Use of the milk protein lactoferrin as a natural antimicrobial in meat products

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Submitted to the Faculty of Graduate Studies

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By

Anas Al-Nabulsi

In Partial Fulfillment of the

Requirements for the Degree of

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FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

OF

DOCTOR OF PHILOSOPHY

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DEDICATION

This thesis is dedicated to my wife Rana and my children Zaid, Abdelrhman and Sanad as well as my mother and my father for their continuous support, encouragement, and numerous sacrifices to provide adequate education for me.

Forward

The thesis is organized to include four manuscripts that have been submitted for journal publication (chapters 3-6). The manuscripts are in various stages of publication which are indicated here. These chapters are presented as originally submitted for publication, with minor changes to format for the purposes of standardizing presentation of this thesis. Chapter 1 gives an overall introduction to the thesis, Chapter 2 is a comprehensive review of related work and a conclusion is presented as Chapter 7 followed by bibliographic references. I, Anas Al-Nabulsi, was the primary author and experimenter for all the work described in the following chapters.

Chapter 3 was originally published as "Effect of bovine lactoferrin against *Carnobacterium viridans*" by Anas A. Al-Nabulsi and Richard A. Holley (2005) in the journal Food Microbiology, 22:179-187.

Chapter 4 was originally published as "Enhancing the bactericidal effects of lactoferrin against *E.coli* O157:H7 by chelation, temperature and NaCl" by Anas A. Al-Nabulsi and Richard A. Holley (2006) in the Journal of Applied Microbiology, 100: 244-255.

Chapter 5 "Temperature sensitive microcapsules containing lactoferrin and their action against *Carnobacterium viridans* on bologna" by Anas A. Al-Nabulsi, Jung H. Han, Zhigiang Liu, Evangelina T. Rodrigues-Vieira and Richard A. Holley was submitted to the Journal of Food Science on Feb.22, 2006.

Chapter 6 "Activity of bovine lactoferrin against *Escherichia coli* O157:H7 strains and meat starter cultures in broth and during dry sausage manufacture following its microencapsulation" by A. Al-Nabulsi and R. Holley. (2006) was submitted to the International Journal of Food Microbiology on Jan. 16, 2006.

Abstract

Initial evaluation of factors influencing the antimicrobial activity of lactoferrin (LF) against the psychrotrophic meat spoilage organism *Carnobacterium viridans* and the pathogen *E.coli* O157:H7 were conducted in All Purpose Tween (APT) or Lauria Broth (LB). NaCl at levels found in cured meats as well as several compounds likely to interact positively with LF against bacteria were examined. Lactoferrin alone (8 mg/ml) killed 4 $\log_{10}$ CFU *Carnobacterium viridans*/ml at 4 °C, 10 °C and 30 °C in both APT and LB broth, but when 2.5% NaCl was added, the antimicrobial effect became bacteriostatic. At 2.5% NaCl (while $\leq$ 160 mM sodium bicarbonate, SB, had no effect on LF activity), the addition of 5 mg/ml sodium hexametaphosphate (SHMP) restored LF lethal action, but only in APT broth and only at 30 °C. Since both 2.5% NaCl or $\leq$ 5% w/v sodium lactate (SL) in LB broth with 0.5% NaCl reduced the antimicrobial activity of LF, it was concluded that increases in medium osmolarity due to salt addition reduced LF access to membrane binding sites following contraction of the cell membrane.

In contrast to *C.viridans*, 2.5% NaCl enhanced the antibacterial activity of LF against *E.coli* O157:H7. Furthermore, the antibacterial activity of LF with 2.5% NaCl at 10 °C was found to be strain dependent. LF alone was bacteriostatic against strains 3081 and LCDC 7283 but 3 other strains grew. Antimicrobial effectiveness of LF was reduced in APT broth, but was enhanced by SB with 2.5% NaCl at 10 °C where 4.0 $\log_{10}$ CFU/ml inoculated cells were killed. The addition of EDTA enhanced the antimicrobial action of the LF-SB combination. SL alone was effective against *E.coli* O157:H7 but a reduction in activity with 2.5% NaCl at 10 °C was reversed by LF. The combinations LF-SHMP and LF-quercetin were more effective at 37 °C and NaCl effects varied.

To overcome the negative effects of divalent cations and other compounds on the antimicrobial activity of LF against the tested organisms, LF was incorporated in two types of microcapsules. First, paste-like microcapsules were prepared as a water-in-oil (W_1/O) emulsion from a mixture of 20% w/v LF in distilled water, 3% w/v SL or 20 mM SB, which was emulsified with an oil mixture of 22% butter fat plus 78% corn oil and 0.1% polyglycerol polyricinoleate. Second, freeze-dried double emulsion ($W_1/O/W_2$), powdered microcapsules were produced following emulsification of paste-like microcapsules in an external aqueous phase (W_2) consisting of a denatured whey protein isolate solution, WPI. The release of LF from the W_1/O microcapsules was dependent on temperature and NaCl concentration. LF was not released from the W_1/O emulsion at $<$ 5.5 °C. At $\geq$ 10 °C, its release was greater from W_1/O microcapsules when suspended in 5% aqueous NaCl than in water, whereas LF release from freeze-dried microcapsules was not controlled by temperature change. Paste-like microcapsules were incorporated in edible WPI packaging film to test the antimicrobial activity of LF against *C. viridans*. The film was applied to the surface of bologna after its inoculation with the organism and stored under vacuum at 4 or 10 °C for 28 d. The growth of *C.viridans* was delayed at both temperatures and microencapsulated LF had greater antimicrobial activity than when unencapsulated. The temperature sensitive property of the W_1/O microcapsules was reduced when they were incorporated in the WPI film.

The antimicrobial activity of LF against five non-pathogenic strains of *E. coli* O157:H7 and 7 meat starter cultures was assessed in a broth system under conditions similar to those used in the production of dry fermented sausages. LF alone was bacteriostatic against *E. coli* O157:H7 strains 0627 and 0628 but 3 other strains grew in

the presence of 2.9% NaCl in LB broth at 13 or 26 °C after 5d or 24h, respectively. However, the addition of EDTA to the reaction mixture enhanced its effects. LF alone did not affect the growth of meat starter cultures in APT broth under the same conditions. However, when LF plus EDTA and SB were used the growth of all meat starter cultures except *Lactobacillus* (*L*) *curvatus* was reduced. The resistant starter culture (*L. curvatus*) plus *Staphylococcus carnosus* were used to ferment sausages, inoculated with *E.coli* O157:H7 strains 0627 and 0628, containing microencapsulated LF (the paste-like or dried powder form) with or without EDTA and SB as well as unencapsulated LF. The reduction of *E. coli* O157:H7 was significantly higher ($p< 0.05$) in treatments containing LF and the largest reduction (4.2 log) was obtained when unencapsulated LF was used. However, some of this reduction in both treatments was due to cell injury (and not lethality) since significantly greater numbers of cells were recovered on APT overlaid with the selective medium ct-SMAC than on ct-SMAC alone. These results suggested that unencapsulated LF can cause significant reductions in the viability of some strains of *E.coli* O157:H7 (if present < 3 log cfu/g) in dry fermented sausages and thus enhance its safety.

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Chapter 1

Introduction

Interest has arisen recently in the possible use of lactoferrin (LF) for the surface decontamination of beef carcasses and its possible use as a natural food preservative. Lactoferrin (LF) is the main iron-glycoprotein present in the milk of various mammals and it exerts an antimicrobial effect against a wide range of Gram-negative and Gram-positive bacteria, fungi, and parasites (Shimazaki 2000). In addition, LF has antioxidant, antiviral, anti-inflammatory, immune-modulating, anti-cancer effects and can promote the growth of probiotic bacteria like *Bifidobacterium* (Aguila and Brock 2001).

LF has been shown to be bacteriostatic due to its ability to bind iron and limit its availability in the growth environment. LF can be bactericidal by binding to the surface of Gram-negative bacteria and causing release of lipopolysaccaride (LPS) (Ellison et al. 1988). In addition, LF is reported to have bactericidal effects against Gram-positive bacteria by binding to lipomannan, which is present on the surface of *Micrococcus luteus* or by binding to proteins on the surface of *Clostridium perfringens* (De Lillo et al. 1997; Tomito et al. 1998).

Although LF has the potential to be used as a natural antimicrobial preservative in the food industry, the success reported in simple broth systems such as peptone or distilled water and buffered phosphate has not been seen in foods because the antimicrobial activity of LF is reduced in the presence of divalent cations (calcium and magnesium) at concentrations between 1-5 mM (Ellison et al. 1988). Divalent cations protect bacteria from LF by inducing changes in its tertiary structure which yield

tetramers with reduced biofunctionality (Shimazaki 2000). Therefore, there is a need to find alternative methods for application of LF to foods that will eliminate the inhibitory effect of cations and stabilize LF activity.

Lactoferricin (LFcin) which contains shorter peptides (< 6 kDa compared to the 80 kDa of LF) generated by porcine pepsin digestion of LF has been shown to exert 9 to 25 times more bactericidal activity than native LF against both Gram-negative and positive bacteria, including strains that were resistant to native LF (Tomito et al.1991; Chantaysakorn and Richter 2000). However, the presence of NaCl or KCl within the range of 25-100mM and 1.0-5.0 mM of $CaCl_2$ or $MgCl_2$ in the growth media also inhibited the antibacterial activity of LFcin (Bellamy et al. 1992b).

Yamauchi et al. (1993) reported that LF is more stable than LFcin in the presence of divalent cations. They noted that more LPS was released by LFcin B (bovine) than by LF from *Salmonella* Typhimurium SL696 in the presence of 2 mM calcium ions, whereas at 10 mM calcium the amount of LPS released was greater when LF was used.

Ellison et al. (1988) reported that bicarbonate could restore the ability of human LF to release LPS from *E.coli* CL99-2 and *Salmonella* Typhimurium SL696 grown in Hanks balanced salt solution (HBSS) containing high concentrations of calcium and magnesium. Naidu (2001) found that the addition of 0.01 M sodium bicarbonate enhanced the ability of immobilized LF to detach *E.coli* O157:H7 from surfaces of beef carcasses. This suggested that bicarbonate could act as a stabilizing agent for LF and serve as a companion anion to chelate metal ions.

Some studies have been done to examine the effect of monovalent cations on the activity of LF, but interactions are not well understood. Bortner et al. (1989) found that

NaCl did not eliminate the antimicrobial effect of LF against *Legionella pneumophila,* but the concentrations used were not mentioned. In addition, 2 and 5 M NaCl did not cause the dissociation of human LF or bovine LF from the surfaces of *Cl. perfringens* and *Shigella flexneri*, respectively (Tigyi et al. 1992; Tomito et al. 1998). The effect of NaCl on the activity of LF should be further investigated because this information is needed to predict the performance of LF in cured meat systems.

The activity of LF was temperature dependent against *L. pneumophila* (Bortner et al. 1986) and *Yersinia pseudotuberculosis* (Salamah and Al-Obaidi 1995b). The antibacterial activity of LF at 37 and 42 °C was lost when the treated samples were incubated at 1°C to 25 °C. Further, Nibbering et al. (2001) reported that expression of bactericidal effects by human LF required bacterial cells to be metabolically active. However, Murdock and Matthews (2002) reported that after 7d in UHT milk at 4 °C there was no difference in numbers of inoculated *Listeria monocytogenes* between untreated control samples and those treated with LF. Thus, the effect of temperature on the activity of LF needs to be investigated further since these studies were either carried out against bacteria that cannot grow at 4°C or incubation for short periods (4h) was used. Therefore, tests of LF at 10 °C against *Carnobacterium viridans* (a meat spoilage organism, Holley et al. 2002) which grows at 4 °C should yield useful information concerning the role of cellular metabolic activity in bacterial sensitivity to LF.

Recently, microencapsulation and controlled-release technology have found broad application in the pharmaceutical, healthfood, paper and cosmetic industries (Reineccius 1995). Microencapsulation is a process in which the sensitive ingredients (or core materials) are entrapped in a protective polymer encapsulating agent (or wall

material) that will allow release of core material at controlled rates under specific conditions (Hogan et al. 2001). Microencapsulation can be beneficial to the material being encapsulated. Firstly, separating reactive or incompatible components stabilizes the core material by protecting it from deterioration or adverse environmental conditions where metal ions, pH, free radicals, enzymes or water in a food may cause instability. Secondly, microencapsulation serves as a delivery system that reduces threshold concentrations necessary to achieve desired effects and extends the reactivity of bioactive ingredients (Dzieak 1988).

The research objectives of the present work were:

1) To investigate the antimicrobial activity of bovine LF against foodborne pathogens *E.coli* O157:H7 and a cured meat spoilage organism, *Carnobacterium viridans* and determine how this activity is affected by growth media (Lauria broth, LB, or All Purpose Tween broth, APT, containing low and moderate cation concentrations, respectively). In addition, the influence of temperature, NaCl concentration and the presence of compounds capable of chelating cations in reaction mixtures were to be studied to better understand factors governing LF action.

2) To develop microscopic capsules containing LF and a food grade metal ion chelating agent for use with cured meat packaging films or as an ingredient, monitor antimicrobial release over a range of temperatures (4 to 25 °C), and study melting characteristics of the encapsulating wall materials.

3) To evaluate the effectiveness of unencapsulated or encapsulated LF incorporated in a whey protein isolate packaging film in extending the shelf-life of a cured meat product (bologna).

4) To evaluate the antimicrobial effectiveness of unencapsulated and encapsulated LF against *E.coli* O157:H7 in dry fermented sausage.

Chapter 2

Review of Literature

2.1 Lactoferrin

The increased consumer demand for all natural food products has put pressure on industry and regulatory agencies to closely examine the potential use of natural antimicrobials in the food industry. Within the last few years, because of the broad range of biological functions of lactoferrin (LF), considerable interest has developed regarding its possible use for the surface decontamination of beef carcasses and subsequently its possible use as a natural food preservative.

Lactoferrin was initially called the red protein of milk, lactotransferrin or lactosiderophilin (Nagasawa et al. 1972; Shimazaki 2000). It is a member of the transferrin family which includes serum transferrin, ovatransferrin and melanotransferrin that play an essential role in controlling the level of free iron in the body fluids of animals (Farnaud and Evans 2003). Lactoferrin is the main iron–binding glycoprotein synthesized by polymorphonuclear leukocytes and glandular epithelial cells. It is found on mucosal surfaces, within specific granules of polymorphonuclear leukoctes, and in milk, saliva, seminal fluid and tears (Shimazaki 2000; Farnaud and Evans 2003). Its concentration is 2-7 mg/ml in human milk with higher concentration in the colostrum which gradually decreases by almost 7 fold in mature milk during lactation. In bovine milk LF ranges from 0.02-0.2 mg/ml, again with higher concentrations in colostrum or in mature milk after mastitic infection (Masson and Heremans 1971; Stopforth et al. 2005).

Lactoferrin exerts an inhibitory effect against wide range of Gram-negative and Gram-positive bacteria (Shimazaki 2000; Farnaud and Evans 2003), fungi (Andersson et al. 2000), parasites(Omata et al. 2001) and viruses (Seganti et al. 2004). In addition, LF has anti-inflammatory, immune-modulating (Legrand et al. 2004), anti-cancer (Tsuda et al. 2000), antioxidant effects (Steijns and van Hooijdonk 2000), and can promote the growth of probiotic bacteria like *Bifidobacterium* (Petschow et al. 1999). In a following section the antibacterial activity of LF will be discussed.

2.1.1 Bovine LF structure

Lactoferrin is monomeric and bilobal, with a molecular mass of 75-83 kDa and is composed of approximately 690 amino acid residues with an iso-electric point (pI) between 8.0-9.0 (Shimazaki 2000; Nadui 2000; Rossi et al 2002; Farnaud and Evans 2003). It has three major structural properties directly related to its biological functions (Aguila and Brock 2001).

Firstly, LF is folded into two similarly-sized globular lobes, the N-lobe and the C-lobe in which each is subdivided into two domains (N1, N2 (N lobe) C1, C2 (C lobe)) (Moore et al. 1997). The N and C lobes are stabilized by intra-chain disulfide bonds and linked by an alpha – helix that provides flexibility (Kanyshkova et al 2001; Stopforth et al. 2005). Each lobe has the capability to bind Fe^{+3} ion with high affinity in the presence of CO_3^{-2} or HCO_3^- anions that may be held electrostatically to an arginyl side- chain which occupies a pocket between the iron and two positively charged amino acid residues. The anions facilitate binding of iron by neutralizing the positive charges prior to iron binding (Rogers et al. 1978; Nadui 2000). The amino acids contributing to iron

binding are Asp 60, Tyr 92, Tyr 192, and His 253 in the N-lobe and Asp 395, Tyr 435, Tyr 528 and His 253 in the C lobe (Kanyshkova et al 2001).

In response to the level of iron saturation, two forms of LF exist, the apo-LF (iron-free) and halo-LF (iron-saturated LF) forms (Steijns and van Hooijdonk 2000). The tertiary structure of apo-LF and halo- LF are different. The N-lobe of apo-LF has an open conformation (with an angle between the N1 and N2 subdomains of 53°), while the C lobe has a closed conformation (C1 and C2 approach each other) (Fig. 2.1). In the halo-LF form both lobes are in a closed conformation (Fig.2.2) (Kanyshkova et al. 2001).

Secondly, the resistance of LF to mucosal protease and low pH may be attributed to a high degree of protein glycosylation (Van Berkel et al. 1995). The glycan chain plays a role in proper folding, maintains the functionally active conformation of the LF molecule and is responsible for reducing its immunogenicity. Glycans may also serve as recognition sites in interactions with viruses and microorganisms and interaction with cell membrane lectins involved in cell adhesion and cell contact inhibition. However, the degree of glycosylation varies among mammalian species. Bovine (b) LF has five potential glycosylation sites where human (h) LF has two such sites (Kanyshkova et al 2001).

Thirdly, the highly basic N- terminal mediates LF binding to several eukaryotic and prokaryotic structures. It has been implicated in bactericidal and anti-viral effects as well as in immunomodulatory and anti-endotoxic action (Naidu 2000; Aguila and Brock 2001).

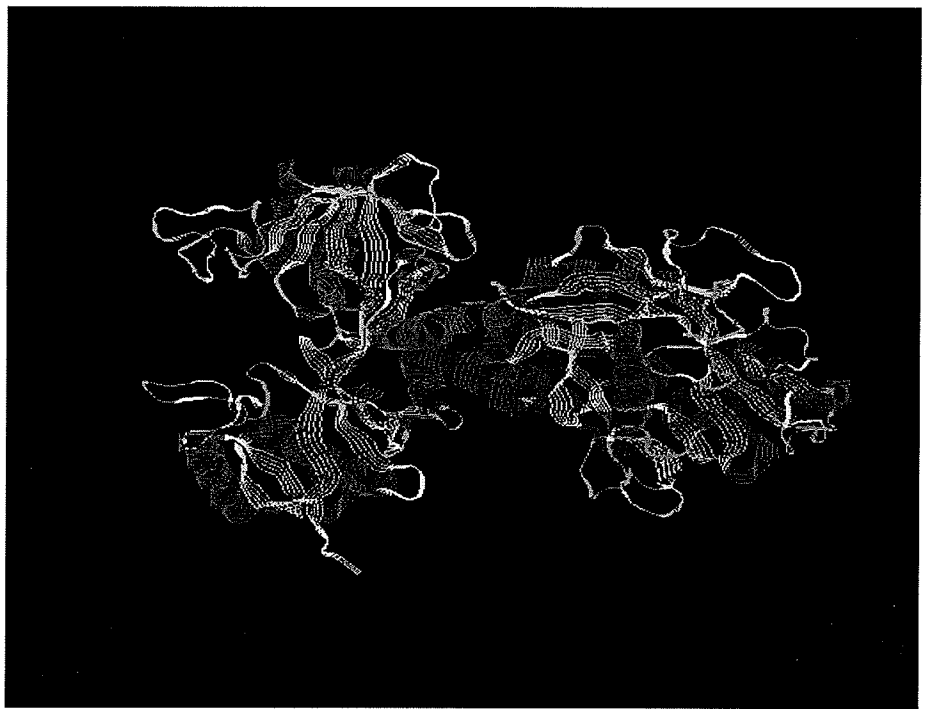

Fig.2.1. Three dimensional structure of apo-LF. Adopted from http://groningen.bio.ucalgary.ca/~hans/apo-lactoferrin.html

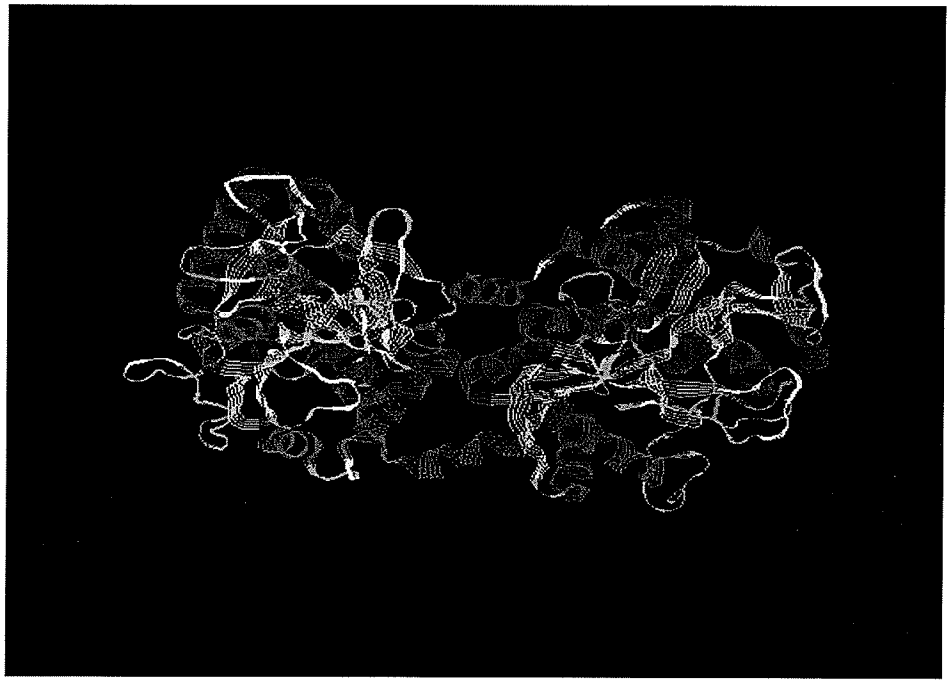

Fig.2.2. Three dimensional structure of halo-LF. Adopted from http://groningen.bio.ucalgary.ca/~hans/apo-lactoferrin.html.

2.1.2 Antimicrobial activity of lactoferrin

LF has been reported to have a wide spectrum of antimicrobial activity against Gram-positive and-negative bacteria, fungi, parasites and viruses. Generally, two modes of action related to its structure have been suggested responsible for its antimicrobial action.

i) The ability of LF to bind iron which results in an iron-deficient medium that suppresses the growth of microorganisms (Naidu 2000).

ii) The ability of the strongly cationic N- terminal to interact with the surface of microorganisms that leads to lethal effects (Bellamy et al. 1992).

2.1.2.1 Bacteriostatic effect

The ability of LF in the presence of bicarbonate to sequester Fe^{3+} from the growth environment has been shown by many researchers to be the primary means whereby LF suppresses growth of microorganisms by inhibition of metabolic activities (Naidu et al 1993; Naidu 2000). Binding of iron is reversible and Fe^{3+} can be released when the pH is < 4.0 (Moore et al.1997).

The bacteriostatic effect produced by LF may be temporary and can be counteracted by bacteria through two defence mechanisms:

1- *E.coli, Neisseria* spp., *Moraxella catarrhalis*, and *Vibro* spp. can produce siderophores during growth under restricted concentrations of iron. These siderophores are iron chelators that can bind ferric ions with high affinity and transport them inside the cell through specific membrane receptors. Production of these siderophores is chromosome

or plasmid-mediated (Griffiths and Williams 1999; Braun and Killmann 1999; Stopforth et al. 2005).

2- Some bacteria such as *Haemophilus influenzae, Actinobacillus pleuropneumonie* and *Bordetella pertussis* can express specific outer membrane receptors that can bind LF and remove iron from it through highly host-adapted specific pathways that facilitate iron uptake or through the production of a Fe^{3+} reductase as is the case with *L. monocytogenes* (Farnaud and Evans 2003; Orsi 2004;Stopforth et al. 2005).

Furthermore, other factors such a high degree of iron saturation of LF or a high citrate/bicarbonate ratio may interfere with the antimicrobial action of LF.

2.1.2.1.1 Degree of iron saturation

The antibacterial activity of LF was found to be dependent upon the degree of iron saturation of the molecule. Payne et al. (1990) found a bacteriostatic effect against *L. monocytogenes* in UHT milk when 30 mg/ml of 18% iron-saturated LF was used, but 46 mg/ml of LF (52% iron-saturated) reduced pathogen viability by only 16% when compared with control samples without LF. Furthermore, inhibitory effects were eliminated by the addition of 0.125 M ferric ammonium citrate. In addition, the bacteriostatic effect of LF against *Yersinia pseudotuberculosis* was reversed by the addition of excess iron to cause Fe^{3+} saturation of LF (Salamah and Al-Obaidi 1995a). In other work, the bacteriostatic effect of apo-LF against *Streptococcus mutans* 6715-13 in Todd Hewitt broth was reversed by saturation of LF with iron (Visca et al. 1989). In addition De Lillo et al. (1997) showed that iron-saturated LF did not have antibacterial activity against *Micrococcus luteus.*

2.1.2.1.2 Citrate/bicarbonate ratio

Naidu (2000) reported that the molar ratio (citrate to apo-LF) was more important in determining antimicrobial activity than the absolute concentration of either component. It was found that a ratio of 75 resulted in 50% growth inhibition of coliform bacteria associated with bovine mastitis while ratios of > 300 resulted in < 10% growth inhibition (Bishop et al. 1976). In another study, Nonnecke and Smith (1984) reported that the addition of bicarbonate to growth media containing 1 mg/ml apo-LF enhanced the inhibition of coliform growth by apo-LF. In contrast, the addition of 2 mg/ml citrate to the same system containing 5 mg/ml apo-LF eliminated the growth inhibition. It was believed that citrate competes with LF for iron and then makes iron available for bacterial growth (Reiter et al. 1975).

2.1.2.2 Bactericidal effect

The bactericidal activity of LF is iron independent and results from the direct interaction of the N-terminal of the LF molecule (which contains nine arginine and lysine residues) with the outer bacterial membrane and leads to lethal effects (Ellison and Giehl 1991; Bellamy et al. 1992a; Naidu 2000).

It has been found that that the negatively-charged lipopolysaccharide (LPS) is the binding site of LF on the Gram-negative bacterial surface (Ellison et al 1988; Ellison and Giehl 1991; Yamauchi et al. 1993). Furthermore, Appelmelk et al. (1994) showed that LF binds to the lipid A component of the LPS. The interaction of LF with LPS resulted in destabilization of the outer membrane thus, altering normal membrane physiology and sensitizing Gram-negative bacteria to hydrophobic molecules.

Another possible LF binding site on the surface of Gram-negative bacteria was also identified by others (Naidu et al. 1993; Erdei et al. 1994). The latter found that LF binds to porin proteins on the surface of *E.coli*, and it was suggested that differences in LF binding affinity were affected by the O chain of the LPS which was believed to be able shield the porins from interaction with LF and thus reduce antibacterial effects. Naidu et al. (1993) reported that LF binding to the surface of an isogenic R (rough) mutant of *S.* Typhimurium increased with a decrease in the amount of LPS polysaccharide moiety present. Furthermore, they showed that a high LF binding mutant was more susceptible to LF.

Recently, in addition to the ability of LF to bind to bacterial surfaces, another possible mechanism for the bactericidal effects of LF has been suggested (Aguilera et al. 2003). The latter reported that LF may interact directly with the cytoplasmic membrane causing selective permeation of ions (K^+) without initially affecting the cellular pH gradient.

Slightly different susceptible target sites were identified for direct interaction of LF with Gram-positive bacteria. De Lillo et al. (1997) concluded that direct interaction of LF with bacterial surfaces was an essential step for initiation of bactericidal effects. They reported that reduction in the viability of *M. luteus* resulted from the ability of LF to bind to lipomannan, a lipoglycan present at the cell surface. They found that a major difference between LF resistant *Micrococcus* strains and sensitive *M. luteus* was the absence of lipomannan from the surface of resistant strains. In another study, proteins present on the surface of *Clostridium perfringens* were identified as binding sites for LF (Tomito et al. 1998).

2.1.2.2.1 Factors that affect the bactericidal activity of LF

2.1.2.2.1.1 Degree of iron saturation

Ellison and Giehl (1991) reported that the combination of hLF and human lysozyme was bactericidal against *Vibrio cholerae* while LF alone was bacteriostatic. However, at high levels of hLF iron saturation both the bacteriostatic and synergistic bactericidal effects of hLF were reduced. Recio and Visser (2000) found that iron-free LF showed greater ability to bind to the surface of *E.coli* cells than the iron-saturated form. This could be explained by the inability of the N-terminal of LF to interact with bacterial surfaces since the N-lobe is closed when it is iron-saturated (Kanyshkova et al 2001).

2.1.2.2.1.2 Effect of divalent cations

In many previously reported studies LF exerted bactericidal effects when assays were performed in low-ionic strength or minimal basal media that did not support bacterial growth (Arnold et al. 1980; Bortner et al. 1986; Salamah et al. 1995b; De Lillo et al. 1997).

Although these studies suggested that LF has the potential to be used as a natural antimicrobial preservative in the food industry, its activity in food systems was reduced in the presence of divalent cations such as Ca^{2+} and Mg^{2+} (Stopforth et al. 2005).

Salamah and Al-Obaidi (1995b) showed that $\leq$ 60 mM Ca^{+2} did not affect the bactericidal activity of hLF against *Y. pseudotuberculosis* but 3 to 32 mM Mg^{+2} decreased its activity in a manner inversely related to Mg^{+2} concentration. In another study, the addition of $CaCl_2$ or $MgCl_2$ reduced the bactericidal action of LF against *L. pneumophila* (Bortner et al. 1989). Furthermore, Ellison and Giehl (1991) showed that

the addition of 1.3 mM $CaCl_2$ or 0.8 mM $MgCl_2$ to bacto-peptone reduced the bactericidal effects of hLF and lysozyme against *E.coli* 5448.

When more complex media that allow bacterial growth were used, it was found that the activity of LF was further reduced. Shin et al. (1998) found the minimum inhibitory concentration of bLF against *E.coli* O157:H7 was 6 mg/ ml in PGY broth (containing 1% bactopeptone, 1% glucose and 0.05% yeast extract) compared to 3 mg/ml in peptone water. Furthermore, ≤20 or ≤40 mg/ml LF did not inhibit the growth of *S. enterica* and *E.coli* O157:H7, respectively, in Tryptose Soy Broth (TSB) while 20 or > 5 mg/ml inhibited their growth in 1% peptone water (Min et al. 2005).

Branen and Davidson (2004) showed that bLF alone was ineffective against *E.coli* O157:H7 and *E.coli* O104:H21, but in combination with monolaurin it was bacteriostatic in TSB against *E.coli* O157:H7. However, no effect was observed when they were used together in 2% fat UHT milk against either strain.

Ellison and Giehl (1991) assessed the effect of different growth media on the activity of a LF and lysozyme mixture against *E.coli* 5448. They found that the activity of the mixture was higher in bacto-peptone than it was in proteose peptone or proteose peptone 3. In contrast, the bactericidal effect was eliminated in proteose peptone 2 or in the more defined WAS II, WAS III or Lauria and Davis media. This was explained by the inverse correlation between the degree of killing and media osmolarity (mOsm) rather than by the relationship between lactoferrin-lysozyme activity and the concentration of cations present, since the lactoferrin-lysozyme activity was diminished in media above 60 mOsm.

There are two possible mechanisms by which the presence of divalent cations in the surrounding environment may result in the reduction of the antibacterial activity of LF.

Firstly, Shimazaki (2000) reported that cations protect bacteria from LF by inducing changes in its tertiary structure which yields tetrameric LF with reduced biofunctionality. Mantel et al. (1994) found that the presence of Ca^{2+} induced the formation of the tetrameric form from LF monomers. Rossi et al. (2002) reported that Ca^{2+} likely binds to the mobile carboxylate groups of the sialic acid residues present in the two glycan chains of the bLF molecule and improves its stability. They found that in the presence of (100μM) of Ca^{2+}, the melting temperature of apo-bLF (iron-free) was 76 °C compared to 67 °C in the absence of Ca^{2+}. However, the stabilizing effect of Ca^{2+} disappeared in the presence of EDTA. This effect was specific to Ca^{2+} since other cations including Mg^{2+} were not able to induce the same effect. In addition, the midpoint of denaturation of bLF was shifted from 2.1 M to 3.3 M guanidine hydrochloride in the presence of Ca^{2+}.

Secondly, divalent cations present in the growth environment increase membrane stability by interacting with the negative charges of the core oligosaccharide chain of LPS molecules (Conghlin et al. 1983).

2.1.3 Synergistic effects of LF

LF has been shown to increase the permeability of the bacterial outer membrane by binding to LPS, thereby sensitizing Gram-negative bacteria to hydrophobic molecules such as rifampicin and actinomycin D.

Ellison and Giehl (1991) reported that the combination of hLF and human lysozyme was bactericidal against *Vibrio cholerae, S.* Typhimurium and *E.coli* while LF alone produced a bacteriostatic effect in 1% proteose peptone 3 broth at 37 °C. By binding to the LPS on the surface of Gram-negative bacteria and increasing permeability or pentration, lysozyme facilitated disruption of the glycosidic linkages of the rigid murein sacculus by lysozyme. In other work, the lethality of human lysozyme toward *E.coli* CL 99 2-1 in 1% bactopeptone medium was increased by the addition of 2 mg/ml bLF (Yamauchi et al 1993).

The susceptibility of *E.coli* strain 11744 to rifampicin was enhanced when hLF was added to broth lacking Ca^{2+}, but the addition of 1.3 mM Ca^{2+} significantly decreased the ability of hLF to sentisitize the bacterium to rifampicin (Ellison et al. 1990). Similarly, Aguilera et al. (2003) reported that hLF caused an increase in outer membrane permeability of *E.coli* to actinomycin D. In addition, the presence of 2 mg/ml LF caused a 16- fold reduction in the minimum inhibitory concentration of vancomycin (8 mg/ml) compared to vancomycin alone (128 mg/ml) against vancomycin resistant strains of *Enterococcus faecalis* (Letich and Willcox 2001).

Ellison et al. (1988) investigated the ability of LF to release LPS from the cell membrane of *E.coli* CL99-2 and *S.* Typhimurium SL696 in Hanks balanced salt solution (HBSS) and in HBSS lacking calcium and magnesium. They reported that LPS was released in higher concentration from cells in HBSS lacking calcium and magnesium, whereas the activity of LF was blocked by the high concentration of calcium and magnesium found in HBSS. The addition of bicarbonate to HBSS without calcium and

magnesium yielded a greater release of LPS. This suggested that bicarbonate could act as "a companion anion" for the chelation of metal ions and stabilization of LF structure.

The synergistic interaction between LF and bicarbonate is believed due to the ability of SB to stabilize LF structure (Naidu 2000). In addition, sodium bicarbonate by itself was reported to have antimicrobial effects under alkaline conditions (pH 8.5) as a result of bicarbonate dissociation to form carbonate (Eq. 1) (Corral et al. 1988; Diez-Gonzalez et al. 2000).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO3^- \leftrightarrow H^+ + CO_3^{2-} \quad \text{..... Eq. 1}$$

The carbonate anions can bind divalent cations and form insoluble complexes that cause destabilization of the outer membrane (Jarvis et al. 2001).

2.1.4 Effect of monovalent cations

Some studies have been done to investigate the effect of monovalent cations (Na^+ and K^+) on the activity of LF, but interactions are not well understood. Viejo-Diaz et al. (2003) reported that the bactericidal effect of hLF against *E.coli* ML-35 in 0.3% bactopeptone was eliminated when the concentration of Na^+ was ≥40 mM or when K^+ was ≥20 mM. On the other hand, Bortner et al. (1989) found that NaCl did not eliminate the antimicrobial effect of LF against *Legionella pneumophila,* but the concentrations used were not mentioned. It was of interest that Naidu et al. (1991) found that 2 M NaCl did not cause the dissociation of hLF from the surfaces of *E.coli* O127. Similarly, 2 and 5 M NaCl did not cause the dissociation of hLF or bLF from the surfaces of *Cl. perfringens* or *Shigella flexneri*, respectively (Tigyi et al. 1992; Tomito et al. 1998). These authors concluded that binding of LF to bacterial cells did not involve electrostatic interaction,

but rather hydrophobic interactions may be responsible for LF binding since 2 to 3 M KSCN was able to dissociate LF from the surfaces of these test organisms. In these studies the effect of NaCl on the viability of cells treated with LF was not examined. Therefore, the effect of NaCl on the activity of LF is uncertain and should be further investigated because this information is needed to predict the performance of LF in cured meat systems.

2.1.5 Inoculum size and strain

Bacteria in the early growth phase were found to be more sensitive to LF than cells in the stationary phase (Arnold et al. 1981). Dionysius et al. (1993) found that the inhibitory effect of LF (0.1 to 1 mg/ml) against 19 strains of enterotoxigenic *E.coli* isolated from piglets was strain dependent in iron-free AOAC bacto-synthetic broth. They found that 10 strains were inhibited when LF concentration was < 0.2 mg/ml and the other 9 strains were variably affected by higher concentrations of LF.

Ellison and Giehl (1991) found that *E.coli* 5448 was more susceptible to the effects of hLF and human lysozyme than *E.coli* CL99-2. Furthemore, they reported that the effect was eliminated when the bacterial inoculum was increased from 5×10^5 to 5×10^7 CFU/ml.

Naidu et al. (1991) reported that hLF bound better than bLF to the cell surface of *E.coli*. In addition, they found that enterotoxigenic *E.coli* (ETEC) strains bound significantly higher amounts of hLF than enteropathogenic *E.coli* (EPEC), enteroinvasive *E.coli* (EIEC), and enterohemorrhagic *E.coli* (EHEC) strains.

Dionysius *et al* (1993) studied the effect of inoculum size of an *E.coli* strain sensitive to apo-LF and an *E.coli* resistant strain. It was found that lethality of the sensitive *E.coli* strain occurred at 5 x 10^3 CFU/ml, but a bacteriostatic effect was achieved at higher bacterial numbers. In contrast, the slight inhibition of the resistant *E.coli* strain was reversed at higher inoculum concentration.

2.1.6 Temperature

The effect of incubation temperature on the antibacterial activity of LF was investigated by Bortner et al. (1986) and Salamah and Al-Obaidi (1995b) against *L. pneumophila* and *Y. pseudotuberculosis*, respectively. They found that the antibacterial activity was temperature dependent since the antibacterial activity of LF at 37 and 42 °C was lost when the treated samples were incubated at 1°C to 25 °C. Bortner et al. (1989) found that hLF was unable to bind to the surface of *L. pneumophila* at 4 °C. In addition, Murdock and Matthews (2002) reported that after 7d in UHT milk at 4 °C there was no difference in numbers of inoculated *L. monocytogenes* between untreated control samples and those treated with LF. At 4 °C they also found with *E.coli* O157:H7 that if low pH (pH 4) was used or if LF was treated with porcine pepsin to create an LF hydrolysate, activity was increased and 1 and 2 log reductions were observed, respectively. Nibbering et al. (2001) found that hLF was bactericidal only if bacterial cells were metabolically active.Thus, the effect of temperature on the activity of LF needs to be investigated further since these studies were either carried out against bacteria that cannot grow at 4°C or incubation for short periods (4h) was used.

