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Lactoferrin in Bovine Intramammary Infection

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ACADEMIC DISSERTATION

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To Pekka and the Boys

A hidden connection is stronger than obvious one.
Heraclitus

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ABSTRACT

Lactoferrin (Lf) is a bioactive molecule found in milk that is expressed in and secreted by mammary epithelial cells. It is also present in the secondary granules of neutrophils. The concentration of Lf in milk depends on the stage of lactation of the cow and infection status of the mammary gland. Lf has many biological functions that relate to bovine intramammary infections. Lf exerts bacteriostatic activity by binding iron and has bactericidal activity that results from the interaction between Lf and bacterium. Lf regulates the inflammatory response by binding to bacterial endotoxin, which inhibits formation of pro-inflammatory cytokines, or indirectly stimulates the host immune system. Lf inhibits intracellular invasion of pathogens and biofilm formation. It has immunoregulatory functions that affect antibody synthesis, production of various cytokines, and complement activation.

Mastitis is the most costly disease of dairy cows, causing decrease in milk quality and safety and marked economic losses for the industry. In addition, this disease affects animal welfare and is one of the main reasons for culling dairy cows. Mastitis is the most common reason for treating dairy cows with antibiotics, but results of the treatments are often poor. Thus, new approaches for mastitis control are urgently needed.

This thesis focuses on the behavior and effects of Lf in the bovine mammary gland. The studies include *in vivo* experiments, using induced mastitis with endotoxin, and two different udder pathogens, as well as *in vitro* experiments using bovine mammary epithelial (BME) cells. Concentrations of bovine Lf (bLf) and citrate in milk were investigated in experimentally induced endotoxin mastitis using the same cows during early- and late-lactation period. The ability of coagulase-negative staphylococci (CNS) isolated from bovine mastitis to adhere to, and invade and replicate intracellularly, and the related effect of bLf, were studied in a BME cell model. Transgenic cows expressing recombinant human Lf (rhLf) in their milk were used in two experiments in which intramammary infection was induced by a major pathogen *Escherichia coli*, and a minor pathogen, *Staphylococcus chromogenes*. Basal concentrations of bLf and rhLf in the milk of the rhLf-transgenic cows were determined during the first months of the lactation period.

Concentration of bLf in milk was significantly higher during the early lactation period than during the late lactation in experimentally induced endotoxin mastitis. The molar ratio of citrate to bLf before and after challenge was also significantly higher during the early lactation period. The molar ratio is more important than the absolute concentration of either component, and a low molar ratio increases resistance of the mammary gland to invading bacteria, coliforms in

particular. The results of this study may partly explain the susceptibility of dairy cows to intramammary infections during early lactation.

All CNS species examined were able to adhere to BME cells, but internalization varied among the strains. The effect of bLf on the adhesion and invasion of the CNS strains was weak, but it significantly decreased intracellular replication rates. Sequestering of free iron by exogenous bLf could inhibit staphylococcal growth. The effect of bLf was not related to the *in vitro* susceptibility of the CNS strain to bLf.

A new approach to prevention of bovine intramammary infection could be represented by genetic engineering, which was tested here using rhLf-transgenic cows. Two different udder pathogens, *E. coli* and *S. chromogenes*, were selected for induction of experimental mastitis in the transgenic and control cows. The bacterial strains used in the experiments were susceptible *in vitro* to bLf; susceptibility to hLf varied such that the *E. coli* strain was intermediately susceptible and the *S. chromogenes* strain fully susceptible. The response of the cows was monitored by determining concentrations of bLf and rhLf in the milk and concentrations of several indicators of inflammation in the milk and blood, such as somatic cell count (SCC), N-acetyl- β -D-glucosamidase (NAGase) activity and acute phase proteins. Expression of rhLf in the milk did not protect the cows from *E. coli* or staphylococcal intramammary infection. All cows became infected in both models. In the *E. coli* model, all cows developed clinical mastitis, but rhLf-cows showed milder systemic signs and lower concentrations of cortisol and haptoglobin in serum than the controls. rhLf-cows were protected from clinical disease after challenge with staphylococci and had a milder inflammatory reaction, which was recorded as lower NAGase activity and lower concentration of serum amyloid A in the milk.

The results of the investigations reported in this thesis show that one aspect of the innate immunity of the mammary gland is mediated by Lf, which has a multifunctional role. The host response of the cow to intramammary infection depends on the stage of lactation, and on the infectious agent and its virulence. The limited positive effects in the experiments using rhLf-transgenic cows do not warrant further development of this type of genetic engineering to increase mastitis resistance in dairy cows. However, Lf could be of potential benefit if it were able to be used to support the immune defense of the bovine mammary udder.

ORIGINAL ARTICLES

This thesis is based on the following original articles, referred to in the text by their Roman numerals I-V:

- I. Hyvönen P., T. Haarahiltunen, T. Lehtolainen, J. Heikkinen, R. Isomäki and S. Pyörälä. 2010. Concentrations of bovine lactoferrin and citrate in milk during experimental endotoxin mastitis in early- versus late-lactating dairy cows. *Journal of Dairy Research* 77:xx-xx. In press.
- II. Hyvönen P., S. Käyhkö, S. Taponen, A. von Wright and S. Pyörälä. 2009. Effect of bovine lactoferrin on the internalization of coagulase-negative staphylococci into bovine mammary epithelial cells under in-vitro conditions. *Journal of Dairy Research* 76:144-151.
- III. Hyvönen P., L. Suojala, J. Haaranen, A. von Wright and S. Pyörälä. 2006. Human and bovine lactoferrins in the milk of recombinant human lactoferrin-transgenic dairy cows during lactation. *Biotechnology Journal* 1:410-412.
- IV. Hyvönen P., L. Suojala, T. Orro, J. Haaranen, O. Simola, C. Røntved and S. Pyörälä. 2006. Transgenic cows that produce recombinant human lactoferrin in milk are not protected from experimental *Escherichia coli* intramammary infection. *Infection and Immunity* 74: 6206-6212.
- V. Simojoki H., P. Hyvönen, T. Orro and S. Pyörälä. 2010. High concentration of human lactoferrin in the milk of rhLf-transgenic cows relieves signs of bovine experimental *Staphylococcus chromogenes* intramammary infection. *Veterinary Immunology and Immunopathology* 136:265-271.

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ABBREVIATIONS

AFLP	amplified fragment length polymorphism
APC	antigen presenting cell
<i>apo-Lf</i>	iron-free lactoferrin
APP	acute phase protein
ATCC	American Type Culture Collection
Ay	Finnish Ayrshire
bLf	bovine lactoferrin
BME cell	bovine mammary epithelial cell
CFU	colony-forming unit
CMT	California Mastitis Test
CNS	coagulase-negative staphylococci
DELFIA	dissociation-enhanced lanthanide fluoroimmunoassay
ELISA	enzyme-linked immunosorbent assay
FepA	ferric enterobactin receptor A
FecA	ferric citrate receptor A
HF	Holstein Friesian
hLf	human lactoferrin
<i>holo-Lf</i>	iron-bound lactoferrin
Hp	haptoglobin
IL	interleukin
IMI	intramammary infection
LBP	LPS-binding protein
Lf	lactoferrin
Lfcin	lactoferricin
LfH	lactoferrin hydolysate
LfR	lactoferrin receptor
LPS	lipopolysaccharide
MAA	milk amyloid A
NAGase	N-acetyl- β -D-glucosamidase

PMN	polymorphonuclear cell
rhLf	recombinant human lactoferrin
SAA	serum amyloid A
SCC	somatic cell count
SUAM	<i>Str. uberis</i> adhesion molecule
Tf	transferrin
TNF- α	tumor necrosis factor alpha

INTRODUCTION

Defense of the bovine mammary gland against invasion of mastitis-causing bacteria is based on several anatomical, cellular, and soluble protective factors. The efficiency of the defense mechanisms determines whether a new intramammary infection (IMI) develops after the bacteria successfully penetrate the teat opening. During early stages of infection, the activity of resident and newly recruited leucocytes plays a critical role in limiting the infection. There are several types of somatic cells, but in a healthy mammary gland they mainly comprise macrophages. Polymorphonuclear neutrophils (PMN) are the predominant cells in mammary tissues of infected glands, and are secreted during inflammation and are able to ingest pathogens through endocytosis and phagocytosis. Collectively these cells constitute the innate cellular host defense system. Compounds released from pathogens, for instance lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria, stimulate the host cells to produce various compounds, such as antibacterial proteins and peptides, proteases, hydrolytic enzymes and free radicals. Phagocytic cells also release enzymes and pro-inflammatory cytokines, which trigger the humoral and cellular response of acute phase proteins and cytokines.

Lactoferrin (Lf) is one of the proteins present in bovine milk, which plays a role in the innate host defense. Lf is found in all external secretions, including milk, tears and saliva, and also in the secondary granules of PMN. Lf is an iron-binding glycoprotein that is structurally closely related to the iron-transport protein transferrin (Tf) in the plasma. Two features distinguish Lf from Tf; Lf contains a strongly basic region, which is very flexible, and has relatively high isoelectric point as well as being strongly cationic, which is a major factor in the ability of Lf to bind to many different cell types and anionic molecules (Baker and Baker, 2009). The concentration of bovine Lf (bLf) in milk is high in the colostrum (Sanchez et al., 1988) and in udder secretions during dry periods (Welty et al., 1976). During IMI, the concentration of bLf increases, and is dependent of the severity of the infection (Kawai et al., 1999).

In IMIs, Lf is both bacteriostatic and bactericidal (Farnaud and Evans, 2003). Bacteriostatic activity of Lf is based on its ability to sequester iron and thereby prevent growth of bacteria. Coliforms have a high requirement for iron, and are more susceptible to Lf than other mastitis-causing bacteria (Bishop et al., 1976; Nonnecke and Smith, 1984b; Rainard, 1986b; Kuttila et al., 2003b). Bactericidal activity results from a direct interaction between Lf and the bacterium. Ellison et al. (1988) showed that Lf binds directly to the outer membrane of Gram-negative bacteria, causing a rapid release of LPS, with increased membrane permeability and damage to the cell wall. The other antimicrobial mechanisms of Lf may be related to the inhibition of bacterial biofilm formation (Singh et al., 2002) or inhibition of intracellular invasion by blocking bacterial adhesion to host cells (Naidu et al., 1990; Naidu et al., 1991). Lf modulates the immune response by decreasing free radical formation and by down-regulating LPS-induced cytokines

(Britigan et al., 1989; Crouch et al., 1992; Haversen et al., 2002). Other mechanisms of inhibition of LPS-induced release of cytokines are interactions between Lf and the receptor sCD14 and the sCD14-LPS complex, terminating their activating functions (Puddu et al., 2009).

Results have been published on *in vivo* studies on the use of bLf alone or with antimicrobials in the treatment and prevention of bovine mastitis. Intramammary bLf-treatment of *Staphylococcus aureus* mastitis combined with penicillin G was shown to increase cure rate as compared with the antibiotic alone, and had also some effect on mastitis caused by a penicillin-resistant isolate (Diarra et al., 2002a; Kai et al., 2002; Komine et al., 2006; Petitclerc et al., 2007). Kuttila et al. (2004) showed in an experimental mastitis model induced with *Escherichia coli* that intramammarily infused bLf had a LPS neutralizing effect, and suggested bLf as an alternative for treatment of severe coliform mastitis.

This dissertation on Lf in bovine IMI represents a continuation of previous studies investigating possible clinical applications of Lf. The dissertation focuses on the use and effects of Lf in treating bovine IMI. *E. coli* and coagulase-negative staphylococci (CNS) among mastitis-causing bacteria, were selected as target pathogens, representing major and minor udder pathogens. The effects of Lf were studied *in vivo* in experimental mastitis models, as well as *in vitro* using a bovine mammary epithelial (BME) cell model. Dairy cows expressing recombinant human Lf (rhLf) in their milk (van Berkel et al., 2002) were used in experimental infection models to test the feasibility of genetic engineering to increase the host defense against mastitis.

REVIEW OF THE LITERATURE

1 Lactoferrin

Lactoferrin (Lf) is an iron-binding glycoprotein of the transferrin (Tf) family discovered in 1939 as “the red protein from milk” (Sørensen and Sørensen, 1939). Lf was first purified from human and bovine milk in 1960 (Johannson, 1960; Groves, 1965). Transferrins are generally recognized for their roles in iron transport and in nonspecific antimicrobial defense (Bullen et al., 1972; Brock, 1980; Schanbacher et al., 1993). Lf is a multifunctional protein with numerous roles, some of which are clearly related to its iron-binding properties and others that appear to be independent of them (Brock, 2002; Farnaud and Evans, 2003; Baker and Baker, 2005; Legrand et al., 2008). Lf is found in the milk of most mammals, including several rodents (Masson and Heremans, 1971). Lf has also recently been identified in rainbow trout eggs (Gonzalez-Chavez et al., 2009).

Lf is present in a variety of tissues and cell types, and its expression is under different regulatory controls. Lf is expressed and secreted by glandular epithelial cells and found in the secondary granules of PMN. It is present in mucosal secretions including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, and in bile, gastrointestinal fluids, urine, blood plasma, amniotic fluid and milk; high concentrations are found in colostrum (Mason et al., 1966). In the secondary granules of PMN, Lf is found in considerable amounts (15 $\mu\text{g}/10^6$ neutrophils), where it plays a significant physiological role (Gonzalez-Chavez et al., 2009).

1.1 Structure of lactoferrin

Lf is a basic, positively charged protein with an isoelectric point (pI) of 8.4-9.0, which is higher than that associated with other members of the transferrin family (pI 5.4-5.9). The highly basic character is distinctive to Lfs and is extremely important to the iron binding activity and the ability to bind many different cell types and anionic macromolecules (Farnaud and Evans, 2003). Bovine Lf (bLf) is an 80-kDa glycosylated, single chain protein of 708 amino acids with a 19 amino acid signal peptides, and with a high degree of homology among species (Goodman and Schanbacher, 1991). Metz-Boutigue et al. (1984) determined the complete amino acid sequence. The three-dimensional structure of Lf was characterized by X-ray crystallography in order to investigate factors that influence the iron binding and release by Tfs. The amino acid sequence of Lf is known for nine animal species: human, pig, horse, cow, buffalo, sheep, goat, camel and mouse (Baker, 2005). The structure of human Lf (hLf) (Anderson et al., 1987; Anderson et al., 1989), rabbit serum Tf (Bailey et al., 1988), chicken and duck ovotransferrins (Kurokawa et al., 1995; Rawas et al., 1996) and bovine Lf (Figure 1) (Moore et al., 1997) show that these proteins

are folded similarly, and have similar iron-binding sites. Transferrins have an extensive amino acid sequence with similarities ranging from a minimum of 65% to nearly 100%. hLf and bLf share 69% sequence homology and are structurally very similar when viewed at the tertiary level (Pierce et al., 1991). The three-dimensional structure of Lf from five animal species is known: human (Anderson et al., 1989), cow (Moore et al., 1997), buffalo (Karthikeyan et al., 1999), horse (Sharma et al., 1998) and camel (Khan et al., 2001), and all share over 90% sequence identity with each other and represent an extremely closely related group.

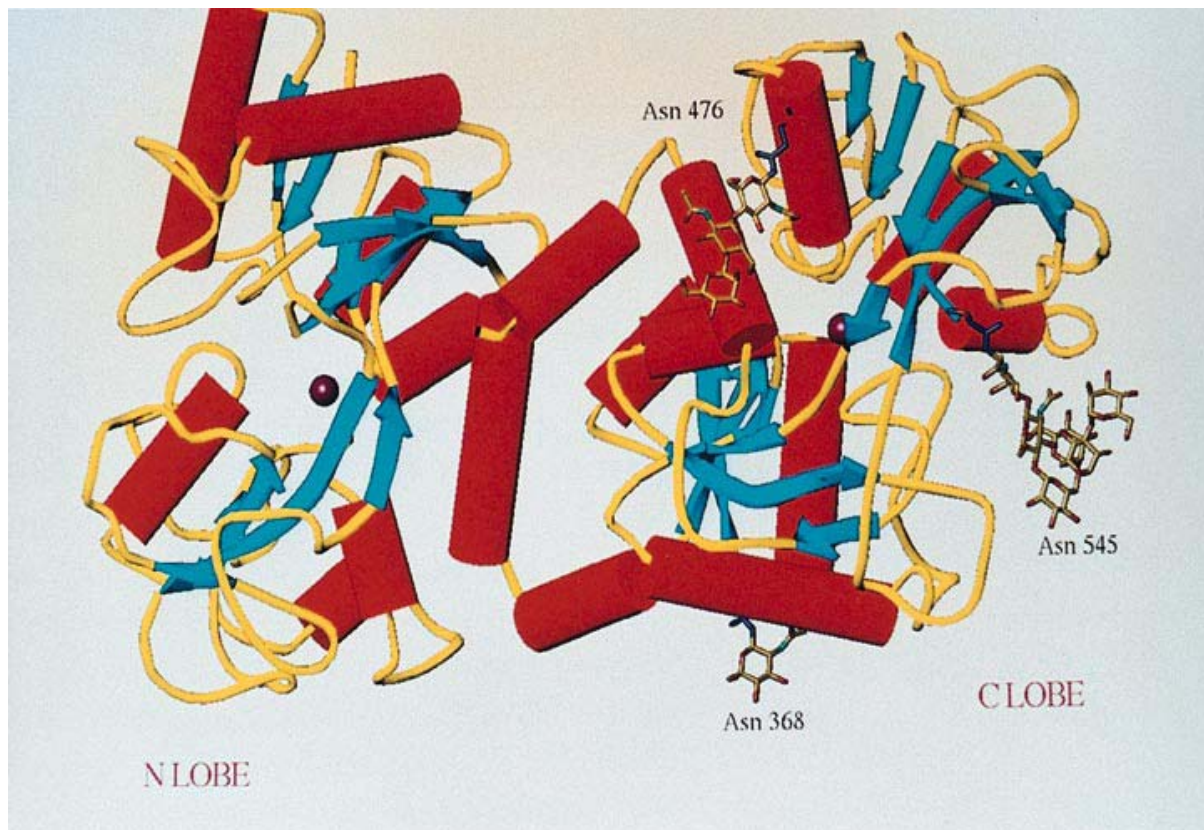


Figure 1. Schematic structure of the bLf molecule with N (left) and C (right) lobes. The β -strands are represented as blue arrows and α -helices as red cylinders. The iron atoms appear as spheres and the three carbohydrate moieties (Asn368, Asn476 and Asn545) attached to the C lobe are presented as bars (Moore et al., 1997, reproduced with the permission of Edward Baker).

The single polypeptide chain is folded into two symmetrical globular lobes representing N- and C-terminal halves of the polypeptide. Both lobes are further subdivided into two domains, N1 and N2, and C1 and C2. These lobes are highly homologous with each other, and are connected by a hinge region between amino acids, containing parts of an α -helix, which provides additional flexibility to the molecule. Lf has two metal binding sites that lie between the two domains in each lobe (Figure 1), and are similar to each other in their capacity to bind reversibly a single ferric iron Fe^{3+} . Four protein ligands and a synergistically bound CO_3^{2-} anion are covalently

bound to the metal ion crosslinking the two domains. This explains the high stability of the structure (Moore et al., 1997).

Despite the similarity in structures among members of the Tf family, considerable differences exist in the surface structure of the proteins, and in antigenicity and cross-reactivity among Lfs (Magnuson et al., 1990). All Lfs are glycosylated, and locations of potential glycosylation sites vary among proteins. The amino acid sequence of bLf has five potential N-glycosylation sites Asn233, Asn281, Asn368, Asn476 and Asn545 (Pierce et al., 1991). The glycan composition of Lf differs among different species such as human, mouse, cow and goat, and chemical studies showed that only four glycan chains are attached, and Asn281 is not used (Spik et al., 1988). Moreover, glycosylation of Lf affects the ability to bind iron (Baker and Baker, 2009). Attachment of carbohydrates to proteins reduces the susceptibility of Lf to proteolysis and thermal denaturation (van Berkel et al., 1996).

Because of reverse iron binding, Lf can exist free of Fe^{3+} (*apo*-Lf) or in association with it (*holo*-Lf) with respectively different three-dimensional conformations (Baker and Baker, 2004). *apo*-Lf has an open conformation while the iron-bound *holo*-form of Lf is a closed molecule, conformationally rigid and very stable; the bound iron is only removed at a low pH or using very strong small-molecule chelating agents. The maximum iron binding capacity of Lf is 1.4 mg/g (Kussendrager, 1993). The metal-free *apo*-form is flexible and more prone to thermal denaturation and proteolysis. The conformational changes of *holo* and *apo* forms of Lf do not affect the molecule surface and thus the surface binding sites for bacterial and viral receptors are unlikely to be affected by the presence or absence of iron ions (Baker and Baker, 2009).

The first helix of Lf forms the major part of the bactericidal domain, described as the lactoferricin (Lfcin) domain, identified by Bellamy et al. (1992b). This bactericidal domain can also serve as a macromolecule binding surface. The strong positive charge at the N-terminal residues, and the adjacent C-terminal end of first helix, form the proposed binding site for bacterial LPS (van Berkel et al., 1997). The properties of Lf that depend on surface features are not necessarily common to Lfs of all species. Lfcin B is released by gastric pepsin cleavage of bLf (Bellamy et al., 1992a) and consists of a positively charged looped peptide with more potent bactericidal and fungicidal activity than the native protein (Bellamy et al., 1993). A second putative antimicrobial domain, lactoferrampin has been identified in the same N1-domain of bLf (van der Kraan et al., 2004). Its antimicrobial activity also differs from that of Lfcin, but physiologically the localization of the peptides in the same N1-domain is important as it allows the protein to bind to the membrane interface and could initiate its bactericidal activity by disturbing membrane integrity (van der Kraan et al., 2004). Lfs of mammals share considerable sequence and structural homology and their functional differences are explained by regional differences in deduced amino acid sequences.

1.2 Functions of lactoferrin

Lf has been ascribed several biological functions (Table 1), including regulation of iron homeostasis, cellular growth and differentiation, host defense against microbial infections, anti-inflammatory activity and protection against cancer development and metastasis (Ward et al., 2005).

Table 1. *In vitro* and *in vivo* studies where different functions of lactoferrin and lactoferricin have been demonstrated

Function		Reference
Antibacterial	Growth inhibition of iron-requiring microorganisms, such as <i>Staphylococcus</i> spp. and <i>Escherichia coli</i> by Lf	Mason et al., 1966; Reiter and Oram, 1967; Reiter et al., 1975
	Bactericidal effect of iron-free hLf	Arnold et al., 1977
	Damage to the outer membrane of Gram-negative bacteria	Ellison et al., 1988
	Iron-regulated Lf-binding outer membrane protein in some bacteria	Schryvers and Morris, 1988
Antiviral	Potent inhibitor effect of bLf in the early phase of human immunodeficiency virus infection (HIV-1)	Harmsen et al., 1995 Berkhout et al., 2004
	Prevented absorption of rotavirus to the target cell by Lf	Superti et al., 1997
	Disturbed entry of poliovirus into target cell by Lf	Marchetti et al., 1999
	Neutralization of HSV-1 and inhibited replication and cell-to-cell spread by hLf	Välilmaa et al., 2009
Antifungal	Fungicidal effect of hLf against <i>Candida albicans</i>	Soukka et al., 1992
	Binding of Lfcin B to <i>C. albicans</i> and lethal effect on them	Bellamy et al., 1993
	Fungicidal effect of hLfcin by forming pores in cell surface of <i>C. albicans</i>	Lupetti et al., 2000
Antiparasitic	Lfcin-B inactivation of the capacity of <i>Toxoplasma gondii</i> to penetrate host cells <i>in vitro</i> and <i>in vivo</i>	Tanaka et al., 1995
	<i>T. gondii</i> has a bLf binding protein	Tanaka et al., 2003
Anti-inflammatory activity	Neutralizing effect of Lf against exogenous pro-inflammatory molecules such as LPS	Miyazawa et al., 1991; Appelmeik et al., 1994

Immunomodulatory activity	Effects of Lf on various immunological functions as a negative feedback mechanism, production of pro-inflammatory cytokines <i>in vivo</i> and <i>in vitro</i> , NK cell activity, complement activation and lymphocyte proliferation	Crouch et al., 1992; Sanchez et al., 1992
Anti-carcinogenic activity	Inhibition of tumor metastasis <i>in vivo</i> by apo-Lf-B and Lfcin-B Induced apoptosis in THP-1 human monocytic tumor cells by bLfcin-B Significant cytotoxic activity of bLfcin-B against three tumor cell lines <i>in vitro</i>	Yoo et al., 1997b Yoo et al., 1997a Eliassen et al., 2002
Enzymatic activity	Proteolytical degradation of the Hap adhesin essential for colonization by Lf	Qiu et al., 1998
Inhibition of biofilm formation	Reduced biofilm formation of <i>S. epidermidis</i> on soft contact lenses Inhibited forming <i>Pseudomonas aeruginosa</i> clusters and biofilms by Lf	Leitch and Willcox, 1999b Singh et al., 2002
Prebiotic effect	Growth promotion of anaerobic bacteria of the genus <i>Bifidobacterium</i> by Lf Accelerated maturation of host defenses against invasive <i>E. coli</i> in nascent small intestine by rhLf and probiotic <i>Lactobacillus</i> GG	Petschow and Talbott, 1991; Petschow et al., 1999 Sherman et al., 2004

The highly cationic character and structural dynamics of the Lf molecule explain many of its proposed roles, and many of its functions depend on the metal-binding capacity and surface properties. The biological activities of Lf depend on the target cells and presence of specific receptors on their surfaces, such as the human Lf receptor (LfR) for iron uptake in the small intestine of infants and the monocyte LfR for the inflammatory response. In addition to small intestinal cells and monocytes, LfRs have been reported to exist in hepatocytes, lymphocytes, platelets, fibroblasts and osteoblastic cells (Suzuki et al., 2005).

