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## Bovine milk immunoglobulins against microbial human diseases

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**Abstract:** Immunoglobulins (Igs), also called antibodies, are present in milk and colostrum of all lactating species. Igs are divided into classes having different physico-chemical structures and biological activities. The major Ig classes in bovine and human milk are IgA, IgG and IgM. Bovine colostrum Igs provide the newborn calf with passive immune protection against microbial infections until the calf's own immune system matures. Colostrum Ig preparations designed for farm animals have been commercially available for many years. Potential health benefits attributed to bovine colostrum have increased manufacture and marketing of colostrum Ig-based dietary supplements also for human use. Furthermore, specific anti-microbial antibodies can be produced into colostrum by immunizing cows with vaccines made of pathogenic microorganisms. These antibodies can be concentrated and used to formulate so-called immune milk preparations. Such preparations have proven effective in prevention of animal and human infections caused, e.g. by rotavirus, *Shigella flexneri*, *Escherichia coli*, *Clostridium difficile*, *Streptococcus mutans*, *Cryptosporidium parvum* and *Helicobacter pylori*. Their therapeutic efficacy, however, seems limited. A few immune milk products have been commercialized and more can be expected in the future for use, e.g. as a supportive means in antibiotic treatments and for prevention of hospital infections.

**Key words:** immunoglobulin, antibody, colostrum, passive immunity, immune milk.

### 10.1 Introduction

It has been recognized for more than 100 years that maternal colostrum and milk offer passive protection to a newborn infant against enteric pathogens, primarily via the transfer of immunoglobulins (Igs) and associated bioactive factors (Ehrlich, 1892). Ruminant neonates are born virtually without Igs and therefore the colostrum Igs are essential for survival. Probably due to this unique function, Igs represent the major protein fraction of

colostrum, accounting for 70–80% of its total protein content (Butler, 1998; Marnila and Korhonen, 2002).

The Ig fraction can be concentrated from colostrum or cheese whey and is marketed commercially as a feed supplement and replacer of colostrum, mainly for neonate calves and pigs, to prevent gastrointestinal infections (Scammell, 2001; Mehra *et al.*, 2006).

The advent of functional foods has increased interest in the bioactive components of bovine colostrum and milk as potential ingredients for health-promoting foodstuffs and even biopharmaceuticals. To this end, scientific and commercial attention has been focused on the development of bovine-derived Ig products that contain specific antibodies targeted for prevention or treatment of microbial infections in humans (Korhonen *et al.*, 1998; Korhonen, 2002). Such preparations have been described as ‘immune milk’. This concept dates back to the 1950s when Petersen and Campbell first suggested that orally administered bovine colostrum from hyperimmunized cows could provide passive immune protection for humans (Campbell and Petersen, 1963). Since then, a large number of animal and human studies have been carried out to demonstrate that these preparations can be effective in the prevention or treatment of human and animal diseases caused by various pathogenic microbes (for reviews see Weiner *et al.*, 1999; Korhonen *et al.*, 2000a; Lilius and Marnila, 2001; Hoerr and Bostwick, 2002; Korhonen and Marnila, 2006; Mehra *et al.*, 2006). This article reviews the current state of knowledge about the properties of bovine Igs, their utilization as ingredients for immune milk preparations, and the application of these preparations for the prevention and treatment of various microbial infections in humans.

## 10.2 Properties of immunoglobulins

### 10.2.1 Structure and physicochemical properties

Igs, which carry the biological function of antibodies, are present in colostrum and milk of all lactating species. In mammals, all five known classes of Igs have been characterized: IgG, IgM, IgA, IgD and IgE. The major Ig classes in both bovine and human milk are IgA, IgG and IgM. The basic chemical structure of all Igs is similar but their biological functions differ, although in principle they all contribute to the major defence mechanism against foreign materials recognized by the body’s immune system. Igs account for up to 70–80% of the total protein content in colostrum, whereas in milk they account for only 1–2% of total protein. IgG1 is the predominant Ig class in bovine lacteal secretions as compared to IgA in human milk (Elfstrand *et al.*, 2002; Marnila and Korhonen, 2002). Table 10.1 provides the concentrations of different Ig classes in bovine colostrum and milk.

Ig molecules of all classes are symmetrical, multi-chain glycoproteins composed of two identical glycosylated heavy chains and two identical

