (Sigma FL-AA) were used in conjunction with a luminometer (Luminoskan TL Plus luminometer, Labsystems Oy, Helsinki, Finland) to evaluate samples for the presence of extracellular and total ATP presence. Intracellular ATP was determined by calculating the difference in the total and extracellular ATP concentrations.

A standard calibration curve of the moles ATP per assay versus the relative light intensity was generated for each experiment using the ATP standard provided in the Sigma kit (FL-AAS) at a 1:10 dilution in the ATP Assay Mix Dilution Buffer (FL-AAB). To generate the standard curve, the ATP standard was then serially diluted to obtain 10^{0} to 10^{-8} dilution series and equal amounts of this dilution series were added with ATP Assay Mix (FL-AAM, enzyme/substrate preparation containing luciferase, luciferin, MgSO4, DTT, EDTA, BSA and tricine buffer salt). Immediately following mixing, the samples were placed in the luminometer and measurements taken. All samples were tested in duplicate and all assays were repeated in triplicate to obtain results for each dilution of the standard.

E. coli O157:H7 cells were grown to mid log phase in PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 min at 5°C. The pellet was re-suspended and washed twice in equal volumes of 50mM HEPES (Fisher Scientific BP-299, 1M HEPES). Cells were re-suspended in one half the original HEPES buffer volume and retained on ice for all studies. For energization studies, cells were re-suspended in one half of their original volume in 50 μM HEPES buffer containing 0.2% glucose and allowed to remain at room temperature for 20 min prior to use for time dependent assays. All cultures were used within 30 min.

To determine background levels of ATP and potential interference within the system samples were prepared with 5000 μg/ml and 10,000 μg/ml LfH in HEPES buffer (*e.g.* antimicrobial buffer mix). Antimicrobial buffer mix was diluted 1:10 in fresh buffer and combined with an equal portion of ATP Assay Mix (enzyme/substrate preparation) in a round cuvette. Measurements were taken immediately. All duplicate samples were tested in triplicate. To determine extracellular levels of ATP, 100 μl of the cell suspension was diluted 1:10 in fresh buffer and combined with an equal portion of ATP Assay Mix in a round cuvette. Measurements were taken immediately. All samples were tested in duplicate and all assays were repeated in triplicate. To determine total levels of ATP (intracellular plus extracellular ATP), 100 μl of the cell suspension was diluted 1:10 in fresh buffer and centrifuged at 10,000 × g for 30 s. The resulting pellet was mixed with 10 μl dimethylsulfoxide (DMSO) to permeabilize the cells. Following 5 min of permeabilization at room temperature, 990 μl fresh buffer was added to the pellet (final volume 1000 μl). Equal portions of the permeabilized cell suspension and ATP Assay Mix were combined in round cuvettes. Measurements were taken immediately. All

samples were tested in duplicate and all assays were repeated in triplicate. Time dependent changes in intracellular and extracellular ATP levels were determined as described above following exposure of the cell suspension to 5000 μg/ml and 10,000 μg/ml LfH.

All ATP data were converted to dry weight of cells according to Lee (1993) and our laboratory protocol (Montville and Chikindas). *E. coli* O157:H7 was incubated for approximately 8 h in 20 ml PYG under agitation and centrifuged at approximately 7,000 × *g* for 10 min. The resulting pellet was removed and washed twice in 50 mM HEPES. The pellet was re-suspended in 2 ml of sterile saline and mixed well. Six aluminum weigh dishes were pre-weighed using an analytical balance (Denver Instruments Company, model TR-64). A volume of 0.5 ml of cell suspension was placed into each of four aluminum dishes and 0.5 ml sterile saline was placed into the remaining two aluminum dishes. The dishes were placed into drying oven at approximately 105^{o}C (VWR Model 1305U) and allowed to dry overnight. After drying, the aluminum dishes were each reweighed immediately following removal from the drying oven. The average weight difference of the saline containing aluminum dishes was subtracted from the weight of each individual aluminum dish containing cells. The dry weight of cells was then converted to cell dry weight (CDW) and used for normalization of all ATP values.

G. Transmembrane potential assay

Relative determination of the transmembrane potential (ΔΨ) in LfH treated *E. coli* O157:H7 were determined using the fluorescent probe 3,3' –dipropyltyiadicarbocyanine iodide (Di-S-C3-(5)) as described by Herranz, *et al.* (2001) and Bruno, *et al* (1992) with

modifications (Schuldiner, 1975, Bruno, *et al.*, 1992, Herranz, *et al.*, 2001). A Spex Industries Fluorolog spectrofluorometer model F1T11 with excitation and emission wavelengths of 643 nm and 666nm respectively with a 10nm slit width and a 700 second assay duration with reading every 0.1 second was used for all assays.

E. coli O157:H7 cells were grown to mid log phase in 50 ml PYG broth and collected by centrifugation at approximately 15,000 × *g* for 5 min at 5°C. The pellet was re-suspended and washed twice in equal volumes of 50mM Potassium HEPES (K-HEPES) buffer (Fisher Scientific BP-299, 1M HEPES) pH 7.0 $\pm$ 0.1 with a final resuspension in 1/100 of the original K-HEPES buffer volume in buffer containing 100 mM KPO_4 (Sigma Chemical Co.), 20% glucose (Sigma Chemical Co.), 5 mM HEPES and 1mM K-EDTA (Sigma Chemical Co) and retained on ice prior to all studies. All cultures were used within 30 min.

Assays were conducted as described above using the process outlined below. A volume of 1980 µl 50 mM K-HEPES-1 mM EDTA and 20 µl concentrated cells in the same buffer was added to a cuvette and placed in the spectrofluorometer. The fluorescence measurements were initiated followed by the addition of 10 µl Di-S-C3-(5) (stock 2mM, final concentration 10 µM in methanol with 1mM K-EDTA) and 10 µl of the cell concentrate in buffer with quick mixing with the pipette. After the stabilization of the fluorescence, 5 µl nigericin (stock 5mM, final concentration 12.5 µM in 95% ethanol with 1 mM K-EDTA) was added with quick mixing using the pipette. Following this, either buffer with 1 mM K-EDTA or LfH in buffer with 1 mM K-EDTA (5000 µg/ml and 10,000 µg/ml) was added to the cuvette. In all assays, 10 µl valinomycin

(stock 2 mM, final concentration 10 μM in 95% ethanol with 1 mM K-EDTA) was added to deplete any residual ΔΨ.

H. Cell viability assay

The viability of *E. coli* O157:H7 cells was determined following treatment with MIC and 2× MIC levels of LfH according to the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Kit # L13153, Invitrogen Molecular Probes, Eugene, Oregon) product protocol and were visualized using an Olympus BH2-RFCA fluorescence microscope fitted with a Pixera camera (Invitrogen Molecular Probes product insert MP 07007, Li, *et al.*, 2002). The LIVE/DEAD® BacLight™ Bacterial Viability assay is based on membrane integrity and as such would demonstrate damage to the membrane caused by LfH. The LIVE/DEAD® BacLight™ Bacterial Viability assay utilizes a mixture of two dyes, SYTO®-9 a membrane-permeable green-fluorescent nucleic acid stain and propidium iodide a membrane-impermeable red-fluorescent nucleic acid stain to differentiate between viable and non-viable cells. The dyes differ in their spectral characteristics and ability to penetrate intact membranes of viable cells. SYTO®-9 labels all cells as it can penetrate both intact and damaged membranes and under fluorescence microscopy the cells will appear green. Propidium iodide cannot penetrate intact membranes and, therefore, will only stain cells with damaged membranes. When the two stains are present in a cell, the propidium iodide stain will quench the fluorescence of the SYTO®-9 and the cells will appear red under fluorescence microscopy. In cases where the cell membrane is only slightly damaged (non-lethal damage), the propidium iodide is

not able to penetrate the membrane (due to size of the molecule) and thus the cell will appear viable (green).

