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Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections

Doctoral Dissertation

Susanna Rokka

Foods

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Supervisor:	Professor Hannu Korhonen, Ph.D.
	Agrifood Research Finland
	Biotechnology and Food Research, Jokioinen, Finland
Reviewers:	Professor Tapani Alatossava, Ph.D.
	University of Helsinki, Helsinki, Finland
	and
	Professor Atte von Wright, Ph.D.
	University of Kuopio, Kuopio, Finland
Opponent:	Professor Harsharn Gill
	Future Farming Systems Research Division
	Department of Primary Industries. Werribee Victoria, Australia
Custos:	Professor Heikki Kallio, Ph.D.
	University of Turku
	Department of Biochemistry and Food Chemistry, Turku, Finland

To Timo,

Matias, Tuomas, and Anni

Bovine colostral antibodies and selected lactobacilli as means to control gastrointestinal infections

Susanna Rokka

MTT Agrifood Research Finland, Biotechnology and Food Research, FI-31600 Jokioinen, Finland, susanna.rokka@mtt.fi

Department of Biochemistry and Food Chemistry, University of Turku

Abstract

Streptococcus mutans and *Helicobacter pylori* belong to the most common bacterial pathogens of humans. They infect more than 50% of the world's population. Further, enterotoxigenic *Escherichia coli* (ETEC) is regarded as the most common bacterial cause of diarrhoea worldwide.

Colostrum is essential for a newborn by providing nutrition but also immunological protection. Immunoglobulins and complement system are considered as the major antimicrobial agents in bovine colostrum. By immunizing a cow it is possible to produce specific antibodies in serum and lacteal secretions against microbes. These antibodies have proven effective in preventing many gastrointestinal microbial diseases. Probiotic bacteria are often used in fermented dairy products because of their beneficial effects on human health. Some probiotic bacteria, especially lactobacilli, can inhibit growth and colonization of pathogens. Probiotic bacteria like *Lactobacillus rhamnosus* GG (LGG) also reduce the side effects of antibiotic treatment.

The aims of this study were to investigate the effects of bovine colostrum and specific colostral antibodies, and some lactic acid bacteria on gastrointestinal infections, especially *H. pylori* induced gastritis and dental caries caused by *S. mutans*. The effect of colostrum on the activity of the complement system in neonatal calves was also studied.

It was possible to increase substantially complement and opsonization activities of serum by feeding colostral whey concentrate to neonatal calves. A colostral immune preparation (IP) containing specific antibodies against *H. felis* prevented but did not eradicate an experimental *H. felis* infection in mice. However, the IP combined with amoxicillin lowered the level of inflammation and colonization more than amoxicillin alone. The specific anti-cariogenic antibodies of IP remained active and functional when added to UHT milk or fermented with LGG and stored for an extended time. Further, the combination of IP and LGG prevented the adherence of *S. mutans* effectively.

Lactobacillus plantarum MLBPL1 isolated from sauerkraut showed anti-*Helicobacter* activity mainly associated with cell wall, from where it can be extracted

into the culture supernatant. Colostral preparations, and acidifying microbes (*L. plantarum* MLBPL1 and LGG) reduced the adhesion of *H. pylori* on human gastric adenocarcinoma cells. They also reduced the IL-8 production of the infected cells. IL-8 secretion is a primary response to *H. pylori* infection.

Supplementation of an antibiotic treatment with a food product containing probiotic lactobacilli and/or IP could offer a potential complementary means to suppress gastrointestinal infections.

Key-words: colostrum, lactic acid bacteria, infections

Abbreviations frequently used in the text

AGS cells	Human gastric adenocarcinoma cells
ATP	Adenosine triphosphate
EGTA	Ethylene glycol tetraacetic acid
ETEC	Enterotoxigenic *Escherichia coli*
FBS	Fetal bovine serum
cfu	Colony forming units
CL	Chemiluminescence
CP	Colostral control preparation
ELISA	Enzyme linked immunosorbent assay
HBSS	Hank's balanced salt solution
IgG	Immunoglobulin G
IFN	Interferon
IL	Interleukin
IP	Colostral immune preparation with specific antibodies
LAB	Lactic acid bacteria
LG21	*Lactobacillus gasseri* OLL2716
LGG	*Lactobacillus rhamnosus* GG
LPS	Lipopolysaccharide
MBL	Mannose binding lectin
NBS	Normal bovine serum
NF-κB	Nuclear factor κB
PBS	Phosphate buffered saline
TGF	Transforming growth factor
TLR	Toll like receptor
TNF	Tumor necrosis factor
UHT	Ultra-high temperature

List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals I-V.

I ROKKA, S., MARNILA, P., NOUSIAINEN, J., KORHONEN, H. 2001. Colostral whey concentrate supplement increases complement activity in the sera of neonatal calves. Journal of Dairy Research 68: 357-367.

II MARNILA, P., ROKKA, S., REHNBERG-LAIHO, L., KÄRKKÄINEN, P., KOSUNEN, T.U., RAUTELIN, H., HÄNNINEN, M.-L., SYVÄOJA, E.-L., KORHONEN, H.J.T. 2003. Prevention and suppression of *Helicobacter felis* infection in mice using colostral preparation with specific antibodies. Helicobacter 8: 192-201.

III WEI, H., LOIMARANTA, V., TENOVUO, J., ROKKA, S., SYVÄOJA, E-L., KORHONEN, H., JOUTSJOKI, V., MARNILA, P. 2002. Stability and activity of specific antibodies against *Streptococcus mutans* and *Streptococcus sobrinus* in bovine milk fermented with *Lactobacillus rhamnosus* strain *GG* or treated at ultra-high temperature. Oral Microbiology and Immunology 17: 9-15.

IV ROKKA, S., PIHLANTO, A., KORHONEN, H., JOUTSJOKI, V. 2006. *In vitro* growth inhibition of *Helicobacter pylori* by lactobacilli belonging to the *Lactobacillus plantarum* group. Letters in Applied Microbiology 43: 508-513.

V ROKKA, S., MYLLYKANGAS, S., JOUTSJOKI, V. 2008. Effect of specific colostral antibodies and selected lactobacilli on the adhesion of *Helicobacter pylori* on AGS cells and the *Helicobacter* induced IL-8 production. Scandinavian Journal of Immunology 68: 280-286.

Contents

1   Introduction

Epithelial cells of skin, lungs and gastrointestinal tract are the first barriers against the attacks by foreign invader like pathogens. The body fluids like saliva, tears, milk, and mucosal secretions contain lysozyme, lactoferrin and other unspecific antibacterial molecules. Gastric and bile acids as well as gastric enzymes are lethal for many bacteria. Complement, cytokines, chemokines, interferons etc. are also included in the defense mechanisms of the innate immune system. Normal flora of the skin and gut are extremely important in keeping the number of possible pathogens low for instance by preventing their colonization. Many microbes have in their membrane structures that receptors of phagocytes recognize and help them to ingest the foreign cells. Some bacteria have a capsule structure that prevents the phagocytes recognizing them. If the innate immune system is insufficient the adaptive immune system is needed. Adaptive immunity recognizes antigenic structures as non-self. Antibodies and the classical route of complement system are central for the adaptive immune system.

Streptococcus mutans and *Helicobacter pylori* belong to the most common bacterial pathogens of humans. They infect more than 50% of the world's population. Further, enterotoxigenic *Escherichia coli* (ETEC) is regarded as the most common bacterial cause of diarrhoea worldwide (Walker et al. 2007).

Common to the infections caused by *S. mutans* and *H. pylori* is that antibiotic treatments are not the choice to treat all people suffering from these disorders. The development of a vaccine against these pathogens has so far not been successful (Aebischer et al. 2008, Loimaranta 1999). Other means to control these pathogens are thus needed. During last decades the use of probiotic bacteria and passive immunization by oral administration of specific antibodies against gastrointestinal diseases has gained a lot of attention.

The concept of probiotics was born in 1907 when Ilya Metchnikoff supposed that Bulgarians live longer than other populations because of their consumption of fermented milk products with viable bacteria. He believed that harmless bacteria of fermented products competed with pathogens. During the past decade there has been a near exponential increase in publications on probiotics (reviewed by Meurman 2005).

The history of the concept "immune milk" started in 1950's when Petersen and Campbell suggested that orally administered bovine colostrum could provide passive immune protection for humans. Since 1980's an increasing number of studies has emerged on the effect of specific milk or colostrum derived antibodies from immunized cows in treatment or prevention of various gastrointestinal diseases (Korhonen and Marnila 2006).

In this study the efficacy of bovine colostrum, specific colostral antibodies, and some lactic acid bacteria were investigated on gastrointestinal infections, especially *Helicobacter* induced gastritis and dental caries. The effect of colostrum on the activity of complement system in neonatal calves was also studied.

2 Review of the literature

2.1 Basic concepts of immunology

2.1.1 Antibodies

Antibodies or immunoglobulins are produced by B-lymphocytes. The basic structure of immunoglobulin molecule is composed of two identical light chains and two identical heavy chain joined together with disulphide bonds (Figure 1). The antigen binding sites are located in the Fab region in the N-terminal "arms" of the Y-shaped flexible molecule. Generally, each antibody can bind specifically to only one antigen. Fc portion interacts with cells of the immune system and activates the complement mediated bacteriolytic reactions. The molecular weight of the basic structure of immunoglobulin molecule is about 160 000 Daltons. IgM is a pentamer of the basic structure. It is the first class of antibodies to appear in the serum after injection of an antigen, and is especially efficient with complement-mediated lysis. IgG is the major antibody in the serum and bovine milk. It has four subclasses differing mainly in the number of disulphide bonds. IgGs are able to inactivate toxins, aggregate and opsonise parasites, and activate the complement system. IgA is a monomer in human serum but dimer in other mammals. The dimeric IgA is the major class of immunoglobulins in external secretions such as saliva, tears, intestinal mucus, and milk, the ruminal milk being an exception with IgG1 as the major class of immunoglobulins. It agglutinates antigens, binds viruses and toxins, and prevents the adhesion of enteropathogenic bacteria to mucosal epithelial cells. The other two classes of immunoglobulins are IgD whose specific role is not known (Vladutiu 2000), and IgE, involved in protection against parasites, as well as in allergic reactions.
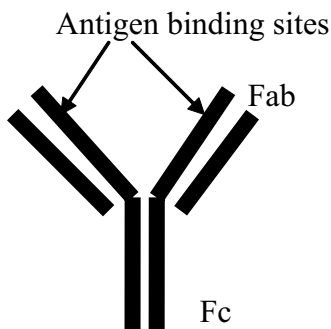
Figure 1. Basic structure of immunoglobulin molecule.

2.1.2 Complement system

The main functions of the complement system are to eliminate foreign pathogens by causing cytolytic membrane lesions, to opsonize microbes, and to mediate an inflammatory reaction. The complement system is present in serum, and also in bovine colostrum (reviewed by Korhonen et al. 2000b). The complement system is composed of more than 20 proteins and regulatory components. Activation of the complement system can occur via three pathways (Figure 2) in a sequential manner by proteolytic cleavages and association of precursor molecules.
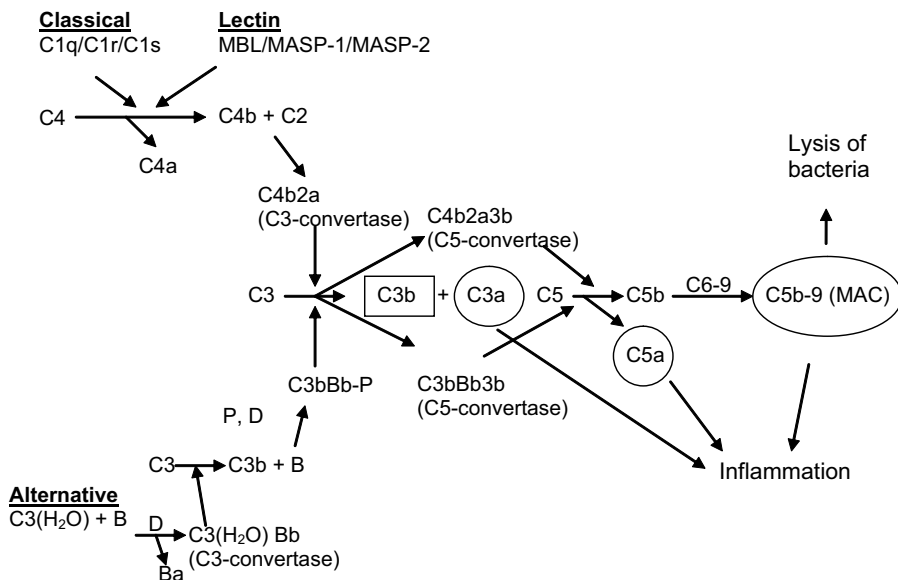
Figure 2. Schematic presentation of the complement cascade (modified from Thurman and Holers 2006). C1-C9 represent complement component molecules, MBL mannose-binding lecting, MASP mannose-binding lectin associated protease, P properdin, MAC membrane attack complex.

The classical pathway of complement uses antibodies, mainly IgM, for the recognition of foreign cells. The binding of the C1 complex to antigen-antibody complexes initiates proteolytic cleavage of complement components C4 and C2 by C1s, leading to the formation of a C4b2a enzyme complex, the C3 convertase of the classical pathway (Markiewski and Lambris 2007, Figure 2). The lectin pathway begins with binding of the complex mannose-binding lectin (MBL) and mannose-binding lectin-associated proteases 1 and 2 to a bacterial cell wall which leads to the formation of the C3 convertase. The alternative pathway is initiated by binding to surfaces lacking complement regulatory proteins. Spon-

taneous hydrolysis of C3 leads to the formation of $C3(H_2O)$ which forms a complex with factor B, followed by the cleavage of factor B within this complex by factor D. The final product of these enzymatic reactions is the $C3(H_2O)$ Bb complex. It is stabilized by properdin and works as a C3 convertase.

