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Healthy Multifunctional Spectra of Milk Glycoproteins and Their Fragments – a Review Article

Atef E. Fayed

Food Science Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

Key words: primary structures of lactoferrin (LF) and glycomacropeptide (GMP); biological, physiological and therapeutic benefits of LF and GMP

The functionalities of glycoprotein lactoferrin (LF) and glycomacropeptide (GMP) were discussed. LF is considered a multifunctional protein. Its absorption in the bowel; immune response; antioxidant, anti-carcinogenic and anti-inflammatory properties; and protection against microbial infection, were the most widely studied functions to date. Besides, promotion of balanced intestinal flora by preventing growth of harmful bacteria and stimulating bifidus, LF helps to secure a correct balance of the intestinal flora. Although, most of the proposed biological activities of LF are related to the binding of iron, the non-iron related functions have been described as well, such as regulation of iron metabolism, prevention of oxidation and control of cell or tissues damage (result of aging).

Likewise, GMP, which is a carbohydrate-containing peptide formed from chymosin or pepsin digestion of κ-casein, exhibits several useful biological activities, including binding of cholera toxin and *E. coli* enterotoxins, inhibition of bacterial and viral adhesions, suppression of gastric secretions, promotion of bifidobacterial growth, and modulation of immune responses. GMP contains no aromatic amino acids and is therefore used for phenylketonuria (PKU) suffering patients.

The carbohydratic parts bound to such glycoprotein or glycopeptide, may act as prebiotics in the intestine and colon.

INTRODUCTION

Milk proteins exert a wide range of nutritional, functional and biological activities. Many milk proteins possess specific biological properties that make these components potential ingredients of health-promoting foods. Increasing attention is being focused on physiologically active peptides derived from milk proteins [Korhonen & Pihlanto, 2006]. Whey, a protein complex derived from milk, is being touted as a functional food with a number of health benefits. The biological components of whey, including lactoferrin (LF), β-lactoglobulin, α-lactalbumin, glycomacropeptide (GMP), and immunoglobulins (Igs), demonstrate a range of immune-enhancing properties. In addition, whey has the ability to act as an antioxidant, antihypertensive, antitumor, hypolipidemic, antiviral, antibacterial, and chelating agent [Gill *et al.*, 2000; Marshall, 2004; Gauthier *et al.*, 2006; and Darewicz *et al*., 2011].

In this respect, LF is considered a multifunctional protein. Its absorption in the bowel; immune response; antioxidant, anti-carcinogenic and anti-inflammatory properties; and protection against microbial infection, were the most widely studied functions to date [Gonzalez-Chavez *et al.,* 2009; Tomita *et al.,* 2009]. Besides, promotion of balanced intestinal flora by preventing growth of harmful bacteria and stimulating bifidus, LF helps to secure a correct balance of the intestinal flora [Tomita *et al.*, 2009]. Although, most of the proposed biological activities of LF are related to the binding of iron, the non-iron related functions have been described by Levay & Viljoen [1995] such as regulation of iron metabolism, prevention of oxidation and control of cell or tissues damage (result of aging).

Likewise, GMP is included among the biologically-active components that have the ability to control the growth of host friendly colonic microflora and to modulate immune functions, thus helping to control infections. GMP may therefore act as an anti-infectious factor, promoting the growth of bifidobacteria while inhibiting the proliferation of pathogens [Bruck *et al.*, 2003a,b; Manso *et al.,* 2004].

In addition, GMP may combat infection by binding to lectins, viruses, and mycoplasma. Its binding of cholera toxin and *Escherichia coli* enterotoxins, inhibition of bacterial and viral adhesions, suppression of gastric secretions and modulation of immune responses were described by Brody [2000]. The effects of GMP on the immune functions are complex, however GMP has been reported to increase the proliferation and phagocytic activity of the macrophage-like cell [Li & Mine, 2004], whereas other authors showed that GMP inhibited the proliferation of several immune cells such as spleen cells and Peyer's patch cells [Otani *et al.,*1995; Manso *et al.,* 2004; Daddaoua *et al.,* 2005].

LACTOFERRIN

LF is a cationic globular glycoprotein occurring naturally in numerous bodily secretions, including milk, tears, mucus,

* Corresponding Author:
E-mail: profateffayed@yahoo.com (Prof. Atef E. Fayed)

blood and saliva. LF is an iron-binding protein that is closely related to the plasma iron-transport protein transferrin. Since its discovery and characterization, a large amount of research has been carried out on its structure and function, and considerable advances in our understanding of its biosynthesis, tissue distribution, and catabolism have been made. Most work has been carried out with either human or bovine lactoferrins, and much of what follows is applicable to lactoferrins from both species [Yamada *et al.,* 1987; Brock, 2002; Marshall, 2004; Adlerova *et al.,* 2008; Tomita *et al.,* 2009].

Structure, tissue distribution and catabolism of lactoferrin

LF is a single-chain glycoprotein with a molecular weight of about 80 kDa. The complete amino acid sequence of bovine LF has been determined and found to contain 689 amino acids, while human LF consists of 691 residues. LF binds two atoms of ferric iron per molecule (Figure 1). The chemical analysis reveals 4N-linked glycans. The sugars found are N--acetyllactosamine, N-acteylgucosamine, galactose, fucose, mannose, and neuraminic acid [Yamada *et al.,* 1987; Pierce *et al.,* 1991; Marshall, 2004; Baker & Baker, 2005].

LF is folded into two lobes that show sequence homology with each other and can each reversibly bind one ferric ion along with a synergistic anion, usually bicarbonate. In these respects it closely resembles transferrin, although its affinity for iron is somewhat higher, allowing iron to be retained at lower pH values. Bovine LF is only 15–20% saturated with iron. Iron-depleted LF, defined as containing less than 5% iron, is referred to as apolactoferrin. Human breast milk contains apolactoferrin [Steijns & Van Hooijdonk, 2000]. The concentration of LF in human milk and colostrums is approximately 2 mg/mL and 7 mg/ mL, respectively, while in bovine milk and colostrums it is approximately 0.2 mg/mL and 1.5 mg/ mL, respectively [Levay & Viljoen, 1995]. LF is a dominant component of whey protein in human breast milk; however, the concentration in most commercial whey protein powders is only 0.35–2.0% of total proteins [Marshall, 2004]. LF is also highly basic with a pI of 8–9, probably due to a unique basic region in the N-terminal region of the molecule that is not found in transferrin. One important consequence of this property is that LF can bind in a "pseudospecific" way to many acidic molecules, including heparin and various cell surface molecules [Lampreave *et al.,* 1990; Gasymov *et al.,* 1999; Zakharova *et al.,* 2000]. As discussed below, these interactions may be relevant to the physiological function of LF. As its name implies, LF was originally isolated from milk, where it can occur in high concentrations, although these vary widely between different species and at different stages of lactation [Masson & Heremans, 1971]. The concentration in blood is normally very low (1 μg/ mL) and probably originates from neutrophil degranulation, because blood concentrations increase during infection or inflammation [Birgens, 1985]. LF in milk will be cat catabolized in the digestive tract of the suckling newborn; the extent to which degradation occurs and the main regions of the tract in which it occurs are uncertain, and probably vary between species and with the age of the neonate. As discussed below and elsewhere in this volume, partial degradation of LF may give rise to peptides ("lactoferricins", LFcins) with biological activities. LF in blood or released by neutrophils is transported to the liver, where it is taken up by specific receptors and catabolized [McAbee & Esbensen, 1991]. The existence of this specific mechanism in the liver probably helps keep blood LF levels low, even in inflammation. It was shown that fed bovine LF is not completely degraded in the gastrointestinal tract, but is retained to some degree, as LFcin-containing peptides [Kuwata *et al*., 2001].

Research data suggest that ingested bovine LF is generally not absorbed in the blood [Wakabayashi *et al*., 2004b], but acts on the intestinal immune system and influences the systemic host-protective system [Wakabayashi *et al*., 2006; Teraguchi *et al.,* 2004].