Particular domains or peptides, such as Lfcin and the novel peptide lactoferrampin, are responsible for the principal antimicrobial activities (Pierce and Legrand, 2009). Peptides produced by enzymatic hydrolysis of hLf (Bellamy et al., 1992a) and bLf (Hoek et al., 1997) are much more effective in killing bacteria than the intact protein. The peptides showed activity against all bacteria tested (*E. coli*, *Klebsiella*, *Pseudomonas*, *Listeria* and *Staphylococcus*), and bLfcin was about nine-fold as effective as hLfcin (Bellamy et al., 1992a). Nibbering et al. (2001) showed that hLf and peptides derived from its N terminus were effective against infections with antibiotic-resistant *S. aureus* and *K. pneumoniae* and the initial two arginines were essential to this activity.

Studies of Lf gene expression have revealed factors related to Lf production and secretion. The bLf gene sequence and identified polymorphisms account for Lf variants and differences in Lf expression (Teng, 2002). While the host defense activities of Lf were originally attributed to the iron-binding property and the immunomodulatory effects to the ability to form complexes with LPS, recent studies suggest that the roles of Lf in host defense against infection are diverse. Lf directly interacts with antigen presenting cells (APCs) such as monocytes and macrophages, and modulates important aspects of APC. At the molecular level it affects expression of soluble mediators (Puddu et al., 2009). Anti-tumoral and anti-metastatic activities could be attributed to the immunomodulatory potential of Lf and its ability to stimulate cytokine production and to promote apoptosis in cancer cells.

1.2.1 Antibacterial activity of lactoferrin

Reiter and Oram (1967) showed that in common with other body fluids, milk contained some bactericidal and bacteriostatic factors, inhibiting growth of *Bacillus subtilis* and *B. stearothermophilus*. The inhibitor was identified as the iron-binding protein lactotransferrin. The inhibitory activity was reversed by ferrous ions, and therefore the bacteriostatic activity of Lf was considered to be primarily due to the depletion of the iron in the medium to a concentration below that required for bacterial growth (Reiter and Oram, 1967; Oram and Reiter, 1968). Iron is an essential nutrient for the growth of almost all bacteria. Within the body, bacteria are often exposed to conditions of low iron, due to the presence of iron binding proteins Tf and Lf, which are normally only 30-40% saturated with iron. The bacteriostatic form of Lf is either apo-Lf or partly saturated Lf because the fully saturated form has no antimicrobial activity (Bishop et al., 1976; Arnold et al., 1982). The effect of Lf can be reversed by adding iron or sodium citrate (Bishop et al., 1976; Nonnecke and Smith, 1984a; Nonnecke and Smith, 1984b).

Bacteria have developed several mechanisms for obtaining iron. In normal situations iron in the body is protein-bound, rather than free, in order to minimize the generation of unwanted free radicals. In response to iron-limited stress, some bacteria synthesize and secrete iron chelators, termed siderophores that bind iron and are capable of removing iron from Tfs (Bullen et al., 2005). These bacteria usually possess membrane proteins that function as receptors for the iron-siderophore complex. For example, *E. coli* produces the iron chelators enterobactin and aerobactin (Bullen et al., 2005) and expresses outer membrane iron receptors such as ferric enterobactin receptor FepA (Lin et al., 1998) and ferric citrate receptor FecA (Lin et al., 1999). Many bacteria have specific outer membrane receptors that directly bind host iron by binding Lf or Tf, and these receptors are often protein-specific and species-specific (Schryvers and Morris, 1988).

Several studies suggest that Lf kills sensitive organisms by mechanisms distinct from sequestering of iron by a direct interaction with the bacterial cell surface (Arnold et al., 1977; Arnold et al., 1980; Arnold et al., 1981; Arnold et al., 1982; Ellison et al., 1988). This

bactericidal mechanism limits the proliferation and adhesion of microbes or kills them. Lf has a tendency to interact with negatively charged bacterial surface components. Arnold et al. reported a direct interaction of apo-Lf with a variety of microorganisms, resulting in inhibition of bacterial growth. Lf caused release of LPS from the cell wall of Gram-negative bacteria, and resulted in increase in permeability of the outer membrane (Ellison et al., 1988). Ellass-Rochard et al. (Elass-Rochard et al., 1995) showed that Lf binds to LPS, and is capable of modulating the host response to LPS (Elass-Rochard et al., 1998). Lf binds to the lipid A portion to LPS with a high affinity, and can inhibit growth of bacteria or kill them, also decreasing the effect of LPS (Appelmeik et al., 1994). In addition to this antimicrobial effect, Lf has been reported to decrease LPS-induced cytokine release (Crouch et al., 1992).

Lf alone is mainly bacteriostatic, but with other antimicrobial proteins such as lysozyme, has a synergistic effect and is bactericidal for Gram-negative bacteria (Ellison and Giehl, 1991). *E. coli* and many other Gram-negative bacteria have surface receptors, pore-forming proteins (porins), which constitute a permeability barrier against nutrients and antibiotics in the bacteria's outer membrane. Lf recognizes these porins and is able to bind to them (Erdei et al., 1994).

It has been suggested that chronic infections are associated with biofilm formation (Costerton et al., 1999; Melchior et al., 2006; Clutterbuck et al., 2007). bLf has been shown to inhibit biofilm growth of *S. epidermidis* on soft contact lenses *in vitro* (Leitch and Willcox, 1999b). Singh et al. (2002) reported that Lf blocked biofilm development of the opportunistic pathogen *Pseudomonas aeruginosa* at lower concentrations than necessary for killing or growth inhibition. Lf stimulates twitching, a specialized form of surface motility, by chelating iron, inhibiting the formation of clusters and biofilm, which generally requires a higher concentration of iron than needed for growth (Weinberg, 2004). B-Lfcin acts synergistically with the antimicrobial agent to increase antimicrobial efficacy *in vitro* against *S. aureus* and *P. aeruginosa* isolates from ocular infections and *in vivo* in corneal infection (Oo et al., 2010). The mechanisms that contribute to synergistic therapy with b-Lfcin are thought to occur via cell membrane interactions with the bacteria, increasing permeability (Naidu et al., 1993; Vorland et al., 1999). The antibacterial activity of b-Lfcin may be enhanced in the presence of antimicrobial factors, such as lysozyme (Facon and Skura, 1996).

1.2.2 Anti-inflammatory and immunomodulatory activities of lactoferrin

In addition to the antimicrobial properties of Lf, many studies have focused on its ability to modulate the inflammatory process and the overall immune response. A number of *in vivo* studies indicated that Lf regulates the immune system: Zagulski et al. (1989) showed a protective effect of Lf during lethal bacteraemia and Lee et al. (1998) in septic shock of piglets by inhibiting LPS from binding to monocytes. Breton-Gorius et al. (1980) reported that people with congenital or acquired Lf deficiency have recurring infections. The protective effect of Lf may involve an inhibition of production of several pro-inflammatory cytokines, including tumor

necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β) and IL-6 (Crouch et al., 1992; Machnicki et al., 1993; Haversen et al., 2002). The anti-inflammatory activity of Lf in response to LPS challenge may also involve inhibition of proinflammatory cytokine synthesis following Lf translocation to the nucleus, where it prevents NF- κ B activation (Haversen et al., 2002). Lf modulates immune response by decreasing free radical formation (Britigan et al., 1989). The results of Kruzel et al. (2006) suggest the utility of Lf in human allergic inflammatory disorders by decreasing accumulation of eosinophils into airways and preventing development of mucin producing cells in mice. At the cellular level, Lf modulates important aspects of antigen presenting cell biology, including migration and cell activation (Puddu et al., 2009). The anti-inflammatory role of Lf has been demonstrated in the gastrointestinal tract: the administration of Lf can reduce gastritis induced *Helicobacter felis* in mice (Dial et al., 2000) and protect gut mucosal integrity during LPS-induced endotoxemia (Kruzel et al., 2000). According to current studies, Lf can act as a potent anti-inflammatory protein at local sites of inflammation including the respiratory and gastrointestinal tracts (Conneely, 2001).

2 Expression of lactoferrin in the milk

Milk is the major source of Lf. Unlike other proteins found in milk, Lf is secreted in abundance by mammary epithelial cells throughout all phases of lactation, including the non-lactating and involuting stages. Lf is present in milk of many species including human, pig, horse, cow, buffalo, sheep, goat, camel, mouse, elephant and alpaca (Stumpf and Welch, 2004; Baker, 2005; Conesa et al., 2008). Human milk is unusually rich in Lf, in contrast to some species such as rat, rabbit, dog and grey seal, which have low or zero concentrations of Lf in their milk (Masson and Heremans, 1971; Conesa et al., 2008). In dog milk, concentrations as low as <0.05 mg/ml have been reported (Masson and Heremans, 1971). Cross-reactivity among Lfs occurs when proteins share part of their amino acid sequence; antibodies against hLf can recognize Lfs from human, sheep and goat milk, but antisera against bLf did not react with hLf (Shimazaki et al., 1991; Conesa et al., 2008).

The concentration of Lf in milk of different species varies: in human milk it is 1-2 mg/ml, in the milk of mouse, mare, sow and guinea pig it ranges from 0.2 to 2 mg/ml, in milk of cows and goats from 0.02 to 0.35 mg/ml and in milk of rat, rabbit and dog it is <0.05 mg/ml (Masson and Heremans, 1971; Nagasawa et al., 1972; Roberts and Bournsell, 1975; Welty et al., 1976; Smith and Schanbacher, 1977; Davidson and Lonnerdal, 1986). The concentration of Lf in human colostrum is 5-7 mg/ml, and in bovine colostrum approximately 0.8 mg/ml (Masson and Heremans, 1971; Smith et al., 1971; Harmon et al., 1975; Sanchez et al., 1988). The concentration of Lf in human and bovine milk is markedly elevated during mammary involution (Welty et al., 1976; Nonnecke and Smith, 1984a; Schanbacher et al., 1993).

Lf and Tf in milk are generally inversely correlated; species with a high Lf content in their milk often have little or no Tf, and vice versa. Human and bovine milk contain lower concentrations

of Tf compared with Lf: the concentration of Tf in human milk is <0.05 mg/ml and that in bovine milk 0.02-0.2 mg/ml (Bullen et al., 1972).

Normal bovine milk contains 30 to 35 g/l protein. Milk proteins can be divided into caseins, which form about 80% of milk proteins, and whey proteins, representing the remaining 20% (Swaisgood, 1992). Lf in milk is associated with the whey fraction; about 70% of Lf remains in the whey and 30% occurs in a complex with casein (Oram and Reiter, 1968). A total of 0.75% of whey is proteins, which consists of β - and α -lactoglobulins (70-90%), immunoglobulins (10-15%), bovine serum albumin (5-6%) proteose-peptone (10-20%), β -caseins (1-2%) and minor proteins <0.5%. Minor proteins include biologically active proteins, e.g. Lf, and the enzymes lactoperoxidase and lysozyme (Korhonen, 1995). Cow's milk contains about 0.03 mg/ml lactoperoxidase (Kussendrager, 1993) and about 0.003 mg/ml lysozyme (Farkye, 1992).

Sequences of the Lf gene promoter have been cloned for various species including human, mouse and cattle (Johnston et al., 1992; Teng, 1994). The complete structure of the bLf encoding gene and its promoter was determined in 1994 by Seyfert, but information available on the bLf promoter is limited compared with that for human and mouse promoter regions (Seyfert et al., 1994). The bLf gene has been mapped to bovine chromosome 22 (Schwerin et al., 1994). Numerous nucleotide polymorphisms were found in this region both within and across cattle breeds (Daly et al., 2006; Arnould et al., 2009). Lf promoter variants may serve as markers for healthier animals and can be used to improve animal breeding (Daly et al., 2006).

The regulation of Lf in the mammary gland differs substantially among species. The activity of the Lf gene is most sensitive to oestrogen stimulation in the human and mouse reproductive organs such as the mammary gland and uterus (Teng et al., 2002b; Teng, 2006). However, the oestrogen response module is not found in the bLf promoter. Concentrations of Lf in human and bovine mammary secretions and milk during development, lactation and involution of the mammary gland differ substantially, but the pattern of Lf concentration changes is similar; involution initiates increase in Lf concentrations similarly in both species (Lonnerdal et al., 1976; Welty et al., 1976). During mammary development, a mitogen-stimulated region identified within the proximal hLf, bLf and mouse Lf promoters, is important for Lf expression (Seyfert et al., 1994). Bovine mammary cells derived from a developing gland produced significantly higher amounts of Lf than did cells from a lactating gland cultured under the same conditions (Talhok et al., 1998). Wang et al. (2005) found that iron, included a dietary supplement, significantly increased Lf expression in the mammary gland.

The regulation of bLf apparently contrasts with that for other milk proteins, including casein and α -lactalbumin in bovine mammary secretions during development, lactation and involution (Smith and Schanbacher, 1977; Talhok et al., 1998). Molenaar et al. (1996) studied bLf gene expression in developing, ductal, resting and regressing parenchymal epithelium of the mammary gland by examining the amount of mRNA. The level of mRNA varied with the state of activity of the secretory cells, and was inversely related to the expression of α -lactalbumin and α_{s1} -casein during pregnancy, lactation, stasis and involution. During lactation, elevated

expression of bLf occurs in the ducts, compared with the mammary epithelial cells, especially in proximal to the teat (Molenaar et al., 1996).

2.1 Lactoferrin in bovine milk

The bLf concentration of mammary secretions varies according to the stage of mammary development and function (Table 2).

Concentration of bLf decreases within two days of calving, from 1-2 mg/ml in colostrum to only 0.01-0.1 mg/ml throughout the remaining lactation. In the involuting mammary gland, concentration of bLf increases substantially to its maximal concentrations, from about 20 mg/ml to 100 mg/ml (Schanbacher et al., 1993). The increase of Lf concentration begins 2 to 4 days after cessation of regular milking and continues at a rate of 1.15 mg/ml per day during the first 14 to 21 days of involution. The maximum bLf concentration is achieved after 3 to 4 weeks of involution. Consequently, a 100-fold increase in bLf concentration is recorded compared to that in normal milk (Welty et al., 1976).

Colostrum is the first natural food for a newborn calf, and contains several biologically active molecules essential for special functions, including growth factors and antimicrobial factors that provide passive immunity and protect against infections during the first week of life. The antimicrobial activity of colostrum is due mostly to immunoglobulins, but colostrum also contains other antimicrobial factors such as Lf, lysozyme and lactoperoxidase. Tsuji et al. (1990) found two different size isoforms, a and b, of the bLf molecule in colostrum. The content of Lf-b was double that of Lf-a, and the antimicrobial activity against *E. coli* was higher for *apo*-Lf-a than for *apo*-Lf-b. The concentration of bLf-a in colostrum was greater than that in the normal milk, and bLf-a could play an important role in the health of the newborn calf (Tsuji et al., 1990).

In dairy cows, concentration of bLf in the colostrum varies considerably within breed and among individuals: multiparous cows had two to three times as high bLf concentration as primiparous cows. In beef breeds, no significant differences in Lf concentrations of colostrum were found between parities (Tsuji et al., 1990). The concentration of bLf in colostrum of dairy breeds was four times as high as in beef breeds (Gaunt et al., 1980).

In contrast, Hagiwara et al. (2003) found that the concentration of Lf in the milk was significantly related to the age of cows but not to the stage of lactation. However, it tended to be higher during the late lactation period as compared with early or middle lactation period (Hagiwara et al., 2003). According to Cheng et al. (2008), bLf is significantly associated with the stage of lactation and daily milk production, but not with parity. Król et al. (2010) reported that a breed had significant effect on a content of lactoferrin, and milk obtained from Simmental and Jersey cows proved to be excellent source of bLf.

Table 2. Concentration of bLf in colostrum, milk and dry period-secretion of a normal, healthy mammary gland

Type of mammary secretion	bLf mg/ml	Reference
Colostrum	1-5 ¹⁾	Smith and Schanbacher, 1977
	0.34 ²⁾	Gaunt et al., 1980
	1.8 ¹⁾	Nonnecke and Smith, 1984a
	0.83 ¹⁾	Sanchez et al., 1988
	2 ⁴⁾ , 0.5 ⁵⁾	Tsuji et al., 1990
	0.34 ¹⁾	Yoshida et al., 2000
	0.25 ¹⁾	Puvogel et al., 2005
Normal milk during lactation	0.09	Senft and Klobasa, 1973
	0.35	Harmon et al., 1975
	0.04-0.25	Welty et al., 1976
	0.01-0.35	Smith and Schanbacher, 1977
	0.24	Gaunt et al., 1980
	0.12	Nonnecke and Smith, 1984a
	0.09	Sanchez et al., 1988
	0.17	Kawai et al., 1999
	0.17	Hagiwara et al., 2003
	0.25	Puvogel et al., 2005
	0.19 ³⁾	Soyeurt et al., 2007
	0.14	Arnould et al., 2009
	0.12	Cheng et al., 2008
Involution period (in relation to drying-off)		
- day of drying-off	0.25	Welty et al., 1976
	0.9	Gaunt et al., 1980
	0.76	Nonnecke and Smith, 1984a
	5.3	Kutilla et al., 2003a
	0.4	Puvogel et al., 2005
	0.49	Newman et al., 2009
- 2 to 4 days later	1.5 – 2.8	Nonnecke and Smith, 1984a
	8.09	Kutilla et al., 2003a
- one week later	0.4 – 1.1	Bushe and Oliver, 1987
	13.5	Nonnecke and Smith, 1984a
- 2 weeks later	20-30	Welty et al., 1976
	13.5	Gaunt et al., 1980
	119	Welty et al., 1976
- 3 to 4 weeks later	17	Persson et al., 1992
	48	Puvogel et al., 2005

¹⁾first milking, ²⁾on day 2 after parturition, ³⁾pLf, ⁴⁾dairy breed, ⁵⁾beef breed

Citrate is a normal constituent of milk, and represents one of the main buffer systems regulating the equilibrium between calcium and hydrogen ions (Faulkner and Peaker, 1982; Walstra, 2006).

Bicarbonate facilitates the binding of iron by Lf (Cheng et al., 2008). The ratio between bLf, bicarbonate and citrate varies during the lactation cycle. The antibacterial effect of Lf is most effective in the dry secretion when bicarbonate concentration is at its highest level and the bLf content of the secretion is also high. Citrate is absorbed from the udder to the blood at the same time as bicarbonate diffuses from the blood to the udder. The molar ratio of citrate to iron-free Lf decreases with udder involution and increases with lactogenesis (Kutilla et al., 2003a).

Heritability of Lf in bovine milk has been investigated. According to Gaunt et al. (1980) the heritability for bLf was 0.44, which would be high enough to be used for selection, but is an unreliable indicator given the large standard error, 0.30. In more recent studies (Soyeurt et al., 2007; Arnould et al., 2009) the estimated heritabilities for milk bLf were 0.2.

3 Lactoferrin and intramammary infection

Mastitis is the most important disease of dairy cows, causing the most significant economic losses (Halasa et al., 2007). Mastitis is an inflammation of the mammary gland that is mostly a response to an intramammary bacterial infection (Schalm, 1977). *S. aureus*, streptococci and *E. coli* are respectively among the most prevalent species of Gram-positive and Gram-negative bacteria that cause bovine clinical mastitis. Disease severity depends on the interaction between the host, the environment and the infectious agent. Frequency of new IMI is greatest during early involution, decreases during mid-stages, and then increases prepartum. In mammary involution, the concentrations of antibacterial components, i.e. leucocytes, immunoglobulins, and lactoferrin increase while fluid volume decreases and thereupon the bovine mammary gland resists the IMI (Nickerson, 1989).

The causative agents in bovine clinical mastitis vary among different countries. In the Nordic countries staphylococci are isolated most commonly and less than 20% of clinical mastitis is caused by coliforms (Nevala et al., 2004; Sorensen et al., 2008; Ericsson Unnerstad et al., 2009). In the United Kingdom and Israel, the proportion of coliform bacteria is higher (Shpigel et al., 1998; Bradley and Green, 2001). IMI caused by *E. coli* typically results in acute clinical mastitis. It is mostly self-limiting and infection is eliminated within a few days, but can be associated with severe clinical signs (Burvenich et al., 2003). During the periparturient period and in early lactation the innate immune defense mechanisms of the cows are suppressed, which makes them more susceptible to IMIs (Pyrälä, 2008). During the dry period, the bovine mammary gland is particularly resistant to infections caused by coliform bacteria (Reiter et al., 1975; Cheng et al., 2008).

Coagulase-negative staphylococci have become the most commonly isolated bacteria in bovine IMIs in many countries (Pitkälä et al., 2004; Tenhagen et al., 2006; Bradley et al., 2007; Koivula et al., 2007). The most common CNS species isolated are *S. chromogenes* and *S. simulans* (Taponen et al., 2006). Differences in pathogenicity may exist among CNS species, but very

little is known about the virulence characteristics of CNS (Zhang and Maddox, 2000). CNS have been considered to be minor pathogens, but that term may not apply to all species (Pyorälä and Taponen, 2009). Some CNS can have similar virulence factors as *S. aureus* (Kuroishi et al., 2003; Anaya-Lopez et al., 2006; Oliveira et al., 2006). CNS infection is commonly seen as a moderately increased somatic cell count in the milk and slightly decreased milk production. They can also cause clinical mastitis with mild to moderate clinical signs (Jarp, 1991; Taponen et al., 2006; Taponen et al., 2007; Simojoki et al., 2009). CNS infections may persist in the mammary gland for several months or over the whole lactation period (Aarestrup and Jensen, 1997; Taponen et al., 2006).

Bacterial adherence seems to be an essential first stage for colonization of the mammary gland by pathogens (Frost, 1975). It enables bacteria to evade host defenses and invade deeper regions of the mammary gland (Finlay and Cossart, 1997). Several udder pathogens, for example *S. aureus*, CNS, *E. coli*, and *Streptococcus uberis*, are able to adhere to and induce their internalization by mammary epithelial cells (Frost, 1975). Persistent coliform mastitis may be a consequence of IMI with *E. coli* strains that are able to invade and survive within mammary epithelial cells, avoiding host defenses (Dogan et al., 2006).

3.1 Lactoferrin in milk of cows with intramammary infection

Concentration of bLf in the milk significantly increases during IMI, and the amount of the increase is related to the severity of the disease (Harmon et al., 1975; Harmon et al., 1976; Harmon and Newbould, 1980). Expression of bLf mRNA is relatively low in the lactating gland but increases markedly during IMI (Table 3) (Molenaar et al., 1996; Schmitz et al., 2004; Bruckmaier, 2005).