C3 convertases generated through various pathways cleave C3 to C3a and C3b. C3b contributes to the formation of the C5 convertase, which cleaves C5 to C5a and C5b. Generated cleavage products function as opsonins augmenting phagocytosis, recruit leucocytes to the sites of inflammation and activate them. The complement cascade leads to membrane attack complex (MAC), the pores generated leading to the death of the bacterial cell (Born and Bhakdi 1986).

All Gram-positive and several Gram-negative organisms are resistant to complement-mediated lysis. In these cases the opsonophagocytosis is the main defense mechanism (Rautemaa and Meri 1999). Opsonization means, "making eatable", that is, augmenting the phagocytizing leukocytes to recognize, bind, activate, ingest, and to kill the pathogen. During opsonization antibodies and/or complement molecules bind to antigens. Phagocytic cells recognize opsonized cells and ingest or destruct them. Bovine colostral antibodies are recognized by human phagocytes and thus have opsonizing activity also in humans (Loimaranta et al. 1999b).

2.1.3 Cytokines and chemokines in gastric infections

Epithelial and innate immune cells, e.g. macrophages, neutrophils and natural killer cells have receptors (like Toll-like receptors (TLR) and nucleotide-binding oligomerization domain (Nod) proteins) that recognize pathogen-associated molecular patterns and mediate immune response (reviewed by Bodger and Crabtree 1998 and O'Keeffe and Moran 2008). Several TLRs with specific actions are known, for instance TLR4 that recognizes lipopolysaccharide (LPS) of Gram-negative bacteria, and TLR2 and TLR5 that recognize bacterial lipopeptides and flagellin, respectively (O'Keeffe and Moran 2008).

In infection, epithelial cells secrete chemokines and cytokines, such as tumor necrosis factor (TNF) $-\alpha$, and interleukins (IL). TNF$-\alpha$, IL-1, IL-6, and IL-8 act as proinflammatory agents. Their production and expression is controlled by a transcription factor, nuclear factor (NF)-κB. The immunoinflammatory reaction is highly important in eliminating pathogens, but this reaction must be controlled to avoid a widespread or chronic inflammation. Immune response is down regulated by anti-inflammatory agents, e.g. IL-10.

Mononuclear phagocytes serve as a source of proinflammatory mediators and as antigen-presenting cells in early immune responses (Bodger and Crabtree

1998). NF-κB and IL-8 production in epithelial cells is dependent on signaling through Nod1 recognition of muropeptides (Viala et al. 2004).

Chemokines are involved in the recruitment and activation of specific immune cells. They can be distinguished to two subfamilies according to the arrangement of two conserved cysteins (Baggiolini et al. 1995). The members of the C-X-C subfamily (e.g. IL-8 and growth-related gene (GRO)-α) have specific chemotactic activity for neutrophils, whereas the members of the C-C subfamily (e.g. RANTES (Regulated on activation, normal T expressed and secreted) and macrophage inflammatory protein (MIP)-1α) have effects on monocytes and lymphocytes (Baggiolini et al. 1995).

T-cells are responsible for cell-mediated immunity and promote activation of many other immune cells and killing virally infected cells. T-cell differentiation is induced by IL-12. T helper cells recognize peptides complexed to MHC class II molecules and secrete cytokines interferon-gamma (IFN-γ) and TNF–α that promote the proinflammatory immune response or IL-4, IL-5, and IL-13 that induce B cells to produce antibodies (O'Keeffe and Moran 2008). Antigen specific regulatory T cells are crucial for the maintenance of tolerance to non-pathogen antigens and for preventing the development of autoimmune diseases. They control and regulate inflammation and bacterial colonization. The suppressive function of regulatory T cells is mediated via the production of IL-10 or transforming growth factor (TGF)-β (O'Keeffe and Moran 2008).

2.2 Significance of colostrum in passive immunity

2.2.1 Composition of bovine colostrum

The milk secreted during the first five to seven days after delivery is called colostrum. The composition of colostrum differs from normal milk (Table 1). The total protein concentration is highest in the first two milkings after delivery (over 15%), and then decreases rapidly (Parrish et al. 1950, Tsioulpas et al. 2007, Kehoe et al. 2007). Lactose content in colostrum is lower than in normal milk, whereas colostrum contains more minerals, e.g. calcium (Tsioulpas et al. 2007). Bovine colostrum contains 100 times more immunoglobulins than normal milk. They make up 70-80% of total protein in colostrum (Larson 1992). The major immunoglobulin classes in bovine colostrum are IgG, IgM, and IgA, IgG being the most abundant (Table 2).

Table 1. Mean values and standard deviation of fat, protein, lactose, and ash of bovine colostrum and milk samples (n=8) from the first 15 days of lactation (data from Tsioulpas et al. 2007).

Time (d) postpartum	Fat (%)	Protein (%)	Lactose (%)	Ash (%)
1	3.55 ± 1.82	16.12 ± 1.64	2.69 ± 0.46	1.18 ± 0.17
2	3.49 ± 1.67	5.43 ± 0.24	3.04 ± 1.23	1.00 ± 0.11
3	4.50 ± 1.54	4.54 ± 0.40	3.52 ± 0.44	0.93 ± 0.05
4	4.26 ± 2.19	4.41 ± 0.31	3.82 ± 0.19	0.92 ± 0.08
5	3.89 ± 1.04	4.23 ± 0.24	4.15 ± 0.24	0.87 ± 0.04
15	3.66 ± 1.22	4.01 ± 0.43	4.32 ± 0.14	0.83 ± 0.04

Colostrum is also a rich source of various antimicrobial factors including lactoferrin, lysozyme, lactoperoxidase and complement components (Korhonen 1977, Korhonen et al. 1995, Table 2). The most abundant and well-characterized growth factors in bovine colostrum are insulin-like growth factors (IGF-1 and IGF-2), insulin, TGF-β1, TGF-β2, and epidermal growth factors (EGF) (Pakkanen and Aalto 1997). Furthermore, milk proteins are a rich source of bioactive peptides (Korhonen and Pihlanto 2006, 2007).

2.2.2 Function of colostrum

The main purpose of colostrum is to offer the newborn adequate nutrition and immunological protection (Ehrlich 1892). It is essential for survival of calves because bovine placenta does not allow the transfer of immunoglobulins in significant amounts (Larson et al. 1980). A sufficient intake of colostral immune components is important for calves in the long term as the overall health of calves fed immunoglobulin concentrate during the fist two days of life was better than in the normally fed control group during the first 30 days of life (Nousiainen et al. 1994). Colostrum intake provokes drastic morphological and functional changes in the gastrointestinal tract of neonates. If IgG_1 concentrations in colostrum are low, high mortality rates of calves due to *E. coli* and other infections may result in. Non-nutrient factors of colostrum also modulate the microbial flora of gastrointestinal tract (reviewed by Blum 2006).

Table 2. Concentration and potential biological functions of major proteins of bovine colostrum (Data from Korhonen and Pihlanto 2007, and Korhonen and Marnila 2006).

Protein	Concentration g/L	Biological functions
Caseins	26	Ion carrier, precursor for bioactive peptides, immunomodulator
β-lactoglobulin	8.0	Retinol carrier, potential antioxidant, precursor for bioactive peptides, binds fatty acids
α-lactalbumin	3.0	Lactose synthesis in mammary gland, Ca carrier, immunomodulator, precursor for bioactive peptides, anticarcinogenic
Immunoglobulins	20-200	
• IgG1 • IgG2 • IgM • IgA	15-180 1-3 3-9 1-6	Specific immune protection, potential precursor for bioactive peptides
Glycomacropeptide	2.5	Antimicrobial, antithrombotic, bifidogenic, gastric regulator
Lactoferrin	1.5	Antimicrobial, antioxidative, anticarcinogenic, anti-inflammatory, iron transport, cell growth regulation, precursor for bioactive peptides, immunomodulator
Lactoperoxidase	0.02	Antimicrobial, synergistic effect with immunoglobulins and lactoferrin
Lysozyme	0.0004	Antimicrobial, synergistic effect with immunoglobulins and lactoferrin
Serum albumin	1.3	Precursor for bioactive peptides

The potential biological functions of major colostral proteins are listed in Table 2. Antibody-complement system is considered as the major antimicrobial agent in bovine colostrum (Korhonen et al. 2000a). The complement system of bovine colostrum is lytic against many pathogenic bacteria *in vitro* (Reiter and

Brock, 1975, Korhonen et al. 1995) but it is not known whether it is effective *in vivo* in the gut of the newborn calf (Korhonen et al. 2000a). Immunoglobulins are able to opsonize bacteria, prevent the adhesion of microbes to surfaces, inhibit bacterial metabolism, agglutinate bacteria, and neutralize toxins and viruses (Korhonen and Marnila 2006). He et al. (2001) suggested that bovine colostrum might alter the human humoral immune responses in an adjuvant-like manner. Colostral growth factors like IGF-1 and IGF-2 can stimulate cell growth as endocrine hormones via the blood and locally (Guimont et al. 1997). Colostral TGF-β has been found to increase IgA and IgG production *in vitro* (Chen and Li 1990).

2.2.3 Production of specific antibodies

The antimicrobial activity of milk can be increased by systemic hyperimmunisation of cows against a defined pathogen or a toxin at the end of the lactation period or during the dry period (Korhonen et al. 2000a,b). Different adjuvants have been used including Freund's complete and incomplete adjuvant. Their use is, however, limited because of possible side effects. Aluminium-hydroxide based adjuvants have proven better for the health status of cows (Korhonen et al. 2000a). Both milk and colostrum have been used as a source of specific antibodies. Colostrum is generally preferred because of its high concentration of IgGs, but on the other hand colostrum is secreted only for a limited time.

Immunoglobulins can be isolated from colostral or milk whey in industrial scale by a number of methods, based on ultrafiltration or a combination of ultrafiltration and chromatography (Korhonen et al. 2000a). The recovery rate of immunoglobulins has varied from 40% to 70% (Elfstrand et al. 2002). With microfiltration (0.1μm) the recovery has reported to be over 90% (for review see Korhonen and Pihlanto 2007). Thermal treatments during processing may influence the stability of immunoglobulins. During storage in ambient or cold temperature the freeze dried immunoglobulin fractions retain their specific activity well for years (Korhonen et al. 2000a, Pant et al. 2007). The shelf life of bovine milk-based products containing antibodies can be prolonged by membrane filtration (Fukumoto et al. 1994) or by fermentation processing.

Eggs are another source for specific antibodies. Immunized hen produce specific immunoglobulin Y (IgY) in the water-soluble part of egg yolk for months. IgY differs from mammal immunoglobulins by not being able to activate the complement system. Specific IgY has been produced and used successfully against a number of different antigens including *E. coli*, *Salmonella*, rotavirus, *S. mutans*, *H. pylori*, *Pseudomonas aeruginosa*, TNF-α, and various fish and other animal pathogens (Kovacs-Nolan et al. 2006).

2.2.4 Applications of immune preparations

The use of specific bovine colostral antibodies for promoting health of humans and animals has been studied since 1950's (reviewed by Korhonen and Marnila 2006). Passive immunization with specific antibodies provides an immediate protection that is available also for persons with impaired immunity (Pant et al. 2007). Colostral and immune milk preparations against enteropathogenic *E. coli*, rotavirus, *Cryptosporidium*, *Shigella flexneri*, *H. pylori*, *Vibrio cholerae*, and caries-promoting streptococci have been studied in controlled clinical trials (see reviews by Korhonen et al. 2000a, and Korhonen and Marnila 2006, Table 3).

Table 3. Selected human studies made with colostral immune preparations.

Target pathogen	Effect	Reference
Escherichia coli	Prevention of infection	Tacket et al. 1988
Escherichia coli	No therapeutic effect	Casswall et al. 2000
Escherichia coli	Lower incidence and shorter duration of diarrhea	Tawfeek et al. 2003
Shigella flexneri	Prevention of infection	Tacket et al. 1992
Shigella dysenteriae	No significant effect	Ashraf et al. 2001
Streptococcus mutans	Reduction of *S. mutans* in dental plaque	Loimaranta et al. 1999a, Shimazaki et al. 2001
Rotavirus	Prevention of infection	Ebina et al. 1985, Davidson et al. 1989
Rotavirus	Decreased severity of diarrhoea	Sarker et al. 1998
Cryptosporidium parvum	Reduction of diarrhoea	Tzipori et al. 1987, Okhuysen et al. 1998
Helicobacter pylori	No eradication, decreased severity	Oona et al. 1997
Helicobacter pylori	No eradication	Casswall et al. 1998
Clostridium difficile	Prevention of a relapse	van Dissel et al. 2005

Oona et al. (1997) studied the effect of bovine immune whey preparation with specific *H. pylori* antibodies in treatment of *H. pylori* positive children. The severity of symptoms and degree of gastric inflammation decreased. The degree of *H. pylori* reduced but total eradication was not observed. These results were consistent with those by Casswall et al. (1998). A prophylactic effect of a colostral immune preparation (IP) having specific anti- *H. pylori* antibodies was

demonstrated in a mouse model. Efficacy of the therapeutic treatment seemed to be related to the concentration of antibodies administered (Casswall et al. 2002). Rotavirus infections have also been successfully prevented and treated with milk preparations from hyperimmunized cows (Ebina et al. 1985, Davidson et al. 1989, Sarker et al. 1998). An anti-rotavirus antibody concentrate is also available commercially (Rota-colostrum®). Pant et al. (2007) combined *L. rhamnosus* GG with specific bovine colostrum-derived immunoglobulins. In an infant mouse model this combination had a significant prophylactic effect against rotavirus diarrhea.

