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Date August 14, 1989

THE EFFICACY OF PHENOLIC COMPOUNDS AND LACTOFERRIN AGAINST *LISTERIA MONOCYTOGENES* IN A MODEL MILK SYSTEM.

A Thesis

Presented for the

Master of Science

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DEDICATION

To my Mother and Father.

Your constant love and support
made this endeavor possible.

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INTRODUCTION

Listeria monocytogenes has been recognized as a human and animal pathogen for over 60 years. Found throughout the environment, this gram-positive bacilli has been isolated from a wide variety of materials, such as soil, water, vegetation, and also sewage (Botzler et al., 1974; Watkins and Sleath, 1981). Even though listeriosis only affects a small portion of the population, the illness still has a high mortality rate, especially among newborns, elderly, and those persons who are immunosuppressed. Symptoms of listeriosis include septicemia, meningitis, meningo-encephalitis, endocarditis, an infectious mononucleosis-like syndrome, and abortions (Gray and Killinger, 1966).

The first confirmed food-borne listeriosis outbreak was in 1981, when 41 cases of listeriosis were linked to consumption of contaminated coleslaw in Nova Scotia, Canada (Schlech et al., 1983). It is now recognized that all raw food products which come in contact with soil or fecal matter have the potential of carrying *L. monocytogenes*. Several outbreaks have been linked to contaminated dairy products (Fleming et al., 1985; Anonymous, 1985). The most recent isolated listeriosis

case was from consumption of microwaved turkey franks (CDC, 1989). The control of *L. monocytogenes* contamination in food processing plants is difficult because of its ability to survive throughout the plant.

In dairy processing plants, the two major sources of *Listeria* are raw milk and fecal material. As the awareness of *L. monocytogenes* in the dairy processing plant has grown, the major concern is how to control its presence (FDA, 1986). Although *Listeria* is controlled effectively through pasteurization (Bradshaw et al., 1985; Bunning et al., 1988; WHO, 1988), post-processing contamination arises when pasteurized product is mixed with raw milk or is in contact with contaminated equipment (FDA, 1986). The ability of *L. monocytogenes* to grow readily in milk at refrigeration temperatures augments this problem (Donnelly and Briggs, 1986; Rosenow and Marth, 1987).

Stricter sanitation practices and utilization of the Hazard Analysis Critical Control Point (HACCP) program are used to help prevent contamination problems. The control measures of the HACCP program are fundamental and could be complimented by secondary controls such as antimicrobial agents.

This study will examine the efficacy of two types of antimicrobial systems against *L. monocytogenes* in a model milk system. The two antimicrobial systems include: a synthetic inhibitor, phenolic compounds, and a natural inhibitor, lactoferrin. The phenolic antimicrobial agents are active over a broad pH range (Busta and Foegeding, 1983) and thus have the potential of being effective under relatively neutral pH mediums of non-cultured dairy products.

The use of a natural antimicrobial system is attractive because of current consumer demands for "all-natural" products (Beuchat and Golden, 1989). Lactoferrin is an iron-binding protein present in milk which has been recognized for its role in preventing enteric infection in newborn infants (Lonnerdal, 1985) and its suggestive role in resistance of the bovine mammary gland to mastitic infections during involution (Breau and Oliver, 1986; Oliver and Bushe, 1987). The increased growth response of *L. monocytogenes* to iron supplemented growth medium (Sword, 1966; Trivett and Myer, 1971; Cowart and Foster, 1985) gives indication that lactoferrin could act as an effective natural antimicrobial system against *L. monocytogenes*.

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PART I

REVIEW OF LITERATURE

CHAPTER I

LISTERIA MONOCYTOGENES

Characteristics and Taxonomy

Listeria monocytogenes is the type species for the genus *Listeria*. It is a gram-positive, non-sporulating, short rod which is aerobic and facultatively anaerobic. When grown at 20°C - 25°C, peritrichous flagella are present on *L. monocytogenes*. These *L. monocytogenes* will display tumbling motility in a wet microscopic examination. Optimum temperature for growth ranges from 30°C - 37°C but the organism is capable of growth at temperatures ranging from 1°C - 45°C. The optimum pH for *L. monocytogenes* is 6.0 - 9.0. On nutrient agar, colonies of *L. monocytogenes* appear small, round, flat, and translucent; under direct light they give off a bluish-gray color.

The genus *Listeria* was placed initially in the family *Corynebacteriaceae* as a single species *L. monocytogenes*. When taxonomic differences were discovered, *Listeria* was moved from the *Corynebacteriaceae* family and reclassified tentatively

as gram-positive, asporogenous, rod-shaped bacteria (Welshimer, 1981).

There are eight known species: *L. monocytogenes*, *L. denitrificans*, *L. grayi*, *L. murrayi*, *L. innocua*, *L. seeligeri*, *L. welshimeri*, and *L. ivanovii*. *Listeria denitrificans* is no longer considered a member of the *Listeria* genus. Appropriate taxonomic assignment will not be made until further studies are completed (Seeliger and Jones, 1986). The similarities between *L. grayi* and *L. murrayi* have generated a proposal for a new genus, *Murraya*. This genus would include *Murraya grayi*, subspecies *grayi*; and *M. grayi*, subspecies *murrayi*. (Seeliger and Jones, 1986).

Listeria monocytogenes and *L. ivanovii* are pathogenic to mice, yet only *L. monocytogenes* is associated consistently with human illness. Both strains are beta- hemolytic on sheep blood agar and are differentiated by their rhamnose-xylose reaction. The Christie-Atkins-Munch-Peterson (CAMP) test is used for species confirmation. *Listeria monocytogenes* hemolysis is enhanced by the presence of *Staphylococcus aureus*, and *L. ivanovii* is enhanced by *Rhodococcus equi* (FDA, 1988). *Listeria monocytogenes* has 13 typed serovars: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e,

and 7 (Seeliger and Hohne, 1979). In human listeriosis cases, 4b is by far the most common serovar isolated.

Incidence of Listeriosis in Humans

Listeriosis is a general term used to define any type of infection initiated by a *Listeria* species. Sources of *L. monocytogenes* are numerous, as the microorganism has a widespread natural environment. It has been isolated from manure, sewage, surface water, soil, vegetation, and the feces of healthy human and animal carriers (Ralovich, 1984). Nyfeldt (1929) was the first to isolate the microorganism from patients who had an infectious mononucleosis-like disease. Since that time the microorganism has been associated with various types of symptoms from mild flu-like occurrence to severe inflammation, meningitis, meningoencephalitis, and encephalitis, all of which can lead to death (Gray and Killinger, 1966).

The ability of *L. monocytogenes* to colonize in the host depends upon the condition of the immune system. When the immune system is depressed by some predisposing factor, *Listeria* is able to cause infections. Several high risk groups include newborns, pregnant women, and elderly people. For these groups the mortality rate

averages approximately 30% (Ralovich, 1984). On a yearly basis the following number of cases per 100,000 population have been reported (McLauchlin et al., 1986):

Country	Percent of Total Population Infected per Year
Germany	3.36×10^{-4}
France	3.27×10^{-4}
USA	6.60×10^{-5}
Canada	6.20×10^{-5}

Even with a higher frequency of listeriosis in European countries, the source of infections has been difficult to trace. This is due to the sporadic onset of the disease and the tedious procedures required to isolate *L. monocytogenes* from any contaminated source. As the collection of isolated case information has increased, the two most commonly documented modes of transmission have been man-to-man and zoonotic. For newborns, man-to-man transmission is the most prevalent (Albritton et al, 1984). Zoonotic transmission occurs from direct contact with an infected animal or from indirect contact

with animal products such as milk, or plants fertilized with infected manure (Hird, 1987).

Food-Related Outbreaks

Two of the largest reported listeriosis outbreaks were in Halle, Germany in 1966 (279 cases) and Anjou, France in 1976 (162 cases). Although no epidemiologic study was completed, the suspected mode of transmission in both outbreaks was the consumption of contaminated raw milk. In the past 10 years, most of the reported common source outbreaks have been associated with food (McLauchlin, 1987):

Place/year	No. of cases	Source
Boston,USA/1979	20	raw vegetables
Auckland, New Zealand/1980	21	seafood (?)
Nova Scotia, Canada/1981	41	coleslaw
Massachusetts, USA/1983	49	milk
Lausanne, Switzerland/83-84	25	soft cheese
California, USA/1985	314	soft cheese

The first epidemiological study was conducted in Nova Scotia, Canada after 34 cases of perinatal listeriosis and seven cases of adult listeriosis were reported between March and September of 1981. The mortality rate was 27% for the infant cases and 33% for the adult meningitis cases. The investigators traced contaminated coleslaw to a farm that housed several listeric sheep. The farmer had used sheep manure for fertilizing cabbage which was later harvested and held in cold-storage. Cold-storage temperatures allowed *Listeria* to thrive and contaminate the coleslaw. Serotype 4b was isolated from patients with listeriosis and from the implicated coleslaw (Schlech et al., 1983). This was the first confirmed outbreak of food-borne listeriosis.

In Massachusetts, between June 30 and August 30 1983, 49 patients were hospitalized with either meningitis, septicemia, or pregnancy complications all due to *L. monocytogenes*. This outbreak had a 29% fatality rate for all adult and perinatal cases. An epidemiological study found a strong association between the consumption of a specific brand of milk and the

onset of infection. However, no *L. monocytogenes* could be isolated from milk or the local dairy plants (Fleming et al., 1985).

The largest outbreak of foodborne listeriosis in the United States happened in Los Angeles and Orange County, California in 1985. Three hundred fourteen cases of listeriosis reported between January 1 and November 2 were linked to the consumption of Jalisco Mexican-style cheese. One hundred eighty-one cases occurred in pregnant Hispanic women and/or their infants. There were no fatal cases among women, but there was a 33% fatality rate among infants. The remaining 133 cases were linked to immunosuppressed persons who had consumed Jalisco cheese. The case-fatality rate for this group averaged 39%. Taking samples of the cheese from implicated production plant showed contamination by *L. monocytogenes*. Further investigation revealed that raw milk had been used in production of the cheese (Anonymous, 1985).

Dairy products are not the only food carrier of *L. monocytogenes*. All raw food products which come in contact with soil or fecal matter have the potential of carrying *L. monocytogenes*. Survival of *L. monocytogenes* in food processing environments presents a high risk of

cross-contamination from raw to processed products. The Centers for Disease Control (CDC) has recognized undercooked hotdogs and undercooked chicken as potential listeria transmitters in many sporadic listeriosis cases (Schwartz et al, 1988). The most recent sporadic listeriosis case, reported in Oklahoma, confirmed this assumption. A cancer patient developed listeriosis from consumption of microwaved turkey franks. *Listeria monocytogenes* was isolated from both the patient and the suspect turkey franks (CDC, 1989).

Control of *Listeria monocytogenes* Contamination

The control of contamination by *L. monocytogenes* in the dairy plant is difficult because of its ability to survive throughout the plant. There are two major sources of *L. monocytogenes*; raw milk and processing facilities.

Listeria is naturally present in raw milk as an intracellular parasite in the cytoplasm of phagocytic leukocytes and also as a fecal contaminant. This contamination can be controlled effectively through pasteurization of raw milk (Bradshaw et al., 1985; Bunning et al., 1988; WHO, 1988). One of the initial

contamination problems in dairy plants was cross connections of transporting pipes which sometimes allowed pasteurized and raw product to mix (FDA, 1986). Complete separation of raw and pasteurized product prevents problems with *L. monocytogenes* from raw milk contaminating processed product (FDA, 1986).

Listeria survives also in the processing plant after being carried in with soil and fecal material from dairy farms. The microorganism has been isolated from plant equipment, ventilation systems, drains, and the clothes and shoes of milk haulers and fieldmen. Presence of *Listeria* in the processing plant environment is most difficult to control as *L. monocytogenes* has been shown to grow readily in milk at refrigeration temperatures (Donnelly and Briggs, 1986; Rosenow and Marth, 1987) Although chemical sanitizers are effective against *L. monocytogenes* on plant equipment (Lopes, 1986), the pervasive environmental sources of *Listeria* leave no margin for neglect in routine sanitation procedures. Emphasis has been placed on Hazard Analysis Critical Control Point (HACCP) program guidelines to identify locations in which *Listeria* may most effectively be controlled (WHO, 1988).