**Table 10.1** Immunoglobulin concentrations in bovine colostrum and milk

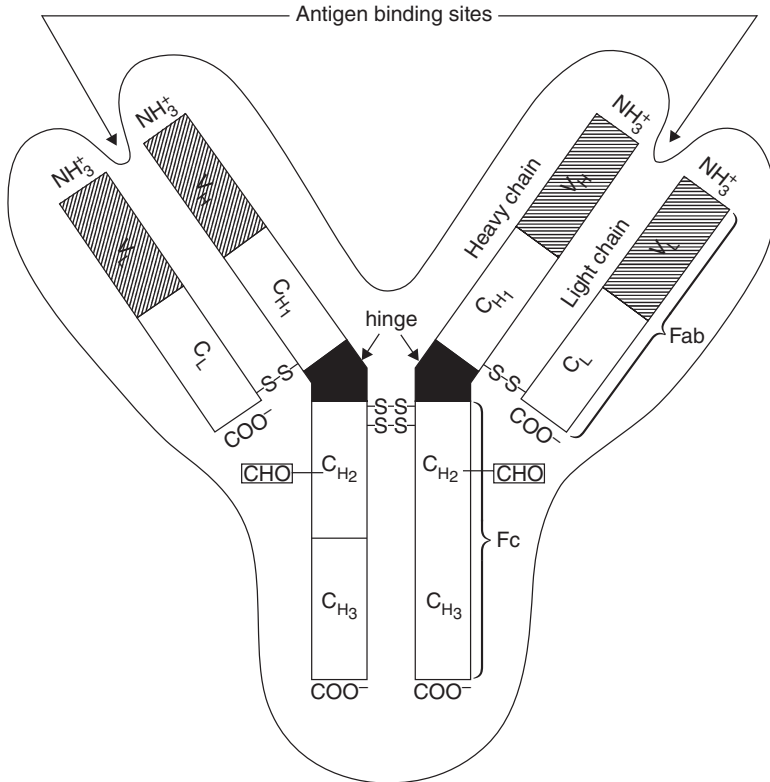
Immunoglobulin class	Molecular mass (kDa)	Concentration (gL <sup>-1</sup> )	
		Milk	Colostrum*
IgG <sub>1</sub>	146–163	0.3–0.6	15–180
IgG <sub>2</sub>	146–154	0.06–0.12	1–3
IgG total		0.15–0.8	20–200
SIgA	385–430	0.05–0.1	1–6
IgM	900	0.04–0.1	3–9

\* = first milking.

Data compiled from Marnila and Korhonen (2002), Elfstrand *et al.* (2002) and Mehra *et al.* (2006).

non-glycosylated light chains. The basic structure of all monomeric Igs is similar (see Fig. 10.1). The IgG class can be considered as a general model of a monomeric Ig. The molecular weight of each light chain is around 23 kDa and of the heavy chains 53 kDa. The molecular weight of the complete Ig molecule varies around 160 kDa. Both the light and heavy chains contain domains referred as constant regions (C<sub>L</sub>, C<sub>H</sub>) and variable regions (V<sub>L</sub>, V<sub>H</sub>). The light chains are attached to the heavy chains by a disulphide bond, and also the two heavy chains are held together by two disulphide bonds near a hinge region which gives the molecule structural flexibility needed in antibody–antigen interactions (Nezlin, 1998a). The two identical antigen-binding sites needed in these interactions are formed by the N-terminal region of one heavy chain and the variable region of one light chain. The V<sub>L</sub>-region determines the immunological specificity. Antigen binding occurs by the interactions between the antigen and these regions. The Ig classes and subclasses are determined by the genes encoding the constant regions of heavy chains (Butler, 1998). The bovine IgG molecule occurs dominantly in two sub-classes: IgG<sub>1</sub> and IgG<sub>2</sub>.

Monomeric IgM and IgA have a similar basic structure to IgG except for the differences in heavy chain structures and the addition of a C-terminal octapeptide to the heavy chain of IgA (Butler, 1998; Nezlin, 1998b). Monomeric IgA occurs in serum, but in milk it is present as a dimer comprising two IgA molecules joined together by a polypeptide J-chain and an additional 75 kD secretory component. This secretory IgA (SIgA) has a molecular weight of about 380 kDa, and is more resistant to proteolysis and therefore more stable in the gastrointestinal tract than antibodies without the secretory component. IgM is a circular pentamer consisting of five subunits similar to those of monomeric IgA or IgG, which are linked together in a circular mode by disulphide bonds and a J chain: the molecular weight of IgM is approximately 900 kDa (see Fig. 10.2). The physicochemical properties of Igs are presented in Table 10.2.

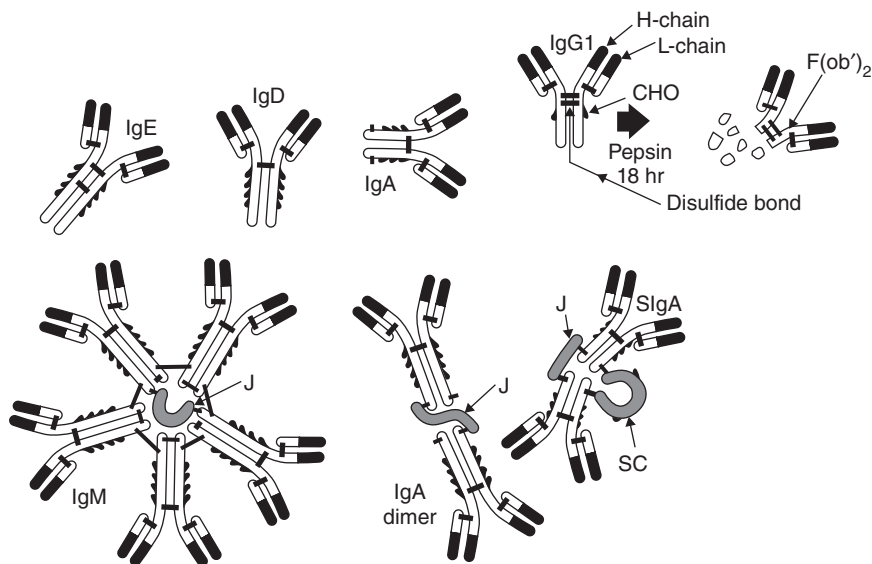


**Fig. 10.1** Schematic diagram of a basic immunoglobulin. From Larson (1992).

Proteolysis of an Ig with papain cleaves it near the hinge region into two antigen binding-sites (Fab-fragments), which are formed by the N-terminal part of one heavy and one light chain, and to the third C-terminal fragment, termed the Fc-part. The differences between Ig classes impact upon the cleavage patterns in enzymatic proteolysis. For instance, cleavage of bovine IgG2a(A2) by pepsin results to two Fab and one Fc parts, whereas in IgG2a(A1) the Fab parts remain together by one disulphur bridge resulting in one  $F(ab')_2$  and one Fc fragment which is cleaved further by pepsin (Heyermann and Butler, 1987).