Immune bovine colostral preparations against enterotoxigenic *E. coli* protected against infection (Tacket et al. 1988, Casswall et al. 2000). An immunoglobulin enriched bovine colostrum preparation, IMMULAC™ (New Zealand Dairy Group, Cambridge, New Zealand), containing antibodies against various bacterial antigens protected mice against ETEC infection (Funatogawa et al. 2002).

Immune bovine colostrum containing specific antibodies against *S. mutans* and *S. sobrinus* was capable of inhibiting the bacterial enzymes producing sticky capsule glycoproteins (Loimaranta et al. 1997), adherence of *S. mutans* cells to saliva-coated hydroxyapatite beads (Loimaranta et al. 1998), and augmenting the recognition, phagocytosis and killing of *S. mutans* by human leukocytes (Loimaranta et al. 1999b). In a short-term human study immune colostrum as a mouth rinse resulted in a higher resting pH in dental plaque and a lower proportion of caries streptococci in plaque microbial flora (Loimaranta et al. 1999a).

16 patients suffering from *Clostridium difficile* diarrhea received immune whey protein concentrate (MucoMilk) after antibiotic treatment. In all but one case, *C. difficile* toxins disappeared from the faeces upon completion of treatment. During a follow-up period none of the patients suffered another episode of *C. difficile* diarrhoea (Dissel et al. 2005). The safety of this product has been demonstrated (Young et al. 2007).

In humans, the oral administration of bovine milk immunoglobulins is generally well tolerated. Local passive immunization with bovine milk antibodies has the advantage of bypassing the host's own immune system. Orally administered immunoglobulins are degraded by intestinal proteases (Reilly et al. 1997) but IgG1 of bovine milk is quite resistant to pepsin cleavage and retains part of its immunological activity in the human intestine (Petschow and Talbott 1994, Roos et al. 1995, Warny et al. 1999). Further, pepsin cleaves IgGs to F(ab′) fragments, but F(ab′) also is able to neutralize antigens.

2.3   Selected gastrointestinal pathogens

2.3.1 *Helicobacter pylori* as a human pathogen

More than 50% of the world's population is infected by *H. pylori*. The infection is more common in developing than developed countries. In some developing countries up to 80% of population is infected by the age of 20 whereas in Finland the rate is less than 10% (Rautelin and Kosunen 2004). Low socioeconomic status, a *H. pylori* positive family member, high density of living, and low household income are associated with the infection (reviewed by Malaty 2007). The infection is usually acquired in childhood, and it rarely disappears spontaneously (Kosunen et al. 1997). Studies on the genetic diversity and geographical variation of *H. pylori* show that humans have carried *H. pylori* ever since they left East-Africa 58,000 3,500 years ago (Linz et al. 2007).

H. pylori infection is the most common cause of gastritis and is involved in peptic ulcer, gastric adenocarcinoma, and primary gastric B-cell lymphoma. 15-20% of those colonized by *H. pylori* develop gastric ulcer disease, and 1% gastric cancer. The pathogenesis depends upon strain virulence, host genetic susceptibility, and environmental cofactors. 65-80% of non-cardia gastric cancer is associated with *H. pylori* (reviewed by Kusters et al. 2006). According to recommendations, a one-week triple therapy using a proton pump inhibitor and two antibiotics is used to treat *H. pylori* infection (Malfertheiner et al. 2007). After eradication the active inflammation rapidly disappears in a few weeks (Valle et al. 1991). Chronic inflammation can persist for months or even years (Veijola et al. 2007).

H. pylori is a Gram-negative spiral shaped human pathogen. It produces urease enzyme, which makes the bacterium resistant to the acidic environment of stomach by neutralizing the gastric acid. Through its flagella it has a good motility to move fast to the mucus layer. *H. pylori* also uses the gastric mucus pH gradient for chemotactic orientation (Schreiber et al. 2004). For the attachment process on the mucous cells *H. pylori* uses complex adhesion molecules like BabA (blood group antigen-binding adhesin) binding to the fucosylated Lewis b (Le[b]) histo-blood group antigen (Ilver et al. 1998), SabA (sialic acid-binding adhesin) binding to the sialyl Lewis x (s-Le[x]) glycosphingolipid (Mahdavi et al. 2002), as well as other outer membrane proteins AlpA, AlpB, and HopZ (Peck et al. 1999) whose receptors are still unknown. The site of adherence is typically the intercellular junction of the epithelial cells.

H. pylori is able to protect itself by evading host defenses (immune evasion). TLRs initiate the innate defence against bacteria and activate the immune cells. TLRs recognize bacterial LPS, yet *H. pylori* LPS has less inflammatory activity than other enteric bacteria (Birkholz et al. 1993). There is a phenotypic variati-

on in molecular mimicry between *H. pylori* LPSs and human gastric epithelial cell surface glycoforms. Environmental factors can induce phase variation of polysaccharides in *H. pylori* that can aid adaptation of the bacterium to its ecological niche (Moran et al. 2002, Bergman et al. 2004).

H. pylori has virulence factors like cytotoxin-associated gene (*cag*)–pathogenicity island (PAI) and the vacuolating cytotoxin (VacA) contributing to the gastric diseases. CagA positive *H. pylori* strains are strongly associated with atrophic antral gastritis (Oksanen et al. 2000). After *H. pylori* adherence to epithelial cells, the type IV secretion system encoded by *cag*-PAI translocates the CagA protein into epithelial cells. Type IV secretion system is also involved in the induction of proinflammatory cytokines or chemokines, such as IL-8 in gastric epithelial cells (Fisher et al. 2001). The presence of an intact type IV secretion system allows *H. pylori* to colonize the gastric corpus. This results in atrophic corpus-dominant gastritis, thus highlighting type IV secretion system and CagA as major risk factors for gastric cancer development (Rieder et al. 2005). Possibly, *H. pylori* lacking a *cag*-PAI actively suppress host cell innate immune responses (Guillemin et al. 2002). Genomic studies from different geographic regions have shown that *cag*-PAI appeared to be disrupted in the majority of patient isolates throughout the world. *CagA* gene was present in 72.8% of strains (Kauser et al. 2004).

H. pylori can be detected by invasive and/or nonivasive tests (reviewed by Logan and Walker 2001). Invasive methods require endoscopy. *H. pylori* can be detected from biopsies by histology, culture, or rapid urease test. Urease test is based on the pH change caused by ammonia liberated by urease enzyme of *Helicobacter*. The tests contain urea and phenol red as a pH indicator. The noninvasive tests include serologic testing, stool antigen test, saliva and urine tests and urea breath testing (UBT). In UBT the patient is given orally urea labeled with nonradioactive ^{13}C or radioactive ^{14}C. In the presense of *H. pylori* the urease metabolizes urea to produce labeled CO_2 that can be measured in the patients' breath. Detection of antibodies by serological tests is a widely used method but it cannot prove ongoing infection due to immunological memory (Kusters et al. 2006). All these tests have their advantages and disadvantages, and there is no single gold standard in the diagnostic of *H. pylori* infection (Malfertheiner et al. 2007).

During last decades the occurrence of *H. pylori* has remarkably declined in industrial countries (Kusters et al. 2006). The rate and risk of *H. pylori* infection is high in cohorts born in the beginning of the 20th century, but is much lower in those born later due to a decrease in the rate of *H. pylori* acquisition in childhood (Sipponen 1997). The infection is curable with antibiotic therapy, but the strains multiresistant to antibiotics are a growing problem worldwide (Egan et al. 2007).

2.3.2 Animal models and cell line studies on *Helicobacter* infection

H. pylori is classified as a host specific pathogen. There are other *Helicobacter* species in different animals that do not infect humans (Fox 2002). *H. felis* was first isolated from gastric mucosa of cats (Lee et al. 1988). It has been extensively used in murine models of *Helicobacter* infection where it can induce gastritis, epithelial cell proliferation, and apoptosis (Gasbarrini et al. 2003, Kusters et al. 2006). During the 1990s the use of a mouse-adapted *H. pylori* strain (SS1) that is able to colonize the murine stomach was described (Lee et al. 1997).

The Mongolian gerbil model is also suitable for studying *H. pylori* infection. In Mongolian gerbils, gastric inflammation is much more evident than in the mouse model. Mongolian gerbils tolerate infection with type I *H. pylori* strains and may develop peptic ulcer or gastric cancer (Rieder et al. 2005). Guinea pigs, piglets, and nonhuman primate animals have also been used as animal models in studying *Helicobacter* infections (reviewed by Kusters et al. 2006).

Human gastric epithelial cell lines like HT29 cells, AGS cells, MKN45 cells, and KatoIII cells can be infected by *H. pylori* (Coconnier et al. 1998, Michetti et al. 1999, Sgouras et al. 2005, Takahashi et al. 2000, Kabir et al. 1997). They are commonly used to study the adhesion of *H. pylori,* the immune responses and gene expression in *H. pylori* infection *in vitro.*

2.3.3 Caries streptococci

Bacteria colonizing the tooth surface cause dental caries, one of the most common infectious diseases in the world. Cariogenic bacteria, mutans streptococci, consist mainly of two species, *Streptococcus mutans* and *S. sobrinus.* They are generally considered to be the principal ethiological agent of dental caries (Loesche 1986). The colonization of mutans streptococci requires the presence of hard surface and thus occurs only after tooth eruption. Cariogenic streptococci adhere to tooth by interacting with the salivary pellicle, co-aggregating with other bacteria, or via sticky polysaccharides they synthesise. The bacteria attached to tooth surface form the dental plaque. The presence of carbohydrates, especially glucose and saccharose supports acid production by *S. mutans* and results in a reduction in environmental pH, which in turn results in an overgrowth of mutans streptococci.

In oral cavity, saliva has a crucial role in maintaining the balance between cariogenic challenges, the environment, and the host defence. Saliva contains a large diversity of specific and non-specific antimicrobial factors. The non-specific defence factors with anti-streptococcal potential are peroxidase, lactoferrin,

histatins, lysozyme, mucins, complement, agglutinins, proteins rich with proline, and carbonic anhydrase (Loimaranta 1999). Specific factors contain immunoglobulins, mainly IgA and IgG.

2.3.4 Enterotoxigenic *Escherichia coli* in calves

Rotavirus, coronavirus, enterotoxigenic *E. coli,* and *Crytosporidium parvum* are the four major pathogens associated with neonatal calf diarrhoea worldwide (Achá et al. 2004). Nonpathogenic *E. coli* are part of the normal microbial flora of calves. Calves become infected with *E. coli* during or shortly after birth by fecal-oral transmission (Smith 1965). There are, however, types of *E. coli* who cause two common diseases of newborn calves namely coli-septicemia in systemic circulation and enteric colibacillosis in the mucosal surface of the small intestine (reviewed by Acres 1985). Virulence factors are crucial for the property of *E. coli* to cause a disease. Enterotoxigenic *E. coli* (ETEC) isolated from calves can produce heat stable toxins. Enterotoxins bind to receptor sites on the membrane of the intestinal epithelial cells and alter normal movement of ions and water across the mucosa by exerting a hormone-like effect on the enterocytes. The infection with ETEC thus leads to acute diarrhoea culminating in dehydration, metabolic acidosis, and death in severe cases (Acres 1985). ETEC contain pili or fimbriae, which allow them to colonize the small intestine by attaching to the epithelial cells. The most common fimbriae observed on ETEC from calves with diarrhea are F5, also named K99, and F41 (Achá et al. 2004).

ETEC causes diarrhoea mainly during the first days of life. Colostrum provides protection against diarrhoea if consumed before the infection occurs. Besides immunoglobulins nonspecific factors like lactoferrin, transferrin, and lactoperoxidase system are crucial for prevention of ETEC-infection (Acres 1985). Lactoferrin and transferrin have bacteriostatic effects against *E. coli* due to their ability to bind iron, which is essential for bacterial growth (Bullen et al. 1972). Milk also contains glycoconjugates, which have been suggested to serve as ligands for bacterial fimbriae and thus prevent the bacterial attachment to mucosa. However, the adhesion of ETEC to colostral gangliosides was considerably weaker than that to gangliosides from the milk (Martin et al. 2003).

2.4   Lactic acid bacteria as antimicrobial agents

2.4.1 Intestinal microbiota

The intestine is the largest immune organ of the body and it is metabolically the most active organ in humans. It provides a protective barrier against incoming bacteria (Ouwehand 2007). The human large intestine contains more than

800 different bacterial species (Bäckhed et al. 2005, Ouwehand 2007). The intestinal microflora is the major driving force in maturation of the immune system after birth (Grönlund et al. 2000, Kalliomäki et al. 2001). Its composition is mainly formed during birth and from the diet of the first month of life, but modifications in some aspects of cellular immunity can be achieved even in the elderly by a supplementation with an immunostimulating probiotic (Gill et al. 2001). In gastric contents the levels of bacteria are usually below 10^3 cfu/g due to the acidic environment. In small intestine the bacterial numbers range from 10^4 to 10^7 cfu/g and in large intestine typically up to 10^{12} cfu/g (for review, see Salminen et al. 1998).

2.4.2 Probiotic bacteria

Fermenting is a traditional food processing technology known for thousands of years. Lactic acid bacteria (LAB) have a dominant role in the fermentation of dairy products, meat, and vegetables to improve their aroma and texture, and to prevent spoilage (Caplice and Fitzgerald 1999).

Probiotics have been defined as live microbial feed supplements that have beneficial effects on the host by improving its intestinal microbial balance (Fuller 1989), or as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO 2002). A majority of probiotic bacteria belong to the families of lactobacilli or bifidobacteria. To benefit human health a probiotic must have good technological properties, it must survive through upper gastrointestinal tract, and it must be able to function in gut environment (Mattila-Sandholm et al. 2002). In commercial dairy products the concentration of probiotics is usually in the range of 10^8-10^9cfu/ml. The safety of probiotics is of prime importance. The safety aspects include the human origin, non-pathogenicity, and antibiotic resistance specifications of the strain (Saarela et al. 2000).