Because it is difficult to completely eliminate environmental sources of *L. monocytogenes*, use of an effective antimicrobial system can be an additional safety factor. Several studies have evaluated efficacy of common antimicrobial agents against *L. monocytogenes* in a synthetic medium (El-Shenawy and Marth, 1988; Berry and Liewen, 1988) and also in actual food products (Ryser and Marth, 1988). These common antimicrobial agents included sorbic, benzoic, and propionic acid and their associated salts (sodium benzoate, potassium sorbate, and sodium propionate). Organic acids were tested also including acetic, citric, lactic, and tartaric acids. Results demonstrated no microbial inhibition at pH levels greater than 5.0.

Several other antimicrobial systems have been evaluated against *L. monocytogenes*. Hydrogen peroxide was tested against *L. monocytogenes* and found to be ineffective (Dominquez et al., 1987). Lysozyme, normally present at low concentrations in milk, has been recognized for its microbial lysis. Hughey and Johnson (1987) found egg white lysozyme to be ineffective against *L. monocytogenes* unless EDTA was added to the growth medium. The bacteriocin, nisin, reportedly

antagonized eight strains of *L. monocytogenes* (Daeschel et al., 1988). Overall, antimicrobial agents which have been evaluated against *L. monocytogenes* have not been shown to be effective under relatively neutral pH ranges of non-cultured dairy products.

CHAPTER II

ANTIMICROBIAL AGENTS

There are many factors which can influence activity of antimicrobial agents (Davidson and Parish, 1989). One of the most important factors is hydrogen ion concentration. Organic acids which are used commonly as antimicrobial agents must exist in an undissociated state to penetrate the microbial cell membrane (Lueck, 1980a). These agents are relatively ineffective in neutral and basic pH systems because they begin to dissociate when the pH is greater than 5.0. Non-cultured dairy products have a neutral pH, approximately 6.5, and require antimicrobial agents effective in neutral or slightly basic environments. One group of compounds which are effective over a very broad pH range are the alkyl esters of p-hydroxybenzoic acid, commonly known as parabens. Of all organic acids and derivates used commonly as antimicrobial agents, parabens warrant the greatest further investigation because they will not dissociate at pH less than 8.5.

Many compounds found naturally in food have antimicrobial activity. Use of a natural antimicrobial system is attractive because of the current consumer demands for "all-natural" products. One potential natural antimicrobial compound in milk is the whey protein, lactoferrin (Schanbacher and Smith, 1975; Reiter, 1978). Lactoferrin is an iron-binding protein which acts as a nonspecific microbial inhibitor in the passive immune system of many mammals. *In vitro* studies have shown lactoferrin to be effective against many coliform bacteria (Stephens et al., 1980; Bishop et al., 1976; Nonnecke and Smith, 1984a). *Listeria monocytogenes* growth increases in the presence of excessive iron (Sword, 1966; Trivett and Myer, 1971). Therefore, lactoferrin has the potential of being effective against *L. monocytogenes*.

Phenolic Compounds

There are three major groups of phenolic compounds which have been shown to have antimicrobial properties. They include: alkyl esters of p-hydroxybenzoic acid (parabens), natural plant phenolic acids, and antioxidants (Davidson and Branen, 1981).

Parabens. Parabens were first synthesized to improve activity of benzoic acid over a broad pH range (Lueck, 1980b). Esterification of the carboxyl group of benzoic acid allows the molecule to remain undissociated up to pH 8.5 versus normal dissociation of benzoic acid at pH 5.0 (Busta and Foegeding, 1983). The degree of inhibition displayed by parabens is related directly to length of their alkyl chain; the longer the chain, the greater the inhibition. However, water solubility is inversely proportional to the number of carbon atoms in the ester group (Davidson, 1983). Therefore, in many products, a combination of methyl and propyl paraben is used to take advantage of the higher water solubility of the methyl ester and the greater inhibitory action of propyl ester (Chichester and Tanner, 1972). Parabens are effective against mold, yeast, and gram-positive bacteria (Davidson, 1983). Gram-negative bacteria have an outer cell membrane which apparently makes it difficult for parabens to penetrate into the cell (Eklund, 1980). When parabens are used at increased concentrations, some sensitivity is shown by gram-negative microorganisms (Davidson, 1983). Parabens are utilized primarily for their antifungal properties in confectionery icings and fillings, syrups, wines, fruit

drinks, and artificially sweetened jams and jellies (Chichester and Tanner, 1972). In the United States the only esters approved as direct food additives by the Food and Drug Administration (FDA) (21 CFR 172, 184) are methyl, propyl, and heptyl.

Natural Plant Phenolic Acids. There is a wide array of phenolic compounds found throughout the plant kingdom. Selected plant phenols have demonstrated bacteriostatic characteristics (Davidson, 1983). All plant phenols have the potential of inhibitory characteristics, based on their common phenolic structure.

There are four major groups of plant phenols: hydroxyquinones; phenolic acids; polyphenolics; and flavonols. Hydroxycinnamic acids belong to the hydroxyquinones group. Derivatives of hydroxycinnamic acid; p-coumaric, ferulic, and caffeic acids, are inhibitory against *Saccharomyces cerevisiae* (Baranowski et al., 1980) and also against several bacteria; *Bacillus cereus, Escherichia coli*, and *Staphylococcus aureus* (Herald and Davidson, 1983).

Polyphenolics are present in rinds and barks of many plants. Tannic acid was the first polyphenolic to be recognized for its fungistatic properties

(Cruickshank and Perrin, 1964). Gallic acid is a derivative of p-hydroxybenzoic acid and is found in several higher plant forms (Towers, 1964). Quercetin is an end-product of hydroxycinnamic acid in plant metabolism. Like tannic acid, it is widely distributed in the rinds and barks of plants (Windhoz, 1983).

Antioxidants. Phenolic antioxidants, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) are all used in preventing autoxidation of lipid products (Davidson, 1983). Since they also have antimicrobial properties, these antioxidants have a potential dual benefit within a food product.

Antimicrobial properties of these compounds exist against both fungi and microorganisms (Klindworth et al., 1979; Davidson and Branen, 1980; Davidson, et al., 1981). Branen et al. (1980) found that potassium sorbate in combination with BHA or TBHQ was synergistic against *S. aureus* and *Salmonella typhi*.

The only drawback in using antioxidants as antimicrobial agents is their lipophilic character (Davidson, 1983). In high fat concentrations, the solubilization of the antioxidant into the lipid phase eliminates its activity against microorganisms which are

present in the water phase. Rico-Munoz and Davidson (1983) showed TBHQ was less affected by the presence of lipid than BHA. This was attributed to the difference in the oil-water partition coefficient of the two compounds.

Mode of Action. Phenolic compounds apparently affect cell function by disrupting the cytoplasmic membrane of the microorganism. Eklund (1980) proposed that leakage of cellular contents was due to neutralization of the normal membrane gradient by parabens. Cytoplasm lost by *Pseudomonas* species after treatment with BHA was measured by radioactive labeling of specific cellular material (Davidson and Branen, 1980). Bargiota et al. (1987) measured changes in membrane fluidity and variation in phospholipid content. The difference in total lipid content of 4 strains of *S. aureus* was related to the difference in strain resistance to the esters of p-hydroxybenzoic acid (Bargiota et al., 1987).

Phenolic compounds also can affect membrane-bound enzyme activity (Rico-Munoz et al., 1987). Inactivation of enzymes disrupts cell membrane functions. ATPase activity of *S. aureus* was measured in the presence of BHA, TBHQ, PG, the hydroxycinnamic acid derivatives, and

methyl and propyl paraben (Rico-Munoz et al., 1987). Inactivation of ATPase activity was dependent upon the specific compound. Rico-Munoz et al. (1987) concluded that the reaction between the phenolic compound and the ATPase activity is based upon the molecular structure and individual properties of each compound.

Lactoferrin

Physical Properties. Whey proteins from milk play a significant role in the passive immune system of many mammals. These proteins act as both specific and nonspecific inhibitors of infectious agents (Schanbacher and Smith, 1975; Reiter, 1978). Specific inhibitors are immunoglobulins and nonspecific inhibitors include both iron-binding proteins and various enzymes (Reiter, 1978). The iron-binding proteins are transferrin (TF), lactoferrin (LF), and conalbumin (CA). Transferrin is found primarily in circulating plasma and lymph where it functions as an iron source for hemoglobin synthesis (Crichton and Charloteaux-Wauters, 1987). Lactoferrin is synthesized by secretory epithelial cells and is also present in polymorphonuclear leukocytes (Masson et al., 1969). It is recognized for its strong bacteriostatic properties (Schanbacher and Smith, 1975; Reiter, 1978).

Conalbumin, although not present in milk, is an iron-binding protein found in egg albumin. It has also been shown to have bacteriostatic properties (Weinberg, 1978).

Iron-binding proteins are glycoproteins and have a molecular weight of approximately 80 kD. All three have similar ferric binding centers. Each glycoprotein molecule has the capacity to bind two atoms of ferric iron in the presence of two bicarbonate anions (Crichton and Charloteaux-Walters, 1987). The specific binding site of LF consists of four amino acid ligands: aspartic acid; histidine; and two tyrosines. Also associated with each site is a bicarbonate atom and water (Anderson et al., 1987). In purification of LF, two polypeptides ("doublet protein"; Rejman, 1988) are isolated and have molecular weights of 83 and 87 kD. Rejman (1988) and Tsuji et al. (1989) attributed part of the size difference to the variation in carbohydrate moieties, however, the physiological significance of the two molecules is not known (Tsuji et al., 1989). Comparison studies of TF and LF show that the binding strength of LF was 300 times greater than TF for ferric iron at pH 6.4-6.7 (Querinjean et al., 1971; Aisen and Leibman, 1972). This gives LF more stability over a

broader pH range. Lactoferrin maintains its iron affinity at pH 4.0, whereas TF losses its iron affinity at pH 5.0 (Groves, 1960). Lactoferrin is secreted in all types of biological fluids, including milk of most mammals. It has been isolated from numerous animal species including; guinea pigs, cows, goats, swine, horses, and mice (Masson and Heremans, 1971). The concentration of LF is variable depending upon the species. Postpartum human milk has approximately 1 mg/ml of LF (Bullen, et al., 1972) versus postpartum bovine milk which has approximately 0.3 mg/ml of LF (Welty et al., 1976).