E. coli O157:H7 cells were grown to mid log phase in 50 ml PYG broth and collected by centrifugation at approximately 15,000 x *g* for 5 min at 5°C. The pellet was re-suspended and washed twice in equal volumes of sterile water (Fisher Scientific Water LC-MS Ultra Pure Chromosolv®) and then re-suspended in one half the original volume of sterile water and retained on ice prior to all studies. All cultures were used within 2 h.

To determine viable cells within a control (negative control), equal portions of the 2× stock solution of SYTO®-9/propidium iodide and the negative control culture were mixed in a microfuge tube and incubated at room temperature in the dark for 15 min. Following incubation, 5 µl of the mixture was placed on three separate microscope slides and covered with a cover slip. Each sample was then observed using a fluorescent microscope; and five random fields (ensuring only to select fields with similar appearing cell quantities on a given slide) were counted from each slide. All assays were repeated.

To determine non-viable cells within a control (positive control), the final sterile water resuspension of the cells, following washes, was replaced with filter sterilized 70% isopropyl alcohol (Fisher Scientific, 2-Propanol HPLC grade) and allowed to incubate at room temperature for 1 h. Following the isopropyl alcohol incubation equal portions of the 2× stock solution of SYTO®-9/propidium iodide and the positive control culture were mixed in a microfuge tube and incubated at room temperature in the dark for 15 min. Samples were processed as described previously.

To determine the effect of LfH on *E. coli* O157:H7 cells the cells were mixed with MIC and 2× MIC levels of LfH and incubated at room temperature for 1 h.

Following incubation equal portions of the 2× stock solution of SYTO®-9/propidium iodide and the treated culture were mixed in a microfuge tube and incubated at room temperature in the dark for 15 min. Samples were then observed using a fluorescent microscope. Five random fields (ensuring only to select fields with similar appearing cell quantities on a given slide) were counted from each of three slides. All assays were repeated.

IV. RESULTS AND DISCUSSION

Generation of *E. coli* O157:H7 spheroplasts and comparison of the antimicrobial activity of Lf and LfH on *E. coli* O157:H7 cells and spheroplasts

The typical cell envelope of Gram-negative bacteria, such as *E. coli* O157:H7, is composed of four layers, the cytoplasmic membrane (inner membrane), the cytoplasm, a rigid peptidoglycan layer and the outer membrane or cell wall. The outer membrane consists of proteins, phospholipids, lipopolysaccharides (LPS) as well as other structures (*e.g.* porins) that transverse the outer membrane (Osborn, *et al.*, 1972, Matsuyama, *et al.,* 1995). Two sites of action for many antimicrobial compounds are the cytoplasmic membrane and the outer membrane (or cell wall). Previous research on Lf has proposed several hypotheses and two of the main hypotheses surrounding the activity of Lf have focused on interactions with the outer membrane, primarily the LPS and porin channels. Research suggested that the antibacterial activity might be due to a destabilization or disruption of the outer membrane due to release of LPS. Moreover, that membrane porin binding may be involved by interference with the transfer of materials in and out of the

cell (Ellison, *et al.*, 1988, Naidu, *et al.,* 1993, Yamauchi, *et al.*, 1993 Erdei, *et al.*, 1994, Sallmann, *et al.,* 1999, Vorland, *et al.*, 1999, Vorland, *et al.*, 1999a, Chapple, *et al.*, 2004, Farnaud, *et al.,* 2004). In testing the first hypothesis, Yamauchi (1993) demonstrated as much as a 58% release of LPS in the presence of Lf (compared to less than 5% for controls) and approximately a 45% release of LPS in the presence of Lfcin. The associated viability studies demonstrated approximately a 1 to 2 log reduction for the whole molecule versus a 4 to 6 log reduction for the peptide. Additionally, other studies have demonstrated similar results concerning the peptide causing a similar release of LPS while having a significantly greater affect on viability (Ellison, *et al.,* 1988, Vorland, *et al.*, 1999, Farnaud, *et al.*, 2004). Similarly, other studies demonstrated that lactoferrin and the peptide both bound to porins and caused a reduction in channel conductance (*e.g.* porin channel interference) (Nadiu, *et al.*, 1993, Erdei, *et al.*, 1994, Sallmann, *et al.*, 1999). These same studies also demonstrated only slight reductions in viability (less than 2 log reduction).

While these two mechanisms appear to demonstrate the antibacterial, activity of Lf neither demonstrates a complete picture of the mechanism of action of Lf. To further elucidate the mechanism of action, studies must be conducted in which those components are removed. The use of cell wall-less forms of bacteria, both L-form and spheroplasts, has been used to study the effects of antimicrobials (Schved, *et al.*, 1994, Freestone, *et al.*, 1998, Stock, *et al.*, 2003) and to demonstrate differences in activity of bacteriocins.

Potential alternate methods involve using other agents, such as chelators (*e.g.* EDTA) to aid the antimicrobial by making the outer membrane more permeable, thus exposing the inner membrane to the antimicrobial (Ellison, *et al.*, 1990, Branen and

Davidson, 2000, Branen and Davidson, 2003, Al-Nabulsi and Holley, 2006, Al-Nabulsi and Holley, 2007). However, the latter option does not reduce or eliminate the potential for outer membrane interaction (*e.g.* LPS removal) nor does it reduce or eliminate the potential for porin channel interference and thus does not adequately allow for the elimination of the outer membrane as a potential or required part of the mechanism of action. In these studies the use of chelators, such as EDTA, provided potential enhancement of the activity of Lf, depending on the concentration of EDTA used. In most experiments, EDTA was used at concentrations greater than 5mM and enhancement of the activity was observed, however at levels below 2mM no significant effect was observed. Alternative approaches involve using multiple antimicrobials to perform similar actions, increasing outer membrane permeability; involving synergistic actions of the compounds (Ellison, 1991, Branen, 2000, Murdock, *et al.* 2007), however, do not eliminate the potential for interaction with the outer membrane.

To reduce or eliminate the potential for interaction of LfH with the outer membrane, via LPS or porin channels, *E. coli* O157:H7 spheroplasts were used (Osborn, *et al*. 1972, Kaback, *et al.*, 1975, Schved, *et al*., 1994, and Gumpert, *et al.*, 1996). Microscopy was used to monitor morphological changes in cells during experiments to generate spheroplasts and photomicroscopy used to document the changes. The experiment results in the partial removal of the outer cell wall and thus the cell looses its rigid structure (Onoda, *et al.,* 1987). Prior to initiating any antimicrobial assays, cultures were examined microscopically for expected morphology. Throughout the spheroplast formation, procedure samples were periodically taken and examined under a microscope. Initially, following incubation of the stock culture, a gram stain was performed to ensure

purity and have a baseline photo for the cellular morphology changes (Figure 1A) and subsequently a second photograph was taken after the culturing in Penn Assay Medium (Figure 1B). Following the transformation period, a third photograph was taken (Figure 1C) and subsequently a fourth following an additional 24 hours (Figure 1D) was taken to determine if the spheroplasts had reverted to cell wall containing *E. coli* O157:H7 cells or remained a stable spheroplast for the time required for the antimicrobial assay.