### 10.2.2 Biological activities

Igs are produced by B lymphocytes and plasma cells diversified from B cells. The soluble Igs function as flexible adaptors between cellular and humoral immunity by binding to antigens and exhibiting one or more effector functions. The Fab parts attach the antigens while other parts (mostly the Fc region) interact with other elements of the immune system.



**Fig. 10.2** Structure of five classes of immunoglobulins. Figure modified from Larson (1992).

**Table 10.2** Properties of bovine immunoglobulins

	IgG <sub>1</sub>	IgG <sub>2</sub>	IgA	SIgA	IgM
<i>Physico-chemical</i>					
Molecular weight (kDa)	146–163	146–154	160	385–430	900
Heavy chain	56–59	54–59	61–63		62–76
Heavy chain type	γ1	γ2	α	α	μ
Number of H- and L-chains	2	2	2	4	10
Structure	Monomer	Monomer	Monomer	Dimer	Pentamer
<i>Immunological</i>					
Opsonization	+++	+	0	0	+++
Complement fixation	++	+	0	0	+++
Agglutination	+	+	++	++	+++

0 = no activity, + = low activity, ++ = moderate activity, +++ = strong activity.  
 Data modified from Marnila and Korhonen (2002).

For many pathogenic organisms, the attachment to the epithelial lining is an obligatory first step in the establishment of colonization and infection. Many pathogens have receptors, e.g. fimbriae for epithelial surfaces. Thus, the prevention of the microbial adhesion to epithelial linings is an

important mechanism of SIgA antibodies in protecting the host (Woof and Kerr, 2006). Colostrum and human milk protect the neonate intestinal mucosa against EPEC infections by inhibiting bacterial adhesion to epithelial cells (Carbonare *et al.*, 1997). This effect has been shown to be mediated by colostrum and milk SIgA (Cámara *et al.*, 1994; Carbonare *et al.*, 1997, 2005; Fernandes *et al.*, 2001; Corrêa *et al.*, 2006), and by oligosaccharides present in human milk (Cravioto *et al.*, 1991). M-cells in intestinal epithelia have specific receptors for SIgA, and antigen-SIgA-receptor complex can be transcytosed by M-cells into lymphoid tissue for antigen presenting and induction of mucosal immune responses (Woof and Kerr, 2006).

The ability of Igs to form cross-links between surface antigens results in a network of cells that can be removed mechanically from the body. This agglutination of microbes reduces their capability to adhere to surfaces. Agglutinated microbes are usually not able to release toxins or to colonize the host. Normal colostrum and milk are known to contain natural antibodies which can agglutinate a large number of pathogenic and non-pathogenic micro-organisms (Bostwick *et al.*, 2000; Korhonen *et al.*, 2000b).

Igs can bind bacterial toxins which, thereafter, can be recognized more effectively by phagocytic leukocytes. Many bacterial toxins must first be actively transported via receptors inside the host cells to cause cell death. Blocking the toxin may prevent its internalization in host cells. Also, Igs can inhibit or reduce the production of toxins and other harmful components by inhibiting bacterial metabolism and by blocking enzymes and receptors. This blocking may also reduce the ability of pathogens to produce structures needed in adherence to epithelia. Specific Igs can protect against viral infections by binding viruses and preventing the virus replication by blocking the receptor mediated internalisation of viruses in the host cells. Specific Igs augment the recognition and phagocytosis of antigens by leukocytes (opsonisation). Divalent or polyvalent binding of an Ig to antigenic structure results in conformational change of the Ig molecule, which process again enables the Fc portion to bind to the corresponding Fc receptors on the leukocyte surface. This receptor binding leads to various immune cell effector functions depending on the cell Ig and leukocyte type. Leukocytes are an integral part of normal milk and colostrum and are of vital importance in defending the mammary gland against pathogens (Korhonen *et al.*, 2000b).

Specific Igs are rarely lethal to micro-organisms but may disturb cellular metabolism by blocking receptors and enzymes, and cause structural alterations leading to immobilization, increased membrane permeability and impaired cell growth. Igs may contribute to the killing of microbes by activating the classical pathway of complement. In blood and tissues, the activation of complement mediated bacteriolytic reactions may be the most important function of Igs, but the significance of this mechanism in milk remains obscure. Bovine and human colostrum contain an active complement system participating in the immune defense of the udder (Butler, 1998).

IgM antibodies, although produced in smaller amounts than IgG, are considerably more efficient than IgG with regard to most of the above activities, including complement fixation, opsonization and agglutination of bacteria. Bovine IgG<sub>2</sub>B is more effective than IgG<sub>2</sub>A in activating the classical pathway of complement (Bastida-Corquera, 1999). Specific IgM can prevent effectively the migration of bacteria by binding the flagellas used in the movement. Bovine IgA does not fix complement or opsonize bacteria, but it agglutinates antigens, neutralizes viruses and bacterial toxins. The SIgA dimer is found in mucosal secretions and its main function is to protect the mucosal barriers by binding microbes and preventing their attachment to the epithelium, and to contribute to the antigen presenting. The milk Igs have also been found to exert a synergistic effect on the activity of non-specific antimicrobial factors in milk, such as lactoferrin and lysozyme, as well as the lactoperoxidase-thiocyanate-hydrogen peroxide system (Loimaranta *et al.*, 1998a; Bostwick *et al.*, 2000).