Role of Iron in Microbial Growth. Iron is essential in maintaining biochemical functions in all microorganisms that utilize iron-containing electron transfer proteins, cytochromes and iron-sulfur proteins (Bagg and Neilands, 1987). Iron may exist in aqueous solution as both ferrous and ferric ions. Ferric iron is extremely insoluble at neutral and alkaline pH and is quickly hydrolyzed and polymerized into ferric hydroxides and oxyhydroxides (Crichton and Charloteaux-Wauters, 1987). Once these compounds are formed, the iron is no longer accessible for metabolic use. To maintain solubility in biological fluids the

glycoproteins are utilized to bind iron. For microorganisms to survive in biological fluids, they must have a mechanism for acquiring essential iron from glycoproteins. This mechanism, known as a siderophore, is a low molecular weight compound synthesized by the organisms (Lankford, 1973). Siderophores are a ferric-specific ligand which possess a high affinity for iron and thus compete with high affinity glycoproteins. There are approximately two hundred different natural siderophores that can be classified into two general classes, hydroxamate and phenolates/catecholates (Crichton and Charloteaux-Wauters, 1987). Five different siderophore molecule have been identified in *Escherichia coli* strain K12: ferrichrome (a hydroxamate); rhodotorulic acid (a dihydroxamate); aerobactin (a plasmid-mediated hydroxamate); Desferal (a ferrioxamine); and enterochelin or enterobactin (a phenolate) (Crichton and Charloteaux-Wauters, 1987). The molecular weight of these compounds varies from 500 to 1000 D. They are too large to allow free diffusion through the small pores in the outer membrane of gram negative organisms. Therefore, each siderophore has a receptor protein (Schwyn and Neilands, 1987) on the outer membrane to bind siderophore and allow cytoplasmic

membrane components to transfer the iron into the cytoplasm. Iron is then released by a mechanism specific for each individual siderophore (Crichton and Charloteaux-Wauters, 1987). Receptor-dependent iron transport is not the only function of siderophores in microorganisms. Siderophores are associated also with the virulence and growth of various organisms (Bagg and Neilands, 1987). Growth of *Listeria monocytogenes* increased markedly when synthetic growth medium was supplemented with iron (Sword, 1966; Trivett and Myer, 1971; Cowart and Foster, 1985). The lethal dosage for infection with *L. monocytogenes* decreased in mice treated after they were injected with ferric or ferrous iron. When Desferal was injected along with iron supplements the lethal dosage increased (Sword, 1966). Increased resistance to infection with Desferal was attributed to its inhibiting *L. monocytogenes* growth. This would allow host defenses to destroy the microorganism.

Bacteriostatic Properties. The efficacy of LF in inhibiting microbial growth is dependent upon the state of the LF molecule and its interaction with other components within a system (Stephens et al., 1980; Brock et al., 1983; Breau and Oliver, 1986). To act as an

effective antimicrobial agent, LF must be in an unsaturated state (Apo-LF) and at a concentration sufficient to bind all free iron present (Bishop et al., 1976; Nonnecke and Smith, 1984a).

During lactation, the concentration of LF varies depending upon lactation stage and type of mammal. In bovine lactation, LF concentration peaks during the dry period (involution) (Nonnecke and Smith, 1984b; Oliver and Bushe, 1987). This increase in concentration is perhaps related to the resistance of the mammary gland to new mastitis infections during involution (Breau and Oliver, 1986; Oliver and Bushe, 1987). The highest concentration of LF in humans occurs after parturition (Masson and Heremans, 1971). Human LF is least saturated in the early stages of lactation (Bullen et al., 1972) which gives it a large amount of iron-binding capacity. Human LF is recognized for its role in protecting newborn infants against enteric infections (Goldman and Smith, 1973; Brock, 1980). Brock (1980) proposed that *in vivo* LF also acts as an iron-absorption control mechanism. This proposal was based upon the inverse relationship between LF concentration and growth rate of an individual species. The more rapid young animals grew the more iron they needed to maintain

growth. In this case, lower LF concentrations were present to allow more iron absorption. Schanbacher and Smith (1975) stated also that the *in vivo* role of LF was not well defined after discovering LF could bind to a microbial cell wall when lysozyme was used to partially digest the wall.

In vitro studies of the antimicrobial properties of LF have shown there are several interacting components which affect inhibitory activity in milk. The three primary components are bicarbonate concentration, citrate concentration, and presence of immunoglobulins (secretory IgA in humans and IgG in bovine species) (Rogers and Synge, 1978; Stephens et al., 1980; Brock et al., 1983; Nonnecke and Smith, 1984b; Oliver and Bush, 1987).

Each binding site on the LF molecule required one bicarbonate anion in order to bind ferric iron (Anderson et al., 1987). Addition of bicarbonate buffers enhanced the degree of inhibition of Apo-LF (Reiter et al., 1975; Nonnecke and Smith, 1984a).

Citrate, like LF, fluctuates during the lactation cycle and varies among animal species. At parturition, human milk contains 2 to 3 mM citrate while bovine milk contains 6 to 8 mM (Peaker and Linzell, 1975). The

antimicrobial action of Apo-LF has been shown to be dependent upon the molar ratio of citrate to LF (Bishop et al., 1976; Nonnecke and Smith, 1984a; Oliver and Bushe, 1987). Oliver and Bushe (1987) found the molar ratio of citrate:LF to be greater than 1000 in normal bovine milk and colostrum and then decreased to approximately 10 in dry secretions. The presence of citrate induces a transport system which can remove iron from LF (Bagg and Neilands, 1987). The citrate-mediated system is similar to siderophores because it has a specific protein receptor on the outer membrane of the bacteria. However, it is not a strong chelator for ferric iron. Consequently, the concentration of citrate must be 20 times greater to form the mononuclear complex (Bagg and Neilands, 1987). *In vitro* studies have shown that at pH 7.4, the citrate-mediated system can be neutralized with an excess concentration of bicarbonate anions (Reiter et al., 1975; Griffiths and Humphreys, 1977).

The presence of immunoglobulins in milk enhances the antimicrobial action of LF (Spik et al., 1978; Stephens et al., 1980; Nonnecke and Smith, 1984b). Stephens et al. (1980) demonstrated this effect was due to antibody specificity of pathogenic microorganisms.

This study exposed both commensal and enteropathogenic strains of *Escherichia coli* to human LF, with and without secretory IgA, in synthetic medium. The inhibitory effect of LF against enteropathogenic *E. coli* was enhanced significantly by IgA. The effect of LF and IgA against commensal strains was not changed significantly from the effect of LF alone. Bovine IgG1 enhanced inhibition against *E. coli* strain pathogenic to calves, but bovine IgG1 and LF tested against human pathogenic strains had no synergist effect.

This review has covered two types of antimicrobial agents which have potential of being effective against *L. monocytogenes* in non-cultured dairy products. The environmental presence of *Listeria* in the dairy processing plant makes it difficult to prevent contamination. Use of an effective antimicrobial agent would serve as a secondary means of controlling outgrowth of *L. monocytogenes* in refrigeration or abuse temperatures if contamination should occur.

The overall objective of the following studies was to determine the efficacy of two types of antimicrobial systems, synthetic and natural, against *L. monocytogenes* in a model milk system. The phenolic compounds are a group of synthetic antimicrobial agents which are active

over a broad pH range (Busta and Foegeding, 1983) and thus have the potential of being effective under relatively neutral pH mediums of non-cultured dairy products.

The use of a natural antimicrobial system is attractive because of current consumer demands for "all-natural" products (Beuchat and Golden, 1989). Milk has several proteins with bacteriostatic properties (Reiter, 1978). Lactoferrin is an iron-binding protein in milk which has been recognized for its role in preventing enteric infection in newborn infants (Lonnerdal, 1985) and its suggested role in resistance of the bovine mammary gland to new mastitic infections during involution (Breau and Oliver, 1986; Oliver and Bushe, 1987). Previous studies (Sword, 1966; Trivett and Myer, 1971; Cowart and Foster, 1985) have shown the growth of *L. monocytogenes* to increase when synthetic growth medium was supplemented with iron. The marked growth response of *L. monocytogenes* to the iron supplements gives indication that lactoferrin could act as an effective natural antimicrobial agent against *L. monocytogenes*.

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PART II

EFFICACY OF PHENOLIC COMPOUNDS AGAINST *LISTERIA MONOCYTOGENES* IN A MODEL MILK SYSTEM

CHAPTER I

PROLUSION

Abstract

The minimum inhibitory concentration (MIC) of several phenolic compounds against eight strains of *Listeria monocytogenes* in tryptose phosphate agar (TPA) was determined. Based upon concentration, the most effective compound was the phenolic antioxidant, tertiary butylhydroquinone (TBHQ), which had a MIC of 64 µg/ml. Among the FDA approved food antimicrobials, the most effective was propyl paraben with a MIC of 512 µg/ml. Propyl paraben and TBHQ were compared to potassium sorbate, a commonly used food antimicrobial, in a model milk system containing 10% non-fat milk solids against *Listeria monocytogenes*, Scott A. Testing conditions included inoculum levels 10 and 1000 CFU/ml and incubation temperatures, 35°C and 4°C. Both propyl paraben and TBHQ caused a significant increase in the lag phase. The extent of antimicrobial activity of TBHQ was dependent upon inoculum size, while that of propyl paraben was not affected by inoculum size.

Introduction

Documented cases of foodborne listeriosis stressed the need for stricter sanitation programs in food processing plants (Schlech et al., 1983; Fleming et al., 1985; Anonymous, 1985). The dairy industry took the first initiative to control contamination of *Listeria monocytogenes* within the processing plant (FDA, 1986). The primary sources of *L. monocytogenes* in the industrial environment are raw milk and dairy farms. *Listeria monocytogenes* is present naturally in raw milk as an intracellular parasite and thrives in soil and fecal material which may be carried into the plant by various workers (FDA, 1986).

Listeria monocytogenes in raw milk is destroyed at pasteurization temperatures (Bradshaw et al., 1985; Bunning et al., 1988; WHO, 1988) but problems occur when the pasteurized products are recontaminated in the plant (FDA, 1986). The psychrotrophic nature of *L. monocytogenes* allows the organism to grow during refrigerated storage, complicating the dairy processors task (Donnelly and Briggs, 1986; Rosenow and Marth, 1987).

Stricter sanitation practices and utilization of the Hazard Analysis Critical Control Point (HACCP) program to uncover and eliminate potential hazardous

areas are both used to help prevent contamination problems. The control measures of the HACCP program are fundamental and could be complimented by secondary controls such as antimicrobial agents. An effective antimicrobial agent could prevent growth of *L. monocytogenes* whenever contamination occurs.

Most common antimicrobial agents have limited use because of their ineffective state in neutral or basic pH (Lueck, 1980a). Organic acids such as: sorbic, benzoic, and propionic, were only effective against *L. monocytogenes* when the medium was acidified below pH 5.0 (El-Shenawy and Marth, 1988; Ryser and Marth, 1988). Phenolic compounds are effective over a broad pH range and therefore potentially useful against *L. monocytogenes* in dairy products.

Our study had two objectives: to determine the inhibitory effect of several phenolic compounds against *L. monocytogenes* in a microbiological medium; and to determine the activity of the most effective inhibitors in a model milk system.

CHAPTER II

MATERIALS AND METHODS

Test Microorganisms

Eight strains of *Listeria monocytogenes* were used in this study: ATCC 19114 (serotype 4a), ATCC 19115 (serotype 4b), ATCC 19116 (serotype 4c), ATCC 19117 (serotype 4d) and ATCC 19118 (serotype 4e), all obtained originally from the American Type Culture Collection, Rockville, MD, and strains Scott A (serotype 4b), F5027 (serotype 1a), and F5067 (serotype 4b), obtained originally from the Centers for Disease Control, Atlanta, GA. All strains were provided by C.W. Donnelly, Dept. Animal Science, The University of Vermont, Burlington. Strains were grown on tryptose phosphate agar slants (TPA; Difco Laboratories, Detroit MI) at 35°C for 24 h. Cultures were stored at 4°C and subcultured every 4 wk.

Test Media

Tryptose phosphate broth (TPB; Difco) was used to grow microorganisms prior to minimum inhibitory concentration (MIC) studies, while TPA was used as the MIC test medium.

The model milk system consisted of a non-fat milk solids suspension (10%) and was prepared by rehydrating the non-fat dry milk (NFDM; Kroger Co., Cincinnati, OH) in deionized water, dispensing in 100 ml portions, and heating in an autoclave at 115°C for 20 min. The approximate final pH of the milk system was 6.4.

Test Compounds

In the MIC study, 12 phenolic compounds were tested including: propyl ester of p-hydroxybenzoic acid (propyl paraben, PP), methyl ester of p-hydroxybenzoic acid (methyl paraben, MP), tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), p-coumaric acid, ferulic acid, caffeic acid, gallic acid, tannic acid, and quercetin. All compounds came from Sigma Chemical Co. (St. Louis, MO) except TBHQ (Fisher Scientific Co., Springfield, NJ) and potassium sorbate (Miles Laboratories, Inc., Elkhart, IN). All compounds except potassium sorbate were solubilized in 95% ethanol to prepare stock solutions. Potassium sorbate was dissolved in deionized water. All solutions were made immediately prior to each experiment and filter-sterilized (0.45 μm).