Once a stable spheroplast culture was formed, antimicrobial assays were performed to determine differences in the activity of Lf or LfH against the spheroplasts. A decrease in antimicrobial activity should be demonstrated in spheroplasts if the outer membrane LPS or porin channels are required as part of the mechanism by which Lf/LfH exert antimicrobial activity.

The assay was similar to the MIC/MLC assay performed previously and utilized intact cells (control) and spheroplast cells. The results demonstrate similar growth patterns for intact control cells and spheroplast cells un treated or treated at MIC levels of Lf and LfH (refer to Figures 2 and 3).

Induced potassium efflux (K^+) from LfH treated *E. coli* O157:H7 cells

Extracellular potassium (K^+) concentrations of *E. coli* O157:H7 cells were determined prior to and after exposure to MIC levels of LfH. Untreated control samples were monitored over time to determine the K^+ efflux and relative amount of efflux due to normal cell culture growth and death. Data indicated that there was approximately a 3 to 5% efflux of K^+ in the culture, most likely associated with normal cell death. The addition of MIC and 2× MIC concentrations of LfH resulted in a rapid K^+ efflux of 18 to

22% (within 1 min) followed by a slow steady increase to approximately 40% within 10 min (Figure 4). This represented a 7 to 8 fold increase over the untreated control samples. Shin (1998) reported that Lfcin interacted with the cytoplasmic membrane resulting in formation of "debris in the cytoplasm". Haukland *et al.* (2001) reported that lactoferricin B was able to cross the cytoplasmic membrane and become localized in the cytoplasm. It is presumed that cationic peptides, such as Lfcin and LfH, potentially form pores, act by thinning the membrane or by destabilizing the membrane permeability causing a loss of intracellular constituents (Matsuzaki, 1998, Heller, *et al.,* 2000, Haukland, *et al.*, 2001). The release of significant amounts of potassium indicates that the membrane is permeabilized and thus the membrane potential may be dissipated as a consequence of the significant loss in cellular ion balance (Ohmizo, *et al.*, 2004).

Loss of intracellular and extracellular ATP from LfH treated *E. coli* O157:H7 cells

Intra-, extra-cellular and total levels of ATP were determined using bioluminescent quantification of ATP, which is based on the luminescence emitted during the conversion of luciferin to oxyluciferin. Light is generated and measured by a luminometer.

E. coli O157:H7 cells were evaluated for the total and extracellular levels of ATP and the intracellular levels were determined from this data. The extracellular and intracellular levels of ATP for both control and treated cells are listed in Figure 5. Extracellular levels of ATP were significantly lower than intracellular ATP levels in control samples both initially and after 30 minutes. Initially, there was approximately 1mM extracellular ATP/g of cells dry weight (CDW) and 27 mM intracellular ATP/g

CDW that was typical for *E. coli* (range 15 – 36 nMol/g). This demonstrated little change over time in untreated control samples, most likely due to normal cell leakage and death (Andersen, 1980, Marr, 1991, Crane, *et al.*, 2005a, Crane, *et al.*, 2005b). Final levels in untreated control samples were approximately 2 to 3 mM extracellular ATP/g CDW and 25 mM intracellular ATP/g CDW. However, the addition of MIC and 2× MIC levels of LfH caused a slow progressive change in ATP concentration. Intracellular levels of ATP decreased nearly 50% from approximately 27 mM/g CDW to 14 mM/g CDW, while extracellular levels increased approximately 5 fold from 2 to 3 mM/g CDW to 9 mM/g CDW. The significant decrease in the intracellular level of ATP coupled with the simultaneous increase in extracellular ATP suggests cell membrane damage was causing a cellular ATP efflux (Figure 5).

Due to the size of the ATP molecule, it cannot freely pass across intact cell membranes. However, if cell membranes are damaged by stress or antimicrobial compounds, the potential for release of ATP exists, if the damage to the membrane is sufficient enough to allow for leakage of large cellular constituents.

When damage to a membrane occurs, a cell can start loosing its constituents such as K^+ or ATP. Minor damage to the inner membrane can result in a loss of certain cellular constituents, such as K^+, but not larger constituents, such as ATP. In each case, there may be changes to the ATP concentrations for a cell. If the damage to the cell is only minor, there may be a change in the intracellular level of ATP as the cell transitions into active transport and utilizes intracellular ATP to pump ions back into the cell to re-stabilize the imbalance created by the leakage of cellular constituents. If the damage is more severe there will be an increase in the extracellular levels of ATP as intracellular

ATP leaks from the cell. Each scenario leads to cell death; however, the mechanism is different. In addition, K^+ leakage is an indicator of loss of membrane potential since Na^+ and K^+ transport are primary builders of the membrane potential within a cell (Bakker and Harold, 1980).

The bioenergetics of a cell is essentially how a cell utilizes chemical and electrical systems to maintain membrane integrity and produce energy. Within a bacterial cell, the cytoplasmic membrane utilizes the energy (*e.g.* ATP) to maintain the balance of the ion gradient and utilizes the ion gradient to produce energy (*e.g.* ATP). When antimicrobials damage this balance, it can lead to a loss of membrane potential and ultimately to death of the cell.

Dissipation of transmembrane electric potential in LfH treated *E. coli* O157:H7 cells

The $\Delta\Psi$ component of the proton motive force was qualitatively examined by utilizing the fluorescent probe Di-S-C3-(5). The addition of MIC and 2× MIC levels of LfH caused a rapid dissipation of $\Delta\Psi$ in *E coli* O157:H7 cells. A similar dissipation was observed in the control assay when valinomycin was added. Valinomycin is a potassium ionophore that has been demonstrated to dissipate residual membrane potential (Figure 15). Cationic peptides have a high affinity for negatively charged surface structures such as lipopolysaccharides on Gram-negative bacteria (Hancock, 1999). Once closely associated with the outer membrane, cationic peptides, such as Lfcin or LfH, may gain access to the cytoplasmic membrane via permeabilization (removal of LPS) or self promoted uptake. In each case, the actual action of the antimicrobial is associated with damage to the cytoplasmic membrane and not the minor damage to the outer membrane.

After the LfH gains access to the cytoplasmic membrane it creates pores that cause a large potassium efflux and a significant change in ATP levels. This causes a disruption in the ion/energy balance maintained by the cytoplasmic membrane (membrane potential). If the damage is severe enough as demonstrated in this study with nearly a 40% efflux of potassium and a 4 to 5 fold increase in extracellular ATP the cell can no longer maintain the membrane potential.

Loss of viability of *E. coli* O157:H7 following treatment with LfH

The viability of *E. coli* O157:H7 cells was determined following treatment with MIC and 2× MIC levels of LfH. The LIVE/DEAD® BacLight™ Bacterial Viability assay is based on membrane integrity and as such would demonstrate damage to the membrane caused by LfH. Cells viewed under a fluorescent microscope appear either red (dead) or green (live).