In order to develop a practical method for pasteurization of LF, Abe *et al.* [1991] found that that Apo-LF is very stable at pH 4.0 and high temperatures. It was considered that heating conditions of pH 4.0 and temperatures of 90 to 100°C for 5 min were suitable as a practical method for pasteurization of LF. Moreover, the possibility of using an UHT method for more effective and efficient pasteurization of LF was demonstrated. Apo-LF treated at pH 2.0 or 3.0 and 100 or 120°C for 5 min was apparently degraded, but the antibacterial activity was equal to or stronger than that of unheated Apo-LF. This result suggests that some of the LF fragments produced by heat under acidic conditions have antibacterial activity.

Lactoferrin receptors

The biological properties of lactoferrin are mediated by specific receptors on the surface of target cells. These receptors are typical for each cell type and can be found, for example, on mucosal epithelial cells, hepatocytes, monocytes, macrophages, polymorphonuclear leukocytes, lymphocytes, trombocytes, fibroblasts, and on some bacteria such as *Staphylococcus aureus* or *Pseudomonas hydrophila* [Levay & Viljoen, 1995; Suzuki & Lonnerdal, 2002; Suzuki *et al.*, 2005; Adlerova *et al.,* 2008]. Some cells have also "main receptors", which enable them to bind not only lactoferrin, but also transferrin or lactoferrins of other species. Besides "classic" receptors, there are also nuclear receptors that bind leukocyte cmDNA [Kanyshkova *et al.*, 2001; Adlerova *et al.,* 2008].

Lactoferrin metabolism

There are two ways in which lactoferrin can be eliminated from the organism: either through receptor-mediated endocytosis of phagocytic cells (macrophages, monocytes, and other cells belonging to the reticuloendothelial system) with subsequent iron transfer to ferritin or through direct uptake by the liver. Endocytosis performed by Kupffer cells, liver endothelial cells, and hepatocytes contributes to lactoferrin removal [Levay & Viljoen, 1995; Adlerova *et al.,* 2008]. Kidneys seem to be involved in the removal of lactoferrin from the circulation since lactoferrin and its fragments, mainly of maternal origin, have been found in the urine of breast-fed infants [Hutchens *et al*., 1991; Adlerova *et al.,* 2008].

Physiological functions of lactoferrin

Because of its close resemblance to transferrin, initial research on LF function was directed towards establishing

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                                    10                                  20
Ala-Pro-Arg-Lys-Asn-Val-Arg-Trp-Cys-Thr-Ile-Ser-Gln-Pro-Glu-Trp-Phe-Lys-Cys-Arg-
                                    30                                  40
Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-
                                    50                                  60
Phe-Ala-Leu-Glu-Cys-Ile-Arg-Ala-Ile-Ala-Glu-Lys-Lys-Ala-Asp-Ala-Val-Thr-Leu-Asp-
                                    70                                  80
Gly-Gly-Met-Val-Phe-Glu-Ala-Gly-Arg-Asp-Pro-Tyr-Lys-Leu-Arg-Pro-Val-Ala-Ala-Glu-
                                    90                                  100
Ile-Tyr-Gly-Thr-Lys-Glu-Ser-Pro-Gln-Thr-His-Tyr-Tyr-Ala-Val-Ala-Val-Val-Lys-Lys-
                                    110                                 120
Gly-Ser-Asn-Phe-Gln-Leu-Asp-Gln-Leu-Gln-Gly-Arg-Lys-Ser-Cys-His-Thr-Gly-Leu-Gly-
                                    130                                 140
Arg-Ser-Ala-Gly-Trp-Ile-Ile-Pro-Met-Gly-Ile-Leu-Arg-Pro-Tyr-Leu-Ser-Trp-Thr-Glu-
                                    150                                 160
Ser-Leu-Glu-Pro-Leu-Gln-Gly-Ala-Val-Ala-Lys-Phe-Phe-Ser-Ala-Ser-Cys-Val-Pro-Cys-
                                    170                                 180
Ile-Asp-Arg-Gln-Ala-Tyr-Pro-Asn-Leu-Cys-Gln-Leu-Cys-Lys-Gly-Glu-Gly-Glu-Asn-Gln-
                                    190                                 200
Cys-Ala-Cys-Ser-Ser-Arg-Glu-Pro-Tyr-Phe-Gly-Tyr-Ser-Gly-Ala-Phe-Lys-Cys-Leu-Gln-
                                    210                                 220
Asp-Gly-Ala-Gly-Asp-Val-Ala-Phe-Val-Lys-Glu-Thr-Thr-Val-Phe-Glu-Asn-Leu-Pro-Glu-
                                    230                                 240
Lys-Ala-Asp-Arg-Asp-Gln-Tyr-Glu-Leu-Leu-Cys-Leu-Asn-Asn-Ser-Arg-Ala-Pro-Val-Asp-
                                    250                                 260
Ala-Phe-Lys-Glu-Cys-His-Leu-Ala-Gln-Val-Pro-Ser-His-Ala-Val-Val-Ala-Arg-Ser-Val-
                                    270                                 280
Asp-Gly-Lys-Glu-Asp-Leu-Ile-Trp-Lys-Leu-Leu-Ser-Lys-Ala-Gln-Glu-Lys-Phe-Gly-Lys-
                                    290                                 300
Asn-Lys-Ser-Arg-Ser-Phe-Gln-Leu-Phe-Gly-Ser-Pro-Pro-Gly-Gln-Arg-Asp-Leu-Leu-Phe-
                                    310                                 320
Lys-Asp-Ser-Ala-Leu-Gly-Phe-Leu-Arg-Ile-Pro-Ser-Lys-Val-Asp-Ser-Ala-Leu-Tyr-Leu-
                                    330                                 340
Gly-Ser-Arg-Tyr-Leu-Thr-Thr-Leu-Lys-Asn-Leu-Arg-Glu-Thr-Ala-Glu-Glu-Val-Lys-Ala-
                                    350                                 360
Arg-Tyr-Thr-Arg-Val-Val-Trp-Cys-Ala-Val-Gly-Pro-Glu-Glu-Gln-Lys-Lys-Cys-Gln-Gln-
                                    370                                 380
Trp-Ser-Gln-Gln-Ser-Gly-Gln-Asn-Val-Thr-Cys-Ala-Thr-Ala-Ser-Thr-Thr-Asp-Asp-Cys-
                                    390                                 400
Ile-Val-Leu-Val-Leu-Lys-Gly-Glu-Ala-Asp-Ala-Leu-Asn-Leu-Asp-Gly-Gly-Tyr-Ile-Tyr-
                                    410                                 420
Thr-Ala-Gly-Lys-Cys-Gly-Leu-Val-Pro-Val-Leu-Ala-Glu-Asn-Arg-Lys-Ser-Ser-Lys-His-
                                    430                                 440
Ser-Ser-Leu-Asp-Cys-Val-Leu-Arg-Pro-Thr-Glu-Gly-Tyr-Leu-Ala-Val-Ala-Val-Val-Lys-
                                    450                                 460
Lys-Ala-Asn-Glu-Gly-Leu-Thr-Trp-Asn-Ser-Leu-Lys-Asp-Lys-Lys-Ser-Cys-His-Thr-Ala-
                                    470                                 480
Val-Asp-Arg-Thr-Ala-Gly-Trp-Asn-Ile-Pro-Met-Gly-Leu-Ile-Val-Asn-Gln-Thr-Gly-Ser-
                                    490                                 500
Cys-Ala-Phe-Asp-Glu-Phe-Phe-Ser-Gln-Ser-Cys-Ala-Pro-Gly-Ala-Asp-Pro-Lys-Ser-Arg-
                                    510                                 520
Leu-Cys-Ala-Leu-Cys-Ala-Gly-Asp-Asp-Gln-Gly-Leu-Asp-Lys-Cys-Val-Pro-Asn-Ser-Lys-
                                    530                                 540
Glu-Lys-Tyr-Tyr-Gly-Tyr-Thr-Gly-Ala-Phe-Arg-Cys-Leu-Ala-Glu-Asp-Val-Gly-Asp-Val-
                                    550                                 560
Ala-Phe-Val-Lys-Asn-Asp-Thr-Val-Trp-Glu-Asn-Thr-Asn-Gly-Glu-Ser-Thr-Ala-Asp-Trp-
                                    570                                 580
Ala-Lys-Asn-Leu-Asn-Arg-Glu-Asp-Phe-Arg-Leu-Leu-Cys-Leu-Asp-Gly-Thr-Arg-Lys-Pro-
                                    590                                 600
Val-Thr-Glu-Ala-Gln-Ser-Cys-His-Leu-Ala-Val-Ala-Pro-Asn-His-Ala-Val-Val-Ser-Arg-
                                    610                                 620
Ser-Asp-Arg-Ala-Ala-His-Val-Lys-Gln-Val-Leu-Leu-His-Gln-Gln-Ala-Leu-Phe-Gly-Lys-
                                    630                                 640
Asn-Gly-Lys-Asn-Cys-Pro-Asp-Lys-Phe-Cys-Leu-Phe-Lys-Ser-Glu-Thr-Lys-Asn-Leu-Leu-
                                    650                                 660
Phe-Asn-Asp-Asn-Thr-Glu-Cys-Leu-Ala-Lys-Leu-Gly-Gly-Arg-Pro-Thr-Tyr-Glu-Glu-Tyr-
                                    670                                 680
Leu-Gly-Thr-Glu-Tyr-Val-Thr-Ala-Ile-Ala-Asn-Leu-Lys-Lys-Cys-Ser-Thr-Ser-Pro-Leu-
                                689
Leu-Glu-Ala-Cys-Ala-Phe-Leu-Thr-Arg.OH
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FIGURE 1. Primary structure of *Bos* lactoferrin (LF). Disulfides occur between the following Cys: 9 and 45, 19 and 36, 115 and 198, 157 and 173, 160 and 183, 170 and 181, 231 and 245, 348 and 380, 358 and 371, 405 and 684, 425 and 647, 457 and 532, 481 and 675, 491 and 505, 502 and 515, 573 and 587, and 625 and 630. The 2 iron-binding sites include (Asp60, Tyr92, Tyr192, His253) and (Asp395, Tyr433, Tyr526, His595). The anion-binding sites are (Arg121, Thr117) and (Arg463, Thr459). The potential glycosylation sites are Asn233, Asn281, Asn368, Asn476, and Asn545, [Farrell *et al.,* 2004].