Minimum Inhibitory Concentration

The MIC for each phenolic compound tested was determined using the agar dilution technique (Barry, 1976). Appropriate amounts of each compound were added to TPA to give final concentrations of 0, 16, 32, 64, 128, 256 and 512 µg/ml, except for quercetin which was evaluated at 6.4, 12.8, 25.6, 51.3, 102.5 and 205 µg/ml. Eight strains of *L. monocytogenes* were grown in TPB at 35°C for 18 h. Approximately 10^6 CFU/ml of each strain were inoculated with a 0.001 ml calibrated loop onto TPA plates containing the desired phenolic compound. The plates then were incubated at 35°C for 18 h. Following incubation, the lowest concentration of each compound which completely prevented outgrowth of the microorganism was termed the MIC for that compound.

Growth Inhibition in a Model Milk System

Four treatments were prepared for the model milk system, a control (1% ethanol), 1000 µg/ml PP, 150 µg/ml TBHQ and 1000 µg/ml potassium sorbate. *Listeria monocytogenes* Scott A was tested at two inoculum levels, 1000 CFU/ml and 10 CFU/ml and two separate incubation temperatures, 35°C and 4°C. Time intervals for monitoring growth were dependent upon incubation temperatures. At 35°C, *L. monocytogenes* were counted

every 3 h for the first 12 h and at 24 h. At 4°C, viable cells of *L. monocytogenes* were determined by counting every 48 h for 10 d. Samples were counted on TPA using the spiral plate counter (Spiral Systems, Cincinnati, OH) for the 1000 CFU/ml inoculum or the standard pour plate method for the 10 CFU/ml inoculum. All experiments were replicated.

Statistical Analysis

For the study of growth inhibition in a model milk system, a randomized complete-block design with microbial growth expressed as $\log_{10}$ CFU/ml was arranged in a split-block arrangement, each replicate representing one block. Contrast statements were used to determine the most effective antimicrobial agent. The overall alpha value was 0.05, yielding an individual alpha value of 0.0169 for each contrast.
The model statement was: G = R + A + (R x A) + T + (A x T), where

G = Growth (Log_{10} CFU/ml) of *L. monocytogenes* in the model milk system.

R = Replication of each assay.

A = Antimicrobial agent, PP, TBHQ, or potassium sorbate.

(R x A) = Interaction between replication and antimicrobial agent.

T = Time of sampling (0, 3, 6, 9, 12 h) or (0, 2, 4, 6, 8, 10 d).

(A x T) = Interaction between antimicrobial agent and time of sampling.

All analysis were conducted using the Statistical Analysis System (SAS Institute Inc., Cary, NC.) .

CHAPTER III

RESULTS

Minimum Inhibitory Concentration

Twelve phenolic compounds including: antioxidants, parabens, and natural plant acids, were tested against eight strains of *L. monocytogenes*. The control was a 1% concentration of ethanol in the test medium, tryptose phosphate agar (TPA). Ethanol showed no effect on growth of eight strains of *L. monocytogenes*. Phenolic compounds demonstrated varying inhibitory effects on the foodborne pathogen (Table 1). There was no difference in susceptibility to the phenolic compounds among the eight strains. The most effective phenolic compounds were the antioxidants. Tertiary butylhydroquinone had the lowest MIC of 64 µg/ml; BHA was effective against *L. monocytogenes* at 128 µg/ml, and PG inhibited growth at 256 µg/ml. Propyl paraben, an approved food antimicrobial agent (CFR, 1988) showed inhibition of *L. monocytogenes* at 512 µg/ml. None of the hydroxycinnamic acid derivatives were effective at the highest concentration tested. Tannic acid had a MIC value of 256 µg/ml.

Growth Inhibition in a Model Milk System

Because there were no differences in the susceptibility among *L. monocytogenes* strains in MIC testing, strain Scott A was chosen to determine potential growth inhibition in a model milk system. Scott A is a clinical strain isolated from the 1983 Massachusetts' listeriosis outbreak associated with milk (Fleming et al., 1985).

Based upon their effectiveness in TPA, the agents PP and TBHQ were chosen to test against *L. monocytogenes* in milk. Potassium sorbate, a common antimicrobial agent, was included to demonstrate the ineffectiveness of the organic salt in a neutral pH medium. Growth of *L. monocytogenes* was evaluated at two inocula, 1000 CFU/ml and 10 CFU/ml. All treatments were incubated at temperatures of 35°C and 4°C. The optimum growth temperature for *L. monocytogenes* is 35°C and the optimum storage temperature for milk products is 4°C.

Growth of *L. monocytogenes* in sterile milk at 35°C was monitored for two initial inoculum levels, 1000 CFU/ml and 10 CFU/ml. After 3 h incubation, counts for all treatments remained stationary (Figure 1). Independent of inocula level, the viable cell count in the ethanol control and the potassium sorbate treatment increased approximately 10-fold every 3 h up to 12 h.

For both inoculum levels, there was no significant difference between the ethanol control and potassium sorbate treatment ($P>0.05$). Both treatments reached counts of approximately 7.0 $\log_{10}$ CFU/ml at 24 h.

Propyl paraben and TBHQ treatment caused the lag phase of *L. monocytogenes* to extend throughout the incubation period. For the 1000 CFU inoculum, there was no difference ($P=0.0927$) in the effect of PP or TBHQ. After 24 h, *L. monocytogenes* reached a maximum of 3.7 $\log_{10}$ CFU/ml in the presence of PP, and 4.8 $\log_{10}$ CFU/ml in the presence of TBHQ. For the 10 CFU inoculum, there was a difference ($P=0.0065$) between the effect of PP and TBHQ. Propyl paraben caused the lag phase of *L. monocytogenes* to extend over the first 12 h, and the microorganism reached a maximum population of 2.7 $\log_{10}$ CFU/ml after 24 h. Tertiary butylhydroquinone also extended the lag phase but after 24 h the viable cell count dropped to less than 10 CFU/ml.

Growth of *L. monocytogenes* in sterile milk at 4°C was monitored for two inocula, 1000 CFU/ml and 10 CFU/ml. After 2 d incubation, viable cell count for all treatments remained stationary (Figure 2). At both inoculum levels, there was a difference ($P<0.05$) between the ethanol control and potassium sorbate treatment. Over the 10 d incubation period, the ethanol control

continued to increase steadily reaching 5.7 $\log_{10}$ CFU/ml for the 1000 CFU inoculum and 3.2 $\log_{10}$ CFU/ml for the 10 CFU inoculum. Potassium sorbate treatment did not grow as steadily. After 10 d incubation, it had reached counts of 4.1 $\log_{10}$ and 2.4 $\log_{10}$ CFU/ml, respectively. Although the potassium sorbate treatment showed some inhibition compared to the ethanol control, PP and TBHQ treatments were more effective (P=0.0001) than the potassium sorbate treatment.

There was no difference (P=0.31) between the PP and TBHQ treatments with 1000 CFU/ml inoculum. Both treatments remained stationary throughout the 10 d incubation period. At the 10 CFU/ml inoculum, TBHQ treatment was more effective (P=0.0039) than the PP. After 4 d incubation, TBHQ treatment decreased the viable cell count to less than 10 CFU/ml. The effect of PP treatment at 10 CFU/ml was equal to its effect at 1000 CFU/ml, extending the lag phase of *L. monocytogenes* throughout the 10 d incubation.

CHAPTER IV

DISCUSSION

Measuring the MIC of twelve phenolic compounds against *L. monocytogenes* indicated there was no difference in susceptibility among the eight strains. Antioxidants demonstrated the lowest MIC values. Maysoon et al. (1984) determined that the susceptibility of *L. monocytogenes* to BHA was dependent upon the ratio of unsaturated to saturated fatty acids in the cell membrane. Using a 50 µg/ml concentration of BHA, Maysoon et al. (1984) found the sensitive *L. monocytogenes* strain contained more unsaturated fatty acid and thus maintained a more fluid cell membrane. Our study found 128 µg/ml of BHA was effective against eight strains of *L. monocytogenes* which indicates higher concentration of BHA may overcome resistance of certain strains.

Parabens (CFR, 1988) are approved food antimicrobial agents and therefore would not require further safety testing before approval for use in a new product. The PP treatment was inhibitory against *L. monocytogenes* at 512 µg/ml in the TPA. This inhibition was demonstrated also in the model milk system. Tannic

acid was the only effective natural plant phenolic acid at the concentrations tested. It has a low solubility in water and ethanol (Windhoz, 1983); therefore tannic acid was not used for further testing.

Compounds chosen for growth inhibition in the model milk system against two controls, potassium sorbate and ethanol, were TBHQ and PP. The effect of environmental conditions were determined by using two inoculum levels (1000 and 10 CFU/ml) and two incubation temperatures (35°C and 4°C). Different incubation temperatures did not change efficacy (power to produce effects) of the antimicrobial agents. Both PP and TBHQ were able to extend the lag phase at both incubation temperatures. The level of inoculum did not change the effect of the PP but did effect the TBHQ treatment. The viable cell count at the 10 CFU/ml inoculum with the TBHQ treatment dropped from the initial inoculation to less than 1 CFU/ml.

In addition to stricter sanitation practices during dairy processing, the use of an effective antimicrobial system against *L. monocytogenes* in dairy products would prevent outgrowth of *L. monocytogenes* contamination. The consistent effectiveness of PP in the model milk system gives the basis for further testing in a formulated dairy product.

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APPENDIXES

APPENDIX A

TABLE 1. Minimum inhibitory concentration of phenolic compounds in tryptose phosphate agar for eight strains of *Listeria monocytogenes*[1].

Compound	MIC[2] ug/ml
Butylated Hydroxyanisole[3]	128
Butylated Hydroxytoluene[3]	>512
Tertiary Butylhydroquinone[3]	64
Propyl Gallate[3]	256
Methyl Paraben[4]	>512
Propyl Paraben[4]	512
Coumaric Acid	>512
Ferulic Acid	>512
Caffeic Acid	>512
Quercetin	>205
Tannic Acid	256
Gallic Acid	>512

[1] ATCC 19114 (serotype 4a), ATCC 19155 (serotype 4b), ATCC 19116 (serotype 4c), ATCC 19117 (serotype 4d), ATCC 19118 (serotype 4e), F5027 (serotype 1a), F5067 (serotype 4b), Scott A (serotype 4b).

[2] The MIC was the lowest concentration of each compound which completely prevented the outgrowth of the microorganism after 18 h of incubation at 35°C.

[3] Approved for use in foods as antioxidants by FDA (21CFR sections 172 and 184) and USDA (9CFR 318.7).

[4] Approved for use in foods as antimicrobial by FDA (21CFR section 184). Only propyl paraben is approved by the USDA (9CFR 318.7).