Zheng et al. (2005) isolated and characterized a fragment of the bLf gene as the promoter region responding to infection. Wellnitz and Kerr (2004) showed that bLf production in a bovine mammary cell culture system was stimulated by infection with *S. aureus* and exposure to LPS. Activity of Lf could be induced by LPS with a dose-dependent pattern in mammary epithelial cells, and the Lf promoter responding to the infection via the NF- κ B pathway (Zheng et al., 2005). This finding provides a potential mechanism of how the Lf gene responds to infection.

The highest concentrations of bLf have been reported in acute coliform mastitis (Kawai et al., 1999; Komine et al., 2005). In clinical mastitis, mean bLf concentration was significantly higher (0.85 mg/ml) than in subclinical mastitis (0.50 mg/ml) (Kawai et al., 1999). Individual variation was high in both groups. The concentration of bLf in milk from quarters infected with *Mycoplasma bovis*, *S. aureus* or streptococci was significantly higher than that from quarters infected with CNS or *Corynebacterium bovis* (Kawai et al., 1999; Hagiwara et al., 2003).

Lf molecules in mastitic secretions from lactating and dry cows differed from those of healthy cows. The *in vitro* iron-binding and antibacterial activity of bLf originating from mastitic secretion was lower than those of bLf from normal milk. Lf molecules from diseased udder showed inflammation-inducing effects such as induction of production of TNF- α and other chemokines, and infiltration of leukocytes (Komine et al., 2005).

Table 3. Mean peak concentration of bLf in milk from cows with spontaneous or experimentally induced intramammary infection caused by different bacteria.

Type of mastitis	bLf mg/ml	Reference
Spontaneous clinical mastitis (Day 3)	1.89 \pm 0.68	Harmon et al., 1975
- Coliform	2.74 \pm 1.41	
- Gram-positive	1.06 \pm 0.35	
Experimental endotoxin mastitis		Harmon and Newbould, 1980
- Day 1	0.20	
- Day 2	1.0	
Spontaneous clinical mastitis	0.85 (0.32-2.27)	Kawai et al., 1999
- <i>S. aureus</i> ¹⁾	0.95	
- <i>E. coli</i> ¹⁾	0.85 ²⁾	
- <i>Str. uberis</i> ¹⁾	0.95	
Spontaneous clinical mastitis¹⁾		Chaneton et al., 2008
- <i>S. aureus</i>	0.21	
- <i>E. coli</i>	0.18	
- <i>Str. uberis</i>	0.29	
Spontaneous subclinical mastitis	0.50 (0.20-1.23)	Kawai et al., 1999
Spontaneous subclinical mastitis	0.51 \pm 0.0025	Hagiwara et al., 2003
- <i>S. aureus</i>	0.65 \pm 0.0019	
- <i>Str. agalactiae</i>	0.80 \pm 0.0017	
- other streptococci	0.61 \pm 0.0025	
- CNS	0.38 \pm 0.0024	
- <i>Corynebacterium bovis</i>	0.24 \pm 0.0036	

¹⁾Concentrations measured from a figure, ²⁾acute *E. coli* mastitis

3.2 Interaction between lactoferrin and mastitis causing bacteria

Coliform bacteria are most susceptible to Lf. Growth of coliform bacteria isolated from mastitis were shown to decrease in media supplemented with more than 1 mg/ml of iron-deprived Lf

(Bishop et al., 1976; Nonnecke and Smith, 1984b; Kutila et al., 2003b). Some isolates of *S. aureus* and *Str. agalactiae* were unaffected (Rainard, 1986b). *S. aureus* is able to grow in an environment with extremely low iron concentration (Trivier and Courcol, 1996) or in the presence of iron chelators such as EDTA (Diarra et al., 2002b). Chaneton et al. (2008) studied the susceptibility of *S. aureus*, *E. coli* and *Str. uberis* isolates from bovine mastitis, and *S. aureus* and *E. coli* strains were demonstrated to be susceptible to bLf (>2 mg/ml), whereas *Str. uberis* strains appeared to be resistant.

Lf-binding proteins have been found in Gram-positive bacteria, for example bLf- and hLf-binding proteins in staphylococci (Naidu et al., 1990; Naidu et al., 1991; Naidu et al., 1992). bLf receptors were distinct from staphylococcal cell surface receptors described for fibronectin and fibrinogen, which may play a role in the adhesion to and colonization of the epithelial surface (Naidu et al., 1991). Naidu et al. (1992) also identified a specific receptor for hLf in *S. aureus*. Lf-binding proteins have been found in *Str. dysgalactiae* subsp. *dysgalactiae* and *Str. agalactiae* isolated from IMI (Park et al., 2002a; Park et al., 2002b). An Lf-binding protein specific for bLf was identified in *Str. uberis* (Fang and Oliver, 1999) and Almeida *et al.* (2006) more recently reported on a novel *Str. uberis* adhesion molecule (SUAM). Lf seems to serve as a bridging molecule between SUAM and Lf receptors located on the surface of mammary epithelial cells, enhancing the internalization of *Str. uberis* into the host cells (Patel et al., 2009a), favoring intracellular survival and persistence of *Str. uberis* (Almeida et al., 2010).

In one *in vitro* study (Diarra et al., 2002a), bLf alone showed only weak inhibitory activity against some strains of *S. aureus*, but when combined with penicillin G a synergistic action was noted. In addition to iron chelation, the bLf-penicillin combination can induce alterations in morphology of *S. aureus*. Addition of iron to the medium had no effect on the inhibition of bacterial growth (Diarra et al., 2002b). Lacasse et al. (2007) showed that Lf and Lfcin reduced β -lactamase activity of *S. aureus* by affecting β -lactamase gene expression. Lf alone did not affect phagocytosis of *S. aureus* by PMN *in vitro*, but in combination with penicillin G it was increased. Invasion of mammary epithelial cells was reduced by Lf alone or in combination with penicillin (Diarra et al., 2003).

4 Practical applications for lactoferrin

4.1 General aspects

Milk proteins have many nutritional, functional and biological functions in humans and animals, and could also be used in practical applications. Lf is one such milk protein. The Lf-derived peptide Lfcin, which is released through proteolysis by pepsin (Korhonen and Pihlanto, 2006), has received much attention recently due to its various roles in host defense (Gifford et al., 2005). bLf is a by-product of the dairy process and as such is readily available. rhLf can be expressed in milk, rice or microorganisms and is also commercially available. Both can be added to foods and different health products (Lonnerdal, 2009). The suggested applications for Lf are

for example from food preservatives to health-promoting foods and supplements, infant foods, iron supplements, pharmaceuticals, sport foods, nutritional foods, chewing gums, healthcare products, such as toothpaste, mouthwashes, and cosmetics (Morinaga Milk Industry CO., Ltd., 2003; Marnila and Korhonen, 2009).

The Food and Drug Administration (FDA) of the United States has assessed Lf and considers it to be generally safe (U.S. Food and Drug Administration, 2001). In the meat industry, Lf can be used as spray applied to carcasses to decrease the growth of contaminant bacteria and to extend the shelf-life (Naidu, 2002). Lf could replace food and feed additives as a preservative, because it is a natural protein of milk. Lf has been used as a supplement in calf feed, to prevent neonatal diarrhea and to increase weight gain (Joslin et al., 2002; Robblee et al., 2003). Calves receiving supplemental bLf in colostrum and milk replacer had fewer days of diarrhea with less serious clinical signs than the control calves. Lf can also stimulate carbohydrate absorption and increase small intestine epithelial cell size (Zhang et al., 2001), resulting a faster growth rate of mice.

4.2 Lactoferrin in the treatment and prevention of bovine mastitis

Kai et al. (2002) compared the efficacy of bLf with antibiotic treatment as a dry-cow treatment in spontaneous staphylococcal mastitis. The cure rate was significantly higher in the bLf group. The locally infused bLf promoted migration of PMNs by inducing the expression of cytokines, and increased the C3 complement in the udder secretion. The authors suggested that bLf could be used as dry-cow therapy to increase cure rates in case of staphylococcal mastitis. In another study, a combination of bLf and cephalosporin was tested as a dry-cow treatment (Komine et al., 2006). The combination therapy inhibited pro-inflammatory cytokine and chemokine production causing an anti-inflammatory effect. Diarra et al. (2002a) showed *in vitro* that bLf acted synergistically with penicillin G and reduced growth of penicillin-sensitive and resistant strains of *S. aureus*. In experimentally induced penicillin-resistant *S. aureus* mastitis, the cure rate was higher for the bLf-penicillin G combination compared with penicillin alone (Petitclerc et al., 2007).

The treatment of spontaneous subclinical mastitis with lactoferrin hydrolysate (LfH) was studied by Kawai et al. (2003). Mastitis was caused by CNS, environmental streptococci, *S. aureus* or *E. coli*. The results of the study suggested that LfH might have therapeutic potential. Kutila et al. (2003b) compared the efficacy of bLf treatment with enrofloxacin in cows with experimentally induced *E. coli* mastitis. No significant differences were found in the outcome from the disease. The concentration of LPS compared with the number of bacteria in milk was significantly lower in Lf than in enrofloxacin-treated cows.

A new approach to prevention and therapy of IMI would be to enhance the immune response by gene manipulation (Bramley, 2002; Maga, 2005; Rainard, 2005; Wheeler, 2007; Laible, 2009). Zhang et al. (2008) used a plasmid-mediated gene transfer technique to enable mammary cells of goats to synthesize and secrete antibacterial peptides including bLfcin. The results showed that

the method enabled secretion of a bioactive form of the antibacterial peptide in the milk, which is a prerequisite for possible use in mastitis prevention.

AIMS OF THE STUDY

The general aim of the work reported in this thesis was to investigate the behavior and effects of lactoferrin in the bovine mammary gland for antibacterial and immunological activity. The studies included *in vitro* experiments with bovine mammary epithelial cells, as well as *in vivo* experiments using induced mastitis models with endotoxin and two different udder pathogens. The specific aims of the studies were as follows:

1. To investigate natural concentrations of bLf and citrate in milk of early- vs. late-lactating dairy cows in an experimental endotoxin mastitis model (I)
2. To investigate the effect of bLf *in vitro* on the internalization of coagulase-negative staphylococci in a mammary epithelial cell model (II)
3. To determine concentrations of human and bovine Lfs in milk of rhLf-transgenic dairy cows during lactation (III)
4. To investigate the clinical course and outcome of experimentally induced mastitis in rhLf-transgenic dairy cows compared with normal dairy cows (IV, V).

MATERIALS AND METHODS

1 Experimentally induced mastitis (I, IV, V)

1.1 Animals

The cows used in the three experiments on induced mastitis are listed in Table 4. The seven Holstein-Friesian (HF) recombinant hLf-transgenic cows were produced and owned by Pharming Group NV, The Netherlands. The transgenic bull was generated through genomic insertion by microinjection of recombinant DNA into the pronucleus of a fertilized oocyte. The rhLf-cows were then produced using an embryo transfer technique (Krimpenfort et al., 1991).

Table 4. Cows used in the three experiments on induced mastitis.

Study	Cows	Parity and lactation state	Udder status
Endotoxin mastitis (I)			
Early lactation	Nine Ay cows	Seven multiparous (two to four lactations) and two primiparous 6th to 15th d of lactation	SCC \leq 150,000/ml
Late lactation	Nine Ay cows	Seven multiparous and two primiparous 137 to 77 day before next parturition	SCC \leq 150,000/ml
Experimental <i>E. coli</i> mastitis (IV)	Seven rhLf-transgenic HF cows Six normal HF cows	Primiparous Median age 39 months Calved 12 days before the trial Primiparous Median age 30 months Calved 18 days before the trial	SCC <100,000/ml SCC <100,000/ml
Experimental CNS mastitis (V)	Six rhLf-transgenic HF cows Six normal HF cows	Primiparous Median age 40 months Calved 4 weeks before the trial Primiparous Median age 30 months Calved 4 weeks before the trial	SCC <100,000/ml SCC <100,000/ml

Seven primiparous, rhLf-transgenic cows were used as experimental animals in studies IV and V, and six normal primiparous HF dairy cows were included as control. Cows were used for experimental intramammary inductions with two different udder pathogens (*E. coli*, CNS). Nine

Finnish Ayrshire (Ay) cows were used in experimental endotoxin mastitis (I) in cross-over model.

The cows were kept in a tie-stall barn and fed according to their energy requirements with good quality hay, silage and concentrated grain. All cows were clinically healthy, had a low somatic cell count (SCC) in their milk (<100,000 cells/ml or $\leq 150,000$ cells/ml) and no bacterial growth in their milk samples before the experiments.

1.2 Clinical observations (I, IV, V)

Cows were examined clinically at each sampling. Clinical status compromised general attitude of the cow, body temperature, rumen function, consistency of udder and milk appearance. Signs were monitored throughout the experiment and were divided into three groups: systemic signs, local signs and milk appearance (Pyörälä et al., 1994). Signs were scored on a three-point scale (1= no signs to 3= severe signs), using also half numbers. The udder was palpated for soreness, swelling, hardness and heat, and the appearance of the milk was assessed visually for clots and flakes, and changes in colour or composition every time the cows were milked. Cows with scores >1 but ≤ 1.5 were recorded as having mild mastitis, those with scores >1.5 but ≤ 2.5 as having moderate mastitis, and those with scores from >2.5 to 3 as having severe mastitis. The milk yield from the infected udder quarter and the total milk yield were measured.

1.3 Ethical considerations

The Ethics Committees of the University of Kuopio (IV, V) and the University of Helsinki (I, IV, V) approved the study protocol and The Board for Gene Technology in Finland approved the use of transgenic animals (III, IV, V).

2 Bacteria (II, IV, V)

E. coli mastitis (IV) was induced as described previously (Pyörälä et al., 1994; Kutila et al., 2004). On average 1700 CFU (range 1500-2300 CFU) of *E. coli* strain FT238 isolated from a cow with clinical mastitis were infused into a single udder quarter of each cow. The bacterial strain was sensitive to bLf *in vitro*, with complete inhibition of growth being achieved at concentrations >1.67 mg/ml (Kutila et al., 2003b). The sensitivity of the strain to hLf *in vitro* was also tested, and the growth was partly inhibited at 1.5 mg/ml.

S. chromogenes strain PAM37 used in experimental CNS mastitis (V) was isolated from bovine clinical mastitis (Taponen et al., 2006). The strain was sensitive to bLf (Kuttila et al., 2003b) and hLf *in vitro*. The inoculate contained approximately 2.1×10^6 CFU in 7 ml saline.

Table 5. *Staphylococcal strains, their origin, clinical signs (scale 1-3) of mastitis of the cows from which the strains were isolated, results of California Mastitis Test (CMT) (scale 1-5) of the milk of the affected quarters, and type of the IMI (persistent/transient) (Taponen et al., 2006; Taponen et al., 2007).*

Strain	Origin	Clinical signs scale 1-3	CMT scale 1-5	Persistent/ transient +/-
CONTROL STRAINS				
<i>S. aureus</i> ATCC 25923, positive control				
<i>S. aureus</i> 298, positive control	clinical mastitis	3	5	
<i>S. epidermidis</i> S808, negative control	cheese			
CNS STRAINS				
<i>S. chromogenes</i> 74	clinical mastitis	1	5	
<i>S. chromogenes</i> PAM 37	clinical mastitis	2	5	
<i>S. simulans</i> 15	clinical mastitis	2	5	
<i>S. chromogenes</i> 117	clinical mastitis	2	5	
<i>S. simulans</i> 2	clinical mastitis	1.5	5	
<i>S. simulans</i> 18	clinical mastitis	1.5	5	
<i>S. simulans</i> 19	clinical mastitis	2	5	
<i>S. chromogenes</i> 22	clinical mastitis	2	5	
<i>S. chromogenes</i> 52	clinical mastitis	2	5	
<i>S. chromogenes</i> 72	clinical mastitis	2	5	
<i>S. epidermidis</i> 221	subclinical mastitis	1	4	+
<i>S. chromogenes</i> 312	subclinical mastitis	1	4	+
<i>S. simulans</i> 198	subclinical mastitis	1	4	+
<i>S. simulans</i> 261	subclinical mastitis	1	4	+
<i>S. chromogenes</i> 93	subclinical mastitis	1	2	+
<i>S. haemolyticus</i> 252	subclinical mastitis	1	2	+
<i>S. cohnii urealyticus</i> 351	subclinical mastitis	1	2	+
<i>S. epidermidis</i> 163	subclinical mastitis	1	1	-
<i>S. haemolyticus</i> 94	subclinical mastitis	1	3	-
<i>S. chromogenes</i> 314	subclinical mastitis	1	1	-
<i>S. chromogenes</i> 98	subclinical mastitis	1	1	-
<i>S. haemolyticus</i> 224	subclinical mastitis	1	1	-

In the internalization assay (II), the 22 strains of CNS (Table 5) used in this study originated from field studies, where the clinical characteristics and outcome from CNS mastitis were investigated (Taponen et al., 2006; Taponen et al., 2007). Ten strains were isolated from clinical mastitis and twelve strains from subclinical mastitis. Of the subclinical samples, seven were from a persistent and five from a transient IMI. Infection was defined as persistent when a staphylococcal isolate with an identical amplified fragment length polymorphism (AFLP) pattern was detected from the same udder quarter in at least three consecutive milk samples taken at intervals of one month.

The strains comprised 10 *S. chromogenes*, 6 *S. simulans*, 3 *S. haemolyticus* and one *S. cohnii urealyticus*. *S. aureus* ATCC 25923, *S. aureus* 298 and *S. epidermidis* S808 were used as control strains in the assay. The positive control strain, *S. aureus* 298, was isolated from milk of a cow with acute clinical IMI (Saari Unit, Faculty of Veterinary Medicine, University of Helsinki) and the negative control strain *S. epidermidis* S808 from cheese (Finnish Food Safety Authority, Helsinki).

3 Endotoxin (I)

In the experimental endotoxin challenge, 100 µg *E. coli* 0111:B4 lipopolysaccharide B (Bacto, Difco Laboratories, Inc., Detroit, MI) diluted into 5 ml of sterile NaCl was infused into the experimental quarter. The same quarter was used at both the challenges during early- and late-lactation.

4 Analytical methods

4.1 Analysis of bovine and human lactoferrin

4.1.1 ELISA (III, IV, V)

Concentration of recombinant hLf was measured by rhLf-specific enzyme-linked immunosorbent assay, ELISA according to the procedure recommended by Pharming. Anti-hLf was absorbed with Sepharoses to remove cross-reacting antibodies (van Berkel et al., 1996). bLf levels were measured using Bovine Lactoferrin ELISA Quantitation KIT (Bethyl Laboratories, Inc. Montgomery, USA). Cross-reactivity of bLf with hLf was tested with bLf and hLf standards (Sigma, St. Louis, USA). The level of detection was 0.008 mg/ml.

4.1.2 DELFIA (I)

In the study I, bLf concentrations were determined using the DELFIA (dissociation-enhanced lanthanide fluoroimmunoassay) (Isomäki, 1999).

4.1.3 Purification of bovine lactoferrin (II)

bLf (Apo-lactoferrin, Biopole SA) was purified according to Karpulus et al. (1987). The concentration of endotoxin was determined with the *Limulus* Amebocyte Lysate assay (BioWhittaker, Walkersville, MD). The LPS concentration before the purification was 4.0 ng/mg and after the purification 0.21 ng/mg.

4.1.4 In vitro susceptibility of CNS strains to bovine lactoferrin (II)

The antibacterial effect of bLf was determined using multichannel turbidometry (Bioscreen instrument, Labsystems, Helsinki, Finland). The final concentrations of bLf (Apo-lactoferrin) in the well of the instrument were 0.1, 0.5, 1.0 and 1.5 mg/ml. The sensitivity was estimated by comparing the growth curve of the strain without bLf with the growth curve of the strain with bLf. The CNS strain was considered sensitive (S) to bLf if the inhibitory effect was <50%, intermediate (I) if it was between 99.9 and 50%, and resistant (R) if it was >100% (the growth of the strain was the same or increased with bLf).

4.2 Analysis of citrate (I)

Citrate concentrations in the milk samples were determined using commercial kit method (Boehringer Mannheim). The molar ratios of citrate and bLf were calculated using the following formula: (average citrate concentration) : (molecular weight of citrate 192 g/mol) / (average Lf concentration) : (molecular weight of Lf 77000 g/mol).

4.3 Internalization assay (II)

A bovine mammary epithelial cell line (BME-UV1) described earlier (Van Oostveldt et al., 2002) was incubated at 37°C in a 5% CO₂ atmosphere. The culture medium was replaced every two to three days. Adhesion, invasion and replication were determined as described by Almeida and Oliver (2001), with some modifications. Analyses were carried out in triplicate for each strain tested including positive and negative controls. Co-cultures were incubated for 30 min (adhesion), 2 h (invasion) and 21 h (replication). Adhesion and invasion, values as percentages, were calculated using the number of adherent/invaded bacteria per original CFU/ml added on monolayers multiplied by one hundred. Replication rate (RR) is the staphylococcal CFU number after 21 h incubation divided by the CFU number of the invaded bacteria.

To test the effect of bLf on the adhesion, invasion and intracellular replication, a series of internalization assays, including bLf (2 mg/ml) in the wells, was also performed.

4.4 Bacterial count (IV, V) and LPS in milk (IV)

Bacterial counts in the milk in the experimentally infected quarters were determined by preparing 10-fold dilution series of the milk in sterile saline. Bacteria were cultured on blood agar at 37°C for 24 hours and counted. The concentrations of LPS in the milk samples at 12 h PC were determined by using the *Limulus* Amebocyte Lysate Test.

4.5 Indicators of inflammation in milk and blood (IV, V)

A modified ELISA method as described by Lehtolainen et al. (2004) was used for the quantification of bovine TNF- α in serum and milk. The detection limit of the ELISA was 0.5 ng/ml for serum and 1.0 ng/ml for milk. Serum cortisol was analyzed using a radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Product Corporation, Helsinki, Finland).

The concentrations of serum amyloid A (SAA) in serum and milk were determined using a commercial ELISA test (Tridelta Development, Wicklow, Ireland). Milk and serum haptoglobin (Hp) concentrations were determined by a method based on the ability of Hp to bind to hemoglobin (Makimura and Suzuki, 1982) and using tetramethylbenzidine as substrate (Alsemgeest et al., 1994). The formed complex was determined photometrically (Labsystem Multiskan MS, LabSystems, Vantaa, Finland).

Milk SCC was measured in Valio Ltd Laboratories, Finland, by a fluoro-optical method using the Fossomatic-instrument (Foss Electric, Hillerød, Denmark). Milk N-acetyl- β -D-glucosamidase (NAGase) activity was measured using the fluorogenic method of Kitchen and co-workers (1978) using a microplate modification developed by Mattila (1985).

4.6 Statistics (I, II, IV, V)

Differences in the concentrations of citrate and lactoferrin and citrate/lactoferrin molar ratios were analyzed using mixed linear model analysis. The cow was considered as a random effect. Interactions between lactation, milking time point and udder quarter were analyzed. All statistical analyses were performed with SPSS 17 for Windows (I).

The correlation of the exposure was analyzed using the Spearman test. For the analysis of the inhibitory effect of bLf on adhesion, invasion or intracellular replication, Student's t-test was used: the data were log transformed and the variations in environmental circumstances were

eliminated using the percentage proportion of the strain with or without bLf. The tests were carried out with SPSS-software 14.0.1. for Windows (II).

In the experimental infections, the effects of time in relation to challenge on the concentrations of measured variables and clinical signs were analyzed statistically using mixed-model ANOVA (IV) (SPSS 11.0, SPSS Inc., Chicago, IL, USA).

Linear random-intercept models were used to compare time trend differences between transgenic and normal animals in milk production data and concentration of SAA in the serum. Differences between the groups were tested with generalized linear mixed models, in which a Poisson distribution was used for the response variables. The cow was included as a random factor. Overall differences in time changes between the groups were tested with an F-test. Logarithmic transformation was used for milk parameters (V).

In all statistical analyses, results were considered as significant when $P < 0.05$.