### 10.3 Production and isolation of immunoglobulins

Traditionally, the globulin fraction was isolated from colostrum whey by precipitation with either ammonium sulphate or ethanol. These chemical methods are, however, economically not suitable for large scale-production of Igs although they yield rather pure Ig fractions. Other challenges in isolation of active Igs from colostrum has been its complex composition and the sensitivity of Igs to heat treatments. With the rapid development of new chromatographic and membrane-separation technologies in the last thirty years, it is now possible to isolate individual milk proteins on a large-scale. Based on these techniques, a great number of pilot- or industrial-scale methods have been developed and patented for fractionation and isolation of Igs from colostrum or cheese whey (for reviews see Korhonen, 2004; Korhonen and Pihlanto, 2007). With these methods, the recovery rate of Igs has varied from 40% to 70% of the level present in the starting material (Elfstrand *et al.*, 2002). Specific chromatographic techniques, such as immobilized metal chelate chromatography, immunoaffinity chromatography and cation-exchange chromatography have been applied to improve the yield and purity of immunoglobulin preparations further (Fukumoto *et al.*, 1994; Akita and Li-Chan, 1998). Microfiltration (MF) combined with ultrafiltration (UF) of bovine, equine and caprine colostrum has led to IgG/total solids purity of more than 90% (Piot *et al.*, 2004). Using cheese whey, a 0.1 µm membrane and the concept of selective membrane separation through pH manipulation, Mehra and Kelly (2004) produced an Ig-rich preparation with a protein composition similar to that produced from colostrum whey. Korhonen *et al.* (1998) used various MF methods, such as UF, MF and reverse osmosis, and a cation-exchange resin as a molecular sieve, to concentrate Igs from colostrum whey. The Ig level of the final freeze-dried



concentrates varied from 45% to 75%. Expanded Bed Adsorption Chromatography (EBAC) is a new technology which has been applied to isolate Igs from cheese whey using an adsorbent with tailored ligand chemistry (Nielsen *et al.*, 2002). EBAC provides significant advantages over conventional packed bed column chromatography, and an Ig-purity from 50% to 70% can be achieved with this up-scalable method.

The technological properties of Igs have been studied extensively, as these proteins are known to affect many dairy processes (see review by Mehra *et al.*, 2006). Igs may inhibit or retard the activity of renneting enzymes of bacterial origin. In normal milk this is not noticed due to the relatively low Ig concentration but in case of mastitic milk, or if the Ig concentration of milk is increased, e.g. by adding colostrum, renneting may be retarded. The antimicrobial properties of Igs may adversely impact upon fermentation processes. A retarded fermentation by dairy starters is noted in colostrum and mastitic milk, which contain increased amounts of Igs. Also, high Ig concentrations may adversely affect the antibiotic residual tests based on microbial growth, causing false positive results (Korhonen, 2004). Igs contribute to cream formation by agglutinating fat globules, which process accelerates the ascent of cream to the surface. This phenomenon is attributed primarily to IgM, which have been termed cryoglobulins or 'cold agglutinins'. The agglutination property of Igs can, however, be inactivated by heat treatment (pasteurization) and mechanical agitation.

Among dairy processes, the properties of Igs are most affected by the thermal treatments. In high temperature/short time (HTST) pasteurization (72°C/15 sec) about 25–40% of the Ig activity is lost, whereas ultra high temperature (UHT) treatment (138°C/4 sec) and evaporation processing destroy most of the specific immune activity of milk due to Ig denaturation (Li-Chan *et al.*, 1995). In contrast, other studies (Mainer *et al.*, 1997, 1999) have reported that bovine milk Igs could resist the HTST pasteurization treatment without affecting their structure. Only 1% of IgG, 2% of IgA, and 14% of IgM concentrations were denatured in laboratory experiments. Also, the HTST pasteurization had little effect on the activity of bovine colostrum IgG as the original rotavirus neutralizing activity was reduced by only 0.5%. In a recent study by McMartin *et al.* (2006), it was demonstrated that a rapid heat inactivation of IgG started at temperatures higher than 65°C, and at 81°C, as much as 90% of the virus neutralization activity of Igs was lost in less than two minutes. On the other hand, heating moderate or high quality colostrum at 60°C for at least 120 min had no effect on mean IgG concentration or titer of neutralizing antibodies against bovine viral diarrhea virus Type 1. In storage stability studies, it has been observed that bovine IgG added into UHT-treated milk retained its specific immune activity for over several months (Fukumoto *et al.*, 1994). Also, Ig molecules seem to retain their specific activity well in milk powder, irrespective of the storage temperature (Mehra *et al.*, 2006). Further, bacterial fermentation of milk by yoghurt or probiotic bacteria have not been found to reduce

significantly the activity of colostral antibodies added in milk during a storage period of 50 days at 4°C. (Wei *et al.*, 2002).