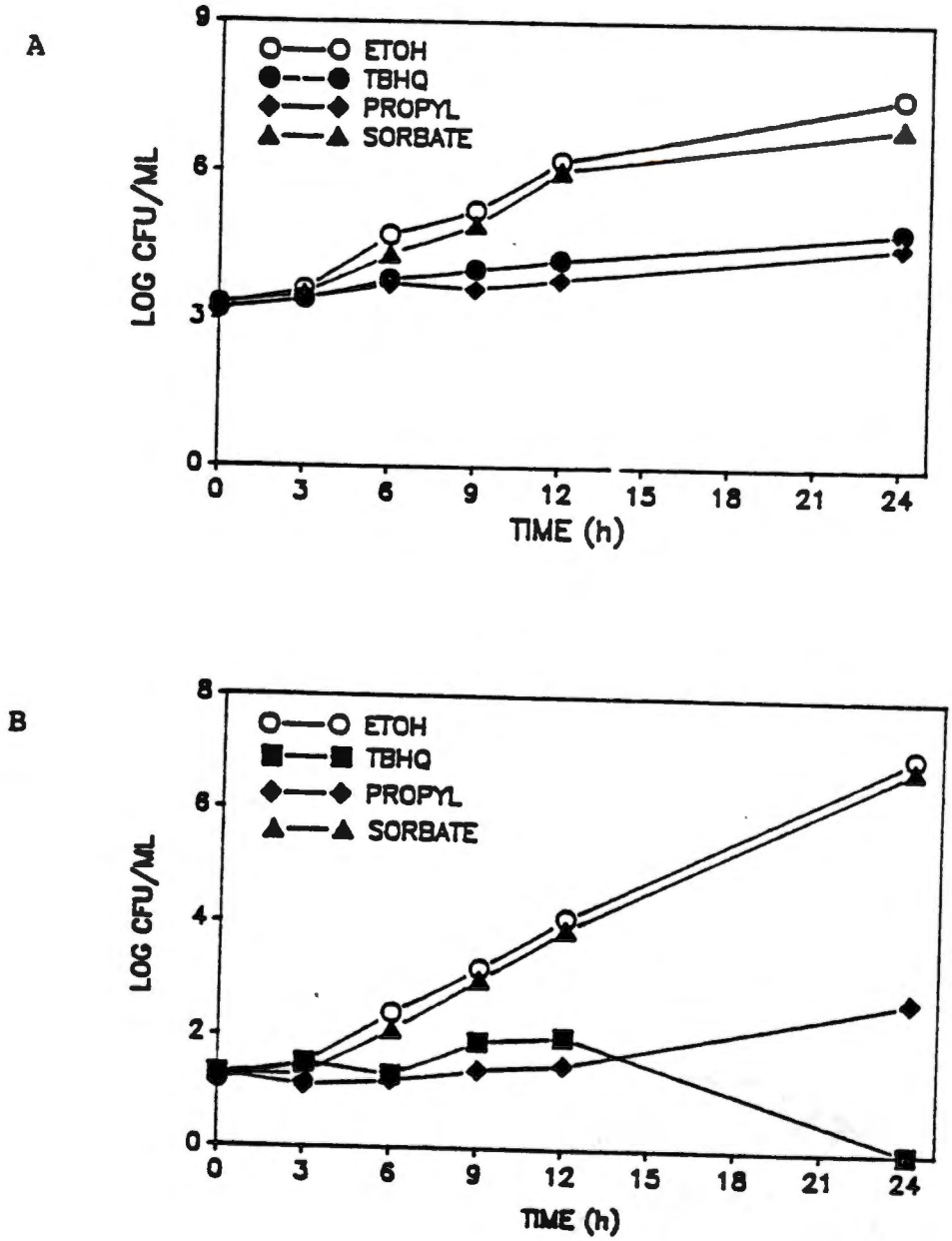


Figure 1. The inhibition of *Listeria monocytogenes* in a model milk system incubated at 35°C for two inocula levels: (A) 10^3 CFU/ml and (B) 10 CFU/ml.

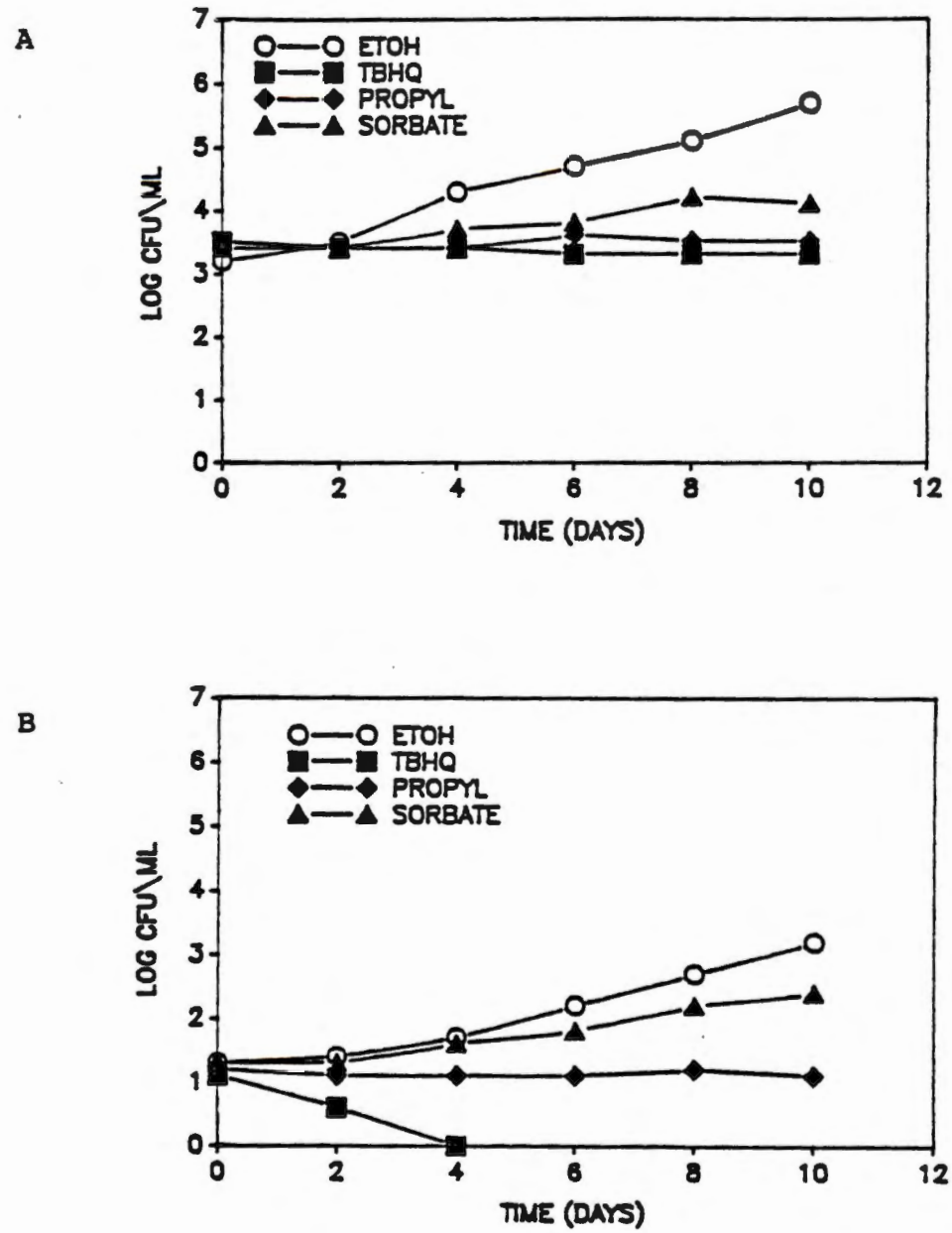


Figure 2. The inhibition of *Listeria monocytogenes* in a model milk system incubated at 4°C for two inocula levels: (A) 10^3 CFU/ml and (B) 10 CFU/ml.

APPENDIX B

Analysis of variance for 1000 CFU/ml inoculum at 35°C.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	31	105.34454306	3.39821107
ERROR	40	6.86794444	0.17169861
CORRECTED TOTAL	71	112.21248750	

MODEL F =	19.79	PR > F = 0.0001	
R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.938795	9.4379	0.41436531	4.39041667

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	2	0.33030833	0.21	0.8163
ANTI[3]	3	25.28621528	10.72	0.0080
ANTI*REP	6	4.71741389	4.58	0.0013
TIME[4]	5	59.67374583	69.51	0.0001
ANTI*TIME	15	15.33685972	5.95	0.0001
CONTRAST STATEMENTS[5]				
P&T V CONT	1	18.25333333	73.53	0.0001
PP V. TBHQ	1	0.44826667	1.81	0.0927
ETOH V SORB	1	0.45926667	1.85	0.0901

[1]Log_{10} CFU/ml.
[2]Replication = 3 (Random variable).
[3]Antimicrobial agents (Random variable).
[4]Time intervals for measuring growth (Fixed variable).
[5]Difference within ANTI treatments at 24 h time.
Individual error rate = 0.017

Analysis of variance for 1000 CFU/ml inoculum at 4°C.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	27	18.82229167	0.69712791
ERROR	20	1.77250000	0.08862500
CORRECTED TOTAL	47	20.59479167	

MODEL F =	7.87	PR > F = 0.0001	
R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.913935	7.9608	0.29769951	3.73958333

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	10.17520833	2.13	0.2409
ANTI[3]	3	7.79729167	31.53	0.0091
ANTI*REP	3	0.24729167	0.93	0.4444
TIME[4]	5	4.06354167	9.17	0.0001
ANTI*TIME	15	6.53895833	4.92	0.0006
CONTRAST STATEMENTS[5]				
P&T V CONT	1	4.35125000	47.58	0.0001
PP V. TBHQ	1	0.02250000	0.25	0.3122
ETOH V SORB	1	2.56000000	27.99	0.0001

[1]Log_{10} CFU/ml.
[2]Replication = 2 (Random variable).
[3]Antimicrobial agents (Random variable).
[4]Time intervals for measuring growth (Fixed variable).
[5]Difference within ANTI treatments at 24 h time.
Individual error rate = 0.017

Analysis of variance for 10 CFU/ml inoculum at 35°C.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	31	220.26430556	7.10530018
ERROR	40	7.09444444	0.17736111
CORRECTED TOTAL	71	227.35875000	

MODEL F =	40.06	PR > F = 0.0001	
R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.968796	19.6261	0.42114263	2.14583333

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	2	2.04083333	5.75	0.5133
ANTI[3]	3	70.07819444	17.10	0.0024
ANTI*REP	6	8.19805556	7.70	0.0001
TIME[4]	5	58.14458333	65.57	0.0001
ANTI*TIME	15	81.80263889	30.75	0.0001
CONTRAST STATEMENTS[5]				
P&T V. CONT	1	117.8133333	326.25	0.0001
PP V. TBHQ	1	2.4066667	6.66	0.0065
ETOH V SORB	1	0.0600000	0.17	0.3430

[1]Log_{10} CFU/ml.
[2]Replication = 3 (Random variable).
[3]Antimicrobial agents (Random variable).
[4]Time intervals for measuring growth (Fixed variable).
[5]Difference within ANTI treatments at 24 h time.
Individual error rate = 0.017

Analysis of variance for 10 CFU/ml inoculum at 4°C.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	27	32.57000000	1.20629630
ERROR	20	1.59000000	0.07950000
CORRECTED TOTAL	47	34.16000000	

MODEL F =	15.17	PR > F = 0.0001	
R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.953454	21.6890	0.28195744	1.30000000

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	1.40083333	9.36	0.0550
ANTI[3]	3	20.71333333	46.12	0.0052
ANTI*REP	3	0.44916667	1.88	0.1649
TIME[4]	5	2.12250000	5.34	0.0028
ANTI*TIME	15	7.88416667	6.61	0.0001
CONTRAST STATEMENTS[5]				
P&T V CONT	1	9.90125000	69.08	0.0001
PP V. TBHQ	1	1.21000000	8.44	0.0039
ETOH V SORBATE	1	0.56250000	3.92	0.0296

[1]Log_{10} CFU/ml.
[2]Replication = 2 (Random variable).
[3]Antimicrobial agents (Random variable).
[4]Time intervals for measuring growth (Fixed variable).
[5]Difference within ANTI treatments at 24 h time.
Individual error rate = 0.017

PART III

INFLUENCE OF BOVINE LACTOFERRIN ON GROWTH OF *LISTERIA MONOCYTOGENES* IN A MODEL MILK SYSTEM

CHAPTER I

PROLUSION

Abstract

The influence of bovine lactoferrin (LF) and Apo-LF on growth of *Listeria monocytogenes* in Ultra-High Temperature (UHT) whole milk was determined. The effect of LF was dependent upon both the degree of iron saturation and concentration. Before iron removal, LF was found to be approximately 52% saturated with iron, and at 23 and 46 mg/ml LF minimal growth inhibition of *L. monocytogenes* was observed. However, after dialysis, Apo-LF iron saturation was reduced to approximately 18%. At 15 and 30 mg/ml Apo-Lf, a bacteriostatic effect against *L. monocytogenes* was observed. Inhibition of growth associated with Apo-LF was abolished when ferric ammonium citrate was added to saturate the iron binding sites of the LF.