RESULTS

1 Lactoferrin in endotoxin mastitis in early- vs. late-lactating cows (I)

Intramammary infusion of endotoxin (LPS) resulted in mild to moderate clinical mastitis in both groups, but in the EL group clinical signs were more severe, and concentrations of indicators of inflammation in the milk were greater (Lehtolainen et al., 2003). Before the intramammary endotoxin challenge, the average bLf concentration of the milk was 0.1 mg/ml (EL) and 0.24 mg/ml (LL). The concentration of bLf in the milk of the experimental quarters started to increase at 8 hours post-challenge (PC) and was still significantly higher than the pre-challenge level on the third day. bLf concentrations in the milk greatly varied among cows, and were higher in LL cows than in EL cows during the whole experimental period (Fig. 2a). The average peak concentrations of bLf in the milk were 0.45 mg/ml (EL) and 1.44 mg/ml (LL). Concentrations of bLf in the milk of the control quarters remained unchanged over the whole experimental period.

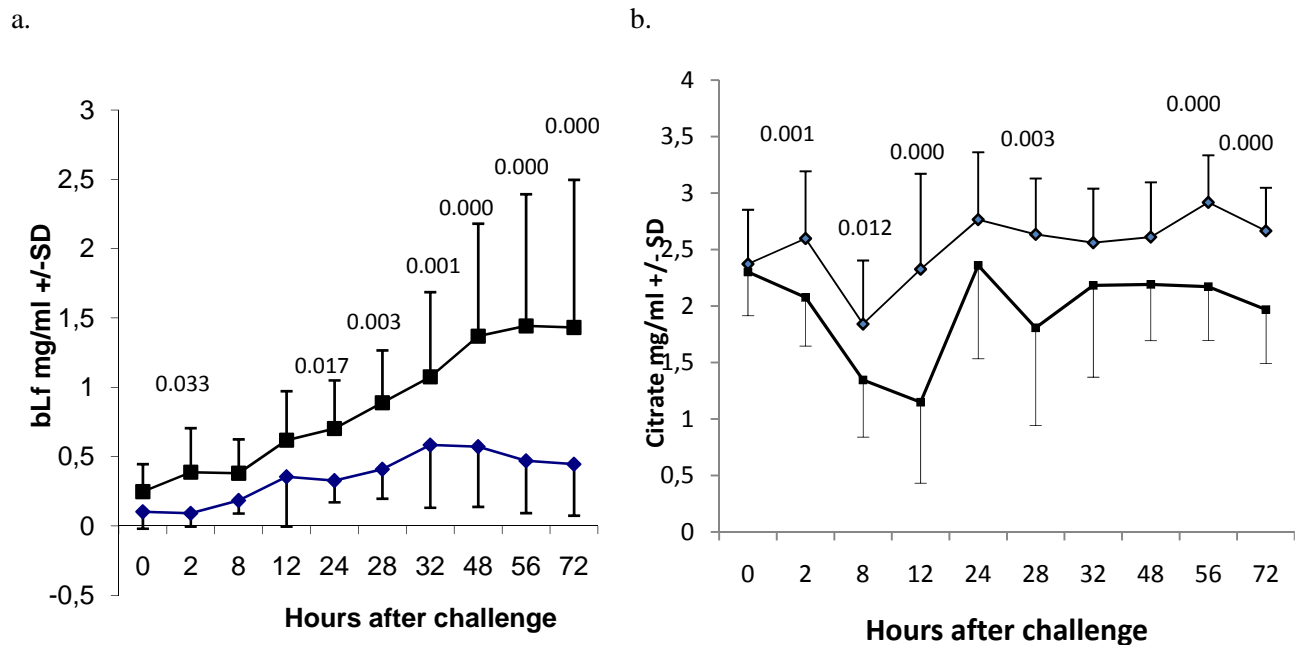


Figure 2. Concentration of a) bLf (mg/ml) and b) citrate (mg/ml) in the milk of udder quarters infused with 100 µg endotoxin (LPS) in early lactating (♦) and late lactating (■) dairy cows (n=9). The significances of the pair-wise comparisons are shown in the figure. Dispersion bars represent SD.

Before challenge, the average citrate concentration was 2.3 mg/ml in the milk of both EL and LL cows. After infusion of endotoxin, concentrations of citrate were higher in the EL cows than in

the LL cows (Fig. 2b), but variation among the cows was high. Citrate concentrations in the milk of the challenged quarters significantly differed between EL and LL groups at 2 h, 8 h, 28 h, 56 h and 72 h PC. Citrate concentrations in the milk of control quarters were higher in EL cows than in LL cows (data not shown). During the first 8-12 h PC, citrate concentrations in the milk of the experimental quarters decreased, but from 24 h PC the differences remained stable.

The molar ratio of citrate to bLf was higher in the milk of the EL cows before the endotoxin challenge. Molar ratios of citrate to bLf in EL and LL cows after endotoxin challenge are shown in Fig. 3. The difference at 2 h PC was statistically highly significant, reflecting the difference prior to challenge.

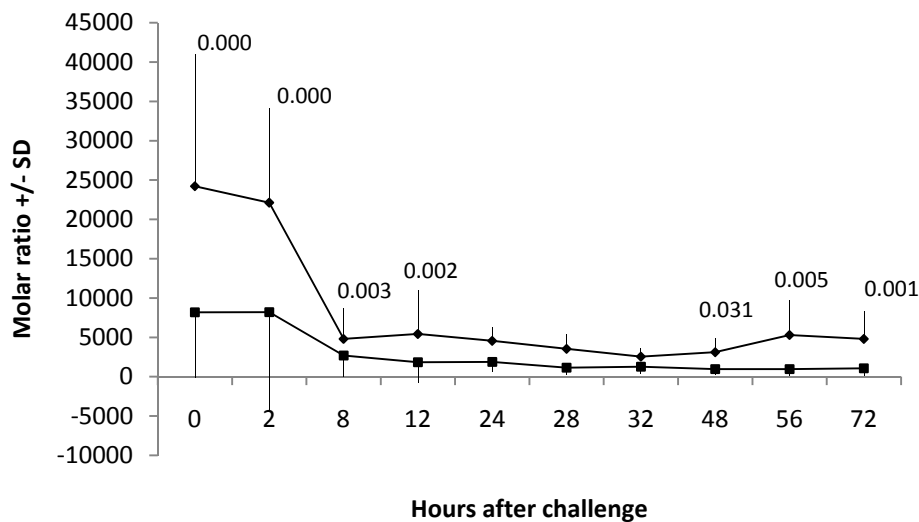


Figure 3. Molar ratios of citrate to bLf in the milk of udder quarters infused with endotoxin (LPS) in early lactating (◆) and late lactating (■) dairy cows (n=9). The significances of the pair-wise comparisons are marked in the figure. Dispersion bars represent SD.

The cross-over model used was valid because no significant differences were found ($P=0.242$) in allocating the cows first to the EL group and then to the LL group and vice versa.

2 Internalization of coagulase-negative staphylococci in the BME cell model (II)

In the BME cell model, all CNS species examined had an adhesive ability equal to that of *S. aureus*, but the internalization capacity varied among staphylococcal strains (Table 6). Adhesion and invasion, as well as the replication rate, were strongly and positively correlated during the exposure. No differences in adhesion, invasion or replication rates were seen between the persistent and transient strains.

Table 6. Adhesion (Adh%), invasion (Inv%), and replication rate (RR) of the staphylococcal strains in bovine mammary epithelial cells BME-UV1 in the absence or presence of bLf (2 mg/ml), and the susceptibility level (S-I-R) of the test strains to bLf.

Strain	Adh%	Adh%	Inv%	Inv%	RR	RR	bLf
		with bLf		with bLf		with bLf	(SIR)*
Control strains							
<i>S. aureus</i> ATCC25923	0.60	0.69	0.52	0.060	86	77	R
<i>S. aureus</i> 298	1.45	0.84	1.30	1.50	15717	93733	I
					5		
<i>S. epidermidis</i> S808	0.86	0.39	0.025	0.0042	42	10	S
CNS strains from clinical mastitis							
<i>S. chromogenes</i> PAM 37	0.49	0.56	0.14	0.014	16737	348	I
<i>S. simulans</i> 15	0.12	0.11	0.004	0.022	219	171	I
<i>S. chromogenes</i> 117	0.56	0.26	0.40	0.035	239	465	I
<i>S. simulans</i> 19	0.15	0.18	0.02	0.051	75	49	I
<i>S. chromogenes</i> 22	0.44	0.15	0.054	0.018	621	285	I
<i>S. chromogenes</i> 52	0.6	0.47	0.10	0.008	435	2456	I
<i>S. chromogenes</i> 72	0.09	0.12	0.006	0.011	295	71	I
CNS strains from subclinical mastitis							
<i>Persistent mastitis</i>							
<i>S. epidermidis</i> 221	0.89	0.90	0.019	0.029	113	84	R
<i>S. chromogenes</i> 312	0.52	0.28	0.023	0.032	102	59	I
<i>S. simulans</i> 198	2.2	2.2	0.020	0.033	77	71	I
<i>S. simulans</i> 261	0.19	0.048	0.001	0.001	706	485	I
<i>S. chromogenes</i> 93	0.44	0.61	0.15	0.011	46	46	I
<i>S. haemolyticus</i> 252	0.80	1.8	0.014	0.012	13	2	S
<i>S. cohnii urealyticus</i> 351	0.59	0.53	0.013	0.067	160	103	S
<i>Transient mastitis</i>							
<i>S. epidermidis</i> 163	0.23	0.10	0.004	0.006	1787	393	R
<i>S. haemolyticus</i> 94	3.4	2.6	0.15	0.015	13450	2339	I
<i>S. chromogenes</i> 314	0.89	0.15	0.10	0.005	337	1867	I
<i>S. chromogenes</i> 98	1.1	1.2	0.17	0.012	10	9	S
<i>S. haemolyticus</i> 224	3.6	1.7	0.06	0.0026	9	100	I

*Sensitivity of the strains to bLf as SIR-classification: S=Sensitive, I=Intermediate and R=Resistant

2.1 Sensitivity of CNS to bovine lactoferrin

The effect of bLf (1.5 mg/ml) on the growth of CNS strains is shown in Table 6 as a percentage of the growth without bLf. The sensitivity was estimated by comparing the growth curve of the strain with and without bLf. The strain was considered to be sensitive to bLf, if the inhibitory effect was <50%, intermediate if it was between 99.9% and 50%, and resistant if it was >100%. Three CNS strains were sensitive to bLf and the inhibitory effect was intermediate for 17 CNS strains. Two strains were resistant.

2.2 Effect of bovine lactoferrin on the internalization

The antagonist effect of bLf on the adhesion and invasion of CNS strains was weak, but bLf significantly decreased intracellular replication and replication rates of CNS (Table 6). No correlation between the *in vitro* susceptibility of the strain to bLf or internalization among clinical signs of mastitis was recorded.

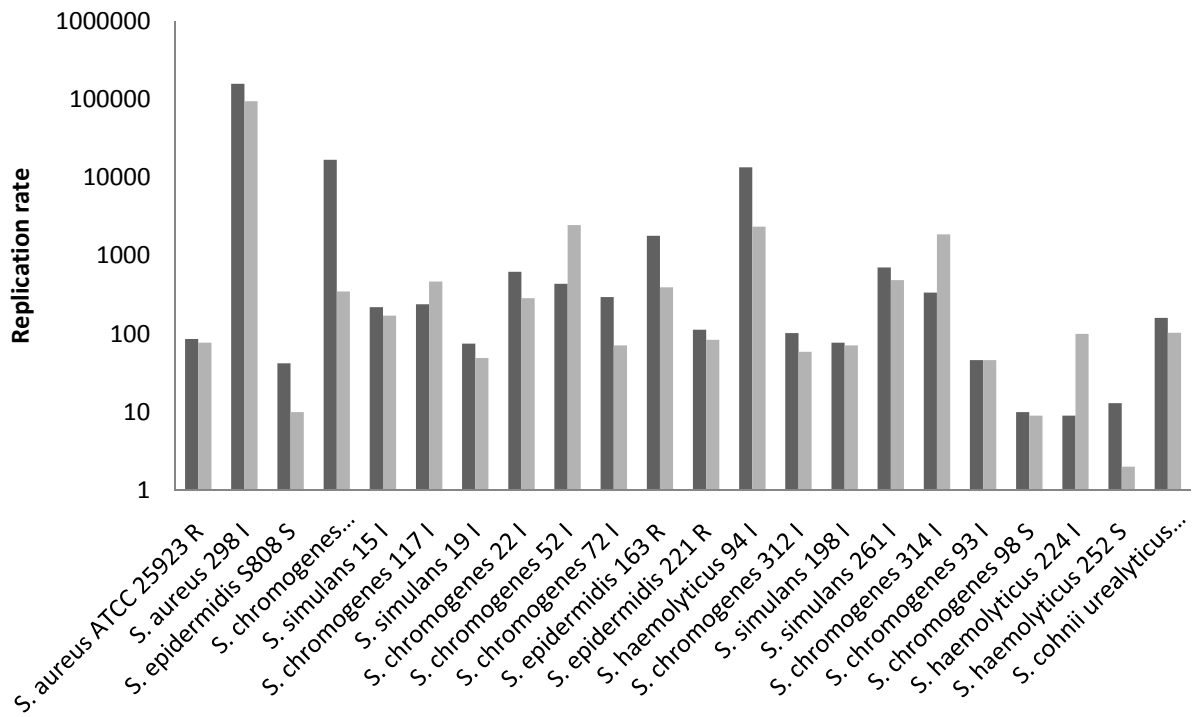


Figure 4. Replication rates of staphylococcal strains in bovine mammary epithelial cells BME-UV1 in the absence (black columns) or presence (gray columns) of bLf (2 mg/ml).

The effect of bLf on adhesion, invasion and intracellular replication was tested with all nineteen CNS strains at a concentration of bLf of 2 mg/ml. bLf did not have a significant effect on the number of adhered bacteria during the exposure. Degree of the adherence established in the

assay did not correlate with the susceptibility of the strain to bLf (Table 6). bLf significantly decreased the intracellular replication ($P=0.013$) and replication rates ($P=0.012$) of the CNS strains.

Susceptibility of the CNS strain to bLf had no significant effect on the intracellular survival of the strain (Fig. 4). For example, *S. aureus* ATCC 25923 resistant to bLf, *S. simulans* 198 party resistant to bLf and *S. cohnii urealyticus* 351 sensitive to bLf had almost equal replication rates with and without bLf in the *in vitro* BME cell assay.

3 Bovine and human lactoferrin (III, IV, V)

The mean bLf and rhLf concentrations of the milk of recombinant hLf-transgenic cows are shown in Figure 5. The level of rhLf was constant (about 2.9 mg/ml) throughout the three-month follow-up period and the differences in rhLf concentrations among the seven cows were not significant (range 2.7 to 3.2 mg/ml). During the first two or three days after calving, rhLf concentrations were slightly lower than later: on average 2.5 mg/ml (from 1.7 to 2.8 mg/ml). The concentration of bLf in colostrum from day 2 was higher, but decreased rapidly during the first days of lactation. The concentration varied among cows (from 0.07 to 0.26 mg/ml). The mean value of bLf during lactation was 0.15 mg/ml (0.01 – 0.45 mg/ml).

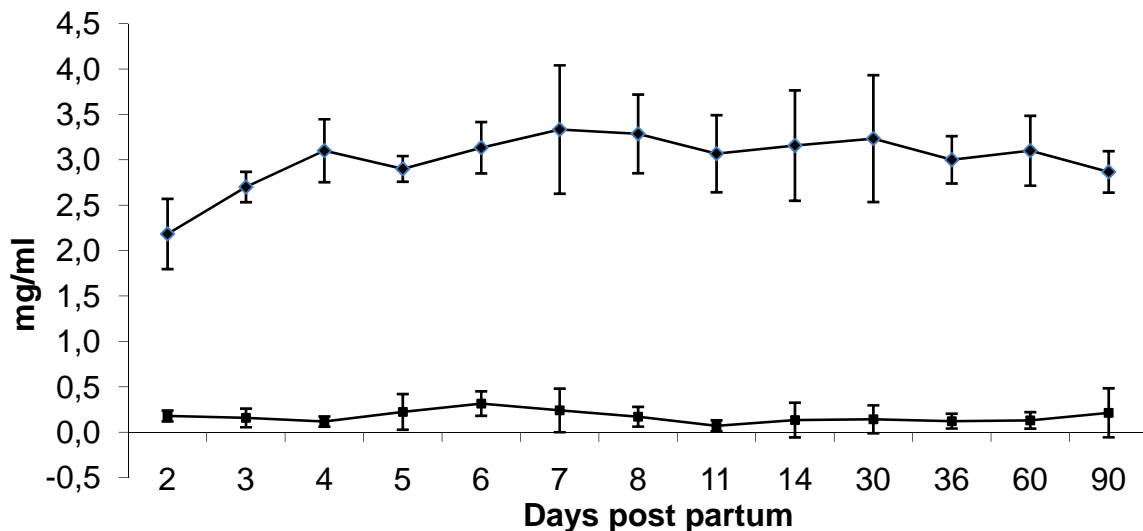


Figure 5. The mean (\pm SEM) concentrations of rhLf (\blacklozenge) and bLf (\blacksquare) (mg/ml) in seven transgenic dairy cows during the first three months of lactation

Total Lf concentrations in the milk of the transgenic and control cows during experimentally induced *E. coli* intramammary infection (IV) are presented in Fig. 6. The mean concentrations of rhLf in the milk ranged from 2.35 to 2.89 mg/ml. No statistically significant differences between concentrations in bLf of the challenged and control quarters were found.

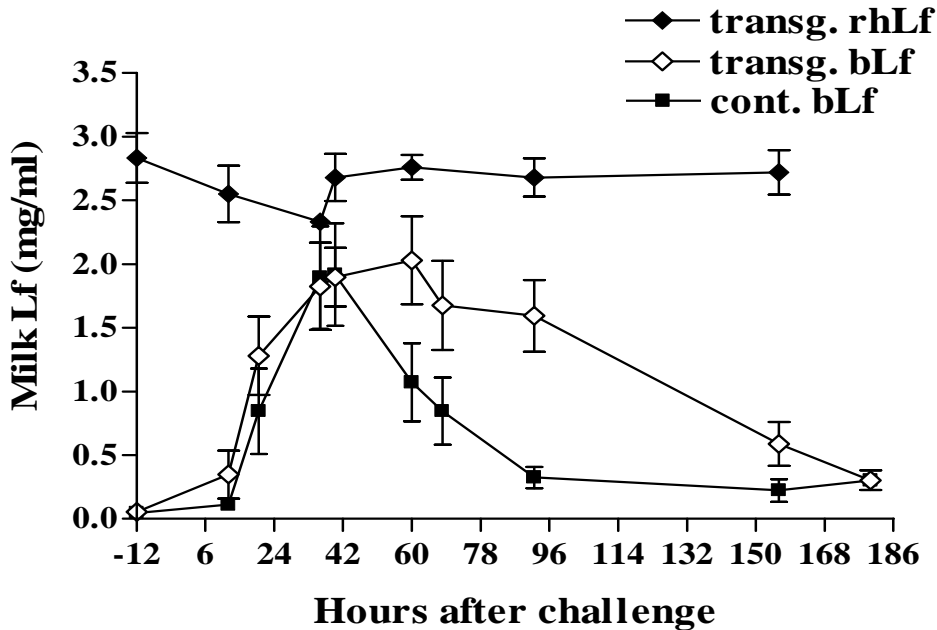


Figure 6. Mean concentrations of rhLf (◆) and bLf (◇) (mg/ml) in milk of challenged quarter of the transgenic cows and bLf (■) (mg/ml) in the control cows after an intramammary infusion of 1700 CFU of *E. coli* into a single udder quarter during the experiment. Data are presented as the means (\pm SEM) of the six transgenic and five control cows.

In the experimentally induced *S. chromogenes* infection model (V), the concentration of bLf in the milk peaked in both groups at 48 h PC; the mean concentration of Lf was 0.27 mg/ml (0.17-0.40 mg/ml) in the transgenic group and 0.36 mg/ml (0.09-0.47 mg/ml) in the control cows. No statistical differences were seen between the groups. The concentration of rhLf in the milk of the transgenic cows remained constant throughout the experimental period (mean 2.6 mg/ml; 2.4-2.8 mg/ml).

The concentrations of bLf in the experimental endotoxin model are shown in chapter 1, Figure 2a.

4 Response of the rhLf-transgenic cows in experimentally induced intramammary infection (IV, V)

In experimentally induced *E. coli* and *S. chromogenes* intramammary infections, all cows in transgenic and control groups became infected and developed mastitis; i.e. expression of recombinant hLf in the milk did not protect the quarters from infection. In general, the clinical response was milder in the transgenic group. In the *E. coli* model, clinical signs became visible 4 hours earlier in the control group ($P=0.006$). In the transgenic group, clinical signs appeared later, within 8-12 hours PC. All transgenic cows and five cows in the control group showed mild to moderate systemic signs; only one cow in the control group exhibited a severe reaction. Mean temperature was lower in the transgenic cows compared with the control cows ($P=0.031$). The transgenic cows suffered significantly less severe systemic clinical signs compared with the control cows ($P=0.020$) and recovered faster ($P=0.008$). Systemic signs in all cows in the transgenic group returned to normal by 24 hours, while the recovery of the control cows lasted over 48 hours. The local signs of the infected quarters and changes in the appearance of milk disappeared within 7 days PC in both groups. No statistically significant differences between the local signs of the groups were found. The daily total milk yield during the experiment did not differ statistically between the groups.

In experimental *S. chromogenes* mastitis, both transgenic and control cows became infected, i.e. hLf present in the milk did not prevent staphylococcal infection. The normal cows showed signs of mild clinical mastitis, but in the transgenic cows, mastitis remained subclinical. Expression of hLf in the milk thus resulted in milder mastitis. None of the cows had a temperature over 39.0°C, and overall, the local signs were scored from 1 to 2. In the control group, slight hardness of the infected quarter and some changes in the milk appearance such as clots in the milk were observed. No differences in quarter milk yield or total milk yield were found between groups. No changes were found in the control quarters at any time points.

In both experiments, bacterial counts in the milk of the challenged quarters peaked in both groups at 8 hours PC. *E. coli* bacteria were eliminated within 3.5 days PC, and no significant differences were observed in the elimination time of bacteria between the transgenic and control cows. In *S. chromogenes* infection, the transgenic cows eliminated bacteria more rapidly ($P<0.05$), and infection remained persistent in one cow in the control group (Fig. 7a).

In *E. coli* and *S. chromogenes* infections, the milk SCC of the challenged quarters increased during the first twelve hours PC, but no significant differences between transgenic and control groups were seen in either of the experimental challenges. In experimental *E. coli* infection, no significant differences were seen in milk NAGase activities between the groups. In *S. chromogenes* infection, NAGase activity of the milk was higher in the control group as

compared with the transgenic group ($P < 0.001$) (Fig. 7b): in the transgenic cows, it remained unchanged throughout the study period.

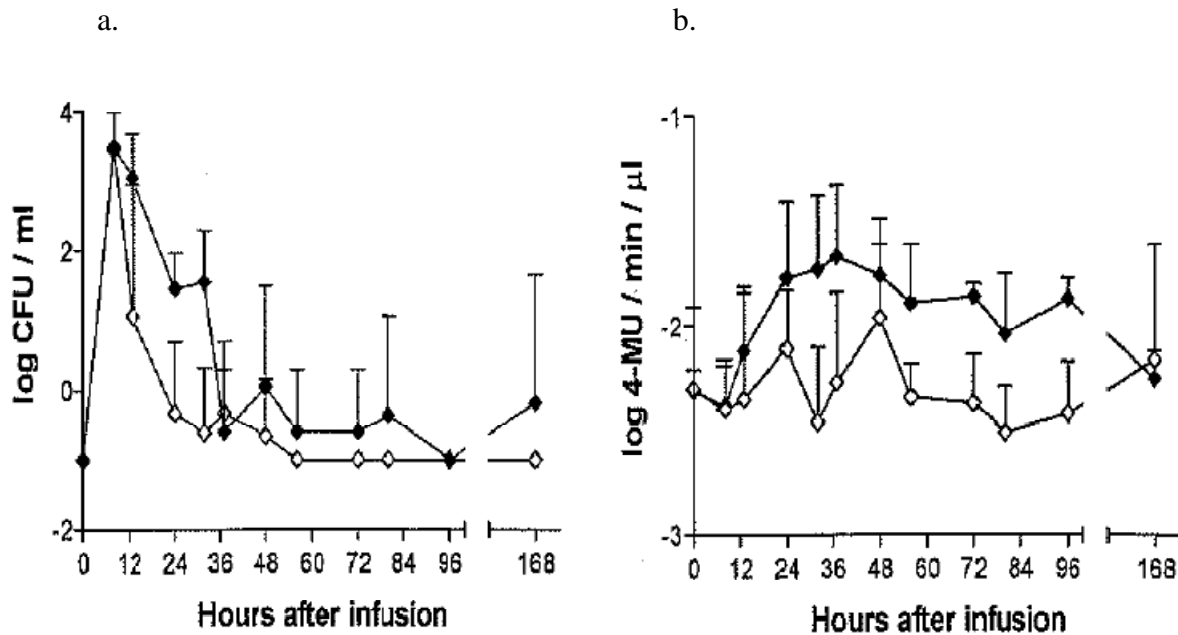


Figure 7. The mean (\pm SEM) concentrations of milk a) bacterial count (log CFU/ml) and b) NAGase activity (MU/min per μ l) of six transgenic cows (\diamond) and five control cows (\blacklozenge) after an intramammary infusion of *S. chromogenes* into a single udder quarter. Statistical significant difference recorded between groups for milk bacterial count and NAGase activity.