functions related to its iron-binding properties: iron absorption, antimicrobial activity, and modulation of iron metabolism during inflammation. However, subsequent research has revealed a large number of other possible functions, many of which do not appear to involve iron-binding. At the same time, its roles in iron absorption, antimicrobial activity, and inflammation have been modified or called into question [Brock, 2002; Farnaud & Evans, 2003].

Plasma levels of LF have been found to be elevated due to release from neutrophils during infection, inflammation, tumor development, and iron overload [Levay & Viljoen, 1995].

Antimicrobial spectrum of lactoferrin

LF has been reported to affect growth and development of a wide range of infectious agents, ranging from viruses to protozoa, by a number of different mechanisms [Naidu, 2000; Baker & Baker, 2005].

Studies on LF have demonstrated its ability to activate natural killer (NK) cells and neutrophils, induce colony-stimulating factor activity, and enhance macrophage cytotoxicity [Nishiya & Horwitz, 1982; Sawatzki & Rich, 1989; Gahr *et al.,* 1991; McCormick *et al.,* 1991;Andres & Fierro, 2010]. LF also appears to have antiviral, antifungal, and antibacterial properties. The antimicrobial effect is likely more potent in organisms that require iron to replicate, as LF has the unique ability to chelate iron in a way that deprives microorganisms of this essential nutrient for growth [Shah, 2000;Andres & Fierro, 2010]. Moreover, Ohashi *et al.* [2003] reported that LF and β-casein in milk might play a role in antiseptic and antiinfectious functions due to cysteine protease inhibition of bacteria and viruses. In additional, LF has the ability to release the outer membrane of gram-negative bacteria, the lipopolysaccharide component, thus acting as an antibiotic [Tomita *et al.,* 2002, 2009; Farnaud & Evans, 2003].

Antibacterial activity

The ability of LF to inhibit bacterial growth *in vitro* was one of the earliest functions described for the protein, and was shown to be due to sequestration of the iron in the medium required for microbial metabolism. The antimicrobial activity is occurring whether as a bacteriostat as a result of iron deprivation, or as a bactericidal agent by direct effect on microbial membranes. Subsequent research has shown that iron sequestration by LF can inhibit growth of many species of bacteria *in vitro* [Weinberg, 1995], although some of bacteria can counteract the inhibitory effect through synthesis of low molecular weight high affinity chelators (siderophores, *i.e.*, iron carriers) or by production of specific LF receptors that can facilitate iron removal from the protein [Gray-Owen & Schryvers, 1996; Yu & Schryvers, 2002]. Adlerova *et al.* [2008] reviewed that LF ability to bind free iron, which is one of the elements essential for the growth of bacteria, is responsible for its bacteriostatic effect. A lack of iron inhibits the growth of iron-dependent bacteria such as *E. coli*. In contrast, LF may serve as iron donor, and in this manner support the growth of some bacteria with lower iron demands such as *Lactobacillus* sp. or *Bifidobacterium* sp., generally considered as beneficial. Nevertheless, some bacteria are able to adapt to the new conditions and release siderophores (iron chelating compounds of bacterial origin) that compete with LF for Fe3+ ions. Some other types of bacteria, including Neisseriaceae family, adapt to new conditions by expressing specific receptors capable of binding LF, and to cause changes in the tertiary structure of the LF molecule leading to iron dissociation.

A second antibacterial mechanism has been described, which is independent of iron-binding and involves the basic N--terminal region of LF. Originally described in 1977 as a bactericidal activity against *Str. mutans* and *Vibrio cholerae* [Arnold *et al.,* 1977], the mechanism was clarified by studies showing that LF can disrupt or possibly even penetrate bacterial cell membranes [Yamauchi *et al.,* 1993], and that the isolated N--terminal basic peptides, named LFcins [Bellamy *et al.,* 1992], were more potent than the intact protein. The LFcin derived from bovine LF was, if anything, more potent than human LFcin, while mouse LF contains additional acidic residues in its N-terminus, and cannot give rise to an active basic LF--cin [Strøm *et al.,* 2000, 2002]. Despite the large amount of research on the mechanisms of these antibacterial effects *in vitro*, their role *in vivo* remains controversial. *In vitro* experiments showing inhibition of growth through iron sequestration cannot mimic the complex interactions occurring during infection *in vivo*, when iron is available from a much wider range of sources, including haemoglobin, to which LF cannot bind. At a clinical level, attempts to mimic the gut flora of breast-fed infants by feeding LF-supplemented cow's milk formulas have had little success [Roberts *et al.,* 1992], and bacterial infection is not a major cause of mortality in patients with primary haemochromatosis [Powell *et al.,* 1994]. New clinical data have now also demonstrated the protective effect against *Helicobacter pylori* (cancer and ulcer inducing bacterium). However, the *in vivo* study in mice reported a protective effect against *H. pylori* infection [Wang *et al.,* 2001; Dial & Lichtenberger, 2002], though this is probably not dependent upon iron sequestration. A protective effect against *H. pylori* has important implications for the development of stomach cancer, which is associated with this organism. The bactericidal mechanism mediated by the basic N-terminal region of LF is sensitive to ionic strength and pH and may not operate well, if at all, under physiological conditions [Bortner *et al.,* 1989]. It is also uncertain whether physiologically relevant concentrations of active LFcin peptides can be generated *in vivo*. However, there remains a potential pharmacological interest in the therapeutic use of LFcins produced on a commercial scale.