The promotion of the host defence systems, modulation of the immune system as well as the competitive exclusion of harmful microbes are thought to be key elements for the mechanisms by which probiotics exert their health effects (for review, see Saxelin et al. 2005). The probiotics are suggested to normalize the intestinal microbiota, have metabolic effects, and immunomodulation potential (Parvez et al. 2006). The probiotic strains differ in their characteristics, and it is therefore likely that selected mechanisms are also strain-specific. Various health benefits from consumption of probiotics are summarized in Table 4.

Some probiotic bacteria interact with immune cells in the mucosa. They can modulate cytokine and chemokine release, and regulate the immune responses in the intestinal mucosa (Isolauri 2001). For example, consumption of *Bifidobacterium lactis* HN019 enhanced the proportions of T cells in the peripheral

circulation, leukocyte phagocytosis, and tumoricidal activity (Gill et al. 2001). *Lactobacillus rhamnosus* GG (LGG) has been extensively studied for its health promoting effects in humans and animals. These benefits include prevention of diarrhea, allergy, alcohol induced liver disease, and colon cancer, reduction of caries risk, and an application as the adjuvant for vaccines (Goldin 1998, Näse et al. 2001, Kalliomäki et al. 2007).

Table 4. Suggested health effects of probiotics (Data from Parvez et al. 2006).

Effect on human health

Strengthening of innate immunity

Alleviating food allergy symptoms in infants

Controlling inflammatory bowel diseases

Lowering serum cholesterol

Improving lactose tolerance

Reducing risk factors for colon cancer

Controlling irritable bowel syndrome

Suppressing endogenous pathogens, e.g. antibiotic-associated diarrhoea

Suppressing exogenous pathogens, e.g. travellers diarrhoea

2.4.3 LAB against *H. pylori*

The *in vitro* inhibition effect of many LAB is associated with lactic acid (Midolo et al. 1995, Aiba et al. 1998). Lactic acid inhibits the urease system of *H. pylori* (Sgouras et al. 2004). Bacterial culture supernatants also exhibit anti-*Helicobacter* effect independent of lactic acid. The culture supernatants of strains of *L. acidophilus*, *Clostridium butyricum, Bacillus subtilis, Weissella confusa* and *Enterococcus faecium* inhibited the growth of *H. pylori* even in neutral pH (Coconnier et al. 1998, Michetti et al. 1999, Takahashi et al. 2000, Pinchuk et al. 2001, Kang et al. 2005, Nam et al. 2002, Tsai et al. 2004). The bactericidal effect of *L. acidophilus* CRL 639 was related to compound released after lysis (Lorca 2001). Lacticin A164 produced by *Lactococcus lactis* subsp. *lactis* was effective against *H. pylori* probably by accelerating cell lysis (Kim et al. 2003). Viable *L. casei* and strains of *L. acidophilus, B. longum, B. lactis,* LGG, *L. plantarum* 299v, *L. gasseri* OLL2716 (LG21), and *L. paracasei* have shown inhibitory activity against *H. pylori* growth *in vitro* (Cats et al. 2003, Sgouras et al. 2004, Wang et al. 2004, Vilaichone et al. 2002, Chatterjee et al. 2003, Hütt et al. 2006, Ushiyama et al. 2003).

W. confusa PL9001, *C. butyricum* and *L. salivarius* inhibited *H. pylori* attachment in MKN45 cells likely mechanism being nonspecific steric hindran-

ce, maybe partially extracellular secretory product (Kabir et al. 1997, Takahashi et al. 2000, Nam et al. 2002). The spent culture supernatants of *L. acidophilus* LB and *E. faecium* decreased the adhesion of *H. pylori* on human cells (Coconnier et al. 1998, Tsai et al. 2004). Selected *L. reuteri* strains inhibited the binding of *H. pylori* to specific glycolipid receptors asialo-GM1 and sulfatide. Strains from intestine or feces but not from fermented molasses exhibited binding to the glycolipids. It was suggested that the glycolipid-binding protein is associated with cell surface (Mukai et al. 2002).

Some probiotic LAB reduce the severity of inflammation in *Helicobacter* infection (Aiba et al. 1998, Sakamoto et al. 2001, Sgouras et al. 2005). IL-8 secretion by AGS and MKN45 cells was reduced with *L. salivarius,* LG21, and *L. johnsonii* La1 spent culture supernatant, but the supernatant did not affect *H. pylori* viability. Suggested mechanism is reducing proinflammatory chemotactic signals responsible for the recruitment of lymphocytes and neutrophils in the lamina propia (Kabir et al. 1997, Ushiyama et al. 2003, Sgouras et al. 2005, Tamura et al. 2006).

LG21, *L. casei* strain Shirota, and *L. salivarius* decreased the number of *H. pylori* in infected mice (Ushiyama et al. 2003, Sgouras et al. 2004, Aiba et al. 1998). Cure of persistent infection with *H. pylori* in mice was demonstrated following infection with *C. butyricum* (Takahashi et al. 2000). Oral treatment with *L. acidophilus* LB-supernatant protected conventional mice against *H. felis* –infection but did not eradicate an existing *Helicobacter* infection (Coconnier et al. 1998). *L. salivarius* prevented *H. pylori* infection in gnotobiotic BALB/c mice and eliminated colonization by *H. pylori* (Kabir et al. 1997). *H. pylori* colonization and inflammation but not apoptosis were reduced in mice treated with live *L. rhamnosus* R0011 and *L. acidophilus* R0052 (Lactofil) in drinking water prior to *H. pylori* SS1 infection. The mechanism was attributed to bactericidal effects (Johnson-Henry et al. 2004).

The effect of *L. johnsonii* La1 on *H. pylori* infection as such or in combination with antibiotic treatment has been carried out in a number of double blind, randomized, controlled clinical trials. According to values of urea breath tests the number of *H. pylori* was reduced following treatment with La1 suggesting that regular intake of La1 contribute to restrict the size of the population of *H. pylori*, and may delay colonization (Michetti et al. 1999, Felley et al. 2001, Cruchet et al. 2003, Gotteland et al. 2003, Pantoflickova et al. 2003). Inflammation and the severity of gastritis also decreased during treatment. It was suggested that La1 could adhere in the stomach and act through a direct bacterial interference, secrete anti-bacterial substances, or to modify immunological response of the host (Felley et al. 2001). Triple therapy supplemented with a fermented milk product containing *L. casei* DN-114 001 (Actimel) enhanced the therapeutic benefit in a randomized double blind controlled study (Sykora et al. 2005). In other hu-

man studies a treatment with *Saccharomyces boulardii, L. brevis, L. acidophilus*, LG21, *B. lactis,* or *L. casei* had a suppressive effect on *H. pylori* colonization (Gotteland et al. 2005, Linsalata et al. 2004, Mrda et al. 1998, Canducci et al. 2000, Sakamoto et al. 2001, Cats et al. 2003, Wang et al. 2004). A strong inhibitory activity against *H. pylori in vitro* does, however, not guarantee a suppressive effect *in vivo* (Wendakoon et al. 2002). Negative results from clinical trials have been reported also by Shimizu et al. (2002), Opekun et al. (2005), and Goldman et al. (2006).

As seen in clinical studies, a regular intake of probiotics does not eradicate *H. pylori*. However, it contributes to maintaining lower concentrations of *H. pylori* in a *H. pylori* positive at-risk population (Gotteland et al. 2005). Further, many studies report on the reduced side effects and increased tolerability during treatment with triple therapy by a supplementation of LGG, *S. boulardi*, *Lactobacillus* spp. & bifidobacteria, *L. reuteri* ATCC 55730, *L. rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS, *L. casei* subsp. *casei* DG, and *C. butyricum* MIYAIRI 588 (Lykova et al. 1999, Armuzzi et al. 2001a, b, Cremonini et al. 2002, Tursi et al. 2004, Myllyluoma et al. 2005, Shimbo et al. 2005, Lionetti et al. 2006).

2.4.4 LAB against *S. mutans*

The literature concerning probiotic bacteria and oral health is still scarce (Twetman and Stecksén-Blicks 2008). The oral cavity becomes colonized by various species of indigenous microflora during the first year of life. Some probiotic bacteria can be found from mouth during and some weeks after an intervention but a permanent colonization does not take place in an established microflora of adults (Meurmann et al. 1994, Busscher et al. 1999, Yli-Knuuttila et al. 2006, Krasse et al. 2006). LAB with probiotic properties can, however, be found in the oral cavity (Meurman and Stamatova 2007). Haukioja et al. (2006) tested the colonization potential of a number of LAB strains and found that lactobacilli showed better adherence than bifidobacteria to oral surfaces.

The treatments with probiotic bacteria have shown beneficial effects on oral microflora by reducing the number of *S. mutans* (Table 5). LGG, *L. casei,* and *L. reuteri* have shown a potential to reduce the risk of caries (Meurman et al. 1994, Näse et al. 2001, Ahola et al. 2002, Busscher et al. 1999, Nikawa et al. 2004). Also consumption of *Bifidobacterium* DN-173 010 resulted in a reduction in salivary mutans streptococci (Caglar et al. 2005). Further, probiotic bacteria can be effective in controlling other oral pathogens like yeasts (Hatakka et al. 2007). More studies are, however, needed to confirm the role of probiotics in oral cavity (Meurman and Stamatova 2007).

Table 5. Controlled clinical intervention studies on the effect of probiotic bacteria on the salivary levels of mutans streptococci (Data from Twetman and Stecksén-Blicks 2008).

Probiotic	Effect on mutans streptococci	Reference
L. rhamnosus GG	Decreased counts	Näse et al. 2001, Ahola et al. 2002
L. reuteri	Decreased counts	Nikawa et al. 2004, Caglar et al. 2006
Lactobacillus spp.	No change in counts	Montalto et al. 2004
Bifidobacteria	Decreased counts	Caglar et al. 2005

3   Aims of the study

The aim of the present study was to investigate the effects of bovine colostrum, specific colostral antibodies, and some lactic acid bacteria on gastrointestinal infections; especially *Helicobacter pylori* induced gastritis and dental caries. The effect of colostrum on the activity of the complement system in neonatal calves was also studied.

The specific aims of the study were

*   To evaluate the effect of a commercial immunoglobulin concentrate made from pooled colostral whey on the complement-mediated bacteriolytic and opsonisation activities in the sera of neonatal calves.

*   To investigate the effectiveness of a bovine colostral control preparation and IP containing specific antibodies against *H. felis* in the prevention and treatment of *H. felis* infection in mice, and to test the efficacy of a combined treatment with specific antibodies and amoxicillin on *H. felis* infection.

*   To investigate whether IP antibodies remain active when stored in UHT -treated or in fermented milk, and whether there is an inhibitory, additive or synergistic effect with LGG in preventing the adherence of *S. mutans* to saliva-coated hydroxyapatite.

*   To test and locate the *in vitro* anti-*Helicobacter* activity of strains belonging to *Lactobacillus plantarum* group.

*   To study the adhesion of *H. pylori* pretreated with LAB and colostral preparations in an AGS cell model and to investigate the effect of LAB and colostral preparations on IL-8 production by *H. pylori* infected AGS cells.

4 Materials and methods

4.1 Bacterial strains, cell lines and culture conditions

The bacterial strains used in this study are listed in Table 6. Detailed descriptions of cultivation media and culture conditions are presented in studies (I-V).

Table 6. The bacterial strains used in the study.

Strain	Source	Study
E. coli strain IH3080	University of Helsinki, Finland	I
E. coli strain JM103 with plasmid pCSS962/pGB3	Lampinen et al. 1992, University of Turku, Finland.	I
H. felis strain CS1		II
H. pylori strain NCTC11637	University of Helsinki, Finland	II, IV-V
Lactococcus lactis MG1363	Gasson 1983	V
L. paraplantarum DSM 10641		IV
L. paraplantarum DSM 10667		IV
L. paraplantarum DSM 14485	Curk et al. 1996	IV
L. pentosus Vege-Start 60	Chr. Hansen A/S, Copenhagen, Denmark	IV
L. plantarum DSM 20174 (ATCC 14917)		IV
L. plantarum MLBPL1	isolated from sauerkraut by MTT	IV, V
L. plantarum Vege-Start 60	Chr. Hansen A/S, Copenhagen, Denmark	IV
Lactobacillus rhamnosus GG (LGG)	ATCC 53103	II, V
S. mutans ATCC 25175 (serotype c)		III

Human AGS-cells (CRL-1739) from American Type Culture Collection (Manassas, VA) were cultured in RPMI 1640 medium + GlutaMAX 1 (Invitrogen, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) in a 5% CO_2 atmosphere at 37°C. For the assays described here, cell suspension was placed in each well of a flat-bottom 96-well CellBind plate (Corning Inc., Corning, NY, USA), and the plate was incubated at 37°C under 5% CO_2 atmosphere for 2-3 days.

4.1.1 Fractions from *L. plantarum* MLBPL1 cultures (IV)

Lactobacilli were cultivated in MRS broth (de Man et al. 1960). Cell-free culture supernatants from LAB were obtained by centrifugation. A whole cell lysate of *L. plantarum* MLBPL1 was obtained by mixing harvested cells with glass beads, and homogenizing by vigorous shaking. Cell wall fragments and intracellular fraction were separated from the whole cell lysate by centrifugation.