Introduction

Antimicrobial agents naturally present in foods have gained attention because of the demand for "all-natural" food products (Beuchaut and Golden, 1989). There are several proteins and enzymes present in milk which have bacteriostatic properties. These include

lactoferrin, lysozyme, and lactoperoxidase (Davidson et al., 1983; Beuchaut and Golden, 1989). Lactoferrin (LF) is an iron-binding protein present in mammalian milk. It is also found in other physiological fluids as well as polymorphonuclear leukocytes (Masson et al., 1969). Each molecule of LF binds two ferric iron cations with two bicarbonate anions (Crichton and Charloteaux-Wauters, 1987).

Lactoferrin is believed to participate in the natural immune defense system present in mammalian milk (Reiter, 1978; Lonnerdal, 1985). *In vitro* studies on the role of human milk in protecting breast-fed infants against enteric infections from enteropathogenic *E. coli* concluded that the interaction of LF and specific immunoglobulins play a significant part in the natural defense system (Spik et al., 1978; Stephens et al., 1980; Brock et al., 1983). Postpartum human milk has approximately 1 mg/ml LF whereas postpartum bovine milk has approximately 0.3 mg/ml LF (Welty et al., 1976). The activity of LF is dependent upon its concentration, its degree of iron saturation (Weinberg, 1978), and the interaction of other milk components (Stephens et al., 1980; Brock et al., 1983; Lonnerdal, 1985). The role of LF in prevention of new coliform intramammary infections in the bovine mammary gland has recently been studied

(Bishop et al, 1976; Nonnecke and Smith, 1984a; Breau and Oliver, 1986; Oliver and Bushe, 1987).

Because infant formulas lack natural defense components of human milk, Reiter (1978) suggested fortifying infant formula with LF and immunoglobulins. The major drawbacks in fortification with LF are complicated and inefficient isolation techniques (Kawakami et al. 1987). Several recent studies (Kawakami et al., 1987; Al-Mashikhi and Nakai, 1987; Al-Mashikhi et al., 1988; Yoshida, 1989) investigated the use of affinity chromatography for large scale isolation of LF from cheese whey. Kawakami et al. (1987) found, using a one-step immunoaffinity column, that pasteurization (65°C for 30 min) of cheddar cheese whey did not significantly effect recovery or iron binding capacity of LF.

Previous studies (Sword, 1966; Trivett and Myer, 1971; Cowart and Foster, 1985) showed a marked increase in growth of *L. monocytogenes* when synthetic growth medium was supplemented with iron. Increased growth of *L. monocytogenes* in an iron-supplemented medium suggests that LF could act as an effective natural antimicrobial agent against *L. monocytogenes*. The iron requirement for growth of *L. monocytogenes*, the result of LF withholding iron from microorganisms, and improved LF

isolation techniques all contribute to the potential for LF being used as a natural antimicrobial agent in dairy products. The purpose of this study was to determine the effect of LF on growth of *L. monocytogenes*.

CHAPTER II

MATERIALS AND METHODS

Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis

The purity of LF was determined using a 10% sodium dodecyl sulfate (SDS) acrylamide slab mini gel electrophoresis procedure (Laemmli, 1970). The LF was dissolved in a solubilizing buffer (20mM Tris acetate, 20% (v\v) glycerol, 20% (w/v) SDS, and 2% (v\v) Beta-mercaptoethanol), and 5 µg were loaded onto the stacking gel. Proteins were separated on the slab gel by electrophoresis at 18 milliamps for 1.5-2.0 h in the presence of a 1% SDS buffer. SDS-polyacrylamide gel electrophoresis (PAGE) molecular weight standards (Bio-rad Laboratories, Richmond, CA) were also run with each LF sample. Standards included: rabbit muscle phosphorylase (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (42,699), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). The slab gel was silver-stained (Bio-Rad Laboratories).

Lactoferrin Preparation

Lactoferrin isolated from bovine colostrum (Sigma Chemical Co., St. Louis, MO) was desaturated using the method of Mazurier and Spik (1980). Acetic acid-sodium acetate buffer (pH 3.8; ionic strength, 0.2 M) containing 40 mM EDTA was used to dissolve LF. A 1% solution of LF and buffer was equilibrated overnight at 4°C. After equilibration, the solution was dialyzed for 3 d against deionized-distilled water. Dialysis tubing had a molecular weight cut-off of 12,000 - 14,000 daltons (Spectrum Medical Industries, Inc., Los Angeles, CA.). The sample was then lyophilized and stored at 4°C.

The amount of iron present in the desaturated sample was measured by an atomic absorption spectrophotometer (Instrumental Laboratories Inc., Wilmington, MA). A 3.0 ml sample was digested in 30.0 ml nitric acid and 3.5 ml perchloric acid, then diluted to a 10.0 ml final volume for measurement. The amount of iron present after dialysis was divided by the theoretical saturation level 1.396 µg Fe/mg protein (Masson and Heremans, 1968) to determine degree of iron saturation.

Test Microorganisms

Six strains of *Listeria monocytogenes*: two raw milk isolates; F5027 (serotype 1a), F5069 (serotype 4b); a high heat resistant isolate, Scott A; and three reference cultures; ATCC 19114 (serotype 4a), ATCC 19115 (serotype 4b), ATCC 19117 (serotype 4d) were all used in the microtiter assay. *Escherichia coli* originally isolated from bovine milk was also used as a positive control. *Listeria monocytogenes* strains were provided by C.W. Donnelly, Dept. of Animal Science, The University of Vermont, Burlington. The *E. coli* strain was provided by S.P. Oliver, Dept. of Animal Science, The University of Tennessee, Knoxville. Each strain was grown in 5 ml tryptose phosphate broth (TPB; Difco laboratories, Detroit, MI) and then streaked on tryptose phosphate agar plates (TPA; Difco) to obtain individual colonies. For each strain, two colonies from the TPA plates were inoculated into 30 ml of UHT milk. *Listeria monocytogenes* strains were incubated at 35°C for 12 h and *E. coli* was incubated in a shaking water bath at 32°C for 6 h. Cells were separated in a centrifuge, and each strain resuspended in 15 ml UHT milk. One-ml aliquots of bacterial cells were mixed with 1 ml of a 50% glycerolsolution in a freezer vial and stored at

-70°C. Viable cell counts for each vial were determined by serial dilution in 0.1% peptone buffer (pH 6.8).

Iron Concentration

To establish initial iron levels, both media (UHT milk and 0.1% peptone diluent) used in the microtiter assay were measured for iron concentration using an atomic absorption spectrophotometer. To minimize iron contamination, all glassware was soaked in 6 N hydrochloric acid bath for 4 h and rinsed 15 times with deionized-distilled water.

Microassay of Bacterial Growth

The microtiter assay system has been described previously (Nonnecke and Smith, 1984a; Nonnecke and Smith, 1984b, Dutt et al, 1986; Breau and Oliver, 1986). Growth inhibition studies were conducted in 96-well, round-bottom microtiter plates (Corning Glass Works, Corning, NY). Each well had a 300 µl capacity. For each test, a total volume of 250 µl was used consisting of 115 µl UHT milk, 125 µl Apo-LF, and 10 µl bacterial inoculum. Samples were measured and dispensed aseptically with micropipettes (Rainin EDP pipetter, Rainin Instrument Co., Inc., Brighton, MA). The stock solution of Apo-LF was dissolved in 0.1% peptone

buffer and filter-sterilized using an Acrodisc (0.2 μm; Gelman Science, Ann Arbor, MI).

It should be noted that the ability of LF to pass through the filter unit changed after iron was removed. No LF was lost when the 52% iron-saturated protein was passed through the filter, whereas approximately 37% Apo-LF was lost during filtration. This change may have been caused by expansion of the protein structure as the iron was released from the binding sites (Anderson et al., 1987). Rosseneu-Motreff et al. (1971) reported that transferrin had a significant conformational change when iron was bound to the protein, making the structure more compact. Future studies with Apo-LF should employ a larger pore-size filter. Bishop et al. (1976) and Nonnecke and Smith (1984a) used a 0.45 μm filtration system.

The concentration (mg/ml) of the stock solution was determined by measuring the absorbance at 280 nm and dividing by the extinction coefficient 1.45 (Castellino et al., 1970). The bacterial inoculum consisted of 50 - 100 CFU/10 μl of UHT milk.

Prepared microtiter plates were covered with a sterile lid and incubated at 35°C for 18 h. After incubation, the number of viable cells in each well was determined in triplicate by the drop method (Meynell and

Meynell, 1965) on TPA plates. All *L. monocytogenes* strains were incubated 18 h. *Escherichia coli* was incubated 12 h. All bacterial strains were incubated at 35°C. Sterile 0.1% peptone buffer was used as diluent.

Statistical Analysis

For each experiment, a randomized complete-block design was used with growth measured as $\log_{10}$ CFU/ml. Each replicate represented one block. Contrast statements were used to determine the most effective concentration of LF. The overall alpha value was 0.05, yielding an individual alpha value of 0.0127 for each contrast. The model statement was:

$$G = C + S + (C \times S) + B \text{ , where}$$

G = Bacterial growth ($\log_{10}$ CFU/ml) in UHT milk

C = Concentration of Apo-LF

S = Strain of *L. monocytogenes* or *E. coli*

(C x S) = Interaction between the concentration and strain

B = Blocks on replications of each assay

All analyses were conducted using the Statistical Analysis System (SAS Institute, Inc., Cary, NC).

CHAPTER III

RESULTS

Purity of the Apo-LF was analyzed by SDS-PAGE. The major component from the bovine colostrum LF (Sigma Chemical Co.) was the LF protein (Figure 1). After measuring the amount of iron present in each LF sample, iron saturation of LF was determined theoretically using 0.025 μmoles Fe/mg of LF (Masson and Heremans, 1968).

Studies were conducted to investigate the influence of LF on growth of *L. monocytogenes* before and after iron was removed from LF. Initial studies examined the effect of LF concentration before iron removal on growth of two *L. monocytogenes* strains, Scott A and ATCC 19115. Approximately 52% of the LF was iron bound. Final concentrations of LF in the assay were: 46, 23, 4.6, and 0.46 mg/ml. The initial inoculum for both strains was approximately 3 $\log_{10}$ CFU/ml (Table 1). The control for both strains, UHT milk without LF, reached an average viable cell count of 10.5 $\log_{10}$ CFU/ml after 18 h incubation at 35°C. Samples with the lower concentrations, 4.6 and 0.46 of mg/ml LF, had viable cell counts equivalent to the control. The two higher concentrations, 46 and 23 mg/ml LF, in comparison to the

control, reduced the growth of both *Listeria* strains 16% and 14%, respectively. There was no difference (P=0.16) in replication of the assay.

The next study examined effects of LF concentration after iron removal (Apo-LF) on growth of *L. monocytogenes* using four different strains; two raw milk isolates, F5069 (serotype 4b) and F5027 (serotype 1a), and two reference cultures, ATCC 19114 (serotype 4a) and ATCC 19117 (serotype 4b). Approximately 18% of Apo-LF was iron bound. Final concentrations of Apo-LF in the assay were: 30, 15, 3, and 0.3 mg/ml. The initial inoculum for all strains was approximately 3 $\log_{10}$ CFU/ml (Table 2). Two controls were used in the assay; UHT milk without LF was a negative control for each strain, and an *E. coli* strain was used as a positive control at each concentration of LF. The UHT milk without Apo-LF showed a 10,000-fold increase in viable cell counts of all test strains after 18 h incubation at 35°C. *Escherichia coli* was inhibited at 30 mg/ml Apo-LF, the viable cell counts being < 10 CFU/ml. Growth inhibition of all *Listeria* strains was observed as the concentration of Apo-LF increased. Apo-LF concentrations, 0.3 and 3 mg/ml, had viable cell counts equivalent to the control, 7.81 $\log_{10}$ CFU/ml. At 15 mg/ml of Apo-LF, growth of all strains of *L.*

monocytogenes was inhibited; the viable cell count remained at the initial inoculum level. Greatest growth inhibition was shown at 30 mg/ml Apo-LF, where all strains had viable cell counts of 1.82 - 2.54 $\log_{10}$ CFU/ml. At 30 mg/ml Apo-LF, there was no difference (P=0.10) in the effect among the 4 strains of *L. monocytogenes* tested. There was no difference (P=0.20) in replication of the assay.