Table 1 indicates that approximately 50% of the cells were killed (non-viable) following 1 h exposure to MIC levels of LfH.

V. CONCLUSIONS

The data presented in this study demonstrate that the pepsin hydrolysate of lactoferrin (LfH) exerts its antimicrobial action on the inner membrane of *E. coli* O157:H7 by forming pores in the membrane, which results in a loss of energy and ion balance leading to a collapse of $\Delta\Psi$ and a loss of cell viability. Future research should focus on determining the potential for a docking molecule or outer membrane binding sites.

VI. ACKNOWLEDGEMENTS

The authors thank John Rejman and the Immu Cell Corporation for providing Lf. The work described in this paper was funded in part by the New Jersey Agricultural Experiment Station.

VII. TABLES AND ILLUSTRATIONS

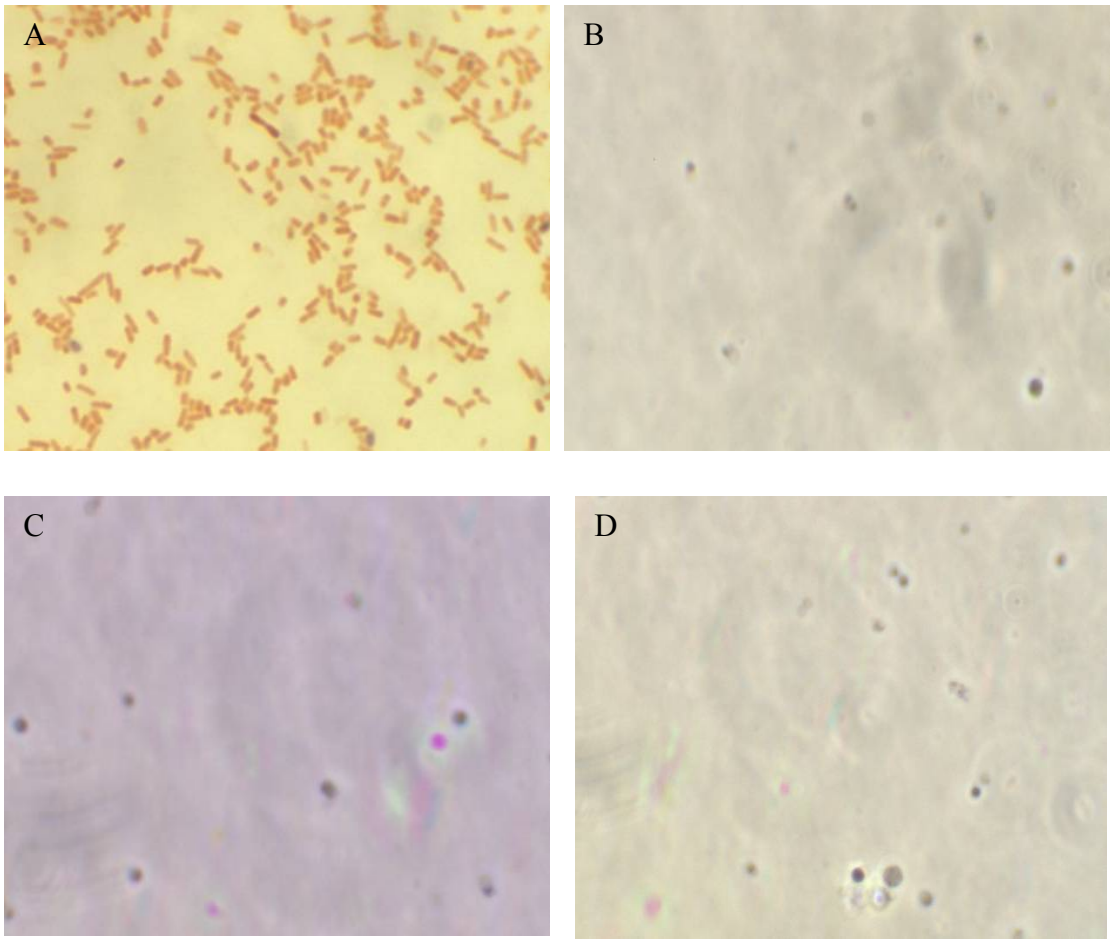

Figure 1: Conversion process from *E. coli* O157:H7 wild type cells to spheroplasts. (A) Gram stain of *E. coli* O157:H7 wild type cells following 18 – 24 hours of incubation. (B) Initial spheroplast formation of *E. coli* O157:H7 wild type cells following 1 treatment, (C) Spheroplast formation of *E. coli* O157:H7 wild type cells following secondary treatments, (D) Confirmation of spheroplast formation of *E. coli* O157:H7 wild type cells following secondary treatments and subsequent over night incubation to determine potential for wild type reversion.