The cell-free culture supernatant of *L. plantarum* MLBPL1 was filtered to obtain fractions <3, >3, <10, and >10 kDa. The pH of the culture supernatant was adjusted to 6.3. The supernatant fluid was then precipitated with ammonium sulphate. The precipitate was collected by centrifugation and dissolved into 10 mmol/1 phosphate buffer, pH 7.0. Samples of the crude and precipitated culture supernatant were incubated at 100°C for 10 min.

Samples of *L. plantarum* MLBPL1 cell-free culture supernatant, precipitated with ammonium sulphate, were treated with α-amylase, pepsin, proteinase K, N-glycosidase F, or neuraminidase. The anti-*Helicobacter* activity of the preparates obtained was tested by measuring inhibition zones on Brucella agar plate as described later.

4.2 Colostral immune preparations

4.2.1 Production of colostral preparations (II, III, V)

The immunization protocol was approved by the Animal Care Committee of MTT Agrifood Research Finland. Pregnant Friesian cows were injected intramuscularly on both sides of the neck with vaccine consisting of 10% (v/v) inactivated bacterial suspension with $Al(OH)_3$ as adjuvant. Immunization was started at the onset of drying off about 2 months before the predicted day of parturition. Four booster injections were given at 1-week intervals starting 2 weeks from the initial injection. The cows were healthy and did not receive any antibiotics during the immunization. The first five milkings after calvings were collected and frozen immediately. The calves were fed with colostrum from other cows of the same herd.

The process for colostral preparations is presented in Figure 3. Colostra were thawed, pooled, and then heated to +37°C. Fat was removed with a separator, casein was coagulated with chymosin, and lactose was hydrolysed with lactase enzyme. Coagulated casein was removed with cheese pressing equipment. Sugars and salts were removed from the whey by chromatographic and membrane-filtering techniques, as described by Loimaranta et al. (1997). The protein

concentrate obtained was sterilized by microfiltration and lyophilised at -45°C to obtain immune preparation (IP). A control preparation (CP) was made similarly from colostra of non-immunized cows.

The total protein content of the preparations was determined by the Kjeldahl method. The IgG content was measured by a chromatographic method as described by Syväoja and Korhonen (1994). Concentrations of IgA and IgM were assessed with RID kits, and IGF-1, TGF-β1, and TGF-β2 by enzyme-linked immune sorbent assay (ELISA) methods.

To inactivate the complement system, samples of IP and CP were incubated at 56°C for 30 minutes. To remove the small molecular weight components and to further enrich the antibody fraction of IP, the powder was dissolved in water and filtered through a 100 kD membrane and lyophilised.
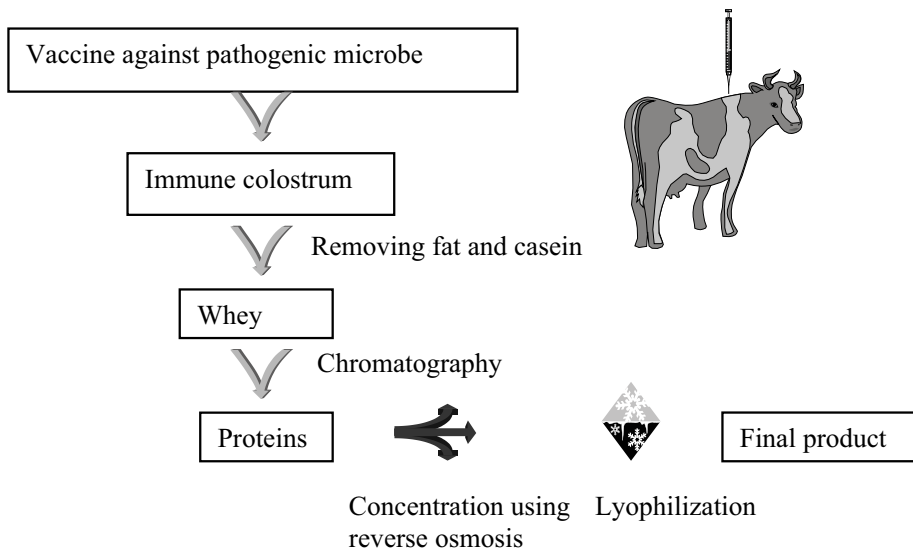
Figure 3. Production of bovine colostral immune preparations with specific antibodies (IP).

4.2.2 Stability of IP and LGG in milk (III)

The ability of bovine antibodies to retain their activity was tested by storing them in a commercial UHT treated milk-based drink for toddlers (Muksu-Milk, Valio Ltd, Turenki, Finland). 5 ml of sterile 15% (w/v) solution of caries IP was injected into the carton to obtain a final concentration of 0.375%. Injected cartons and uninjected control cartons were incubated in triplicate at three diffe-

rent temperatures: 5°C, 21°C, and 30°C. The antibody concentrations and the ability of antibody-supplemented Muksu-Milk to inhibit the adherence of *S. mutans* cells to salivacoated hydroxyapatite were measured, as described below, at the beginning of the test and after storage for 1 and 2 months.

Fresh cow's milk, supplemented with IP was fermented with LGG and stored for 7 weeks in order to evaluate the possible effects of fermentation and storage of antibodies together with LAB on antibody titres and functions (Figure 4). Fresh milk was first centrifuged to remove fat, large casein particles, bacteria, and somatic cells. IP and CP were mixed with pasteurized milk to obtain a final IP concentration of 5% (w/v). After LGG inoculation the milk samples were incubated at 37°C for 48h, and stored at 4°C. To assess the effect of low pH on antibodies and LGG, both IP milk and non-IP milk were also fermented in 50 mM HEPES buffer to maintain pH 7 during fermentation and storage. pH, lactic acid content, and number of viable cells were determined during the fermentation and storage periods. Aliquots of IP containing samples were analysed for antibody titres by ELISA.

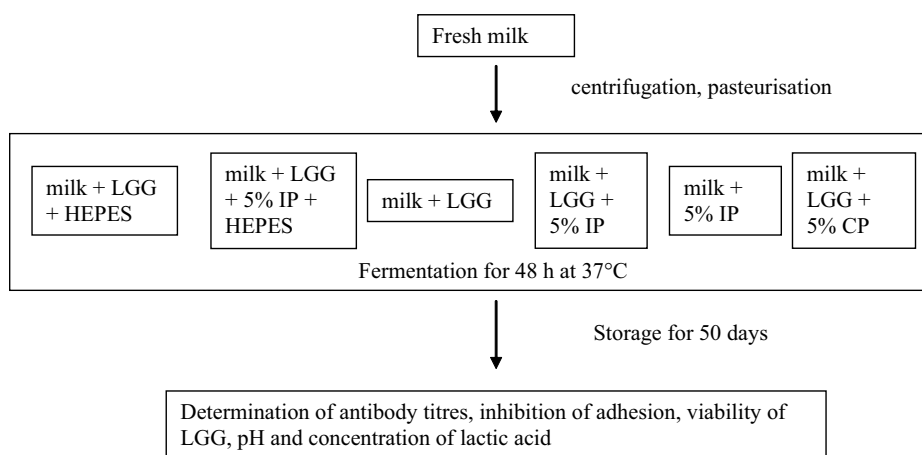Figure 4. Study design for the fermentation of the anti-caries immune preparation (IP) or the control preparation (CP) with *L. rhamnosus* GG (LGG) (10^7 cfu/ml).

4.3   Analytical methods

4.3.1 Determination of specific antibodies (I, II, III)

Specific antibodies in bovine colostrum, milk, and serum samples were measured by ELISA. Briefly, microtitre plates were coated with the antigen. After

overnight incubation at 4°C, the wells were washed with PBS containing 0.05% Tween 20 (PBST) and rinsed three times with deionized water. Diluted samples were added to the antigen-coated plates. After 90 min incubation at 37°C the wells were washed as above and a secondary anti-bovine IgG or anti-mouse IgG antibody with alkaline phosphatase conjugate was added for 90 min at 37°C. After washing, the plates were developed with p-nitrophenylphosphate disodium salt in diethanolamine-$MgCl_2$ buffer, pH 10.0. The absorbance values at 405 nm were recorded with a Multiskan MCC/340 spectrophotometer (Thermo Labsystems, Vantaa, Finland). Endpoint titres of specific antibodies were determined at a cut-off level of 0.5 from OD_{405}.

4.3.2 *In vitro H. pylori* growth inhibitory activity (II, IV)

The bactericidal activity of colostral preparations was measured by a plate-counting method, as described by Korhonen *et al.* (1995). Briefly, a sample containing $2x10^7$ bacteria/ml was prepared by mixing *H. pylori* suspension, sample (IP, CP, and heated preparations), and 5% FBS with or without active complement. The test samples were incubated under microaerophilic conditions at 37°C for 2 hours. After that, 10-fold dilutions (from 10^{-2} to 10^{-8}) were made from samples, and cultured on Brucella agar plates. Colony forming units (cfu) were counted.

Inhibition of the growth of *H. pylori* by LAB culture supernatants and cellular fractions obtained as described above was screened as in Wendakoon et al. (1998). Briefly, *H. pylori* suspension containing ca. 10^8 bacteria was spread on Brucella agar plates. Wells were cut with a sterile straw and filled with a LAB sample. Amoxicillin trihydricum (Orion Diagnostica, Espoo, Finland) in concentrations of 0.2–1 mg /1 was used as a positive control, and MRS broth as a negative control. Plates were incubated at 37°C under microaerophilic conditions for 3 days, after which the diameters of the inhibitory zones were measured.

4.3.3 Infection studies with AGS cells (V)

H. pylori bacteria were harvested from 1-2 days solid cultures and resuspended into PBS to a concentration of ca. 10^9–10^{10} cfu/ml. Cells of the grown LAB cultures were counted in Bürker's chamber, and the concentration of cultures was adjusted to 10^{10} cfu/ml with PBS. Samples of the grown LAB cultures were inactivated by heating at 80°C for 20 min. Before infection of AGS cells on microtitre plates, *H. pylori* were pretreated with different concentrations of CP, IP, or LAB for 40 minutes at 37°C. An AGS cell-to-bacteria ratio of 1:40 was used in the infection. Adhesion test was carried out as described by Shmuely

et al. (2004). After 2 hours infection AGS cells were washed with PBS. Urease test was performed by adding 100 µl of urease test solution (7 mM phosphate buffer pH 6.8, 110 mM urea, 10 mg/l phenol red) into each well of the microtitre plate. After a reaction time of 10-30 min, absorbance values at 540 nm were recorded with a Multiscan MCC spectrophotometer. The relative adhesion level was calculated by dividing the absorbance value of the sample with the absorbance value of PBS control containing the same amount of *H. pylori*.

For the determination of IL-8, the culture media of AGS cells were collected after 20 h infection, centrifuged, and kept at −20°C until assayed for IL-8. IL-8 levels were determined by ELISA (Bender Medsystems, Vienna, Austria) according to the manufacturer's protocol. Absorbance values at 450 nm were measured with a Multiscan MCC spectrophotometer.

4.3.4 Adherence to hydroxyapatite (III)

The effect of pretreatment with fermented IP and UHT milk samples on the adherence of mutans streptococci to hydroxyapatite was studied as described in Loimaranta et al. (1998). Briefly, rehydrated spheroidal hydroxyapatite beads (BDH Chemicals Ltd, Poole, UK) were treated for 60 min with human parotid saliva diluted 1:2 with adhesion buffer (1.0 mM potassium phosphate, 50 mM KCl, 1.0 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH 6.5). Uncoated sites were blocked with 0.5% human albumin (Sigma). Saliva-coated beads were treated with the sample before the addition of *S. mutans* labelled with L-[^{35}S]methionine (NEN Research Products, Du Pont, France). Unbound bacteria were removed by washing with the adhesion buffer and the bound activity was measured by liquid scintillation counter (Microbeta, Wallac, Finland).

4.3.5 Measurement of complement-dependent *E. coli* bacteriolytic activity (I)

The serum samples of newborn calves were from a previous experiment carried out by Nousiainen et al. (1994). Briefly, twenty-one calves were divided into three groups, and given one of three feeding regimens on the first day of life. The control group was fed normal pooled whole milk and pooled colostrum with total immunoglobulin intake 19.5 g. The Treat1 and Treat2 groups were fed normal pooled whole milk, pooled colostrum, and an immunoglobulin concentrate giving a total immunoglobulin intake of 52.7 and 119.0 g, respectively. From day 2 the calves were fed similarly. Blood samples were drawn on days 2, 7, 14, and 30 after birth. The sera were stored at -20°C for analysis.

Bacteriolytic activity was measured by a method modified from that described by Virta et al. (1995, 1998). The assay is based on a complement-sensitive

nonencapsulated *E. coli* strain JM103 containing a luciferase structural gene. In the presence of luciferine, luciferase converts the chemical energy associated with ATP into green light, which can be measured with a luminometer. Thus, the amount of light emitted is proportional to the number of metabolically active cells (Griffiths 1996). The survival of bacteria in the serum samples was calculated relative to that in heat-inactivated (56°C, 30 min) FBS. The results were determined as a serum dilution with a chemiluminescence emission 50% of that of blank samples containing a dilution buffer instead of a serum sample. This dilution represents a lethal dose 50% value (LD50) for the serum sample.

The serum samples were thawed and diluted serially with Hank's balanced salt solution (HBSS) containing heat-inactivated FBS. Samples in duplicates were incubated with 3.6×10^6 cells of *E. coli* JM103 in microtitre plate wells for 90 min at 37°C. After that, 0.25 mM D-luciferine (Bio-Orbit, Turku, Finland) in 0.1 M disodiumcitrate (pH 5.0) was added and the bioluminescence emission was measured for 1 hour with a Luminoskan EL1 luminometer (Labsystems, Helsinki, Finland).