To demonstrate that growth inhibition of *L. monocytogenes* was related directly to iron availability in the medium, iron was added to the growth medium in an attempt to abolish inhibition by Apo-LF. The amount of iron added to the medium was determined by calculating the theoretical number of iron binding sites for 50 mg/ml LF. Apo-LF (30 mg/ml) was added to both UHT milk and UHT milk with 0.125 M ferric ammonium citrate. The pH of the UHT milk was 6.4 without iron supplementation and 6.7 with the addition of iron. Because there was no difference in the 4 strains of *L. monocytogenes* tested originally, one milk isolate and one reference culture strain was evaluated. Two controls were used; UHT milk and UHT milk plus excess iron. Both controls increased 10,000-fold after 18 h incubation at 35°C (Table 3). Apo-LF (30 mg/ml) without ferric ammonium citrate was bacteriostatic against both strains of *Listeria*,

reducing the viable cell counts below the initial inoculum, 3 $\log_{10}$ CFU/ml. The same bacteriostatic effect was observed with 30 mg/ml Apo-Lf in the previous study. However, when 0.125 M ferric ammonium citrate was added to 30 mg/ml Apo-LF growth inhibition of *L. monocytogenes* was abolished. The viable cell counts were equivalent to controls. There was no difference (P=0.94) in replication of the assay.

CHAPTER IV

DISCUSSION

Influence of bovine LF on growth of *L. monocytogenes* was dependent upon iron saturation and concentration. Before iron removal, LF was approximately 52% iron saturated. At this iron level, concentrations of 23 and 46 mg/ml were needed to reduce *Listeria* growth. Even at these high concentrations, growth inhibition was minimal. After iron removal, Apo-LF was only 18% iron saturated and a greater effect was observed on bacterial growth. As the Apo-LF concentration increased, a bacteriostatic effect was observed. At 15 mg/ml Apo-LF, viable cell counts remained at the initial inoculum, 3 $\log_{10}$ CFU/ml. At 30 mg/ml LF, as much as a 10-fold reduction from the initial inoculum level was present.

Inhibition of growth associated with Apo-LF was abolished when excess ferric ammonium citrate was added to saturate iron binding sites on the LF molecule. This reversal of growth inhibition indicates that bacteriostasis resulted from LF withholding free iron necessary for microbial growth.

The 52% iron saturated LF at 46 mg/ml theoretically had 518 nmoles iron-binding sites per ml, which was 70 times the amount of iron present in the milk. Apo-LF, at 18% iron saturation and 30 mg/ml, theoretically had 598 nmoles iron binding sites per ml, which was 81 times the amount of iron present in the milk. Even though iron removal did not change the theoretical iron binding capacity of the LF, inhibitory effects were different. Nonnecke and Smith (1984a) also found iron-saturated LF to be ineffective against growth of coliform bacteria, but the degree of iron saturation was not reported.

Previous studies have also found bacterial growth inhibition in the presence of Apo-LF. (Nonnecke and Smith, 1984a; Bishop et al., 1976). Nonnecke and Smith (1984a) found that Apo-LF at concentrations of 15 and 30 mg/ml were less inhibitory than 5 mg/ml. Bishop et al. (1976) found 5 mg/ml Apo-LF to be the most effective concentration. However, both Nonnecke and Smith (1984a) and Bishop et al. (1976) used a synthetic medium which contained 1.8 - 2.7 μM iron, whereas in the present study UHT milk was used as the growth medium and contained 7.5 μM iron. Bishop et al. (1976) calculated the theoretical number of iron binding sites for 5 mg/ml at 107 nmoles of iron-binding sites per ml, or 50 times the amount of iron present in the synthetic medium.

With 7.5 μM iron in UHT milk, concentrations of 15 and 30 mg/ml Apo-LF were required to provide an iron-binding capacity of 41 and 81 times the amount of iron present in the UHT milk. Therefore, the higher concentration of Apo-LF had the same iron binding capacity as the lower concentrations used in previous studies (Nonnecke and Smith, 1984a; Bishop et al., 1976).

There are several other proteins and enzymes present in milk that have bacteriostatic properties (Reiter, 1978) and could influence bacteriostatic behavior of LF. Immunoglobulins are a part of the antimicrobial system in milk. They have been shown *in vitro* to enhance the bacteriostatic effect of LF (Spik et al., 1978; Stephens et al., 1980; Nonnecke and Smith, 1984b). Stephens et al. (1980) demonstrated that this *in vitro* effect was based on antibody specificity for a microbial pathogen. Bovine IgG1 in combination with LF enhanced inhibition against a strain of *E. coli* that was pathogenic to calves, but bovine IgG1 and LF tested against human pathogenic strains had no synergistic effect.

Lysozyme is also present in milk, and can be effective in inhibiting microbial growth (Reiter, 1978). Milk lysozyme has been shown to be more effective than albumin lysozyme unless a chelator is added to the

albumin (Vakil et al., 1969). Hughey and Johnson (1987) used albumin lysozyme against 4 strains of *L. monocytogenes* and found no inhibitory action without the addition of 1 mM EDTA. The required presence of a chelating agent for albumin lysozyme to lyse microbial cells suggests that the combination of milk lysozyme and lactoferrin could provide a synergistic antimicrobial effect.

The survival of microorganisms in the presence of iron-binding proteins such as LF depends upon their ability to synthesize iron-sequestering compounds. Such compounds are generally termed siderophores. There are over two hundred natural siderophores that have been classified into two general categories, hydroxamates and phenolates (or catecholates) (Crichton and Charloteaux-Wauters, 1987). The ability of microorganisms to produce a siderophore is considered to be a virulence factor (Weinberg, 1971; Finkelstein et al., 1983). Another iron-sequestering system similar to siderophores is the citrate-mediated system found in *E. coli* (Bagg and Neilands, 1987). Citrate is not a very effective chelating agent unless present in high concentrations. However, it is considered a high-affinity iron carrier for *E. coli* because of the specific receptor on the outer membrane of the bacteria (Bagg and Neilands,

1987). The antimicrobial action of LF has been shown to be dependent upon the molar ratio of citrate to LF for coliform bacteria (Bishop et al., 1976; Nonnecke and Smith, 1984a; Oliver and Bushe, 1987). Oliver and Bushe (1987) found the molar ratio of citrate to LF to be > 1000 in both normal bovine milk and colostrum, and then decrease to approximately 10 in dry secretions. Reiter et al. (1975) and Griffiths and Humphreys (1977) were able to neutralize the citrate-mediated system *in vitro* with an excess concentration of bicarbonate anions. The specific mechanism of iron acquisition for *L. monocytogenes* is not known. Cowart and Foster (1985) were not able to identify an iron transport system, but they only assayed for two general compounds, hydroxamates and phenolates.

Results of the present study indicate Apo-LF is bacteriostatic against *L. monocytogenes* grown in UHT milk. Further investigations could improve inhibitory effects against *L. monocytogenes* when other milk components such as immunoglobulins and lysozyme are included with Apo-LF. A major drawback to further evaluation is cost of LF and the limited commercial availability (Kawakami et al., 1987). Improving the cost efficiency of LF isolation and development of low-cost LF sources, such as cheese whey, would enhance the

potential use of lactoferrin as a future natural antimicrobial agent against *L. monocytogenes*.

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APPENDIXES

APPENDIX A

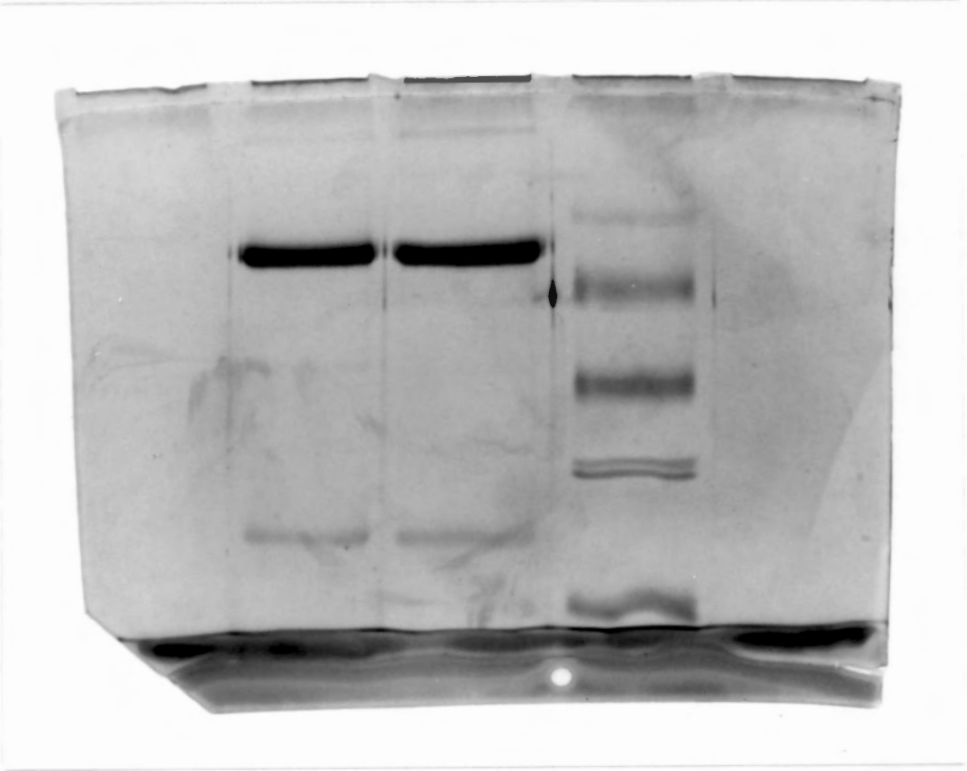


Figure 1. Purity of bovine lactoferrin (Sigma Chemical Co.) on a 10% sodium dodecyl sulfate mini-slab gel.
(A) Lactoferrin before dialysis
(B) Lactoferrin after dialysis
(C) Molecular weight standards: 97,400, 66,200, 42,699, 31,000, 21,500, 14,400.

TABLE 1. Influence of 52% iron saturated LF on growth of *Listeria monocytogenes* in UHT Milk.[a]

Strain	LF (mg/ml)[b]				
	46	23	2.3	0.23	0
Scott A	8.68	9.01	9.81	10.1	10.6
SEM	0.06	0.06	0.02	0.12	0.05
ATCC 19115	8.91	9.06	10.1	10.5	10.3
SEM	0.20	0.20	0.021	0.14	0.13

Initial inoculum approximately $\log_{10}$ 3.0 CFU/ml.

[a] Data are expressed as mean $\pm$ SEM $\log_{10}$ CFU/ml.
[b] For all concentrations N=6.

TABLE 2. Influence of 18% iron saturated Apo-LF on growth of *Listeria monocytogenes* in UHT Milk.[a]

Strain	Apo-LF (mg/ml)[b] 30	15	3.0	0.3	0	Initial Inoculum
Milk isolates						
F5027	2.54	3.24	8.01	8.73	8.34	4.05
SEM	0.05	0.05	0.04	0.03	0.03	
F5069	2.01	4.84	7.59	7.79	7.36	3.53
SEM	0.17	0.04	0.05	0.05	0.03	
Ref. cultures						
ATCC 19114	1.82	3.19	7.69	8.18	7.96	3.60
SEM	0.04	0.13	0.07	0.14	0.17	
ATCC 19117	2.24	4.27	7.71	8.00	7.58	3.52
SEM	0.15	0.18	0.08	0.04	0.03	
Milk isolate						
E. coli	<1.00	8.83	8.94	8.84	8.97	3.15
SEM	0.00	0.03	0.02	0.08	0.02	

[a] Data expressed as mean ± SEM $\log_{10}$ CFU/ml.
[b] Mean based on N=2 for 30 mg/ml and N=6 for all other concentrations.