Several studies have revealed that LF plays a direct role in the body's defense against pathogens, including findings that individuals more susceptible to infection have lower levels of neutrophil LF [Breton-Gorius *et al.,* 1980; Boxer *et al.,* 1982; Baker & Baker, 2005]. In an open, randomized, single-center study of 150 individuals with diagnosed *H. pylori* infection, patients were given antibiotics at varying doses and durations (range 7–10 days) in conjunction with 200 mg encapsulated LF [Di Mario *et al.,* 2003]. Analysis of the study revealed 100%-eradication of *H. pylori* in the group using the seven-day antibiotic course with the addition of LF. Other research reported the anti-infective effects of oral LF in animals with *H. pylori* gastric infection, *Staphylococcus aureus* systemic infection and *E. coli* urinary tract infection [Wakabayas-

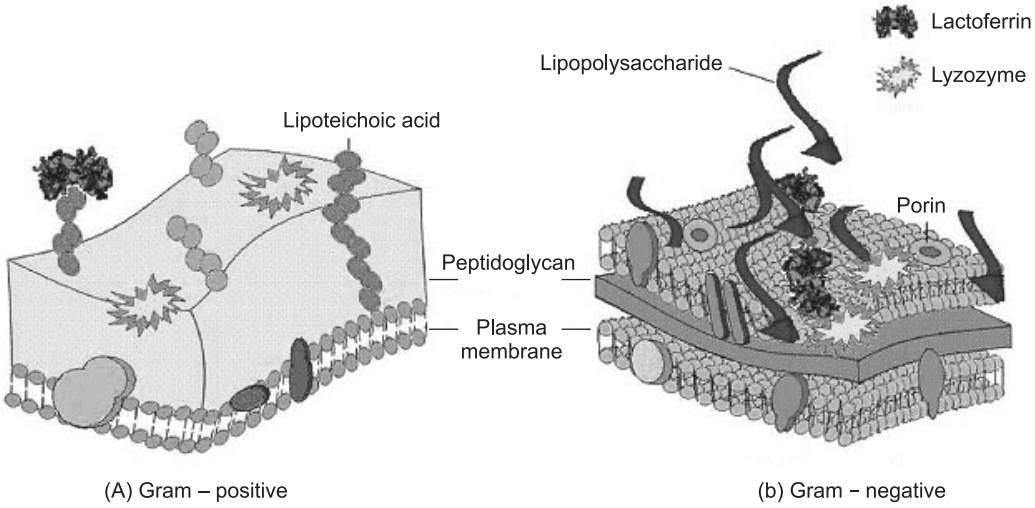

FIGURE 2. Mechanism of antibacterial action of lactoferrin (LF). (A) Gram-positive bacteria: LF is bound to negatively charged molecules of the cell membrane such as lipoteichoic acid, neutralising wall charge and allowing the action of other antibacterial compounds such as lysozyme. (B) Gram-negative bacteria: LF can bind to lipid A of lipopolysaccharide, causing liberation of this lipid with consequent damage to the cell membrane.

hi *et al.*, 2006]. LF also improves some symptoms of *H. pylori* gastric infection [Okuda *et al.*, 2005] and increases the eradication rate of triple therapy against *H. pylori* in the stomach [Di Mario *et al*., 2003]. The suppressive effect of bovine LF against *H. pylori* was examined using two clinical tests. Yoghurt or yoghurt with LF was administered to a *H. pylori* positive group of 25 people (including healthy people and patients with digestive disease) for eight weeks. Both groups had significantly decreased urine breath test (UBT). A comparison test involving 59 *H. pylori* positive people showed significant effects of LF and no side effect [Imoto *et al.,* 2004].

On the other hand, the "classic" seven-day triple antibiotic group had a success rate of 76.9%, while the 10-day treatment group demonstrated a 70.8-percent success rate. In a small study, 12 children suffering from chronic pharyngitis were administered a combination of 500 mg erythromycin three times daily and 100 mg bovine LF in a gargle. All children were tested positive for Group A Streptococci. After 15 days of treatment, fewer intracellular Group A Streptococci were found compared to a children-group treated with antibiotics alone [Ajello *et al.,* 2002]. In a concise review, it has been discussed the bacteriostatic and bacteriocidal activity of LF exhibited against a number of organisms, including *E. coli, Salmonella typhimurium, Shigella dysenteriae, Staphylococcus aureus, Listeria monocytogenes, Bacillus stearothermophilus, B. subtilis,* and *Micrococcus luteus* [Batish *et al.,* 1988; Payne *et al.,* 1990; Saito *et al*., 1991; Yamauchi, 1992; Shah, 2000; Bessler *et al.,* 2006]. Suzuki *et al.* [1989], Shah [2000] and Baker & Baker [2005] further discussed that LF, when in combination with lysozyme, is a more potent bacteriostatic agent against *Pseudomonas aeruginosa, L. monocytogenes*, and *E. coli.* Recently, Gonzalez-Chavez *et al*. [2009] pointed out a description explaining the antibacterial mechanism of LF as shown in Figure 2.

Antiviral activity

LF may prevent entry of viruses, like herpes and Human Immunodeficiency Virus (HIV) into mammalian cells. Research also demonstrated the beneficial effects of oral LF in other animal infection models, including herpes virus skin infection, and influenza virus pneumonia [Wakabayashi *et al*., 2004a; Shin *et al*., 2005]. Shin *et al.* [2005] suggested the potential of oral administration of LF to attenuate pneumonia in influenzavirus-infected mice through the suppression of infiltration of inflammatory cells in the lung

LF can reduce infectivity of a number of different viruses, predominantly *in vitro* systems. Again, mechanisms are uncertain, but probably involve blocking of cell–virus interactions as a result of LF's propensity to bind to acidic molecules, rather than iron-mediated effects on host cells. For example, both LF and LFcin block entry of cytomegalovirus into fibroblasts [Andersen *et al.,* 2001], whereas only the intact protein inhibits hepatitis C, this being achieved by virus neutralization [Ikeda *et al.,* 2000]. Anti-herpesvirus activity is mediated mainly by the N-lobe, but the C-lobe and even other members of the transferrin family also demonstrate some activity [Siciliano *et al.,* 1999; Giansanti *et al.,* 2002], suggesting that antiviral activity may be a property that developed early in the evolution of the transferrin family of proteins. Further work on mechanisms and *in vivo* effects will be required to determine whether LF genuinely has a role in the pathogenesis of viral infections. New clinical data have now also demonstrated the protective effect against *Heamophilus influenzae* (flu) and chronic hepatitis C. Initially it was found that bovine LF prevented hepatitis C virus (HCV) infection *in vitro* in a human hepatocyte line [Ikeda *et al.,* 1998]. A pilot study was conducted on 11 patients with chronic HCV. Each patient received either 1.8 or 3.6 g bovine LF daily for eight weeks. In patients with low pretreatment viral loads of HCV, decreases in HCV RNA and serum alanine transaminase were observed. In patients with higher pretreatment HCV viral loads, the levels did not change significantly [Tanaka *et al.,* 1999]. A dose--response trial of 45 individuals with HCV was conducted at doses of 1.8, 3.6, and 7.2 g LF daily for eight weeks [Tanaka *et al.,* 2000]. A virological response was observed in only four patients, although HCV RNA was still detectable. Eight patients

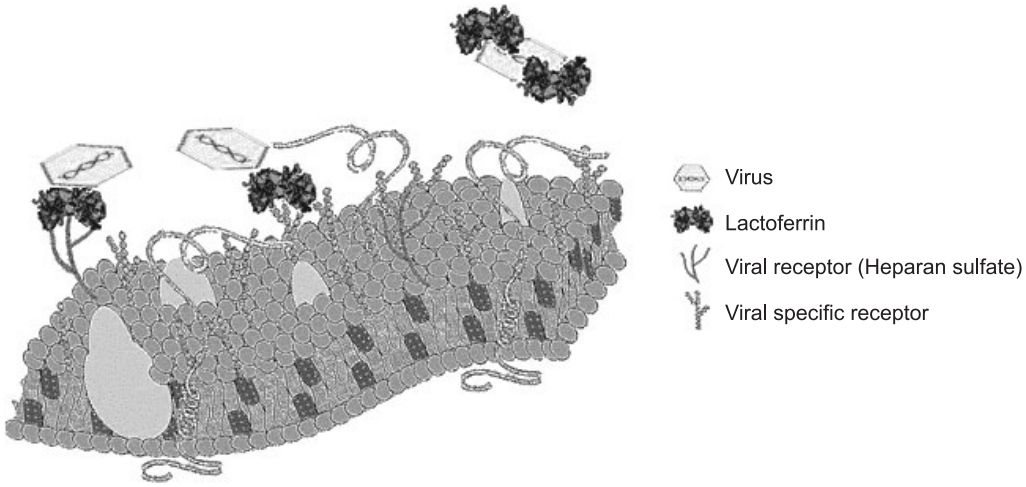

FIGURE 3. Mechanism of antiviral action of lactoferrin (LF). LF can be linked to the viral particle and to glycosaminoglycans, specific viral receptors or heparan sulfate to prevent internalisation of the virus into the host cell.