## 10.4 Applications of immunoglobulins

### 10.4.1 Immunoglobulin and immune milk preparations

The progress in understanding the mechanisms of Ig mediated immune functions and the rapid development of industrial fractionation technologies have raised interest in developing formulations supplemented with bovine colostral or cheese whey derived Igs. Most of the current commercial Ig products are prepared from colostrum of non-immunized cows by removing the fat, followed by microfiltration or pasteurization under conditions that retain the biological activity of Igs. These products are usually in the form of spray-dried and freeze-dried powders, but some are in the form of filtered colostral whey liquids or concentrates. Dried products include whole colostrum powder, skim powders, skim colostrum protein concentrate and colostrum whey concentrates. Some of above preparations have been tested clinically for certain physiological functions or prevention/treatment of microbial infections. Accordingly, a few products boast specific health or nutrition function claims, such as boosting immunity against microbial infections or speeding recovery from physical endurance exercises. (Scammel, 2001; Kelly, 2003; Tripathi and Vashishtha, 2006). However, in most cases, the clinical evidence related to these products is very limited or not available.

The specificity of natural antibodies found in milk and colostrum of different cows reflects the wide spectrum of antigens the animals have encountered in the past in their environment and in ingested feedstuffs (Korhonen *et al.*, 2000a; Kelly, 2003). The antibody titre against certain antigenic pathogens or structure, e.g. virulence factor, can be raised up to several hundred times by immunizing the cow before parturition with vaccines containing the antigens (Korhonen *et al.*, 1995). The resulting immune colostrum and products made thereof have fundamentally different antimicrobial properties and efficacies against pathogens than normal colostrum and these two concepts have, therefore, to be differentiated. This is emphasized by the fact that the normal colostrum and preparations made from it are, in most countries, regarded as food or dietary supplement, whereas the Ig containing preparations from immunized cows are often regarded as pharmaceuticals, e.g. in the EU and USA, or their regulatory status is not defined (Scammel, 2001; Hoerr and Bostwick, 2002; Mehra *et al.*, 2006). Table 10.3 lists commercial Ig and immune milk preparations developed over the last decade.

### 10.4.2 Proteolysis of immunoglobulins in the gastrointestinal tract

It is well known that the low pH of gastric acid reduces significantly the activities of ingested Igs. Furthermore, the ingested Igs are subjected to

**Table 10.3** Commercial colostrum and immune milk products

Product	Company	Claimed health benefits
Intact™	Numico RA (Australia)	Immune enhancing, athletic performance
Gastrogard-R™ (from immunized cows)	Northfield Laboratories, (Australia)	Prevents diarrhea caused by rotavirus in infants and children <4 years
PRO-IMMUNE 99	GalaGen Inc., (USA)	Prevents scours caused by <i>E. coli</i> in calves.
Proventra™	GalaGen Inc., (USA)	Boosts immunity and enhances body's natural resistance
Lactimmunoglobulin Biotest	Biotest Pharm GmbH (Germany)	Product for treatment of diarrhea in AIDS patients
ColostrumGold™ liquid Colostrumune™ powder	Sterling Technology, Inc (USA)	Immune system booster
First Defence <sup>R</sup> (from immunized cows)	Immucell (USA)	Reduces mortality and morbidity from scours caused by <i>E.coli</i> K99 <sup>+</sup> and coronavirus in calves
Anti-CD WPC (from immunized cows)	MucoVax Ltd (Netherlands)	Prevents relapse of <i>C. difficile</i> diarrhea
Glycomax™ Immunoglobulin	Probiotec Nutritionals Ltd (Australia)	Improves immunity against microbes and improves health and wellbeing
ColoPlus™	ColoPlus Ltd. (Sweden)	Alleviates HIV-associated diarrhea

Data modified from Mehra *et al.* (2006).

degradation by intestinal proteases. In the stomach, the enzymatic hydrolysis by pepsin, fragments the IgG molecule to F(ab')<sub>2</sub>, Fab/c, pFc and Fv fragments (Nezlin, 1998a). In the small intestine, trypsin, chymotrypsin, carboxypeptidase and elastase initially degrade the antibodies to F(ab')<sub>2</sub>, Fab and Fc fragments. The secretory piece of milk SIgA protects this Ig form against digestion by proteolytic enzymes. Of the SIgA present in human colostrum, 20–80% passes undegraded through the gut of the human infant. Also, bovine IgG<sub>1</sub> makes an exception since it is rather resistant to trypsin. Bovine colostrum and mastitic milk contain a compound which inhibits trypsin activity. The resulting F(ab')<sub>2</sub> and Fab fragments retain at least part of the neutralizing and adhesion inhibiting activities in the intestine. Pacyna *et al.* (2001) supplemented whole milk with immune colostrum containing specific Igs against rotavirus. When 100 ml of this supplemented whole milk was administered to 105 children three times a day for six days, the anti-rotavirus Ig activity was detected by the ELISA method in 521 of 602 fecal specimens obtained during the study. Kelly *et al.* (1997)

demonstrated that 10%–30% of orally administered bovine anti-*Clostridium difficile* Ig concentrate could be recovered from the stools of human infants and adults in the form of F(ab')<sub>2</sub> and Fab fragments. The survival of IgG increased remarkably by encapsulation with gelatin.