2.1.7 Heat stability

Paulsson et al. (1993) demonstrated that the thermal stability of LF in water was dependent on the degree of iron saturation of LF. They examined the thermal denaturation of different forms of LF in water at pH 7.2 by using differential scanning calorimetry (DSC). Native LF (the iron saturation was 25.2mg/100g LF) showed two denaturation temperatures, 65 and 92 °C, whereas apo-LF (iron-free) denatured at 71 °C and for halo-LF(iron-saturated) denaturation occurred at 93 °C. In addition, they studied the effect of pasteurization and UHT treatments on the ability of LF to interact with bacteria. Pasteurization did not affect the ability of native and iron-saturated LF to bind to various bacterial species, whereas UHT treatment decreased this interaction. Therefore, LF can tolerate mild heat treatments without affects on its activity and can be used in food products that receive pasteurization heat treatment.

Abe et al. (1991) investigated the effect of pH on the heat stability of LF. They reported that LF was more heat stable at low pH, especially around 4.0, whereas neutral and basic pH enhanced its thermal denaturation.

2.1.8 Safety

The toxicity of LF towards cultured HT-29 and Caco-2 cells derived from human colonic carcinoma cells lines was assessed by Antonini et al. (1997) after 1h incubation of these cells with different concentrations of apo-LF, iron-saturated LF and manganese-saturated LF. The health of the intestinal cells was scored by examination of cell morphology, viability and yield. It was found that up to 4 mg/ml of apo-and iron-saturated LF did not produce any damage to either type of cells, whereas changes in these parameters were observed at 2 mg/ml of Mn-LF. Naidu (2000) reported that no side

effects were noted when 20g LF/L of milk for 14 d or 20g LF/ kg diet for 13 weeks was orally administrated to mice or rats, respectively. In addition it was reported that regular LF consumption by adults or children will be in the range of 2 or 30g/L of mature milk or colostrum, respectively.

2.1.9 Food Applications

LF is available in two forms: a liquid form and a spray dried powder (Stopforth et al 2005). Since a number of factors influence LF antimicrobial action such as the presence of divalent cations, the bicarbonate/citrate ratio and the need for high concentrations of LF, other approaches have been used to broaden the range of LF antimicrobial action (Stopforth et al. 2005).

In an attempt to overcome the difficulty of applying native LF to meat surfaces and maintain its activity, Naidu (2001) used a mixture of 1% "immobilized" (Im) activated LF (ALF) and native LF in a ratio of 1:1 to maintain LF structural stability and bio-functional specificity in order to inhibit *E.coli* O157:H7 attachment to beef tissue. He found that the efficiency of microbial detachment of *E.coli* O157:H7 by washing beef tissue with a regular sanitizing sequence (a procedure that included 5 washing steps of 10 sec each, consisting of spraying with: water, 2% lactic acid, hot water (180°F, for 30 sec), water and 2% lactic acid, consecutively) was improved from 72.2% to 99.6% when 1% Im-LF/LF was used in each step of the regular sanitizing sequence. It was also found that immobilized LF could be effectively used in coating beef tissue by electrostatic spray dispersion, with an aqueous suspension or solution of dehydrated powder containing immobilized LF. ALF was believed to act as a blocking agent, which interfered with microbial adhesion/colonization, causing detachment of live or dead microorganisms

from biological surfaces, which inhibited microbial growth/multiplication, and neutralized the activity of endotoxins (Naudi 2002).The use of ALF on fresh beef was approved by the U.S. Department of Agriculture to prevent bacterial contamination during processing (Naidu 2003).

Recently, another possible application was reported which involved the addition of LF to infant milk formula in order to inhibit the growth of pathogenic microorganisms and promote the growth of bifidobacteria (Shimazaki 2000). Several researchers have found that LF can bind to the surface of bifidobactera and promote their growth (Petschow et al. 1999; Kim et al. 2002; Kim et al 2004). The outcome is a delicate balance of interactions since Griffiths et al. (2003) found that the presence of *Bifidobacterium infantis* enhanced the inhibitory effect of LF against *E.coli* O157:H7 in Reinforced Clostridial Broth. Therefore, LF might be used with probiotic bacteria to enhance the safety of fermented foods.

The use of LF with other compounds able to chelate divalent cations such as EDTA (Vaara 1992) in food systems was investigated by Murdock and Matthews (2002). However, they found that the addition of EDTA did not enhance the activity of LF against *E.coli* O157:H7 in UHT milk.

The effects of other chelating agents that are added to food formulations such as sodium hexametaphosphate, SHMP (Denyer and Maillard 2002) and lactate (Alakomi et al. 2000) on the antibacterial activity of LF has not been yet investigated. SHMP is widely used in food processing for emulsification, moisture retention, leavening, chelating cations, buffering and improving the tenderness of processed meat (Vaara and Jaakkola 1989). The antimicrobial activity of SHMP was reported to be stronger against

Gram-positive than Gram-negative bacteria due to its ability to destabilize the relatively unprotected cell membrane of the former by binding divalent cations and increasing the permeability of hydrophobic molecules (Knabel et al. 1991; Denyer and Maillard 2002). Vaara and Jaakkola (1989) reported that the minimum inhibitory concentration of rifampin against *E.coli* was reduced 10 fold in the presence of 3 mg/ml SHMP. In addition, the uptake of 1-N-phenylnaphthylamine (NPN) (a hydrophobic molecule) by *E.coli* O157:H7 was increased when SHMP was used (Helander and Mattila-Sandholm 2000). Therefore, LF may be useful in inhibiting the growth of *E.coli* O157:H7 in foods where SHMP is used as an ingredient such as in cured meat products.

Lactic acid or sodium lactate, which are also used as antimicrobials in the food industry, can act to increase permeability of the Gram-negative bacterial outer membrane while reducing cytoplasmic pH (Alakomi et al. 2000), and may have potential value for use in improving LF antimicrobial activity.

2.2 Lactoferricin

Pepsin hydrolysis of LF produced active peptides characterized by having bactericidal activity against a wide range of Gram-positive and negative bacterial cells as well as against mold and yeast. The active peptides of human LF are named lactoferricin H (LFcin H) and those from bovine LF are named lactoferricin B (LFcin B) (Bellamy et al. 1992b; Branen and Davidson 2000).

Tomito et al. (1991) were able to generate potent antimicrobial peptides with lower molecular weight (< 6 kDa compared to LF 80 kDa) by porcine pepsin digestion of bovine LF. These were found to be 9 to 25 times more bactericidal than native LF against several Gram-negative and-positive bacteria including strains that were resistant to native

LF (Chantaysakorn and Richter 2000). De Lillo et al. (1997) reported that *Micrococus radiophilus, M. roseus and M. varians* which were resistant to apo-LF were sensitive to the LF-hydrolysate. In other work it was found that only 0.08 and 0.23 mg/ml LF-hydrolysate were required to inhibit the growth of *L. monocytogenes* IDF-lb and *E.coli* IID-861. Inhibition of the same organisms required 1.6 and 2.0 mg/ml of LF, respectively (Tomito et al. 1991).

The bactericidal activity of LFcin B is independent of iron concentration and this may be explained by its ability to penetrate the cell membrane (Tomito et al. 1991 and Facon and Skura 1996). The mode of action of LFcin B may involve its amphiphatic structure, suggesting that it may interact with biological membranes. The primary sequence of LFcin B contains many hydrophobic residues clustered to one side, while the majority of positively charged side chains are positioned outside this area, with the exception of Lys2. The hydrophobic residues could interact with fatty acyl groups of the lipid bilayer and the positively charged Arg and Lys side chains may interact with phosphodiester groups facilitating binding to the surface of Gram-negative and-positive bacteria (Hwang et al. 1998).

Many studies have reported that the antimicrobial activity of LFcin is reduced in the presence of divalent cations such as calcium and magnesium at concentrations between 1-5 mM, and monovalent cations, sodium and potassium at concentrations $\leq$ 100 mM.

Bellamy et al. (1992b) and Wakabayashi et al. (1992) found that the presence of NaCl or KCl within the range of 25-100 mM (or 1.0-5.0 mM $CaCl_2$ or $MgCl_2$) in the growth media inhibited the antibacterial activity of LFcin B. The protective effect of

adding these salts was found to vary depending on the target strain. It may be that these salts interact with membrane constituents in a strain dependent manner rather than acting to promote the formation of inactive aggregates of LFcin B (Bellamy et al. 1992b).

Similarly, increasing the complexity of the growth media will result in reducing the antibacterial activity of LFcin B. Branen and Davidson (2000) found that the antibacterial activity of a lactoferrin digest (LFD) was much lower in TSB than it was in PYG against *E.coli* O157:H7 ATCC 43895, *E.coli* O104:H21 MPHL 94-56815, *S.* Enteritidis ATCC 13076, *L. monocytogenes* Scott A and *L. monocytogenes* ATCC 19115. The reduced activity of LFD against *S.* Enteritidis ATCC 13076 in TSB compared to PYG has been attributed to the presence of higher concentrations of Ca^{2+} and Mg^{2+} in TSB. The activity of LFD was improved in TSB by adding EDTA in combination with lysozyme (Facon and Skura 1996). The addition of EDTA to TSB enhanced the inhibitory effect of LFD against *E.coli* O157:H7 and *E.coli* O104:H21 and yielded the same minimum inhibitory concentration of LFD in PYG. Therefore, EDTA in TSB chelated the cations and restored the inhibitory effects of LFD. Another reason for the reduced activity of LFD in TSB (compared to PYG) is that PYG does not support bacterial growth as well as TSB, which gives LFD the opportunity to be more effective against slowly growing cells (Branen and Davidson 2000).

Many studies have been carried out to assess the potential applications of LFcin B in the food industry. Facon and Skura (1996) showed that LFcin B alone or in combination with lysozyme was ineffective against *S.* Enteritidis in chicken skin extract. The effectiveness of LFcin B against enterohemorrhagic *E.coli* O157:H7 in ground beef was studied at 4 and 10 °C by Venkitanarayanan et al. (1999). It was found that only a 0.8

log_{10} CFU/g reduction was achieved when LFcin B was applied to the ground beef and stored for 0–5 days at 4 C° or 0-3 days at 10 C°. Therefore, the level of reduction observed in the ground beef may not be of practical significance. These studies indicated that the antimicrobial activity of LFcin B alone without a cation chelating agent (such as EDTA) in food products which have high concentrations of calcium and magnesium will be reduced.

Chantaysakorn and Richter (2000) found that LFD did not inhibit the growth of *E.coli* in carrot juice. They found that calcium, magnesium and sodium were responsible for the loss of LFD antimicrobial activity but this activity was regained by removing the effect of these cations from carrot juice by dialysis. This indicated that LFcin B could be useful in food products that contain low cation concentrations.

Divalent cations are belived to diminish the antimicrobial activity of LF by formation of tetramers that adversely affect LF structural integrity (Shimazaki 2000). Also, these cations increase bacterial resistance toward LFcin B by stabilizing the cell membrane. In addition Yamauchi et al. (1993) found that at 10 mM calcium in the growth media, the amount of LPS released from *S.* Typhimurium SL696 by LF was higher than that released by LFcin B.

Also, these studies showed that monovalent cations reduce the antimicrobial activity of LFcin, but there has been no research carried out to assess the effect of sodium ions on LF activity at concentrations similar to those present in cured meat (> 2.5% NaCl).

2.3 Dry fermented sausages

Dry fermented sausages were first produced in the Mediterranean region. They derive their name from the Latin term *salsus* meaning salted (Ricke et al. 2001). They are produced by fermentation of carbohydrates in meat and contain added fat, salt, nitrate/ nitrite, spices and a reductant such as isoascorbate which are mixed, stuffed, ripened (fermented) and dried in environmentally controlled chambers, and thus are preserved without heat treatment (Roca and Incze 1990). The synergistic or hurdle effects of salt, nitrite/ nitrate, low pH (< 5) and the low moisture/protein ratio (< 3.1:1.0), expressed and measured as water activity (a_w) as well as the low oxidation-reduction potential are responsible for the preservation of dry fermented sausage (Roca and Incze 1990; Clavero and Beuchat 1996; Pidcock et al. 2002).

The manufacture of dry and semi-dry sausages involves discrete steps (Roca and Incze 1990; Varnam and Sutherland 1995; Ricke et al. 2001; Pidcock et al. 2002) which are indicated below. Dry sausages have a moisture/protein ratio of 1.9:1 while semi-dry sausages, which require refrigeration, have a moisture protein ratio of 3.1:1 (Muthukumarasamy 2006).

i) Selection of raw material

First, meat of high quality should be used with low numbers of bacteria. Fat, salt, nitrate and/or nitrite, spices, glucose, other seasonings and starter cultures are selected on the basis of the type of the sausage to be produced and the level of lactic acid desired in the final product. Meat ingredients and fat should be chilled to -2 °C before chopping. To ensure good organoleptic characteristics, fresh fat should be used to avoid changes in the flavour as a result of oxidation.

ii) Sausage batter preparation

Fat is chopped to 6 mm diameter, 7 $\log_{10}$ cfu/g starter culture (usually a lactic acid bacterium, LAB, like *Pediococcus pentosaceus* plus *Staphylococcus carnosus*) are added, mixed, and pork plus beef are added and chopped. Then $\leq 0.2\%$ glucose plus 3% (w/w) salt, cure (200ppm NO_2 + NO_3) plus sodium isoascorbate (500ppm) are mixed and further reduction in the particle size to 3 mm is carried out. Finally, the meat batter is vacuum stuffed into moisture permeable collagen or natural casings to allow drying.

iii) Smoking, ripening and drying

The stuffed meat batter undergoes fermentation at 24-30 °C and high humidity (98%) for < 65h during which the meat pH is reduced from 5.8 to 5.3. Meat proteins are denatured and a gel is formed as a result of lactic acid production by the *Pediococcus* (LAB) starter culture. The *Staphylococcus* (or *Micrococcus*) culture reduces nitrate to nitrite and produces catalase to destroy H_2O_2 produced by the LAB. These actions stabilize meat colour and reduce risk from pathogen growth. The time-temperature relationship during the fermentation period is regulated by Agriculture and Agri-Food Canada (1992). The "degree-hour" guidelines specify the maximum time that the sausage meat batter can be held > 15.6° C before a pH of 5.3 is reached during fermentation. These guidelines were originally developed to eliminate the growth of *Salmonella* and *Staphylococcus aureus*, and have been effective. During fermentation smoke is applied which reduces surface mould growth and can improve product flavour, and as a result it extends shelf-life as well. After the pH is reduced below the isoelectric point (5.3) of meat protein, meat is dried to achieve the required moisture/protein ratio and establish shelf-life stability. Drying should be performed under conditions (time, temperature and

relative humidity) to allow the evaporation of moisture from the centre of the product at the same rate that it migrates from the surface. In practice, relative humidity (RH) and temperature are reduced by 2% and 2 °C every 12h for several days until 88% RH and 13 °C are reached. Further drying at 13 °C and 77% RH are continued for 25d. Finished products have a pH $\leq$ 5.3, a water activity of 0.86 and are shelf stable at room temperature.

2.3.1 Safety of dry fermented sausages

Dry fermented sausages have an excellent safety record which results from hurdles such as low pH, low water activity, presence of nitrite, NaCl and sometimes other inhibitory metabolites produced by starter cultures during the production of these products (Incze 1998; Barbuti and Parolari 2002). Unfortunately, these hurdles were not sufficient to prevent the occurrence of a major outbreak of foodborne illness in Washington and California in 1994, which resulted from the consumption of sliced dry fermented sausage contaminated with *E.coli* O157:H7 (CDC 1995a). In south Australia another outbreak was attributed to *E.coli* O111:NM in a low-temperature-cooked fermented sausage in 1995 (CDC 1995b). Again in 1999, the Canadian Food Inspection Agency issued a health hazard alert recalling Hungarian salami as a result of potential *E. coli* O157:H7 contamination (CFIA, 1999).

As a consequence of the first outbreak, the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) developed guidelines that fermented sausage processors demonstrate a 5 log unit reduction in the numbers of enterohemorrhagic *E.coli* that survive processing (Incze 1998). Later the Canadian Food Inspection Agency (CFIA) Meat Hygiene Directive (1999) adopted the FSIS rule on

fermented sausage manufacture to control *E. coli* O157:H7. The proposed FSIS "performance standard for the production of processed meat and poultry products" validated manufacturing process requires that one of the following 5 options be used:

1- A hold and test program be established for finished product (30 samples per lot with zero positive) to demonstrate *E.coli* O157:H7 absence.

2- A heat treatment be applied at 63 °C for 4 min.

3- Companies develop and validate a 5 D process (5 log reduction in the pathogenic population).

4- Companies use combined treatments that would ensure equivalent safety.

5- Companies use raw ingredients prepared under a HACCP program that verifies the presence of ≤ 1 *E.coli* O157:H7/125g meat and that a 2 D (2 log) inactivation occurs during fermentation and drying steps.

These regulations stimulated researchers to investigate the effect of alternative processing parameters during dry fermented sausage manufacture to reduce the survival of *E. coli* O157:H7. A number of studies showed that *E.coli* O157:H7 survived dry fermented sausage manufacture, and only a 1-2 log reduction was consistently obtained during its production (Glass et al. 1992; Hinkens et al. 1996; Faith et al. 1998). Glass et al. (1992) showed that *E.coli* O157:H7 survived in fermented sausage containing 3.5% NaCl prepared with either starter culture or with the indigenous lactic acid bacteria. Acid adaptation of *E.coli* O157:H7 was suggested to be the reason for the ability of *E. coli* O157:H7 to survive the dry fermented sausage process (Calicioglu et al. 1997).

Conner and Kotrola (1995) showed that *E. coli* O157:H7 was able to grow $\geq$ 2-4 $\log_{10}$ cfu/ml at 25 °C in TSB broth acidified with lactic acid to a final pH of 4.7. Indeed,

Jordan and Davies (2001) and Casey and Condon (2002) reported that *E.coli* O157:H7 was able to regulate its internal pH and counteract the bactericidal effects of lactic acid in the presence of NaCl in the growth medium. The expression of acid shock proteins at pH < 5.5-6.0 has been suggested to enhance the ability of *E.coli* O157:H7 to survive in low pH environments (Heyde and Portalier 1990). In addition, the acid tolerance of *E. coli* O157:H7 was suggested to contribute to its low infective dose (Gorden and Small 1993). In addition, it was not surprising that *E. coli* O157:H7 can tolerate the presence of NaCl in dry fermented sausage. Several studies conducted before the onset of the first outbreak of illness from dry fermented sausage showed that *E.coli* O157:H7 grew in the presence of higher concentrations of NaCl at low temperatures. Glass et al. (1992) reported that *E.coli* O157:H7 grew well in TSB containing NaCl concentrations up to 2.5% at 37 °C. In addition, Effat et al. (2001) found that *E.coli* O157:H7 was able to grow in TSB containing ≤ 6% NaCl at 37 °C and 7 °C.

Since the fermentation and drying conditions used in commercial production were not sufficient to generate the 5 log reduction required in *E.coli* O157:H7 numbers to meet the guidelines set by the USDA FSIS, researchers started to look for other alternatives to control the survival of *E.coli* O157:H7 in these products.

2.3.2 Methods to control *E. coli* O157:H7 in dry fermented sausages.

2.3.2.1 Use of heat

Hinkens et al. (1996) reported that heating pepperoni chubs to an instantaneous internal temperature of 63 °C or at 53 °C for 60 min was sufficient to obtain the 5 D log reduction. In another study, Caliciolglu et al. (1997) found that fermentation to pH 4.6 and a post fermentation heat treatment of summer sausage to an internal temperature 54

°C reduced the viability *E. coli* O157:H7 by > 5 log cfu/g. In contrast, without heat only a 3.2 log reduction was achieved at pH 5.0.

While post fermentation heating was effective in destruction of *E.coli* O157:H7 and complied with the guidelines of the USDA, it negatively affected sensory quality and products no longer resembled uncooked fermented sausages. For example, it was found that heating pepperoni at 58.3 °C for 61.3 min altered the texture and taste of products compared to non-heat treated pepperoni (Riordan et al. 2000). Similar results were found by others, and when these factors were considered along with the added cost of the heating step plus the loss of raw product identity, thermal treatment became an unpopular solution (Johnson et al. 2000; Lahti et al. 2001).

2.3.2.2 Ionizing radiation

Samelis et al. (2005) showed that ionizing radiation of frozen meat and fat trimmings at 4kGy was sufficient to deliver a 5 log reduction of *E.coli* O157:H7 prior to sausage fermentation. Radiation at 2 kGy achieved a 5 log reduction of the pathogen as early as the end of fermentation (7d at 23 °C and 96% RH).

However, the effect of radiation on the sensory qualities of dry fermented sausages has not been investigated, but should be, since irradiation is known to adversely affect sensory qualities of food by enhancing oxidative processes that lead to rancidity, colour defects and flavour problems (Farkas 2001).

2.3.2.3 Starter cultures

Lahti et al. (2001) compared the ability of two different commercial starter cultures to inhibit the growth of *E.coli* O157:H7 in dry fermented sausage. They found that the Müller RM 52 culture containing *Lactobacillus curvatus* Lb3 and *Staphylococcus*

carnosus was able to reduce the numbers of *E.coli* O157:H7 by 5 log during fermentation at 17-23° C for 15 d and subsequent drying at 15-17° C for 34 d. On the other hand, only 2.8 log reductions were noted when a starter culture containing *Pediococcus acidilactici* PA-2, *Lb. bavaricus* MI-410 and *S. xylosus* was used. Unfortunately, the fermentation conditions used in this study did not comply with the current regulations of AAFC regarding required time- temperature conditions and can not be safely used by industry. It is unlikely that different bacterial starter cultures alone can prevent *E.coli* O157:H7 survival during fermented sausage manufacture.

2.3.2.4 Natural antimicrobials in dry fermented sausages

The ability of a microencapsulated combination of the essential oils oregano and thyme at 0.3% (w/w) each, in 10% (w/w) colloidal starch at a ratio of 1:1 to inhibit *E. coli* O157:H7 during fermented sausage manufacture was investigated by Gagné et al. 2003. After 4 weeks storage at room temperature following manufacture, a > 5 log reduction in *E. coli* O157:H7 viability was obtained. However, holding fermented dry sausages at room temperature may accelerate oxidative deterioration of fat and adversely affect the sensory quality of sausage (Stiebing, 1999). Recently, Chacon et al. (2006) showed that 500 ppm allyl isothiocyanate (AIT), when encapsulated in gum acacia, was acceptable to panellists, and reduced the viability of *E.coli* O157:H7 > 5 $\log_{10}$ units in dry fermented sausages after 28d with drying at 13 °C. Therefore, microencapsulation of natural antimicrobials may contribute to enhanced efficacy against this pathogen in dry fermented sausage.

2.4. Microencapsulation

Microencapsulation and controlled-release technology have found broad application in the pharmaceutical, healthfood, paper and cosmetic industries (Reineccius 1995). Microencapsulation is a process in which sensitive ingredients (core or active agents) are entrapped in a protective polymeric encapsulating material (wall or coating substance) that allows the release of the active agents at controlled rates under specific conditions (Hogan et al. 2001a).

Microencapsulation systems are beneficial for the following reasons. Firstly, they protect and stabilize the core material from deterioration or adverse environmental conditions such as metal ions, pH, free radicals, enzymes or water in a target environment that could include food. Secondly, they reduce threshold concentrations of active agents necessary to achieve the desired effects. Thirdly, microencapsulation extends the reactivity period of bioactive ingredients which can increase over-all product quality (Dzieak 1988).

Wall materials can be designed to release the core material after a defined time (delayed release), for a defined time at a defined rate (prolonged release) or at a defined location (target release) within living animal (human) or in other environments (Depypere et al. 2003). Wall material is chosen based on desired physical and chemical properties such as mechanical strength, glass/melting transition, film-forming ability, low viscosity at higher concentrations and its effective re-dispersion behavior to facilitate release of the core material (Trubiano and Lacourse 1988; Depypere et al. 2003). The wall material should be insoluble and non-reactive with the core material, i.e., a water-insouble polymer is used to encapsulate an aqueous core material. Choice of wall

material is also made by taking into account the required final active agents performance and the desired release mechanism (Brazel 1999; Depypere et al. 2003). By selecting the appropriate wall material, the release of microencapsulated ingredients in food systems can be triggered by temperature or moisture change, a phase change in the wall material (melting), response to changes in pH, ionic strength, enzymatic action or mechanical disintegration of the microcapsules (Lee and Rosenberg 2000a). Carbohydrates, natural and modified gums or proteins can be used as wall materials for preparing water soluble microcapsules (Gibbs et al. 1999). However, carbohydrates and related products lack emulsification properties and cannot be used as wall materials without use of a surface active agent (Hogan et al. 2001b). On the other hand, whey proteins have good emulsification, gelation and film forming properties (Lee and Rosenberg 2000a). In addition, whey protein isolates (WPI) have been reported to provide effective protection against oxidation of encapsulated lipids during storage under conditions that are known to promote their oxidation (Kim and Morr 1996).

There are many techniques available for microencapsulation of food ingredients such as spray drying, extrusion, fluidized bed drying, coacervation, liposome entrapment and multiple emulsion technology (Augustin et al. 2001). Multiple emulsion technology is a microencapsulation strategy that can be used for preserving sensitive ingredients. Emulsions are heterogeneous systems that contain a fine dispersion of one liquid in a second largely immiscible liquid, and exist in the form of droplets which are thermodynamically instable (Garti and Aserin 1996; Cramp et al. 2004). Furthermore, double emulsions, an emulsion within an emulsion, are more complex systems and can be defined as liquid or semisolid dispersions that have chemically similar internal and

external phases, as well as an intermediate immiscible phase, which separates the inner and outer phases (Garti 1997a; Rodrfguez-Huez et al. 2005). Double emulsions are in reality are multiple emulsions because of their dynamic nature resulting from their heterogeneous stability. Two forms of double emulsions have been described. First in an oil-in-water-in-oil ($O_1/W/O_2$) emulsion, O_1 is entrapped in an aqueous phase (W) containing a surface active agent with a high hydrophilic-lipophilic balance (HLB) to form an O_1/W emulsion, that in turn is dispersed in a second oil phase (O_2) containing another surface active agent with a low HLB to form the $O_1/W/O_2$ emulsion (Avendano-Gomez et al. 2005). A second type is a water-in-oil-in-water ($W_1/O/W_2$) emulsion containing both W/O and O/W simple emulsions. In these, small water droplets (W_1) are entrapped inside an oil phase (O) containing a surface active agent with a low HLB which are dispersed in a continuous water phase (W_2) containing another surface active agent with a high HLB (van der Graaf et al. 2005). It should be noted that HLB is a semi-empirical scheme that is used to classify emulsifiers based on the ratio of the weight of the hydrophilic portion of the molecule to the lipophilic portion (McClements 1999). Hydrophilic emulsifiers with an HLB > 8 are used to stabilize oil-in-water emulsions while hydrophobic emulsifiers with an HLB < 7 are used to stabilize water-in-oil emulsions. However, those with an HLB of around 7 can be used for both type of emulsions (Dickinson 1992).

Because these systems contain a "reservoir phase" inside droplets of dissimilar phase, they can serve to prolong the release of active ingredients. Since their first description 80 years ago, multiple emulsions have many applications in different disciplines and have been used as delivery vehicles in foods, cosmetics, pharmaceuticals,

and for agricultural chemicals, as red blood substitutes, in separation processes and as solvent reservoirs in drug overdose treatments (Dogru et al. 2000; Wen and Papadopoulos 2000; Jiao and Burgess 2003). However, control over active agent release is poor and this characteristic plus their low stability during storage compared to simple W/O or O/W emulsions have meant that multiple emulsions have been of limited value to the pharmaceutical and food industry for ingredient delivery. Unpredictable release of active agents from these emulsions can result from coalescence of the internal aqueous phase, coalescence of the multiple oil droplets, rupture of the oil layer and migration of the surfactant from one interface to another (Florence and Whitehill 1981; Opawale and Burgess 1998). Instability can also develop from the immiscibility of the dispersed and continuous phases and high interfacial tension. When the dispersed phase is broken into droplets, the surface energy is increased. This increase in the interfacial free energy causes thermodynamic instability of the dispersed phase, leading to droplet coalescence (Jiao et al. 2002). Garti (1997b) suggested three possible approaches to improve stablity and slow internal aqueous phase release: stabilization of the internal interface of the inner emulsion; selection of a more appropriate oil phase with increased viscosity; or stabilization of the aqueous phase. Phase stabilization can be achieved with surface active agents. In addition, Musashino et al. (2001) suggested that dehydration of multiple emulsions may be used as a strategy to enhance their stability.

2.4.1 Methods for multiple emulsion ($W_1/O/W_2$) preparation.

Two principal methods are used for the preparation of multiple emulsions. Both of these involve the preparation of a W_1/O emulsion followed by the addition of this emulsion to another external phase to create the $W_1/O/W_2$ emulsion.

1- Turbulence-based methods

The primary emulsion (W_1/O) is prepared under high shear conditions to obtain small droplets (0.5-2.0 μm). The second emulsification is carried out under lower shear conditions to avoid the disruption of the internal droplets. A $W_1/O/W_2$ emulsion is created by adding the primary emulsion to another external aqueous phase with a droplet size of 10-60 μm by controlled agitation (Garti 1997b). The stability and the release rate of the internal aqueous phase are dependent on the shear stress used to prepare the double emulsion (van der Graf et al. 2005). This method is suitable if the dispersed phase viscosity and/or volume fraction is large (Joscelyne and Trägårdh 2000). However, this method is not suitable for shear-stress sensitive ingredients (Lambrich and Schubert 2005).

2- Membrane-based method

This is relatively a new technique in which the dispersed phase is pressed through a membrane with pores of uniform size into a continuous phase containing a surfactant (Fig. 2.3) (Joscelyne and Trägårdh 2000; Nakashima et al. 2000). The primary emulsion (W_1/O) is prepared by allowing the aqueous phase to pass through a hydrophobic membrane into the oil phase. The primary emulsion is then passed under pressure through a hydrophilic membrane with a pore size larger than the hydrophobic emulsion droplets into the external aqueous phase to yield the $W_1/O/W_2$ emulsion (Joscelyne and Trägårdh 2000). The main advantage from this method is the production of emulsion droplets of defined and uniform size which are controlled by both the size of membrane pores and the surfactant concentration (Rayner and Trägårdh 2002). Another benefit is that this method requires low energy consumption (van der Graaf et al 2005). On the other hand,

this procedure is time-consuming compared to turbulence-based methods (Okochi and Nakano 2000).

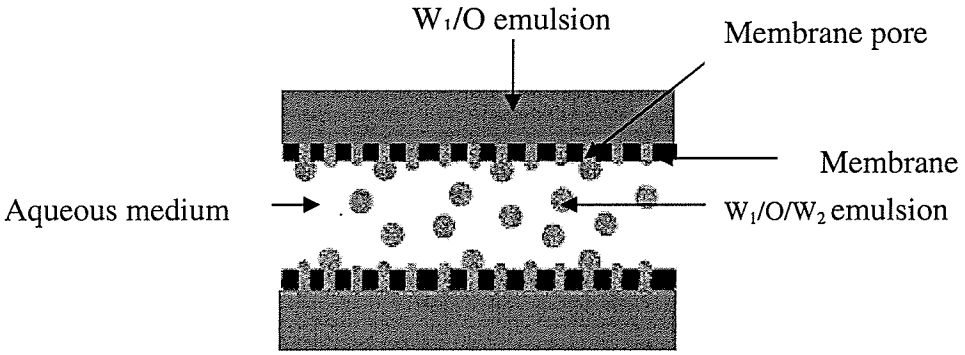

Fig. 2.3: Principles of membrane emulsification (adopted from Nakashima et al. 2000).

2.4.2 Mechanism of release from emulsions

Three mechanisms can be involved in release of active agents from emulsions (Benichou et al. 2004).

First, water and water soluble molecules can be transported by reverse micellization and be involved in spontaneous emulsification. In this process reverse micelles and emulsified water droplets are formed at the W_1/O interface. Water in reverse micelles diffuses through the bulk oil from the W_1/O to the O/W_2 interface. It is this feature that makes double emulsions "multiple emulsions".

Second, water and water soluble material can be transported via hydration of the surfactant (Wen and Papadopoulos 2001). Hydration of the hydrophilic portion of a hydrophobic surfactant can occur at the W_1/O interface in the presence a low salt concentration. The hydrated surfactant can then diffuse through the thin oil layer from the W_1/O to the O/W_2 interface. At high salt concentration dehydration occurs at the O/W_2 interface (Colinart et al. 1984; Wen and Papadopoulos 2000 and 2001). It is worth

nothing that the rate of water transport is higher by this mechanism than the previously described method.

Thirdly, water diffusion can be responsible for active agent release from microcapsules. Water movement through the oil layer in $W_1/O/W_2$ emulsions, to or from the internal aqueous phase, is controlled by the difference in the osmotic pressure between the two aqueous phases (Yan and Pal 2001). If osmotic pressure is higher in the internal phase, water will pass from the external to the internal phase resulting in swelling and eventual rupture of the oil layer. However, if the osmotic pressure is higher in the external phase, water will move to the external phase from the internal phase and lead to its shrinkage and destabilization (Jager-Lezer et al. 1997).

The diffusion of water was found to be dependent on the concentration and type of lipophilic emulsifier used. When surfactants were used that had high molecular weight, the transport of water via inverse micellar formation could not be induced even under iso-osmotic conditions (Jager-Lezer et al. 1997). When the concentration of lipophilic emulsifier was increased, the swelling capacity of the W_1/O emulsion increased but the droplet size decreased. Thus, the release of the internal aqueous phase was delayed. In contrast, increasing the volume fraction of the internal phase decreased its swelling capacity, and as a result water release was accelerated (Yan and Pal 2001).

As previously described two emulsifiers are used in the production of $W_1/O/W_2$ emulsions, and one must be hydrophobic and the other hydrophilic. Higher concentration of the hydrophobic emulsifier in the oil phase and lower concentrations of the hydrophilic emulsifier in the outer water phase are needed to stabilize these emulsions (Garti and Bisperink 1998). The concentration of the hydrophilic emulsifiers must be optimized

carefully because concentrations greater than the optimal in the outer phase can cause destabilization of $W_1/O/W_2$, emulsions because the oil film is then easily ruptured (Pay et al. 2002). In some cases the two emulsifiers can interact at the external O/W_2 interface and interfere with the overall stabilizing performance of the surfactants (Opawale and Burgess 1998). This interaction can be avoided by using a combination of surfactants with high HLB in the W_2 phase (Kanouni et al 2002).

2.4.3 Interfacial (surface) tension

The narrow region which separates the water and oil phases, called the interfacial region, constitutes a small fraction of the total volume of an emulsion, but plays a major role in its stability (McClements 2005).

Because of hydrophobic effects, the interaction of oil with water is thermodynamically unfavorable at the interface. It is therefore necessary to supply free energy to the system in order to increase the contact area between oil and water molecules. The amount of energy that must be supplied to achieve emulsion stability is proportional to the increased contact area between oil and water created in the emulsion (Norde 2003).

One method used to increase the free energy or decrease the surface tension is the addition of an emulsifier or surface active agent. Choice of emulsifier is based on its hydrophilic-lipophilic balance and the physical chemical properties of the dispersed phase (Walstra 2003).

The emulsifier will accumulate at the interface separating the oil and water phases when the free energy of the adsorbed state of the emulsifier is lower than when in a non-adsorbed state. Under these conditions the non-polar part of the emulsifier will rearrange

itself in the oil layer while the polar part will orient itself in the water phase, and as a result the number of unfavorable thermodynamic interactions between the oil and water phases will be reduced. In addition, at higher concentrations of emulsifier at the interface there will be greater reduction in the interfacial tension (McClements 2005). Therefore in double emulsions, the concentration of hydrophobic emulsifier used and adsorbed at the first interface must be carefully chosen to allow the formation of microcapsules but should not be so high that the release of the encapsulated active ingredient is prevented.

Chapter 3

Effect of bovine lactoferrin against *Carnobacterium viridans*

3.1 Abstract

Lactoferrin (LF) alone (8 mg/ml) was able to kill 4 $\log_{10}$ CFU/ml of *C. viridans* at 4 °C, 10 °C and 30 °C in All Purpose Tween (APT) or Lauria broth (LB). In the presence of 2.5% NaCl in each broth system, LF activity shifted from a bactericidal to bacteriostatic effect at all tested temperatures. The addition of sodium bicarbonate (SB) up to 0.16M did not alter the activity of LF in LB broth containing 2.5% NaCl. However, when 5 mg/ml sodium hexametaphosphate (SHMP) was used with 2.5% NaCl, LF regained some of its bactericidal activity at 30 °C in APT but not in LB broth. This reversal did not occur in APT at 4 or 10 °C. Except for the observation with SHMP at 30 °C, neither sodium lactate (SL) nor SHMP enhanced the bactericidal activity of LF in LB or APT broth containing < 2.5% NaCl. It was suggested that 2.5% NaCl in LB and APT broths increased media osmolarity and prevented LF access to binding sites on the contracted cell membrane. This is the first report showing a bactericidal effect of unmodified LF at neutral pH and refrigeration temperatures.

Key words: Lactoferrin, *Carnobacterium*, bactericidal action, natural antimicrobial

3.2 Introduction

Interest has arisen recently in the possible use of lactoferrin as a natural antimicrobial agent in food. Lactoferrin (LF) is the main iron-glycoprotein present in the milk of various mammals and it exerts an antimicrobial effect against a wide range of Gram-negative and Gram-positive bacteria, fungi, and parasites (Shimazaki 2000). In addition, LF has antioxidant, antiviral, anti-inflammatory, immune-modulating, anti-

cancer effects and can promote the growth of probiotic bacteria like *Bifidobacterium* (Aguila and Brock 2001).

LF has been shown to be bacteriostatic due to its ability to bind iron and limit its availability in the growth environment. LF can be bactericidal by binding to the surface of Gram-negative bacteria and causing release of lipopolysaccaride (LPS) (Ellison et al. 1988). In addition, LF is reported to have bactericidal effects against Gram-positive bacteria by binding to lipomannan, which is present on the surface of *Micrococcus luteus* or binding to proteins on the surface of *Clostridium perfringens* (De Lillo et al. 1997; Tomito et al. 1998).

The antibacterial activity of LF is dependent upon its concentration, the degree of iron saturation of the molecule as well as its interaction with mineral media constituents. Payne et al. (1990) found that 46 mg/ml of LF (52% iron-saturated) reduced the viability of *Listeria monocytogenes* in UHT milk 16% when compared with control samples, but a bacteriostatic effect was achieved when 30 mg/ml of 18% iron-saturated LF was used. When EDTA was added to UHT milk the activity of LF against *Escherichia coli* O157:H7 or *L. monocytogenes* was not enhanced (Murdock and Matthews 2002). Salamah and Al-Obaidi (1995) reported that $\leq$ 60 mM Ca^{+2} did not affect the bactericidal activity of human LF against *Yersinia pseudotuberculosis* but 3 to 32 mM Mg^{+2} decreased activity in a manner inversely related to its concentration. High concentrations of divalent cations in growth media can protect bacteria from LF by causing the latter to change its tertiary structure, forming tetramers that adversely affect LF biofunctionality and by increasing the stability of the cell membrane (Shimazaki 2000). Ellison et al. (1988) reported that bicarbonate could restore the ability of human LF to release LPS

from *E.coli* CL99-2 and *Salmonella* Typhimurium SL696 grown in Hanks balanced salt solution (HBSS) containing high concentrations of calcium and magnesium. Naidu (2001) found that the addition of 0.01 M sodium bicarbonate enhanced the ability of immobilized LF to detach *E.coli* O157:H7 from surfaces of beef carcasses. This suggested that bicarbonate could act as stabilizing agent for LF and serve as a companion anion to chelate metal ions. Ellison and Giehl (1991) reported that the combination of LF and lysozyme was bactericidal against *Vibrio cholerae, S.* Typhimurium and *E.coli* while each protein alone produced a bacteriostatic effect. Some work has been done to study the effect of monovalent cations on the activity of LF, but interactions are not well understood. Bortner et al. (1989) found that NaCl did not eliminate the antimicrobial effect of LF against *Legionella pneumophila,* but the concentrations used were not mentioned. In addition, 2 and 5 M NaCl did not cause the dissociation of human LF or bovine LF from the surfaces of *Cl. perfringens* and *Shigella flexneri*, respectively (Tigyi et al. 1992; Tomito et al. 1998).