had a virological response, a 50% or greater decrease in HCV RNA and ended after eight weeks of treatment. There were no significant variations in dose-dependant responses. Recently, Pan *et al.* [2006] has revealed that bovine LF is more effective against viral infections than human LF. Apo-LF is less effective than the iron-saturated LF. Antiviral effects of LFcin and other peptides liberated from LF are weaker than those of intact LF. Moreover, the beneficial effects of LF on rotaviral gastroenteritis were shown [Egashira *et al.,* 2007]. Moreover, Gonzalez-Chavez *et al*. [2009] described an explanation for the antiviral mechanism of LF as shown in Figure 3.

Antifungal activity

LF has also demonstrated antifungal activity toward *Candida albicans* [Jones *et al.,* 1994; Viejo-Diaz *et al.,* 2004] and *Tinea pedis* (athlete's foot) a type of dermatophytosis [Yamauchi *et al*., 2000]. LF facilitates the cure of dermatophytosis and decreases fungal abundance in the skin [Wakabayashi *et al.*, 2000]. Research also demonstrated the beneficial effects of oral LF in other animal infection models including oral candidiasis [Wakabayashi *et al*., 2004a; Shin *et al*., 2005].

Antiparasitic activity

The role of LF in parasitic diseases is not well defined and may involve multiple mechanisms. Preincubation of *Toxoplasma gondii* and *Eimeria stiedai* sporozoites with bovine LF peptides reduced their infectivity in animal models [Omata *et al.,* 2001], suggesting an effect of basic peptides on parasite membrane integrity and/or interaction with host tissues. Likewise, incubation of fibroblasts with LF reduces the ability of *Plasmodium berghei* to bind to surface acidic molecules [Shakibaei & Frevert, 1996]. Other reported antiparasitic activities appear to involve interference with parasite iron acquisition, *e.g*., by *Pneumocystis carinii* [Cirioni *et al.*, 2000], while for other parasites such as *Tritrichomonas foetus* [Tachezy *et al.,* 1996], LF appears to act as a specific iron donor and could thus be expected to enhance infection. Given that many parasitic infections involve mucosal tissues where LF is likely to be present, further studies of the role of LF in parasitic infections would be worthwhile.

Promotion of balanced intestinal flora

Through the promotion of balanced intestinal flora by preventing growth of harmful bacteria and stimulating bifidus, LF helps to secure a correct balance of the intestinal flora. LF may serve as iron donor, and in this manner support the growth of some bacteria with lower iron demands such as *Lactobacillus* sp. or *Bifidobacterium* sp., generally considered as beneficial [Adlerova *et al.,* 2008]. Companies that market nutritional products containing LF suggest dosage levels ranging from 10–300 mg *per* day. This is in addition to the 100–125 mg of LF that an average adult already consumes *per* day in dairy products. Oral administration of LF suppressed the overgrowth of Enterobacteriaceae, *Streptococcus* sp., and *Clostridium* sp., and translocation of intestinal bacteria, including Enterobacteriaceae [Teraguchi *et al*., 1994, 1995;Tomita *et al.*, 2009].

Concerning infant formula and infantile colic, creating a substitute for mother's milk has proved to be challenging. It is estimated that a nursling infant ingests about 3 g LF daily during the first week of life [Reiter, 1985], whereas a calf drinking two liters of milk a day ingests about 2 g LF daily. It is well accepted that nursing infants have a much richer gut flora than do the bottle-fed infants, particularly with Bifidobacteria and Lactobacilli [Walzem *et al.,* 2002]. Such flora is normally associated with an increased resistance to colonization of the digestive tract with pathogenic bacteria [Van Hooijdonk *et al.,* 2000]. It was determined that the addition of LF to a feeding formula increased levels of Bifidobacteria in bottle-fed babies. The levels of Bifidobacteria in formula-fed babies that were supplemented with LF were not as high as those found in breast-fed babies. In addition, Bifidobacteria in formula-fed babies took up to three months to develop, while Bifidobacteria developed more rapidly in nursing infants [Roberts *et al.,* 1992]. Administration of infant formula containing 1 mg/mL of LF increased the ratio of *Bifidobacterium* sp., but decreased that of Enterobacteriaceae and *Clostridium* sp., in the feces of low-birth weight infants [Kawaguchi *et al.,* 1989].

Recently, Kim *et al*. [2004] found *in vitro* that the growth of *Lb. acidophilus* was stimulated by bovine Holo-LF but

not by Apo-LF. With bifidobacteria, bovine LF stimulated growth of three strains: *B. breve*, *B. infantis* and *B. bifidum* under certain conditions. Both apoprotein and holoprotein had similar effects. However, *B. longum* growth was not affected by LF. Thus, the mechanism of stimulating growth of bifidobacteria may be different from that of *Lb. acidophilus*. By far-western blotting using biotinylated LF and horseradish peroxidase-conjugated streptavidin, LF-binding proteins were detected in the membrane protein fraction of *Lb. acidophilus*, *B. bifidum*, *B. infantis* and *B. breve*. The molecular weights of LF-binding proteins of *Lb. acidophilus* were estimated to be 27, 41 and 67 kDa, and those of the three bifidobacterial strains were estimated to be 67–69 kDa. However, no such LF-binding components were detected in the membrane fraction of *B. longum*. It is interesting that the appearance of LF--binding proteins in the membrane fraction of these species corresponds to their growth stimulation by LF.

Lactoferrin and inflammation

LF demonstrates anti-inflammatory and immunity properties by protection of lymphoctes against free iron, binding of lipopolysaccharides and activation of cells involved in the anti-inflammatory response [Anonymous, 2003].

Apart from a possible role in modulating iron homeostasis during inflammation, there is now a substantial amount of work indicating that LF may directly regulate the inflammatory response. It has been known for some time that LF can bind to bacterial endotoxin (lipopolysaccharide, LPS), a major mediator of inflammatory responses in bacterial infections [Miyazawa *et al.,* 1991]. As a result, interaction of LPS with receptors is disrupted and downstream events such as upregulation of inflammatory cytokines are reduced [Baveye *et al.,* 1999]. Another potential anti-inflammatory role of LF is through the sequestration of "free" iron at inflammatory foci, such as rheumatoid joints, thus preventing catalysis of the production of damaging free radicals [Trif *et al.,* 2001 and Guillen *et al.,* 2000]. LF can also reduce cutaneous inflammation by inhibiting migration of Langerhans cells [Griffiths *et al.,* 2001]. These anti-inflammatory effects of LF are probably initiated following release of LF from neutrophils, and could thus be viewed as a further manifestation of the role of neutrophils in inflammation.

Lactoferrin and tumorigenesis

Over the years a number of reports have suggested that LF has an antitumour role *in vitro*. The mechanisms implicated have been varied, and regulation of NK cell activity [Damiens *et al.,* 1998], modulation of expression of G1 proteins [Damiens *et al.,* 1999], inhibition of VEGF(165)- mediated angiogenesis, and enhancement of apoptosis [Yoo *et al.,* 1997] have all been reported. None of these seem to implicate LF's iron-binding activity. There have also been a number of animal studies showing that LF can inhibit development of experimental tumors [Ushida *et al.,* 1998;Tsuda *et al.,* 2002]. Yoo *et al.* [1998] demonstrated that LF has the ability to inhibit metastasis of primary tumors in mice with cancer.