The experimental *E. coli* IMI induced both local and systemic TNF- α responses in the cows. Higher TNF- α concentrations were found in the milk of the transgenic group at hours 16 and 20 PC (220.4 ± 50.3 ng/ml) compared with the control group (130.5 ± 24.2 12 PC and ± 14.9 ng/ml 16 PC) but the difference was not significant. In serum, the TNF- α concentration peaked at 12 hours PC, and returned to the baseline level by 24 hours PC. One of the control cows with severe systemic signs had the highest concentration of serum TNF- α at 12 hours PC, but the mean concentrations of serum TNF- α did not statistically differ between groups.

In *E. coli* mastitis, the serum cortisol concentrations of the transgenic group returned to normal by 36 hours, but in the control group they stayed elevated and did not return to baseline levels by 7 days PC (Fig. 8a). Concentrations of the milk SAA and Hp in the milk did not differ statistically between the groups. In both groups serum SAA peaked at 60 hours PC; the maximum concentration was on average 282.4 mg/l in the transgenic group and 376.9 mg/l in the control group during the *E. coli* experiment. The same pattern was recorded for serum Hp concentrations, but the difference was significant at 8 hours and 168 hours PC (Fig. 8b).

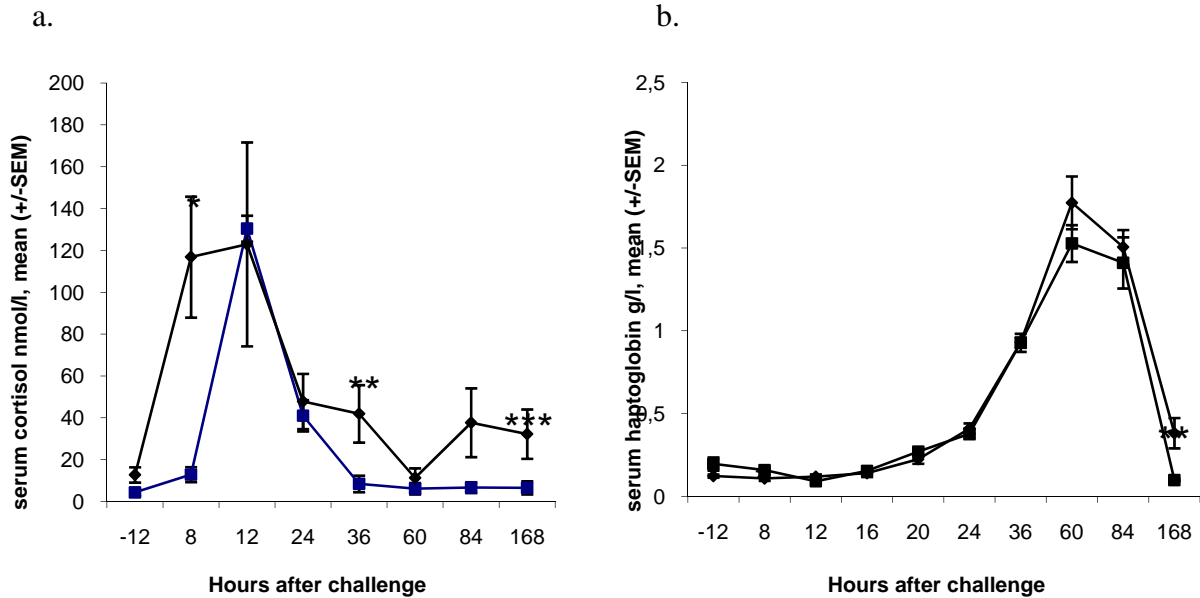


Figure 8. Mean (\pm SEM) serum a) cortisol (nmol/l) and b) Hp (g/l) concentrations in the transgenic (■) and control (◆) cows after an intramammary infusion of 1700 CFU of *E. coli* into a single udder quarter during the experiment. Data are presented as the means \pm SEM of the six transgenic and five control cows. The *P* values in a) were 0.003 (*), 0.031 (**) and 0.048 (***) in cortisol and in b) 0.035 (*) and 0.029 (**) in serum Hp.

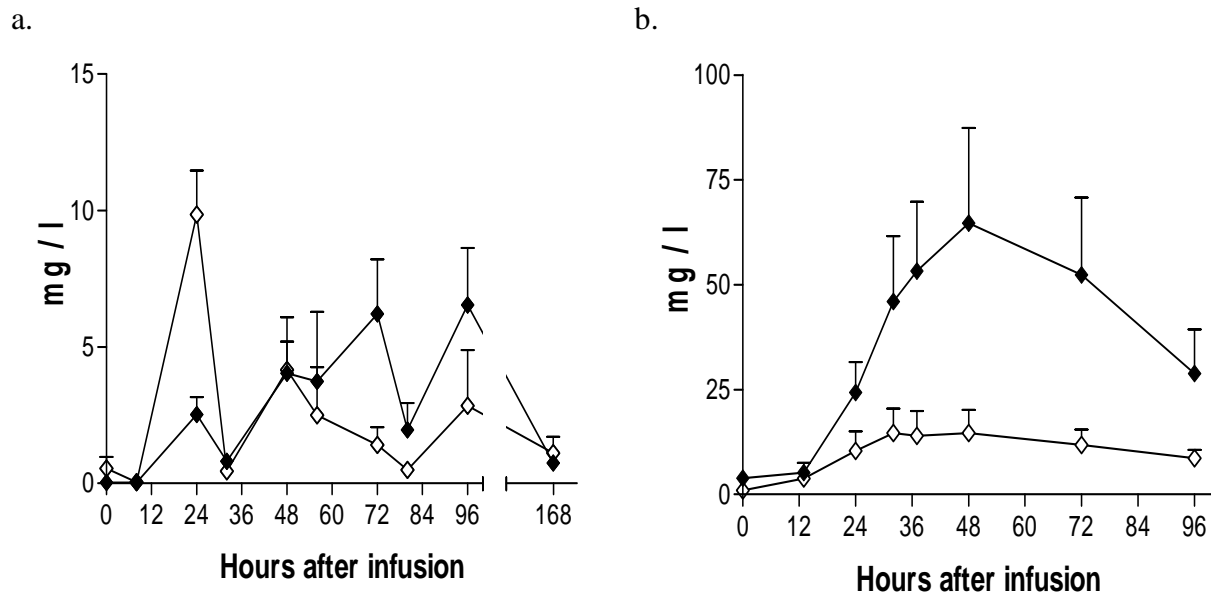


Figure 9. The mean (\pm SEM) concentrations of acute phase protein a) MAA (mg/l) in milk and b) SAA (mg/l) in serum with six transgenic cows (◇) and five control cows (◆) after an intramammary infusion of *S. chromogenes* into a single udder quarter. Statistical difference occurred between groups with MAA.

The concentration of milk amyloid A (MAA) during experimental staphylococcal infection was lower in the rhLf group ($p < 0.01$), but began to increase earlier (Fig. 9a). In the transgenic cows, SAA peaked at 34 h PC and in the control cows at 46 h PC (Fig. 9b). In three cows of the control group, concentration of SAA rose to 90 mg/l, indicating a clear systemic response. The difference between the groups was not statistically significant, probably due to the large variation among the individual cows. Mean SAA was lower throughout the study period and never exceeded 20 mg/l in the experimental group.

DISCUSSION

1 Lactoferrin in bovine intramammary infection

Lf in milk is expressed and secreted by mammary epithelial cells and is found in the secondary granules of neutrophils (Masson et al., 1969). Numerous biological functions are known for Lf that relate to bovine intramammary infections, as described in the literature review included in this thesis. Lf is one of the key elements of the innate immune system of the mammary gland. Concentration of Lf in milk varies according to with lactation period, being high in colostrum and dry-period secretion. Concentration of Lf also depends on the infection status of the mammary gland; the concentration increases during mastitis, varying according to the causal bacteria and the severity of the infection (Harmon et al., 1975; Kawai et al., 1999; Hagiwara et al., 2003; Lee et al., 2004; Chaneton et al., 2008). There is substantial variation on Lf levels among individual cows, as was also demonstrated in our studies.

Previous studies have shown that the susceptibility of bacteria to Lf varies according to bacterial species and strains. Most bacteria require iron for growth, but the iron content of normal mammary milk is low. Iron is largely bound to bLf (Bishop et al., 1976). Consequently, the amount of free iron in normal milk is too low to support the growth of bacteria. During mastitis, in the presence of bicarbonate leaking from blood following increased permeability, Lf sequesters free ferric ions present in milk (Sordillo and Streicher, 2002). bLf in its *apo* form shows marked bacteriostatic activity *in vitro* against mastitic bacteria, the most susceptible species being *E. coli* (Nonnecke and Smith, 1984b; Rainard, 1986a). However, Dionysius et al. (1993) reported that the inhibition of growth of enteric *E. coli* was strain-dependent, and Sanchez and Watts (Sanchez and Watts, 1999) did not report an inhibitory effect of bLf alone on *E. coli* strains isolated from bovine mastitis. Bacteriostatic activity of bLf is not necessarily seen *in vivo*, although *in vitro* the strains were apparently susceptible to Lf. This indicates that mechanisms other than simple iron sequestration may be involved in the antibacterial action of Lf.

2 Behavior of lactoferrin and citrate in endotoxin mastitis (I)

Concentrations and dynamics of bLf and citrate in the milk were studied in the same cows during early and late lactation periods using an endotoxin mastitis model (study I). The concentration of bLf was 0.3 mg/ml at 24 hours PC and 0.5 mg/ml one day later in EL cows, and significantly higher, 0.7 and 1.4 mg/ml, respectively, in the late lactating cows. The response to endotoxin was significantly more severe in EL than in LL cows (Lehtolainen et al., 2003). A more severe response in newly calved cows was also observed in an experimental *E. coli* mastitis model (Hill et al., 1979; Shuster et al., 1996; Mehrzad et al., 2002). In our study, a slightly lower initial

number of blood and milk PMN were recorded in the EL cows, but they increased faster and reached higher levels in EL cows than in LL cows. Neutrophils are the predominant cell type found in mammary tissues and secretions during the early stage of inflammation. bLf is produced by mammary epithelial cells and leukocytes. Besides the iron-binding ability, Lf binds to lipid A of LPS and thus inhibits the effect of endotoxin (Appelmelk et al., 1994; Ellass-Rochard et al., 1995). Here, the concentration of bLf was higher in LL than that in EL cows, despite the higher PMN numbers in the milk of EL cows. In the endotoxin model, inflammation in the quarter is often of short duration and epithelial damage is transient, if it occurs at all. The inflammation was more severe in the EL cows and perhaps the lower concentration of Lf in the milk in them could be due to decreased synthesis of Lf by the epithelial cells. The involution that developed very probably was the basic reason for the higher concentrations of bLf in LL cows. At that time the milk yield also decreased, diminishing the dilution effect of milk on the soluble components. The higher bLf concentration in the milk may have been the major contributor to the milder inflammatory response seen in the LL cows.

In one earlier study of endotoxin-induced mastitis, the concentration of bLf was 0.20 mg/ml before induction and 1.0 mg/ml on the next day, but the period of lactation was not defined (Harmon and Newbould, 1980). Comparison of the concentrations of bLf among various studies differs substantially, as do the methods, sampling times, and lactation periods. Furthermore, analyzing Lf concentration is more difficult in mastitic milk than in normal milk because the consistency of the milk is abnormal and samples need to be diluted, which reduces the accuracy of analysis.

Schmitz et al. (2004) studied short-term changes in mRNA expression of various inflammatory factors and milk proteins, including Lf using a LPS-induced mastitis model. Lf mRNA expression increased within 3 hours after LPS infusion and peaked at 6 hours PC. In a cell culture model, Lf mRNA expression of LPS-treated milk PMNs increased 1-2 hours after the induction (Prgomet et al., 2005). In a study using BME cells, mRNA expression of Lf differed between heat-inactivated *S. aureus* and *E. coli* bacteria; one hour after the challenge expression of Lf mRNA was significantly higher in the *E. coli* infected cells than in the cells infected with *S. aureus* (Griesbeck-Zilch et al., 2008). In our study, concentration of bLf in the milk increased in a linear fashion during the first 12 hours PC, but was lower than record in our *E. coli* infection model (study IV).

In our study, the concentration of citrate in the milk first increased after the endotoxin challenge, then decreased and was at its lowest 8 hours PC in EL cows. Decrease in the concentration of citrate in the milk was more apparent in LL cows, and was at its lowest 12 hours PC. Our results agree with those of a previous study (Guidry et al., 1983) using an endotoxin mastitis model and ten EL or LL cows, in which the concentration of citrate in milk decreased significantly in the EL cows and was at its lowest 7 to 10 hours PC. The results on the effect of mastitis on the

concentrations of citrate are controversial. Sloth et al. (2003) did not establish any differences between cows with healthy or infected quarters. Garnsworthy et al. (2006) came to the conclusion that the variation in concentration of citrate in the milk was not associated with health status of the udder. The concentration of citrate in milk has been found to vary significantly throughout lactation (Banks et al., 1984). The main conclusion to be drawn from our results was that lactation stage affected the trend of decreasing citrate concentration in the milk after endotoxin challenge, but that no large changes in relation to time were noted in either group.

The molar ratio of citrate to Lf is more important than the absolute concentration of either component (Bishop et al., 1976). The molar ratio decreases towards the involution of the udder (Kuttila et al., 2003a) and during mastitis (Bishop et al., 1976), and increases along with lactogenesis (Peaker and Linzell, 1975). In principle, the lower the ratio, the more resistant the udder should be against coliform infections and the faster bacteria should be eliminated from the udder. In our study, before the endotoxin challenge the molar ratio of citrate to bLf in the milk was significantly higher in the EL group than in the LL group because of the higher concentration of bLf in the milk of LL cows. The difference remained significant, but decreased during the first 24 hours PC. The higher molar ratio in newly-calved cows may expose them to IMI, and may be one explanation for the well-documented susceptibility and high proportion of mastitis in cows post partum and during early lactation (Burton and Erskine, 2003; Pyörälä, 2008). A tendency towards higher concentrations of bLf in the milk in late-lactating cows, compared with early or mid-lactating cows, was reported earlier (Hagiwara et al., 2003). In our study, molar ratios among cows varied greatly during the first hours PC, but the concentration of citrate in the milk was always higher in EL cows compared with LL cows. It can be concluded that the concentration of bLf and molar ratio of citrate to bLf in the milk probably affect the susceptibility of the bovine udder to IMI during different lactation periods.

3 The effect of bovine lactoferrin on staphylococcal internalization in the BME cell model (II)

Bacterial infection *in vivo* is a complex process, the first step being an intimate colonization of epithelial cells through non-specific physical interactions, and development of specific ligands to host receptors (Van Belkum et al., 2002). The development of CNS intramammary infection and potential virulence factors of CNS are poorly understood. CNS mastitis is generally mild but can persist in the udder for long periods, and the factors promoting persistence of infection are not known (Aarestrup and Jensen, 1997; Taponen et al., 2006). Several pathogens of the bovine mammary gland are capable of adhering to and inducing their internalization by mammary epithelial cells (Matthews et al., 1994; Almeida et al., 1996; Almeida and Oliver, 2001; Anaya-Lopez et al., 2006; Dogan et al., 2006). Studies on CNS adhesion have focused on bacterial aggregation and binding to biomaterials, as well as on the role of specific adhesins and slime production (Kloos and Bannerman, 1994). Production of capsular polysaccharide adhesin and

slime has been suggested essentially to determine the persistence of bacteria in biofilms in *S. aureus* infection (Cucarella et al., 2004). The interaction between CNS and BME cells was explored in the study of Almeida and Oliver (2001). The results for three different CNS species isolated from the milk of cows with mastitis showed that they were able to adhere to and be internalized in bovine mammary cells.

In our study, the antagonistic effect of bLf on the adhesion and invasion of CNS strains was weak, but bLf significantly decreased intracellular replication and replication rates of CNS. As do all bacteria, staphylococci need iron for growth, and bLf has an ability to bind iron, which is then not available to the bacteria. However, our results showed that the limitation of iron by bLf was not relevant to the adhesion and invasion processes of CNS because the degree of adherence did not correlate with *in vitro* inhibition of bLf during bacterial growth. Diarra et al. (2003) reported that bLf alone, or in combination with penicillin, reduced the invasion of mammary epithelial cells by *S. aureus*, but the mechanism remained unclear. In contrast, the *Str. uberis* adhesion molecule (SUAM) has an affinity for Lf, and bLf serves as a molecular bridge for internalization of *Str. uberis* in BME cells (Patel et al., 2009b). Lf may enhance phagocytosis of *S. aureus* by bovine PMNs and decrease the invasion of BME cells (Diarra et al., 2003). The other possible mechanism suggested by Naidu et al. (1990) is that Lf-binding to staphylococci could interfere with its capacity to adhere to the extracellular matrix protein. Sequestrering of iron by Lf not only inhibits microbial growth but also reduces oxidative stress resulting from inflammation (Kruzel et al., 2006). Kruzel et al. (2007) showed that Lf significantly decreased intracellular reactive oxygen species (ROS) concentrations in a dose-dependent manner. This immunomodulatory function of Lf may explain the decreased intracellular replication of CNS strains.

All CNS species studied here (study II) had an adhesive ability equal to that of *S. aureus* isolated from clinical mastitis, but the capacity for internalization varied among the strains. Biofilm formation is involved in bacterial adherence to host epithelial cells and considered as the most important virulence trait of staphylococci, and essential for the persistence of staphylococcal infection (Aguilar et al., 2001; Cucarella et al., 2004). Oliveira et al. (2006) suggested that biofilm formation might constitute an important selective advantage for bacterial persistence in the udder, but not for the severity of mastitis. According to the present results, CNS strains originating from subclinical mastitis had an enhanced ability to adhere to BME cells in comparison with strains isolated from clinical mastitis. This finding supports the suggestion that bacterial adhesion and biofilm formation are associated with development of subclinical intramammary infection. CNS strains from persistent IMI had a lower adhesion capacity than the strains isolated from transient infections, which does not promote the idea that persistent strains adhere more intensively than transient strains. No differences were found between the persistent and transient CNS strains regarding invasion and replication rates. Our material was limited to only seven persistent and five transient strains, and the variation among strains was high. The

results are thus not conclusive, and more studies are needed to explore the association between persistence of infection and internalization capacity of CNS. Dogan et al. (2006) studied *E. coli* strains from persistent and transient IMI, and suggested that the invasion of mammary epithelial cells and intracellular survival play an important role. Possibly the replication time of 24 hours in our study was too short for representative replication to take place. The pathogenesis of *E. coli* and staphylococcal intramammary infections probably also differs. In addition to bacterial characteristics, the host response may be significant or even decisive in determining the persistence of the intramammary infection. This is, however, an aspect that is very difficult to study with *in vitro* models. Extrapolation of *in vitro* findings directly to *in vivo* circumstances should be done cautiously (Van Belkum et al., 2002). Expression of adhesins is strongly affected by the environment, including the chemical composition of the growth medium. Staphylococci may show stronger adhesion to damaged udder tissue cells than to cultured host cells used in the *in vitro* models. The cultured cells may, for instance, express different receptors to cells in the mammary gland (Cifrian et al., 1994).

4 The effect of expression of rhLf in the milk on experimentally induced intramammary infection (IV, V)

To the best of our knowledge, our studies (IV, V) were the first to explore the possible resistance of rhLf-transgenic dairy cows to mastitis. The high content of Lf in the milk of the transgenic cows was a good starting point to study the possible protective effects of indigenous Lf on the progress of intramammary infection. The concentration of rhLf in milk of the transgenic cows was constant (about 2.9 mg/ml) throughout the first trimester of the lactation period. The differences in rhLf concentrations between the seven cows were not significant. According to van Berkel et al. (2002), in hormone-induced lactation of various transgenic animal lines, rhLf concentrations in the milk were between 0.3 and 2.8 mg/ml. The expression pattern seen here probably reflected the fact that the cows used were of the same transgenic line, resulting in smaller variation. The molecules of hLf and bLf are structurally very similar when viewed at the tertiary level, and they share 69% sequence homology (Pierce et al., 1991). Recombinant and natural hLf are of similar structure and functional properties *in vitro* (van Berkel et al., 2002; Thomassen et al., 2005). However, rhLf glycans may possess specific features that must be taken into account when interpreting results from *in vitro* and *in vivo* experiments (Legrand et al., 1997). For the induced mastitis experiments, *E. coli* and CNS were selected, representing two very different types of mastitic bacteria. They are respectively a major pathogen causing severe disease, and a so-termed minor pathogen.

The concentration of bLf in the milk of the transgenic cows was higher after calving, but decreased rapidly during the first days of lactation. The mean concentration of bLf during the lactation period was 0.15 mg/ml, but the variation among cows was quite high. Before experimental infection, the mean concentrations of bLf in the milk of transgenic and control

cows were equal (0.05 mg/ml). These results agree with those of previous studies on normal cows (Harmon et al., 1975; Welty et al., 1976; Smith and Schanbacher, 1977; Sanchez et al., 1988). Mean rhLf concentrations in the milk were similar in experimentally induced *E. coli* and CNS mastitis. Concentrations of bLf in the milk substantially differed according to the bacterial species, and were almost ten times as high in *E. coli* mastitis as in CNS mastitis. Concentration of bLf in *E. coli* mastitis peaked at 36 to 60 hours PC and the mean maximum concentration was from 1.8 to 2.0 mg/ml. In CNS mastitis, concentration of bLf peaked at 48 hours PC and the mean peak concentration was 0.27 mg/ml.

Both strains of *E. coli* and *S. chromogenes* used in experimentally induced infections were susceptible to bLf *in vitro* (Kuttila et al., 2003b). The *E. coli* strain was intermediately sensitive and the *S. chromogenes* strain completely sensitive to rhLf (1.5 mg/ml) *in vitro*. The CNS strain used, *S. chromogenes* PAM37, was also tested in an internalization assay (study II), and had the highest replication rate and adhesion and invasion percentages among the CNS strains. This strain originates from clinical mastitis with moderate clinical signs and a high milk SCC. The results of the internalization assay revealed the potential virulence of this CNS strain, which was capable of replicating in bovine mammary epithelial cells. The experimental model confirmed the capacity of this strain to induce intramammary infection and in normal cows even clinical mastitis. The results from the two models thus supported each other.

No significant differences were observed in the elimination times of bacteria between the transgenic and normal cows in experimentally induced *E. coli* IMI, but in the CNS infection the transgenic cows eliminated bacteria more rapidly than the control cows. In experimentally induced *S. chromogenes* IMI, the rhLf-transgenic cows showed no clinical signs, unlike the control cows, which developed mild clinical mastitis. As previously stated, *S. chromogenes* was fully sensitive to hLf, which could partly explain the differences in the elimination rates of bacteria. In the *E. coli* model, NAGase activity of the milk was not statistically different between the groups, whereas in the CNS model NAGase activity was lower in the transgenic group. NAGase is released from neutrophils and injured epithelial cells, and NAGase activity of milk reflects the inflammatory reaction and tissue damage in the mammary gland (Kitchen et al., 1978). It is known that Lf inhibits the secretion of some bacterial exoproteins, which cause tissue damage (Diarra et al., 2002b). This could be one potential advantageous effect of indigenous Lf, but the difference was only seen in the CNS infection. Inflammatory reaction in the *E. coli* infection may have been too strong to see any differences in milk NAGase activities, as they were high in both groups.