The activity of LF was temperature dependent against *L. pneumophila* (Bortner et al. 1986) and *Y. pseudotuberculosis* (Salamah and Al-Obaidi 1995). The antibacterial activity of LF at 37 and 42 °C was lost when the treated samples were incubated at 1°C to 25 °C. Bortner et al. (1989) found that human LF was unable to bind to the surface of *L. pneumophila* at 4 °C. Further, Nibbering et al. (2001) reported that expression of bactericidal effects by human LF required bacterial cells to be metabolically active. Murdock and Matthews (2002) reported that after 7d in UHT milk at 4 °C there was no difference in numbers of inoculated *L. monocytogenes* between untreated control samples and those treated with LF at neutral pH.

The objectives of the present study were to investigate the antimicrobial activity of bovine LF against a cured meat spoilage organism, *Carnobacterium viridans* and determine how this activity is affected by growth media (Lauria broth, LB, or All Purpose Tween broth, APT) which contained different cation concentrations. The influence of temperature, NaCl concentration and the presence of compounds capable of chelating cations in reaction mixtures were studied to better understand factors governing LF action.

3.3 Materials and Methods

3.3.1 Preparation of culture and test media

Carnobacterium viridans MPL-11 was originally isolated from spoiled cured meat (Holley et al. 2002) and was kept frozen in glycerol. It was maintained on APT agar slants at 4 °C and transferred monthly to maintain viability. Working cultures were prepared by growth in 10 ml APT broth incubated at 30 °C for 2d. For tests, 100 μl was transferred to 100 ml APT broth and incubated at 30 °C until the absorbance ($A_{620\ nm}$) reached 0.045 which corresponded to 7.4 $\log_{10}$ CFU/ml. This was approximately the mid-exponential phase of growth. The culture was added to test media (APT or LB) to give a final concentration of approximately 4 $\log_{10}$ CFU/ml.

Bovine lactoferrin (Bioferrin 2000) was obtained from Glanbia Nutritionals (Glanbia Ingredients, Inc., Monroe, WI). Stock solutions of LF were prepared to give final concentrations of 8, 16 and 32 mg/ml by dissolving LF in distilled water and filter sterilizing (0.22 μm syringe filter, Fisher Scientific, Fairlawn, NJ) prior to addition to sterilized growth media.

Stock solutions of sodium bicarbonate, SB (J.T Baker, Phillipsburg, NJ) were prepared to give final concentrations of 0.005 to 0.16 M at 2-fold increments. Sodium hexametaphosphate, SHMP (Sigma, St.Louis, MO) was prepared to give final concentrations of 1, 3 and 5 mg/ml by dissolution in distilled water and sterilization at 121 °C for 15 min. Sodium lactate, SL (Sigma) stock solutions were prepared to give final concentrations of 1, 3 and 5% (w/v) by dissolution in distilled water and sterilization by filtration (0.22 μm, Fisher Scientific) before addition to heat-sterilized growth media. Double strength LB containing 10g/l tryptone (Difco, division of Becton Dickinson, Sparks, MD), 5g/l yeast extract (Difco), 1g/l glucose (Mallinckrodt, Paris, KY) and 5g/l NaCl (Fisher Scientific) and double strength APT (46.2g/l) broth (Difco) were used to assess the activity of LF. To investigate the effect NaCl on the activity of LF, double strength broths above were prepared to yield 0.5, 1.0, 1.5, 2.0 and 2.5% NaCl at testing. Standard formulations for cured meat products often include 2.5% NaCl. The pH of each growth medium was measured with an Accumet Basic pH meter (Fisher Scientific). Calcium, magnesium, sodium, potassium and iron content in both LB and APT broth containing 0.5 and 2.5% NaCl were determined by Inductively Coupled Plasma -Optical Emission Spectrometry, (ICP-OES; Varian Liberty 200, Varian Canada Inc. Mississauga, ON) by G. Morden, Dept. Geological Sciences, University of Manitoba.

3.3.2 Antimicrobial assay

Antimicrobial assays were carried out following the procedure outlined by Davidson and Parish (1989). Microcentrifuge tubes (1.5 ml flat top microcentrifuge tubes, Fisher Scientific) used contained the following: 250µl growth medium, 250µl distilled water, 250µl SHMP, 250µl SL, or 250µl SB, 500 µl LF and 500 µl culture

diluted in same growth medium and then the tubes were incubated at 30, 10, or 4 °C. At regular intervals ≤ 16 h for tubes incubated at 30 °C and up to 10 d for tubes incubated at 10 and 4 °C, a 100μl sample was taken to determine the viable cells present in each tube by serially diluting each sample in 0.1% peptone water and plating in duplicate on APT agar using the spiral plate method (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA) To investigate the effect of possible changes in cell membrane permeability (fatty acid composition) on LF activity, cells were grown at 4 and 10 °C before the addition to tubes containing LF. Test media with LF were tempered to 30, 10, or 4 °C, culture was added and the antimicrobial assays were carried out at 30, 10 and 4 °C as described above.

3.3.3 Statistical analysis

All data were analyzed by Statistical Analysis System (version 8.1) software (SAS Institute, Inc., Cary, NC). Analyses of variance by the General Linear Models procedure and Duncan's multiple range tests were used to find significant differences ($p<$ 0.05) between treatments at a particular time.

3.4 Results

In LB broth containing 0.5% NaCl, there was no difference in the lethality of LF at 8, 16 or 32 mg/ml after 2h at 30 °C, but the level of surviving cells was lower with 32 mg/ml (Table 3.1). Again, the effect at 10 °C was also concentration dependent with higher concentrations of LF yielding fewer survivors. No viable organisms were found at 12h (0.5d) of treatment (Table 3.2). LF at all concentrations used produced 4 log reductions after 6h at 4 °C (Table 3.3).

In APT broth containing 0.5% NaCl the effect of LF was both temperature and concentration dependent. At all three temperatures lethal effects of LF developed more

slowly in APT than in LB but were still evident (Tables 3.1, 3.2, 3.3). Higher concentrations of LF yielded fewer surviving cells at all test temperatures. For cells previously adapted to 10 and 4 °C before being challenged with LF, there was no difference in the susceptibility of the cells to LF in LB broth at all incubation temperatures. The same trend was noticed in APT except for cells grown at 10 °C and challenged with 8 and 16 mg/ml LF at 30 °C which were more resistant, and 16 h (compared to 4h, Table 3.1) was required for their elimination. However, in the same treatment when 32 mg/ml LF was used no differences were found in LF resistance of cells grown at the lower temperatures. To evaluate changes in LF potency at higher NaCl levels, a concentration of 32 mg/ml LF was chosen for further study.

3.4.1 Effect of NaCl on the activity of LF

As the concentration of NaCl was increased from 0.5 to 2.5%, the lethality of LF decreased. The rapid onset of the bactericidal effect that was noted at 0.25h in LB containing 0.5% NaCl (Table 3.1) took 16h to achieve in the presence of 1.0% NaCl and only bacteriostatic effects were found ≥ 1.5% NaCl when tested at 30 °C (Fig. 3.1). A similar result was obtained at 4 and 10 °C, but the maximum lethality was reduced to 2.4 log and took 10d to develop in the presence of 1.0% NaCl. Again only bacteriostatic effects were found at NaCl levels of 1.5% (Fig. 3.2).

In APT broth, the reduced activity of LF was more obvious at increased NaCl levels and at lower temperatures. In the presence of 1.0% NaCl only a 2 log reduction was found, but at 2.5% NaCl and at 30 °C *C.viridans* grew by 1.2 log CFU/ml within 16h (Fig. 3.1). At 1.5 and 2.0% NaCl, LF was bacteriostatic in APT. At lower temperatures in the presence of 1% NaCl, LF was also bacteriostatic and at ≥ 1.5 % NaCl inhibitory

effects were lost (Fig. 3.3). Similarly, the increase in cell survival at 10 °C was greater than at 4 °C in the presence of 1.5 and 2.0% NaCl.

3.4.2 SMHP

Sodium hexametaphosphate in LB containing 0.5% NaCl had a negative effect upon the lethal activity of LF and did not prevent the interference with LF activity caused by higher (2.5%) NaCl concentrations (Fig. 3.4). Greater neutralization of LF action by SHMP was evident at 10 °C and was most pronounced at 4 °C (Fig. 3.5). In contrast, when APT was tested, SHMP interacted synergistically with LF to kill *C.viridans* in the presence of 2.5% NaCl at 30 °C (Fig. 3.6). However, this partial restoration of LF activity by SHMP was eliminated at both lower temperatures examined and its addition to APT containing 0.5% NaCl yielded reduced LF activity as found in LB broth (Fig. 3.7).

3.4.3 Sodium lactate

The influence of 1 to 3% SL upon LF action against *C.viridans* was examined at 30 °C or 10 °C with ≤ 2.5% NaCl. By itself 1% SL was bacteriostatic (Fig. 3.8) and caused a slight reduction in viability at 3 and 5% in APT and LB containing ≤ 2.5% NaCl at 30 °C over 16h . There was an antagonistic interaction between SL and LF in both growth media at 0.5% NaCl and the bactericidal effect of LF when used alone became bacteriostatic in the presence of SL (Fig. 3.8). At 10 °C and ≤ 2.5% NaCl similar results were found.

3.4.4 Sodium bicarbonate

In contrast with SHMP and SL, SB did not affect the action of LF against *C.viridans* in LB containing 0.5% NaCl at 30 °C. However, ≤ 0.16 M SB did not enhance

the activity of LF in the presence of 2.5% NaCl in LB at 30 °C (Fig. 3.9) and similar results were found at 10 °C or 4 °C . By itself SB was not inhibitory to *C.viridans* at the concentrations used.

3.4.5 Mineral content of LB and APT broths

Results from mineral analysis of the two media are shown in Table 3.4. APT broth contained substantially higher levels of potassium, calcium, magnesium and iron than LB.

3.4.6 pH of growth media

The pH of growth media before SL, SHMP, or SB were added was 6.7 ± 0.1. The pH of LB was between 7.2 and 8.5 when 0.02 to 0.16 M SB was added. At the highest concentrations used both SL and SHMP reduced the pH of APT to 6.1 and 6.2, respectively, and of LB to 5.8 and 6.1, respectively.

5.0 Discussion

Lactoferrin is believed to exert bacteriostatic effects through chelation of iron, but rapid bactericidal effects have been reported as a result of its binding to bacterial cells. Such a mechanism could explain its rapidly lethal action against *C.viridans* in media tested here. De Lillo et al. (1997) reported that the bactericidal effects of LF against *M. luteus* resulted from the ability of LF to bind to lipomannan, a lipoglycan present at the cell surface. The major difference between LF resistant *Micrococcus* strains and *M. luteus* was the absence of lipomannan from the surface of resistant strains. LF activity in the present study was found to be dependent on growth media composition, with greater activity occurring in LB broth. The increased resistance of *C.viridans* toward LF in APT broth may be explained by the higher concentration of cations in this medium. APT broth

contained 14 times more magnesium and iron and 3 times more calcium than LB. In another work, Payne et al. (1990) reported that there was a bacteriostatic effect of LF against *L. monocytogenes* in UHT milk that contained the same concentration of iron as LB broth (7.5 μM), but the inhibitory effect was eliminated by the addition of 0.125 M ferric ammonium citrate. Similarly, Shin et al. (1998) indicated that the minimal inhibitory concentration of LF against *E.coli* O157:H7 in PYG broth was twice as high as in buffered peptone water.

Our results have demonstrated that LF activity against *C.viridans* is reduced at lower temperature but this is the first report showing bactericidal action of LF at neutral pH and refrigeration temperatures. In contrast to previous research with *L. pneumophila* and *Y. pseudotuberculosis* (Bortner et al. 1986; Salamah and Al-Obaidi 1995), LF was found to be bactericidal in both broths at 10 and 4 °C against *C.viridans*, but complete elimination of viable organisms took longer at lower temperatures. Nibbering et al. (2001) concluded that expression of bactericidal effects by human LF required metabolically active bacterial cells. However, Murdock and Matthews (2002) found that LF was inactive against *L. monocytogenes* cells during 7d incubation at 4 °C in UHT milk, a temperature at which the organism can grow. However, at 4 °C they also found with *E.coli* O157:H7 that if low pH (pH 4) was used or if LF was treated with porcine pepsin to create LF hydrolysate, activity was increased and 1 and 2 log reductions were observed, respectively. Bortner et al. (1989) found that LF was unable to bind to the surface of *L. pneumophila* cells at 4 °C. Results from the present study showed that there was no difference between the bactericidal action of LF against cells grown at 4 °C or 10 °C and challenged with LF at 30 °C and that found when cells were grown at 30 °C before

challenge with LF. We concluded that membrane fluidity, normally higher in cells grown at the lower temperatures (Berry and Foegeding 1997) had little influence upon susceptibility to lethal effects and therefore binding of LF by *C.viridans*. Whether metabolic activity in target cells is needed for bactericidal action by LF is still unclear since *C.viridans* grows, albeit slowly, at refrigeration temperatures. It is certainly possible that access to these binding sites on the cell surface is temperature dependent. Arnold et al. (1980) found that *Streptococcus mutans* became resistant to LF when grown in Brain Heart Infusion Broth containing 1.0% sucrose before being challenged with LF in saline. The authors concluded that the presence of extracellular, capsular polysaccharide prevented access by LF to binding sites on cell surfaces.

As the concentration of NaCl was increased in the growth media, the antimicrobial activity of LF against *C.viridans* was shifted from a bactericidal to a bacteriostatic effect. Limited additional information is provided by the work of Naidu et al. (1991b) and Tigyi et al. (1992) who found that 2 M NaCl did not cause the dissociation of human LF from the surfaces of *E.coli* O127 and *S. flexneri*, respectively, and Tomito et al. (1998) who reported that binding of bovine LF to the surface of *Cl. perfringens* was unaffected by the presence of 5 M NaCl. Tigyi et al. (1992) and Tomito et al. (1998) concluded that binding of LF to bacterial cells did not involve electrostatic interaction, but rather they suggested that hydrophobic interaction may be responsible for LF binding since 2 to 3 M KSCN was able to dissociate LF from the surfaces of these test organisms. In these two studies the effect of NaCl on the viability of cells treated with LF was not examined. However, binding of LF to the cell surface does not always result in lethal events. Bacteria such as *Haemophilus influenzae, Actinobacillus*

pleuropneumonie and *Bordetella pertussis* can express membrane bound proteins that complex with lactoferrin under iron-restricted conditions and facilitate iron uptake (Farnaud and Evans 2003).

The results are in agreement with those of Kiyosawa (1991) who reported 61.1 % survival of *Clostridium innocuum* challenged with LF in saline containing 150 mM NaCl, but only 37.1% survived in deionized water. It is more likely then, that electrostatic interaction may be involved in the binding of LF to the surface of *C.viridans* to yield its bactericidal effect. From the present results and those of others, it is apparent that the nature of LF interaction with the cell surface is dependent on the target organism and the conformation of LF and the relative strength of electrostatic or hydrophobic interactions that occur in specific environments. The LF molecule has both hydrophobic and hydrophilic regions (Naidu 2000). It is important to keep in mind that the nature of LF binding sites can be different in different organisms. In *S. flexneri* and *Cl. perfringens* binding sites were protein in nature but they consisted of carbohydrate in *M.luteus* (Tigyi et al. 1992; Tomito et al. 1998; DeLillo et al. 1997).

Sodium bicarbonate has been reported to protect LF from divalent cations by maintaining its structural integrity (which is essential for its ability to bind to the cell surface or to chelate iron), but in our experiments SB $\leq$ 160 mM did not improve LF activity in the presence of 2.5% NaCl in LB with low cation levels. Therefore, it is probable that sodium did not affect the structural stability of LF.

In an attempt to overcome the reduced activity of LF in the presence of NaCl at high concentrations, SHMP or SL in addition to SB were added separately to reaction mixtures. An antagonistic interaction was found between LF and both SL and SHMP in

both growth media at lower NaCl concentration, while SB had no effect. However, the sodium content in 160 mM SB, 1% SL and 5 mg/ml SHMP was estimated to be 160, 89 and 49 mM, respectively and their addition caused further increases in the sodium content of test media. Ellison and Giehl (1991) reported that the activity of LF and lysozyme were reduced as the osmolarity of the media increased. They found that when the osmolarity became > 60 *mOsmol,* activity was lost even though test media contained: ≤ 37 mM Na^{+}, ≤ 0.01 mM Ca^{+2}, ≤ 0.01 mM Mg^{+2}, and ≤ 0.01 mM Fe. However, these concentrations were less than the concentrations found in the present study in LB or APT containing 0.5% NaCl. Thus, the reduced activity of LF in the presence of higher concentrations of NaCl and both SL and SHMP could be attributed to the increased osmolarity of the media which can lead to reduction of cell volume and contraction of the cell membrane. This in turn may prevent LF access to target sites on the cell membrane. Furthermore, Naidu et al. (1991a) reported that LF binding capacity at the surface of *Staphylococcus aureus* was lower in skim milk, broth media or media rich in salt or carbohydrate (mannitol salt agar or staphylococcus medium 100) compared to blood or nutrient agar. Differences in binding appeared to be due to differences in the osmolarity between the two groups of nutrient materials.

The synergistic interaction between LF and SHMP in APT broth containing 2.5% NaCl could be explained by the ability of SHMP to chelate the higher levels of calcium and magnesium in APT and allow the destabilization of the cell membrane and improve LF performance. The absence of this effect at 10 °C and 4 °C may reflect reduced interference by Ca^{2+} with the metabolic activity of *C.viridans* at reduced temperatures.

In the present study when SL was added to APT and LB broths containing 0.5% NaCl, pH values reached 6.15 and 5.81, respectively, and this was accompanied by a reduction in LF activity. However, it was unlikely that the lower pH alone was responsible for reduced LF activity. Bortner et al. (1989) and Salamah and Al-Obaidi (1995) found that decreasing the pH of growth media to 5 increased the sensitivity of bacteria to LF. The reduced activity of LF in the presence of SL could explain the resistance of *Lactobacillus casei* towards LF (Arnold et al. 1980). This could mean that under suitable conditions LF and LAB may be used in combination to control Gram-negative foodborne pathogens and spoilage bacteria. In order for LF to be bactericidal in cured meats, which often contain $\geq$ 2.5% NaCl, the interference shown by NaCl will need to be overcome.

Acknowledgements

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Table 3.1

Survival of *C.viridans* (Log_{10} cfu/ml) after treatment with lactoferrin in LB and APT broth containing 0.5% NaCl at 30 °C.

	LB broth				APT broth			
Lactoferrin [1]	0	8	16	32	0	8	16	32
Time (h)								
0	4.14a [2]	4.14a	4.14a	4.14a	4.16e	4.16e	4.16e	4.16e
0.25	4.24a	2.54b	2.39b	2.20c	4.18e	4.08e	4.15e	4.05e
0.5	4.54a	2.41b	2.20bc	2.08c	4.20e	4.07e	4.11e	4.07e
1	4.56a	1.90b	1.70c	1.60c	4.24e	4.13e	4.14e	4.08e
2	4.60a	nv b [3]	nv b	nv b	4.49e	3.82f	3.33g	3.18g
4	4.93a	nv b	nv b	nv b	5.10e	nv f	nv f	nv f
16	5.80a	nv b	nv b	nv b	5.90e	nv f	nv f	nv f

1- (mg/ml).

2- Means from each broth in the same row with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of three experiments.

3- No viable cells were detected.Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 30 °C for growth.

Table 3.2
Survival of *C.viridans* (Log_{10} cfu/ml) after treatment with lactoferrin in LB and APT broth containing 0.5% NaCl at 10 °C.

	LB broth				APT broth			
Lactoferrin [1]	0	8	16	32	0	8	16	32
Time (day)								
0	4.14a [2]	4.14a	4.14a	4.14a	4.16e	4.16e	4.16e	4.16e
0.25	4.66a	1.77b	0.99c	nv d [3]	4.30e	4.17ef	4.12ef	4.07f
0.5	4.89a	nv b	Nv b	nv b	4.90e	4.05f	4.08f	4.10f
1	5.27a	nv b	Nv b	nv b	5.34e	4.15f	4.04f	3.32f
2	5.40a	nv b	Nv b	nv b	6.61e	3.48f	3.43f	nv g
5	7.04a	nv b	Nv b	nv b	8.36e	nv f	nv f	nv f
10	7.70a	nv b	Nv b	nv b	8.92e	nv f	nv f	nv f

1- (mg/ml).
2- Means from each broth in the same row with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of three experiments.
3- No viable cells were detected.Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 30 °C for growth.

Table 3.3
Survival of *C.viridans* (Log_{10} cfu/ml) after treatment with lactoferrin in LB and APT broth containing 0.5% NaCl at 4 °C.

	LB broth				APT broth			
Lactoferrin [1]	0	8	16	32	0	8	16	32
Time (day)								
0	4.14a [2]	4.14a	4.14a	4.14a	4.16e	4.16e	4.16e	4.16e
0.25	4.30a	nv b [3]	nv b	nv b	4.17e	4.11e	4.11e	4.07e
0.5	4.50a	nv b	nv b	nv b	4.31e	4.10e	4.12e	4.13e
1	4.79a	nv b	nv b	nv b	4.63e	4.13f	3.97f	3.40g
2	5.19a	nv b	nv b	nv b	5.01e	4.13f	4.11f	3.37g
5	5.96a	nv b	nv b	nv b	6.45e	3.24f	2.23g	nv h
10	7.06a	nv b	nv b	nv b	7.82e	nv f	nv f	nv f

1- (mg/ml).
2- Means from each broth in the same row with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of three experiments.
3- No viable cells were detected. Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 30 °C for growth

Table 3.4

Mineral content[1] of LB and APT broths containing different concentrations of NaCl.

	Na^+		K^+		Ca^{+2}		Mg^{+2}		Fe	
Growth Medium	ppm	mM	ppm	mM	ppm	mM	ppm	mM	ppm	mM
LB[2]	569	24.74	167	4.28	0.22	0.0055	3.81	0.1568	0.50	0.0090
LB+ 0.5% NaCl	1783	77.52	245	6.28	0.21	0.0053	3.39	0.1395	0.45	0.0081
LB+ 2.5% NaCl	9822	427.04	312	8.00	0.21	0.0053	2.91	0.1198	0.38	0.0069
APT[3] + 0.5% NaCl	1835	79.78	1265	32.43	0.55	0.0138	50.38	2.0733	6.39	0.1156
APT + 2.5% NaCl	10050	436.95	1613	41.36	0.51	0.0128	43.18	1.7770	6.23	0.1127

1- Mineral analysis was conducted by by Gregg Morden in the Dept. Geological Sciences, University of Manitoba using Inductively Coupled Plasma -Optical Emission Spectrometry.

2-Lauria broth.

3- All purpose tween broth contains 0.5% NaCl in its commercial formula.

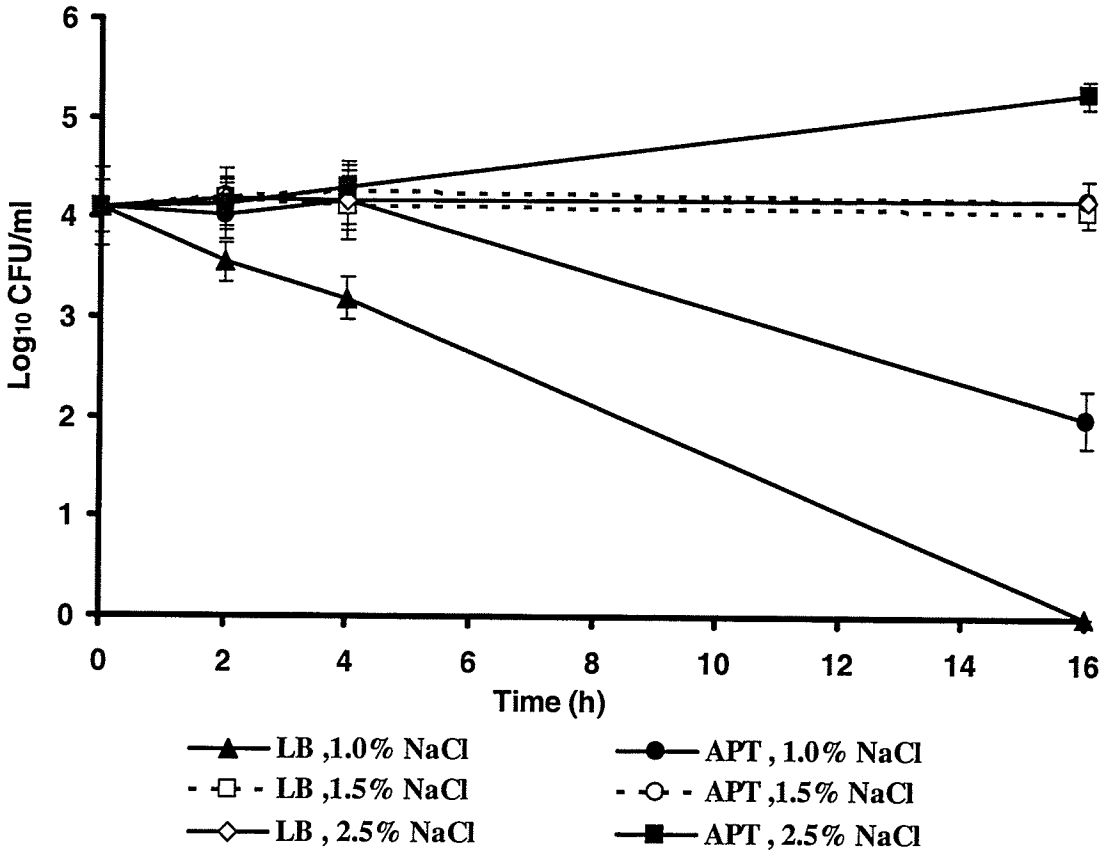

Fig 3.1
Effect of 32mg/ml lactoferrin against *C.viridans* in All Purpose Tween (APT) and Lauria Broth (LB) containing 1.0, 1.5 or 2.5% NaCl at 30 ° C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

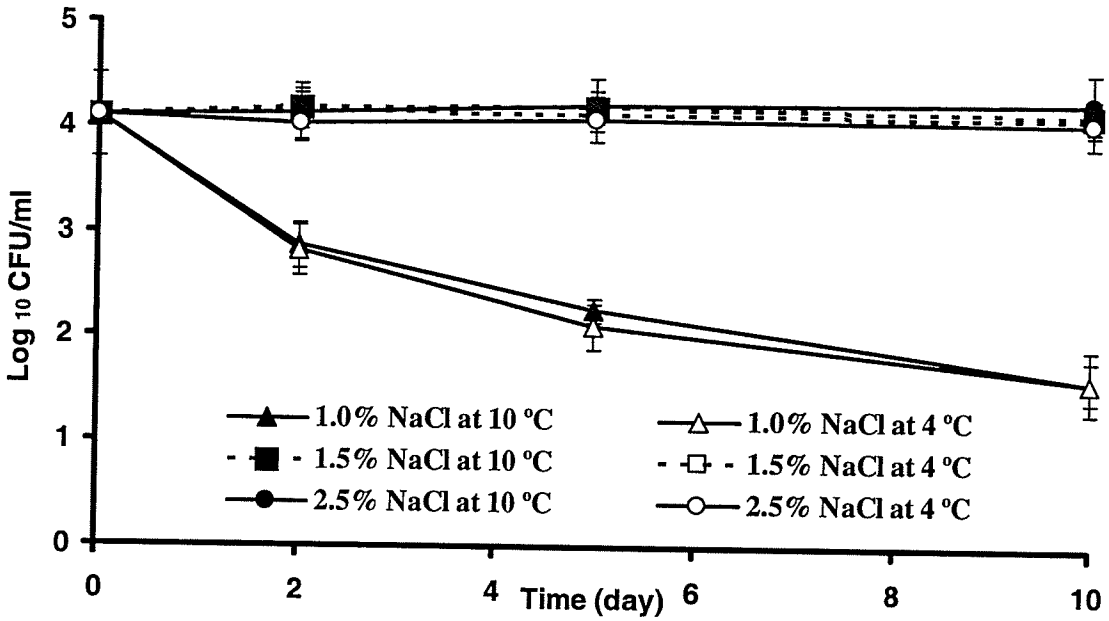

Fig 3.2
Effect of 32mg/ml lactoferrin against *C.viridans* in LB broth containing 1.0, 1.5 or 2.5% NaCl at 4 or 10 ° C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

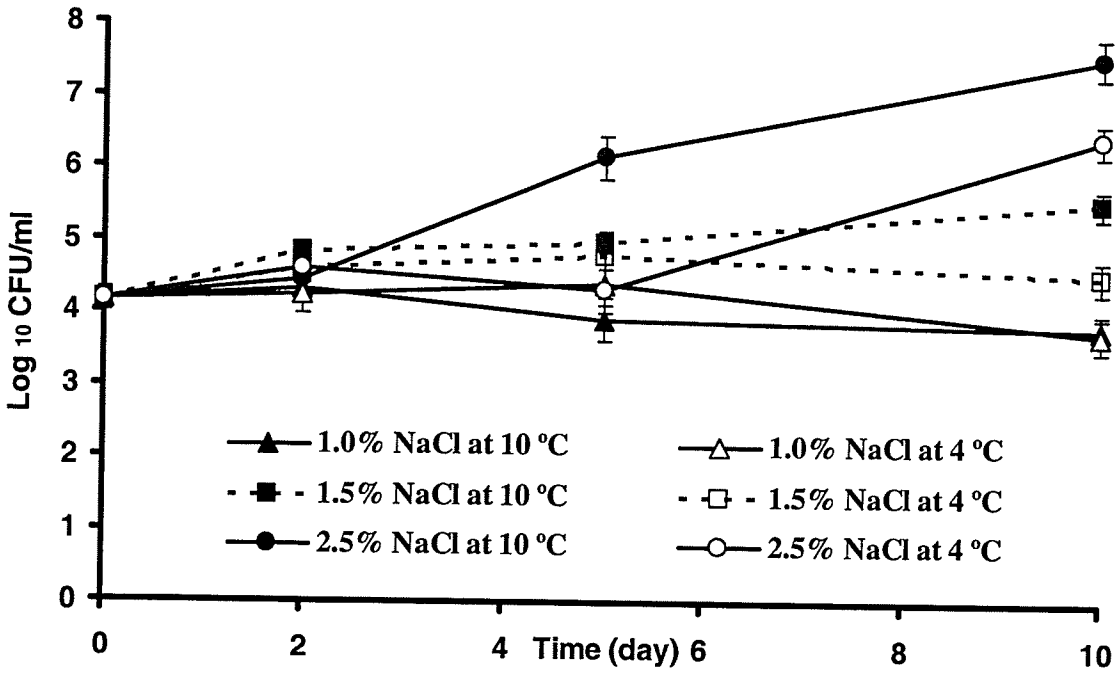

Fig 3.3
Effect of 32mg/ml lactoferrin against *C.viridans* in APT broth containing 1.0, 1.5 or 2.5% NaCl at 4 or 10 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

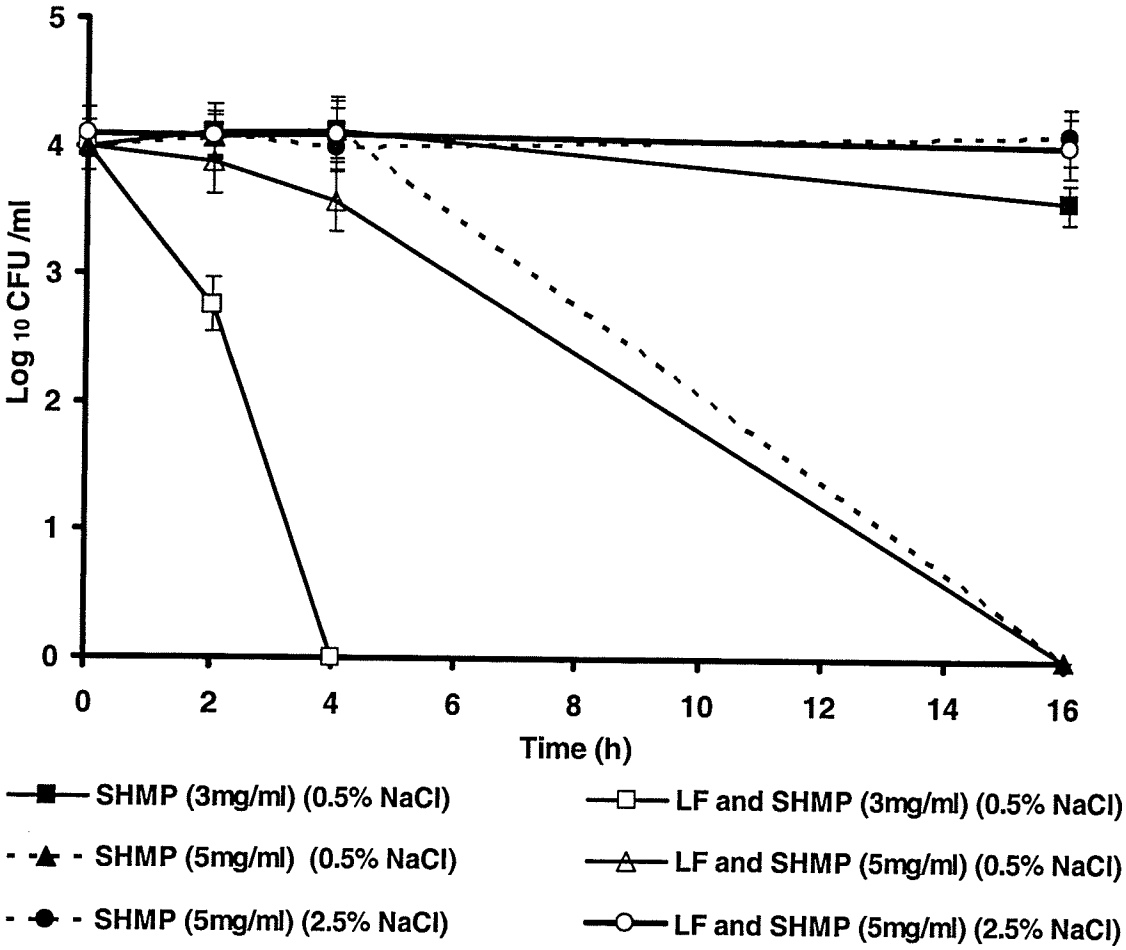

Fig 3.4
Effect of 32mg/ml lactoferrin (LF) and sodium hexametaphosphate (SHMP) against C.*viridans* in LB broth containing 0.5 or 2.5% NaCl at 30 ° C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

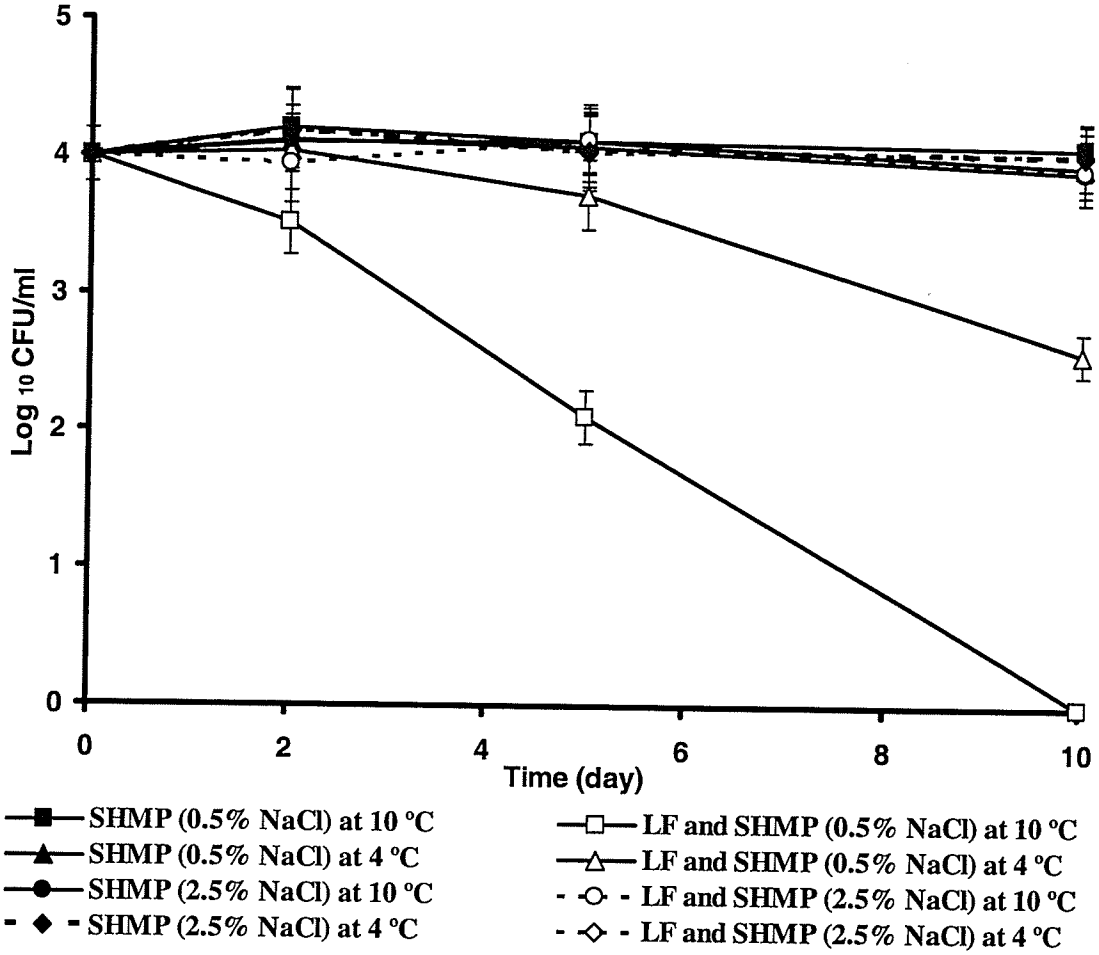

Fig. 3.5. Effect of 32mg/ml lactoferrin (LF) and 5 mg/ml sodium hexametaphosphate (SHMP) against *C.viridans* in LB containing 0.5 or 2.5% NaCl at 10 and 4 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