The alternative pathway of complement was analysed by blocking the classical pathway with 10 mM EGTA in Ca^{2+}- and Mg^{2+}-free HBSS. The inactivation of *E. coli* was measured as described above.

4.3.6 Opsonisation and phagocyte activation (I)

A complement-insensitive *E. coli* strain IH3080 was used as a model for serum opsonisation activity against bacteria. A mixture in HBSS containing 1% of serum sample from calves and 2×10^9 *E. coli* was shaken for 35 minutes at 37°C. After centrifugation, the pellet was suspended in HBSS and stored at -20°C. The control samples contained heat-inactivated FBS instead of serum samples.

The phagocyte oxidative burst activity was measured as luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) enhanced chemiluminescence (CL) emission. The reaction mixture consisted of 2.5×10^4 leukocytes isolated from the blood of a healthy calf as described by Robinson (1993), and 0.4 mM luminol in 0.1% gelatin-HBSS. The CL reaction was stimulated with *E. coli* cells opsonised either with heat-inactivated FBS or with the serum samples (2.5×10^6 cells/well). The CL response was measured in four replicates at 90 s intervals for 1 hour at 37°C with a luminometer. The opsonisation capacities are expressed as the relative increase in the oxidative burst activity of the phagocytising leukocytes evoked by opsonised *E. coli* as compared to that of nonopsonised *E. coli*.

4.3.7 C3 content (I)

The relative content of the complement C3 component in the sera was estimated by the radial immune diffusion method. In brief, anti-bovine C3 serum (Cappel, Organon Teknika, Durham, NC, USA) was diluted 1:80 in 2% Sea-Kem LE agarose in Mancini buffer and poured on petri dishes. After cutting wells with a diameter of 3 mm, serum sample was applied to each well. After 72 h incubation at +4°C in a humid chamber, the precipitation rings were stained with PhastGel Blue R (Pharmacia LKB Biotechnology, Uppsala, Sweden) and the diameter of the precipitant was measured. FBS was used as a standard.

4.4   Infecting mice with *H. felis* (II)

The protocols for the infection and treatment of the mice were approved by the local Animal Care and Ethics Committee. Balb/c mice were obtained from the Laboratory Animal Centre of the University of Helsinki, and SJL mice from the Laboratory Animal Centre of the University of Turku. *H. felis* CS1 was cultured on Brucella agar as described above, and suspended in PBS. The mice were infected on three consecutive days by oral administration of 0.1 ml PBS containing 10^8 cells of *H. felis*. Uninfected mice were used as healthy controls. The mice were maintained in conventional conditions and allowed free access to non-sterile food and water.

The *H. felis* status of infected mice was assessed by bacteriological stainings (Gram and acridine orange stains), gastric histology (Giemsa and hematoxylin-eosin stains), and by measurement of anti- *H. felis* antibodies in sera by the ELISA method.

4.4.1 Treatment of gastritis in mice (II)

The efficacy of IP, CP, complement, and amoxicillin against murine *H. felis* infection was tested in three consecutive trials (Figure 5, II). 10 + 17 mice served as infected controls. In the preliminary experiments, the amoxicillin trihydricum (Orion Diagnostica, Espoo, Finland) dosage of 40 µg/g for 6 days was shown to eradicate *H. felis* from some but not all of the animals (data not shown). Thus, it was assumed that this was a proper dosage to test the combined effect of IP and amoxicillin treatments. In trial 1 the mice were killed 4-5 weeks after the end of the treatment. Since no eradication but only a lowered level of colonization was expected the mice were killed immediately after the treatment in trials 2 and 3 in order to prevent reinfections or spread of *H. felis*.

Infection of mice with *H. felis*

Trial 1	Trial 2	Trial 3
IP (n=19) CP (n=10) administered for 14 days two times per day	IP + NBS (n=8) CP + NBS (n=10) Inact. IP (n=10) Inact. CP (n=10) NBS (n=6) administered for 16-18 days three times per day	IP + NBS (n=20) IgG of IP + NBS (n=15) Amoxicillin (n=8) IgG of IP + NBS + amoxicillin (n=10) administered for 28-29 days three times per day

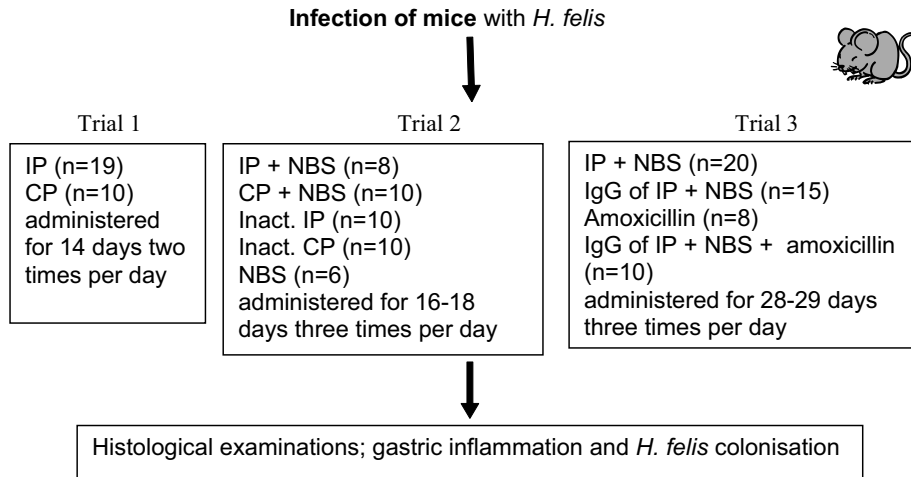Histological examinations; gastric inflammation and *H. felis* colonisation

Figure 5. Study design for treating *H. felis* infected mice with colostral immune preparation (IP) containing specific antibodies against *H. felis*, control preparation (CP), complement source (NBS, normal bovine serum), complement inactivated IP and CP, concentrated IP (IgG of IP), or amoxicillin. Infected and uninfected mice without treatment were used as infected and healthy controls. The time between infection and treatment was 1 week in trial 1, and 2 months in trials 2 and 3.

4.4.2 Preventing infection with immune and control preparation (II)

The prophylactic efficacies of IP containing specific antibodies against *H. felis*, and CP were studied by giving Balb/c mice IP or CP orally. The administration of colostral preparations started two days before, and ended five days after the *H. felis* administration. Infected controls received phosphate buffered saline (PBS) instead of IP or CP. Uninfected mice served as healthy controls. The mice were killed from seven to eight weeks after the *H. felis* inoculation. Blood samples were taken from the orbital venous plexus and the stomachs were dissected and prepared for the assessment of *H. felis* status, colonization and inflammation.

4.5 Statistical analyses

Data concerning infection status of mice were analysed with exact chi-square test. The results for the inhibition of adhesion of *S. mutans* to hydroxyapatite particles were analysed with Wilcoxon's non-parametric sign test. For calves, the covariance structure of the repeated measurements was chosen by comparing several potential structures using Akaike's information criterion (Wolfin-

ger 1996). Data concerning the adhesion of *H. pylori* and IL-8 production by AGS cells were analysed using analysis of variance for randomised complete block design by considering each replicate as a blocking factor and the combination of the sample, its activity level, and concentration as a treatment factor. Pairwise comparisons were performed using two-sided t-type tests. The parameters of the models were estimated by the restricted maximum likelihood estimation method and degrees of freedom were calculated using the Kenward-Roger method (1997). The data were analyzed using SAS Statistical software for Windows releases 6.12, 8.01, and 9.1.3 and SAS Enterprise Guide release 4.1 (SAS Institute Inc., Cary, NC, USA).

5   Results

5.1   Composition and stability of colostral preparations (II, III, V)

Cows tolerated the immunization process well, and no severe side effects were observed. Before immunization, the levels of specific antibodies in the sera of the cows were negligible. The immunization protocol resulted in the development of a specific antibody response in all cows (Figure 6). Typical compositions of colostral preparations are presented in Table 7.
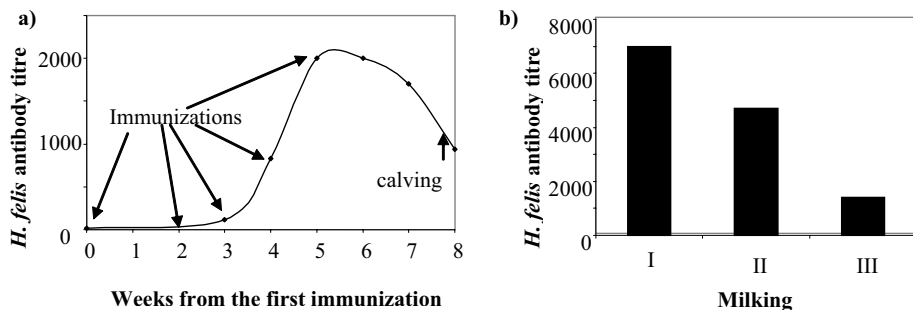

Figure 6. Typical titres of *H. felis* specific IgG antibodies in the serum (a) and in the three first milkings after calving (b) of an immunized cow.

IP did not contain any cross-reactive antibodies against LGG, whereas specific activities towards *S. mutans, H. pylori*, and *H. felis* of respective IPs were high. The antibody titre for *H. pylori* -specific IgG were still on a high level after 10 years storage at -20°C. The concentrations of anti-caries IgG antibodies of IP remained unchanged during storage at 5°C, 21°C, and 30°C for 2 months in UHT milk drink. The presence of live LGG or HEPES had no significant effect on IP antibodies during the 50-day storage at 4°C. However, during this long storage period, there was a steady decline in titres from 10 days after the beginning of storage, and after 50 days about 30% of the original activity was left. During the 50 days storage at 4°C, IP did not have any notable effect on the growth of LGG.

Table 7. Major composition of colostral preparations. N= number of preparations analyzed.

	n	Immune preparation	Control preparation
protein	3	68-90% (w/w)	71-91% (w/w)
IgG	3	42-52% (w/w)	44-52% (w/w)
IgA	1	7% (w/w)	3.2% (w/w)
IgM	1	4.3% (w/w)	4.9% (w/w)
IGF-1	1	1.2 mg/g	0.22 mg/g
TGF-β1	1	0.11 mg/g	0.13 mg/g
TGF-β2	1	1.5 mg/g	1.3 mg/g

5.2 Dependence of the bactericidal activity of colostral preparations on the presence of active complement (II)

In vitro, both CP and IP were bactericidal against *H. pylori.* Colostral preparations reduced the *H. pylori* cfu by two orders of magnitude from 10^7 to 10^5. Similar incubation with normal bovine serum and colostral preparations supplemented with serum killed all the *H. pylori* in the samples. The bactericidal activity was solely dependent on the presence of active complement in the solutions. When the preparations were heated to 56°C for 30 min, no bactericidal activity was detected. The bactericidal activity was restored by the addition of a complement source (FBS).

5.3 Substantial increase of the complement activities of serum by feeding colostral whey concentrate to neonatal calves (I)

Two groups of neonatal calves were fed different amounts of immunoglobulin concentrate for the first two feedings. The sera of both immunoglobulin concentrate groups had 2-3 times higher bacteriolytic activity than the control group of both the classic ($P<0.005$) and alternative ($P<0.0005$) pathways of complement at days 2 and 7 after birth (Figure 7). At day 30 there were no differences between the groups except, in the Treat2 group, where the activity of the classic pathway was still higher at day 30 than in the controls ($P=0.03$). No differences in the complement component C3 contents between the groups were observed.

The C3 levels increased significantly in all groups during the study ($P<0.0001$). In both immunoglobulin concentrate treated groups the initial *E. coli* JM103-specific antibody levels were high and decreased towards the control values in the course of time.

The group fed highest amount immunoglobulins had the highest serum *E. coli* IH3080 opsonisation capacity. At day 30, the capacities of the control group had risen to the same level as those of the immunoglobulin concentrate treated groups.
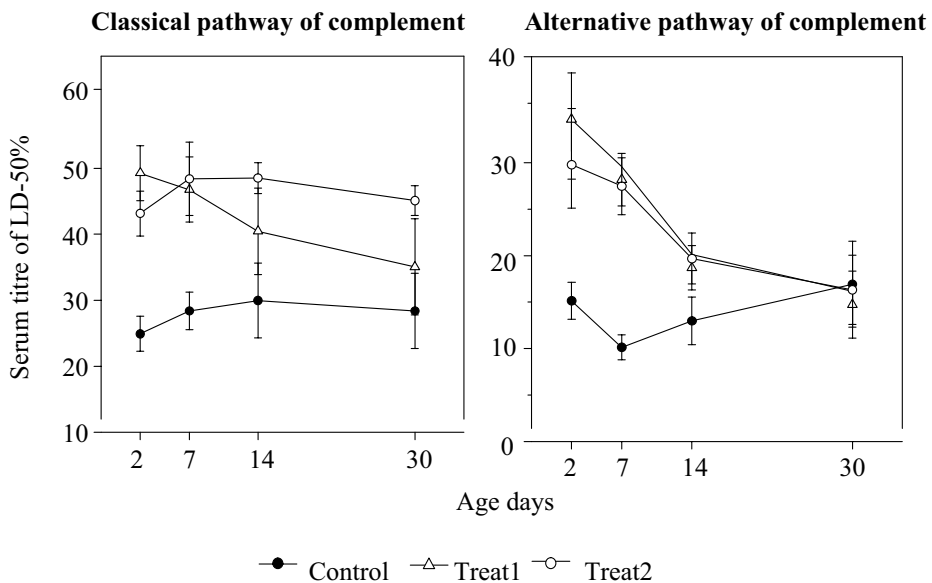

Figure 7. The effect of immunoglobulin concentrate fed on first two feedings on the serum complement activities of calves during the first 30 days of life. Bars represent estimated means ±SE.