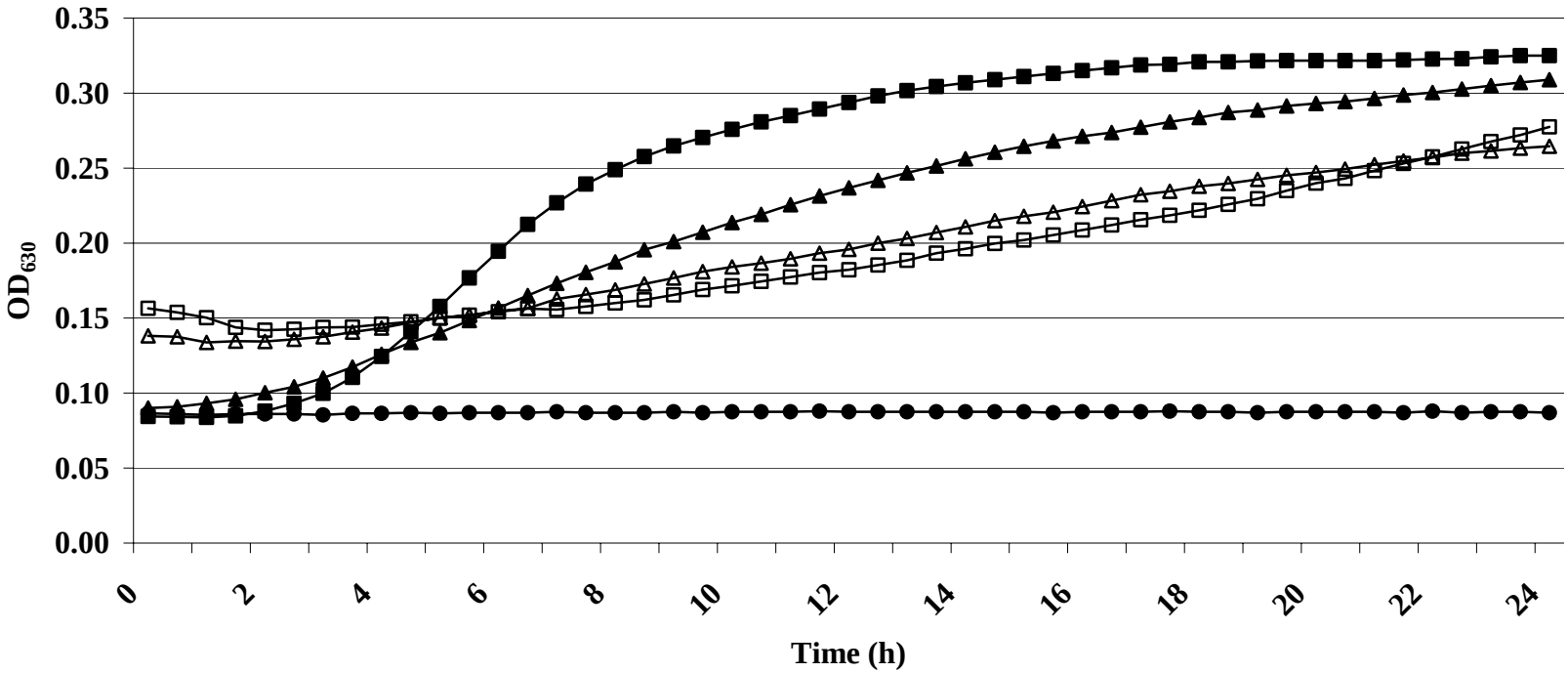

Figure 2: Comparison of growth of Wild Type *E. coli* O157:H7 and Spheroplast cells alone and in the presence of Lf.

■ - O157:H7 Wild Type ▲ - O157:H7 Spheroplast ● - Negative Control

□ - O157:H7 WT with Lf △ - O157:H7 Spheroplast with Lf

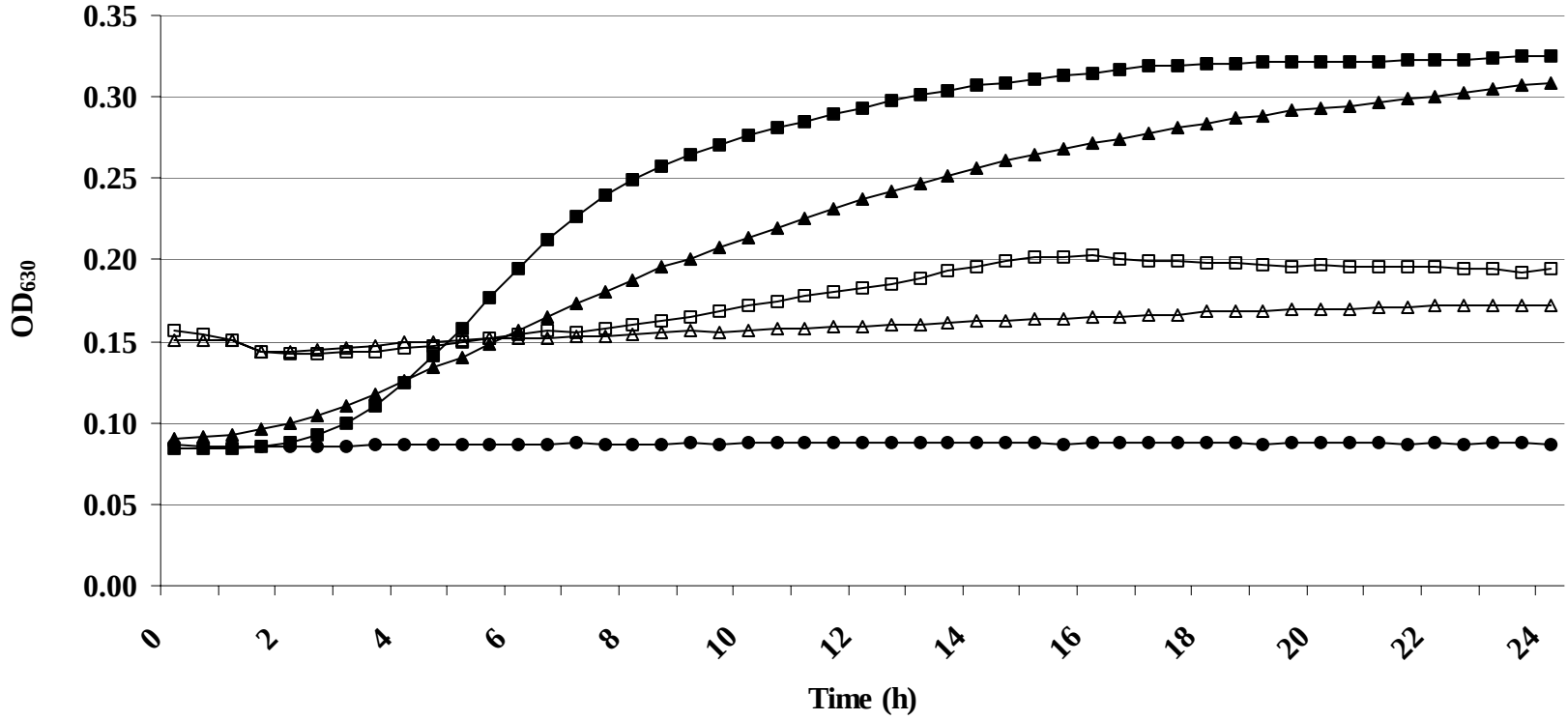

Figure 3: Comparison of growth of Wild Type *E. coli* O157:H7 and Spheroplast cells alone and in the presence of LfH.