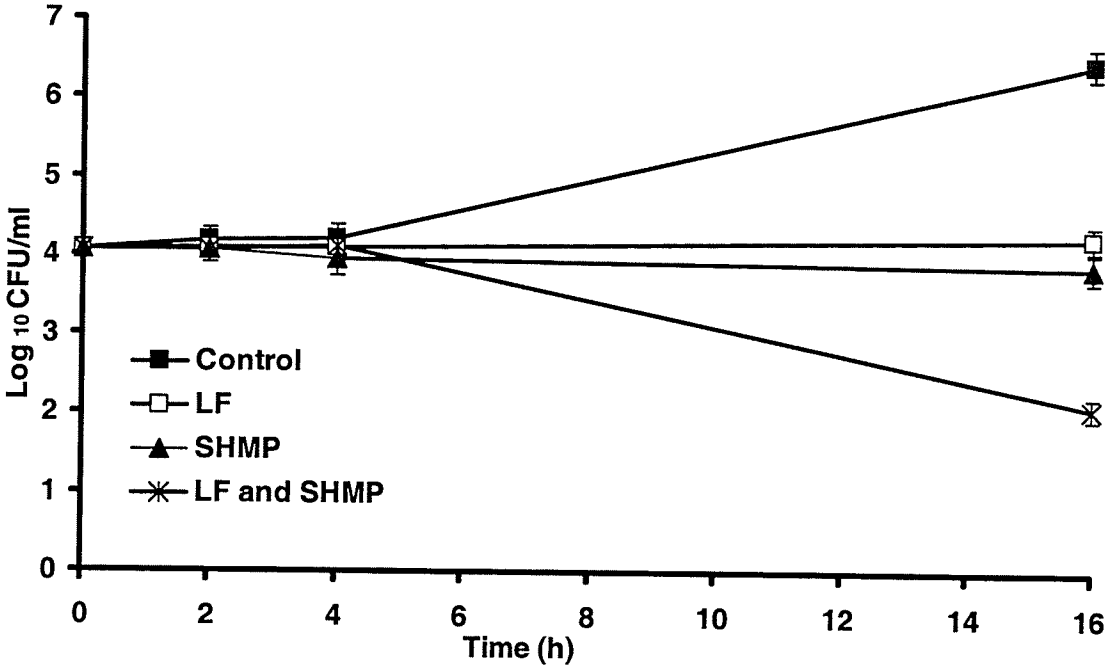

Fig. 3.6
Effect of 32 mg/ml lactoferrin (LF) and 5 mg/ml sodium hexametaphosphate (SHMP) against *C.viridans* in APT containing 2.5% NaCl at 30 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

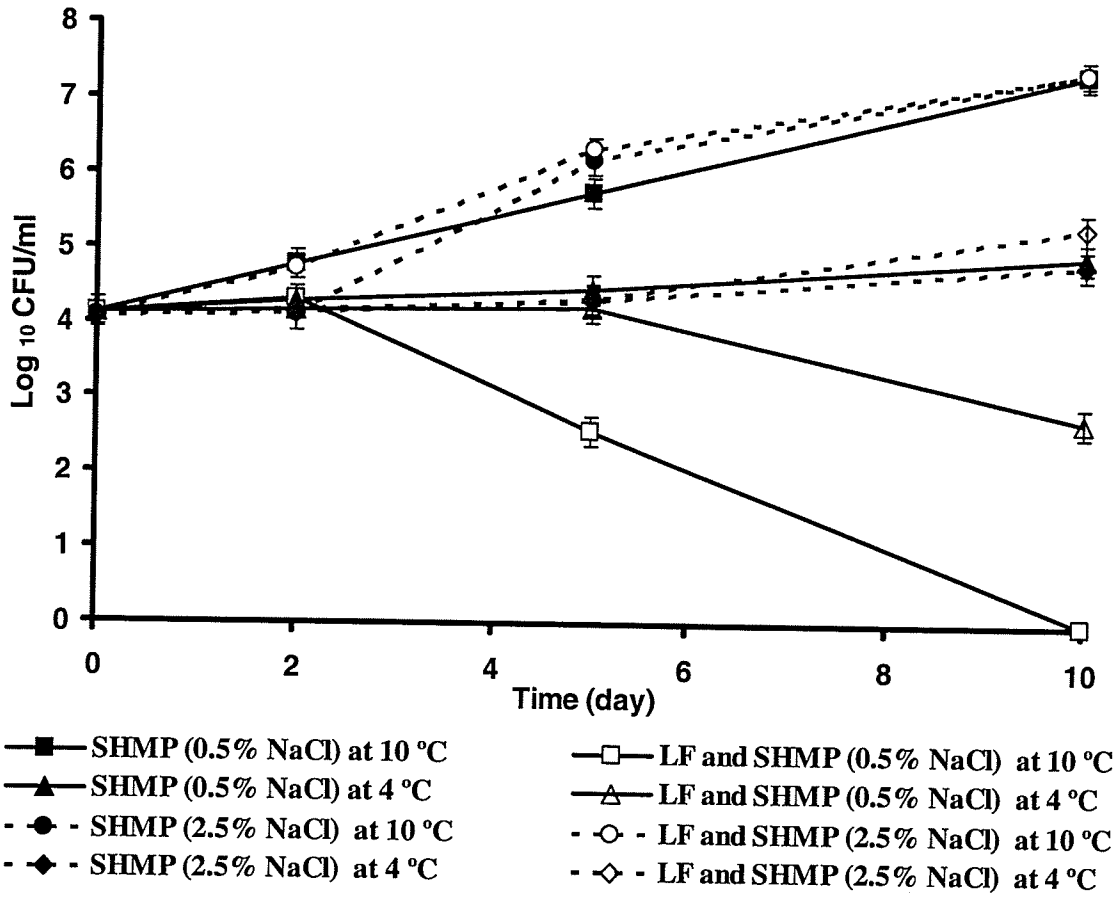

Fig 3.7
Effect of 32 mg/ml lactoferrin (LF) and 5 mg/ml sodium hexametaphosphate (SHMP) against *C.viridans* in APT broth containing 0.5 or 2.5% NaCl at 10 and 4 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

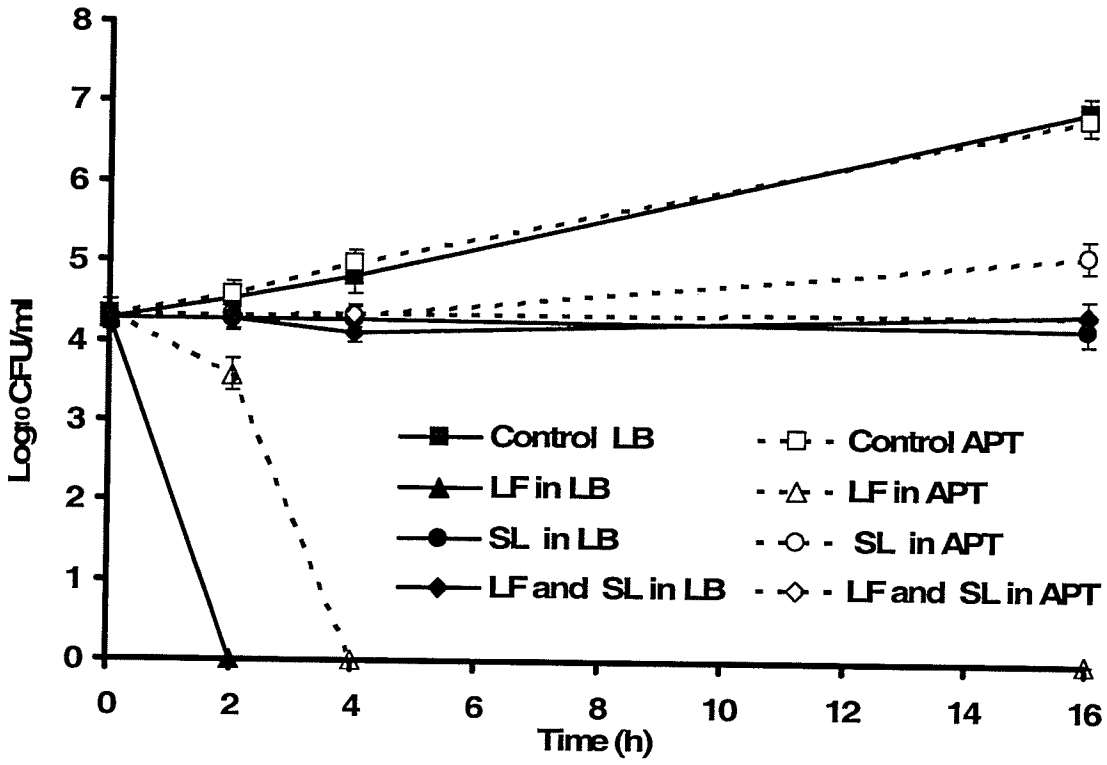

Fig. 3.8
Effect of 32 mg/ml lactoferrin (LF) and 1.0% sodium lactate (SL) against *C.viridans* in LB or APT containing 0.5% NaCl at 30 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

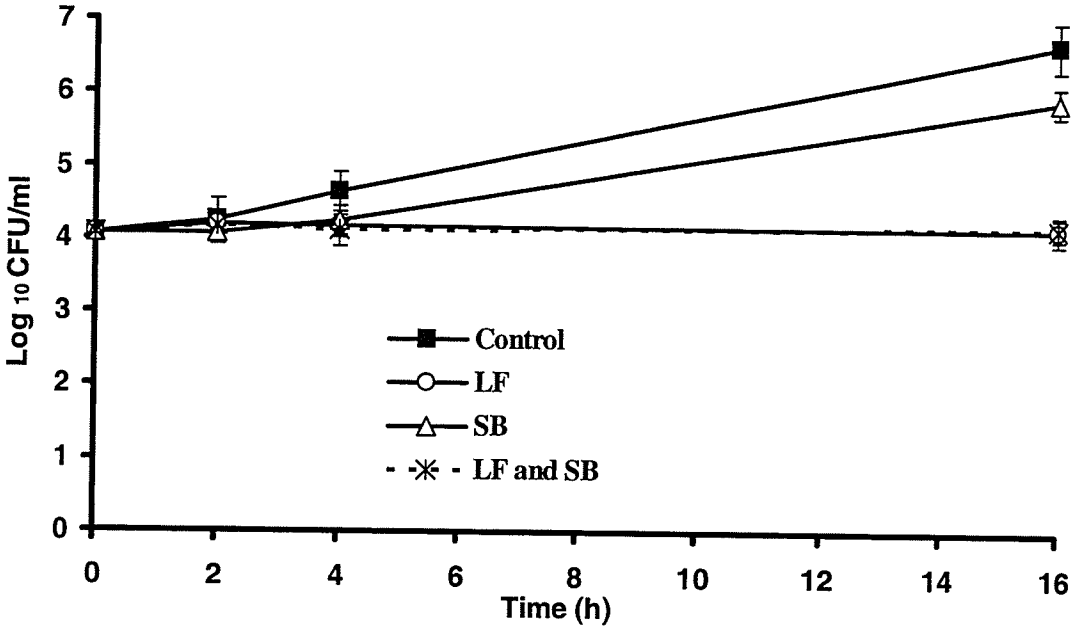

Fig 3.9
Effect of 32 mg/ml lactoferrin (LF) against *C.viridans* in LB broth containing 2.5% NaCl in the presence of 0.16 M sodium bicarbonate (SB) at 30 °C. Results shown are the means of two experiments Vertical bars indicate 95% confidence limit.

Chapter 4
Enhancing the antimicrobial effects of bovine lactoferrin against *E.coli* O157:H7 by cation chelation, NaCl and temperature

4.1 Abstract:

Aim: To evaluate the effect of NaCl, growth medium and temperature on the antimicrobial activity of bovine lactoferrin (LF) against *E.coli* O157:H7 in the presence of different chelating agents.

Methods and results: LF (32 mg/ml) was tested against *E.coli* O157:H7 strain 3081 in Luria broth (LB) and All Purpose Tween (APT) broth with metal ion chelators sodium bicarbonate (SB), sodium lactate (SL), sodium hexametaphosphate (SHMP), ethylene diamine tetraacetic acid (EDTA) or quercetin at 0.5 and 2.5% NaCl at 10 and 37 °C. LF and the chelators were tested against 4 other *E.coli* O157:H7 strains in LB at 2.5% NaCl and 10 °C. LF alone was bacteriostatic against strains 3081 and LCDC 7283 but other strains grew. Antimicrobial effectiveness of LF was reduced in APT broth but enhanced by SB at 2.5% NaCl and 10 °C where 4.0 $\log_{10}$ CFU/ml inoculated cells were killed. EDTA enhanced antimicrobial action of the LF-SB combination. SL alone was effective against *E.coli* O157:H7 but a reduction in its activity at 2.5% NaCl and 10 °C was reversed by LF. The combinations LF-SHMP and LF-quercetin were more effective at 37 °C and NaCl effects varied.

Conclusions: LF plus SB or SL were bactericidal toward the same 3/5 *E.coli* O157:H7 strains and inhibited growth of the others at 2.5% NaCl and 10 °C.

Significance and Impact of the Study: The combination of LF with either SL or SB shows potential for reducing viability of *E.coli* O157:H7 in food systems containing NaCl at reduced, but growth permissive temperature.

Key words: *E.coli* O157:H7, lactoferrin, sodium bicarbonate, sodium lactate, EDTA, antimicrobial, chelation.

4.2 Introduction:

High profile foodborne illness outbreaks and increased consumer demand for all natural food products have necessitated the further development of effective antimicrobial preservative systems for the meat industry. Within the last few years, considerable interest has developed regarding the possible use of bovine lactoferrin (LF) for the surface decontamination of beef carcasses and subsequently its possible use as a natural food preservative. Lactoferrin is the main iron –binding glycoprotein present in the milk of various mammals that exerts an antimicrobial effect against many Gram-negative and Gram-positive bacteria, fungi, and parasites (Shimazaki 2000; Farnaud and Evans 2003). In addition, it can promote the growth of probiotic bacteria like *Bifidobacterium* (Aguila and Brock 2001).

Lactoferrin is structurally monomeric, bilobal (C and N lobe), with a molecular mass of about 80 kDa. It has been shown to be bacteriostatic due to the capability of each lobe to bind the Fe^{3+} ion with high affinity in the presence of carbonate or bicarbonate anions, even at low pH values and thus suppress bacterial growth by producing an iron deficient environment. Furthermore, it can also be bactericidal against Gram-negative and Gram-positive bacteria following binding of the N-terminal of the molecule to bacterial surfaces, and with Gram-negative bacteria LPS is released from the outer membrane (De Lillo et al. 1997; Dionysius and Milne 1997; Nibbering et al. 2001).

Studies have indicated that LF has the potential to be used as a natural antimicrobial preservative in the food industry (Salamah and Al-Obaidi 1995; Murdock

and Matthews 2002). Unfortunately, the success in simple broth systems such as peptone or distilled water and buffered phosphate has not been achieved in food or complex media because the antimicrobial activity of LF appears to be reduced in the presence of divalent cations like Ca^{2+} and Mg^{2+} at concentrations between 1-5 mM (Ellison et al. 1988). Shimazaki (2000) reported that divalent cations protect bacteria from LF by inducing changes in its tertiary structure yielding a tetrameric form of LF with reduced biofunctionality and at the same time generating bacterial cell membranes with increased stability.

To enhance the antimicrobial activity of LF, work has been conducted to determine whether compounds such as metal ion chelators could overcome the effect of divalent cations. Ellison et al. (1988) reported that when bicarbonate was added to a broth system, it served as a "companion" anion preventing induction of LF tetramers by Ca^{2+} and Mg^{2+} by increasing monomeric LF stability. They found that the addition of bicarbonate to Hanks balanced salt solution (HBSS) containing high concentrations of Ca^{2+} and Mg^{2+} enhanced the ability of human (h) LF to release LPS from the surface of *Escherichia coli* CL99-2 and *Salmonella* Typhimurium SL696. Naidu (2001) found that the addition of 0.01 M sodium bicarbonate improved the ability of "immobilized" LF to prevent *E.coli* O157:H7 from attaching and growing on beef carcass surfaces, although the mechanism responsible was not fully described. In another study, the addition of ethylene diamine tetraacetic acid (EDTA), a strong cation chelator, did not enhance the activity of LF against *E.coli* O157:H7 in UHT milk (Murdock and Matthews 2002).

Other chelating agents such as sodium hexametaphosphate (Denyer and Maillard 2002), SHMP, and lactate (Alakomi et al. 2000) may act to enhance LF activity against

E. coli O157:H7. SHMP is widely used in food processing for emulsification, moisture retention, leavening, buffering and improving the tenderness of processed meat (Vaara and Jaakkola 1989). In addition, SHMP has been reported to have antimicrobial activity against Gram-negative bacteria by binding divalent cations which causes increased permeability of the cell membrane to hydrophobic molecules (Knabel et al. 1991; Denyer and Maillard 2002). Lactic acid or sodium lactate which are also used as antimicrobials in the food industry can act to increase permeability of the Gram-negative bacterial outer membrane while reducing cytoplasmic pH (Alakomi et al. 2000).

Studies on the effects of NaCl on LF antimicrobial activity have generated controversial results. A better understanding of the interaction between the two compounds in foods containing high concentrations of NaCl (2.5%) such as cured meat products would be useful. Bortner et al. (1989) reported that NaCl did not eliminate the antimicrobial effect of LF against *Legionella pneumophila* in distilled water, but the concentrations used were not mentioned. In other work , 2 mol l^{-1} NaCl did not affect the ability of hLF to interact with the *E. coli* cell surface (Naidu et al. 1991). Al-Nabulsi and Holley (2005) found that increasing the concentration of NaCl from 77 to 427 (mM Na^+) in LB broth shifted the activity of LF against *Carnobacterium viridans* from a bactericidal to a bacteriostatic effect. To further illustrate the complex effect of NaCl on LF activity, Viejo-Diaz et al. (2003) found the bactericidal effect of hLF against *E.coli* ML-35 in 0.3% bactopeptone was eliminated when the concentration of Na^+ was $\geq$40 mM or K^+ was $\geq$20 mM.

Other studies have examined the effect of temperature on the antimicrobial activity of lactoferrin. Salamah and Al-Obaidi (1995) reported that the antibacterial

activity of hLF against *Yersinia pseudotuberculosis* was higher when treated samples were incubated at 37 and 42 °C than at 1°C to 25 °C. The ability of hLF to bind to the surface of *L. pneumophila* was lost when the temperature was reduced to 4 °C (Bortner et al.1989). In addition, Nibbering et al. (2001) reported that hLF was bactericidal only when bacterial cells were metabolically active. However, Murdock and Matthews (2002) found that 1 and 2 $\log_{10}$ reductions in numbers of inoculated *E. coli* O157:H7 could be achieved in UHT milk after 7d at 4 °C if low pH (pH 4) was used or if LF was hydrolyzed with porcine pepsin. Since cations interfere with the antimicrobial effectiveness of LF hydrolyzates more than with non-hydrolyzed LF (Yamauchi et al. 1993), the present study examined factors affecting the antimicrobial activity of the intact LF molecule.

The objective of the present study was to investigate the effect of NaCl and temperature in the presence of different chelating compounds that may be used in cured meat formulations as well as the chelator quercetin, (a bi-flavonoid) on the antimicrobial activity of LF against *E.coli* O157:H7 in Luria broth, LB, or All Purpose Tween broth, APT, which contain low or medium concentrations of cations, respectively, compared to cured meat products.

4.3 Materials and Methods

4.3.1Preparation of cultures

E.coli O157:H7 strain 3081 (animal isolate) was obtained from Dr. W. C. Cray, National Animal Disease Center, Ames IA. *E. coli* O157:H7 strain LCDC 7110 (human isolate) and *E.coli* O157:H7 strain LCDC 7283 (hamburger steak isolate) were provided by the Laboratory Centre for Disease Control, Ottawa ON. *E. coli* O157:H7 strain E318N

(human isolate) was provided by Dr. S. Read, Health of Animals Laboratory, Agriculture Canada, Guelph ON and *E.coli* O157:H7 strain CRIFS # 828 was obtained from Dr. M. Griffiths, University of Guelph, ON. All cultures were kept as frozen glycerol stocks. They were maintained on APT agar slants at 4 °C and transferred monthly to maintain viability. Working cultures were prepared by growing a single colony in 10 ml APT broth incubated at 37 °C for 24h. For tests, 100 μl was transferred to 100 ml APT broth and incubated at 37 °C until the optical density ($O.D_{620\ nm}$) reached 0.180 which corresponded to 7.4 $\log_{10}$ CFU/ml. This was approximately the mid-exponential phase of growth. The cultures were added to test media (APT or LB) to give a final concentration of approximately 4 $\log_{10}$ CFU/ml by dilution in the same growth media.

4.3.2 Bovine lactoferrin

Bovine lactoferrin (Bioferrin 2000) was provided by Glanbia Nutritionals (Glanbia Ingredients, Inc., Monroe, WI). The iron saturation of LF was 14mg /100g as determined by Glanbia. A stock solution of LF was prepared to give a maximum final concentration of 32 mg/ml in test media by dissolving LF in distilled water and filter sterilizing (0.22 μm syringe filter, Fisher Scientific, Fairlawn, NJ) before addition to the test media.

4.3.3 Preparation of chemicals

Stock solutions of sodium bicarbonate, SB (Sigma, St.Louis, MO), were prepared to give final concentrations of 0.02, 0.04 and 0.16 mM. Sodium hexametaphosphate, SHMP (Sigma), was prepared by dissolution in distilled water to yield final concentrations of 1, 3 and 5 mg/ml. EDTA disodium salt (Fisher Scientific) was prepared by dissolution in distilled water to yield a final concentration of 0.5 mg/ml. SB, SHMP

and EDTA were sterilized at 121 °C for 15 min. Sodium lactate, SL (Sigma), stock solutions were prepared by dissolution in distilled water to give final concentrations of 1, 3 and 5% (w/v) and were sterilized by filtration (0.22 μm, Fisher Scientific) prior to addition to heat-sterilized growth media. Quercetin (Sigma) was dissolved in 95% ethanol and a stock solution was prepared to give a final concentration of 250 μg/ml at 4.75% ethanol in test broth.

4.3.4 Test Media

Double strength APT (46.2g /l) broth (Difco) and double strength LB containing 10g/ l tryptone (Difco, division of Becton Dickinson, Sparks, MD), 5g/ l yeast extract (Difco), 1g/ l glucose (Mallinckrodt, Paris, KY) and 5g/ l NaCl (Fisher Scientific) containing 0.5% or 2.5% (w/v) NaCl were used to evaluate LF activity. The pH of each growth medium was measured with an Accumet Basic pH meter (Fisher Scientific).

4.3.5 Antimicrobial assay

The antimicrobial assays were carried out in two stages:

i) The effect of media and metal chelating agents on the activity of LF was characterized in depth against *E.coli* O157:H7 strain 3081 to establish test parameters for use with the other strains. These tests were done in both LB and APT broths.

ii) The antimicrobial activity of LF was evaluated against each of the five strains of *E.coli* O157:H7 in LB broth containing either 0.5% or 2.5% NaCl at 10 or 37 °C.

The antimicrobial assays were carried out following the procedure described by Al-Nabulsi and Holley (2005). Microcentrifuge tubes (1.5 ml flat top tubes, Fisher Scientific) used contained the following: 250 μl double strength growth medium, 250 μl of treatments (distilled water, SB, SL or SHMP), 500 μl LF and 500 μl culture diluted

in the same growth medium and then the tubes were incubated at 37 or 10 °C. When quercetin was used, 250 µl double strength growth medium, 75 µl of the stock solution and 175µl of distilled water were added to give a final concentration of 250µg ml^{-1} of quercetin and 4.75 % (v/v) ethanol. In addition, 500 µl LF and 500 µl culture diluted in the same growth medium were used and the tubes were incubated as previously described. At intervals of 2, 4 and 16 h for tubes incubated at 37 °C and at 0, 2, 5 and 10 d for tests at 10 °C, a 100µl sample was withdrawn from each tube to enumerate the viable cells. This was done by serially diluting each sample in 0.1% peptone water and plating in duplicate on APT agar using the spiral plate method (Autoplate 4000, Spiral Biotech, Inc., Bethesda, MD). Agar plates were incubated at 37 °C for 48h. Bacterial injury was assessed by plating selected LF-NaCl treatments on both APT and cefixime-tellurite sorbitol McConkey agar (ct-SMAC) (Zadik et al. 1993). Plates were incubated at 37 °C for 48h and the difference in viable numbers on the two agars represented the injured population.

4.3.6 Statistical analysis

All data were analyzed by Statistical Analysis System (version 8.1) software (SAS Institute, Inc., Cary, NC). Analyses of variance by the General Linear Models procedure and Duncan's multiple range tests were used to find significant differences ($p<$ 0.05) between treatments at sampling times.

4.4 Results

LF by itself had no antimicrobial activity in LB broth containing 0.5% (77mM) NaCl at 37 °C against the tested strains in the present study (data not shown). The antimicrobial activity of LF was enhanced with some strains either by increasing the

concentration of NaCl to 2.5% (427mM) or by lowering the temperature. At 37 °C in LB containing 2.5% NaCl, *E.coli* O157:H7 strain E318N was more susceptible than the other strains with a one log reduction observed at 16h, while the viable numbers of *E.coli* O157:H7 strain CRIFS # 828 increased by 1.7 $\log_{10}$ CFU/ml after 16h. LF showed bacteriostatic activity against the other tested strains under the same conditions (Fig. 4.1).

At 10 °C in LB with 0.5% NaCl, *E.coli* O157:H7 strain 3081 was initially most susceptible to LF, but all strains grew during 10d incubation (Fig. 4.2). In the presence of 2.5% NaCl, a bacteriostatic effect was noted with *E.coli* O157:H7 strains 3081 and LCDC 7283. With the other strains there was an increase in viable numbers which ranged from 1 to 2 $\log_{10}$ CFU/ml (Fig. 4.3).

4.4.1 Effect of cations in APT broth

The antimicrobial activity of LF that was noted in LB broth with 2.5% NaCl at 10 °C was reduced when *E.coli* O157:H7 strain 3081 was challenged with LF in APT broth. Although again at 37 °C LF was not effective, even in the presence of 2.5% NaCl (data not shown). At 10 °C the viable numbers were 1.5 and 2.1 $\log_{10}$ CFU/ml less than the control in APT containing 0.5 or 2.5% NaCl, respectively (Fig. 4.4).

4.4.2 Sodium bicarbonate

A synergistic interaction occurred between LF and SB under some conditions which yielded improved antimicrobial activity of these agents against *E.coli* strain 3081. This interaction was stronger in the presence of 2.5% NaCl at lower temperature. A 2 or 3 $\log_{10}$ CFU/ml reduction was noted in LB containing 0.5 or 2.5% NaCl, respectively, at 37 °C when 160 mM SB was added to LF (Table 4.1a). At 10 °C, with 0.5% NaCl, no viable cells were detected after 10d when 160 mM SB was used with LF, but only 20

mM of SB was required to obtain a 4.0 $\log_{10}$ CFU/ml reduction after 2d in LB broth containing 2.5% NaCl (Table 4.1b). Identical results were obtained with *E.coli* O157:H7 strain LCDC 7283. *E.coli* O157:H7 strain LCDC 7110 was reduced by 1.8 $\log_{10}$ CFU/ml, but a bacteriostatic effect was noted with *E.coli* O157:H7 strains E318N and CRIFS # 828 when challenged under the same test conditions (Table A 3.1).

The combination of SB and LF was less effective in APT broth than in LB (with 2.5% NaCl) against *E.coli* O157:H7 strain 3081. The higher concentration of SB (160 mmol/ml) and a longer incubation time (5d) were required to give a 4.0 $\log_{10}$ CFU/ml reduction at 10 °C (Fig. 4.5) while a bacteriostatic effect was observed at 37 °C (Fig. 4.6).

While EDTA alone at 0.5 mg/ml was bacteriostatic against *E.coli* O157:H7 strain 3081 in LB at 0.5 and 2.5% NaCl (Figs. 4.7 and 4.8), when combined with only 5 mM SB and 16 mg/ml LF, the mixture caused a 4.0 $\log_{10}$ CFU/ml reduction in 4h at 37 °C and 0.5% NaCl (Fig. 4.7). An increase of SB to 10 mM was necessary to maintain the bactericidal effect when 2.5% NaCl was used (Fig. 4.8). EDTA was not tested at 10 °C or used against the other strains.

4.4.3 Sodium lactate

SL alone at $\geq$ 3% (w/v) showed substantial antimicrobial activity against *E.coli* O157:H7 strain 3081, however, this activity was reduced in most cases by increasing the concentration of NaCl or by decreasing the temperature. The negative effect of higher NaCl concentration and lower temperature on the antimicrobial activity of SL was overcome by an additive (possibly synergistic) interaction between LF and SL. The 4

$\log_{10}$ CFU/ml reduction noted in LB by 3% SL was restored at the higher salt concentration after 16h or 5d at 37 or 10 °C, respectively (Tables 4.2a and 4.2b).

In LB broth containing 2.5% NaCl at 10 °C a 4.0 $\log_{10}$ CFU/ml reduction was noted when 3% (w/v) SL and LF were tested together against *E.coli* O157:H7 strains LCDC 7110 and LCDC 7283. Only a 0.6 or 1.4 $\log_{10}$ CFU/ml reduction was found when the combination was tested against *E.coli* O157:H7 strains CRIFS # 828 and E318N, respectively (Table A 3.1).

In APT broth the combination of 3% (w/v) SL with LF had greater activity against strain 3081 at the higher NaCl level at 10 °C. Only 2d was required to obtain the 4.0 $\log_{10}$ CFU/ml reduction (Fig. 4.5) while only a 1.9 $\log_{10}$ CFU/ml reduction occurred at 37 °C under the same test conditions (Fig. 4.6).

4.4.4 Sodium hexametaphosphate

Neither LF nor SHMP alone or together were effective against *E.coli* O157:H7 strain 3081 at either temperature in LB with 0.5% NaCl. Additive or possibly synergistic interactions between LF and SHMP occurred with 2.5% NaCl at both temperatures. A 4.0 $\log_{10}$ CFU/ml reduction was obtained after 4h in LB containing 2.5% NaCl at 37 °C when LF was combined with $\geq$ 3 mg/ml SHMP (Table 4.3a). However, at 10 °C a reduction of only 1.9 $\log_{10}$ CFU/ml was found when LF was used with 5 mg/ml SHMP (Table 4.3b). Similarly at 10 °C, a 0.7 to 1.0 $\log_{10}$ CFU/ml reduction was noted after 10d with the other strains (Table A 3.1).

The combination of 5 mg/ml SHMP with LF was initially effective against *E.coli* O157:H7 strain 3081 in APT broth at 10 °C with 2.5% NaCl (Fig. 4.5), but the organism recovered after 8d. At 37 °C the combination was ineffective against this strain (Fig. 4.6).

4.4.6 Quercetin

A synergistic interaction between LF and quercetin occurred in LB broth. This interaction was stronger at higher temperature. In LB containing 0.5% NaCl, when LF was combined with quercetin a 4.0 $\log_{10}$ CFU /ml reduction was obtained after 2h at 37 °C, but it took 4h in LB containing 2.5% NaCl (Table 4.4a). In contrast, at lower temperature, only a 1.8 $\log_{10}$ CFU/ml reduction was observed after 10d in the presence of 0.5% NaCl and a 4 $\log_{10}$ CFU/ml reduction took < 5d at 10 °C in LB broth containing 2.5 % NaCl (Table 4.4b). A similar trend was observed with the other strains as with strain 3081 in LB broth containing 2.5 % NaCl at 10 °C (Table A 3.1).

In APT broth containing LF and quercetin plus 2.5% NaCl, a 1.1 or 4.0 $\log_{10}$ CFU/ml reduction of *E.coli* O157:H7 strain 3081 was noted after 5d at 10 °C or 16h at 37 °C, respectively (Figs. 4.5 and 4.6).

4.4.7 pH of growth media

The pH of LB and APT before the addition of SL, SHMP, or quercetin ranged from 6.5-6.6 at both NaCl concentrations. SL and SHMP reduced the pH of LB to 5.8 and 6.1, respectively, while SB raised the pH of LB to 8.5. When quercetin was added to LB the pH was between 6.4-6.9. APT broth resisted changes in pH slightly better than LB. The pH of APT was 6.1 after the addition of SL and SHMP and it became 7.1 when SB was added. In addition, the pH was between 6.3-6.4 when quercetin was added to APT.

4.5 Discussion

E.coli O157:H7 strains used in this study showed resistance toward LF at 37 °C and low NaCl concentration. While strain E318N was most susceptible to LF in LB with 2.5% NaCl at 37 °C, strains E318N and CRIFS # 828 showed greater resistance to most

other treatments including LF plus SB and LF with SL than strains 3081, LCDC 7110, and LCDC 7283. Perhaps it was not surprising that Lee et al. (2004) also found 11 strains of *E.coli* isolated from mastitic milk were resistant to > 50 mg/ml LF in BHI broth. In the present study the range of responses of *E.coli* O157:H7 strains to LF with or without chelators observed and the results reported by others could be related to differences in strain susceptibility, the extent of LF purity, its iron saturation levels, and difference in amounts of cations present in the media used. In many of the previous studies where LF had bactericidal or bacteriostatic effects, the test media used did not support bacterial growth (Bortner et al. 1989; Salamah and Al-Obaidi 1995). It is possible that nutritional stress may have contributed to antimicrobial susceptibility. Both APT and LB supported *E.coli* O157:H7 growth. Dionysius et al. (1993) found that the inhibitory effect of LF (0.1 to 1 mg/ml) against 19 strains of enterotoxigenic *E.coli* isolated from piglets was strain dependent in iron-free AOAC bacto-synthetic broth. They found that 10 strains were inhibited when LF concentration was < 0.2 mg/ml and the other 9 strains were variably affected by higher concentrations of LF.

Another possible reason for the resistance of some stains used in the present study might be that they may have a low binding affinity for LF, since binding of LF to bacterial surfaces is a requirement for initiation of lethal effects. In the present study, this may have been true for the strain CRIFS # 828, but strain E318N which was most susceptible to the antimicrobial effects of LF (at 37°C and 2.5% NaCl), was one of two strains more resistant to LF plus SB or SL. Naidu et al. (1991) reported that human lactoferrin (hLF) bound better than LF to the surface of *E.coli* cells. In addition, they found that enterotoxigenic *E.coli* strains bound significantly higher amounts of hLF than

other pathogenic *E.coli* strains. Erdei et al. (1994) identified porins to be the sites where LF binds to the surface of *E.coli* and they suggested that differences in LF affinity was affected by the O chain of the LPS which was believed to be able shield the porins from interaction with LF. In another study it was found that the major difference between LF resistant *Micrococcus* strains and a sensitive strain of *M. luteus* was the presence of lipomannan (lipoglycan) at the surface of resistant strains (De Lillo et al. 1997). Therefore, low binding affinity caused by a number of factors can protect bacteria from the antimicrobial action of LF.

In the present study it was found that increased concentrations of NaCl in the growth media and lower temperature improved the antimicrobial activity of LF against *E.coli* O157:H7. These observations are in contrast to results with *Carnobacterium viridans* (Al-Nabulsi and Holley 2005) where it was found that the bactericidal effect of LF became bacteriostatic when the NaCl concentration in LB and APT broths was increased from 0.5% to 2.5%. The enhanced activity of LF in the present study at 2.5% NaCl (427 mM Na^+) may have resulted directly from stress and injury caused by NaCl to *E.coli* O157:H7. It was found that the degree of injury in samples treated at 10 °C with LF alone was higher in LB containing 2.5% NaCl. No viable cells were recovered on ct-SMAC agar from samples treated with only LF in LB containing 2.5% NaCl at 10 °C, and under the same conditions at 0.5% NaCl numbers recovered on ct-SMAC were 2.5 $\log_{10}$ CFU/ml less than those recovered on APT agar (Fig. A1). Furthermore it is unlikely that NaCl at the concentrations used interfered with LF binding to the cell surface (Naidu et al. 1991).

The greater activity of LF against *E.coli* O157:H7 strain 3081 in LB than in APT broth could be explained by the lower concentrations of divalent cations and iron in LB. It was found that APT broth contained 14x more magnesium, 3x more calcium and 15x more iron than LB broth (Al- Nabulsi and Holley 2005). Shin et al. (1998) found the minimum inhibitory concentration of LF against *E.coli* O157:H7 was 6 mg/ml in PGY broth (containing 1% bactopeptone, 1% glucose and 0.05% yeast extract) compared to 3 mg/ml in peptone water.

The SB plus LF combination was bactericidal against 3 *E. coli* O157:H7 strains (3081, LCDC 7110 and LCDC 7283) and was bacteriostatic against the others. It was suggested by Naidu (2000) that SB could enhance the antimicrobial activity of LF against *E.coli* O157:H7 by stabilizing LF structure. The greater inhibitory activity of LF plus SB when higher concentrations of SB were used could have resulted from the greater dissociation of SB at pH 8.5 to form carbonate (Corral et al. 1988; Diez-Gonzalez et al. 2000).

Carbonate anions can bind divalent cations, form insoluble complexes and cause destabilization of the outer bacterial membrane (Jarvis et al. 2001). This in turn can enable LF to interact directly with the cytoplasmic membrane causing selective permeation of ions (K^+) without initially affecting the pH gradient (Aguilera et al. 2003). This possibility can be supported by the results obtained when 0.5 mg/ml EDTA was added to the reaction mixture. EDTA can also bind divalent cations that stabilize the outer bacterial membrane and increase its permeability (Vaara 1992). Furthermore, the addition of EDTA lowered concentrations of LF plus SB needed to produce a 4.0 $\log_{10}$ CFU/ml reduction. EDTA alone did not exhibit lethal effects against *E.coli* O157:H7

strain 3081 although growth was inhibited. This can be explained by the ability of EDTA to form insoluble complexes with divalent cations at pH 6.8 which is near its pKa value of 6.2 (Boland et al. 2004).

The substantial lethal activity of SL against *E.coli* O157:H7 found in the present study was reduced at both the higher NaCl concentration and at lower temperature. These results are in agreement with those of Jordan and Davies (2001) and Casey and Condon (2002) who reported that the bactericidal effects of lactic acid were reduced in the presence of NaCl in the growth medium because *E.coli* O157:H7 is better able to regulate internal pH under these test conditions. Jordan and Davies (2001) found that the bactericidal effect of 1.5% (w/v) lactic acid at pH 5 was eliminated when $\geq$ 4 % (w/w) NaCl was added to TSB. Further, McWilliam Leitch and Stewart (2002a, b) reported that the antimicrobial efficiency of lactic acid against *E.coli* O157:H7 was reduced at 5 °C. This was believed due to either reduced permeability of resistant strains caused by alteration of the fatty acid composition of the bacterial membrane or occurred as a result of an increase in the dissociation of lactic acid in the broth at lower temperatures.

The combination of LF with SL in the present study had improved antimicrobial activity over SL alone against *E.coli* O157:H7 at low temperature or in the presence of 2.5% NaCl. The enhanced activity of SL in the presence of LF could also be explained in part by an increase in membrane permeability caused by LF. In turn, lactate may facilitate the interaction of LF with the cytoplasmic membrane by making the outer membrane more permeable and lowering the internal pH (Alakomi et al. 2000). The latter suggested that the increased permeability resulted from weakening of the molecular

interactions of outer membrane components due to protonation of anionic constituents such as carboxyl and phosphate groups.

The bactericidal effect of LF in combination with SHMP in the presence of NaCl may involve SHMP destabilization of the outer membrane of *E.coli* O157:H7, allowing LF to interact with the cytoplasmic membrane. Vaara and Jaakkola (1989) reported that the minimum inhibitory concentration of rifampin against *E.coli* was reduced 10 fold in the presence of 3 mg/ml SHMP. In addition, the uptake of 1-N-phenylnaphthylamine (NPN) (a hydrophobic molecule) by *E.coli* O157:H7 was increased when SHMP was used (Helander and Mattila-Sandholm 2000). Therefore, LF may be useful in inhibiting the growth of *E.coli* O157:H7 in foods where SHMP and NaCl are used as ingredients such as in cured meat products. However, LF plus SHMP was poorly effective at 10 °C, limiting usefulness of this combination for refrigerated perishable foods. The reduced activity of SHMP in combination with LF compared to SB plus LF may be explained by the reduced ability of SHMP to chelate divalent cations at pH 6.1, since the pKa of SHMP is 7.6 (Boland et al. 2004).