A mouse study revealed that LF had the ability to regulate levels of tumor necrosis factor (TNF) and interleukin 6 (IL-6), thus decreasing inflammation and, ultimately, mortality [Machnicki *et al.,* 1993].

The preventive effects of LF feeding on colon carcinogenesis in rats were studied. It was clarified the cancer-preventive effects of LF in various organ-specific cancer models [Sekine *et al*., 1997; Tsuda *et al*., 2002]. They also showed that LF, LF-pepsin-hydrolyzate (LFhyd), and LFcin B have anti-metastatic effects [Iigo *et al*., 1999]. Recently, their group found in a randomized, double-blind, placebo-controlled study that oral LF may inhibit progression of colorectal polyps. Administration of LF at 3 g/day for 1 year showed a tendency to suppress colorectal adenomas of less than 5 min in diameter compared with that of placebo administration [Kozu *et al*., 2006].

Immunomodulatory activity

LF has a wide range of effects on the immune system, both *in vivo* and *in vitro*, and these have been discussed in earlier reviews [Levay & Viljoen 1995; Brock 1998; Baveye *et al.,* 1999]. However, it is difficult to see any clear trend evolving, and most observations are phenomenological, without any clue to underlying mechanisms. Most are probably unrelated to LF's iron-binding capacity, although recent studies indicate that LF may induce type 1 T cell responses by modulating iron supply to the spleen [Brock, 2002]; previous work has shown that iron deprivation favours a Th1 response [Mencacci *et al.,* 1997]. Many immunological mechanisms are critically dependent upon cell–cell interactions; the number and affinity of interactions between two cells can often affect the nature of downstream events. The ability of LF to bind to cell surfaces is likely to affect these parameters, and could thus give rise to altered immune responses. Further work will be needed to test this hypothesis, but at present it is difficult to see any clear-cut role for LF as an immunomodulatory agent.

Fed LF is not completely degraded in the gastrointestinal tract, but is retained to some degree, as LFcin-containing peptides [Kuwata *et al*., 2001]. Ingested LF is generally not absorbed in intestine into the blood [Wakabayashi *et al*., 2004b], but acts on the intestinal immune system and influences the systemic host-protective system [Wakabayashi *et al*., 2006; Teraguchi *et al.,* 2004].

Enhancement of iron transport and absorption

Due to its high affinity for iron, LF is an excellent iron carrier and increases the bioavailability of iron [Anonymous, 2003].

The structural and biochemical resemblance to transferrin suggested that LF might play a fundamental role in iron metabolism as an iron-transport molecule. However, despite much research into the interaction of LF with cells and tissues, there is still no good evidence that it plays any role as an iron finding, showing that LF-knockout mice have normal parameters of iron metabolism and transporter or indeed is involved in "mainstream" iron metabolism [Ward *et al.,* 2002]. Recent reinforce this conclusion. This is perhaps not surprising: under normal conditions LF is present in very low concentrations, if at all, in blood and tissues. In contrast, during inflammation, increased release of LF from neutrophils might be expected to impinge on iron metabolism, and in-

deed it was suggested many years ago that LF contributed to the hypoferraemia of inflammation by removing iron from transferrin and shuttling it back to macrophages [Van Snick *et al.,* 1974]. However, the slow interchange of iron between transferrin and LF at physiological pH argues against such a mechanism. LF might however contribute to local accumulation of iron at sites of inflammation, where lower pH could favour iron exchange from transferrin. In conclusion, current evidence suggests that while LF plays no major role in normal iron homeostasis, it may contribute to alterations in iron metabolism during infection and inflammation. In addition, the iron-binding function of LF may contribute to other physiological functions [Brock, 2002].

Natural antioxidant

LF is a non-enzymatic antioxidant [Yamada *et al.,* 1987]. As an iron scavenger, LF prevents the formation of free radicals, which trigger oxidation processes; thus LF may reduce the susceptibility to aging processes and disease [Anonymous, 2003].

Enzymatic activities

Ribonuclease activity

Several years ago, LF was reported to exist in multiple isoforms, some of which possessed ribonuclease activity [Furmanski *et al.,* 1989]. The molecular differences in these isoforms were not investigated and, although some further reports confirmed this activity [Devi *et al.,* 1994;Ye *et al.,* 2000], others have shown that the activity is weak and may be due to formation of a complex between LF and the low molecular weight ribonuclease present in milk and other biological fluids [Sorrentino *et al.,* 1999]. It is not clear what physiological role such an activity might perform.

Protease activity

More recently it has been reported that LF possesses serine protease activity, with specificity for the IgA1 protease and Hap adhesin of *Haemophilus influenzae* [Qiu *et al.,* 1998]. The activity was present in recombinant as well as native LF, ruling out the possibility of contamination by milk enzymes, and furthermore the active site has been identified and activity abrogated by site-directed mutagenesis. Thus this activity appears to be based on much firmer scientific ground than the ribonuclease activity, and may represent a new type of antimicrobial function for LF [Brock, 2002].

Autoantibodies to lactoferrin

Although not strictly a physiological function, it should be noted that there have been a large number of reports [Caradonna *et al.,* 2000; Okazaki *et al.,* 2000] of autoantibodies to LF in patients with various autoimmune diseases. These are now recognized as a component of antineutrophil cytoplasmic antibodies (ANCA). They might arise through cross-priming by bovine LF during infancy [Brock *et al.,* 1998], though this remains unproven. Interest has focused primarily on the diagnostic value of anti-lactoferrin ANCA, and although they could in theory interfere with functions such as iron binding or LF–cell interactions, there is no clear evidence that they have any pathological role in autoimmune disease [Brock, 2002].

GLYCOMACROPEPTIDE

Glycomacropeptide (GMP), arising from cleavage of κ-casein by chymosin or pepsin [Farrell *et al.,* 2004], exhibits several useful biological activities, including binding of cholera toxin and *E. coli* enterotoxins, inhibition of bacterial and viral adhesions, suppression of gastric secretions, promotion of bifidobacterial growth, and modulation of immune responses [Brody, 2000].

The increased interest due to health-promoting aspects of GMP will spark successful food use applications to take advantage of the biological activities. Articles have appeared in food processing magazines [Steijns, 1996; Clare, 1998; LaBell, 1998] extolling the benefits of GMP as a nutraceutical. GMP contains no aromatic amino acids and is therefore used for phenylketonuria (PKU) diets [Smithers *et al.,* 1991; Lim *et al.,* 2007; Van Calcar *et al.,* 2009; Ney *et al.,* 2009]. These individuals lack the ability to metabolize phenylalanine making GMP an amino acid source which they can tolerate [Nielsen & Tromholt, 1994]. It is therefore not surprising that there is a keen and growing interest in exploiting GMP for use in the food industry. GMP provides good palatability and functional properties imparting favorable mouth-feel and flavor to foods, which many existing food preparations used for PKU diets lack [Marshall, 2004]. GMP supplementation of infant formula led to increase zinc absorption in infant rhesus monkeys [Kelleher *et al.,* 2003]. Initial attempts to incorporate GMP into meringues, biscuits and apple jelly [Marshall, 1991] met with limited success. Snow Brand Milk Products has received a patent on a stabilizer/viscosifier which contains GMP [Brody, 2000].