#### **10.4.3 Efficacy of immune milk preparations against microbial pathogens**

The concept of 'immune milk', i.e. the transfer of passive immunity by milk Igs, dates back to the 1950s. Since then, a great number of clinical studies have been published on the efficacy of various immune and non-immune milk Ig preparations in the prevention and treatment of various gastrointestinal microbial diseases. Recently, these studies have been reviewed by Mehra *et al.* (2006), Korhonen and Marnila (2006) and Hammarström and Weiner (2008). Thus, the protocols and results of these numerous studies are not discussed in detail in this article. Briefly, the general tendency of these studies has been that orally administered bovine milk or colostrum Igs have, in most cases, proved to be effective in the prevention of orally mediated infections. Colostrum or milk Igs can effectively agglutinate bacteria (Xu *et al.*, 2006), and bovine colostrum and colostrum preparations prevent effectively the attachment of a pathogen to the cells in epithelial lining. However, since bovine colostrum contain more than one adhesion inhibiting factor, the importance of specific Igs is often not known. Palmeira *et al.* (2001) observed that inhibition of enteropathogenic *E. coli* adherence to HEp-2 cultured cells by bovine colostrum was not caused by IgG fraction and was mediated by a high molecular weight fraction. However, in some studies the inhibition of pathogenic adhesion to epithelial cells has been attributed to presence of specific Igs. Casswall *et al.* (2002) showed that a specific bovine immune colostrum preparation blocked almost 90% of *Helicobacter pylori* attachment to human gastric mucosal tissue *in vitro* and 95% of binding to Lewis b glycoconjugate, while a control colostrum preparation from nonimmunized cows was inefficient. Doyle *et al.* (1993) reported that hyperimmune bovine colostrum Ig inhibited *Cryptosporidium parvum* adherence to epithelial MDCK cells and infectivity in an *in vitro* assay, and this inhibition was correlated with the protective capacity of the bovine colostrum *in vivo*. No significant adhesion inhibition was observed for control preparation from colostrum of sham immunized cows. Bojsen *et al.* (2007) studied the efficacy of bovine macromolecular whey protein fractions in prevention of infection of two human intestinal cell lines (Caco-2 and FHs 74 Int) by four different rotavirus strains (Wa, RRV, YM, RF). The major component of the protein fraction that inhibited effectively the rotavirus infectivity *in vitro* was shown to be bovine IgG. The same fraction was the only one affecting rotavirus shedding in an *in vivo* mouse model.

In the treatment of already established infections, promising therapeutic effects have been reported, mainly in such diseases where the infection is

maintained through a reattachment and reinfection, e.g. inside the oral cavity or the gastrointestinal lumen, and where the secretion of toxins or other inflammatory compounds is involved, which can be neutralized by the specific colostral Igs. Diseases caused by enterotoxigenic *Escherichia coli* strains, *Cryptosporidium parvum*, *Candida spp.*, *Shigella flexneri* and rotavirus are examples. As instances of clinical studies, a closer look is taken hereunder at recent progress in developing bovine immune milks targeted against *Streptococcus mutans* and *Clostridium difficile*. Table 10.4 provides data on recent clinical studies carried out with colostral Ig and immune milk preparations.

#### *Streptococcus mutans immune milk*

The pathogenesis of dental caries caused by *Streptococcus mutans* involves a series of attachment and binding events that lead to accumulation of cariogenic bacteria sufficient to cause the caries. Immunization strategies against streptococcal adhesins or glucosyltransferase enzymes (GTFs) have been shown to effectively interfere with the pathogenesis of mutans streptococci (Smith *et al.*, 2001). Blocking of the activity of *S. mutans* glucan binding proteins serves another effective strategy. Since humans cannot be immunized actively against caries bacteria due to the risk of side effects, bovine colostral antibodies have been studied in local passive immunization to prevent dental caries (for a review see Koga *et al.*, 2002).

An immune milk preparation from cows immunized with a *S. mutans* and *Streptococcus sobrinus* whole cell vaccine inhibited *in vitro* glucosyltransferase and fructosyltransferase enzyme activities of *S. mutans* (Loimaranta *et al.*, 1997), promoted aggregation of *S. mutans*, and inhibited adherence of the bacteria to saliva-coated hydroxyapatite particles (Loimaranta *et al.*, 1998b). The same immune preparation resulted in a higher resting pH in dental plaque of adult volunteers as compared to control groups, when used as a mouth rinse for three days (Loimaranta *et al.*, 1999). After the rinsing period with the immune product, the relative number of mutans streptococci had decreased significantly as compared to the controls.

Chicken egg yolk Igs obtained from immunized hens with *S. mutans* glucan binding protein had a clear protective effect against *S. mutans* infection and caries development in a rat model (Smith *et al.*, 2001). Mítoma *et al.* (2002) immunized cows with a fusion protein prepared by a fusion of a saliva-binding alanine-rich region of a cell surface protein antigen (Pac) and a glucan binding domain of the glucosyltransferase-I cell surface protein from *S. mutans*. The immune colostrum preparation effectively prevented dental caries development in a rat model when given as concentrate once a day for 55 days, together with cariogenic diet. Shimazaki *et al.* (2001) examined the effect of immune colostrum containing Igs against the same fusion protein on adult subjects after an antibiotic (cetylpyridinium chloride) treatment. The immune preparation inhibited significantly