Quercetin was better able to enhance the activity of LF in LB containing 0.5% NaCl at 37 °C than the other chelating agents used. However, ethanol at the concentration used to dissolve quercetin was inhibitory to *E.coli* O157:H7 strain 3081, particularly in 2.5% NaCl at 10 °C. Enhanced lethality occurred upon addition of quercetin and when LF was added to this mixture its antimicrobial effectiveness was increased further. While it is possible that quercetin acted to improve LF action by chelation of metal cations (Arima et al. 2002), the usefulness of quercetin in food systems is limited by its poor solubility.

Results presented demonstrated that increased NaCl concentration and lower temperature improved the antimicrobial effect of LF toward *E.coli* O157:H7. LF plus SL showed enhanced activity against *E.coli* O157:H7 strain 3081 than when used individually. In addition, the combination of LF and SB was more effective at lower temperature than when SHMP was used with LF. The combination of LF with either SL or SB has potential for reducing the viability of *E.coli* O157:H7 strains in situations where NaCl is used at higher concentrations. While the addition of quercetin to LF was able to enhance the activity of LF against all the tested strains at 10 °C in the presence of 2.5% NaCl, its poor solubility is a disadvantage. Use of LF with SB or SL in refrigerated foods where *E.coli* O157:H7 is a potential health threat is likely to provide additional safety.

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Table 4.1a
Effect of bovine lactoferrin (LF) and sodium bicarbonate (SB) on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 37 °C.

	NaCl							
	0.5%				2.5%			
Time (h)	0	2	4	16	0	2	4	16
Control *	4.00a‡	5.33a	6.95a	9.08a	4.00a	4.41a	4.71a	8.82a
LF†	4.00a	5.20ab	5.92b	9.00a	4.00a	4.28ab	4.33b	4.04b
SB (20 mM[1])	4.00a	5.05ab	6.20b	9.17a	4.00a	4.17ab	4.40b	9.07a
SB (20 mM[1])+ LF	4.00a	4.90b	5.11c	6.44b	4.00a	3.87c	4.99a	4.07b
SB (40 mM)	4.00a	4.86b	6.08b	9.09a	4.00a	4.00bc	4.35b	9.11a
SB (40 mM)+LF	4.00a	4.89b	5.20c	4.42c	4.00a	3.80c	4.19b	4.19b
SB (160 mM)	4.00a	4.17c	4.13d	8.87a	4.00a	2.69d	2.90c	4.04b
SB (160mM)+LF	4.00a	3.67d	2.87e	2.04d	4.00a	1.84e	2.07d	1.00c

Table 4.1b
Effect of bovine lactoferrin (LF) and sodium bicarbonate (SB) on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 10 °C.

	NaCl							
	0.5%				2.5%			
Time (day)	0	2	5	10	0	2	5	10
Control *	4.00a‡	5.55a	8.29a	9.20a	4.00a	2.23a	4.22a	7.98a
LF†	4.00a	3.62b	5.54b	7.06b	4.00a	1.45b	3.44b	3.83c
SB (20 mM[1])	4.00a	3.50b	4.47c	6.52c	4.00a	1.30b	2.67c	4.52b
SB (20 mM[1])+ LF	4.00a	3.64b	4.23cd	5.56d	4.00a	nv c	nv d	nv d
SB (40 mM)	4.00a	3.55b	3.89e	3.87e	4.00a	nv c	nv d	nv d
SB (40 mM)+LF	4.00a	3.54b	4.11de	3.59e	4.00a	nv c	nv d	nv d
SB (160 mM)	4.00a	2.73c	1.00f	nv f	4.00a	nv c	nv d	nv d
SB (160mM)+LF	4.00a	2.22d	Nv§ g	nv f	4.00a	nv c	nv d	nv d

* No LF
† 32 mg/ml
‡ Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.
§ No viable cells were detected. Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 37 °C for growth.

Table 4.2a
Effect of bovine lactoferrin (LF) and sodium lactate (SL) on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 37 °C.

	NaCl							
	0.5%				2.5%			
Time (h)	0	2	4	16	0	2	4	16
Control *	4.00a‡	5.33a	6.95a	9.08a	4.00a	4.41a	4.71a	8.82a
LF†	4.00a	5.20a	5.92b	9.00a	4.00a	4.28a	4.33b	4.04d
1% SL	4.00a	5.02ab	6.14b	8.90a	4.00a	3.87b	4.76a	8.24a
1% SL + LF	4.00a	4.72b	5.46c	8.54b	4.00a	3.81b	4.11c	4.56c
3% SL	4.00a	nv §e	nv d	Nv c	4.00a	3.62c	3.14d	1.80e
3% SL + LF	4.00a	2.66d	nv d	Nv c	4.00a	3.26d	2.52e	nv f
5% SL	4.00a	nve	nv d	Nv c	4.00a	3.43cd	3.13d	nv f
5% SL + LF	4.00a	3.59c	nv d	Nv c	4.00a	3.44cd	3.01d	nv f

Table 4.2b
Effect of bovine lactoferrin (LF) and sodium lactate (SL) on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 10 °C.

	NaCl							
	0.5%				2.5%			
Time (day)	0	2	5	10	0	2	5	10
Control *	4.00a‡	5.55b	8.29a	9.20a	4.00a	2.23a	4.22a	7.98a
LF†	4.00a	3.62d	5.54c	7.06b	4.00a	1.45b	3.44b	3.83c
1% SL	4.00a	6.20a	7.97b	9.10a	4.00a	2.29a	4.16a	6.29b
1% SL + LF	4.00a	5.29c	7.94b	9.00a	4.00a	1.54b	2.87c	4.04c
3% SL	4.00a	1.70d	nv§ d	Nv c	4.00a	2.23a	1.38d	nv d
3% SL + LF	4.00a	1.00e	nv d	Nv c	4.00a	1.49b	Nv e	nv d
5% SL	4.00a	1.65d	nv d	Nv c	4.00a	1.52b	1.30d	nv d
5% SL + LF	4.00a	1.00e	nv d	Nv c	4.00a	1.69b	Nv e	nv d

* No LF
† 32 mg/ml
‡ Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.
§ No viable cells were detected. Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 37 °C for growth.

Table 4.3a
Effect of bovine lactoferrin (LF) and sodium hexametaphosphate (SHMP) on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 37 °C.

	NaCl							
	0.5%				2.5%			
Time (h)	0	2	4	16	0	2	4	16
Control *	4.00a‡	5.33a	6.95a	9.08a	4.00a	4.41a	4.71a	8.82a
LF†	4.00a	5.20ab	5.92c	9.00ab	4.00a	4.28a	4.33b	4.04c
SHMP (1mg ml^{-1})	4.00a	4.91bc	5.87c	8.90ab	4.00a	2.18cd	1.90d	5.56b
SHMP (1mg ml^{-1}) + LF	4.00a	5.25ab	6.52b	8.92ab	4.00a	2.76b	2.51c	5.55b
SHMP (3mg ml^{-1})	4.00a	4.84bc	6.21bc	8.57bc	4.00a	2.39c	1.95d	1.80c
SHMP (3mg ml^{-1}) + LF	4.00a	4.86bc	4.77d	8.32c	4.00a	1.90d	nv§ e	nv e
SHMP (5mg ml^{-1})	4.00a	4.62c	4.92d	8.53bc	4.00a	2.76b	1.84d	nv e
SHMP (5mg ml^{-1}) + LF	4.00a	4.51c	4.81d	8.79abc	4.00a	2.44bc	nv e	nv e

Table 4.3b
Effect of bovine lactoferrin (LF) and sodium hexametaphosphate (SHMP) on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 10 °C.

	NaCl							
	0.5%				2.5%			
Time (day)	0	2	5	10	0	2	5	10
Control*	4.00a‡	5.55a	8.29a	9.20a	4.00a	2.23b	4.22b	7.98a
LF†	4.00a	3.62e	5.54g	7.06c	4.00a	1.45c	3.44c	3.83c
SHMP (1mg ml^{-1})	4.00a	5.09b	7.37b	9.05ab	4.00a	2.93a	4.61a	8.20a
SHMP (1mg ml^{-1}) + LF	4.00a	4.22d	5.87f	8.75b	4.00a	2.34b	4.04bc	6.92b
SHMP (3mg ml^{-1})	4.00a	4.57c	6.23de	8.90ab	4.00a	2.26b	3.94bc	7.95a
SHMP (3mg ml^{-1}) + LF	4.00a	4.41cd	6.44d	8.85ab	4.00a	2.20b	3.82c	6.66b
SHMP (5mg ml^{-1})	4.00a	4.59c	5.96ef	8.95ab	4.00a	2.04b	3.35c	6.59b
SHMP (5mg ml^{-1}) + LF	4.00a	4.53cd	6.81c	8.75b	4.00a	0.65d	1.45e	2.10d

* No LF
† 32 mg/ml
‡ Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.
§ No viable cells were detected. Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 37 °C for growth.

Table 4.4a
Effect of bovine lactoferrin (LF), ethanol and quercetin on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 in LB broth containing 0.5 or 2.5% NaCl at 37 °C.

	NaCl							
	0.5%				2.5%			
Time (h)	0	2	4	16	0	2	4	16
Control *	4.00a‡	5.33a	6.95a	9.08a	4.00a	4.41a	4.71a	8.82a
LF†	4.00a	5.20a	5.92b	9.00a	4.00a	4.28a	4.33b	4.04b
Ethanol**	4.00a	4.56b	5.06c	8.76ab	4.00a	2.66c	2.66d	2.25c
Ethanol+ Quercetin††	4.00a	4.35bc	4.79c	8.43b	4.00a	3.02b	3.02c	2.00d
Ethanol+ LF	4.00a	4.07c	2.49d	nv c	4.00a	1.30e	Nv d	nv e
Quercetin‡‡ + LF	4.00a	nv§ d	nv e	nv c	4.00a	1.60d	Nv d	nv e

Table 4.4b
Effect of bovine lactoferrin (LF), ethanol and quercetin on the viability (log_{10} CFU/ml) of *E.coli* O157:H7 strain 3081 LB broth containing 0.5 or 2.5% NaCl at 10 °C.

	NaCl							
	0.5%				2.5%			
Time (h)	0	2	5	10	0	2	5	10
Control *	4.00a‡	5.55a	8.29a	9.20a	4.00a	2.23a	4.22a	7.98a
LF†	4.00a	3.62c	5.54b	7.06b	4.00a	1.45c	3.44b	3.83b
Ethanol**	4.00a	4.00a	3.90c	3.36c	4.00a	2.17ab	1.85c	nv c
Ethanol+ Quercetin††	4.00a	3.81bc	3.54d	2.66d	4.00a	1.87b	1.30d	nv c
Ethanol+ LF	4.00a	3.99b	3.85cd	2.49d	4.00a	2.17ab	nv e	nv c
Quercetin‡‡+ LF	4.00a	3.59c	2.99e	2.21e	4.00a	1.84b	nv e	nv c

* No LF
† 32 mg/ml
‡ Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.
§ No viable cells were detected. Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 37 °C for growth.
** 4.75%
†† 250 μg/ml.
‡‡ Quercetin contained 4.75% ethanol.

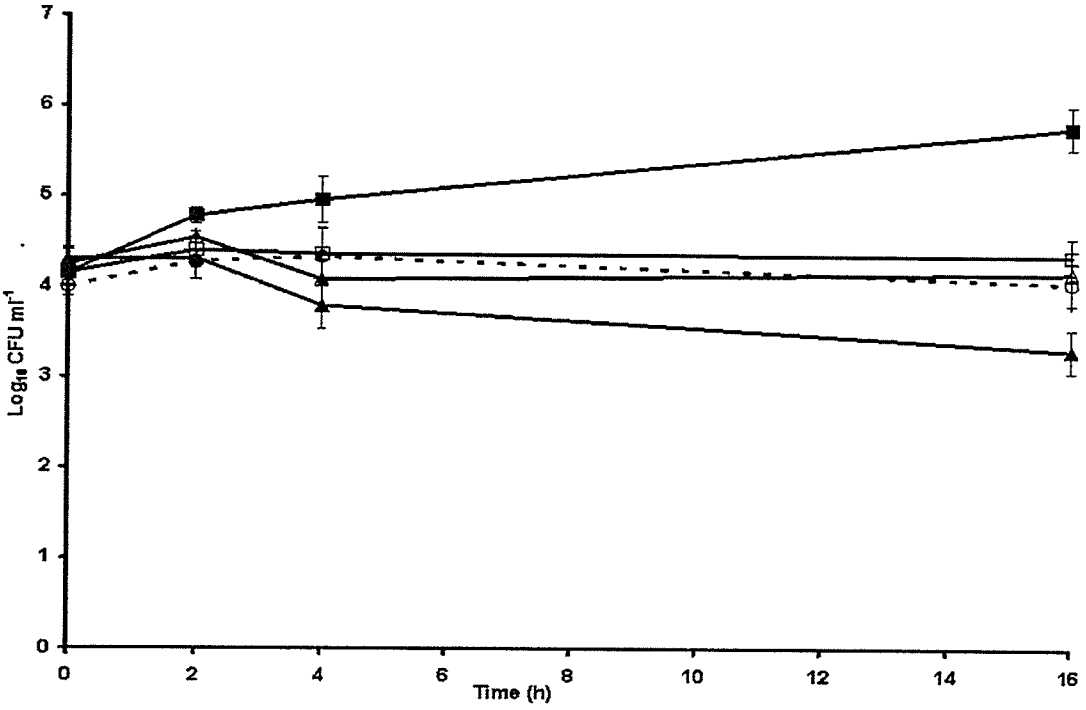

Fig. 4.1
Effect of 32 mg/ml LF on viability of *E.coli* O157:H7 strains (○) 3081, (□) LCDC 7110, (△) LCDC 7283, (▲) E318N and (■) CRIFS # 828 in LB broth containing 2.5 % NaCl at 37 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

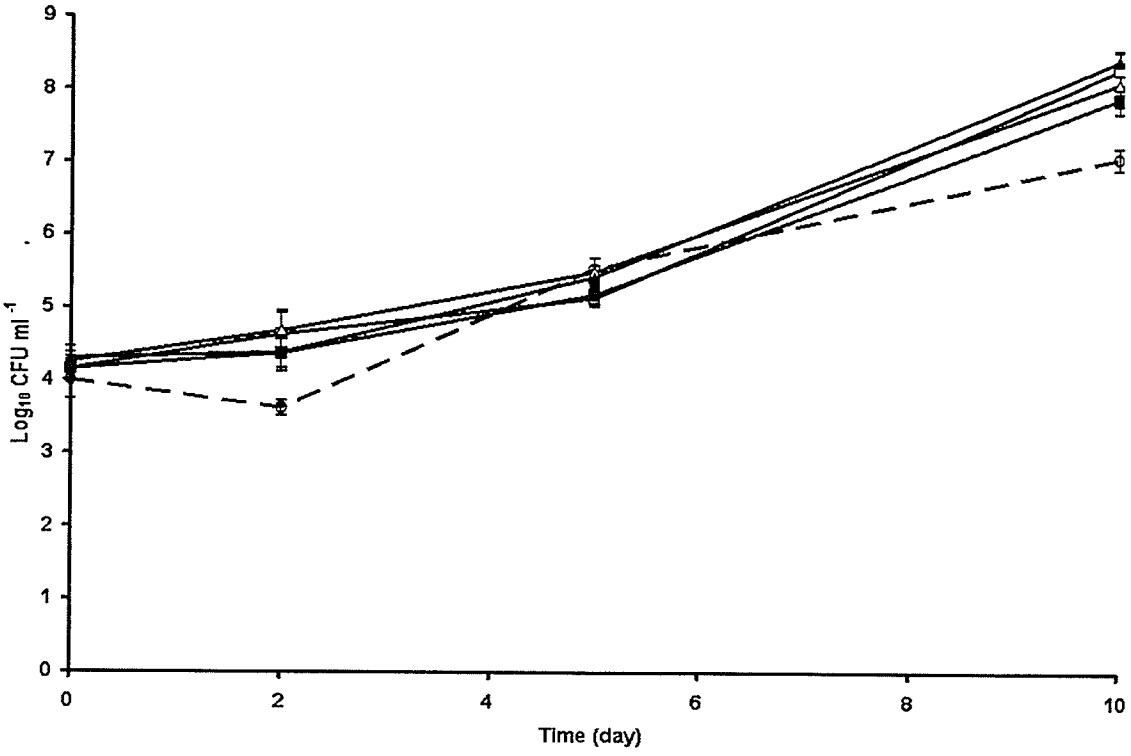

Fig. 4. 2
Effect of 32 mg/ml LF on viability of *E.coli* O157:H7 strains (○) 3081, (□) LCDC 7110, (△) LCDC 7283, (▲)E 318N and (■) CRIFS # 828 in LB broth containing 0.5 % NaCl at 10 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

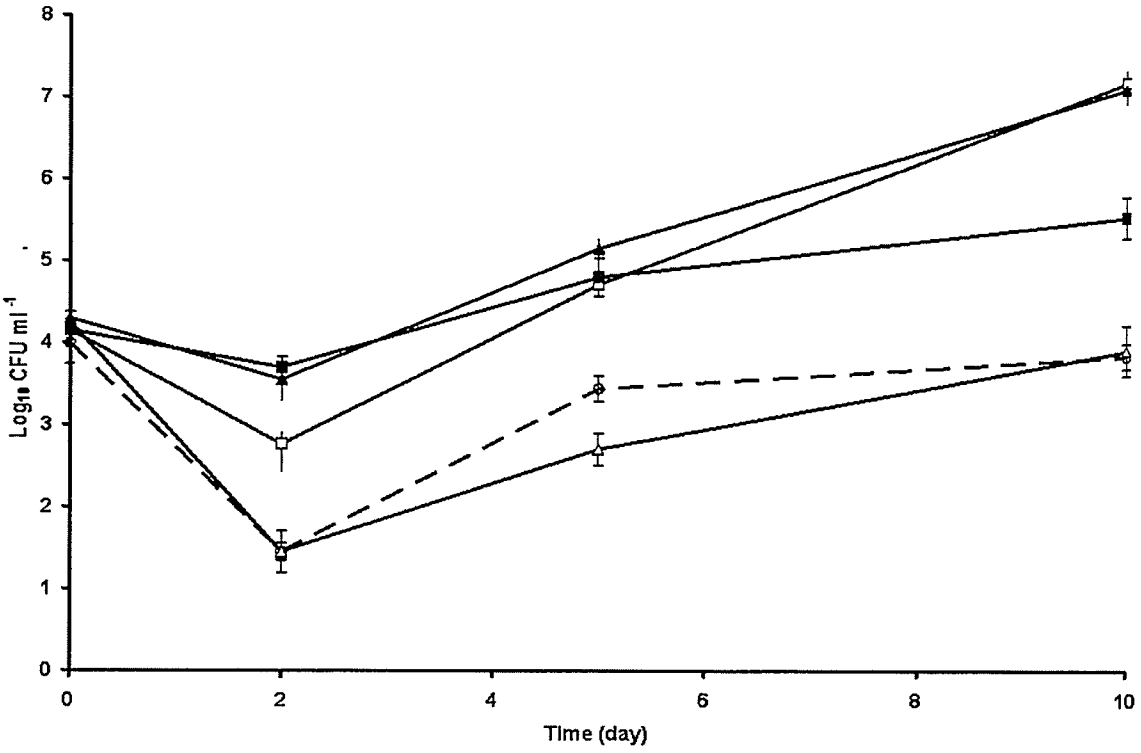

Fig. 4.3
Effect of 32 mg/ml LF on viability of *E.coli* O157:H7 strains (○) 3081, (□) LCDC 7110, (△) LCDC 7283, (▲)E 318N and (■) CRIFS # 828 in LB broth containing 2.5 % NaCl at 10 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

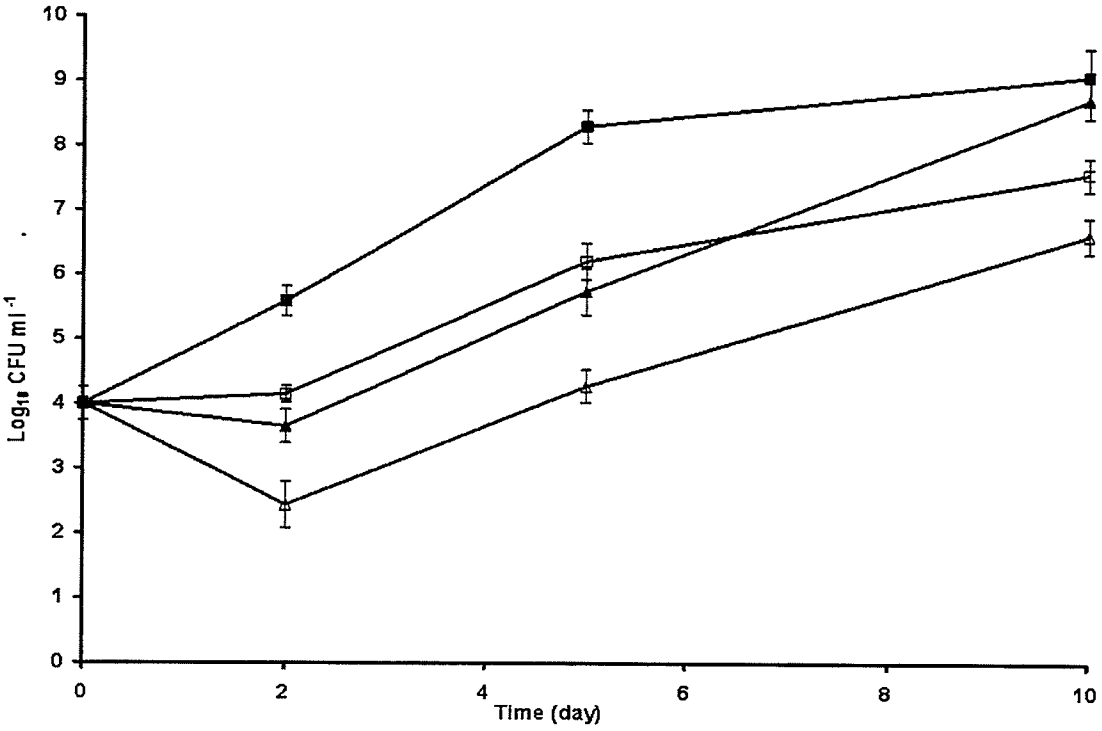

Fig. 4.4

Effect of LF on viability of *E.coli* O157:H7 strain 3081at 10 °C in APT broth: (■) control containing 0.5% NaCl, (□) 32mg/ml LF in APT containing 0.5% NaCl, (▲) control containing 2.5% NaCl,(△) 32mg/ml LF in APT containing 2.5% NaCl. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

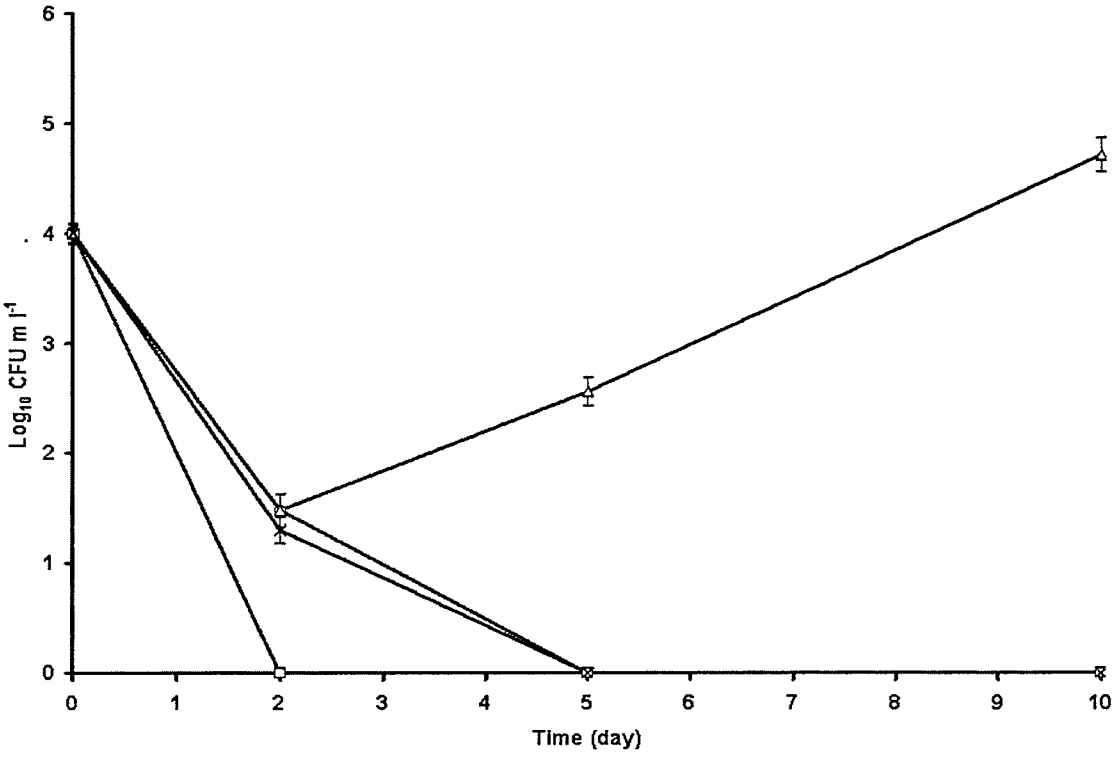

Fig. 4.5

Effect of (○) 32 mg/ml LF and 160 mM SB, (□) 32 mg/ml LF and 3% (w/v) SL, (△) 32 mg ml^{-1} LF and 5 mg/ml SHMP and (*) 32 mg/ml LF and 250 μg/ml quercetin against *E.coli* O157:H7 strain 3081 in APT broth containing 2.5% NaCl at 10 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

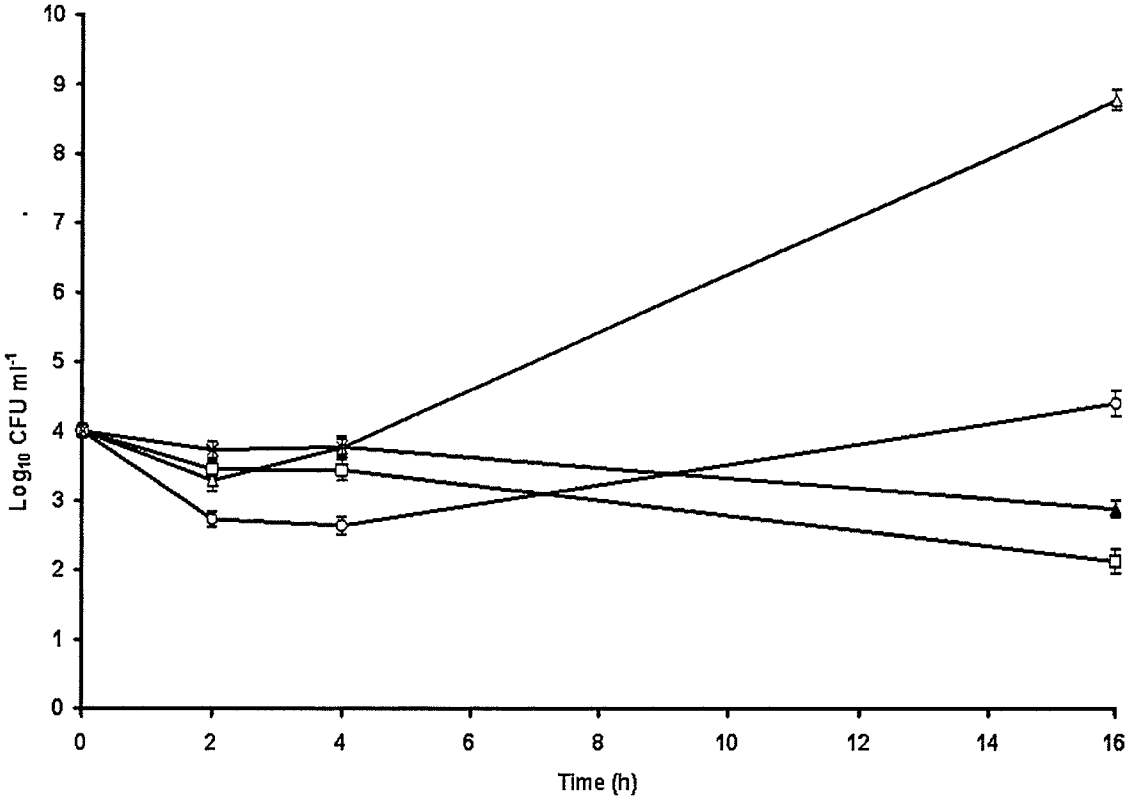

Fig. 4.6

Effect of (○) 32mg/ml LF and 160 mM SB, (□) 32 mg/ml LF and 3% (w/v) SL, (△) 32 mg/ml LF and 5 mg/ml SHMP and (▲) 32 mg/ml LF and 250 μg/ml quercetin against *E.coli* O157:H7 strain 3081 in APT broth containing 2.5% NaCl at 37 °C. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

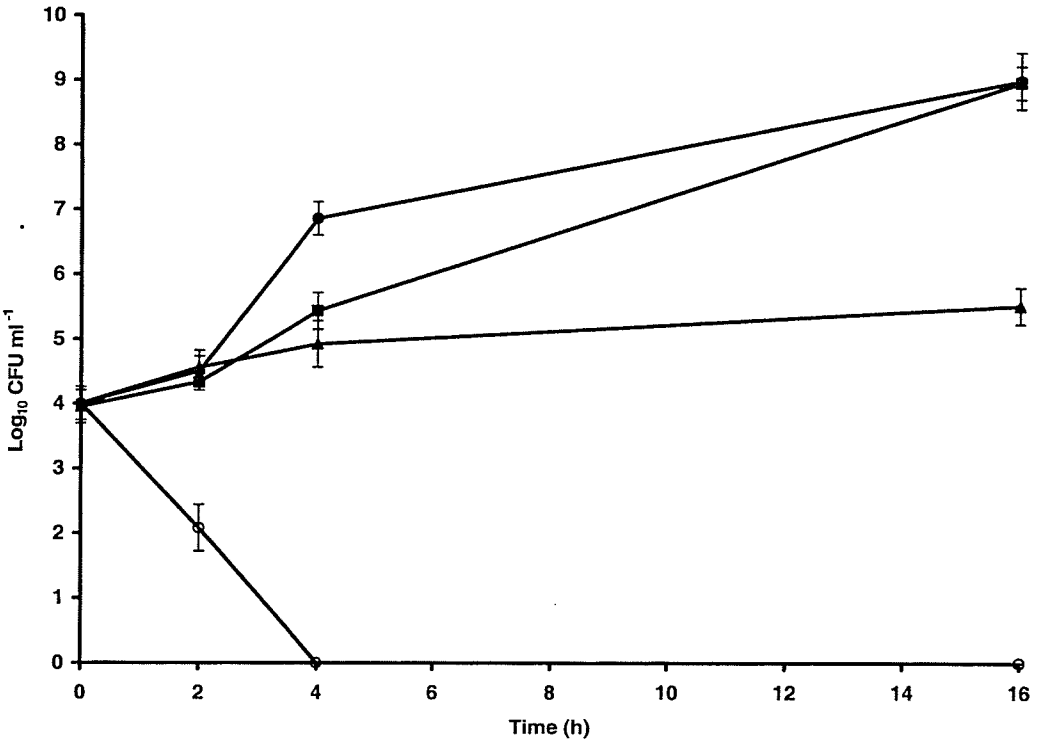

Fig. 4.7
Effect of bovine lactoferrin (LF), sodium bicarbonate (SB) and EDTA against *E.coli* O157:H7 strain 3081 in LB containing 0.5% NaCl at 37 °C: (●) control without inhibitors, (■) 16 mg/ml LF and 5 mM SB, (▲) 0.5 mg/ml EDTA, (○) 16 mg/ml LF, 5 mM SB and 0.5 mg/ml EDTA. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

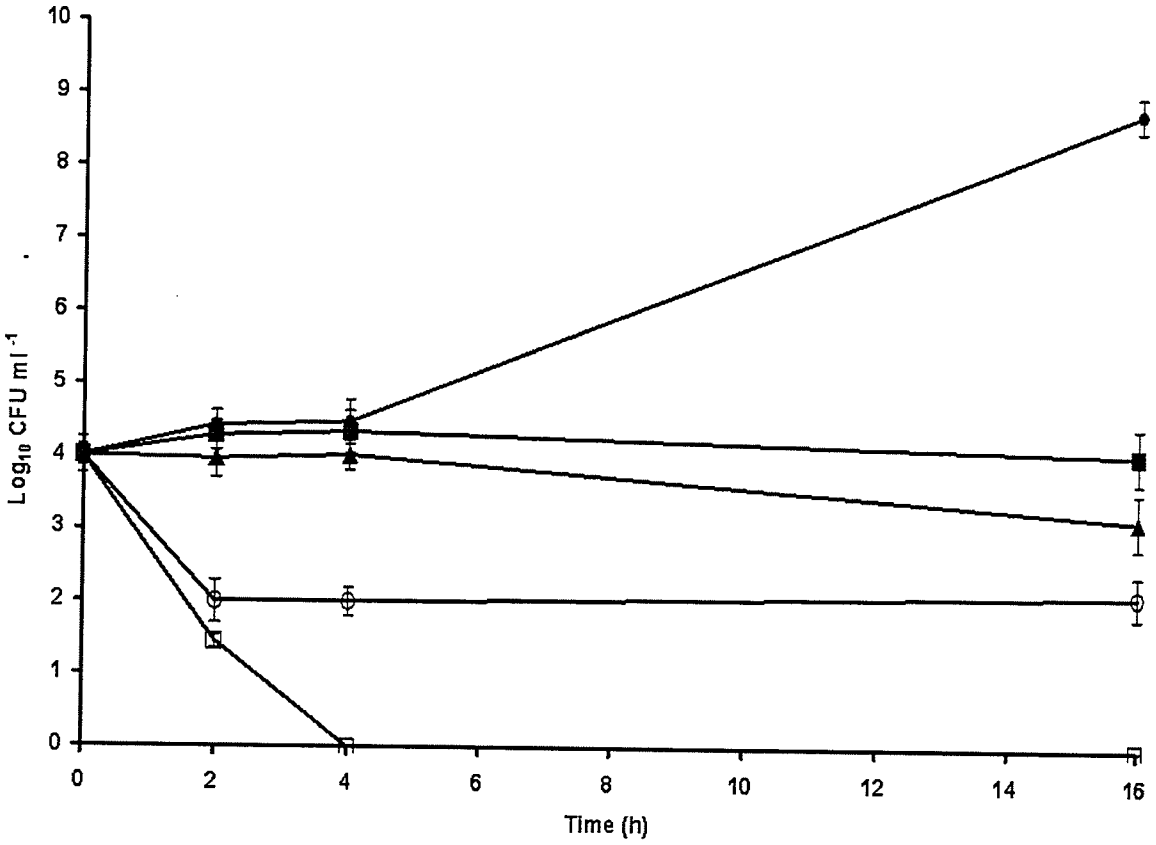

Fig. 4.8
Effect of bovine lactoferrin (LF), sodium bicarbonate (SB) and EDTA against *E.coli* O157:H7 strain 3081 in LB containing 2.5% NaCl at 37 °C: (●) control without inhibitors, (■) 16 mg/ml LF and 10 mM SB , (▲) 0.5 mg/ml EDTA, (○) 16 mg/ml LF, 5 mM SB and 0.5 mg/ml EDTA,(□) 16 mg/ml LF, 10 mM SB and 0.5 mg/ml EDTA. Results shown are the means of two experiments. Vertical bars indicate 95% confidence limit.

Chapter 5

Temperature sensitive microcapsules containing lactoferrin and their action against *Carnobacterium viridans* on bologna

5.1 Abstract:

Lactoferrin (LF) was encapsulated in two types of emulsion to protect it from contact with agents like divalent cations which interfere with its antimicrobial activity. First, paste-like microcapsules were prepared as a water-in-oil (W_1/O) emulsion from a mixture of 20% w/v LF in distilled water, 3% w/v sodium lactate or 20 mM sodium bicarbonate which was emulsified with an oil mixture of 22% butter fat plus 78% corn oil and 0.1% polyglycerol polyricinoleate. Second, freeze-dried double emulsion ($W_1/O/W_2$), powdered microcapsules were produced following emulsification of paste-like microcapsules in an external aqueous phase (W_2) consisting of a denatured whey protein isolate solution, WPI. The release of LF from the W_1/O microcapsules was dependent on temperature and NaCl concentration. LF was not released from the W_1/O emulsion at < 5.5 °C. Its release was greater from W_1/O microcapsules when suspended in 5% aqueous NaCl than in water at ≥ 10 °C, whereas LF release from freeze-dried microcapsules was not controlled by temperature change. Paste-like microcapsules were incorporated in edible WPI packaging film to test the antimicrobial activity of LF against a meat spoilage organism *Carnobacterium viridans*. The film was applied to the surface of bologna after its inoculation with the organism and stored under vacuum at 4 or 10 °C for 28 d. The growth of *C.viridans* was delayed at both temperatures and microencapsulated LF had greater antimicrobial activity than when unencapsulated. The temperature sensitive property of the W_1/O microcapsules was reduced when they were incorporated into a WPI film.

Key words: lactoferrin, microencapsulation, double emulsion, *Carnobacterium*, antimicrobial.

5.2 Introduction

The globalization of the world economy has resulted in meat and meat products being shipped further distances to reach foreign markets. The social and economic impacts of foodborne illness outbreaks, and uncertain achievement of desired shelf life of perishable refrigerated foods have led to work on the development of more effective antimicrobial preservatives for the industry (Ouattara and others 1997; Naidu 2000).

Natural antimicrobials have gained attention because of the demand for preservative-free food products (Payne and others 1990). Included as natural antimicrobials are several milk proteins and enzymes that inhibit the growth of various intestinal pathogens and which protect children against gastroenteritis (Paulsson and others 1993). Among these is lactoferrin (LF), the main iron–binding glycoprotein present in the milk of mammals, which inhibits many bacteria, fungi, and parasites (Shimazaki 2000). It also has antioxidant, antiviral, anti-inflammatory, immune-modulating, and anti-cancer activities, and can promote the growth of probiotic bacteria like *Bifidobacterium* (Naidu 2000; Aguila and Brock 2001).

Although many studies have indicated that LF has the potential to be used as a natural antimicrobial in foods, success in laboratory broth systems has not been observed in food because its antimicrobial activity is reduced by divalent cations like calcium and magnesium (Ellison and others 1988; Al-Nabulsi and Holley 2006). Additionally,

monovalent cations like Na^+ can reduce its bactericidal effects (Al-Nabulsi and Holley 2005).

Recently, microencapsulation and controlled-release technology have found broad applications in the pharmaceutical, healthfood, paper and cosmetic industries (Reineccius 1995). Microencapsulation results from a process in which sensitive ingredients (or core materials) are protected from deterioration or adverse environmental conditions by entrapment in a protective polymer encapsulating agent (or wall material). The wall material will allow release of core material at predictable rates under specific conditions (Hogan and others 2001a). Microencapsulation can serve as a delivery system to reduce the threshold concentrations of active agents necessary to achieve desired sensory or therapeutic effects, and extends the effective biological reactivity of ingredients (Dzieak 1988).