■ - O157:H7 Wild Type ▲ - O157:H7 Spheroplast ● - Negative Control

□ - O157:H7 WT with LfH △ - O157:H7 Spheroplast with LfH

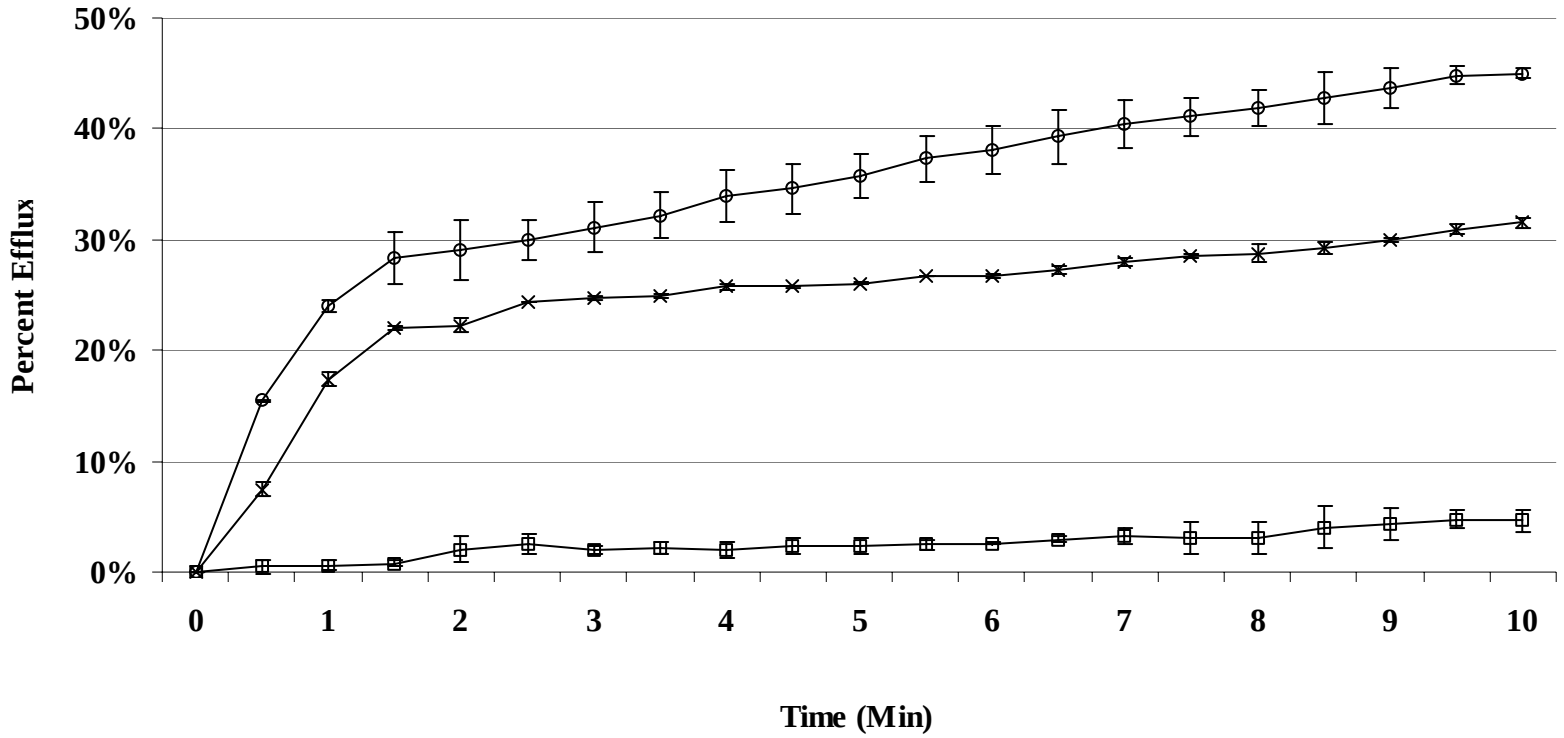

Figure 4: Time dependent Potassium Efflux in *E. coli* O157:H7 cells alone and in the presence of LfH.

□ - Untreated cellular K^+ efflux × - 5,000 μg/ml exposure ○ – 10,000 μg/ml exposure

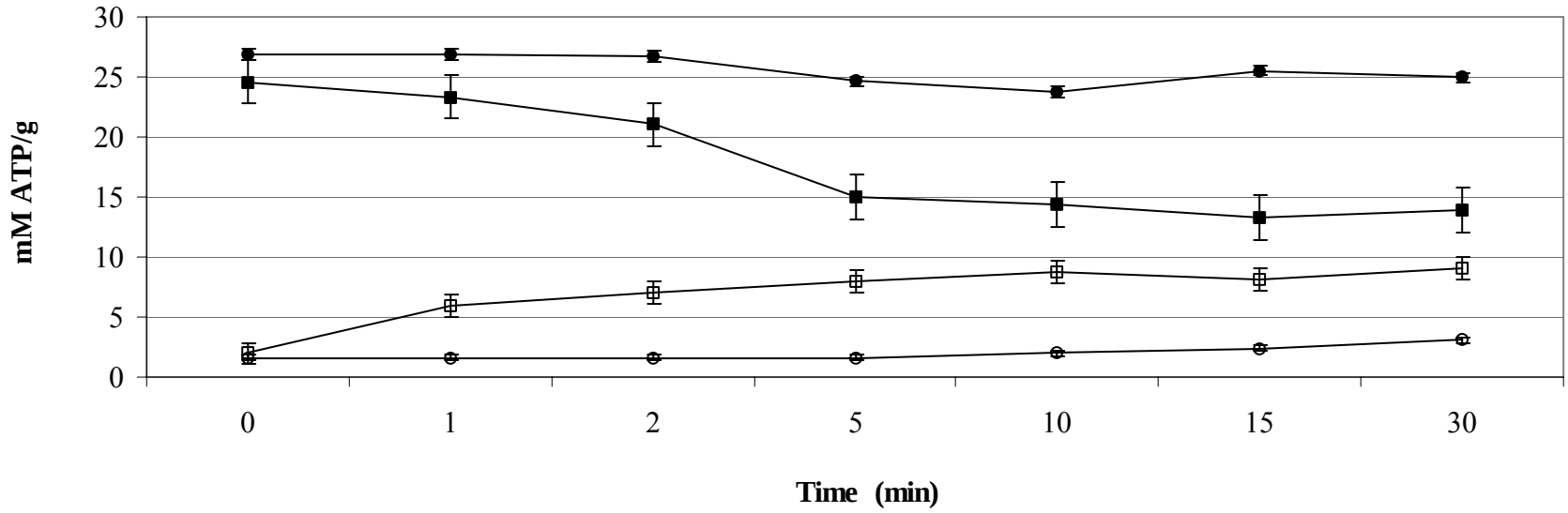

Figure 5: Time dependent Intracellular and Extracellular levels of ATP in *E. coli* O157:H7 cells alone and in the presence of LfH.