**Table 10.4** Recent *in vivo* studies on the efficacy of orally administered immunoglobulin preparations on health

Antigen used in immunization	Disease or condition	Treatment regimen	Treatment effect	Reference
<i>S. mutans</i> and <i>S. sobrinus</i> whole cell vaccine	Dental caries	Mouth rinse 3 times daily for 3 days (human volunteers)	Higher resting pH and smaller proportion of <i>S. mutans</i> in dental plaque	Loimaranta <i>et al.</i> (1999)
Virulence factors of <i>S. mutans</i>	Dental caries	Mouth rinse twice per day for 14 days by 10 ml of immune milk (human volunteers)	Inhibited recolonization of <i>S. mutans</i> after antibiotic treatment	Shimazaki <i>et al.</i> (2001)
<i>Helicobacter felis</i> whole cell vaccine	Gastritis	0.2 ml of immune whey (7.5% IgG) before infection or 3 times daily for 4 weeks in treatment of infected mice	Prevented infection in non-infected mice and decreased gastric inflammation and colonization in readily infected mice	Marnila <i>et al.</i> (2003)
<i>Helicobacter pylori</i> urease	Gastritis	150 ml of yogurt with 1% avian IgY and probiotic bacteria 3 times daily for 4 weeks	Decreased values in urea breath test indicating decrease in colonization	Horie <i>et al.</i> (2004)
No immunization	EHEC-colitis	Colostrum preparation IMMULACT <sup>®</sup> to mice ad libitum (around 300 mg per day) for 3 weeks	Decreased rapidly EHEC colonization and decreased attachment to cecum walls and decreased mortality in mouse	Funatogawa <i>et al.</i> (2002)
Polyvalent or monovalent <i>E. coli</i> vaccine	Diarrhea	IgG supplemented baby formula, daily dose 0.5 g of IgG per kg of body weight	Lower incidence of diarrhea and shorter duration of diarrhea episodes in human infants during follow-up period for 6 months	Tawfeek <i>et al.</i> (2003)
<i>Shigella dysenteriae</i> antigen I	Shigellosis	100 ml orally 3 times per day for 3 days in combination with antibiotics	No significant difference in any clinical parameter	Ashraf <i>et al.</i> (2001)
No immunization	HIV-associated diarrhea	Colostrum preparation ColoPlus <sup>®</sup> to HIV-associated diarrhea patients for 7 weeks	Substantial decrease in stool frequency and in fatigue and increase in body weight	Florén <i>et al.</i> (2006)

**Table 10.4** *Cont'd*

Antigen used in immunization	Disease or condition	Treatment regimen	Treatment effect	Reference
Cocktail of 17 strains of pathogenic diarrhea bacteria	<i>E. coli</i> and <i>Salmonella</i> diarrhea	Orally 10 mg of specific Ig per day for 10 days starting 6 or 8 days before infection	Prevented enteroinvasive <i>E. coli</i> and <i>Salmonella typhi</i> diarrhea and normalized immunological parameters	Xu <i>et al.</i> (2006)
Cocktail of 17 strains of pathogenic diarrhea bacteria	<i>E. coli</i> , <i>Salmonella</i> and <i>Shigella</i> diarrhea	Orally once per day for 10 days starting 6 or 8 days before infection	Decreased the clinical signs of diarrhea and supported splenic NK-cell functions	Huang <i>et al.</i> (2008)
No immunization	Murine rotavirus infection in mouse	Milk IgG fraction orally at time of infection and 12 hours post infection	Decreases rotavirus shedding in stools	Bojsen <i>et al.</i> (2007)
<i>Clostridium difficile</i> toxin and <i>C. difficile</i> whole cells	<i>C. difficile</i> diarrhea	Orally for 2 weeks as supportive treatment after antibiotic treatment	<i>C. difficile</i> toxins eradicated from 15 of 16 patients and no relapses in any patient during 11-month follow-up period	Van Dissel <i>et al.</i> (2005)
No immunization	Mild hypercholesterolemia	Orally 5 g of blood derived IgG daily for 3 or 6 weeks	Both total cholesterol and LDL levels decreased from baselines	Earnest <i>et al.</i> (2005)
No immunization	Upper respiratory tract infections	60 g of colostral protein daily for 8 weeks	Reduced significantly incidence of self-estimated symptoms of respiratory infections but no difference in duration	Brinkworth and Buckley (2003)
No immunization	Endotoxemia due to abdominal surgery	Colostral product Lactobin® 52 g daily in 4 doses orally for 3 days before surgery	Lower levels of endotoxin and endotoxin neutralizing capacity in blood suggesting reduced endotoxemia due to surgery	Bölke <i>et al.</i> (2002a)
No immunization	Endotoxemia due to coronary surgery	Colostral product Lactobin® 42 g daily doses orally for 2 days before surgery	Lower levels of CRP but no effect on perioperative endotoxemia	Bölke <i>et al.</i> (2002b)

Parts of this table compiled and edited from Korhonen *et al.* (2000) and Mehra *et al.* (2006).

recolonization of *S. mutans* in the saliva and plaque as compared to the control group, and the ratios of *S. mutans* to total streptococci in saliva and plaque were lower than in the control group. Wei *et al.* (2002) studied the combined effect of specific colostrum Ig preparation against caries streptococci with the probiotic bacterium *Lactobacillus rhamnosus* GG, ATCC 53103 (LGG). LGG added in milk was earlier shown to reduce the risk of caries in day-care children (Näse *et al.*, 2001). The LGG-bacteria and specific Igs in LGG-fermented milk synergistically inhibited adhesion of *S. mutans* to saliva-coated hydroxyapatite particles. The Igs remained active in UHT milk and in LGG fermented milk over the whole period of expected shelf-life of these products. Thus, the rinsing with bovine immune whey indicates favourable effects on human dental plaque by controlling *S. mutans* in the human oral cavity and it may be beneficial to combine specific bovine milk antibodies against mutans streptococci to probiotic LGG-containing milk products.