Structure of glycomacropeptide

When κ-casein is treated with chymosin during cheesemaking, the protein is hydrolysed into para-κ-casein (residues 1–105), which remains with the curd, and GMP (residues 106–169), which is removed with the whey [Van Hooydonk *et al.,* 1984; Farrell *et al.,* 2004]. GMP is a kind of glycosyl phosphopeptide containing 64 amino-acid residues that is a protein present in whey at 10–15%. GMP is high in branched chain amino acids and lacks the aromatic amino acids including phenylalanine, tryptophan, and tyrosine (Figure 4). Its low molecular weight of 8000 Da makes it difficult to visualize with Coomassie Blue stain in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Many researchers [Tran & Baker, 1970; Fiat *et al.,* 1972; Jolles *et al.*, 1972; Fournet *et al.,* 1975; 1979; Doi *et al.*, 1979; Van Halbeek *et al.,* 1980; Saito *et al.,*1981; Saito & Itoh, 1992] contributed to information about the saccharide structures in GMP. In mature cow's milk it has been established that five saccharides are found:

(1) Monosaccharide GalNAc – O - R
(2) Disaccharide Gal b1 ! 3 GalNAc – O – R
(3) Trisaccharide NeuAc a2 ! 3 Gal b1 ! 3 GalNAc – O - R
(4) Trisaccharide Gal b1 ! 3 NeuAc a2 ! 6. Gal- NAc – O - R
(5) Tetrasaccharide NeuAc a2 ! 3 Gal b1 ! 3 (NeuAc a2 ! 6. GalNAc – O - R

where, Gal: galactose; GalNAc: N-acetylgalactosamine, and NeuAc: sialic acid.

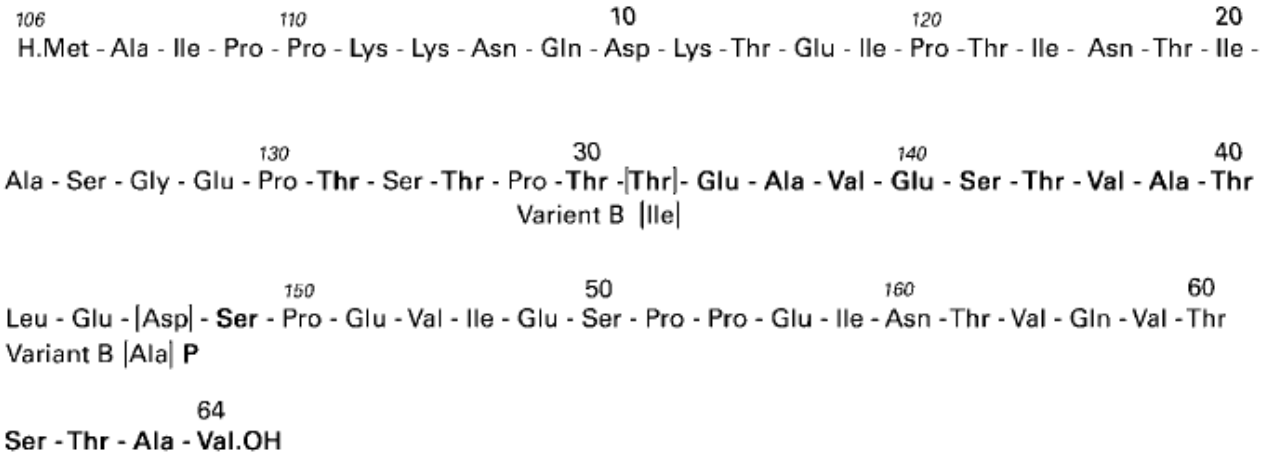

FIGURE 4. Primary structure of bovine GMP variant A [Eigel *et al.,* 1984; Whitney, 1988]. The enclosed amino acid residues are the sites corresponding to mutational differences in the B variant. The small italicized numbers refer to the amino acid residue sequence numbering based upon κ-casein. Sites of glycosylation and phosphorylation are boldfaced. Note that there are eleven negatively charged and three positively charged amino acid residues.

Six other saccharides have been delineated in cow's colostrum [Saito *et al.*, 1981; Fiat & Jolles, 1989]; Nacetylglucosamine (GlcNAc) and fucose (Fuc) have been identified as constituents in these saccharides. It is important to draw a distinction between bovine and human GMP oligosaccharides. The latter contain no NeuAc and have Gal, GlcNAc, and Fuc as terminal sugars on their oligosaccharides [Fiat & Jolles, 1989]. Of the fourteen human milk GMP oligosaccharides which have been identified, only disaccharide II is found in milk GMP from both species. There is an asialotetrasaccharide which is found in both bovine colostrum and human milk. But the point of saccharide attachment is not uniform [Eigel *et al.,* 1984; Whitney, 1988; Fiat & Jolles, 1989]. Several reports indicate that multiple saccharide substituents may be linked on a single peptide chain [Doi *et al.,* 1980; Otani *et al.*, 1995]. In contrast to this variability, one phosphate moiety in GMP is always at serine 44 (Ser149 of the κ-casein chain) though non-phosphorylated fractions have been found [Fiat *et al.,* 1981; Eigel *et al.*, 1984; Whitney, 1988; Farrell *et al.,* 2004].

Biological activities

Ability to bind cholera toxin and *E. coli* enterotoxins

Cholera toxin produced by *Vibrio cholerae* consists of an A subunit and five B subunits. The B subunits form the attachment site which binds to oligosaccharides on cell walls. Once bound, the A subunit activates adenylate cyclase in cells, which results in a loss of cellular water which causes diarrhoea and possibly death [Holmgren, 1981]. It has been shown that the receptor is a oligosaccharide ganglioside GM1 [Van Heyningen, 1974], which is not identical to GMP, but other glycoproteins such as fetuin and glycophorin which have oligosaccharides similar to GMP inhibit cholera toxin [Sugii & Tsuji, 1990; Schengrund & Ringler, 1989]. Kawasaki *et al.* [1992] have shown that GMP is capable of binding cholera toxin. Normal Chinese hamster ovary (CHO)-K1 cells are spherical. In the presence of cholera toxin, CHO-K1 cells take on a spindle shape. As little as 20 ppm GMP is enough to cause considerable rounding of CHO-K1 cells and 100 ppm GMP result in almost completely rounded CHO-K1 cells, which indicates that GMP has bound to cholera toxin. When the GMP was treated with sialidase, which hydrolyses the sialic acids, complete loss of cholera toxin inhibiting activity occurred. The peptide chain must also participate in the binding as partial loss of cholera toxin inhibiting activity occurred after treatment with proteases.

Isoda *et al.* [1999] carried the work further to other bacterial toxins. They obtained similar inhibitions against *E. coli* heat labile enterotoxins LT-I and LT-II (associated with colonization factor antigen CFA/I and CFA/II, respectively) in the CHO-K1 model. Additionally, the ability of the GMP to protect mice against diarrhoea caused by the toxins was evaluated. Feeding 1 mg GMP per day protected 100% of the mice against cholera toxin and LTII, and 80% of the mice against LT-I.

Inhibition of bacterial and viral adhesion

Many bacteria and viruses bind themselves to their hosts as a part of the colonization process. Binding to the intestine or other mucosal surfaces is achieved by adhesins, capsular material on the bacterial cell surface or hair-like fimbriae orpili which are specific for the various ceramide and ganglioside glycoconjugates which make up epithelial cell membranes [Simon, 1996]. Considerable research has been done to characterize the nature of the adhesins and their receptors and it may be possible to find substances which have sufficient similarity to the receptors that they block the receptor and thereby inhibit colonization [Ofek & Sharon, 1990]. The haemagglutination assay is often used to screen for compounds which prevent bacterial or viral binding to cell receptor sites. The assay detects the ability of the bacterium or virus to bridge between erythrocytes (red blood cells) and cross-link or agglutinate the erythrocytes. If the adhesin is bound to the compound in preference to the receptor site, agglutination will not occur. Thus, haemagglutination inhibition is a sign that the compound has potential to prevent bacterial colonization. Neeser *et al.* [1988a] have been investigat-

ing the mechanism by which milk components prevent dental caries. They evaluated the role of GMP in inhibiting adhesion of cariogenic bacteria (*Str. mutans, Str. sanguis, Str. sobrinus* and *Actinomyces viscosus*) to oral surfaces. Haemagglutination by *Str. mutans, Str. sanguis* and *Act. viscosus* is prevented by GMP with disaccharide II [Neeser *et al.,* 1988a]. Using saliva-covered hydroxyapatite beads, as a saliva covered tooth model, binding of bacteria in the presence of GMP was measured. GMP prevented binding of *Str. sobrinus* and *Str. sanguis*, but not *Act. viscosus* [Neeser *et al.,* 1994]. A further proof of binding was obtained using GMP-gold conjugates which could be seen attached to *Str. sanguis* bacteria by electron microscopy. A 23 kDa glycoprotein from *Str. sanguis* which binds to buccal (cheek) epithelial cells was identified [Neeser *et al.,* 1995]. Trisaccharide III is on the glycoprotein. Further, *Str. mutans* and *Str. sobrinus* binding to salivary pellicle (the thin layer of salivary protein and glycoprotein which quickly adheres to a freshly cleaned tooth) could be prevented by GMP [Schupbach *et al.,* 1996]. They believe that the mechanism by which GMP reduces dental caries is by changing the microbial composition of dental plaque from streptococci to less cariogenic Actinomyces. Incorporating GMP in gum or toothpaste is a method of preventing dental plaque and caries [Neeser, 1991a,b]. Xylitol and GMP appear to have a synergistic effect in not only preventing caries but also remineralizing teeth [Zhang & Shapiro, 1998]. Recently, Nejad *et al.* [2009] confirmed the protective effect of GMP against dental erosion.