Untreated Cellular ATP Levels

● - Intracellular, ○ – Extracellular

Treated Cellular ATP Levels

■ - Intracellular, □ - Extracellular

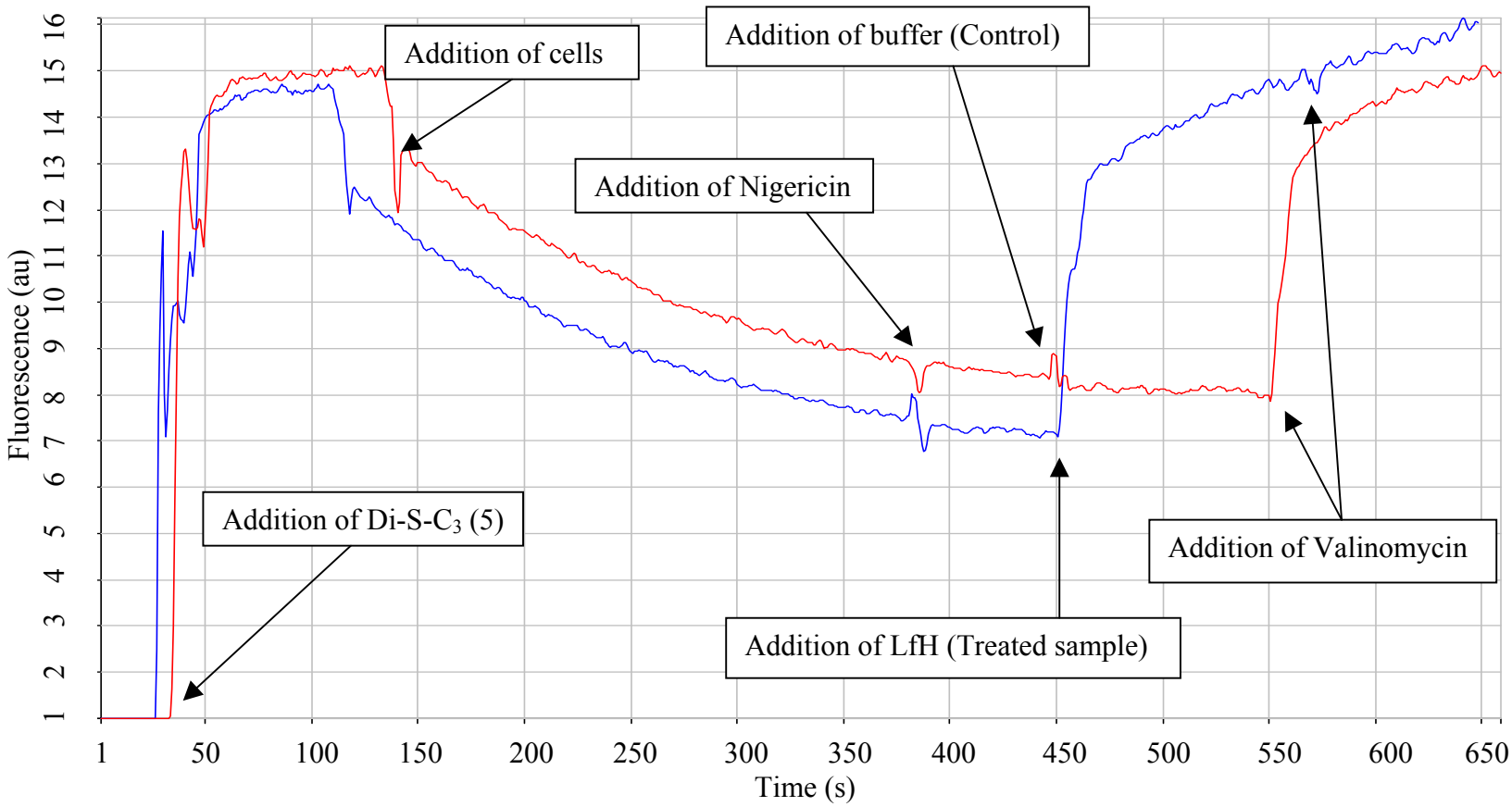

Figure 6: Determination of Membrane Potential (ΔΨ) in *E. coli* O157:H7 cells alone and in the presence of LfH.

Control in red (upper line), Treated sample in blue (lower line)

Table 1. Viability of *E. coli* O157:H7 after exposure the LfH.

	% Viable	% Non-Viable
Negative Control (No treatment)	91%	9%
Positive Control (Treated with 70% IPA for 1 h)	8%	92%
Sample (Treated with LfH at MIC level for 1 hour)	56%	44%

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CHAPTER 7

I. DISCUSSION

The goal of this research was to provide further understanding of the mechanism(s) of action of LfH on *E.* coli O157:H7. Prior to initiating the research project there had been a significant amount of research completed on the activity of Lf, Lfcin and LfH against Gram-positive and Gram-negative bacteria. However, no work completely elucidated the mechanism of action of any of these compounds. This specific research project was undertaken to develop further understanding of the mechanism of action of the hydrolysate under the hypothesis, "The pepsin hydrolysate of Lactoferrin (LfH) acts on the inner membrane of *E. coli* O157:H7 causing disruption in the membrane stability which ultimately leads to cell death". The data presented in this work supported this hypothesis by demonstrating that LfH acts on the inner membrane causing a loss of cellular constituents (*e.g.* K^+ and ATP) leading to a loss of membrane potential and ultimately a loss of cell viability (*e.g.* death).

The pepsin hydrolysate (LfH) was selected for this work for several reasons that make LfH potentially beneficial for use in foods. First, the intact protein, Lf, while possessing antimicrobial activity also has the potential to bind divalent cations and upon binding divalent cations (*e.g.* Fe^+, Ca^+, Mg^+) Lf loses much if not all of its antimicrobial activity (Bellamy et *al*., 1993, Naidu, A. S., 2000, Shimazaki, K., 2000). Peptides produced by different digestion methods yield different results. For example, peptides produced by papain, actinase AS protease P, protease A or biprase yield peptides that

have lost their ability to bind divalent cations, an advantage, also have lost their antibacterial activity (Tomita, *et al.*, 1994a, Tomita, *et al.*, 1994b, Hoek, *et al.*, 1997). Treatment with pepsin yields antibacterial peptides, corresponding to a region of the surface helix near the N-terminus. The peptides lost the ability to bind divalent cations but retain or have increased antimicrobial activity (Bellamy *et al*., 1993, Hoek, *et al.*, 1997, Hwang, *et al.*, 1998, Naidu, 2000, Shimazaki, 2000). Second, Venkitanarayanan, *et al.*, (1999) demonstrated little to no decrease in activity at pH 5.5 versus 7.2 in broth and ground beef and Appendini and Hotchkiss (2000) demonstrated that the LfH retained its activity as low as pH 3.5. Additionally, many studies demonstrated no loss or an increase in activity at refrigerated temperatures (Saito, *et al.*, 1994, Venkitanarayanan, *et al.*, 1999, Appendini and Hotchkiss, 2000, Murdock and Matthews, 2002). Third, the purified peptides, lactoferricin (Lfcin) were demonstrated to have significantly greater antimicrobial activity than Lf alone. Thus, LfH, which contains the peptides, should have increased activity over Lf (Dionysius and Milne 1997, Shin *et al*., 1998). Finally, as Lf itself is considered GRAS there is an increased likelihood that the minimal processing (and/or modifications) of Lf to generate LfH might lead to greater acceptance by industry and government regulatory agencies (Sofos *et al*. 1998).

Much research has been completed to date on the antimicrobial activity of Lf and Lfcin on Gram-positive and Gram-negative bacteria. Additionally, many studies demonstrated that LfH has a more potent antimicrobial activity than Lf. One of the dilemmas has been the ability to demonstrate activity in foods. Data generated demonstrated Lf to be antimicrobial against *L. monocytogenes*, but not Gram-negative bacteria, in ultra-high temperature (UHT) pasteurized milk as well as against *E. coli*

O157:H7 in sausage batters and in combination with lysozyme or EDTA in edible chitosan food films (Payne *et al*., 1990, Payne *et al*., 1994, Al-Nabulsi and Holley, 2006, Brown, *et al.*, 2007). Research demonstrated that Lfcin, the purified peptides, exhibit antibacterial activity against *E. coli*, *Ps. fluorescens*, *L. monocytogenes* and *Bacillus cereus* at concentrations 4–80 times lower than the native molecule in broth systems (Dionysius and Milne 1997, Shin *et al*., 1998, Venkitanarayanan *et al*., 1999). Finally, Chantaysakorn and Richter (2000) showed LfH to inhibit the growth of *E. coli* in carrot juice. Data in this study supported research demonstrating that LfH was antimicrobial towards *E. coli* O157:H7 and *L. monocytogenes* in UHT spiked milk.