TABLE 3. Influence of Apo-LF on growth of *Listeria monocytogenes* in UHT Milk following addition of ferric ammonium citrate.[a]

Strain	Initial inoculum	Mean Growth (Log CFU/ml)[b] UHT milk Control	UHT milk + iron	Apo-LF + iron	Apo-LF 30 mg/ml
Milk isolate					
F5069	3.60	7.40	7.50	6.82	2.13
SEM		0.03	0.04	0.05	0.02
Ref. culture					
ATCC 19114	3.30	7.65	7.85	7.45	1.72
SEM		0.02	0.05	0.02	0.13
Milk isolate					
E. coli	3.00	8.91	8.85	7.76	1.00
SEM		0.02	0.04	0.02	0.00

[a] Data expressed as mean ± SEM log CFU/ml.
[b] Mean based on N=2 for 30 mg/ml LF and N=6 for all other samples.

APPENDIX B

THEORETICAL SATURATION OF APO-LACTOFERRIN

IRON CONCENTRATION OF LF (LOT # 029F8030)

Before dialysis = 0.152 µg Fe/ ml solution

After dialysis = 0.055 µg Fe/ ml solution

0.1% peptone diluent = 0.036 µg Fe/ ml solution

Percent Iron Removed from LF during Dialysis:

$$\frac{0.152 \text{ ug Fe}}{\text{ml sol'n}} \times \frac{\text{ml}}{0.21 \text{ mg LF}^1} = 0.724 \text{ µg Fe / mg LF}$$

$$\frac{0.055 \text{ ug Fe}}{\text{ml sol'n}} \times \frac{\text{ml}}{0.22 \text{ mg LF}^1} = 0.250 \text{ µg Fe / mg LF}$$

[1] Protein concentration calculated by taking absorbancy at 280 nm and dividing by the extinction coefficient, 1.45.

Percent Fe Saturation of Apo-LF:

Masson & Heremans (1968) determined 0.025 umoles of Fe were present for every mg LF at saturation.

$$\frac{0.025 \text{ µmoles Fe}}{\text{mg LF}} \times \frac{55.85 \text{ µg Fe}}{\text{µmoles Fe}} = 1.369 \text{ µg Fe / mg LF}$$

Theoretical saturation for LF lot # 039F8030:

BEFORE DIALYSIS $\frac{0.724 \text{ µg Fe}}{1.396 \text{ µg Fe}} \times 100 = 52 \%$ saturation

AFTER DIALYSIS $\frac{0.25 \text{ µg Fe}}{1.396 \text{ µg Fe}} \times 100 = 17.9 \%$ saturation

THEORETICAL IRON BINDING CAPACITY OF LACTOFERRIN

PART I. Influence of LF concentration before and after dialysis on growth of *Listeria monocytogenes* in UHT milk.

Amount of Iron Present in Assay:

$$\text{UHT milk} = \frac{0.852\ \mu g\ Fe}{ml} \times \frac{0.115\ ml}{well} = 0.098\ \mu g\ Fe$$

$$\text{0.1\% peptone} = \frac{0.036\ \mu g\ Fe}{ml} \times \frac{0.135\ ml}{well} = 0.0049\ \mu g\ Fe$$

$$\text{Total Fe in assay} = \frac{0.098\ \mu g + 0.0049\ \mu g}{0.250\ ml} = 0.411\ \mu g/ml$$

$$\frac{0.411\ \mu g\ Fe}{ml\ sol'n} \times \frac{\mu moles\ Fe}{55.85\ \mu g} \times \frac{1000\ ml}{L} = 7.73\ \mu M$$

Iron Binding Capacity of LF

LF[a] AT 52% IRON SATURATION

Theor. (mg/ml)	Actual (mg/ml)	Unsat.[1] (mg/ml)	IBS[2] (nmol/ml)	IBC[3] (μM IBS:μM Fe)
50	46	22	518	70X
25	23	11	259	35X
5	4.6	2.2	26	3.5X
0.5	0.46	0.22	3	0.35

[a] The actual concentration of LF BEFORE and AFTER filter sterilization was 92 mg/ml.

APO-LF[b] AT 18% IRON SATURATION

Theor. (mg/ml)	Actual (mg/ml)	Unsat. (mg/ml)	IBS (nmol/ml)	IBC (μM IBS: μM Fe)
50	31	25.4	598	81X
25	15.5	12.7	299	41X
5	3.1	2.6	60	8X
0.5	0.3	0.26	6	0.8X

[b] 37% loss of protein during filtration. Concentration of Apo-LF AFTER filter-sterilization was 61.7 mg/ml.

- -

1 [Actual conc.-(Actual conc.x % saturated)] = unsat. conc.

2 IBS (iron binding sites) is nmoles of iron theoretically bound by the LF.

Calculations were as follows:

$$\frac{\text{mg LF}}{\text{ml}} \times \frac{\text{mmole LF}}{85{,}000\ \text{mg LF}} \times \frac{2\ \text{Fe binding sites}}{\text{molecules LF}} \times \frac{6.024 \times 10^{23}\ \text{molecules}}{\text{mole}}$$

$$\frac{1\ \text{g}}{1000\ \text{mg}} \times \frac{\text{moles}}{6.024 \times 10^{23}} \times \frac{10^{9}\ \text{nmoles}}{\text{mole}} = \frac{\text{nmoles of Fe bound}}{\text{ml of LF}}$$

3 IBC (iron binding capacity) = (IBS μM* / 15.9 μM Fe in assay)

*Converted nmoles IBS to μM IBS as follows:

$$\frac{\text{nmoles Fe}}{\text{ml}} \times \frac{10^{3}\ \text{ml}}{\text{L}} \times \frac{\mu\text{mole}}{10^{3}\ \text{nmoles}} = \mu\text{M binding sites of LF}$$

PART II. Abolishment of *Listeria monocytogenes* growth inhibition by Apo-LF.

Amount of Ferric Ammonium Citrate for saturation of LF.

$$\frac{0.025\ \mu mole\ Fe}{mg\ LF} \times \frac{50\ mg\ LF}{ml} \times \frac{ml}{10^3\ \mu l} = \frac{0.00125\ \mu mole}{\mu l} \times \frac{250\ \mu l}{well}$$

$$= \frac{0.3125\ \mu mole\ Fe}{well} \times \frac{mole}{10^6\ \mu mole} = 3.125 \times 10^{-7} \times 100\text{-fold extra}$$

$$= 3.125 \times 10^{-5}\ mole/\ well$$

A stock sol'n of 3.125×10^{-4} mole and then 1:10 dilution with the UHT milk to give final concentration.

$$\frac{3.125 \times 10^{-4}\ mole}{L} \times \frac{353\ g}{mole} \times \frac{L}{10^3\ ml} = \frac{0.00011\ g}{ml} = \frac{0.110\ mg}{ml}$$

Amount of iron present in assay:

$$\text{UHT milk} = \frac{0.852\ \mu g\ Fe}{ml} \times \frac{0.115\ ml}{well} = 0.098\ \mu g\ Fe$$

$$\text{0.1\% peptone} = \frac{0.052\ \mu g}{ml} \times \frac{0.135\ ml}{well} = 0.007\ \mu g\ Fe$$

$$\text{Total Fe in assay} = \frac{0.098\ \mu g + 0.007\ \mu g}{0.250\ ml} = 0.420\ \mu g\ Fe$$

$$\frac{0.420\ \mu g\ Fe}{ml} \times \frac{\mu moles\ Fe}{55.85\ \mu g} \times \frac{1000\ ml}{L} = 7.52\ \mu M$$

Iron Binding Capacity of APO-LF

18% Iron Saturation

Theor. (mg/ml)	Actual (mg/ml)	Unsat. (mg/ml)	IBS (nmol/ml)	IBC (μM IBS: μM Fe)
50	33.3	27.3	642	85X

Actual concentration of APO-LF AFTER filter-sterilization was 66.5 mg/ml LF.

APPENDIX C

Analysis of variance for influence of 52 % saturated LF.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	10	26.56345478	2.65634548
ERROR	46	5.16235926	0.11222520
CORRECTED TOTAL	56	31.72581404	

MODEL F =	23.67	PR > F = 0.0001	
R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.837282	3.4399	0.33500030	9.73877192

SOURCE*	DF	TYPE III SS	F VALUE	PR > F
CONC[2]	4	25.007404075	5.71	0.0001
STRAIN[3]	1	0.30615474	2.76	0.1054
C*S	4	0.83479279	1.86	0.1337
REP[4]	1	0.22555741	2.01	0.1630

* All fixed variables.
[1]Log CFU/ml.
[2]Lactoferrin concentration (mg/ml).
[3]*L. monocytogenes*.
[4]Replication = 2.

Analysis of variance for Influence of Apo-LF .

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	25	684.61983231	27.38479329
ERROR	104	2.19401077	0.02109626
CORRECTED TOTAL	129	686.81384308	
MODEL F =		1298.09	PR > F = 0.0

R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.996806	2.1127	0.14524551	6.87476923

SOURCE*	DF	TYPE III SS	F VALUE	PR > F
CONC[2]	4	525.43815641	6226.68	0.0
STRAIN[3]	4	24.86490286	294.66	0.0001
C*S	16	99.47503590	294.71	0.0001
REP[4]	1	0.03588923	1.70	0.1950
CONTRAST STATEMENTS[5]				
L.M. V. E.C.	1	6.4496033	303.69	0.0001
STRAIN DIFF.	1	0.0352666	1.66	0.1002
ISOLATES	1	2.85187500	134.29	0.0001
REFERENCE	1	0.42940833	20.22	0.0001

* All fixed variables.
[1]Log CFU/ml.
[2]Lactoferrin concentration (mg/ml).
[3]*L. monocytogenes* or *E. coli*.
[4]Replication = 2.
[5]Difference within STRAIN treatment at 30 mg/ml LF.
Individual error rate = 0.013

Analysis of variance for Abolishment of LF activity.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: GROWTH[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	12	190.92343943	15.91028662
ERROR	46	0.31694023	0.00689000
CORRECTED TOTAL	58	191.24037966	

MODEL F =	2309.18	PR > F = 0.0001	
R-SQUARE	C.V.	ROOT MSE	GROWTH MEAN
0.998343	1.1392	0.08300605	7.28627119

SOURCE*	DF	TYPE III SS	F VALUE	PR > F
TREATMENTS[2]	3	163.08009000	7889.69	0.0001
STRAIN[3]	2	2.87644391	208.74	0.0001
C*S	6	4.97923135	120.45	0.0001
REP[4]	1	0.00004310	0.01	0.9373

* All fixed variables.
[1]Log CFU/ml.
[2]With and without supplementation of Fe^{3+} ammonium citrate.
[3]*L. monocytogenes* or *E. coli*
[4]Replication = 2

VITA

Kimberli Dale Payne was born on May 3, 1964 in Manhattan, Kansas. Her parents were Henry Dale and Patricia Arnold Payne. After graduating from Labette County High School in 1982, she entered Northeastern Oklahoma A&M College in the Fall of 1982 and received an Associate of Science Degree in Medical Technology, Spring of 1984. She then attended Kansas State University and completed a Bachelor of Science Degree in Food Science, Spring of 1987. Receiving a Graduate Research Assistantship at the University of Tennessee - Knoxville, the author began her graduate studies in the Fall of 1987. She was awarded a Master of Science Degree in Food Technology and Science in December of 1989.

The author is a member of the Institute of Food Technology, as well as two honor societies: Gamma Sigma Delta and Sigma Xi.