The antibacterial effect of Lf against Gram-positive bacteria is based on binding to the staphylococcal membrane, decreasing its negative charge and making it more vulnerable to lysozyme, the bactericidal protein present in milk, which then cleaves peptidoglycans from the cell wall (Leitch and Willcox, 1999a). Another mechanism for Lf activity against staphylococci

could be its ability to inhibit biofilm formation, because to form biofilm some bacteria require higher levels of iron than for the vegetative growth phase (Weinberg, 2004). The inhibition of biofilm formation would support the elimination of bacteria by the host, or make it more suitable for antimicrobial treatment (Flanagan and Willcox, 2009).

The systemic inflammatory response in *E. coli* mastitis is mediated mainly by LPS (Carroll et al., 1964; Bannerman et al., 2003), which is the initiating factor of acute phase response (APR) that results in the observed systemic and local signs (Hirvonen et al., 1999; Burvenich et al., 2003). The ability of Lf to bind LPS and down-regulate LPS-induced cytokines has been considered to be one of the immunomodulatory functions of Lf (Legrand et al., 2004). Lf is one of the proteins with high-affinity for LPS, but it does not completely neutralize LPS activity. Lipid A can still be active even after the Lf-LPS-complex has been formed (Na et al., 2004). Our findings of reduced clinical signs in the transgenic animals in the *E. coli* model may indicate that elevated concentration of total Lf inhibited the activity of LPS to some extent, but not completely. The mean concentration of LPS in the milk of cows in the transgenic group was lower than in the milk of those in the control group, but the difference was not significant. Lf competes with LPS-binding protein (LBP) for binding to LPS and might interfere with interaction of LPS and CD14 (Legrand et al., 2005). Lf is also believed to act like LBP during the inflammatory activation of macrophages (Na et al., 2004). The LPS-neutralizing activity of Lf in the milk may depend on the presence and concentration of other LPS-binding proteins, and not solely on the concentration of Lf.

When searching for possible reasons for the failure of the expressed rhLf in the milk to prevent intramammary infections, some issues need to be considered. Komine et al. (2005) found that bLf molecules in mastitic secretions differ from those in milk of healthy cows: Lf originating from healthy secretions had better iron-binding ability and antibacterial activity *in vitro* than Lf from mastitic secretions, but the latter showed inflammation-inducing effects such as induction of pro-inflammatory cytokines and chemokines. These results may explain why the antibacterial effect of Lf *in vitro* is not necessarily similar to that *in vivo*. Expression of rhLf was almost constant during both experimentally induced IMIs, and rhLf molecules apparently did not change like bLf from the mastitic secretions in the cited study. The rhLf gene is under the control of bovine α S1-casein promoter and the expression of rhLf occurs in the mammary gland (Brink et al., 2000). The concentration of α S1-casein decreases in milk during IMI (Schanbacher et al., 1993), but the expression of casein did not decrease significantly during LPS-induced mastitis (Schmitz et al., 2004). Concentrations of bLf and casein in the milk during mammary development, lactation and involution are reciprocal (Schanbacher et al., 1993). Consequently, rhLf did not respond to experimentally induced IMI. bLf is released from the specific granules of neutrophils, and thus the concentration of bLf in milk is also related to the number of neutrophils present in the milk (Hagiwara et al., 2003), and increases depending on the causal agent of IMI.

In studies on experimentally induced LPS and *E. coli* mastitis, the increase of systemic cortisol concentrations in mildly and moderately affected cows was transient (Jackson et al., 1990; Hirvonen et al., 1999), but in the most severely affected cows cortisol concentrations remained elevated for a long time (Hirvonen et al., 1999). In our study, all cows responded to the challenge with increased concentrations of cortisol in the serum, but in the control cows the concentration rose significantly earlier and remained elevated for a longer time than in the transgenic cows, indicating a stronger inflammatory reaction. This may be explained through the binding of rhLf to LPS and the partial inhibition of the effects of LPS.

Lf down-regulates the secretion of pro-inflammatory cytokines that stimulate host response, which is reflected by an increase in the concentrations of acute phase proteins (APP) like SAA, MAA and Hp. Cytokines such as TNF- α and acute phase proteins SAA, MAA and Hp, can be measured from milk and blood, and they reflect the severity of the cow's response (Alsemgeest et al., 1994; Appelmelk et al., 1994; Hirvonen et al., 1999; Blum et al., 2000). In our *E. coli* experiment, SAA and Hp concentrations in serum started to increase shortly after the challenge and remained lower in the transgenic cows than in the control cows. SAA and Hp peaked earlier in the milk than in the serum in both groups. This was probably a result of the rapid local production of these proteins in the mammary gland, as described previously (Hiss et al., 2004). Hp binds harmful molecules such as hemoglobin as well as debris produced after tissue damage (Baumann and Gauldie, 1994), and thus can help restrict the spread of infection by limiting the free iron, and perhaps other nutrients, available for *E. coli*. This is also a function of Lf and Hp may complement the actions of Lf.

A rise in the serum TNF- α level reflects the severity of systemic signs (Hirvonen et al., 1999; Blum et al., 2000). Here, mean peak concentration of TNF- α in the milk was somewhat higher in the transgenic group, but the difference between groups was not significant. Local production of TNF- α in the udder was strong, in particular in the transgenic cows, and higher concentrations of TNF- α than in serum were noted in the milk in both groups. Blum et al. (2000) observed that peak plasma TNF- α concentrations were up to 20-fold lower than maximal concentrations reached in milk in *E. coli* induced mastitis, in agreement with our study. In our study, the serum TNF- α concentration in the transgenic group was almost 100-fold lower and in the control group 30-fold lower than the maximal concentrations in the milk. The differences in the ratios can be explained by the higher TNF- α concentrations in the milk of rhLf-transgenic cows, which showed less severe systemic signs.

In the *S. chromogenes* model, the significantly lower concentrations of MAA in the milk in transgenic cows support observation of reduced tissue damage, which was also reflected in the lower NAGase activities in the milk. The SAA concentrations were remarkably high in three cows of the control group, indicating a clear systemic response that was not seen in the transgenic group. The relevance of the anti-inflammatory effect of Lf is of less clinical

importance in mild CNS mastitis than in more severe *E. coli* mastitis (Burvenich et al., 2004). As regards pathogenesis research, it is perhaps easier to detect possible differences between different treatment groups in a mild model such as CNS infection, where the inflammatory reaction is not so extreme.

The model using rhLf-transgenic cows did not provide protection against *E. coli* or CNS infection, in contrast to the case of experimentally induced *S. aureus* infection in lysostaphin-transgenic cows (Wall et al., 2005). That study, the first using transgenic cows in mastitis research, used three transgenic cows expressing lysostaphin in their milk. Of the udder quarters challenged, only 14% in the transgenic cows became infected, whereas for the non-transgenic cows 71% of the quarters were infected. Increased milk somatic cell counts, elevated body temperatures and induced acute phase proteins were found in all control cows but not in the transgenic cows. On our study with hLf, the only significant effects seen were reductions in the severity of the inflammatory reaction and the faster elimination of bacteria in the staphylococcal model. Lactoferrin has broad and non-specific antimicrobial activity, unlike lysostaphin, that is targeted against staphylococci. Lf would thus have an advantage over lysostaphin as a molecule for use in genetic engineering. Our results on rhLf-transgenic cows hold some promise but are not unequivocal. Only one strain of each bacterial species per time can be tested in experimental mastitis models, and further experiments would be needed for more comprehensive results to be generated. Although even the more successful lysostaphin experiments have not resulted in any breakthroughs in the use of genetic engineering in mastitis control, it could be one means among others to combat mastitis successfully. Further studies in this field are needed.

According to the former and our results the role of Lf in the course of intramammary infection in dairy cows is multifunctional and provides protection against infections. Lf decreased *in vitro* intracellular replication of mastitis-causing bacteria. Low Lf concentrations in milk were associated with increased susceptibility to mastitis in early lactation. Recombinant hLf in milk exhibited some positive effects, such as less severe clinical signs and faster recovery of the cows, milder inflammation and more efficient elimination of bacteria in experimental infections with *E. coli* and CNS.

CONCLUSIONS

1. The concentration of bLf in the milk is low during early lactation, and consequently the molar ratio of citrate to bLf is lower than during late lactation. These indigenous factors may contribute to the greater susceptibility of the udder to intramammary infections during early lactation, particular to infections by coliform bacteria.
2. The antagonistic effect of bLf on the adhesion and internalization of CNS strains in BME cells was weak and strain-dependent, but bLf significantly decreased intracellular replication. All CNS species had an adhesive ability equal to that of *S. aureus*, but the capacity for internalization varied among CNS species and strains.
3. The susceptibility of the staphylococcal strains to bLf *in vitro* was not correlated with the ability of the strains to adhere to, invade or replicate intracellularly in BME cells. However, the limited number of strains tested must be taken into consideration when interpreting the results.
4. The concentration of rhLf in the milk of rhLf-transgenic dairy cows was constant during the first trimester of lactation. It remained at a constant level during the experimentally induced infections with *E. coli* and *S. chromogenes*. The concentration of bLf in the milk of rhLf-transgenic cows was the same as that in the milk of normal dairy cows.
5. The high total concentration of Lf, mostly consisting of recombinant hLf, in the milk of rhLf-transgenic cows, did not protect them from experimentally induced intramammary infections. In experimentally induced *E. coli* infection, the transgenic cows suffered from significantly less severe systemic clinical signs compared with the control cows, and they recovered faster. In experimentally induced *S. chromogenes* intramammary infection, the transgenic cows showed a significantly milder inflammatory response than the normal cows, which was reflected as lower concentrations of indicators of inflammation in the milk. They also eliminated the bacteria more rapidly. These positive but limited effects in the two infection models observed in the rhLf-transgenic dairy cows are not likely to lead to further practical applications of this type of genetic engineering.

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REFERENCES

- Aarestrup, F. M. and N. E. Jensen. 1997. Prevalence and duration of intramammary infection in danish heifers during the peripartum period. *J. Dairy Sci.* 80:307-312.
- Aguilar, B., B. Amorena and M. Iturralde. 2001. Effect of slime on adherence of *Staphylococcus aureus* isolated from bovine and ovine mastitis. *Vet. Microbiol.* 78:183-191.
- Almeida R. A., J. R. Dunlap and S. P. Oliver. 2010. Binding of host factors influences internalization and intracellular trafficking of *Streptococcus uberis* in bovine mammary epithelial cells. *Vet. Med. Int.* 2010:319192.
- Almeida, R. A., D. A. Luther, H. M. Park and S. P. Oliver. 2006. Identification, isolation, and partial characterization of a novel *Streptococcus uberis* adhesion molecule (SUAM). *Vet. Microbiol.* 115:183-191.
- Almeida, R. A., K. R. Matthews, E. Cifrian, A. J. Guidry and S. P. Oliver. 1996. *Staphylococcus aureus* invasion of bovine mammary epithelial cells. *J. Dairy Sci.* 79:1021-1026.
- Almeida, R. A. and S. P. Oliver. 2001. Interaction of coagulase-negative staphylococcus species with bovine mammary epithelial cells. *Microb. Pathog.* 31:205-212.
- Alsemgeest, S. P., H. C. Kalsbeek, T. Wensing, J. P. Koeman, A. M. van Ederen and E. Gruys. 1994. Concentrations of serum amyloid-A (SAA) and haptoglobin (HP) as parameters of inflammatory diseases in cattle. *Vet. Q.* 16:21-23.
- Anaya-Lopez, J. L., O. E. Contreras-Guzman, A. Carabez-Trejo, V. M. Baizabal-Aguirre, J. E. Lopez-Meza, J. J. Valdez-Alarcon and A. Ochoa-Zarzosa. 2006. Invasive potential of bacterial isolates associated with subclinical bovine mastitis. *Res. Vet. Sci.* 81:358-361.
- Anderson, B. F., H. M. Baker, E. J. Dodson, G. E. Norris, S. V. Rumball, J. M. Waters and E. N. Baker. 1987. Structure of human lactoferrin at 3.2-A resolution. *Proc. Natl. Acad. Sci. U. S. A.* 84:1769-1773.
- Anderson, B. F., H. M. Baker, G. E. Norris, D. W. Rice and E. N. Baker. 1989. Structure of human lactoferrin: Crystallographic structure analysis and refinement at 2.8 A resolution. *J. Mol. Biol.* 209:711-734.
- Appelmelk, B. J., Y. Q. An, M. Geerts, B. G. Thijs, H. A. de Boer, D. M. MacLaren, J. de Graaff and J. H. Nuijens. 1994. Lactoferrin is a lipid A-binding protein. *Infect. Immun.* 62:2628-2632.
- Arnold, R. R., M. Brewer and J. J. Gauthier. 1980. Bactericidal activity of human lactoferrin: Sensitivity of a variety of microorganisms. *Infect. Immun.* 28:893-898.
- Arnold, R. R., M. F. Cole and J. R. McGhee. 1977. A bactericidal effect for human lactoferrin. *Science.* 197:263-265.
- Arnold, R. R., J. E. Russell, W. J. Champion, M. Brewer and J. J. Gauthier. 1982. Bactericidal activity of human lactoferrin: Differentiation from the stasis of iron deprivation. *Infect. Immun.* 35:792-799.

- Arnold, R. R., J. E. Russell, W. J. Champion and J. J. Gauthier. 1981. Bactericidal activity of human lactoferrin: Influence of physical conditions and metabolic state of the target microorganism. *Infect. Immun.* 32:655-660.
- Arnould, V. M., H. Soyeurt, N. Gengler, F. G. Colinet, M. V. Georges, C. Bertozzi, D. Portetelle and R. Renaville. 2009. Genetic analysis of lactoferrin content in bovine milk. *J. Dairy Sci.* 92:2151-2158.
- Bailey, S., R. W. Evans, R. C. Garratt, B. Gorinsky, S. Hasnain, C. Horsburgh, H. Jhoti, P. F. Lindley, A. Mydin and R. Sarra. 1988. Molecular structure of serum transferrin at 3.3-Å resolution. *Biochemistry.* 27:5804-5812.
- Baker, E. N. 2005. Lactoferrin: A multi-tasking protein par excellence. *Cell Mol. Life Sci.* 62(22): 2592-30.
- Baker, E. N. and H. M. Baker. 2009. A structural framework for understanding the multifunctional character of lactoferrin. *Biochimie.* 91:3-10.
- Baker, E. N. and H. M. Baker. 2005. Molecular structure, binding properties and dynamics of lactoferrin. *Cell Mol. Life Sci.* 62(22):2592-30.
- Baker, H. M. and E. N. Baker. 2004. Lactoferrin and iron: Structural and dynamic aspects of binding and release. *Biometals.* 17:209-216.
- Banks, W., J. L. Clapperton, A. K. Girdler and W. Steele. 1984. Effect of inclusion of different forms of dietary fatty acid on the yield and composition of cow's milk. *J. Dairy Res.* 51:387-395.
- Bannerman, D. D., M. J. Paape, W. R. Hare and E. J. Sohn. 2003. Increased levels of LPS-binding protein in bovine blood and milk following bacterial lipopolysaccharide challenge. *J. Dairy Sci.* 86:3128-3137.
- Baumann, H. and J. Gauldie. 1994. The acute phase response. *Immunol. Today.* 15:74-80.
- Bellamy, W., M. Takase, H. Wakabayashi, K. Kawase and M. Tomita. 1992a. Antibacterial spectrum of lactoferricin B, a potent bactericidal peptide derived from the N-terminal region of bovine lactoferrin. *J. Appl. Bacteriol.* 73:472-479.
- Bellamy, W., M. Takase, K. Yamauchi, H. Wakabayashi, K. Kawase and M. Tomita. 1992b. Identification of the bactericidal domain of lactoferrin. *Biochim. Biophys. Acta.* 1121:130-136.
- Bellamy, W., H. Wakabayashi, M. Takase, K. Kawase, S. Shimamura and M. Tomita. 1993. Killing of candida albicans by lactoferricin B, a potent antimicrobial peptide derived from the N-terminal region of bovine lactoferrin. *Med. Microbiol. Immunol. (Berl).* 182:97-105.
- Berkhout, B., R. Floris, I. Recio and S. Visser. 2004. The antiviral activity of the milk protein lactoferrin against the human immunodeficiency virus type 1. *Biometals.* 17:291-294.
- Bishop, J. G., F. L. Schanbacher, L. C. Ferguson and K. L. Smith. 1976. In vitro growth inhibition of mastitis-causing coliform bacteria by bovine apo-lactoferrin and reversal of inhibition by citrate and high concentrations of apo-lactoferrin. *Infect. Immun.* 14:911-918.
- Blum, J. W., H. Dosogne, D. Hoeben, F. Vangroenweghe, H. M. Hammon, R. M. Bruckmaier and C. Burvenich. 2000. Tumor necrosis factor-alpha and nitrite/nitrate responses during acute mastitis induced by *Escherichia coli* infection and endotoxin in dairy cows. *Domest. Anim. Endocrinol.* 19:223-235.

- Bradley, A. J. and M. J. Green. 2001. Aetiology of clinical mastitis in six somerset dairy herds. *Vet. Rec.* 148:683-686.
- Bradley, A. J., K. A. Leach, J. E. Breen, L. E. Green and M. J. Green. 2007. Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Vet. Rec.* 160:253-257.
- Bramley, A. J. 2002. Is there a role for transgenics in udder health? Page 3 in National Mastitis Council Annual Meeting Proceedings.
- Breton-Gorius, J., D. Y. Mason, D. Buriot, J. L. Vilde and C. Griscelli. 1980. Lactoferrin deficiency as a consequence of a lack of specific granules in neutrophils from a patient with recurrent infections. Detection by immunoperoxidase staining for lactoferrin and cytochemical electron microscopy. *Am. J. Pathol.* 99:413-428.
- Brink, M. F., M. D. Bishop and F. R. Pieper. 2000. Developing efficient strategies for the generation of transgenic cattle which produce biopharmaceuticals in milk. *Theriogenology.* 53:139-148.
- Britigan, B. E., D. J. Hassett, G. M. Rosen, D. R. Hamill and M. S. Cohen. 1989. Neutrophil degranulation inhibits potential hydroxyl-radical formation. relative impact of myeloperoxidase and lactoferrin release on hydroxyl-radical production by iron-supplemented neutrophils assessed by spin-trapping techniques. *Biochem. J.* 264:447-455.
- Brock, J. H. 2002. The physiology of lactoferrin. *Biochem. Cell Biol.* 80:1-6.
- Brock, J. H. 1980. Lactoferrin in human milk: Its role in iron absorption and protection against enteric infection in the newborn infant. *Arch. Dis. Child.* 55:417-421.
- Bruckmaier, R. M. 2005. Gene expression of factors related to the immune reaction in response to intramammary *Escherichia coli* lipopolysaccharide challenge. *J. Dairy Res.* 72:120-124.
- Bullen, J. J., H. J. Rogers and L. Leigh. 1972. Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br. Med. J.* 1:69-75.
- Bullen, J. J., H. J. Rogers, P. B. Spalding and C. G. Ward. 2005. Iron and infection: The heart of the matter. *FEMS Immunol. Med. Microbiol.* 43:325-330.
- Burton, J. L. and R. J. Erskine. 2003. Immunity and mastitis. Some new ideas for an old disease. *Vet. Clin. North Am. Food Anim. Pract.* 19:1-45.
- Burvenich, C., E. Monfardini, J. Mehrzad, A. V. Capuco and M. J. Paape. 2004. Role of neutrophil polymorphonuclear leukocytes during bovine coliform mastitis: Physiology or pathology? *Verh. K. Acad. Geneesk. Belg.* 66:97-150; discussion 150-3.
- Burvenich, C., V. Van Merris, J. Mehrzad, A. Diez-Fraile and L. Duchateau. 2003. Severity of *E. coli* mastitis is mainly determined by cow factors. *Vet. Res.* 34:521-564.
- Bushe, T. and S. P. Oliver. 1987. Natural protective factors in bovine mammary secretions following different methods of milk cessation. *J. Dairy Sci.* 70:696-704.
- Carroll, E. J., O. W. Schalm and J. Lasmanis. 1964. Experimental coliform (*Aerobacter aerogenes*) mastitis: Characteristics of the endotoxin and its role in pathogenesis. *Am. J. Vet. Res.* 25:720-726.

- Chaneton, L., L. Tirante, J. Maito, J. Chaves and L. E. Bussmann. 2008. Relationship between milk lactoferrin and etiological agent in the mastitic bovine mammary gland. *J. Dairy Sci.* 91:1865-1873.
- Cheng, J. B., J. Q. Wang, D. P. Bu, G. L. Liu, C. G. Zhang, H. Y. Wei, L. Y. Zhou and J. Z. Wang. 2008. Factors affecting the lactoferrin concentration in bovine milk. *J. Dairy Sci.* 91:970-976.
- Cifrian, E., A. J. Guidry, C. N. O'Brien, S. C. Nickerson and W. W. Marquardt. 1994. Adherence of *Staphylococcus aureus* to cultured bovine mammary epithelial cells. *J. Dairy Sci.* 77:970-983.
- Clutterbuck, A. L., E. J. Woods, D. C. Knottenbelt, P. D. Clegg, C. A. Cochrane and S. L. Percival. 2007. Biofilms and their relevance to veterinary medicine. *Vet. Microbiol.* 121:1-17.
- Conesa, C., L. Sanchez, C. Rota, M. D. Perez, M. Calvo, S. Farnaud and R. W. Evans. 2008. Isolation of lactoferrin from milk of different species: Calorimetric and antimicrobial studies. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 150:131-139.
- Conneely, O. M. 2001. Antiinflammatory activities of lactoferrin. *J. Am. Coll. Nutr.* 20:389S-395S; discussion 396S-397S.
- Costerton, J. W., P. S. Stewart and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. *Science.* 284:1318-1322.
- Crouch, S. P., K. J. Slater and J. Fletcher. 1992. Regulation of cytokine release from mononuclear cells by the iron-binding protein lactoferrin. *Blood.* 80:235-240.
- Cucarella, C., M. A. Tormo, C. Ubeda, M. P. Trotonda, M. Monzon, C. Peris, B. Amorena, I. Lasa and J. R. Penades. 2004. Role of biofilm-associated protein bap in the pathogenesis of bovine *Staphylococcus aureus*. *Infect. Immun.* 72:2177-2185.
- Daly, M., P. Ross, L. Giblin and F. Buckley. 2006. Polymorphisms within the lactoferrin gene promoter in various cattle breeds. *Anim. Biotechnol.* 17:33-42.
- Davidson, L. A. and B. Lonnerdal. 1986. Isolation and characterization of rhesus monkey milk lactoferrin. *Pediatr. Res.* 20:197-201.
- Dial, E. J., J. J. Romero, D. R. Headon and L. M. Lichtenberger. 2000. Recombinant human lactoferrin is effective in the treatment of *Helicobacter felis*-infected mice. *J. Pharm. Pharmacol.* 52:1541-1546.
- Diarra, M. S., D. Petitclerc, E. Deschenes, N. Lessard, G. Grondin, B. G. Talbot and P. Lacasse. 2003. Lactoferrin against *Staphylococcus aureus* mastitis. lactoferrin alone or in combination with penicillin G on bovine polymorphonuclear function and mammary epithelial cells colonisation by *Staphylococcus aureus*. *Vet. Immunol. Immunopathol.* 95:33-42.
- Diarra, M. S., D. Petitclerc and P. Lacasse. 2002a. Effect of lactoferrin in combination with penicillin on the morphology and the physiology of *Staphylococcus aureus* isolated from bovine mastitis. *J. Dairy Sci.* 85:1141-1149.
- Diarra, M. S., D. Petitclerc and P. Lacasse. 2002b. Response of *Staphylococcus aureus* isolates from bovine mastitis to exogenous iron sources. *J. Dairy Sci.* 85:2141-2148.
- Dionysius, D. A., P. A. Grieve and J. M. Milne. 1993. Forms of lactoferrin: Their antibacterial effect on enterotoxigenic *Escherichia coli*. *J. Dairy Sci.* 76:2597-2606.