Double emulsion technology is another form of microencapsulation used for protecting sensitive ingredients (Augustin and others 2001). Water-in-oil-in-water ($W_1/O/W_2$) emulsions are liquid or semi-solid dispersions characterized by having chemically similar internal and external aqueous phases (W_1 and W_2, respectively) and have an intermediate immiscible lipid phase (O) that separates the two aqueous phases (Rodríguez-Huez and others 2004). These $W_1/O/W_2$ emulsion systems have small water droplets (W_1) entrapped inside an oil phase (O) containing a surface active agent with low hydrophilic-lipophilic balance (HLB), that in turn are dispersed in a continuous water phase (W_2) containing another surface active agent with high HLB (van der Graaf and others 2005). Because these emulsions have a "reservoir" phase inside droplets of another phase, these systems can be used to prolong the release of hydrophilic active ingredients.

Often described as multiple emulsions, these systems have a variety of applications such as being used as delivery vehicles for food, drugs and cosmetics and serving as solvent reservoirs in drug overdose treatments (Dogru and others 2000; Jiao and others 2002). However, $W_1/O/W_2$ emulsions are infrequently used for drug or food ingredient delivery because of their low stability during storage compared to simple W/O or O/W emulsions (Opawale and Burgess 1998). One method which can be used to enhance the stability of multiple emulsions is dehydration (Musashino and others 2001).

Microcapsules prepared from multiple emulsions are sensitive to a number of "triggers" that can be used for release of core materials. These include: moisture, temperature, pH, ionic strength, enzymatic reaction, or physical stress of various types (Lee and Rosenberg 2000a). Temperature-catalyzed release of core materials from multiple emulsions can be achieved by controlling the melting temperature of the lipid wall material that makes up the intermediate phase. In addition, lipids, carbohydrates, natural gums and proteins can be used as wall materials for preparing water soluble microcapsules (Gibbs and others 1999). However, starch and related products lack emulsification activity and cannot be used as wall materials without the addition of a surface active agent (Hogan and others 2001b). On the other hand, whey proteins have good emulsification, gelation and film forming properties (Lee and Rosenberg 2000a).

The primary objective of this study was to overcome the interference by cations or other agents with the antibacterial effects of LF through its microencapsulation. Microencapsulation of LF could maximize its contact with pathogenic and spoilage bacteria if the microcapsules were applied at the meat surface where most microorganisms are to be found (Gill and Holley 2000). Two different forms of

microcapsules were developed in this study: a) a paste-like mixture of microcapsules prepared from a water-in-oil (W/O) emulsion and b) freeze-dried W/O/W microcapsules prepared using double emulsion technology. A secondary objective was to incorporate the primary W/O LF emulsion inside a whey protein isolate (WPI) edible packaging film and evaluate the inhibitory activity of the LF-WPI film on the growth of the meat spoilage bacterium *Carnobacterium viridans.*

5.3 Materials and Methods

5.3.1 Bovine lactoferrin (LF)

Bovine lactoferrin (Bioferrin 2000) was obtained from Glanbia Nutritionals (Glanbia Ingredients, Inc., Monroe, WI, USA). LF stock solutions (20% w/v) were prepared in distilled water with or without 3% (w/v) sodium lactate (27 mM), SL (Fisher Scientific, Fairlawn, NJ, USA) or 20 mM sodium bicarbonate, SB (Sigma Chemical, St. Louis, MO, USA) . The pH of LF solutions was measured with an Accumet Basic pH meter (Fisher Scientific).

5.3.2 Wall Material

Corn oil (Mazola, ACH Food Companies Inc., Memphis, TN, USA) and commercial, organic, non-salted butter were purchased locally at retail. Butter was melted and left overnight at 50 °C in a separatory funnel to allow separation of butter fat from water soluble ingredients.

To be useful as a delivery vehicle in temperature-abused foods the wall material of the primary emulsion (W/O) was designed to be solid at 4 °C and liquid at 10 °C. Different proportions of corn oil and butter fat (85:15, 80:20, 78:22, and 75:25, respectively) were mixed together at 25 °C for 1 min at 1600 rpm using an Omni Mixer

(Sorvall, Marietta, GA, USA). After these mixtures were held at -40 °C for 5 min, their melting points were determined using a differential scanning calorimeter (Micro DSC III Calorimeter, Setaram Instrumentation, Caluire, France). Small amounts (30-40 mg) of the lipid samples were placed onto an aluminium pan and sealed under a continuous flow of dry N_2 gas. The heating rate was 1 °C/min from -20 to 40 °C.

5.3.3 Preparation of primary emulsion

A primary emulsion of LF in oil (22% butter fat: 78% corn oil) containing 0.1% (v/v) polyglycerol polyricinoleate (PGPR, Palsgaard, Juelsminde, Denmark) was prepared at 25 °C by adding the internal aqueous phase containing 20% (w/v) LF with 3% (w/v) SL or 20 mM SB drop-wise to the oil mixture to achieve a 1:1 volume ratio of oil:aqueous phase, and emulsified using the Omni Mixer at 4800 rpm for 5 min. The emulsion was solidified at -40 °C for 5 min.

5.3.4 Preparation of dried microcapsules

Dried powder consisting of microencapsulated LF with metal chelating agents SL or SB was produced by freeze drying. The primary W/O emulsion produced as described above was added at 25 °C to an external aqueous phase containing 30% (w/v) whey protein isolate, WPI (Davisco Foods International Inc., Le Sueur, MN, USA) and 0.02% (w/v) xanthan gum (Sigma). The volume ratio of WPI plus xanthan gum was 40:60. A multiple emulsion ($W_1/O/W_2$) was prepared at a mixing rate of 3200 rpm for 3 min with the Omni Mixer. Two hundred ml of each emulsion was spread over individual aluminium trays (230 mm diameter x 44 mm , previously covered with wax paper) forming a layer approximately 15 mm thick. Emulsions were frozen at -40 °C for < 12h, placed in a freeze dryer (Model 10-146 MP; Virtis Corp., Gardiner, NY, USA), and held

at -5 °C and 0.5 mm Hg atmospheric pressure for < 72 h. Dried emulsions were gently ground manually using a mortar and pestle.

5.3.5 Packing fraction

The maximum volume of aqueous LF solution that could be physically entrapped inside the oil phase was determined by measuring the initial viscosity of the primary emulsion directly after its preparation as described above using a rheometer at 20 °C (AR 2000, TA Technologies, NewCastle, DE). The W/O volume ratio where an abrupt change in viscosity was observed (caused by phase inversion) was considered to indicate the maximum volume of LF that could be entrapped inside the oil phase.

5.3.6 Measurement of LF released from microcapsules

5.3.6.1 Primary emulsion

After the emulsion was solidified by freezing, 2.1g of the W/O paste was suspended in a test tube containing 20 ml of either water or 5% NaCl at pH 5.6 ± 0.1 previously adjusted to 25, 10, or 4 °C. The test tubes containing the paste were sampled at intervals of 0h, 1d, 3d and 7d by withdrawal of a 2 ml sample with a 10 ml syringe equipped with a 21 G needle. The solution withdrawn was filtered to eliminate the emulsion droplets by replacing the needle with a filter (0.22 μm pore diameter, Fisher Scientific). The concentration of LF released from the microcapsules into the solution was determined spectrophotometrically by the Lowry method at 600 nm (Stoscheck 1990).

5.3.6.2 Freeze-dried emulsion

Lyophilized powder (4.0 g) from each emulsion was placed in a screw-cap test tube (150 x 20 mm) containing 20 ml of either water or 5% NaCl at pH 5.6 ± 0.1

previously adjusted to 25, 10, or 4 °C. The test tubes containing the lyophilized powder were sampled after 7d by withdrawal of a 3 ml sample with a 10 ml syringe equipped with a 21 G needle. The solution withdrawn was filtered at two stages to eliminate the emulsion droplets by replacing the needle with two differently sized filters (0.45 and 0.22 μm pore diameter, respectively). The concentration of LF released from the microcapsules into the solution was determined by HPLC (Elgar and others 2000).

HPLC analysis was performed using a Waters 2695 Separations Module coupled with a Waters 2996 photodiode array detector (Waters, Mississauga, ON, Canada). The separation was carried out using a 1 ml Resource RPC column (Pharmacia Biotech, Piscataway, NJ, USA) at 25 °C. The composition of mobile phases was: A) 0.1% (v/v) tetrafluoric acetate (TFA) (Fisher Scientific) in Milli-Q water; and B) 0.09% (v/v) TFA in 90 % acetonitrile (Sigma) solution. The elution gradient of B in A was as follows: 1-6 min, 20-40%; 6-16 min, 40-45%; 16-19 min, 45-50%; 19-20 min, 50%; 20-23 min, 50-70%; 23-24 min, 70-100%. The injection volume was 10 μl and running time was 30 min.

5.3.7 Size distribution of the primary and double emulsion droplets

A drop (about 0.2 mL) of freshly prepared emulsion was placed on a microscope slide at 25 °C, 40% relative humidity and observed using a phase-contrast microscope (Zeiss photomicroscope, Oberkochen, Germany) with an F100/1.25 oil lens. Images were captured by a digital camcorder (DCR-PC330, Sony Corporation, Tokyo, Japan) connected to the microscope through a binocular adapter. Droplet size was measured using an objective micrometer (100 units/ mm) and data from captured images were processed using an image analysis program (SigmaScan Pro 5.0, Systat Software, Inc.,

Point Richmond, CA). At least 100 droplets in each emulsion were measured to assess droplet size distribution.

5.3.8 Bacterial inhibition assay

5.3.8.1 Preparation of edible film using emulsion technology

WPI solution (10% w/v) was heated at 90 °C for 30 min in a water bath to denature the protein, and glycerol (Sigma) was added to the denatured WPI solution to achieve a 70:30 ratio of WPI:glycerol. The WPI the solution was held overnight at 4 °C. The edible film was prepared by adding the primary emulsion (W/O) prepared as described above to the external aqueous phase (WPI solution) at ratio of 30:70 of primary emulsion:WPI with mixing at 3200 rpm for 3 min using an Omni Mixer. Then 8.0 g from the mixture was spread over a sterile Petri dish and dried at 4 °C for 24h. Three different primary LF emulsions were incorporated in the WPI to yield different films as follows: LF dissolved in distilled water, LF plus 3% w/v SL or LF plus 20 mM SB.

5.3.8.2 Antimicrobial assay in meat

Carnobacterium viridans ATCC BAA-336 (MPL-11) which had been isolated from spoiled bologna (Holley and others 2002) and stored frozen in glycerol was used. It was maintained on All Purpose Tween (APT) agar (Difco, division of Becton Dickinson, Sparks, MD, USA) slants at 4 °C and transferred monthly to maintain viability. Working cultures were prepared by growth in 10 ml APT broth incubated at 30 °C for 2d. For tests, 100 μl culture was transferred to 100 ml APT broth and incubated at 30 °C for 20h. Then the culture was diluted $1:10^6$ in 0.1% peptone water and 200 μl was used to inoculate bologna slices.

The effectiveness of the paste-like emulsion incorporated in WPI packaging film prepared as described above on the growth of *C.viridans* on bologna was evaluated following the procedure of Peirson and others (2003).

Vacuum packaged sliced bologna was obtained within a week of manufacture directly from Maple Leaf Consumer Foods Mississauga, ON, Canada. The bologna contained pork and/or beef, mechanically separated chicken, water, salt, sodium phosphate, sodium erythorbate and smoke. Commercial 500g packages were opened and two unseparated adjacent slices were used together. Only the upper surface of one slice was inoculated with bacteria. *C.viridans* in 200 μl 0.1% peptone water was spread with a glass rod over the bologna surface to yield approximately 2.5 $\log_{10}$ cfu/cm^2. WPI film was placed on the surface of each inoculated bologna slice and the slices were packed in WinPak Deli *1 bags (WinPak, Winnipeg, MB, Canada), heat sealed under vacuum in a Bizerba Model GM 2002 vacuum-packing machine (Bizerba Canada, Mississauga, ON, Canada), and held at 4 or 10 °C for 28 d. Deli *1 bags were made of laminated Nylon/EVOH/polyethylene films and had an oxygen transmission rate of 2.3 cm^3/m^2 /24h/ 23 °C and were 0.064 mm thick. Six treatments were set up for the WPI films (Table 5.1). At intervals of 0, 3, 7, 14, 21 and 28 d, a 6.28 cm^2 area (approximately 2g) of bologna and WPI film were cut from the inoculated upper slice using a sterile cork borer, added to 18 ml 0.1% peptone water in a sterile stomacher bag (Filtra-bag, VWR International, Edmonton, AB, Canada), and macerated using a mortar. This sample dispersion was serially diluted in 0.1% peptone water and 100μl of appropriate dilutions was plated on Cresol Red Thallium Acetate Sucrose Inulin (CTSI) agar and incubated at 25 °C for 2d followed by 2 d incubation at 8 °C to enumerate *C.viridans* (Wasney et al.

2001). APT agar plates incubated for 3 d at 30 °C were used to enumerate total bacteria present. The pH was measured using an Accumet Basic™ pH meter (Fisher) using a 10^{-1} dilution of chopped bologna in distilled water after treatment for 2 min with a Stomacher 400 (Seward Laboratory, London, UK)

5.3.9 Statistical analysis

All data were analyzed by Statistical Analysis System (version 8.1) software (SAS Institute, Inc., Cary, NC). Duncan's multiple range tests were used to find significant differences ($p< 0.05$) between means of each treatment at a particular time.

5.4 Results and Discussion

5.4.1 Physical properties

5.4.1.1 Melting point of lipid mixture and appearance of emulsions

The results from DSC analyses showed that as the butter fat percentage increased the melting temperature of the mixture increased (data not shown). It was noted that the solid-liquid phase transition of the oil phase at ratios of 85:15, 80:20, 78:22, and 75:15 corn oil:butter fat were 2.0, 4.5, 5.5, and 7.0 °C, respectively. Therefore for the oil phase, a ratio of 78:22 corn oil:butter fat was selected for emulsion preparation. Solidification of the oil phase as a result of fat crystallization at <5.5 °C should prevent the internal water phase from destabilization by reducing oil droplet coalescence (McClements 1999). The paste-like emulsions resulting from the incorporation of the internal aqueous phase were cream in color while the freeze-dried powder from the multiple emulsions was white.

5.4.1.2 Packing fraction of the primary emulsion

The relationship between viscosity and the ratio of the internal phase to the oil phase volume is shown in Fig. 5.1. It was noted that the maximum internal aqueous phase

possible when LF was dissolved in DW or 3% SL was 55% of the total emulsion volume, but this was increased to 60% when LF was dissolved in 20 mM SB (Fig. 5.1). Clément and others (2000) found that the stability of a concentrated W/O emulsion was decreased by increasing the internal phase volume fraction up to 90%. Destabilization resulted from a decrease in thickness of the wall material as the core volume increased (Lee and Rosenberg 2000b). In addition, Yan and Pal (2001) suggested that increasing the volume fraction of the internal phase decreased emulsion swelling capacity and thus accelerated its destabilization. Therefore, a 50% volume ratio was selected to maximize LF content in the emulsion and provide marginal emulsion stability, but still allow LF release as the oil phase melted.

The higher packing fraction of LF when dissloved in SB could be explained by the ability of SB to enhance the structural stability of LF thus allowing the hydrophobic part of LF to be adsorbed at the interface of the oil and water and enhance the emulsion stability.

5.4.1.3 Size distribution

Regardless of the composition of the internal aqueous phases, all primary emulsions (Fig. 5.2a) contained aqueous droplets with rather uniform size (around 1 μm), characterized by a narrow distribution in peak frequency of occurrence (Fig. 5.3a). Droplets of internal LF solutions with SB or SL were smaller (median 0.8μm) than those with DW (median 1.2μm), probably because the higher surface tensions of the former solutions due to the electrolytes present allow formation of smaller droplets (MacDonald and others 1996; Clément and others 2000). In addition we found that when the PGPR concentration was increased > 1%, droplet size was reduced to < 0.1μm diameter.

In contrast with the primary emulsion, the internal LF droplets in $W_1/O/W_2$ multiple emulsions showed a bimodal size distribution (Fig. 5.3b). Although the first major peak diameter frequency occurred around 1μm as in Fig. 5.3a, a second peak appeared at 2.5 μm and for SL there was a third at > 3 μm. These may have been caused by the coalescence of dispersed aqueous droplets (Ficheux and others 1998) due to the lower mixing speed used (3200 rpm), or could have developed as water diffused from the outer to inner phases in response to osmotic pressure (Mezzenga and others 2004). The oil capsules in $W_1/O/W_2$ emulsions (Fig. 5.2b) also exhibited a bimodal size distribution, with the first major peak diameter frequency around 4μm, and a second peak diameter frequency around 11μm (Fig. 5.3c). From informal calculation, an oil capsule in the $W_1/O/W_2$ emulsions contained at least 4 aqueous LF droplets, showing that the method used for preparing the multiple emulsions was effective.

5.4.2 Release of LF from paste-like capsules

The extent of LF release from microcapsules containing LF with or without SL or SB when suspended in 5% NaCl was significantly greater at both 10 and 25 °C than when microcapsules were suspended in water (Table 5.2). This may have been caused by the higher osmotic pressure of the NaCl solution, which caused water diffusion from the microcapsules to the exterior. As a result, the W/O droplets would be expected to shrink (Jager-Lezer and others 1997). These results are consistent with those of Bjerregaard and others (1999) who reported that glucose release from microcapsules was higher as the osmotic strength of the receptor buffer increased.

When microcapsules were suspended in water there was greater LF release during storage at 25 °C than at 10 °C. This was probably due to the greater fluidity of the lipid

membranes at the higher temperature, yielding greater instability. When SL was included in microcapsules stored at 25 °C in water, LF release was greatest. It is suspected that in this treatment an increase in internal osmotic pressure may have caused water to flow inside capsules (Yan and Pal 2001) and led to swelling, capsule instability and rupture (Jager-Lezer and others 1997). The lower release of LF from microcapsules containing SL and SB when suspended in NaCl at 25 °C may have resulted from a stabilizing effect on osmotic pressure by the salts added to the microcapsules. At 10 °C there was no effect of SL or SB on LF release.

The negligible release of LF from microcapsules stored at 4 °C was desirable and probably due to the solidification of the hydrophobic wall material at this temperature. This meant that this emulsion would retain LF during proper refrigerated storage, and if it were included in food packages, would not be released (would remain active) until storage temperature were raised above 5.5 °C. On the other hand, the paste-like emulsions need careful handling and storage; therefore, a dried emulsion was developed in the present study.

5.4.3 Release from dried capsules

The amount of LF released from the lyophilized powder (Fig. A2) was measured only at 7d since LF release from the primary emulsion occurred in few treatments and only at 25 °C and ≥3d. In contrast to the barely measurable release of LF at 4 °C from paste-like microcapsules, 60-76% of LF was released from the dried powder emulsion at 4 °C after 7d (Table 5.3). This could have resulted from freezing of the internal aqueous phase during preparation for freeze drying which may have disrupted the oil layer. Clément and others (2000) noted that the expansion of water droplets in an internal phase

of a W/O emulsion during ice formation caused deformation of the oil layer. Clausse and others (1999) reported that 50% of the internal pure water droplets in an emulsion were frozen within 14h when they were cooled to -30 °C. The process followed in the present work involved exposure of the emulsion to -40 °C for 12h before drying at -5 °C. It is likely that partial freezing of the aqueous phase led to its destabilization. Another factor contributing to the fast release of LF at 4 °C from the freeze-dried powder may have been the emulsification action of WPI upon the hydrophobic emulsifier PGPR in the oil layer at the external O/W_2 interface which may have caused $W_1/O/W_2$ emulsion instability. This has been reported to cause rupture of the oil film in emulsions (Opawale and Burgess 1998; Kanouni and others 2002; Pays and others 2002). Unfortunately, lower concentrations of PGPR in the present study did not allow adequate emulsion formation. In contrast, higher concentrations of PGPR were found to cause an unacceptable delay in the release of LF at 10 °C, because the swelling capacity of the internal emulsion was increased and the droplet size (< 0.1 μm) was reduced. Alternately, when the WPI concentration was reduced below 30% it was difficult to obtain a powder after lyophilization. Therefore, further investigation is required to develop the desired temperature-catalyzed release of LF from the multiple emulsion.

5.4.4 Antimicrobial activity of paste-like microcapsules in a meat system

The incorporation of antimicrobials in packing film has been reported to improve antimicrobial activity by concentrating the agents at contaminated surfaces (Ouattara and others 2000). In the present study the bologna slices were checked before inoculation and did not contain *C.viridans*. The viability of *C. viridans* after 3d of storage at 4 °C was reduced below the detection limit (0.8 $\log_{10}$ cfu/ml) on the surface of bologna treated

with LF microencapsulated with SB or SL. A similar result was obtained at 7d when the microcapsules contained only LF (Table 5.4a). However, the reduction in bacterial viability may have been due to injury caused by LF since *C. viridans* cells were able to recover and grow after 14d. Nonetheless at 28d, the numbers of *C. viridans* were significantly lower ($p< 0.05$) on bologna surfaces treated with the WPI film containing microencapsulated LF. Reduced numbers of *C.viridans* on bologna treated with films containing microencapsulated LF stored at 4 °C were unexpected. At this temperature LF was not released from microcapsules for 7d (Table 5.2). In explanation, it is possible that during WPI film production some instability developed in the W_1/O emulsion. During film preparation microcapsules were added directly to the WPI solution without pre-cooling at -40 °C for 5 min to ensure lipid crystallization. This omission plus the added step involving mixing with WPI solution at 3200 rpm for 3 min, which would have caused shear and perhaps localized frictional heating, could have contributed to emulsion instability in the film. Greater reductions in *C.viridans* numbers at 4 °C than at 10 °C may have been due to greater activity of LF against this organism at 4 °C than higher temperatures (Al-Nabulsi and Holley 2005). At 10 °C, the WPI film containing microencapsulated LF reduced the number of *C. viridans* 0.7-1.4 $\log_{10}$ cfu/cm^2 more than WPI film containing unencapsulated LF after 28d (Table 5.4b). The largest reduction (1.4 log) was obtained when microencapsulated LF and SL was used.

The total number of bacteria in the samples treated with microencapsulated LF incorporated in WPI films was significantly lower at 4 or 10 °C up to 14d (Table 5.5a and b). However, there was no significant difference between the treatments at 10 °C when the samples were stored > 21d (Table 5.5b). At 4 °C, the total numbers of bacteria were

significantly lower ($p< 0.05$) in treatments with WPI film containing microencapsulated LF up to 14d (Table 5.5a).

In the present study it was noted that when microencapsulated LF was incorporated in WPI film, LF had higher antimicrobial activity than when unencapsulated in the WPI film. Furthermore, Limjaroen and others (2003) found that following incorporation of 2.5% LF without encapsulation into Saran® F-310 resin, LF had no activity against *L. monocytogenes* on trypticase soy agar at 35 °C. Therefore, use of microencapsulated LF in packaging films may protect LF from inactivation and enhance its antimicrobial activity by allowing its release from the film directly onto the surface of bologna. Incorporation of LF without encapsulation directly into the packaging film may have negatively influenced LF stability.

5.5 Conclusion

The results obtained in the present study demonstrated that microencapsulation can enhance the antimicrobial activity of LF in cured meat. They also suggest that temperature-catalysed release of otherwise sensitive antimicrobial agents from microcapsules may provide another option to address undesirable temperature fluctuations during food product distribution. We expect these results will lay the foundation for additional work where natural antimicrobials will be used with or instead of conventional preservatives to improve the shelf-life and safety of perishable foods.

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Table 5.1
Experimental design used for challenge studies of lactoferrin against *C.viridans* on the surface of bologna.

Treatment	*C.viridans*		Agent concentration in film		
			mg/cm^2 LF	mM/cm^2 SB	% SL
Control	+	-	-		
1	+	WPI[1]	-		
2	+	WPI + LF[2]	4.2		
3	+	WPI + microencapsulated LF[3]	4.2		
4	+	WPI + microencapsulated LF[4]	4.2	0.016	
5	+	WPI + microencapsulated LF[5]	4.2		0.24

1- Whey protein isolate film.
2- lactoferrin dissolved in distilled water and added to WPI to form a film.
3- lactoferrin paste-like microcapsules W_1/O; lactoferrin in distilled water was encapsulated in oil (78% corn oil + 22% butter fat with 0.1% polyglycerol polyricinoleate, PGPR), and added to WPI to form a film.
4- lactoferrin paste-like microcapsules; lactoferrin in 20 mM sodium bicarbonate (SB) in water was encapsulated in oil (78% corn oil + 22% butter fat with 0.1% PGPR), and added to WPI to form a film.
5-lactoferrin paste-like microcapsules; lactoferrin in 3% w/v sodium lactate (SL) was encapsulated in oil (78% corn oil + 22% butter fat with 0.1% PGPR), and added to WPI to form a film.

Table 5.2
Release of LF from W/O microcapsules suspended in water or 5% NaCl at pH 5.6 and different temperatures when the capsule core material contained distilled water (DW), 20 mM sodium bicarbonate (SB), or 3% w/v sodium lactate (SL) as a solute.

			0h		1d		3d		7d	
Emulsion	Temp.	Soln.	Mg/ml[1]	%[2]	mg/ml	%	mg/ml	%	mg/ml	%
LF in DW	25 °C	Water	0.00 a	0.00%	0.07 b	0.82%	0.90 b	10.51%	3.00 cd	35.05%
LF in DW	25 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.89 b	10.40%	7.50 a	87.62%
LF in 20 mM SB	25 °C	Water	0.00 a	0.00%	0.28 a	3.27%	1.14 ab	13.32%	5.15 b	60.16%
LF in 20 mM SB	25 °C	5% NaCl	0.00 a	0.00%	0.05 b	0.58%	1.46 ab	17.06%	5.31b	62.03%
LF in 3% SL	25 °C	Water	0.00 a	0.00%	0.07 b	0.82%	1.68 a	19.63%	7.66 a	89.49%
LF in 3% SL	25 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.16 c	1.87%	5.80 b	67.76%
LF in DW	10 °C	Water	0.00 a	0.00%	0.02 b	0.23%	0.06 c	0.70%	2.03 de	23.71%
LF in DW	10 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.01 c	0.12%	7.12 a	83.18%
LF in 20 mM SB	10 °C	Water	0.00 a	0.00%	0.02 b	0.23%	0.06 c	0.70%	3.21 c	37.50%
LF in 20 mM SB	10 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.01 c	0.12%	7.72 a	90.19%
LF in 3% SL	10 °C	Water	0.00 a	0.00%	0.03 b	0.35%	0.05 c	0.58%	1.93 e	22.55%
LF in 3% SL	10 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.11 c	1.29%	7.41a	86.57%
LF in DW	4 °C	Water	0.00 a	0.00%	0.00 b	0.00%	0.00 c	0.00%	0.01 f	0.12%
LF in DW	4 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.00 c	0.00%	0.06 f	0.70%
LF in 20 mM SB	4 °C	Water	0.00 a	0.00%	0.01 b	0.12%	0.02 c	0.23%	0.09 f	1.05%
LF in 20 mM SB	4 °C	5% NaCl	0.00 a	0.00%	0. 00 b	0.00%	0.00 c	0.00%	0.00 f	0.00%
LF in 3% SL	4 °C	Water	0.00 a	0.00%	0.01 b	0.12%	0.02 c	0.23%	0.03 f	0.35%
LF in 3% SL	4 °C	5% NaCl	0.00 a	0.00%	0.00 b	0.00%	0.00 c	0.00%	0.06 f	0.70%

1- mg/ml LF released into solution.

2- Total percentage of LF released into the solution.

The concentration of LF inside the emulsion was calculated to be 8.15 mg/ml at 0h as follows: the initial concentration of LF used to prepare the emulsion was 163mg/ml. The primary emulsion was prepared at a ratio of 50:50 LF to the oil phase. Therefore, the concentration of LF in the primary emulsion was 163 x 0.5 = 81.5 mg/ml. Then 2.1g of the emulsion was suspended in 20 ml of water or 5% NaCl. As a result, the LF concentration in the emulsion was 81.5 x (2.1/20) = 8.56 mg/ml.

3- Means from each test in the same column with the same letters are not significantly different ($p>0.05$). Values are the means of three experiments

Table 5.3

Release of LF from W/O/W dried microcapsules suspended in water or 5% NaCl at pH 5.6 and different temperatures when the primary emulsion (W/O) core material contained distilled water (DW), 20 mM sodium bicarbonate (SB), or 3% w/v sodium lactate (SL) as a solute.

			7d	
Emulsion	Temp.	Soln.	mg/ml	%
LF[1] in DW	25 °C	Water	9.92[2] edf [3]	72.89% [4]
LF in DW	25 °C	5% NaCl	12.04 a	88.46%
LF in 20 mM SB	25 °C	Water	8.07 h	59.29%
LF in 20 mM SB	25 °C	5% NaCl	9.45 efgh	69.43%
LF in 3% SL	25 °C	Water	11.02 abcd	80.97%
LF in 3% SL	25 °C	5% NaCl	11.76 ab	86.41%
LF in DW	10 °C	Water	11.11 abcd	81.63%
LF in DW	10 °C	5% NaCl	11.44 abc	84.06%
LF in 20 mM SB	10 °C	Water	8.05 h	59.15%
LF in 20 mM SB	10 °C	5% NaCl	9.76 edfg	71.71%
LF in 3% SL	10 °C	Water	11.69 ab	85.89%
LF in 3% SL	10 °C	5% NaCl	11.49 abc	84.42%
LF in DW	4 °C	Water	10.12 cdef	74.36%
LF in DW	4 °C	5% NaCl	10.34 bcde	75.97%
LF in 20 mM SB	4 °C	Water	8.23 h	60.47%
LF in 20 mM SB	4 °C	5% NaCl	8.43 gh	61.94%
LF in 3% SL	4 °C	Water	8.88 fhg	65.25%
LF in 3% SL	4 °C	5% NaCl	8.84 fgh	64.95%

1- The concentration of LF in the emulsion (W_1/O) was calculated to be 13.61 mg/ml after preparation as follows: the initial concentration of LF used to prepare the emulsion was 163 mg/ml. The primary emulsion was prepared at a ratio of 50:50 LF to the oil phase. Therefore, the concentration of LF in the primary emulsion was 163 x 0.5 = 81.5 mg/ml. Then the double emulsion (W/O/W) was prepared at a ratio of 40:60 primary emulsion:WPI, yielding 81.5 x 0.4 = 32.6 mg/ml LF in the double emulsion before drying. Then 4.0 g of the dried powder (weight of dried powder was 47.9% of the initial weight of the double emulsion) was suspended in 20 ml of water or 5% NaCl. As a result, the LF concentration in the emulsion was 32.6/0.479 x 0.2 = 13.61 mg/ml.

2- mg/ml LF recovered in solution

3- Means from each test in the same column with the same letters are not significantly different ($p>0.05$). Values are the means of three experiments.

4- Percent recovery of LF in solution

Table 5.4
Survival of *C.viridans* (log_{10} cfu/cm^2) inoculated on the bologna surface after treatment with lactoferrin or microencapsulated lactoferrin incorporated in WPI packaging film[1] stored at 4 or 10 °C for 28 d.

	0d	3d	7d	14d	21d	28d
a- Cells recovered 4 °C						
Control	2.41 bc[2]	3.15 a	2.69 a	2.75 a	2.85 a	4.77 a
T1	2.30 c	3.19 a	2.54 a	2.62 a	2.58 a	5.03 a
T2	2.28 c	1.80 c	2.75 a	2.56 a	2.80 a	4.93 a
T3	2.65 ab	2.28 b	ND[3] b	ND b	1.93 b	4.17 b
T4	2.77 a	ND d	ND b	ND b	1.80 b	3.52 c
T5	2.10 c	ND d	ND b	ND b	1.80 b	3.71 bc
b- Cells recovered at 10 °C						
Control	2.41 bc	3.73 a	4.59 ab	4.33 ab	6.45 a	6.93 a
T1	2.30 c	3.33 b	4.98 a	4.48 ab	6.56 a	6.83 a
T2	2.28 c	3.23 b	5.08 a	4.54 a	6.37 a	6.45 b
T3	2.65 ab	2.35 c	3.23 d	4.22 ab	5.63 b	5.75 c
T4	2.77 a	2.74 c	3.13 d	3.82 b	4.97 c	5.43 c
T5	2.10 c	2.45 c	3.61c	4.16 b	5.47 b	5.05 d

1- Treatments are described in Table 1. Cells were recovered on CTSI agar.
2- Means from each sampling time in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of 6 observations.
3- No cells were detected on CTSI agar. The minimum detection limit was 0.80 log cfu/cm^2.

Table 5.5
Total bacterial numbers (log_{10} cfu/cm^2) recovered from bologna slices challenged with unencapsulated or microencapsulated lactoferrin incorporated in WPI packaging film[1] stored at 4 or 10 °C for 28d.

	0d	3d	7d	14d	21d	28d
a- Cells recovered 4 °C						
Control	4.13 d[2]	3.94 a	4.58 a	5.98 a	6.84 a	7.36 a
T1	4.31 bc	3.91 a	3.94 b	4.49 b	6.03 b	7.02 b
T2	4.48 ab	4.11 a	3.93 b	3.80 c	5.41 c	7.41a
T3	4.53 a	3.29 b	2.78 c	2.75 d	5.14 c	7.38 a
T4	4.32 bc	2.86 c	3.03 c	2.44 d	5.07 c	6.66 c
T5	4.25 cd	3.53 b	3.67 b	2.49 d	5.32 c	6.94 cb
b- Cells recovered at 10 °C						
Control[1]	4.13[2] d	5.29 a	6.82 a	8.05 a	7.97 a	8.16a
T1	4.31 bc	4.54 c	7.12 a	7.99 a	7.75 a	7.92a
T2	4.48 ab	5.04 ab	7.12 a	8.03 a	8.07 a	8.09a
T3	4.53 a	3.59 d	5.75 b	7.12 b	7.73 a	8.17a
T4	4.32 bc	3.68 d	5.15 c	7.01 b	7.98 a	8.06a
T5	4.25 cd	4.70 bc	5.12 c	7.32 b	7.77 a	8.14a

1- Treatments are described in Table 1. Cells were recovered on APT agar.
2- Means from each sampling time in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of 6 observations.

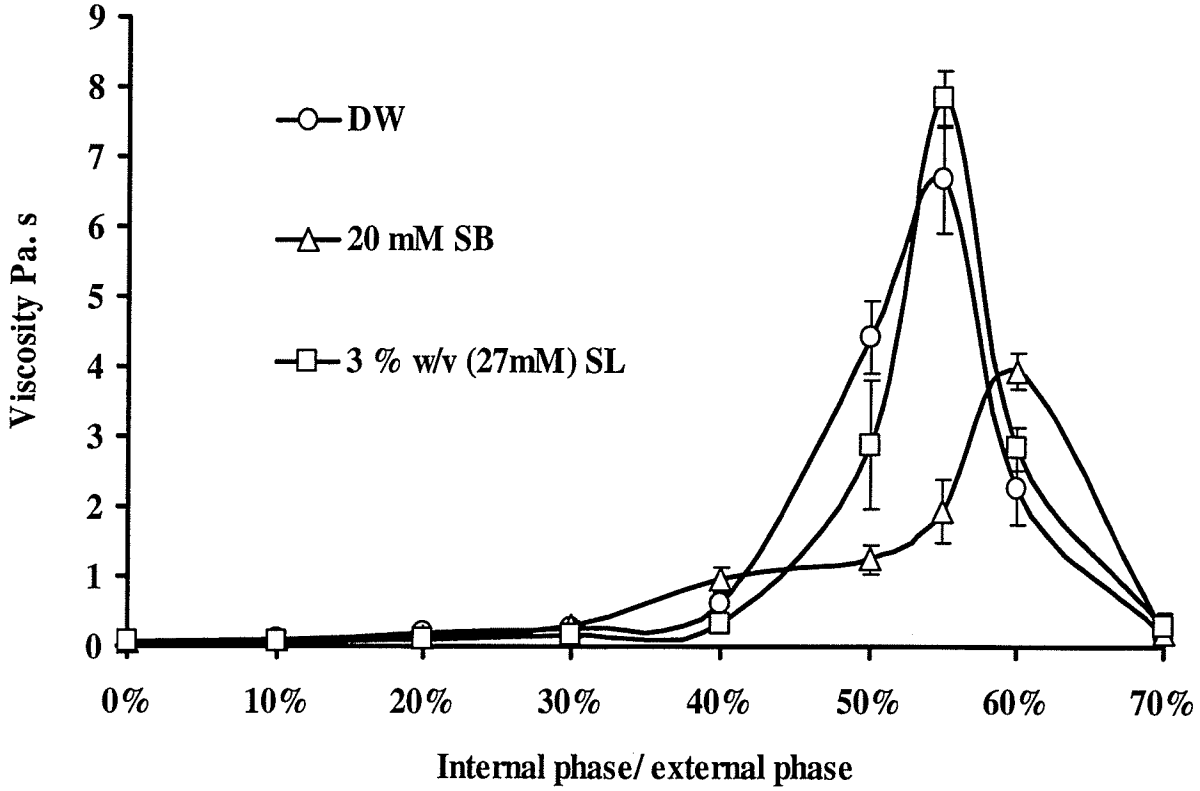

Fig. 5.1
Volume fraction percentage of 20% (w/v) LF dissolved in distilled water,(DW), sodium bicarbonate (SB) or sodium lactate (SL) that was successfully entrapped in the oil phase (78% corn oil + 22% butter fat) at 20 °C without destruction of the emulsion as measured by decrease (phase inversion) of emulsion viscosity. Each point is the mean of three experiments and error bars define the standard deviation.