Other research demonstrated that Lf, Lfcin or LfH could act synergistically with other compounds to enhance the activity of each compound to produce a similar or greater antimicrobial effect at lower concentrations. Synergy of Lf, Lfcin or LfH with antifungals, antibiotics, lysozyme or other chemical and physical barriers has been extensively documented (Sanchez and Watts, 1999, Vorland, *et al.*, 1999a, Kuipers, *et al.*, 1999, Ellison, 1994, Branen and Davidson 2000, Masschalck, *et al.*, 2001, Murdock and Matthews 2002, Branen and Davidson 2004). However, little work has been done on the activity of Lf, Lfcin or LfH with antimicrobials traditionally ineffective against Gram-negative bacteria due to the outer membrane. Studies within this work demonstrated that nisin; a bacteriocin typically not effective against Gram-negative bacteria has enhanced effects when combined with Lf or LfH. Jointly, nisin and Lf exhibited enhanced activity against .*E. coli* O157:H7 and *L. monocytogenes* as demonstrated by Fractional Inhibitory Concentration (FIC) indices of 0.35. Nisin and Lf had 4-fold and 10-fold decreased MICs, respectively, towards *E. coli* O157:H7. These

data collectively demonstrate that Lf and potentially LfH act to permeabilize the Gram-negative outer cell wall and then either Lf, nisin or both are able to access the inner membrane causing a loss of viability.

The second part of this project was to utilize the antimicrobial data previously generated and determine if the outer membrane was a required part of the mechanism of action as hypothesized previously. Numerous reports focused on activity against the outer membrane as a step in the mechanism of action. For example, Hancock and Chapple (1999) indicated that Lf and Lfcin, like any cationic molecule, have high affinities for binding to negatively charged structures on the outer membranes of bacteria (*e.g.* LPS) (Hancock and Chapple, 1999) and cause a release of these materials resulting in decrease stability of the cell and thus a loss of viability. Additional studies focused on porins, which are integral membrane proteins responsible for transporting materials from the extracellular environment to the periplasmic space. In this hypothesis, Lf, Lfcin of LfH would block the porins ability to transport materials resulting in a loss of viability (Sallmann, *et al.*, 1999). However, the presence and survivability of cell wall-less forms of bacteria offer a possibility that this may not be the mechanism site. Research by Freestone, *et al.* (1998), Hoischen, *et al.* (2002), Marvin and Wilholt (1987) and Onoda, *et al.* (1987) demonstrated that spheroplast (*e.g.* cell-wall less bacteria) can remain viable in culture for extended periods of time. Specifically Onoda, *et al.* (1987) demonstrated only minor percentages of reversion to "normal-shaped cells" following as much as 4 to 5 days incubation in a medium lacking the necessary spheroplast inducing agents (*e.g.* penicillin, EDTA, lysozyme) (Onoda, *et al.*, 1987, Onoda, *et al.*, 1988). Additionally, Gumpert and Hoischen (1998) and Kujau, *et al.* (1998) have L-forms of *E. coli* LWF+ and LWF- and

Proteus mirabilis LVI that have been cultivated and remained cell-wall-less for more than 30 years (Gumpert and Hoischen, 1998, Kujau, *et al.,* 1998).

The results from our work indicate that the spheroplast *E. coli* O157:H7 used were stable for at least 48-72 hours as determined by microscopy; well beyond the time required to complete the experimental assay's used in the present study. Additionally, the data generated indicated that the spheroplast *E. coli* O157:H7 cells responded similarly to cell wall containing *E. coli* O157:H7 cells in the presence of Lf and LfH, OD_{600} difference of 0.02 controls versus 0.02 and 0.03 respectively. Additionally, there was an enhanced inhibitory effect in the presence of LfH versus Lf as demonstrated previously in MIC studies. These data support the hypothesis proposed for this study that while there may be some antimicrobial action against the outer membrane it is not the site of action. If binding or association with the outer membrane were a necessary step in the mechanism of action, a change in the inhibitory effects caused by Lf and LfH would be expected in the presence of the spheroplast cells, which lack significant portions of the outer membrane. Finally, demonstrating similar antimicrobial activity in spheroplasts and intact cells indicates that the mechanism of action is more likely associated with a site other than one on the outer membrane.

The final part of this research was to determine the mechanism by which LfH caused a loss of viability in *E. coli* O157:H7 and the potential association of the mechanism of action with the inner membrane. In this portion of the work, the effect that LfH had on the inner membrane of *E. coli* O157:H7 was evaluated to better understand the mechanism of action that leads to cell death. Experiments were designed to determine the impact on the membrane integrity through evaluations of the changes

energy balance (ATP concentrations) and chemical or ion balance (*e.g.* potassium concentration, K^+ efflux), that would lead to the collapse of the membrane potential and ultimately the cell death.

Many bacteriocins and antibiotics have been shown to damage the cytoplasmic membrane causing a loss of membrane potential and thus leading to cell death (Chikindas, *et al*., 1995, Chung, *et al.*, 2000, Herranz, *et al.*, 2001, Penyige, *et al.*, 2002, Musse and Merrill, 2003, Li, *et al.*, 2005, Yang, *et al.*, 2006). Additionally, the damage to the inner membrane may result in partial or complete loss of membrane potential ($\Delta\Psi$), loss of small cellular constituents (*e.g.* K^+ efflux) or changes in ATP levels both intracellular and extracellular. Therefore, it is important to evaluate several aspects of cellular activity to determine the level of damage to the cell and the potential reason for cell death. This work involved evaluating the changes in intra- and extra-cellular ATP levels to determine if ATP was being hydrolyzed in the cell as a result of membrane damage (*e.g.* active transport) or if ATP was leaking out of the cell. Second, was an evaluation of K^+ efflux as an indicator of membrane damage and possibly the loss $\Delta\Psi$. Finally, we evaluated $\Delta\Psi$ as a qualitative measured of membrane potential. The results of this research suggest that LfH caused a disruption in membrane integrity. Following exposure of *E. coli* O157:H7 to MIC levels of LfH significant changes were observed in intracellular ATP, extracellular ATP, K^+ efflux and $\Delta\Psi$. There was approximately a 35 to 45% increase in K^+ efflux demonstrating some damage had occurred to the inner membrane. Additionally, there was approximately a 50% decrease in intracellular ATP. These two could be indicative of a loss of K^+ and the cell initiating active transport to compensate for the loss by utilizing ATP to pump K^+ back into the cell. However, this

coupled with an approximately 4-fold increase in extracellular ATP indicates that ATP was also leaking out of the cell. Finally, the data from the membrane potential experiments indicates that there was a dissipation of the membrane potential following exposure to LfH.

The data presented in this study demonstrate that LfH exerts its antimicrobial action on the inner membrane of *E. coli* O157:H7 by forming pores in the membrane, which lead to a loss of energy and ion balance materials (K^+ and ATP). This leads to a collapse of the membrane potential ($\Delta\Psi$) and a loss of cell viability.

II. FUTURE WORK

Results of the present study suggest that the activity of LfH is linked to damage of the inner membrane that ultimately leads to a loss of viability. The data from the spheroplast studies indicated that interaction with the outer membrane was not an integral part of the mechanism of action. However, the potential for an outer membrane receptor that may influence the activity of Lf, LfH or Lfcin exists. Several studies have been completed that indicate Lf, LfH or Lfcin bind to negatively charged surface structures on both Gram-positive and Gram-negative bacteria (Naidu, 1992, de Lillo, 1997Fang, 1999, Prinz, 1999, Ogunnariwo, 2001). Further exploration of LfH binding to bacteria could reveal a means to enhance the activity or further elaborate on the differences in inhibition seen in similar organisms. For example, data presented in this work demonstrated 2-4 fold differences in inhibition in *E. coli* and *Salmonella* species. The potential exist for

this difference in activity to be associated with the peptides ability to bind to the outer membrane and be in close association with the cell thus increasing its activity.

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