5.4   Growth inhibition of *H. pylori* by *L. plantarum* MLBPL1 (IV)

The inhibitory effect of culture supernatants of seven *L. plantarum* -related lactobacilli against *H. pylori* was screened by measuring the diameters of inhibitory zones on plate. All members of the *L. plantarum* group showed some anti-*Helicobacter* activity, but the culture supernatants of the strains *L. plantarum* MLBPL1, isolated from sauerkraut, and *L. pentosus* from a commercial starter Vege-Start 60 showed the clearest inhibition. The inhibitory activities of MLBPL1 cells cultivated in cucumber juice, carrot juice or whey were similar to the inhibitory activity of MLBPL1 cells cultivated in MRS broth. In addition

to the culture supernatant, whole cells as well as cell lysate and cell wall fragments of *L. plantarum* MLBPL1 contained anti-*Helicobacter* activity. The intracellular fraction did not significantly inhibit the growth of *Helicobacter*.

The active substance of the culture supernatant from a MRS-grown *L. plantarum* MLBPL1 culture was concentrated by ammonium sulphate precipitation. This precipitate retained its anti-*Helicobacter* activity after 10 min treatment at 100°C, and at neutral pH, whereas the crude culture supernatant lost its activity if pH was raised. It was not possible to remove the anti-*Helicobacter* activity of MLBPL1 precipitate by incubation with any of the proteolytic enzymes tested. On the contrary, incubation with N-glycosidase F slightly increased the anti-*Helicobacter* activity of MLBPL1. The substance with anti-*Helicobacter* activity in the cell-free culture supernatant was found to be between 3,000 and 10,000 daltons in size.

5.5 Reduction of the adherence of *H. pylori* and *S. mutans* by colostral preparations and live LAB (III, V)

Fresh milk and toddler's milk based drink without IP slightly enhanced the adherence of *S. mutans* to saliva-coated hydroxyapatite, but products containing anti-caries-IP displayed significantly lower adherence of *S. mutans* to saliva-coated hydroxyapatite. There were no marked differences between different temperatures. IP with specific *H. pylori* antibodies, but also CP reduced the adhesion of *H. pylori* on AGS-cells in a dose-dependent way. The anti-adhesive activity was present also when the complement system of colostral preparations was inactivated by heating, but not as strong as with active IP and CP.

LGG-fermented milk without antibodies also inhibited the adherence of *S. mutans* by about 40% (Figure 8). This inhibition was dependent on the presence of LGG cells in milk since the supernatant of these milk samples did not inhibit adherence. However, there was a trend that LGG-fermented milk containing IP was even more effective ($P = 0.062$) in preventing adherence of *S. mutans* than LGG-fermented milk without IP in spite of decline in specific antibody titres. The presence or absence of HEPES buffer did not affect these adhesion phenome but the number of viable LGG cells remained higher (over 10^8 cfu/ml) during the long storage period if no HEPES was added (III).

Live lactobacilli reduced the adhesion of *H. pylori* on AGS cells by approximately 50% when LAB concentration was 10^{10} cfu/ml (p<0.05). Heat killed LAB had a non-significant effect on adhesion.
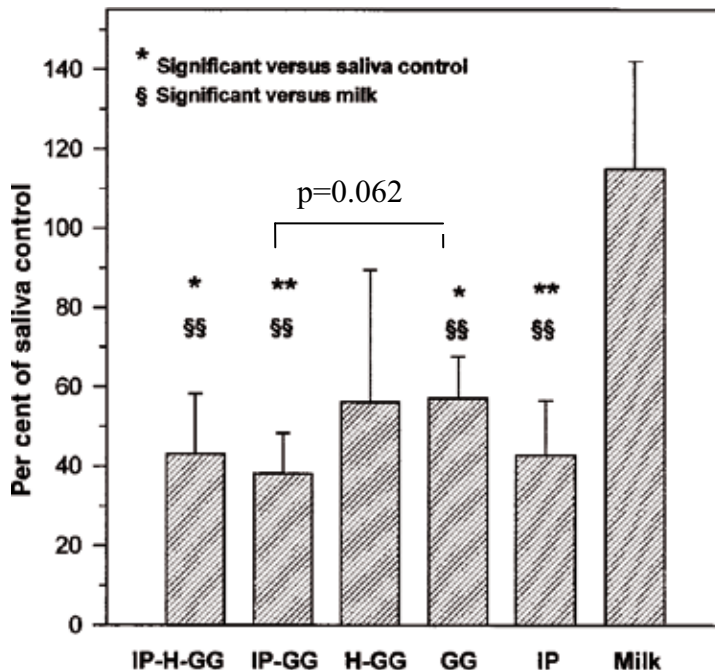
Figure 8. Effects of *L. rhamnosus* GG fermented milks on the adherence of *S. mutans* to salivacoated hydroxyapatite compared with saliva alone (approx. 1×10^8 cells adhered; taken as 100%). IP=immune preparation, H= HEPES and GG= *L. rhamnosus* GG, Milk=fresh milk. Bars represent estimated means ±SE.

5.6 Reduction in the IL-8 production of AGS cells after infection by *H. pylori* pretreated with live lactobacilli or colostral preparations (V)

IL-8 concentration in the growth medium of AGS cells after 20 h incubation was 540±150 pg/ml. After the infection of AGS cells by *H. pylori* the IL-8 level rose up to about 10 fold (5500±1600 pg/ml) (Figure 9). The presence of IP, CP, or live LAB did not affect the IL-8 levels produced by uninfected AGS cells. On the contrary, heat killed LAB at a concentration of 10^{10}/ml raised the IL-8 level of uninfected AGS cells significantly. Pretreatment of *H. pylori* with live *L. plantarum* MLBPL1, LGG, or *Lactococcus lactis* cells before infection of AGS cells prevented the IL-8 rise when concentration of added LAB was 10^{10}/ml (p<0.0001) (Figure 9a). The prevention was also statistically significant with LGG at a concentration of 10^9/ml (p=0.0382), and *L. lactis* at concentrations of 10^9/ml (p=0.0026) and 10^8/ml (p=0.0116). The heat-inactivated LAB were shown to be less effective in reducing the IL-8 production induced by *H. pylori* infection in AGS cells than live LAB (Figure 9b). IP and CP also lowered the IL-8 level of AGS cells infected with *Helicobacter* back to the normal

level. Heat inactivated CP needed a higher concentration than the other colostral samples for this reduction.
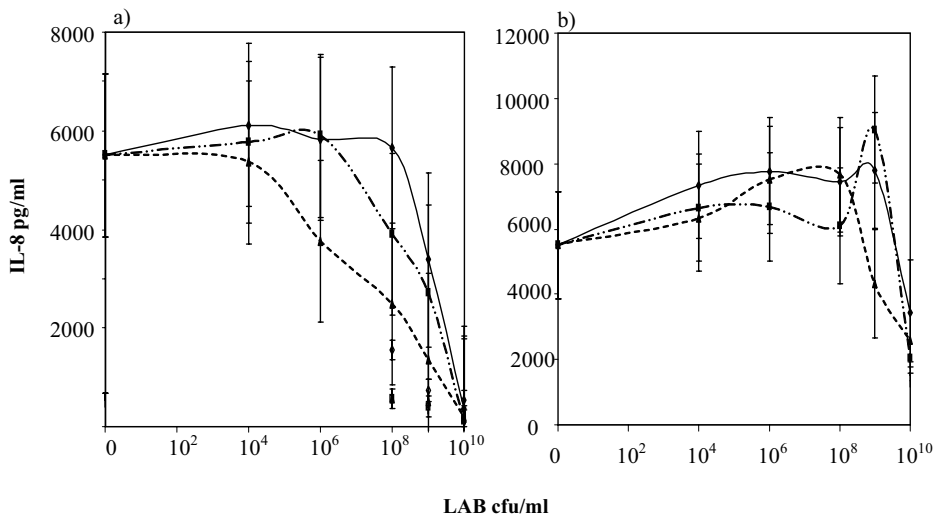

Figure 9. The IL-8 production of AGS-cells treated for 20 hours with *H. pylori* and live (a) or heat killed (b) lactic acid bacteria (LAB) at concentrations from 10^4 to 10^{10}cfu/ml. Levels of IL-8 (pg/ml) in the culture medium of AGS-cells were determined by ELISA test. Bars represent estimated means ±SE; n= 2-6. ――― *Lactobacillus plantarum* MLBPL1, ··‒·· *Lactobacillus rhamnosus* GG, - - - *Lactococcus lactis.*

5.7 The effect of specific antibodies of IP in the prevention and treatment of an experimental *H. felis* infection (II)

All mice in the infected control group in Trial 1, 70% in Trial 2, and 79% in Trial 3 were *H. felis* positive, whereas all animals in the healthy control groups remained *H. felis* negative. The levels of *H. felis* antibodies in the sera of mice correlated well with the presence or absence of *H. felis* infection but not with the degree of colonization or inflammation in the gastric antrum. The SJL strain developed more severe inflammation and a higher grade of *H. felis* colonization than Balb/c mice used in Trial 1.

The IP had a significant (P=0.0014) preventive effect on *H. felis* infection, given the absence of visible bacteria in gastric antra and corpusa. In the group receiving prophylactic immune preparation (IP) *H. felis* bacteria were detected in 7 out of 17 mice (41%), whereas all the mice given control preparation or PBS were infected (Table 8).

Table 8. Prevention of experimental *Helicobacter felis* infection in mice with colostral immune preparation.

	Rate (%) of infection		
	H. felis positive	*H. felis* negative	p values
immune preparation	41%	59%	0.0014
control preparation	100%	-	n.s.
infected controls	100%	-	

The administration of colostral preparations did not eradicate an established *H. felis* infection. A slight decrease of *H. felis* colonization and of the degree of inflammation of the gastric antrum was observed, but not in all trials (Table 9).

A two-week treatment with IP had a significant (P=0.029) and with CP a trend-setting (P=0.091) reducing effect on *H. felis* colonization of the antra in Trial 1. However, only two of the 19 mice treated with IP were *H. felis* negative while all the mice treated with CP remained *H. felis* positive.

The only therapeutic effect on infected SJL mice was seen in animals treated with IP containing both specific antibodies and active serum complement in Trial 2 but not in Trial 3 (Table 9). In this group (Trial 2) the grade of inflammation in antrum was lower than in the infected controls. In the other treatment groups there was a tendency towards a heavier *H. felis* colonization as compared to infected controls. In the corpusa of mice treated with complement inactivated IP or serum supplemented CP the grade of colonization was significantly higher. The removal of small molecules from IP did not abolish the effect increasing *H. felis* colonization even though the increasing effect of IgG was not statisitically significant (p=0.13).

A 6-day treatment with amoxicillin decreased the grade of *H. felis* colonization in antrum with a trend-setting (P=0.056) significance when compared to infected controls. The combination of filtered and serum supplemented immune preparation, and amoxicillin treatment resulted in a significant (P=0.0005) decrease in the colonization of antrum. The difference between these two groups was not significant.

Table 9. Effect of colostral immune preparation and complement on *H. felis* infection in mice as compared to infected controls.

Treatment	Trial	Colonization		Inflammation
		corpus	antrum	antrum
IP	1	n.s.	↓ *	n.s.
CP	1	n.s.	↓ º	n.s.
IP + complement (NBS)	2	n.s.	n.s. (↑)	↓ º
CP + complement (NBS)	2	↑*	n.s.	n.s.
Complement inactivated IP	2	↑*	n.s. (↑)	n.s.
Complement inactivated CP	2	n.s. (↑)	n.s. (↑)	n.s.
Complement (NBS)	2	n.s. (↑)	n.s.	n.s.
IP + complement (NBS)	3	n.s.	↑*	n.s. (↑)
IgG of IP + complement (NBS)	3	n.s.	n.s. (↑)	n.s. (↑)
Amoxicillin	3	n.s.	↓ º	n.s. (↓)
IgG of IP + NBS + amoxicillin	3	n.s.	↓ ***	↓**

↓= reduction, ↑= increase. º p<0.1, * p<0.05, ** p<0.01, *** p<0.001, n.s. = statistically non-significant. IP= colostral immune preparation with specific *Helicobacter felis* antibodies, CP= colostral control preparation, NBS= normal bovine serum.

6 Discussion

6.1 The role of colostral complement in gastrointestinal infections

In the present study the efficacies of colostral immunoglobulin preparations and selected lactic acid bacteria on gastrointestinal infections were investigated. The regulation of complement activity by colostrum is of vital importance for the health of a calf. In study (I) it was demonstrated that the ingestion of colostral immunoglobulin concentrate increases the complement and opsonisation activities in the sera of neonatal calves. As the concentration of complement component C3 was not higher in the sera of calves supplemented with colostral concentrate than in the sera of control calves, it was concluded that no considerable amounts of complement C3 protein was transferred from the cow to the calf by absorption from colostrum.

Enterotoxigenic *E. coli* is a common cause of diarrhea and other infectious diseases in calves during the first weeks of life. The *E. coli* strains JM103 sensitivite to complement and IH3080 resistant to complement lysis were used in study (I) as model organisms. They are not known to be natural pathogens of calves but the complement and opsonization activities against these strains reflect well the functions of serum complement and antibodies against bacterial pathogens in general. Antibody-dependent, naturally occurring bactericidal complement activity has a significant role against coliform infections in calves (Carroll and Crensaw 1976). Ingestion of colostral immunoglobulin concentrate increased the level of *E. coli* specific antibodies in the blood, and thus caused the higher activity in the classic pathway of complement (I).