- Dogan, B., S. Klaessig, M. Rishniw, R. A. Almeida, S. P. Oliver, K. Simpson and Y. H. Schukken. 2006. Adherent and invasive *Escherichia coli* are associated with persistent bovine mastitis. *Vet. Microbiol.* 116:270-282.
- Elass-Rochard, E., D. Legrand, V. Salmon, A. Roseanu, M. Trif, P. S. Tobias, J. Mazurier and G. Spik. 1998. Lactoferrin inhibits the endotoxin interaction with CD14 by competition with the lipopolysaccharide-binding protein. *Infect. Immun.* 66:486-491.
- Elass-Rochard, E., A. Roseanu, D. Legrand, M. Trif, V. Salmon, C. Motas, J. Montreuil and G. Spik. 1995. Lactoferrin-lipopolysaccharide interaction: Involvement of the 28-34 loop region of human lactoferrin in the high-affinity binding to *Escherichia coli* 055B5 lipopolysaccharide. *Biochem. J.* 312 (Pt 3):839-845.
- Eliassen, L. T., G. Berge, B. Sveinbjornsson, J. S. Svendsen, L. H. Vorland and O. Rekdal. 2002. Evidence for a direct antitumor mechanism of action of bovine lactoferricin. *Anticancer Res.* 22:2703-2710.
- Ellison, R. T., 3rd and T. J. Giehl. 1991. Killing of gram-negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest.* 88:1080-1091.
- Ellison, R. T., 3rd, T. J. Giehl and F. M. LaForce. 1988. Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infect. Immun.* 56:2774-2781.
- Erdei, J., A. Forsgren and A. S. Naidu. 1994. Lactoferrin binds to porins OmpF and OmpC in *Escherichia coli*. *Infect. Immun.* 62:1236-1240.
- Ericsson Unnerstad, H., A. Lindberg, K. Persson Waller, T. Ekman, K. Artursson, M. Nilsson-Ost and B. Bengtsson. 2009. Microbial aetiology of acute clinical mastitis and agent-specific risk factors. *Vet. Microbiol.* 137:90-97.
- Facon, M. J. and B. J. Skura. 1996. Antibacterial activity of lactoferricin, lysozyme and EDTA against *Salmonella enteritidis*. *Int. Dairy Journal.* 6:303-313.
- Fang, W. and S. P. Oliver. 1999. Identification of lactoferrin-binding proteins in bovine mastitis-causing *Streptococcus uberis*. *FEMS Microbiol. Lett.* 176:91-96.
- Farke, N. Y. 1992. Indigenous enzymes in milk. other enzymes. 339-397 in *In Advanced Dairy Chemistry. Vol. 1. Proteins.* Fox, P.E. ed. Indigenous enzymes in milk. Other enzymes. Elsevier Science, Cambridge.
- Farnaud, S. and R. W. Evans. 2003. Lactoferrin--a multifunctional protein with antimicrobial properties. *Mol. Immunol.* 40:395-405.
- Faulkner, A. and M. Peaker. 1982. Reviews of the progress of dairy science: Secretion of citrate into milk. *J. Dairy Res.* 49:159-169.
- Finlay, B. B. and P. Cossart. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science.* 276:718-725.
- Flanagan, J. L. and M. D. Willcox. 2009. Role of lactoferrin in the tear film. *Biochimie.* 91:35-43.
- Frost, A. J. 1975. Selective adhesion of microorganisms to the ductular epithelium of the bovine mammary gland. *Infect. Immun.* 12:1154-1156.

- Garnsworthy, P. C., L. L. Masson, A. L. Lock and T. T. Mottram. 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. *J. Dairy Sci.* 89:1604-1612.
- Gaunt, S. N., N. Raffio, E. T. Kingsbury, R. A. Damon Jr, W. H. Johnson and B. A. Mitchell. 1980. Variation of lactoferrin and mastitis and their heritabilities. *J. Dairy Sci.* 63:1874-1880.
- Gifford, J. L., H. N. Hunter and H. J. Vogel. 2005. Lactoferricin: A lactoferrin-derived peptide with antimicrobial, antiviral, antitumor and immunological properties. *Cell Mol. Life Sci.* 62(22):2588-89.
- Gonzalez-Chavez, S. A., S. Arevalo-Gallegos and Q. Rascon-Cruz. 2009. Lactoferrin: Structure, function and applications. *Int. J. Antimicrob. Agents.* 33:301.e1-301.e8.
- Goodman, R. E. and F. L. Schanbacher. 1991. Bovine lactoferrin mRNA: Sequence, analysis, and expression in the mammary gland. *Biochem. Biophys. Res. Commun.* 180:75-84.
- Griesbeck-Zilch, B., H. H. Meyer, C. H. Kuhn, M. Schwerin and O. Wellnitz. 2008. *Staphylococcus aureus* and *Escherichia coli* cause deviating expression profiles of cytokines and lactoferrin messenger ribonucleic acid in mammary epithelial cells. *J. Dairy Sci.* 91:2215-2224.
- Groves, M. L. 1965. Preparation of some iron-binding proteins and alpha-lactalbumin from bovine milk. *Biochim. Biophys. Acta.* 100:154-162.
- Guidry, A. J., M. Ost, I. H. Mather, W. E. Shainline and B. T. Weinland. 1983. Sequential response of milk leukocytes, albumin, immunoglobulins, monovalent ions, citrate, and lactose in cows given infusions of *Escherichia coli* endotoxin into the mammary gland. *Am. J. Vet. Res.* 44:2262-2267.
- Hagiwara, S., K. Kawai, A. Anri and H. Nagahata. 2003. Lactoferrin concentrations in milk from normal and subclinical mastitic cows. *J. Vet. Med. Sci.* 65:319-323.
- Halasa, T., K. Huijps, O. Osteras and H. Hogeveen. 2007. Economic effects of bovine mastitis and mastitis management: A review. *Vet. Q.* 29:18-31.
- Harmon, R. J. and F. S. H. Newbould. 1980. Neutrophil leukocyte as a source of lactoferrin in bovine milk. *Am. J. Vet. Res.* 41:1603.
- Harmon, R. J., F. L. Schanbacher, L. C. Ferguson and K. L. Smith. 1976. Changes in lactoferrin, immunoglobulin G, bovine serum albumin, and alpha-lactalbumin during acute experimental and natural coliform mastitis in cows. *Infect. Immun.* 13:533-542.
- Harmon, R. J., F. L. Schanbacher, L. C. Ferguson and K. L. Smith. 1975. Concentration of lactoferrin in milk of normal lactating cows and changes occurring during mastitis. *Am. J. Vet. Res.* 36:1001-1007.
- Harmsen, M. C., P. J. Swart, M. P. de Bethune, R. Pauwels, E. De Clercq, T. H. The and D. K. Meijer. 1995. Antiviral effects of plasma and milk proteins: Lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication *in vitro*. *J. Infect. Dis.* 172:380-388.
- Haversen, L., B. G. Ohlsson, M. Hahn-Zoric, L. A. Hanson and I. Mattsby-Baltzer. 2002. Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-kappa B. *Cell. Immunol.* 220:83-95.

- Hill, A. W., A. L. Shears and K. G. Hibbitt. 1979. The pathogenesis of experimental *Escherichia coli* mastitis in newly calved dairy cows. *Res. Vet. Sci.* 26:97-101.
- Hirvonen, J., K. Eklund, A. M. Teppo, G. Huszenicza, M. Kulcsar, H. Saloniemi and S. Pyörälä. 1999. Acute phase response in dairy cows with experimentally induced *Escherichia coli* mastitis. *Acta Vet. Scand.* 40:35-46.
- Hiss, S., M. Mielenz, R. M. Bruckmaier and H. Sauerwein. 2004. Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary hp mRNA expression. *J. Dairy Sci.* 87:3778-3784.
- Hoek, K. S., J. M. Milne, P. A. Grieve, D. A. Dionysius and R. Smith. 1997. Antibacterial activity in bovine lactoferrin-derived peptides. *Antimicrob. Agents Chemother.* 41:54-59.
- Isomäki, R. 1999. Separation of Whey Antimicrobial Proteins and Development of Bovine Lactoferrin Immunoassays. Licentiate thesis, University of Oulu, Department of Process and Environmental Engineering, 89 p.
- Jackson, J. A., D. E. Shuster, W. J. Silvia and R. J. Harmon. 1990. Physiological responses to intramammary or intravenous treatment with endotoxin in lactating dairy cows. *J. Dairy Sci.* 73:627-632.
- Jarp, J. 1991. Classification of coagulase-negative staphylococci isolated from bovine clinical and subclinical mastitis. *Vet. Microbiol.* 27:151-158.
- Johansson, B. G. 1960. Isolation of an iron containing red protein from human milk. *Acta Chem. Scand.* 14:510-512.
- Johnston, J. J., P. Rintels, J. Chung, J. Sather, E. J. Benz Jr and N. Berliner. 1992. Lactoferrin gene promoter: Structural integrity and nonexpression in HL60 cells. *Blood.* 79:2998-3006.
- Joslin, R. S., P. S. Erickson, H. M. Santoro, N. L. Whitehouse, C. G. Schwab and J. J. Rejman. 2002. Lactoferrin supplementation to dairy calves. *J. Dairy Sci.* 85:1237-1242.
- Kai, K., Y. Komine, K. Komine, K. Asai, T. Kuroishi, T. Kozutsumi, M. Itagaki, M. Ohta and K. Kumagai. 2002. Effects of bovine lactoferrin by the intramammary infusion in cows with staphylococcal mastitis during the early non-lactating period. *J. Vet. Med. Sci.* 64:873-878.
- Karpulus, T. E., R. J. Ulevitch and C. B. Wilson. 1987. A new method for reduction of endotoxin contamination from protein solutions. *J. Immunol. Methods.* 105:211-212-220.
- Karthikeyan, S., M. Paramasivam, S. Yadav, A. Srinivasan and T. P. Singh. 1999. Structure of buffalo lactoferrin at 2.5 Å resolution using crystals grown at 303 K shows different orientations of the N and C lobes. *Acta Crystallogr. D Biol. Crystallogr.* 55:1805-1813.
- Kawai, K., S. Hagiwara, A. Anri and H. Nagahata. 1999. Lactoferrin concentration in milk of bovine clinical mastitis. *Vet. Res. Commun.* 23:391-398.
- Kawai, K., H. Nagahata, N. Y. Lee, A. Anri and K. Shimazaki. 2003. Effect of infusing lactoferrin hydrolysate into bovine mammary glands with subclinical mastitis. *Vet. Res. Commun.* 27:539-548.
- Khan, J. A., P. Kumar, M. Paramasivam, R. S. Yadav, M. S. Sahani, S. Sharma, A. Srinivasan and T. P. Singh. 2001. Camel lactoferrin, a transferrin-cum-lactoferrin: Crystal structure of

- camel apolactoferrin at 2.6 Å resolution and structural basis of its dual role. *J. Mol. Biol.* 309:751-761.
- Kitchen, B. J., G. Middleton and M. Salmon. 1978. Bovine milk N-acetyl-beta-D-glucosaminidase and its significance in the detection of abnormal udder secretions. *J. Dairy Res.* 45:15-20.
- Kloos, W. E. and T. L. Bannerman. 1994. Update on clinical significance of coagulase-negative staphylococci. *Clin. Microbiol. Rev.* 7:117-140.
- Koivula, M., E. A. Mäntysaari, A. Pitkälä and S. Pyörälä. 2007. Distribution of bacteria and seasonal and regional effects in a new database for mastitis pathogens in Finland. *Acta Agric. Scand. A.* 57:89-96.
- Komine, K., Y. Komine, T. Kuroishi, J. Kobayashi, Y. Obara and K. Kumagai. 2005. Small molecule lactoferrin with an inflammatory effect but no apparent antibacterial activity in mastitic mammary gland secretion. *J. Vet. Med. Sci.* 67:667-677.
- Komine, Y., K. Komine, K. Kai, M. Itagaki, T. Kuroishi, H. Aso, Y. Obara and K. Kumagai. 2006. Effect of combination therapy with lactoferrin and antibiotics against staphylococcal mastitis on drying cows. *J. Vet. Med. Sci.* 68:205-211.
- Korhonen, H. 1995. Whey as raw material for development of new products for human nutrition and health; review. 102:207-219. In *milk in nutrition. Effects of production and processing factors.* Nordisk Jordbruksforskarens förening. Report 102. pp. 327.
- Korhonen, H. J. T. and A. Pihlanto. 2006. Bioactive peptides: Production and functionality. *International Dairy Journal.* 9:945-960.
- Krimpenfort, P., A. Rademakers, W. Eyestone, A. van der Schans, S. van den Broek, P. Kooiman, E. Kootwijk, G. Platenburg, F. Pieper and R. Strijker. 1991. Generation of transgenic dairy cattle using 'in vitro' embryo production. *Biotechnology (N. Y.)* 9:844-847.
- Król J., Z. Litwińczuk, A. Brodziak and J. Barłowska. 2010. Lactoferrin, lysozyme and immunoglobulin G content in milk of four breeds of cows managed under intensive production system. *Pol. J. Vet. Sci.* 13:357-361.
- Kruzel, M. L., J. K. Actor, I. Boldogh and M. Zimecki. 2007. Lactoferrin in health and disease. *Postepy Hig Med Dosw.* 61:261-267.
- Kruzel, M. L., A. Bacsi, B. Choudhury, S. Sur and I. Boldogh. 2006. Lactoferrin decreases pollen antigen-induced allergic airway inflammation in a murine model of asthma. *Immunology.* 119:159-166.
- Kruzel, M. L., Y. Harari, C. Y. Chen and G. A. Castro. 2000. Lactoferrin protects gut mucosal integrity during endotoxemia induced by lipopolysaccharide in mice. *Inflammation.* 24:33-44.
- Kuroishi, T., K. Komine, K. Kai, M. Itagaki, J. Kobayashi, M. Ohta, S. Kamata and K. Kumagai. 2003. Concentrations and specific antibodies to staphylococcal enterotoxin-C and toxic shock syndrome toxin-1 in bovine mammary gland secretions, and inflammatory response to the intramammary inoculation of these toxins. *J. Vet. Med. Sci.* 65:899-906.
- Kurokawa, H., B. Mikami and M. Hirose. 1995. Crystal structure of diferric hen ovotransferrin at 2.4 Å resolution. *J. Mol. Biol.* 254:196-207.

- Kussendrager, K. 1993. Lactoferrin and lactoperoxydase. bioactive milk proteins. International Food Ingredients. 6:17-21.
- Kuttila, T., S. Pyörälä, L. Kaartinen, R. Isomäki, K. Vahtola, L. Myllykoski and H. Saloniemi. 2003a. Lactoferrin and citrate concentrations at drying-off and during early mammary involution of dairy cows. J. Vet. Med. A Physiol. Pathol. Clin. Med. 50:350-353.
- Kuttila, T., S. Pyörälä, H. Saloniemi and L. Kaartinen. 2003b. Antibacterial effect of bovine lactoferrin against udder pathogens. Acta Vet. Scand. 44:35-42.
- Kuttila, T., L. Suojala, T. Lehtolainen, H. Saloniemi, L. Kaartinen, M. Tähti, K. Seppälä and S. Pyörälä. 2004. The efficacy of bovine lactoferrin in the treatment of cows with experimentally induced *Escherichia coli* mastitis. J. Vet. Pharmacol. Ther. 27:197-202.
- Lacasse, P., K. Lauzon, M. S. Diarra and D. Petitclerc. 2008. Utilization of lactoferrin to fight antibiotic resistant mammary gland pathogens. J. Anim. Sci. 86:66-71.
- Laible, G. 2009. Enhancing livestock through genetic engineering--recent advances and future prospects. Comp. Immunol. Microbiol. Infect. Dis. 32:123-137.
- Lee, N. Y., K. Kawai, I. Nakamura, T. Tanaka, H. Kumura and K. Shimazaki. 2004. Susceptibilities against bovine lactoferrin with microorganisms isolated from mastitic milk. J. Vet. Med. Sci. 66:1267-1269.
- Lee, W. J., J. L. Farmer, M. Hilty and Y. B. Kim. 1998. The protective effects of lactoferrin feeding against endotoxin lethal shock in germfree piglets. Infect. Immun. 66:1421-1426.
- Legrand, D., V. Salmon, B. Goddeville, M. Benaissa, Y. Plancke and G. Spik. 1997. Structural determination of two N-linked glycans isolated from recombinant human lactoferrin expressed in BHK* cells. 111-117 in Lactoferrin. Interactions and Biological Functions. T. W. Hutchens and B. Lönnerdal eds. Humana Press, Totowa, New Jersey, USA.
- Legrand, D., E. Ellass, M. Carpentier and J. Mazurier. 2005. Lactoferrin: A modulator of immune and inflammatory responses. Cell Mol. Life Sci. 62(22):2549-59
- Legrand, D., E. Ellass, A. Pierce and J. Mazurier. 2004. Lactoferrin and host defence: An overview of its immuno-modulating and anti-inflammatory properties. Biometals. 17:225-229.
- Legrand, D., A. Pierce, E. Ellass, M. Carpentier, C. Mariller and J. Mazurier. 2008. Lactoferrin structure and functions. Adv. Exp. Med. Biol. 606:163-194.
- Lehtolainen, T., C. Rontved and S. Pyörälä. 2004. Serum amyloid A and TNF- α ; in serum and milk during experimental endotoxin mastitis. Vet. Res. 35:651-659.
- Lehtolainen, T., S. Suominen, T. Kuttila and S. Pyörälä. 2003. Effect of intramammary *Escherichia coli* endotoxin in early- vs. late-lactating dairy cows. J. Dairy Sci. 86:2327-2333.
- Leitch, E. C. and M. D. Willcox. 1999a. Elucidation of the antistaphylococcal action of lactoferrin and lysozyme. J. Med. Microbiol. 48:867-871.
- Leitch, E. C. and M. D. Willcox. 1999b. Lactoferrin increases the susceptibility of *S. epidermidis* biofilms to lysozyme and vancomycin. Curr. Eye Res. 19:12-19.
- Lin, J., J. S. Hogan and K. L. Smith. 1999. Antigenic homology of the inducible ferric citrate receptor (FecA) of coliform bacteria isolated from herds with naturally occurring bovine intramammary infections. Clin. Diagn. Lab. Immunol. 6:966-969.

- Lin, J., J. S. Hogan and K. L. Smith. 1998. Inhibition of in vitro growth of coliform bacteria by a monoclonal antibody directed against ferric enterobactin receptor FepA. *J. Dairy Sci.* 81:1267-1274.
- Lonnerdal, B. 2009. Nutritional roles of lactoferrin. *Curr. Opin. Clin. Nutr. Metab. Care.* 12:293-297.
- Lonnerdal, B., E. Forsum and L. Hambræus. 1976. A longitudinal study of the protein, nitrogen, and lactose contents of human milk from Swedish well-nourished mothers. *Am. J. Clin. Nutr.* 29:1127-1133.
- Lupetti, A., A. Paulusma-Annema, M. M. Welling, S. Senesi, J. T. van Dissel and P. H. Nibbering. 2000. Candidacidal activities of human lactoferrin peptides derived from the N terminus. *Antimicrob. Agents Chemother.* 44:3257-3263.
- Machnicki, M., M. Zimecki and T. Zagulski. 1993. Lactoferrin regulates the release of tumour necrosis factor alpha and interleukin 6 in vivo. *Int. J. Exp. Pathol.* 74:433-439.
- Maga, E. A. 2005. Genetically engineered livestock: Closer than we think? *Trends Biotechnol.* 23:533-535.
- Magnuson, J. S., J. F. Henry, T. T. Yip and T. W. Hutchen. 1990. Structural homology of human, bovine, and porcine milk lactoferrins: Evidence for shared antigenic determinants. *Pediatric Research.* 28:176-181.
- Makimura, S. and N. Suzuki. 1982. Quantitative determination of bovine serum haptoglobin and its elevation in some inflammatory diseases. *Nippon Juigaku Zasshi.* 44:15-21.
- Marchetti, M., F. Superti, M. G. Ammendolia, P. Rossi, P. Valenti and L. Seganti. 1999. Inhibition of poliovirus type 1 infection by iron-, manganese- and zinc-saturated lactoferrin. *Med. Microbiol. Immunol.* 187:199-204.
- Marnila, P. and Korhonen, H. 2009. Lactoferrin for human health. P. 290-307 in *Dairy-derived ingredients: Food and nutraceutical uses*. Ed. M. Gorredig. Woodhead Publishing Limited, Cambridge, UK.
- Mason, P. L., J. F. Heremans and C. Dive. 1966. An iron-binding protein common to many external secretions. *Clin. Chim. Acta.* 14:735-739.
- Masson, P. L. and J. F. Heremans. 1971. Lactoferrin in milk from different species. *Comp. Biochem. Physiol. B.* 39:119-129.
- Masson, P. L., J. F. Heremans and E. Schonke. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. *J. Exp. Med.* 130:643-658.
- Matthews, K. R., R. A. Almeida and S. P. Oliver. 1994. Bovine mammary epithelial cell invasion by *Streptococcus uberis*. *Infect. Immun.* 62:5641-5646.
- Mattila, T., T. Honkanen-Buzalski and M. Sandholm. 1985. Depressed bacterial growth in whey during endotoxin induced mastitis. *Zentralbl. Veterinarmed. B.* 32:85-92.
- Mehrzdad, J., L. Duchateau, S. Pyörälä and C. Burvenich. 2002. Blood and milk neutrophil chemiluminescence and viability in primiparous and pluriparous dairy cows during late pregnancy, around parturition and early lactation. *J. Dairy Sci.* 85:3268-3276.

- Melchior, M. B., H. Vaarkamp and J. Fink-Gremmels. 2006. Biofilms: A role in recurrent mastitis infections? *Vet. J.* 171:398-407.
- Metz-Boutigue, M. H., J. Jolles, J. Mazurier, F. Schoentgen, D. Legrand, G. Spik, J. Montreuil and P. Jolles. 1984. Human lactotransferrin: Amino acid sequence and structural comparisons with other transferrins. *Eur. J. Biochem.* 145:659-676.
- Miyazawa, K., C. Mantel, L. Lu, D. C. Morrison and H. E. Broxmeyer. 1991. Lactoferrin-lipopolysaccharide interactions. Effect on lactoferrin binding to monocyte/macrophage-differentiated HL-60 cells. *J. Immunol.* 146:723-729.
- Molenaar, A. J., Y. M. Kuys, S. R. Davis, R. J. Wilkins, P. E. Mead and J. W. Tweedie. 1996. Elevation of lactoferrin gene expression in developing, ductal, resting, and regressing parenchymal epithelium of the ruminant mammary gland. *J. Dairy Sci.* 79:1198-1208.
- Moore, S. A., B. F. Anderson, C. R. Groom, M. Haridas and E. N. Baker. 1997. Three-dimensional structure of diferric bovine lactoferrin at 2.8 Å resolution. *J. Mol. Biol.* 274:222-236.
- Morinaga Milk Industry CO., Ltd. 2003. Biological functions of lactoferrin: Basic research and applications. Fourth edition.
- Na, Y. J., S. B. Han, J. S. Kang, Y. D. Yoon, S. K. Park, H. M. Kim, K. H. Yang and C. O. Joe. 2004. Lactoferrin works as a new LPS-binding protein in inflammatory activation of macrophages. *Int. Immunopharmacol.* 4:1187-1199.
- Nagasawa, T., I. Kiyosawa and K. Kuwahara. 1972. Amounts of lactoferrin in human colostrum and milk. *J. Dairy Sci.* 55:1651-1659.
- Naidu, A. S., M. Andersson and A. Forsgren. 1992. Identification of a human lactoferrin-binding protein in *Staphylococcus aureus*. *J. Med. Microbiol.* 36:177-183.
- Naidu, A. S., M. Andersson, J. Miedzobrodzki, A. Forsgren and J. L. Watts. 1991. Bovine lactoferrin receptors in *Staphylococcus aureus* isolated from bovine mastitis. *J. Dairy Sci.* 74:1218-1226.
- Naidu, A. S., J. Miedzobrodzki, M. Andersson, L. E. Nilsson, A. Forsgren and J. L. Watts. 1990. Bovine lactoferrin binding to six species of coagulase-negative staphylococci isolated from bovine intramammary infections. *J. Clin. Microbiol.* 28:2312-2319.
- Naidu, S. 2002. Activated lactoferrin - A new approach to meat safety. *Food Technology.* 56:40-45.
- Naidu, S. S., U. Svensson, A. R. Kishore and A. S. Naidu. 1993. Relationship between antibacterial activity and porin binding of lactoferrin in *Escherichia coli* and *Salmonella typhimurium*. *Antimicrob. Agents Chemother.* 37:240-245.
- Nevala, M., S. Taponen and S. Pyörälä. 2004. Bacterial etiology of bovine clinical mastitis - data from Saari ambulatory clinic in 2002-2003. *Suomen Eläinlääkärilehti (Finnish Veterinary Journal)*. 110:363-369.
- Newman, K. A., P. J. Rajala-Schultz, J. Lakritz and F. J. DeGraves. 2009. Lactoferrin concentrations in bovine milk prior to dry-off. *J. Dairy Res.* 76:426-432.