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Fig. 5.2a

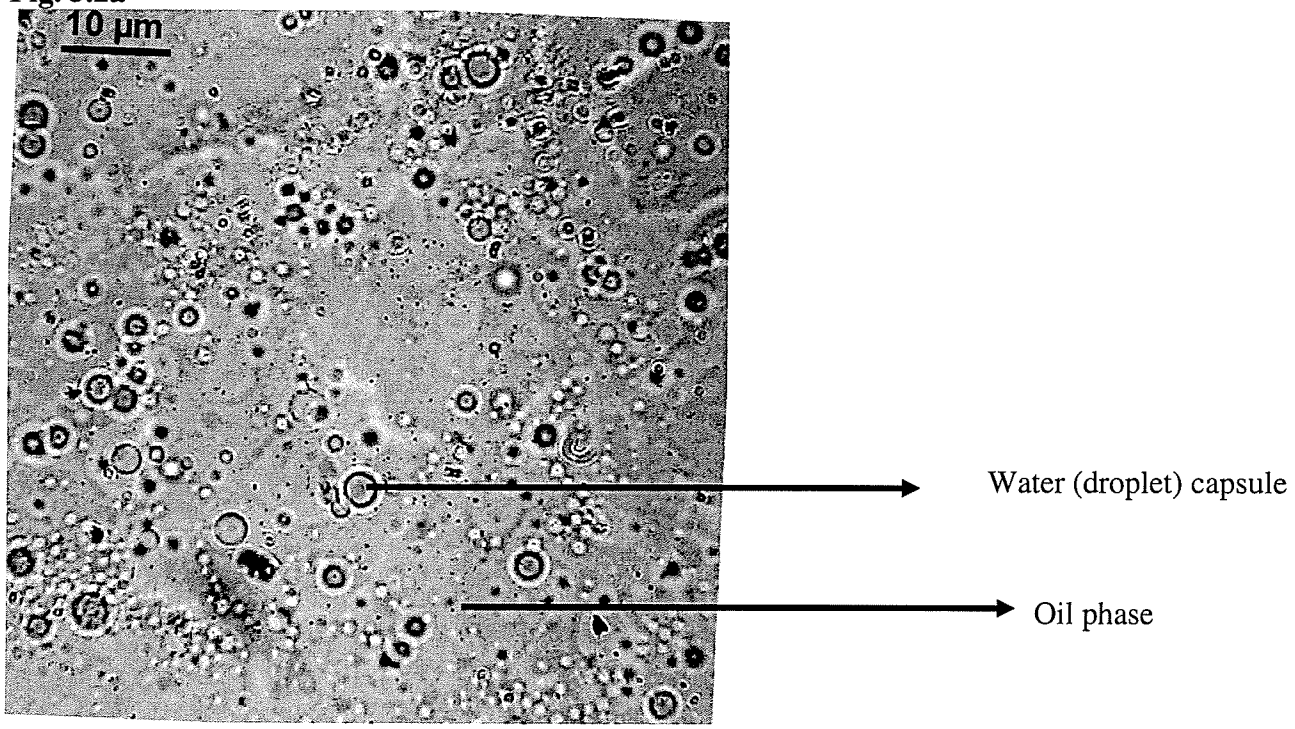

Fig. 5.2b

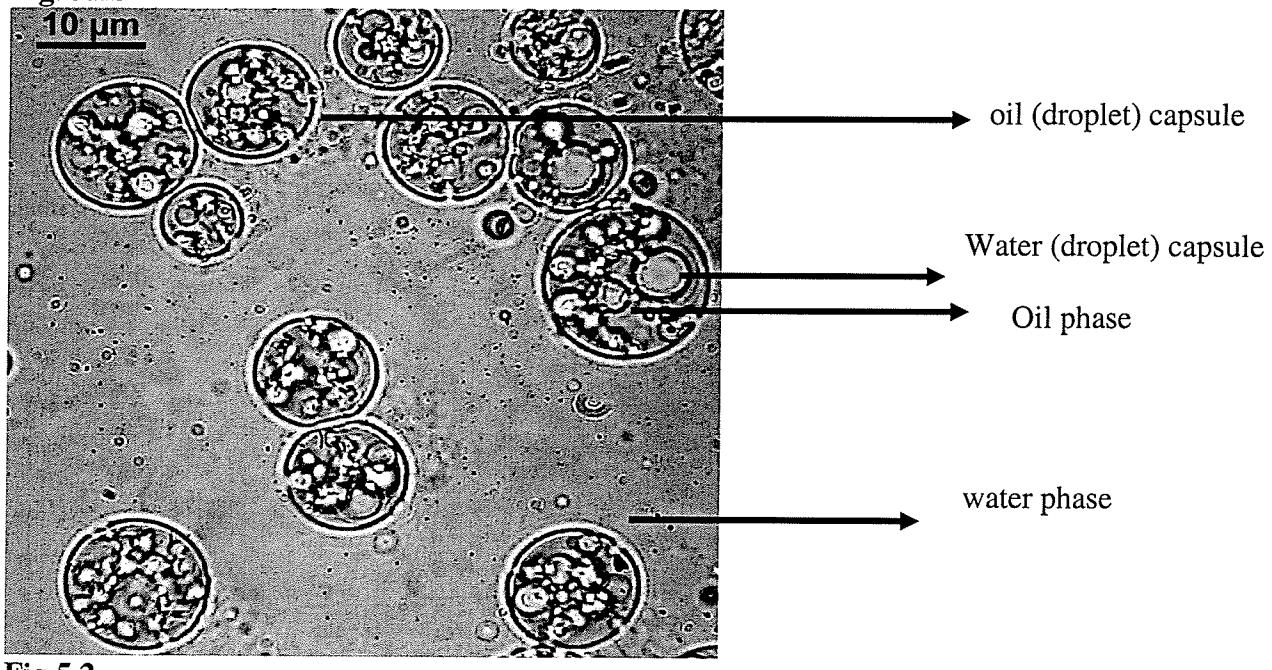

Fig.5.2

a) 20% (w/v) LF dissolved in distilled water and entrapped in the oil phase (78% corn oil + 22% butter fat + 0.1% PGPR); b) 20% (w/v) LF dissolved in distilled water entrapped in the oil phase (78% corn oil + 22% butter fat + 0.1% PGPR) in 30% WPI and 0.02% xanthan gum. The photographs were taken with a digital camcorder (Sony, DCR-PC330) using a phase-contrast microscope with F100/1.25 oil lens.

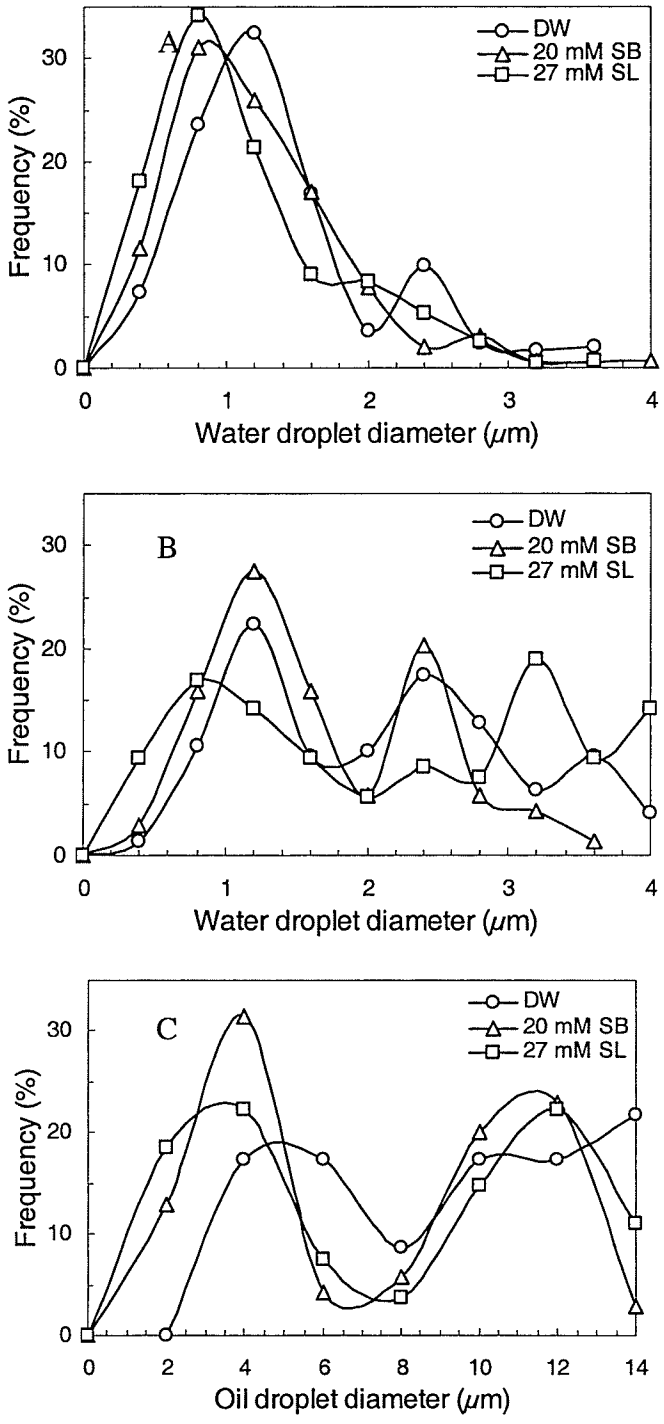

Fig. 5.3
Size distribution of droplets containing LF in two types of emulsion: a) size distribution of internal water phase droplets in W/O emulsions; b) size distribution of water (internal) phase droplets in W/O/W emulsions; c) size distribution of oil capsules in W/O/W emulsion droplets.

Chapter 6

Effect of lactoferrin with or without microencapsulation on *Escherichia coli* O157:H7 survival in dry fermented sausages

6.1 Abstract:

The effect of lactoferrin (LF) alone or with different chelating agents against the growth of 5 strains of *E. coli* O157:H7 and 7 meat starter cultures was evaluated in LB and APT broth, respectively, containing 2.9% NaCl at 13 or 26 °C. LF alone was bacteriostatic against *E. coli* O157:H7 strains 0627 and 0628 but other strains grew. Antimicrobial effectiveness of LF was enhanced by EDTA but LF alone did not affect the growth of meat starter cultures in broth. However, when LF plus EDTA and sodium bicarbonate (SB) were used the growth of all meat starter cultures except *Lactobacillus* (*L*) *curvatus* was reduced. During dry sausage manufacture with *L. curvatus* and *Staphylococcus carnosus* starter cultures, the effects of two types of microencapsulated LF (a paste-like and a dried powder form) with or without EDTA and SB as well as unencapsulated LF were tested on the viability of *E. coli* O157:H7. The reduction of *E. coli* O157:H7 was significantly higher ($p< 0.05$) in treatments containing LF and the largest reduction (4.2 log) was obtained when unencapsulated LF was used. However, some of this reduction in both treatments was due to cell injury (and not lethality) since significantly greater numbers of cells were recovered on APT overlaid with the selective medium ct-SMAC than on ct-SMAC alone.

Key words: Dry fermented sausages, *E. coli* O157:H7, lactoferrin, microencapsulation.

6.2 Introduction

Since 1982 *E. coli* O157:H7 has been implicated in foodborne illness outbreaks following of consumption of undercooked beef, unpasteurized milk and apple juice, yogurt, cheese, water and fresh salads (Willams et al. 2000). Despite inherent hurdles to growth of enterotoxigenic *E. coli* present in dry fermented sausage such as low pH, water activity (a_w), redox potential and high levels of NaCl (Pidcock, Heard and Henriksson 2002), outbreaks of illness caused by these organisms in fermented sausage were reported in the US (1994), Australia (1995) and Canada (1999) (Sumner, Ross, Jenson and Pointon 2005). As a result, the United States Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) developed guidelines requiring fermented sausage processors to demonstrate a 5-log unit reduction in the numbers of enterohemorrhagic *E. coli* during processing (Incze 1998). This stimulated work to investigate the effect of processing steps during dry fermented sausage manufacture on the survival of *E. coli* O157:H7. Prior and subsequent studies showed that *E. coli* O157:H7 survived dry fermented sausage manufacture and only a 1-2 log reduction was consistently obtained during its production (Hinkens et al. 1996; Faith, Parniere, Larson, Lorang, Kaspar and Luchansky 1998). One option used for elimination of *E. coli* O157:H7 was the application of a post-fermentation heat treatment (53 °C for 60 min; 63 °C instantaneous internal), but these treatments negatively affect the organoleptic quality of dry fermented sausage (Hinkens et al. 1996; Calicioglu, Faith, Buege and Luchansky 1997; Samelis, Kakouri, Savvaidis, Riganakos and Kontominas 2005).

Another alternative to thermal treatment for controlling the growth of foodborne pathogens is the use of synthetic or natural antimicrobial agents. Natural antimicrobials

have gained attention because of consumer desire for all-natural food products (Payne, Oliver and Davidson 1994). Subsequently, interest at the laboratory level in the possible use of lactoferrin (LF) as a natural antimicrobial in food increased. Lactoferrin is the main iron –binding glycoprotein present in milk and it has antimicrobial activity against wide range of Gram- positive and-negative bacteria, fungi, and parasites (Farnaud and Evans 2003). Furthermore, LF has antioxidant, antiviral, anti-inflammatory, immune-modulating, anti-cancer action and it can enhance the growth of probiotic bacteria like *Bifidobacterium* (Naidu 2000; Aguila and Brock 2001).

Several studies indicated the potential value of using LF as a natural antimicrobial in the food industry (Salamah and Al-Obaidi 1995; Murdock and Matthews 2002). However, the presence of divalent cations like Ca^{2+} and Mg^{2+} at 1-5 mM reduced its antimicrobial activity. This was believed due to the induction of changes in its tertiary structure, yielding a less effective tetrameric form of LF, while cations at the same time increased the stability of target bacterial cell membranes (Coughlin, Tonsager and MaGroaty 1983; Ellison, Giehl and LaForce 1988; Shimazaki 2000).

The outcome following antimicrobial challenge is a delicate balance of interactions. For example, Kim et al. (2004) reported that the presence of an LF binding protein on the surface of *Bifidobacterium* spp. and *Lactobacillus acidophilus* promoted their growth. In another study, Griffiths et al. (2003) found that the presence of *Bifidobacterium infantis* enhanced the inhibitory activity of LF against *E. coli* O157:H7 in reinforced clostridial broth.

Microencapsulation and controlled-release technology have found broad applications in the pharmaceutical, healthfood, paper and cosmetic industries (Reineccius

1995). Microencapsulation is a process in which sensitive ingredients (or core materials) are protected from deterioration or adverse environmental conditions by entrapment in a protective polymer encapsulating agent (or wall material) that will allow release of core material at controlled rates under specific conditions (Dzieak 1988; Hogan, McNamee, O'Riordan and O'Sullivan 2001). Recently, Chacon, Muthukumarasamy and Holley (2006) showed that when microencapsulated, otherwise highly volatile allyl isothiocyanate (AIT) reduced the viability of *E. coli* O157:H7 > 5 $\log_{10}$ units in dry fermented sausages.

The objectives of the present study were first to assess the antimicrobial activity of LF against five non-pathogenic strains of *E. coli* O157:H7 as well as meat starter cultures in a broth system under conditions similar to those used in the production of dry fermented sausages. Secondly, tests were designed to determine whether free and microencapsulated LF were able to reduce the levels of *E. coli* O157:H7 by at least 5 $\log_{10}$ units during dry fermented sausage manufacture.

6.3 Materials and Methods

6.3.1Effect of LF against E. coli *O157:H7 and meat starter cultures*

6.3.1.1 Bovine lactoferrin

Bovine lactoferrin (Bioferrin 2000) was provided by Glanbia Nutritionals (Glanbia Ingredients, Inc., Monroe, WI). Stock solutions of LF were prepared by dissolution in distilled water to yield 6 or 32 mg/ml LF in test media and were filter sterilized (0.22 μm syringe filter, Fisher Scientific, Fairlawn, NJ) before addition to the heat-sterilized test media. The iron saturation of LF was 14mg/100g as determined by

Glanbia which is less than in its normal physiological state (15-20% saturated) (Steijns and van Hooijdonk 2000).

6.3.1.2 EDTA and sodium bicarbonate

A stock solution of EDTA, disodium salt (Fisher Scientific) was prepared by dissolution in distilled water to yield a final concentration of 500 ppm. Sodium lactate, SL (Sigma, St.Louis, MO) and sodium bicarbonate, SB (Sigma) stock solutions were prepared in distilled water to give final concentrations of 1.5% (w/v) and 5mM, respectively, in test media. EDTA, SL or SB were sterilized by filtration (0.22 μm, Fisher Scientific) prior to addition to heat-sterilized growth media.

6.3.1.3 Test media

Lauria broth (LB) containing low levels of divalent cations (Al-Nabulsi and Holley 2005) was used for preliminary tests to evaluate the effect of LF against *E. coli* O157:H7. LB broth contained 10g /l tryptone (Difco, division of Becton Dickinson, Sparks, MD), 5g /l yeast extract (Difco), 1g /l glucose (Mallinckrodt, Paris, KY) and 29g /l NaCl (Fisher Scientific). To test the effect of LF against meat starter cultures All Purpose Tween broth, APT, (Difco) with 2.9% NaCl (containing a medium concentration of cations compared to cured meat products) was used (Al-Nabulsi and Holley 2005). The pH of each growth medium was measured with an Accumet Basic pH meter (Fisher Scientific)

6.3.1.4 Antimicrobial assay

The antimicrobial activity of 32mg/ml LF alone or with either 1.5% SL or 500ppm EDTA was evaluated against strains of non-pathogenic *E. coli* O157:H7 in LB broth containing 2.9% NaCl at 13 or 26 °C. These strains (3581, 0304, 0627, 0628) which had

mutated and become non-pathogenic (verotoxigenic negative), included a non-motile strain (1840) and were provided by Rafig Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Science Centre for Human and Animal Health, Winnipeg, MB. *E. coli* O157:H7 strains were maintained in trypticase soy broth (TSB) (BBL, Becton Dickinson, Sparks, MD) and sub-cultured twice in APT broth at 37°C for 18h before use. For experiments the cultures were centrifuged at 7,800 xg for 10 min at 10 °C (Sorvall RC-5, Du Pont, Newtown, CT), washed and suspended in 0.1% peptone water to achieve 7.4 $\log_{10}$ cfu/ml following standardization at an optical density of at 600 nm (Ultrospec 2000, Pharmacia Biotech, Baie d'Urfe, QC). The cultures were diluted in 0.1% peptone water and added to double strength LB to give a final concentration of approximately 4 $\log_{10}$ cfu/ml in single strength LB.

The antibacterial activities of LF alone or 500 ppm EDTA plus 5 mM SB as well as the 3 agents together were evaluated under the same conditions in APT broth containing 2.9% NaCl, against isolated commercial meat starter cultures used in the production of dry fermented sausage (Holley and Blaszyk 1998). *Lactobacillus curvatus* UM 133L (RM-53, Rudolff Müller GmbH, Pohlheim, Germany), *L. plantarum* UM 131L (Rosellac A, Institut Rosell Inc., Montreal, QC) , *L. plantarum* UM 134 L (Duploferment 66, Rudolff Müller GmbH), *Pediococcus pentosaceus* UM 127P (Rosellac A, Institut Rosell Inc.), *P. pentosaceus* UM 116P (Trumark LTII M Rector Foods Ltd., Mississauga, ON) and *P . acidilactici* UM 104P (Diversitech LPH, Rudolff Müller GmbH) were maintained in deMan Rogosa Sharpe (MRS) broth (BBL, Becton Dickinson, Sparks, MD) containing 20% glycerol at -70° C. *Staphylococcus carnosus* (UM 110, Trumark LTII, Rector Foods Ltd.) was maintained in tryptic soy broth (TSA,

BBL, Becton Dickinson) containing 20% glycerol at -70° C. The starter cultures were sub-cultured twice in APT by incubation at 37° C for 24h. Before use in experiments the cultures were centrifuged at 7,800 xg for 10 min at 10 °C, washed and suspended in 0.1% peptone water to achieve 9.0 $\log_{10}$ cfu/ml by standardization using optical density measurement at 600 nm. The cultures then were diluted in 0.1% peptone water and added to APT broth to give a final concentration of 5-6 $\log_{10}$ cfu/ml.

Antimicrobial assays were carried out following the procedure described by Al-Nabulsi and Holley (2006). Microcentrifuge tubes (1.5 ml flat top tubes, Fisher Scientific) used contained the following: 250µl double strength growth medium, 250µl of treatment (distilled water, EDTA, SL or SB), 500 µl LF and 500 µl culture diluted in same growth medium, and then the tubes were incubated at 13 or 26 °C. At intervals of 0, 2, 4 and 24h for tubes incubated at 26 °C and 0, 6, 48 and 120h for tubes incubated at 13 °C, a 100µl sample was taken to determine the viable cells present in each tube by serially diluting each sample in 0.1% peptone water and plating in duplicate on APT agar using the spiral plate method (Autoplate 4000, Spiral Biotech, Inc., Norwood, MA). To assess *E. coli* O157:H7 injury, a 100µl sample was surface plated on cefixime-tellurite sorbitol McConkey agar (ct-SMAC) (Zadik, Chapman and Siddons 1993). Plates were incubated at 37 °C for 48h and the difference in viable numbers on the two agars represented the injured population.

6.3.2 Microencapsulation procedure

Microencapsulation of LF was done at two levels of complexity; in water-in-oil and in a water-in-oil-in-water emulsion.

6.3.2.1 Paste- like microcapsules as a water- in- oil (w/o) emulsion.

A primary emulsion of LF in oil (22% butter fat: 78% corn oil) was prepared at 25 °C by adding an internal aqueous phase containing LF to the oil mixture. This was done by adding 20% (w/v) LF in distilled water drop-wise to the oil mixture which contained 0.1% (v/v) polyglycerol polyricinoleate, PGPR, (PALSGAARD, Denmark) as an emulsifier to a final LF:oil phase ratio of 50%:50% and emulsified using an Omni mixer (Omni-mixer, Ivan Sorvall Inc., Norwalk, CT) operated at 4800 rpm for 5 min. The emulsion was then placed at -20 °C for 10 min to allow solidification before it was added to the sausage batter.

Another mixture prepared as a primary emulsion as previously described included EDTA and SB with LF. The EDTA and SB were dissolved in distilled water to yield a final concentration 250 ppm and 2.5 mM, respectively, in the meat batter (Table 6.1).

6.3.2.2 Preparation of dried microcapsules created from a water-in-oil-in-water, W/O/W, multiple emulsion.

Dried powder consisting of microencapsulated LF alone or with EDTA and SB was produced by freeze-drying. This was done by adding the primary emulsion produced in the first step above at 25 °C to an external aqueous phase containing 30% (w/v) whey protein isolate, WPI (Davisco Foods International Inc. Le Sueur, MN, USA) and 0.02% xanthan gum (Sigma) at a ratio 40:60, primary emulsion: WPI. The multiple core emulsion (W/O/W) was prepared at a moderate mixing rate (3200 rpm for 3 min) as described by van der Graaf, Schroën and Boom (2005). Two hundred ml of each emulsion was spread over individual aluminum plates (230 mm diameter x 44 mm deep lined with wax paper) forming a layer approximately 15 mm thick. Emulsions were frozen at -40 °C for < 12h, placed in a freeze dryer (Model 10-146 MP; Virtis Corp.,

Gardiner, NY) and held at -5 °C and 0.5 mm Hg atmospheric pressure for < 72 h. Dried emulsions were chopped using a food processor (Model FP 1000-04, Black and Decker, Brockville, ON) for 15s to form the microencapsulated powder.

6.3.3 Dry fermented sausage production

6.3.3.1 Bacterial strain preparation

Dry cured sausages were fermented using *L. curvatus* (UM 133L) and *S. carnosus* (UM 110M) which were isolated from lyophilized commercial meat starter cultures. *L. curvatus* and *S. carnosus* were sub-cultured twice in MRS or TSA broth, respectively, and incubated at 37° C for 24h before use in dry fermented sausage. The cultures were centrifuged at 7,800g for 10 min at 10 °C, washed and suspended in 0.1% peptone water to achieve 9.0 $\log_{10}$ cfu/ml by standardization at $OD_{600\ nm}$ values of 2.37 or 1.84 for *L. curvatus* or *S. carnosus*, respectively. The two cultures were added to yield 7.0 $\log_{10}$ cfu/g meat batter.

E. coli O157:H7 strains 0627 and 0628 were sub-cultured twice at 37°C for 18 h before use in experiments. After the last incubation, each *E. coli* O157:H7 culture was centrifuged at 7,800g rpm for 10 min at 10 °C, washed and suspended in 0.1% peptone water to achieve 8 log cfu/ml by standardization at an $OD_{600\ nm}$ of 2.16. The organisms were mixed to obtain an equal number of cells of each strain in the cocktail with a final inoculation level of 6.0 $\log_{10}$ cfu/g sausage meat.

6.3.3.2 Dry fermented sausage manufacture for E. coli O157:H7 challenge with LF and microencapsulated LF.

Dry fermented sausage was produced as described by Muthukumarasamy and Holley (2006). Seven salami batches of 10 kg each were prepared in the Food Science

Department pilot plant. Sausage batters were formulated from -2 °C tempered 85% lean beef, 90% lean pork and pork fat (purchased from a local wholesaler) at ratio in the recipe of 17.59, 60.67 and 17.59 % (w/w), respectively, by chopping in a pre-cooled (1°C) Titane 40 rotating bowl meat cutter (Dadaux, Bersaillin, France). *L. curvatus* and *S. carnosus* were added to reach a final level of 7.2 and 6.6 $\log_{10}$ cfu/g sausage batter, respectively. All sausage batters (except controls) were also inoculated with the two strain cocktail of non-pathogenic *E. coli* O157:H7 (0627 and 0628) to yield 5.8 $\log_{10}$ cfu/g. Cervelat spice mix (0.44% w/w , Hermann Laue Spice Co. Inc, Uxbridge, ON) was added to these batters. Rapidur (0.69% w/w, a proprietary mixture of corn syrup solids and dextrose), 0.31% (w/w) pickle cure concentrate containing 6.25 % $NaNO_2$, which gave a final concentration of 193 ppm of nitrite, plus 1% $NaHCO_3$ and 500 ppm sodium erythorbate (Canada Compound Western Ltd., Winnipeg, MB) and 2.9% (w/w) NaCl were added and chopped ($\leq$ 5 min) to achieve a granular (3mm) consistency of meat and fat particles. Then LF or microencapsulated LF was added to sausage batters as outlined in Table 6.1. Fibrous casings (55 mm diameter, Kalle GmbH & Co. Wiesbaden, Germany) were pre-soaked in lukewarm water (40° C for 30 min) before use. Sausage batters at 4°C were stuffed with a piston stuffer (Mainca Model EM30, Equipamientos Carnicos, S. L. Barcelona, Spain) into the casings to achieve a final weight of about 500g. Sausages were transferred to a single cage smoke house (Allroundsystem Rondette with Titan controller, Maurer AG, Reichenau, Germany) and processed by fermentation at 26° C and 88% RH for $\leq$ 65h to reach a pH of < 5.3. Thereafter fermentation temperature and RH were decreased step-wise (6x12h intervals, where temperature and RH were reduced by 2° C and 2.2 % RH) from 26 to 14° C and 88 % to 75% RH over 72 h with intermittent

smoking for 30 min at 48 and 60 and 72h. The degree-hour guidelines of Agriculture and Agri-Food Canada (1992) were followed when calculating the permitted time and temperature combinations during fermentation. Fermentation was followed by drying at 13° C and 75% RH for 25 d.

In treatment 3 (Table 6.1) the pH did not drop to < 5.3 at end of the maximum 65h fermentation period defined by AAFC (1992). To provide additional time for sausages to reach pH 5.3, they were cooled to 4 °C and soaked in a brine solution containing 3% NaCl and 0.3 % glucose for 3d and then held at 13 °C and 90% RH until the pH reached ≤ 5.3. Sausages in this treatment required 10d instead of 3d to reach pH 5.3 which was considered the end of fermentation. Subsequently, for this treatment with the temperature still at 13 °C, smokehouse RH was reduced stepwise as previously described and sausages were dried at 75% RH for 25d.

6.3.3.3 Sampling of sausages

Dry fermented sausages were sampled at 0, 1, 2, 3, 6, 9, 15, 21 and 28 d during fermentation and ripening for pH, a_w, *E. coli* O157:H7, *L. curvatus, S. carnosus* and total numbers of bacteria. For microbiological sampling 11g of internal meat from cross-sectioned sausage were mixed with 99 ml of 0.1% peptone water and homogenized in a sterile stomacher bag (Filtra-bag, VWR International, Edmonton, AB) for 3 min with a Stomacher 400 (Seward Laboratory, London, UK). The total number of bacteria, *L. curvatus* and *S. carnosus* cells were determined by serial dilution in 0.1% peptone water and plated on APT agar, deMan Rogosa Sharpe (MRS) and Mannitol Salt agar (MSA) (BBL, Becton Dickinson, Sparks, MD) , respectively, using the spiral plater. *E. coli* O157:H7 numbers were determined by spiral plating the diluted samples on ct-SMAC.

All plates were incubated at 37 °C for 24-48h aerobically. At the end of drying samples were also plated on APT agar and incubated for 3h at 37 ° C, then the plates were overlaid with an equal volume of ct-SMAC and incubated at 37° C for 24-48h to detect the presence of injured cells.

6.3.3.4 pH and water activity

The pH of triplicate samples of sausage meat was directly measured on cross-sectioned sausages using a pH meter (Sentron Titan pH meter equipped with a lancefet probe). The a_w of sausage slices was analysed in triplicate (Novasina AW Sprint TH 500 a_w measuring unit, Axair AG, Pfäffikon, Switzerland).

6.3.5 Statistical Analysis

All data were analysed using Statistical Analysis System (version 8.1) software (SAS Institute, Cary, NC). Analysis of variance by the general linear model (GLM) and Duncan's multiple range tests were used to find significant differences ($p<0.05$) among treatments.

6.4 Results

6.4.1 Effect of bovine LF against E. coli *O157:H7 in broth*

E. coli O157:H7 strains 0627 and 0628 were more susceptible than the other strains used toward LF alone or LF with SL or EDTA at 26 °C in LB broth (Table 6.2a). LF was bacteriostatic against strains 0627 and 0628 whereas the other strains (although inhibited by 1.2-1.5 $\log_{10}$ cfu/ml compared to the control) were able to grow. In the presence of SL, LF exerted a bacteriostatic effect against strains 0627, 1840 and 0628, but cells of strains 3581 and 0304 increased by 1.2 and 1.6 $\log_{10}$ cfu/ml, respectively, after 16h under the same conditions. A greater bacteriostatic effect against strains 0627,

0628 and 1840 was noted when LF was combined with EDTA (Table 6.2a). Furthermore, the degree of injury caused by EDTA with LF was greater with these strains (Table 6.2b). The extents of lethality and injury caused by LF plus EDTA to cells of *E. coli* O157:H7 strains 0627 and 0628 were higher at 13 °C (Table 6.3a and b). Therefore, these two strains were selected to study the effect of LF on the survival of *E. coli* O157:H7 in dry fermented sausage.

6.4.2 Effect of bovine LF against meat starter cultures in broth

LF by itself did not inhibit any of the starter cultures in APT broth at either temperature. In several instances which were more frequent at 13 °C LF stimulated bacterial growth (Table 6.4 and 6.5). When EDTA + SB were included in reaction mixtures most starter cultures were inhibited. *L. curvatus* UM 133L was not inhibited at 26 °C and was least affected at 13 °C. On the basis of these results the latter organism was selected for use as the acidulating starter culture for dry fermented sausage manufacture and it was paired with *S. carnosus* UM 110 for colour development (Holley and Blaszyk 1998).

6.4.3 Physicochemical quality of dry sausages

All salami treatments achieved the guidelines for fermentation safety (degree-hour factor) established by Agriculture and Agri-Food Canada (1992). That is, the time above 15 °C required to reach pH 5.3 was < 65h. It should be noted that for treatment 3 it was necessary to extend fermentation an additional 7d at < 15 °C to achieve the required pH. For consistency when data were plotted in Figs. 6.1 and 6.2, day 3 represented the end of fermentation for all tests including treatment 3. The initial pH of the salami batter before fermentation ranged from 5.73 to 5.87 and this declined to 5.10 ± 0.04 at the end

of fermentation. In addition, no significant decrease in pH was noted at end of drying (28d) when it remained at 5.0 (Fig 6.1). An exception was treatment 3 where the pH increased slightly to 5.2 at the end of drying.

The a_w dropped from 0.97 ± 0.01 at the start of fermentation to 0.94 ± 0.01 65h later and further dropped to 0.87 ± 0.01 after 25d of drying (Fig 6.2). Again, treatment 3 was an exception where the final a_w of sausages was 0.90.

6.4.4 Survival of E. coli *O157:H7 in dry fermented sausages*

The raw meat used in dry fermented sausage production was checked and did not contain *E. coli* O157:H7. *E. coli* O157:H7 viability was reduced by one log during fermentation and there was no significant difference ($p > 0.05$) among the different treatments at this point (Table 6.6). Additional reductions in the viability of *E.coli* O157:H7 occurred by the end of drying and these reductions were significantly greater ($p < 0.05$) in treatments containing LF (Table 6.6). The largest reduction (4.2 log) was obtained when unencapsulated LF was used alone. Results from samples plated on APT agar overlaid with ct-SMAC showed that part of this reduction occurred as a result of cell injury. The lethality of this treatment was only 3 $\log_{10}$ when injured cells were taken into account.

6.4.5 Effect of unencapsulated and microencapsulated LF on starter culture performance in dry fermented sausages

L. curvatus numbers increased during the first day of fermentation, but later decreased slightly and remained between 6.31-7.03 $\log_{10}$ cfu/g at the end drying (Table A 4.1). Similarly, the numbers of *S. carnosus* increased during fermentation and then decreased (1.0 – 2.8 $\log_{10}$ cfu/g) by the end of drying in all treatments (Table A 5.1).

6.4.6 Effect of unencapsulated and microencapsulated LF on total bacterial numbers in dry fermented sausages

The total viable bacterial numbers in the salami batter directly after formulation were 7.6 ± 0.1 $\log_{10}$ cfu/g (Table A4). At the end of drying there was some variation in numbers in the treatments which ranged from 6.49-8.32 $\log_{10}$ cfu/g (Table A 6.1).

6.5 Discussion

As found previously (Al-Nabulsi and Holley 2006) the antimicrobial activity of LF against *E. coli* O157:H7 in broth in the present study was strain dependent. Differences in strain susceptibility may be explained by the ability of LF to bind to bacterial surfaces since binding is a requirement for initiation of its lethal effects (De Lillo, Quiros and Fierro 1997). Naidu, Svensson, Kishore, and Naidu (1993) reported that *E. coli* strains with low LF binding recovered from LF-mediated bacteriostatic effects while strains with high LF affinity failed to recover. Erdei, Forsgren, and Naidu (1994) identified porins to be the sites where LF bound to the surface of *E. coli* and they suggested that differences in LF affinity were affected by the O chain of the LPS which was believed to be able shield the porins from interaction with LF. In addition, Naidu et al. (1993) found that LF binding to the surface of an isogenic R mutant strain of *Salmonella* Typhimurium increased with a decrease in the size of the LPS polysaccharide moiety, and they found the high LF binding mutant was more susceptible to LF. Susceptible strains of *E.coli* O157:H7 used in the present study did not exhibit the R phenotype.

The addition of EDTA to broth reaction mixtures enhanced the activity of LF. This could be explained by the ability of EDTA to form insoluble complexes at pH 6.8

(which is near its pKa value of 6.2) with divalent cations, preventing their stabilization of the outer bacterial membrane and increasing its permeability (Coughlin et al. 1983; Vaara 1992; Boland, Davidson, Bruce and Weiss 2004). This in turn may enable LF to interact directly with the cytoplasmic membrane causing selective permeation of ions (K^+) without initially affecting the cellular pH gradient (Aguilera, Quiros and Fierro 2003). In addition, Al-Nabulsi and Holley (2006) showed that when sodium bicarbonate was added to LF and EDTA a 4 log reduction was obtained that may have been due to the enhancement of LF stability by bicarbonate.

In the present study it was found that LF inhibited the growth of *E. coli* O157:H7 without affecting the growth of meat starter cultures. However, in combination with EDTA + SB some inhibitory activity of LF against meat starter cultures tested was found in APT broth. Thus, it was important to monitor the effects of LF against starter cultures during dry fermented sausage manufacture.

Lactic acid in combination with NaCl is commonly used to preserve food in conjunction with storage at refrigeration temperatures, but these interventions are not sufficient to dependently eliminate foodborne pathogens. Glass, Loeffelholz, Ford, and Doyle (1992) showed that *E. coli* O157:H7 survived in fermented sausage containing 3.5% NaCl prepared with either starter culture or with the indigenous lactic acid bacteria. Acid adaptation of *E. coli* O157:H7 was suggested to be the reason for the ability of *E. coli* O157:H7 to survive the dry sausage manufacturing process (Calicioglu et al. 1997). Indeed, Jordan and Davies (2001) and Casey and Condon (2002) reported that *E. coli* O157:H7 was able to regulate its internal pH and counteract the bactericidal effects of lactic acid in the presence of NaCl in the growth medium. Another possible reason for *E.*

coli O157:H7 being able to survive sausage drying at lower temperatures could be a reduction in membrane permeability caused by alteration of its fatty acid composition. In addition, an increase in the dissociation of lactic acid at lower temperatures may also contribute to bacterial resistance (McWilliam Leitch and Stewart 2002a, b).

A reduction of *E. coli* O157:H7 numbers occurred within the first 3d of fermentation in all treatments as a consequence of the meat pH drop. However, *E. coli* O157:H7 viability was reduced continuously in treatments containing unencapsulated and microcapsulated LF during the drying process. The enhanced activity of LF during the ripening process could have resulted from a number of factors acting additively or synergistically with LF. These could include the lower storage temperature (13 °C), pH ≤5.3, the presence of > 2.9% NaCl, and ≤ 200ppm nitrite. It was found in a previous study (Al-Nabulsi and Holley 2006) that *E. coli* O157:H7 cells stressed as a result exposure to increased NaCl levels and lower temperatures were more susceptible to LF.

In broth studies reported here SL alone caused injury to *E. coli* O157:H7 cells, which was greater at 26 °C than 13 °C. While SL enhanced the antimicrobial activity of LF against *E. coli* O157:H7, greater reductions were sought. Therefore, for sausage experiments SB was substituted for SL on the basis of previous results (Al-Nabulsi and Holley 2006). Unfortunately, when SB was not encapsulated (treatment 3) its high pH and buffering capacity delayed meat fermentation by a week and slowed the drying process.

Microencapsulation can protect sensitive ingredients in the core from adverse environmental effects, and by careful design of wall materials, release of core materials can be sustained (delayed) or even directed toward either hydrophobic or hydrophilic

phases in foods to which microcapsules are added (Lee and Rosenberg 2000). Two types of microcapsules were used in the present study to evaluate these options since it was possible that *E. coli* O157:H7 cells might not be uniformly distributed throughout the sausage meat/fat matrix. The first type were paste-like microcapsules characterized by a hydrophobic surface, while the second type had a dry powder consistency and were comprised of microcapsules with a hydrophilic surface. Each was designed, respectively, to target either the adipose tissue surface or aqueous phase of the dry fermented sausage where *E. coli* O157:H7 might be located.

In separate trials the release of LF from the paste-like microcapsules was found to be < 11% after 3d or > 90% after 7d at 25 °C in aqueous medium containing 5% NaCl (data not presented). The release of LF from the dried microcapsules under the same conditions was >75% after 7d (Al-Nabulsi et al. unpublished). Therefore, the lower level of antimicrobial activity shown by microencapsulated LF was unexpected. This was probably due in part to the dilution of LF which occurred when microcapsules were formulated into the meat. While 32 mg/ml LF was used in some broth tests, the maximum final level of LF that could be used in sausages to which LF- containing microcapsules were added was 1.8 -3 mg/g meat (Table 6.1).

The viability of meat starter cultures used in the present study was not seriously affected by treatments as indicated by the normal rate of pH reduction in dry fermented sausage, except in treatment 3 which contained unencapsulated LF, SB and EDTA. The later could be explained by the high pH of the SB stock solution (9.5) and its buffering capacity. In all treatments, starter culture and total bacterial numbers were not substantially affected by the use of LF in the sausage formulation. Results obtained here

are consistent with other work (Holley, Wittmann and Kwan 1988) which showed the predominant component of the microflora recovered on non-selective media from fermented sausages were Gram-positive lactic acid bacteria.

The unencapsulated LF treatment was able to cause a significant reduction in numbers of *E. coli* O157:H7 recoverable from dry sausage after processing. The combined lethality of processing and treatment was ≤ 3 $\log_{10}$ cfu/g. Cell injury in response to LF treatment was apparent (a further one $\log_{10}$ cfu/g reduction), but LF was not capable of generating the required 5 $\log_{10}$ cfu/g reduction in *E. coli* O157:H7 viability. It is also recognized that in these tests, 3 strains of *E. coli* O157:H7 which showed resistance to LF in broth tests were not included in the sausage challenge. While microencapsulation of LF did not improve the antimicrobial performance of LF in the tests reported here, the results suggested that LF can cause significant reduction in the viability of some strains of *E. coli* O157:H7 in fermented sausage.