The cause for the higher activity of the antibody-independent alternative pathway of the complement system as well as a higher opsonisation capacity in the calves fed immunoglobulin concentrate was supposed to be the complement proteins and/or lectins. Mannose-binding lectin (MBL) is able to trigger the lectin pathway of complement activation by binding to sugar groups on the pathogen surface (Turner 1996). Also, bovine conglutinin is known to show opsonizing activity towards *E. coli* (Holmskov 2000). MBL is present in human colostrum (Trégoat et al. 2002). *H. pylori* activates the lectin pathway of complement (Kuipers 2003). MBL deficiency does, however, not increase the risk of chronic gastritis associated with *H. pylori* infection (Bak-Romaniszyn et al. 2006, Worthley et al. 2007).

The antibody-complement system of bovine colostrum is known to kill *H. pylori* effectively (Korhonen et al. 1995, Early et al. 2001). This bacteriolytic activity was also present in our colostral preparations (II), which killed *H. pylori*

in vitro. In study (II) the effects of inactivation and supplementation of the serum derived complement system in the treatment of *H. felis* infection was tested in a mouse model. IP and CP supplemented with serum as a source for active complement system did, however, not decrease the rate of *H. felis* colonization. In one of the trials IP supplemented with serum reduced the degree of inflammation in the antra of *H. felis* infected mice, but this result could not be repeated in the next trial. However, the results obtained support the suggestion that an active complement system might be important for the efficacy of the IP in the treatment of gastritis.

6.2 Colostral antibodies in protection against infections

Colostral immunoglobulins are an essential part of the protection of calves against infections as the serum of a newborn calf is essentially free of immunoglobulins. The *E. coli*-specific antibody levels in the sera of calves fed immunoglobulin concentrate (I) correlated with the total IgG concentrations in the sera as reported by Nousiainen et al. (1994). In study (II) it was demonstrated that more than 25% of antibodies were active after 30 min incubation in a simulated gastric juice containing HCl and pepsin at pH 4.0. It can thus be assumed that in humans a substantial proportion of administered bovine IgG1 remains immunologically active long enough to reach the gastric mucosa and to attach to *Helicobacter*. Further, the pH of gastric juice of gastritis patients treated with proton pump inhibitors is often relatively high, and immunoglobulins have been recovered in their gastric juice (Meining et al. 2002).

In mice, the administration of colostral preparations did not eradicate an established *H. felis* infection, but the treatment with IP reduced the *H. felis* colonization in the antra of Balb/c mice, the CP giving a rather similar but statistically less significant result (II). In study (II) there was a tendency towards a higher *H. felis* colonization rate in mice treated with IP than in infected controls. It was suggested that milk components others than immunoglobulins might be responsible for this effect. However, concentration of the immunoglobulin fraction in order to abolish the *H. felis* colonization increasing effect did not have any effect.

In study (II) it was tested whether the IP could enhance the effect of the treatment of *Helicobacter* infection with an inadequate antibiotic dosage. The IP supplemented with serum and combined with amoxicillin lowered the levels of inflammation and colonization significantly as compared to the infected controls whereas the treatment with amoxicillin alone had a trend setting effect on colonization and none on inflammation.

The IP with specific anti-*Helicobacter* antibodies was able to protect the mice against experimental *H. felis* infection (II). This preventive effect could not be

demonstrated in the mice receiving CP concluding that the preventive effect is dependent on the presence of specific antibodies. Similar IP with specific anti-caries antibodies used in study (III) inhibited the adherence of cariogenic strep-tococci to saliva-coated hydroxyapatite beads, and CP had a significantly lower effect than IP. However, according to results in study (V), both IP and CP redu-ced *H. pylori* adhesion to AGS cells in a dose dependent manner, and there was no significant difference between IP and CP.

In studies (I, II, III and V) we found that other antimicrobial factors than anti-bodies and complement system are involved in the protection against infections. Dietary factors are reported to influence the severity of *Helicobacter*-induced gastritis and gastric cancer, low intake of vegetables and fruit, and high intakes of salt and processed meat being risk factors (Rocco and Nardone 2007, Epplein et al. 2008). Bovine lactoferrin or lactoferrin plus probiotics added to triple the-rapy improved the eradication rates in the treatment of *H. pylori* infection (Di Mario et al. 2003, de Bortoli et al. 2007). Lysozyme and lactoperoxidase also act against pathogens (Pakkanen and Aalto 1997, Zimecki and Kruzel 2007). Milk components such as κ-casein (Vacca-Smith et al. 1994), caseinomacro-peptide also called glycomacropeptide, and caseinophosphopeptide (Schupbach et al. 1996) may reduce the adherence of *S. mutans* and *S. sobrinus*. However, the fresh milk and UHT milk products in our study slightly enhanced bacte-rial adherence (III). Similarly, in the study by Shimazaki et al. (2001) the num-ber of *S. mutans* cells in saliva and plaque increased in the control group recei-ving nonimmune milk. The mechanism for the dose dependent anti-adhesion mechanism of IP and CP in the study (V) is suggested to be in other colostral components than specific antibodies against *H. pylori*. Also factors related to the properties of the host, or the type or phase of the infection may influence the efficacy of IP (II).

The idea for combining IP and probiotic bacteria was for the first time introdu-ced in study (II). Recently, Pant et al. (2007) reported about a successful prophy-lactic use of a combination of probiotic *L. rhamnosus* GG and specific colostral antibodies against rotavirus in a mouse model.

6.3   LAB in prevention of caries and *H. pylori* infection

The anti-*Helicobacter* activity of seven LAB strains belonging to the *L. planta-rum* group was screened *in vitro* (IV). All members of the *L. plantarum* group showed some anti-*Helicobacter* activity, but the culture supernatants of the strains *L. plantarum* MLBPL1, isolated from sauerkraut, and *L. pentosus* from a commercial starter Vege-Start 60 showed the clearest inhibition.

In this study, the active substance of *L. plantarum* MLBPL1 was further cha-racterized. Even though lactic acid inhibits the urease activity of *H. pylori*, se-

veral studies have shown other substances secreted by LAB to be behind the mechanism of antibacterial effects (Hamilton-Miller 2003, Coconnier et al. 1998, Michetti et al. 1999). The substance with anti-*Helicobacter* activity in the culture supernatant of *L. plantarum* MLBPL1 was found to be between 3000 and 10 000 Daltons in size (IV). Thus, the effect of organic acids and other low molecular mass compounds was excluded. The anti-*Helicobacter* activity was heat resistant and retained its activity at neutral pH. According to the results, the main activity seemed to be associated with the cell wall, from where it is probably extracted into culture supernatant. It was not possible to remove the anti-*Helicobacter* activity of MLBPL1 by incubation with proteolytic enzymes. Therefore, it seems that the anti-*Helicobacter* effect observed is not explained by an already known bacteriocin. On the contrary, incubation with N-glycosidase F increased the anti-*Helicobacter* activity of MLBPL1, suggesting that a component of a larger molecule could be the active compound, which gains the full activity after cleavage from a carrier substance. Further studies are, however, needed to identify the active compound.

LGG is known to inhibit the growth of cariogenic streptococci by producing antistreptococcal substances (Meurman et al. 1995). The inability of LGG to ferment sucrose or lactose limits its growth in milk but greatly increases its potential as a food probiotic against cariogenic streptococci, particularly if combined with specific antibodies against *S. mutans* and *S. sobrinus* as shown in (III). LGG is also known to adhere on human gastric epithelial cells (Tuomola and Salminen 1998). *L. plantarum* MLBPL1, LGG and *L. lactis* were all able to prevent *H. pylori* adhesion on AGS cells and suppress the IL-8 production of *H. pylori* infected AGS cells similarly independent of their ability to adhere to gastric epithelium (V). *L. johnsonii* La1 and *L. acidophilus* strain LB secrete heat-stable substances able to decrease *H. pylori* adhering to gastric cells (Michetti et al. 1999, Coconnier et al. 1998). In the study (V), however, the heat inactivation of LAB cells reduced the anti-adhesive activity. This suggests the occurrence of another anti-adhesive mechanism in addition to those secreted components.

6.4 The effect of colostral preparations and LAB on inflammatory response

In spite of effectiviness of IP in passive immunization (II) the role of antibodies is apparently only marginal for the protective immunity in *Helicobacter* infection, the cellular immunity being more important. The production of IL-8 by gastric epithelial cells plays a significant role in the initial response to *H. pylori* infection by promoting inflammation and tissue damage locally and attracting leucocytes to the sites of inflammation (O'Keeffe and Moran 2008). In the study (V) the suppressive effect of bovine colostral preparations on the IL-8 produc-

tion by *H. pylori* induced AGS cells was demonstrated. The reduction of IL-8 production might have caused the slight decrease in the degree of inflammation of the gastric antrum observed in *Helicobacter* infected mice treated with IP in study (II). Colostral preparations were effective in reducing the *H. pylori* induced IL-8 production also at low concentrations.

Live LAB did not affect the IL-8 levels of uninfected AGS cells. Reduction in IL-8 concentration by LAB happened only after *H. pylori* infection. This suggests that LAB act on IL-8 secretion induced by *H. pylori* infection in the host cell. It is known that lactobacilli can affect the IL-8 production of intestinal epithelial cells *in vitro*, when IL-8 secretion is stimulated with TNF-α. Other studies have also demonstrated that LAB do not affect IL-8 production without the recruitment of proinflammatory cytokines (Ko et al. 2007, McCracken et al. 2002, Zhang et al. 2005). We also showed that heat killed LAB had a weak suppressive effect on IL-8 production of *H. pylori* infected cells, but increased the IL-8 level of uninfected AGS cells (V). Together, these results suggest that the active factor affecting the immune response of AGS cells might be in the cell wall material of heat killed LAB. Live LAB were more effective in reducing *H. pylori* induced IL-8 production in host cells than heat inactivated, suggesting that physiological response from LAB is required to suppress *H. pylori* induced IL-8 activation.

Control of bacterial adhesion and IL-8 production by LAB or colostral products could have a suppressive effect on *Helicobacter* infection by reducing pro-inflammatory cytokine expression and thus avoiding a chronic inflammation.

6.5   Some technological aspects on the use of IP and LAB

Bovine milk is a good medium for functional products containing, for example, specific antibodies. The colostral preparations are composed mainly on proteins, the majority being IgG (Table 7). The rest of colostral preparations consist of sugars and minerals (data not shown). The specific antibodies retained their activity for more than 10 years when stored at –18°C like shown also by Pant et al. (2007). In study (III) when IP was stored in UHT milk at different temperatures, the pH and the IgG concentration remained stable for up to 2 months. ELISA test and adherence assay provided further evidence that the specific antibodies in UHT milk also remain functionally active during the storage. We also found that the metabolism of LGG did not influence the number of antibodies during fermentation. Both fermented and unfermented IP could significantly inhibit the adherence of *S. mutans*, as could normal LGG-fermented milk (III).

Several factors, such as utilization of IgG as a substrate for bacterial growth (Jansen et al. 1994) or proteolytic cleavage of IgG (Brock et al. 1977, de Rahm

and Isliker 1977), may contribute to reductions in IgG activities. The degradation of IgG1 is also dependent on temperature. Foley et al. (1978) reported only minimal breakdown of IgG during fermentation, as also found in our study in the presence of LGG. The fact that the antibody titres declined during storage in fermented milk (III) may be related to the metabolism of LGG and the subsequent pH decline in IP.

The anti-*Helicobacter* activity of several probiotic LAB strains used in dairy products has been reported in a number of *in vitro* studies and clinical trials, and the results have been encouraging (Hamilton-Miller 2003). The majority of the LAB tested for anti-*Helicobacter* activity has been exemplars of species with minor importance as starters or strains used for probiotic purposes. Technological properties of commercial probiotic culture preparations are often problematic. They are sensitive, and may affect the sensory properties of the product. So, it is common to add them to a completed product. On the contrary, *L. plantarum* has been traditionally used for acidification of foodstuffs and can be used as starter in the production of a wide variety of fermented foods. In study (IV), the anti-*Helicobacter* activity was present also in cultivations performed in different food matrices. Consequently, the production of a fermented food with anti-*Helicobacter* activity would be easy and cost-efficient, because no additional components besides the food matrix and the starter culture will be needed to complete the process.

7   Conclusions and future aspects

In the present study bovine colostral antibodies and lactic acid bacteria as means to control gastrointestinal infections were investigated *in vitro* and in animal models. The main results and conclusions of the study were:

•   In neonatal calves, complement and opsonization activities of serum were increased substantially by feeding colostral whey concentrate.

•   Specific colostral antibodies effectively prevented *Helicobacter* infection in a mouse model but they were not able to eradicate an established *Helicobacter* infection. Colostral antibodies seemed to increase the therapeutic effect of amoxicillin on *H. felis* infection.

•   Colostral antibodies retained their activity when stored in UHT-milk drink or fermented with LGG. Combining specific colostral antibodies and probiotics proved promising in preventing the adherence of *S. mutans* to saliva-coated hydroxyapatite.

•   Anti-*Helicobacter* activity was found in seven strains belonging to *L. plantarum* group. The activity of *L. plantarum* MLBPL1 was located to cell wall.

•   LAB and colostral preparations were able to inhibit the adhesion of *H. pylori* in a cell model, and to suppress IL-8 production by *H. pylori* infected AGS cells.

In future, the combining of LAB and IP needs to be further studied. However, the authorities in European Union limit the use of IP in food as products containing antibodies are considered as drugs. Products, supplemented by specific bovine colostral antibodies or LAB, might be useful in the prevention of human dental decay during early childhood, or in the prevention of *Helicobacter* infection in endemic areas. IP could also contribute beneficially to the effect of the antibiotics and alleviate the inflammatory effects in clinically asymptomatic subjects infected by *H. pylori* and, in the best case, suppress the development of a chronic inflammation. Further *in vivo* studies are, however, needed to verify the beneficial effects of LAB and IP on gastrointestinal infections.

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Susanna Rokka

8   References

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