#### *Clostridium difficile immune milk*

Colonization of *Clostridium difficile* in the intestine may cause severe infectious diarrhea and colitis. The most important virulence factors are the toxins A and B, which are associated with the development of the disease. Outbreak of *C. difficile* infection often results from antibiotic treatments. In animal models, bovine immune colostrum preparations have been effective in the treatment of experimental *C. difficile* diarrhea. Van Dissel *et al.* (2005) used a preparation made of milk from cows immunized against *C. difficile* toxins and whole cell *C. difficile* as a supportive treatment for two weeks after a standard antibiotic treatment in an uncontrolled cohort study. Nine of 16 patients had a history of relapsing *C. difficile* diarrhea. After the regimen in 15 patients, the *C. difficile* toxins had disappeared from feces, and during the follow-up period of 11 months, none of the patients had another episode of *C. difficile* diarrhea. Numan *et al.* (2007) assessed the efficacy of *C. difficile* immune milk preparation in aiding the prevention of relapses in *C. difficile* patients. The immune milk was administered orally 5 g/day, divided into three equal doses for 14 days starting two weeks after standard treatment with oral metronidazole or vancomycin. In 109 disease episodes, 11 (about 10%) were followed by a relapse, whereas in contemporary controls the relapse rate was 20–25%. Young *et al.* (2007) evaluated the safety of the same immune milk administered to 77 *C. difficile* patients, similarly as in the previous study. Adverse effect monitoring, physical examinations and haematological and biochemical assessments showed no adverse effects in this group. Mattila *et al.* (2008) immunized cows with a whole-cell vaccine made of two toxigenic *C. difficile* strains and the immune whey preparation was made from the colostrum of these cows. In a controlled double-blind randomized study, 18 patients with *C. difficile* associated diarrhea received orally 1.6 g of Ig in 200 ml volume twice per day for 2 weeks, and 20 patients received 400 mg of metronidazole twice per day. A



*C. difficile* culture and toxin test was made on days 0, 14 and 28, respectively. At day 14, 100% of the metronidazole group and 83% of the immune colostrum group, respectively, responded positively to the treatment. The authors concluded that the immune colostrum treatment is somewhat less effective than the standard treatment with antibiotics but, on the other hand, it does not cause antibiotic resistance problems and does not alter the normal colonic bacterial flora as antibiotics do. In future, by optimizing the dosage, the treatment time, and the antigens used in immunizing the cows, the efficacy of bovine milk Ig preparations could be improved and this treatment strategy could also be used as a supportive treatment together with conventional antibiotics.

## 10.5 Future trends

As a result of progress made in membrane separation and chromatographic techniques it is now possible to isolate Igs from bovine colostrum and cheese whey on a large scale. This progress has facilitated and boosted manufacture of Ig concentrates as dietary supplements or ingredients. Some of these preparations are being marketed with health or nutrition function claims attached, even though the clinical evidence is limited or not reported at all. More scientific research is therefore needed in this field to substantiate the claimed health benefits. As for immune milk preparations, there are already a few commercial products on the market with quite well established clinical evidence about the efficacy. The main limitation of the clinical use of bovine milk antibodies for humans is that, as proteins of another species, the Igs can be used only orally against gastrointestinal pathogens. An interesting attempt to overcome this limitation is a trans-chromosomal calf that has been cloned for producing humanized polyclonal Igs. As a result of five sequential genetic modifications and seven consecutive cloning events, this calf was reported to have full human IgG 10–20% of total serum IgG. After hyperimmunization of this calf with an anthrax protective antigen, both full human IgG and chimeric IgG were found to be effective in a toxin-neutralization assay (Kuroiwa *et al.*, 2009).

The regulatory status regarding products containing specific Igs may be considered as the main reason why products (for instance those containing specific Igs against cariogenic bacteria) are not on the market in most countries. The regulatory status of immune colostrum preparations was undetermined for a long time and for some years these products have been classified as pharmaceuticals in the EU and USA. In the case of diseases that can be controlled also by conventional means, e.g. dental caries, this regulatory approach has slowed down the development of immune milk preparations in many countries. However, in the case of severe diseases caused by bacterial strains resistant to antibiotics, such as *C. difficile*, the regulatory status of a pharmaceutical has delayed but not hampered completely the progress of such product development (Hoerr and Bostwick,

2002; Mattila *et al.*, 2008). Currently, the US Food and Drug Administration (FDA) have accepted the safety of hyperimmune milks on the basis of clinical studies that show no adverse health effects from these products (Gingerich and McPhillips, 2005; Krissansen, 2007).

Incidences of diarrhea and mortality rates among children in many areas of developing countries are high. The prevention of diarrhea would improve the nutritional status and health in general. The emergence of antibiotic resistant pathogen strains puts emphasis on the need to develop alternative ways to prevent and treat gastrointestinal infections. In developed countries, the control of gastrointestinal microbial flora has become an integral part of health promotion. It is concluded that synergistic effects of Igs with probiotics and other milk bioactives, such as lactoferrin, may open new prospects for developing novel means to prevent microbial diseases by nutritional intervention.

## 10.6 References

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