Acknowledgments

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Table 6.1
Experimental design used for challenge studies of lactoferrin against *E. coli* O157:H7 in dry fermented sausages [1]

Treatments	*E. coli* O157:H7[2]	Lactoferrin	mg lactoferrin /g sausage batter	ppm EDTA	mM SB
Control	-	-	-	-	-
1	+	-	-	-	-
2	+	LF[3]	6.0	-	-
3	+	LF + EDTA+ SB[4]	6.0	500	5.0
4	+	Paste like microcapsules of LF [5]	3.0	-	-
5	+	Dried powder microcapsules of LF [6]	1.8	-	-
6	+	Dried powder microcapsules of LF with EDTA and SB [7]	1.8	250	2.5

1- Meat starter cultures (7.2 log cfu/g *L. curvatus* and 6.6 log cfu/g *S. carnosus*) were added to all treatments.
2- 5.8 log cfu/g sausage batter
3- lactoferrin dissolved in distilled water and added to sausage batter.
4- lactoferrin , ethylene diamine tetraacetic acid (EDTA) and sodium bicarbonate (SB) dissolved in distilled water and added to sausage batter.
5- Water in oil emulsion; lactoferrin in distilled water was encapsulated in oil (78% corn oil + 22% butter fat with 0.1% polyglycerol polyricinoleate, PGPR).
6- Water-in-oil-in-water emulsion; lactoferrin in distilled water was encapsulated in oil (78% corn oil + 22% fat butter containing 0.1% PGPR) in 30% w/v whey protein isolate (WPI).
7- Water-in-oil-in-water emulsion; lactoferrin, ethylene diamine tetraacetic acid (EDTA) and sodium bicarbonate (SB) in distilled water were encapsulated in oil (78% corn oil + 22% fat butter with 0.1% PGPR) in 30% (w/v) WPI.

Table 6.2

Effect of 32 mg/ml lactoferrin alone or with 1.5% (w/v) sodium lactate or 500 ppm EDTA against non- pathogenic strains of *E.coli* O157:H7[1] (log_{10} cfu/ml) in LB broth containing 2.9% NaCl at 26 °C.

	E.coli O157:H7 strains														
	3581			0304			1840			0627			0628		
	2h	4h	16h	2h	4h	16h	2h	4h	16h	2h	4h	16h	2h	4h	16h
a- Cells recovered on APT agar															
Control[2]	3.94[3]a	4.24a	7.89a	4.52a	4.49a	8.14a	4.02a	4.42a	8.36a	3.92a	4.36a	7.91a	3.95a	4.38a	8.19a
LF [4]	3.88a	3.90b	6.26b	4.44a	4.25ab	6.62b	4.03a	4.26ab	7.12b	3.86a	4.08ab	4.29c	3.86a	4.17ab	4.12cd
SL [5]	3.92a	4.08ab	5.75c	4.01b	4.11b	6.21bc	4.09a	4.08ab	5.42c	3.95a	4.01ab	5.79b	3.86a	4.07ab	6.12b
SL + LF	3.93a	3.93b	5.21d	3.91b	4.08b	5.65c	3.98a	4.06ab	4.21d	3.90a	3.94b	3.93d	3.91a	3.95ab	4.48c
EDTA [6]	3.84a	3.90b	3.80e	4.02b	4.08b	4.35b	4.04a	4.07ab	3.98de	3.94a	3.97b	5.74b	3.86a	3.90ab	3.94d
EDTA + LF	3.94a	3.88b	3.64e	3.96b	4.01b	3.86e	4.09a	4.01ab	3.37f	3.94a	3.89b	3.29e	3.86a	3.83b	3.36e
b- Cells recovered on ct-SMAC agar															
Control	3.80a	3.87a	7.57a	3.92a	4.09a	7.60a	3.24a	3.89a	7.52a	3.03a	3.64a	6.89a	3.65a	3.56ab	7.03a
LF	3.78a	3.75a	5.91b	3.76a	4.00a	6.18a	3.11ab	3.75a	6.31b	3.44a	3.73a	3.14c	3.61a	3.83a	3.82c
SL	3.72a	3.59a	5.11c	3.41ab	3.51b	4.96b	3.43a	3.15b	4.05c	3.11a	3.39ab	4.53b	3.04b	3.30b	4.81b
SL + LF	3.72a	3.71a	4.85c	3.86ab	3.45b	4.59b	3.21ab	3.07b	3.20d	3.30a	3.04b	3.41c	3.30ab	2.95b	4.05c
EDTA	3.61ab	2.62c	0.00[7]d	3.61ab	2.65c	0.00c	2.97b	3.01b	0.00e	2.52b	2.79b	0.00d	2.38c	2.15c	0.00e
EDTA + LF	3.54b	3.19b	0.00d	3.06b	2.75c	0.00c	2.33c	2.11c	0.00e	2.36b	1.30c	0.00d	2.13c	0.00d	0.00e

1- The number of *E.coli* O157:H7 cells at 0 time was 4.00 $\log_{10}$cfu/ml.

2-No lactoferrin.

3- Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.

4- lactoferrin.

5- Sodium lactate.

6- Ethylene diamine tetraacetic acid

7- Minimum detection limit was 0.60 log cfu/ml.

Table 6.3

Effect of 32 mg/ml lactoferrin alone or with 1.5% (w/v) sodium lactate or 500 ppm EDTA against non-pathogenic strains of *E.coli* O157:H7[1] (log_{10} cfu/ml) in LB broth containing 2.9% NaCl at 13 °C.

	E.coli O157:H7 strains														
	3581			0304			1840			0627			0628		
	6h	48h	120h	6h	48h	120h	6h	48h	120h	6h	48h	120h	6h	48h	120h
a- Cells recovered on APT agar															
Control[2]	3.99a[3]	5.30a	8.54a	4.21a	5.24a	8.55a	4.01a	5.81a	8.77a	3.96a	4.84a	8.73a	3.88a	5.48a	8.45a
LF[4]	3.95a	5.03a	7.57b	4.01a	4.69b	7.91b	3.95a	5.11b	4.74d	3.84a	3.98b	4.18d	3.84a	3.80c	4.32c
SL[5]	3.95a	4.50b	7.80b	4.03a	5.08a	7.76b	3.96a	5.14b	7.95b	3.94a	4.76a	6.75b	3.93a	4.57b	7.53b
SL + LF	3.96a	4.59b	6.29c	3.99a	4.82b	6.53c	4.09a	5.03b	6.96c	3.90a	4.34ab	5.77c	3.92a	4.61b	3.42d
EDTA[6]	4.02a	3.58c	3.18d	4.00a	3.92c	2.98d	4.06a	3.72c	2.86e	3.82a	3.26c	2.80e	3.87a	3.66c	2.91e
EDTA + LF	3.94a	3.02d	2.56e	4.18a	3.04e	1.95e	3.99a	3.18d	2.48e	3.85a	2.56d	2.00f	3.77a	3.03d	2.06f
b- Cells recovered on ct-SMAC agar															
Control	3.57a	4.43a	8.45a	3.50a	4.99a	8.43a	3.64a	5.01a	8.00a	3.24a	4.34a	7.58a	2.58a	4.68a	7.78a
LF	3.66a	3.57b	7.22b	2.99ab	4.61a	7.64b	3.05b	4.56ab	3.65d	2.78ab	3.57b	3.63c	2.65a	3.18b	3.53c
SL	3.30ab	4.02a	6.21c	3.30a	3.48b	5.29c	2.99b	4.32b	6.92b	2.81ab	3.51b	5.78b	2.15b	3.24b	4.40b
SL + LF	3.52ab	3.67b	5.15d	2.76b	3.76b	5.15c	2.45cd	4.27b	5.22c	2.08b	2.88c	4.02c	2.56a	3.13b	3.44c
EDTA	3.23b	0.00[7]c	0.00e	2.96b	0.00c	0.00e	2.69bc	0.00c	0.00e	2.38b	0.00d	0.00e	2.45ab	0.00c	0.00d
EDTA + LF	2.90b	0.00c	0.00e	2.13c	0.00c	0.00e	2.15d	0.00c	0.00e	1.30c	0.00d	0.00e	1.60c	0.00c	0.00d

1- The number of *E.coli* O157:H7 cells at 0 time was 4.00 log_{10} cfu/ml.

2-No lactoferrin.

3- Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.

4- lactoferrin.

5- Sodium lactate.

6- Ethylene diamine tetraacetic acid

7- Minimum detection limit was 0.60 log cfu/ml.

Table 6.4
Effect of lactoferrin alone or with EDTA plus SB against meat starter cultures at 26 °C in APT broth containing 2.9% NaCl.

	0h	2h	4h	24h
***L. curvatus* UM 133L**				
Control[1]	5.88a[4]	5.90a	6.11a	9.49a
LF[2]	5.88a	5.69a	5.90a	9.51a
EDTA + SB[3]	5.88a	5.80a	5.88a	8.87b
EDTA + SB + LF	5.88a	5.78a	5.99a	8.97ab
***L. plantarum* UM 131L**				
Control	5.11a	5.14a	5.56a	9.52a
LF	5.11a	5.16a	5.40a	9.38a
EDTA + SB	5.11a	5.09a	5.36a	8.58b
EDTA + SB + LF	5.11a	5.21a	5.40a	8.63b
***L. plantarum* UM 134L**				
Control	5.75a	5.83a	6.08a	8.42a
LF	5.75a	5.87a	5.93a	8.43a
EDTA + SB	5.75a	5.80a	5.78a	7.28b
EDTA + SB + LF	5.75a	5.74a	5.75a	7.44b
***P. pentosaceus* UM 116P**				
Control	6.05a	5.97a	5.95a	8.59b
LF	6.05a	5.88a	5.86a	9.04a
EDTA + SB	6.05a	5.97a	5.86a	5.95c
EDTA + SB + LF	6.05a	5.93a	5.98a	6.10c
***P. pentosaceus* UM 127P**				
Control	5.72a	5.62a	5.68a	8.94a
LF	5.72a	5.61a	5.65a	9.35a
EDTA + SB	5.72a	5.59a	5.61a	6.85c
EDTA + SB + LF	5.72a	5.54a	5.57a	7.25b
***P. acidilactici* UM 104P**				
Control	5.59a	5.67a	5.63a	8.44a
LF	5.59a	5.70a	5.59a	8.58a
EDTA + SB	5.59a	5.61a	5.56a	5.65b
EDTA + SB + LF	5.59a	5.63a	5.39a	5.64b
***S. carnosus* UM 110**				
Control	6.08a	6.20a	6.22a	8.59b
LF	6.08a	6.17a	6.16a	9.18a
EDTA + SB	6.08a	6.05a	6.19a	6.30c
EDTA + SB + LF	6.08a	6.08a	6.16a	6.39c

1- No lactoferrin
2- 6 mg/ml lactoferrin
3- 500 ppm ethylene diamine tetraacetic acid (EDTA) + 5 mM sodium bicarbonate (SB)
4- Means in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of two experiments.

Table 6.5
Effect of lactoferrin alone or with EDTA plus SB against meat starter cultures at 13 °C in APT broth containing 2.9% NaCl.

	0h	6h	48h	120h
***L. curvatus* UM 133L**				
Control[1]	5.88a[4]	5.81a	6.58a	8.86a
LF[2]	5.88a	5.81a	6.40ab	9.08a
EDTA + SB[3]	5.88a	5.83a	6.00b	8.23b
EDTA + SB + LF	5.88a	5.73a	6.06b	8.26b
***L. plantarum* UM 131L**				
Control	5.11a	5.24a	6.80a	8.84a
LF	5.11a	5.08a	6.28b	8.93a
EDTA + SB	5.11a	5.11a	5.87bc	7.93b
EDTA + SB + LF	5.11a	5.11a	5.73c	8.14b
***L. plantarum* UM 134L**				
Control	5.75a	5.68a	5.92a	7.52b
LF	5.75a	5.70a	6.00a	8.04a
EDTA + SB	5.75a	5.72a	5.94a	6.19c
EDTA + SB + LF	5.75a	5.82a	5.94a	6.30c
***P. pentosaceus* UM 116P**				
Control	6.05a	5.70a	5.72a	8.95a
LF	6.05a	5.63a	5.84a	9.06a
EDTA + SB	6.05a	5.63a	5.77a	5.84b
EDTA + SB + LF	6.05a	5.62a	5.84a	5.85b
***P. pentosaceus* UM 127P**				
Control	5.72a	5.67a	5.69a	8.07a
LF	5.72a	5.61a	5.64a	8.17a
EDTA + SB	5.72a	5.56a	5.62a	5.46b
EDTA + SB + LF	5.72a	5.62a	5.57a	5.57b
***P. acidilactici* UM 104P**				
Control	5.59a	5.97a	6.00a	8.06b
LF	5.59a	6.00a	6.05a	8.77a
EDTA + SB	5.59a	5.63a	6.01a	6.22c
EDTA + SB + LF	5.59a	5.92a	5.97a	6.24c
***S. carnosus* UM 110**				
Control	6.08a	5.95a	6.36a	8.36b
LF	6.08a	5.89a	6.43a	9.28a
EDTA + SB	6.08a	5.84a	6.14a	6.12c
EDTA + SB + LF	6.08a	5.97a	6.09a	6.03c

1- No lactoferrin
2- 6 mg/ml lactoferrin
3- 500 ppm ethylene diamine tetraacetic acid (EDTA) + 5 mM sodium bicarbonate (SB)
4- Means in the same column with the same letters are not significantly different ($p > 0.05$). Tabulated values are the means of two experiments.

Table 6.6
Recovery of *E. coli* O157:H7 from dry fermented sausage during processing when challenged with unencapsulated or microencapsulated lactoferrin.

	Processing time (day)									
	log_{10} cfu/g recovered on ct-SMAC agar									log_{10} cfu/g recovered on APT/ ct-SMAC
Treatment[1]	0	1	2	3	6	9	15	21	28	28
Control	0.00b[2]	0.00b	0.00c	0.00c	0.00d	0.00c	0.00e	0.00d	0.00d	0.00e
1	5.76a	4.86a	4.79ab	4.72ab	4.36bc	4.00b	3.90c	3.42ab	3.22b	4.52b
2[3]	5.79a	4.84a	4.53b	4.45b	4.34bc	3.62b	3.48d	1.46c	1.65c	2.74d
3[3]	5.83a	5.13a	4.92a	5.02a	4.22c	3.73b	4.49b	4.31a	4.07a	5.09a
4[4]	5.65a	4.93a	4.60ab	4.43b	4.53abc	4.03b	3.67cd	2.00c	1.85c	3.88c
5[4]	5.75a	5.16a	4.69ab	4.66ab	4.65ab	4.99a	4.49b	2.74bc	2.26c	4.53b
6[4]	5.90a	5.01a	4.95a	4.64ab	4.75a	4.59a	4.93a	2.33bc	2.09c	4.42b

1- Treatments are described in Table 1.
2- Means from each sampling time in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of 6 observations.
3- Unencapsulated lactoferrin.
4- Microencapsulated lactoferrin.

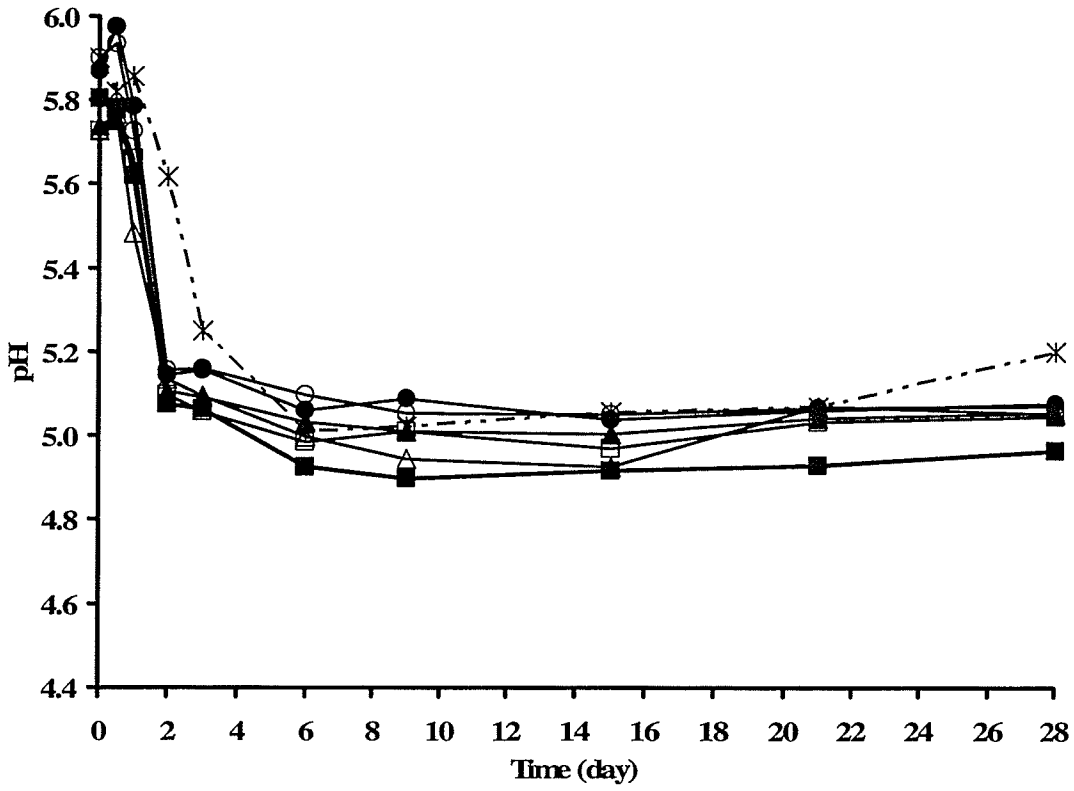

Fig. 6.1
Changes in meat pH during dry fermented sausage manufacture; fermentation at 26 °C for 3d and then drying at 13 °C for 25 d. (■) control (without *E.coli* O157:H7 and without LF); (□) *E. coli* O157:H7 without LF; (▲) LF; (*) LF + EDTA+ SB, for this treatment day 3 on the x-axis is 10 d after stuffing; (△) paste-like microcapsules of LF; (○) dried powder microcapsules of LF; and (●) dried powder microcapsules of LF, EDTA and SB. Results shown are the means of 3 values

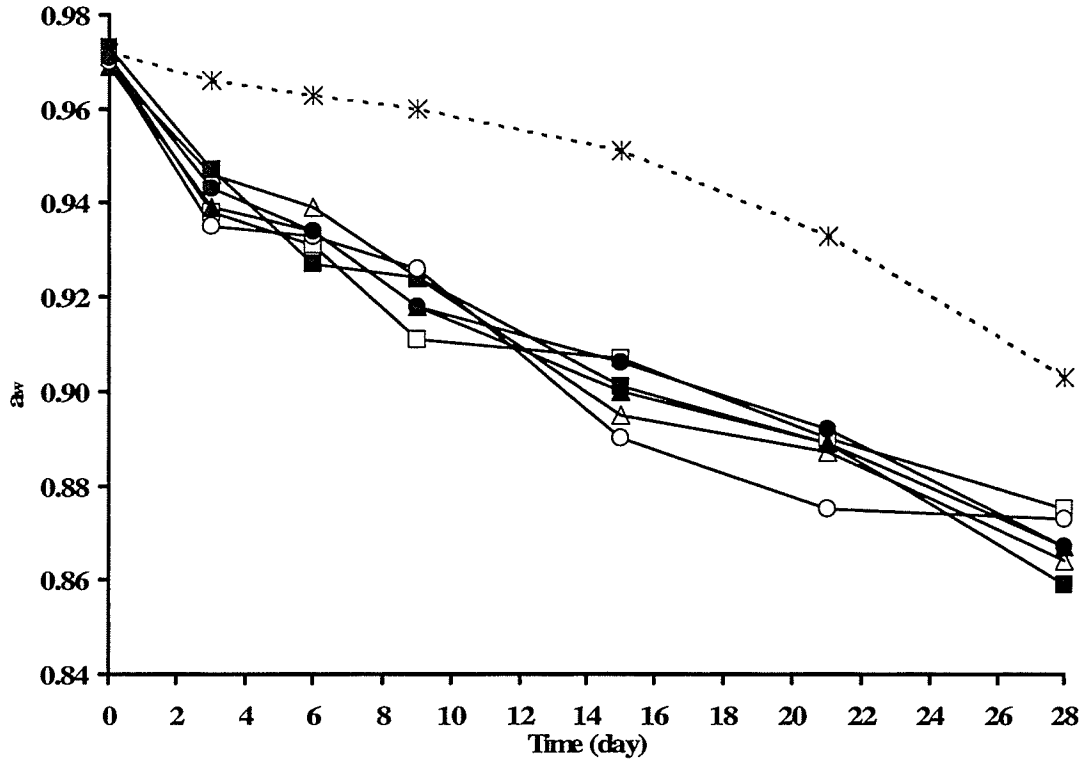

Fig. 6.2

Changes in water activity (a_w) of meat during dry fermented sausage manufacture; fermentation at 26 °C for 3d and then drying at 13 °C for 25 d. (■) control (without *E.coli* O157:H7 and without LF); (□)*E. coli* O157:H7 without LF; (▲) LF; (*) LF + EDTA+ SB, for this treatment day 3 on the x-axis is 10 d after stuffing; (△) paste-like microcapsules of LF; (○) dried powder microcapsules of LF; and (●) dried powder microcapsules of LF, EDTA and SB. Results shown are the means of 3 values.

Chapter 7

Overall Conclusion

The results in Chapter 3 and 4 have demonstrated for the first time a bactericidal effect of unmodified LF at neutral pH and refrigeration temperatures (10 and 4 °C) against *C.viridans* and *E.coli* O157:H7. This is in contrast with previous research that reported that LF activity is temperature dependent and is reduced at lower temperatures.

Previous reports showed that LF had antimicrobial effects in simple broth that would not support bacterial growth. LF activity was reduced when divalent cations were added to these broths or when antimicrobial assays were performed in complex media. The experiments in the present study were conducted in Lauria broth, LB, or All Purpose Tween broth, APT, which contain low or medium concentrations of cations, respectively, compared to cured meat products. The results obtained in Chaper 3 and 4 showed that divalent cations reduced the antibacterial activity of LF and this was believed due to either increasing the stability of the cell membrane or by binding to LF and forming less active tetramers. This reduced activity is in agreement with previous research.

The effect of NaCl on the antimicrobial activity of LF depends on the organism challenged. As NaCl concentration increased the activity of LF against *C.viridans* decreased, but increased NaCl concentrations sensitized *E.coli* O157:H7 toward LF. This could be explained by the ability of C.v*iridans* but not *E.coli* O157:H7 to tolerate the increased osmolarity of the salt containing growth media. Increasing the osmolarity of the

growth media causes reduction of cell volume and contraction of the bacterial cell membrane which may obstruct LF binding sites there. This conclusion is supported by the reduced activity of LF in LB containing 0.5% NaCl when SL or SHMP were added. In addition, *E.coli* O157:H7 does not tolerate NaCl well. This may lead to the conclusion that the effect of monovalent cations on the activity of LF is organism dependent and LF may act synergistically or additively with other compounds that can induce bacterial stress. This effect is different from the effect of divalent cations which change the conformational structure of LF and reduce its biofunctionality.

During the present work in an attempt to enhance LF antimicrobial activity against tested organisms, different chelating agents were used. However, the best chelating agent for use with LF to achieve lethal effects depended on the targeted organism. When SHMP was used, the antimicrobial activity of LF against *C.viridans* was enhanced only in APT broth. On the other hand, if *E.coli* O157:H7 was the target, the combination of LF with either SL or SB showed potential for reducing its viability in food systems containing NaCl, at reduced but growth permissive temperature.

In Chapter 4 and 6 the results showed variation in the susceptibility of 10 strains of *E.coli* O157:H7 toward LF. The strain dependent antibacterial activity of LF could be explained by the differing ability of LF to bind to the cell membrane, which seems to be a precondition in order for LF initiate bactericidal effects.

In Chapter 6 the results showed that LF by itself did not affect the growth of meat starter cultures in APT broth under conditions similar to those used in the manufacture of

dry fermented sausages. This could mean that under suitable conditions LF and LAB may be used in combination to control Gram-negative foodborne pathogens and spoilage bacteria cured meats products, which often contain ≥ 2.5% NaCl.

To overcome the effect of divalent cations on the antimicrobial activity of LF against the tested organisms, LF and metal chelating agents were encapsulated using W/O emulsion technology to generate paste-like microcapsules. The results of LF release profiles > 4 °C showed that LF might be used to inhibit pathogens and spoilage bacteria which grow at abuse temperature during refrigerated distribution and storage of food products. However, these paste like emulsions need careful handling and storage, therefore freeze-dried powdered microcapsules were produced using double emulsion ($W_1/O/W_2$) technology.

In Chapter 5 it was shown that when paste-like microcapsules containing LF were incorporated in WPI film, LF was more effective against *C.viridans* than when uncapsulated. Therefore, use of microencapsulated LF in packaging films may protect LF from inactivation and enhance its antimicrobial activity by allowing its release from the film directly onto the surface of packaged food like bologna.

Since increased concentrations of NaCl improved the activity of LF against *E.coli* O157:H7, the inhibitory activity of unencapsulated and microencapsulated LF (both as paste-like and dried powder forms) with or without EDTA and SB were tested in dry fermented sausages. The results in Chapter 6 showed that unencapsulated LF was able to cause a significant reduction in numbers of *E. coli* O157:H7 recoverable from dry sausage after processing. The combined lethality of processing and treatment was ≤ 3

$\log_{10}$ cfu/g. Cell injury in response to LF treatment was apparent (a further one $\log_{10}$ cfu/g reduction), but LF was not capable of generating the required 5 $\log_{10}$ cfu/g reduction in *E. coli* O157:H7 viability.

The results obtained provide new information on factors which limit the antimicrobial effectiveness of LF in broth and cured meats. Improvement in the stability of microcapsules containing LF may enhance its antimicrobial effectiveness. Restoration of temperature-catalyzed release of LF from dried microcapsules by preventing emulsion rupture with a cryoprotectant in the W_1 phase should address the instability issue. Low temperature and low shear stress are key elements that must be controlled during incorporation of emulsions into films to preserve microcapsule integrity. Since the N-terminal of LF is responsible for its lethal effects and can be "concentrated" by pepsin cleavage to form lactoferricin, effort should be made to microencapsulate lactoferricin even though it is more susceptible to cation interference than LF. Nonetheless, the resistance of some strains of *E.coli* O157:H7 to LF will limit its value for control over these organisms in meat and other foods. The development of a new natural antimicrobial hurdle using LF to address post thermal processing contamination of ready-to-eat meat could otherwise be a valuable alternative secondary strategy. The microencapsulation procedure used is novel and the technology may be useful for other natural antimicrobials where stability is also problematic. It is expected that these results will lay the foundation for additional work where natural antimicrobials will be used with or instead of conventional preservatives to improve the shelf-life and safety of perishable foods.

Appendix 1

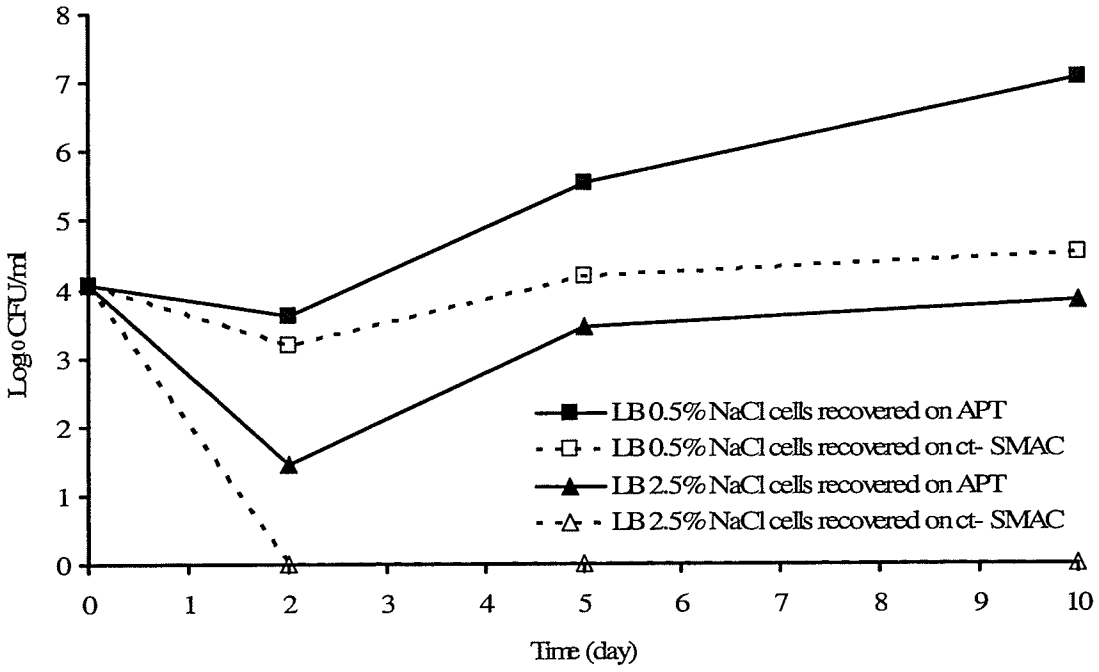

Fig. A1
Total numbers of *E.coli* O157:H7 strain 3081 cells recovered on APT or ct-SMAC after challenged with 32 mg/ml LF at 10 °C. Results shown are the means of two experiments.

Appendix 2

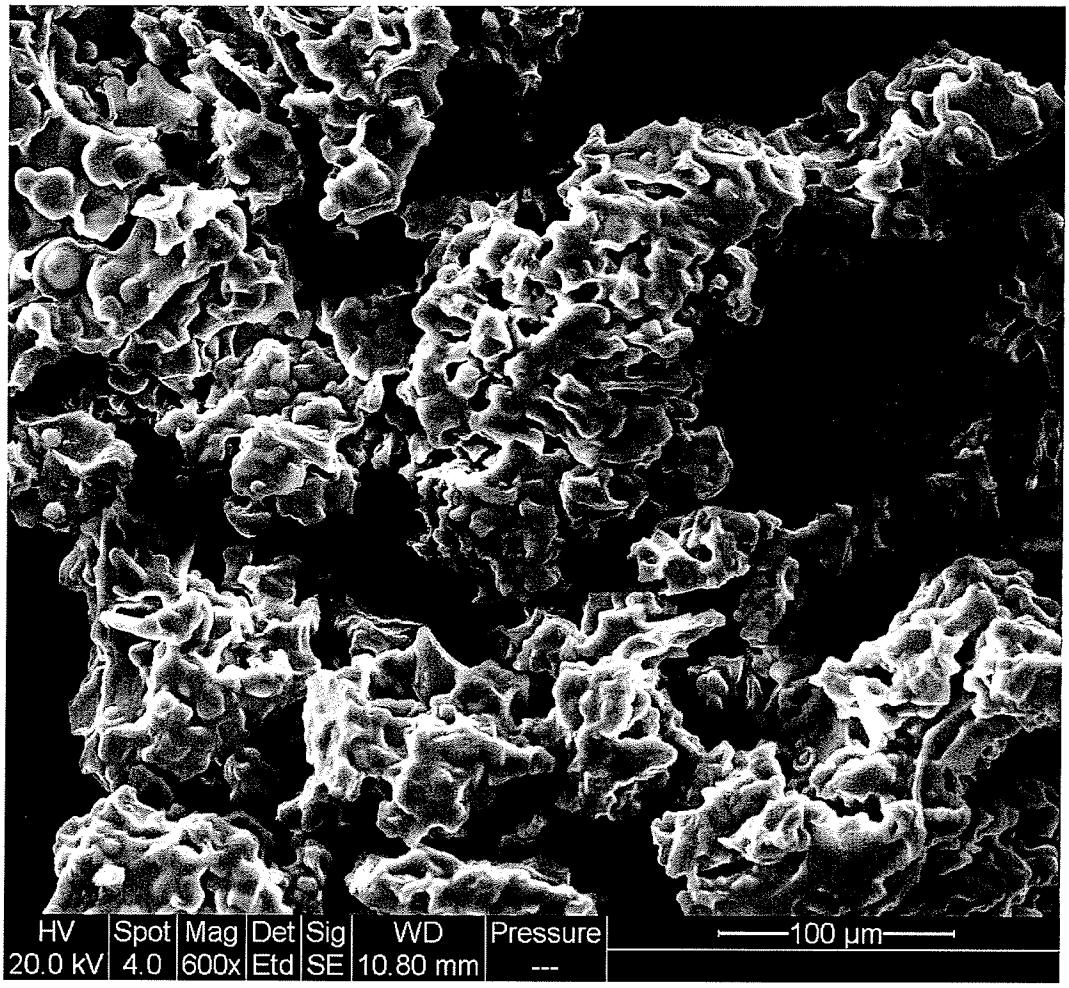

Fig. A2
Scanning electron micrograph showing the appearance of freeze-dried microcapsules at 600 X magnification. Microcapsule morphology and surface structures were assessed using a cold stage scanning electron microscope (FEI, Quanta 200, Hillsboro, OR, USA) at an accelerating voltage of 5 to 15 kV. Microcapsules were mounted on stubs using double-sided sticky carbon tape. Stubs were then coated with a gold-palladium layer (15 nm thick) using a SEM sputter coater (Hummer VII Sputter Coater, Anated Ltd, Alexandria, VA, USA). Electron micrographs were taken at magnifications of 600 X.

Appendix 3
Table A 3.1
Comparison between the effect of 32 mg/ml LF alone or in combination with 20 mM SB, 3% SL, 5 mg/ml SHMP, 4.75% ethanol or 250 μg/ml Quercetin on the viability of *E.coli* O157:H7 (log_{10} CFU/ ml) strain 3081 or LCDC 7110, LCDC 7283, E 318N and CRIFS # 828 in LB broth containing 2.5% NaCl at 10 °C.

	E.coli O157:H7														
	3081			LCDC 7110			LCDC 7283			318N			CRIFS # 826		
Treatments	2d	5d	10d	2d	5d	10d	2d	5d	10d	2d	5d	10d	2d	5d	10d
20 mM SB	1.3	2.67	3.52	0.89	2.67	6.90	1.75	1.30	4.15	2.14	2.71	3.07	3.03	4.72	6.88
20 mM SB+LF	Nv [1]	Nv	Nv	2.04	0.89	2.24	0.00	0.00	0.00	2.63	3.05	4.36	3.81	3.55	3.95
3% SL	2.23	1.38	Nv	1.99	2.78	3.45	1.15	1.00	0.89	3.03	3.32	3.91	3.39	3.62	4.01
3% SL + LF	1.49	Nv	Nv	Nv	Nv	Nv	Nv	Nv	Nv	1.04	1.84	2.60	2.74	2.54	3.47
SHMP (5mg/ml)	2.04	3.35	6.59	3.41	3.82	4.29	1.30	1.68	4.05	3.13	4.51	4.07	3.49	3.91	3.87
SHMP (5mg/ml)+LF	0.65	1.45	2.1	2.57	2.99	3.66	2.25	2.36	3.28	0.80	3.52	3.60	2.90	3.01	3.18
Ethanol	2.17	1.85	Nv	2.73	2.53	2.10	0.80	2.61	2.22	2.34	2.85	2.58	3.15	2.90	2.44
Ethanol+LF	2.17	Nv	Nv	3.15	1.37	2.58	3.10	2.87	2.60	1.30	3.20	2.79	3.27	2.83	2.54
Quercetin	1.87	1.3	Nv	1.41	2.53	1.84	1.69	2.27	1.95	2.17	1.30	2.10	1.75	2.67	1.78
Quercetin and LF	1.84	Nv	Nv	3.07	2.00	Nv	3.04	2.31	Nv	3.30	2.21	Nv	2.84	2.58	Nv

1- No viable cells were detected. Tests showing no growth were transferred (0.1 ml) to 10 ml control medium and monitored 48h at 37 °C for growth.

Appendix 4

Table A 4.1

Recovery of *Lb. curvatus* (log_{10} cfu/g) from dry fermented sausage during processing when challenged with unencapsulated or microencapsulated lactoferrin.

	Processing time (day)								
Treatment[1]	0	1	2	3	6	9	15	21	28
Control	7.43a[2]	7.71a	7.19c	6.72d	6.80e	6.41d	6.46c	6.50d	6.31d
1	7.31a	7.96a	7.79ab	7.88a	7.72ab	7.34b	7.38a	7.19bc	6.71bcd
2[3]	7.40a	7.69a	7.22c	7.32b	7.16cd	6.75c	6.64c	6.93c	6.57cd
3[3]	7.28a	7.41b	7.48bc	7.35b	7.48bc	6.82c	6.96b	7.54a	7.03ab
4[4]	7.26a	7.77a	7.64ab	7.53b	7.43bc	7.53a	7.28a	7.04bc	6.78abc
5[4]	7.27a	7.67a	7.18c	7.02c	7.01de	6.86c	7.04b	7.07bc	6.89abc
6[4]	7.17a	7.47b	7.93a	7.86a	7.92a	7.26b	7.23a	7.23b	7.20a

1- Treatments are described in Table 1.

2- Means from each sampling time in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of 6 observations.

3- Unencapsulated lactoferrin.

4- Microencapsulated lactoferrin

Appendix 5

Table A 5.1

Recovery of *S. carnosus* (log_{10} cfu/g) from dry fermented sausage during processing when challenged with unencapsulated or microencapsulated lactoferrin.

	Processing time (day)								
Treatment[1]	0	1	2	3	6	9	15	21	28
Control	6.75a[2]	7.12a	7.09a	7.02a	6.99a	7.04a	6.58a	6.61a	5.75a
1	6.71a	7.21a	7.01ab	6.95ab	6.75bc	6.61c	5.79b	5.06cd	3.89b
2[3]	6.63a	7.15a	6.94abc	6.93ab	6.78b	6.85ab	6.53a	5.34b	5.86a
3[3]	6.66a	7.16a	7.02ab	6.84bc	6.62cd	5.78d	5.46c	4.95cd	3.99b
4[4]	6.62a	7.07a	6.94bc	6.71d	6.71bc	6.65bc	6.65a	5.31bc	4.03b
5[4]	6.62a	7.05a	6.85cd	6.79cd	6.56de	6.44c	6.72a	5.56de	6.01a
6[4]	6.65a	7.04a	6.74d	6.55e	6.45e	5.93d	5.78b	5.66e	4.02b

1- Treatments are described in Table 1.

2- Means from each sampling time in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of 6 observations.

3- Unencapsulated lactoferrin.

4- Microencapsulated lactoferrin

Appendix 6

Table A 6.1

Total bacterial numbers (log_{10} cfu/g) recovered from dry fermented sausage during processing when challenged with unencapsulated or microencapsulated lactoferrin.

	Processing time (day)								
Treatment[1]	0	1	2	3	6	9	15	21	28
Control	7.56b[2]	7.92b	7.30c	6.69e	6.58e	6.60f	6.43d	6.53d	7.32c
1	7.58ab	8.15a	8.51a	8.42a	8.28a	8.14b	8.39a	8.24a	8.32a
2[3]	7.72a	7.82bc	7.10c	7.03d	6.92d	6.86e	6.60d	6.78cd	6.49e
3[3]	7.60ab	7.54d	7.61b	7.59c	7.48c	7.06d	7.88b	7.63b	8.02 b
4[4]	7.61ab	7.86bc	7.65b	7.55c	7.73b	7.55c	7.37c	7.03c	7.04d
5[4]	7.59ab	7.76bc	7.23c	7.05d	7.06d	6.93de	7.13c	6.88cd	6.73e
6[4]	7.54b	7.72c	8.29a	8.15b	8.23a	8.29a	8.25a	7.75b	8.13ab

1- Treatments are described in Table 1.

2- Means from each sampling time in the same column with the same letters are not significantly different ($p>0.05$). Tabulated values are the means of 6 observations.

3- Unencapsulated lactoferrin.

4- Microencapsulated lactoferrin

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