- Nibbering, P. H., E. Ravensbergen, M. M. Welling, L. A. van Berkel, P. H. van Berkel, E. K. Pauwels and J. H. Nuijens. 2001. Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic-resistant bacteria. *Infect. Immun.* 69:1469-1476.
- Nickerson, S. C. 1989. Immunological aspects of mammary involution. *J. Dairy Sci.* 72:1665-1678.
- Nonnecke, B. J. and K. L. Smith. 1984a. Biochemical and antibacterial properties of bovine mammary secretion during mammary involution and at parturition. *J. Dairy Sci.* 67:2863-2872.
- Nonnecke, B. J. and K. L. Smith. 1984b. Inhibition of mastitic bacteria by bovine milk apolactoferrin evaluated by in vitro microassay of bacterial growth. *J. Dairy Sci.* 67:606-613.
- Oliveira, M., R. Bexiga, S. F. Nunes, C. Carneiro, L. M. Cavaco, F. Bernardo and C. L. Vilela. 2006. Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. *Vet. Microbiol.* 118:133-140.
- Oo, T. Z., N. Cole, L. Garthwaite, M. D. Willcox and H. Zhu. 2010. Evaluation of synergistic activity of bovine lactoferricin with antibiotics in corneal infection. *J. Antimicrob. Chemother.*
- Oram, J. D. and B. Reiter. 1968. Inhibition of bacteria by lactoferrin and other iron-chelating agents. *Biochim. Biophys. Acta.* 170:351-365.
- Park, H. M., R. A. Almeida, D. A. Luther and S. P. Oliver. 2002a. Binding of bovine lactoferrin to *Streptococcus dysgalactiae* subsp. *dysgalactiae* isolated from cows with mastitis. *FEMS Microbiol. Lett.* 208:35-39.
- Park, H. M., R. A. Almeida and S. P. Oliver. 2002b. Identification of lactoferrin-binding proteins in *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus agalactiae* isolated from cows with mastitis. *FEMS Microbiol. Lett.* 207:87-90.
- Patel, D., R. A. Almeida, J. R. Dunlap and S. P. Oliver. 2009a. Bovine lactoferrin serves as a molecular bridge for internalization of *Streptococcus uberis* into bovine mammary epithelial cells. *Vet. Microbiol.* 137:297-301.
- Patel, D., R. A. Almeida, J. R. Dunlap and S. P. Oliver. 2009b. Bovine lactoferrin serves as a molecular bridge for internalization of *Streptococcus uberis* into bovine mammary epithelial cells. *Vet. Microbiol.* 137:297-301.
- Peaker, M. and J. L. Linzell. 1975. Citrate in milk: A harbinger of lactogenesis. *Nature.* 253:464.
- Persson, K., A. Carlsson, C. Hambleton and A. J. Guidry. 1992. Immunoglobulins, lysozyme and lactoferrin in the teat and udder of the dry cow during endotoxin-induced inflammation. *Zentralbl. Veterinarmed. B.* 39:165-174.
- Petitclerc, D., K. Lauzon, A. Cochu, C. Ster, M. S. Diarra and P. Lacasse. 2007. Efficacy of a lactoferrin-penicillin combination to treat β -lactam-resistant *Staphylococcus aureus* mastitis. *J. Dairy Sci.* 90:2778-2787.
- Petschow, B. W. and R. D. Talbott. 1991. Response of *Bifidobacterium* species to growth promoters in human and cow milk. *Pediatr. Res.* 29:208-213.

- Petschow, B. W., R. D. Talbott and R. P. Batema. 1999. Ability of lactoferrin to promote the growth of *Bifidobacterium* spp. in vitro is independent of receptor binding capacity and iron saturation level. *J. Med. Microbiol.* 48:541-549.
- Pierce, A., D. Colavizza, M. Benaissa, P. Maes, A. Tartar, J. Montreuil and G. Spik. 1991. Molecular cloning and sequence analysis of bovine lactotransferrin. *Eur. J. Biochem.* 196:177-184.
- Pierce, A. and D. Legrand. 2009. Advances in lactoferrin research. *Biochimie.* 91:1-2.
- Pitkälä, A., M. Haveri, S. Pyörälä, V. Myllys and T. Honkanen-Buzalski. 2004. Bovine mastitis in Finland 2001--prevalence, distribution of bacteria, and antimicrobial resistance. *J. Dairy Sci.* 87:2433-2441.
- Prgomet, C., H. Sarikaya, R. M. Bruckmaier and M. W. Pfaffl. 2005. Short-term effects on pro-inflammatory cytokine, lactoferrin and CD14 mRNA expression levels in bovine immunoseparated milk and blood cells treated by LPS. *J. Vet. Med. A Physiol. Pathol. Clin. Med.* 52:317-324.
- Puddu, P., P. Valenti and S. Gessani. 2009. Immunomodulatory effects of lactoferrin on antigen presenting cells. *Biochimie.* 91:11-18.
- Puvogel, G., C. R. Baumrucker, H. Sauerwein, R. Ruhl, E. Ontsouka, H. M. Hammon and J. W. Blum. 2005. Effects of an enhanced vitamin A intake during the dry period on retinoids, lactoferrin, IGF system, mammary gland epithelial cell apoptosis, and subsequent lactation in dairy cows. *J. Dairy Sci.* 88:1785-1800.
- Pyörälä, S. 2008. Mastitis in post-partum dairy cows. *Reprod. Domest. Anim.* 43 Suppl 2:252-259.
- Pyörälä, S., L. Kaartinen, H. Käck and V. Rainio. 1994. Efficacy of two therapy regimens for treatment of experimentally induced *Escherichia coli* mastitis in cows. *J. Dairy Sci.* 77:453-461.
- Pyörälä, S. and S. Taponen. 2009. Coagulase-negative staphylococci-emerging mastitis pathogens. *Vet. Microbiol.* 134:3-8.
- Qiu, J., D. R. Hendrixson, E. N. Baker, T. F. Murphy, J. W. St Geme 3rd and A. G. Plaut. 1998. Human milk lactoferrin inactivates two putative colonization factors expressed by *Haemophilus influenzae*. *Proc. Natl. Acad. Sci. U. S. A.* 95:12641-12646.
- Rainard, P. 2005. Tackling mastitis in dairy cows. *Nat. Biotechnol.* 23:430-432.
- Rainard, P. 1986a. Bacteriostasis of *Escherichia coli* by bovine lactoferrin, transferrin and immunoglobulins (IgG1, IgG2, IgM) acting alone or in combination. *Vet. Microbiol.* 11:103-115.
- Rainard, P. 1986b. Bacteriostatic activity of bovine milk lactoferrin against mastitic bacteria. *Vet. Microbiol.* 11:387-392.
- Rawas, A., H. Muirhead and J. Williams. 1996. Structure of diferric duck ovotransferrin at 2.35 Å resolution. *Acta Crystallogr. D Biol. Crystallogr.* 52:631-640.
- Reiter, B. and J. D. Oram. 1967. Bacterial inhibitors in milk and other biological fluids. *Nature.* 216:328-330.

- Reiter, B., J. H. Brock and E. D. Steel. 1975. Inhibition of *Escherichia coli* by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and serum resistant strain of *E. coli*. *Immunology*. 28:83-95.
- Robblee, E. D., P. S. Erickson, N. L. Whitehouse, A. M. McLaughlin, C. G. Schwab, J. J. Rejman and R. E. Rompala. 2003. Supplemental lactoferrin improves health and growth of holstein calves during the preweaning phase. *J. Dairy Sci.* 86:1458-1464.
- Roberts, T. K. and J. C. Bournnell. 1975. The isolation and characterization of lactoferrin from sow milk and boar seminal plasma. *J. Reprod. Fertil.* 42:579-582.
- Sanchez, L., P. Aranda, M. D. Perez and M. Calvo. 1988. Concentration of lactoferrin and transferrin throughout lactation in cow's colostrum and milk. *Biol. Chem. Hoppe Seyler*. 369:1005-1008.
- Sanchez, L., L. Lujan, R. Oria, H. Castillo, D. Perez, J. M. Ena and M. Calvo. 1992. Synthesis of lactoferrin and transport of transferrin in the lactating mammary gland of sheep. *J. Dairy Sci.* 75:1257-1262.
- Sanchez, M. S. and J. L. Watts. 1999. Enhancement of the activity of novobiocin against *Escherichia coli* by lactoferrin. *J. Dairy Sci.* 82:494-499.
- Schalm, O. W. 1977. Pathologic changes in the milk and udder of cows with mastitis. *J. Am. Vet. Med. Assoc.* 170:1137-1140.
- Schanbacher, F. L., R. E. Goodman and R. S. Talhouk. 1993. Bovine mammary lactoferrin: Implications from messenger ribonucleic acid (mRNA) sequence and regulation contrary to other milk proteins. *J. Dairy Sci.* 76:3812-3831.
- Schmitz, S., M. W. Pfaffl, H. H. Meyer and R. M. Bruckmaier. 2004. Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. *Domest. Anim. Endocrinol.* 26:111-126.
- Schryvers, A. B. and L. J. Morris. 1988. Identification and characterization of the human lactoferrin-binding protein from *Neisseria meningitidis*. *Infect. Immun.* 56:1144-1149.
- Schwerin, M., S. Solinas Toldo, A. Eggen, R. Brunner, H. M. Seyfert and R. Fries. 1994. The bovine lactoferrin gene (LTF) maps to chromosome 22 and syntenic group U12. *Mamm. Genome.* 5:486-489.
- Senft, B. and F. Klobasa. 1973. Untersuchung uber die konzentration an lactoferrin in kolostralmilch und reifer milch von kuhen. *Milchwissenschaft.* 28:750-752.
- Seyfert, H. M., A. Tuckoricz, H. Interthal, D. Koczan and G. Hobom. 1994. Structure of the bovine lactoferrin-encoding gene and its promoter. *Gene.* 143:265-269.
- Sharma, A. K., S. Karthikeyan, S. Sharma, S. Yadav, A. Srinivasan and T. P. Singh. 1998. Structures of buffalo and mare lactoferrins. Similarities, differences, and flexibility. *Adv. Exp. Med. Biol.* 443:15-21.
- Sherman, M. P., S. H. Bennett, F. F. Hwang and C. Yu. 2004. Neonatal small bowel epithelia: Enhancing anti-bacterial defense with lactoferrin and *Lactobacillus* GG. *Biometals.* 17:285-289.

- Shimazaki, K., N. Kawano and Y. C. Yoo. 1991. Comparison of bovine, sheep and goat milk lactoferrins in their electrophoretic behavior, conformation, immunochemical properties and lectin reactivity. *Comp. Biochem. Physiol. B.* 98:417-422.
- Shpigel, N. Y., M. Winkler, G. Ziv and A. Saran. 1998. Clinical, bacteriological and epidemiological aspects of clinical mastitis in Israeli dairy herds. *Prev. Vet. Med.* 35:1-9.
- Shuster, D. E., E. K. Lee and M. E. Kehrl Jr. 1996. Bacterial growth, inflammatory cytokine production, and neutrophil recruitment during coliform mastitis in cows within ten days after calving, compared with cows at midlactation. *Am. J. Vet. Res.* 57:1569-1575.
- Simojoki, H., T. Orro, S. Taponen and S. Pyörälä. 2009. Host response in bovine mastitis experimentally induced with *Staphylococcus chromogenes*. *Vet. Microbiol.* 134:95-99.
- Singh, P. K., M. R. Parsek, E. P. Greenberg and M. J. Welsh. 2002. A component of innate immunity prevents bacterial biofilm development. *Nature.* 417:552-555.
- Sloth, K. H., N. C. Friggens, P. Lovendahl, P. H. Andersen, J. Jensen and K. L. Ingvarsen. 2003. Potential for improving description of bovine udder health status by combined analysis of milk parameters. *J. Dairy Sci.* 86:1221-1232.
- Smith, K. L., H. R. Conrad and R. M. Porter. 1971. Lactoferrin and IgG immunoglobulins from involuted bovine mammary glands. *J. Dairy Sci.* 54:1427-1435.
- Smith, K. L. and F. L. Schanbacher. 1977. Lactoferrin as a factor of resistance to infection of the bovine mammary gland. *J. Am. Vet. Med. Assoc.* 170:1224-1227.
- Sordillo, L. M. and K. L. Streicher. 2002. Mammary gland immunity and mastitis susceptibility. *J. Mammary Gland Biol. Neoplasia.* 7:135-146.
- Sorensen, L. P., B. Guldbandsen, J. R. Thomasen and M. S. Lund. 2008. Pathogen-specific effects of quantitative trait loci affecting clinical mastitis and somatic cell count in Danish holstein cattle. *J. Dairy Sci.* 91:2493-2500.
- Sørensen, M. and S. P. L. Sørensen. 1939. The proteins in whey. *C.R. Trav. Lab. Carlsberg, Ser. Chim.* 23:55.
- Soukka, T., J. Tenovuo and M. Lenander-Lumikari. 1992. Fungicidal effect of human lactoferrin against *Candida albicans*. *FEMS Microbiol. Lett.* 69:223-228.
- Soyeurt, H., F. G. Colinet, V. M. Arnould, P. Dardenne, C. Bertozzi, R. Renaville, D. Portetelle and N. Gengler. 2007. Genetic variability of lactoferrin content estimated by mid-infrared spectrometry in bovine milk. *J. Dairy Sci.* 90:4443-4450.
- Spik, G., B. Coddeville and J. Montreuil. 1988. Comparative study of the primary structures of sero-, lacto- and ovotransferrin glycans from different species. *Biochimie.* 70:1459-1469.
- Stumpf and Welch. 2004. Secretory and defensive functions of the duct system of the lactating mammary gland of the African elephant (*Loxodonta africana, proboscidea*). *Zoomorphology.* 123:155-167.
- Superti, F., M. G. Ammendolia, P. Valenti and L. Seganti. 1997. Antiroviral activity of milk proteins: Lactoferrin prevents rotavirus infection in the enterocyte-like cell line HT-29. *Med. Microbiol. Immunol.* 186:83-91.

- Suzuki, Y. A., V. Lopez and B. Lonnerdal. 2005. Mammalian lactoferrin receptors: Structure and function. *Cell Mol. Life Sci.* 62(22):2560-75.
- Swaigood, H. E. 1992. Chemistry of the caseins. P. 63-110 in In. *ADV. Dairy Chem. Vol. 1. Proteins. Chemistry of the caseins.* Elsevier Applied Science, Canbridge.
- Talhok, R. S., R. L. Neiswander and F. L. Schanbacher. 1998. Effect of substratum on growth, cell morphology and lactoferrin synthesis and secretion in bovine mammary cell culture. *Tissue Cell.* 30:226-235.
- Tanaka, T., Y. Abe, W. S. Kim, X. Xuan, H. Nagasawa, I. Igarashi, H. Kumura and K. Shimazaki. 2003. The detection of bovine lactoferrin binding protein on *Toxoplasma gondii*. *J. Vet. Med. Sci.* 65:1377-1380.
- Tanaka, T., Y. Omata, A. Saito, K. Shimazaki, K. Yamauchi, M. Takase, K. Kawase, K. Igarashi and N. Suzuki. 1995. *Toxoplasma gondii*: Parasitocidal effects of bovine lactoferrin against parasites. *Exp. Parasitol.* 81:614-617.
- Taponen, S., J. Koort, J. Björkroth, H. Saloniemi and S. Pyörälä. 2007. Bovine intramammary infections caused by coagulase-negative staphylococci may persist throughout lactation according to amplified fragment length polymorphism-based analysis. *J. Dairy Sci.* 90:3301-3307.
- Taponen, S., H. Simojoki, M. Haveri, H. D. Larsen and S. Pyörälä. 2006. Clinical characteristics and persistence of bovine mastitis caused by different species of coagulase-negative staphylococci identified with API or AFLP. *Vet. Microbiol.* 115:199-207.
- Teng, C. T. 2006. Factors regulating lactoferrin gene expression. *Biochem. Cell Biol.* 84:263-267.
- Teng, C. T. 2002. Lactoferrin gene expression and regulation: An overview. *Biochem. Cell Biol.* 80:7-16.
- Teng, C. T. 1994. Lactoferrin gene promoter in human and mouse. Analogous and dissimilar characteristics. *Adv. Exp. Med. Biol.* 357:183-196.
- Teng, C. T., W. Gladwell, C. Beard, D. Walmer, C. S. Teng and R. Brenner. 2002. Lactoferrin gene expression is estrogen responsive in human and rhesus monkey endometrium. *Mol. Hum. Reprod.* 8:58-67.
- Tenhagen, B. A., G. Koster, J. Wallmann and W. Heuwieser. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J. Dairy Sci.* 89:2542-2551.
- Thomassen, E. A., H. A. van Veen, P. H. van Berkel, J. H. Nuijens and J. P. Abrahams. 2005. The protein structure of recombinant human lactoferrin produced in the milk of transgenic cows closely matches the structure of human milk-derived lactoferrin. *Transgenic Res.* 14:397-405.
- Trivier, D. and R. J. Courcol. 1996. Iron depletion and virulence in *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 141:117-127.
- Tsuji, S., Y. Hirata, F. Mukai and S. Ohtagaki. 1990. Comparison of lactoferrin content in colostrum between different cattle breeds. *J. Dairy Sci.* 73:125-128.
- U.S. Food and Drug Administration. 2001. GRAS notice no. GRN 000077.

- Välilä, H., J. Tenonuo, M. Waris and V. Hukkanen. 2009. Human lactoferrin but not lysozyme neutralizes HSV-1 and inhibits HSV-1 replication and cell-to-cell spread. *Viol. J.* 6:53.
- Van Belkum, A., M. Kools-Sijmons and H. Verbrugh. 2002. Attachment of *Staphylococcus aureus* to eukaryotic cells and experimental pitfalls in staphylococcal adherence assays: A critical appraisal. *J. Microbiol. Methods.* 48:19-42.
- van Berkel, P. H., M. E. Geerts, H. A. van Veen, M. Mericskay, H. A. de Boer and J. H. Nuijens. 1997. N-terminal stretch Arg2, Arg3, Arg4 and Arg5 of human lactoferrin is essential for binding to heparin, bacterial lipopolysaccharide, human lysozyme and DNA. *Biochem. J.* 328 (Pt 1):145-151.
- van Berkel, P. H., H. A. van Veen, M. E. Geerts, H. A. de Boer and J. H. Nuijens. 1996. Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: A study with glycosylation-site mutants. *Biochem. J.* 319 (Pt 1):117-122.
- van Berkel, P. H., M. M. Welling, M. Geerts, H. A. van Veen, B. Ravensbergen, M. Salaheddine, E. K. Pauwels, F. Pieper, J. H. Nuijens and P. H. Nibbering. 2002. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nat. Biotechnol.* 20:484-487.
- van der Kraan, M. I., J. Groenink, K. Nazmi, E. C. Veerman, J. G. Bolscher and A. V. Nieuw Amerongen. 2004. Lactoferrampin: A novel antimicrobial peptide in the N1-domain of bovine lactoferrin. *Peptides.* 25:177-183.
- Van Oostveldt, K., G. M. Tomita, M. J. Paape, A. V. Capuco and C. Burvenich. 2002. Apoptosis of bovine neutrophils during mastitis experimentally induced with *Escherichia coli* or endotoxin. *Am. J. Vet. Res.* 63:448-453.
- Vorland, L. H., H. Ulvatne, O. Rekdal and J. S. Svendsen. 1999. Initial binding sites of antimicrobial peptides in *Staphylococcus aureus* and *Escherichia coli*. *Scand. J. Infect. Dis.* 31:467-473.
- Wall, R. J., A. M. Powell, M. J. Paape, D. E. Kerr, D. D. Bannerman, V. G. Pursel, K. D. Wells, N. Talbot and H. W. Hawk. 2005. Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection. *Nat. Biotechnol.* 23:445-451.
- Walstra, P. 2006. Milk components. p. 17-108 in *Dairy Science and Tehnology*. 2.th ed. P. Walstra, J. T. M. Wouters and T. J. Geurts eds. CRC Press, Taylor & Francis Group, Boca Raton, FL, USA.
- Wang, Y., Y. Tu, F. Han, Z. Xu and J. Wang. 2005. Developmental gene expression of lactoferrin and effect of dietary iron on gene regulation of lactoferrin in mouse mammary gland. *J. Dairy Sci.* 88:2065-2071.
- Ward, P. P., E. Paz and O. M. Conneely. 2005. Multifunctional roles of lactoferrin: A critical overview. *Cell Mol. Life Sci.* 62(22):2540-8.
- Weinberg, E. D. 2004. Suppression of bacterial biofilm formation by iron limitation. *Med. Hypotheses.* 63:863-865.
- Wellnitz, O. and D. E. Kerr. 2004. Cryopreserved bovine mammary cells to model epithelial response to infection. *Vet. Immunol. Immunopathol.* 101:191-202.

- Welty, F. K., K. L. Smith and F. L. Schanbacher. 1976. Lactoferrin concentration during involution of the bovine mammary gland. *J. Dairy Sci.* 59:224-231.
- Wheeler, M. B. 2007. Agricultural applications for transgenic livestock. *Trends Biotechnol.* 25:204-210.
- Yoo, Y. C., R. Watanabe, Y. Koike, M. Mitobe, K. Shimazaki, S. Watanabe and I. Azuma. 1997a. Apoptosis in human leukemic cells induced by lactoferricin, a bovine milk protein-derived peptide: Involvement of reactive oxygen species. *Biochem. Biophys. Res. Commun.* 237:624-628.
- Yoo, Y. C., S. Watanabe, R. Watanabe, K. Hata, K. Shimazaki and I. Azuma. 1997b. Bovine lactoferrin and lactoferricin, a peptide derived from bovine lactoferrin, inhibit tumor metastasis in mice. *Jpn. J. Cancer Res.* 88:184-190.
- Yoshida, S., Z. Wei, Y. Shinmura and N. Fukunaga. 2000. Separation of lactoferrin-a and -b from bovine colostrum. *J. Dairy Sci.* 83:2211-2215.
- Zagulski, T., P. Lipinski, A. Zagulska, S. Broniek and Z. Jarzabek. 1989. Lactoferrin can protect mice against a lethal dose of *Escherichia coli* in experimental infection in vivo. *Br. J. Exp. Pathol.* 70:697-704.
- Zhang, J., L. Li, Y. Cai, X. Xu, J. Chen, Y. Wu, H. Yu, G. Yu, S. Liu, A. Zhang, J. Chen and G. Cheng. 2008. Expression of active recombinant human lactoferrin in the milk of transgenic goats. *Protein Expr. Purif.* 57:127-135.
- Zhang, P., V. Sawicki, A. Lewis, L. Hanson, J. H. Nuijens and M. C. Neville. 2001. Human lactoferrin in the milk of transgenic mice increases intestinal growth in ten-day-old suckling neonates. *Adv. Exp. Med. Biol.* 501:107-113.
- Zhang, S. and C. W. Maddox. 2000. Cytotoxic activity of coagulase-negative staphylococci in bovine mastitis. *Infect. Immun.* 68:1102-1108.
- Zheng, J., J. L. Ather, T. S. Sonstegard and D. E. Kerr. 2005. Characterization of the infection-responsive bovine lactoferrin promoter. *Gene.* 353:107-117.

ORIGINAL ARTICLES