Bacterial adhesion offers a field ripe for further GMP application, but there are some caveats related to specificities. Neeser *et al.* [1988b] evaluated GMP as a haemagglutination inhibitor for CFA/I and CFA/II expressing *E. coli* which are associated with the toxins discussed above (LT-I and LT-II). N-linked glycoproteins with trisaccharide III were active, but GMP and other O-linked glycoproteins were not. Thus, GMP inhibits toxin binding but not bacterial binding. There are reports that trisaccharide III prevents haemagglutination of other *E. coli* strains [Parkkinen *et al.,* 1986; Liukkonen *et al.,* 1992]. Also, proteins with O-linked trisaccharide III and tetrasaccharide IV have modest activity in binding of *Mycoplasma gallisepicum* [Glasgow & Hill, 1980] and *Myco. pneumonia* [Loomes *et al.,* 1984] which are associated with an autoimmune disorder. There should be evaluations of GMP in these applications. Recently, Bruck *et al.* [2006] found that milk supplemented with α-lactalbumin and GMP might be effective in inhibiting associations of the pathogens *E. coli* (EPEC), *Salmonella typhimurium*, and *Shigella flexneri* to intestinal cells.

Kawasaki *et al.* [1993] demonstrated that GMP inhibits haemagglutination by four strains of human influenza virus. As little as 80 ppm was effective. Dosako *et al.* [1992] found 10 ppm concentrations of GMP prevent Epstein-Barr virus from inducing morphological transformations in peripheral blood lymphocytes. Recently, Pan *et al.* [2006] has also reported that GMP interferes with infection by some viruses.

The preventive effects of GMP against intestinal infection were investigated by Nakajima *et al.* [2005], and conjugates of GMP with xylooligosaccharides (XOS) and carboxymethyldextran (CMD) were prepared by the Maillard reaction to enhance the effect of GMP. GMP showed the ability to bind to *S. enteritidis* and enterohemorrhagic *E. coli* O157: H7 (EHEC O157). This binding ability was decreased by a sialidase treatment and completely eliminated by periodate oxidation. They indicated that such carbohydrate moieties as sialic acid in GMP are involved in binding to *S. enteritidis* and EHEC O157. The preventive effect of GMP on the adhesion of pathogenic bacteria to Caco-2 cells was also investigated. GMP showed an inhibitory effect on the adhesion of EHEC O157 in a dose-dependent manner, although it was not a potent inhibitor of the adhesion of Salmonella infection. However, in the case of Salmonella infection, GMP-XOS and GMP-CMD significantly suppressed IL-8 production which was the index of infection. These results indicated GMP to be a promising agent for preventing intestinal infection.

Suppression of gastric secretions

GMP inhibits gastric secretions and slows stomach contractions in dogs. When dogs were intravenously injected with 10–15 mg of bovine GMP, their gastric secretions became less acidic and motions at the gastric fundus and the duodenum were reduced [Stan & Chernikov, 1982]. Rat derived GMP had the same effect [Vasilevskaya *et al.,* 1977]. Further experiments showed that a pepsin digest of GMP produced two active fractions. The stronger of these was a 700–2000 Da peptide fraction [Stan & Chernikov, 1979]. Activity of the two fractions was resistant to proteolysis with pepsin, trypsin and chymotrypsin. It was also established that GMP peptides could reduce gastric acid secretions by half and blood serum gastrin levels by 8% [Aleinik *et al.,* 1986]. Further experiments were carried out in France at the INRA laboratories. Guilloteau *et al.* [1987] found that intravenous injection of GMP afforded no inhibition of gastric secretions or changes of digestive hormone blood plasma levels in preruminant calves. However, they did find that feeding GMP at levels similar to those experienced in normal feeding resulted in inhibition of gastric secretion during the first and second hour after feeding [Guilloteau *et al.,* 1994]. Feeding at five times the normal feeding level resulted in no effect. Beucher *et al.* [1994b] found that feeding one GMP fraction, stimulated the intestinal hormone cholecystokinin which, in turn, regulates gastrointestinal functions. Non-glycosylated GMP had no effect on the basal cholecystokinin level and B variant (which varies from the A variant by two amino acids) glycosylated GMP had only a slight stimulating effect in the rat [Beucher *et al.,* 1994a]. The A variant with a terminal sialic acid [Yvon *et al.,* 1994] exhibited the largest stimulation which indicates that both the peptide chain and the carbohydrate structure are important for stimulating gastric secretions. Stan *et al.* [1983] proposed that it is necessary for the GMP molecule to enter the blood in order to cause gastric acid inhibition. Yvon *et al.* [1994] demonstrated that GMP acts by triggering receptors on the intestinal mucosa.

Promotion of bifidobacterial growth

Bifidobacteria predominate in the lower intestine and are thought to inhibit pathogenic bacterial growth and thereby afford protection from gastrointestinal diseases [Faure *et al.,* 1984]. Gyorgy *et al.* [1954a] found the first evidence of a bi-

fidobacterial growth promoting factor in human colostrums and human milk. The activity in human milk was about half that of colostrum on a dry weight basis. They also found that bovine colostrum has about one-tenth of the activity and bovine milk has one-hundredth of the activity of human colostrum on a dry weight basis [Gyorgy *et al.,* 1954b]. In a search to identify the bifidus factor, Gyorgy *et al.* [1974] found that GlcNAc and oligosaccharides with terminal GlcNAc promote bifidobacterial growth. Sialidase treatment of α_1-acid glycoprotein resulted in an increase in bifidobacterial growth and they proposed that this is due to cleavage of the terminal NeuAc exposing a GlcNAc. Since that time the elusive bifidus factor has been sought in bovine milk in general and GMP in particular. Kehagias *et al.* [1977] found some bifidobacterial growth promoting activity in a fraction obtained from a sulphuric acid treatment of whole casein. The preparation was ill defined. They had evidence that it was perhaps κ-casein derived but not similar to GMP. Bezkorovainy *et al.* [1979] found glycopeptide from a bovine milk casein chymotryptic digest to have one-tenth the growth-promoting activity of human milk solids. Azuma *et al.* [1984] compared human and bovine GMPs and found that acidity generation with the latter was one-third as large as the acidity generation with the former. The situation appears to be quite complex with conflicting data which are not favourable to bovine GMP as a specific bifidus growth promoter. Poch & Bezkorovainy [1988] evaluated several promoter candidates, including bovine casein digest and bovine milk whey, on eight bifidus species. Each of the candidates promoted growth of one or more bifidus species. In further work they showed that any growth-promoting activity of κ-casein goes with the para-κ--casein rather than the GMP when rennet-treated κ-casein is fractionated [Poch & Bezkorovainy, 1991]. Petschow & Talbott [1991] reported that growth promoting activity for some bifidus species is present in cow milk ultrafiltration permeate as well as retentate. This would rule GMP out because, as noted above, oligomerization prevents GMP from passing through an ultrafiltration membrane except at a very low pH. Research by Proulx *et al.* [1992] would lead one to believe that the amino acid portion is critical for bifidus growth but other (patented) works [Idota, 1996; Yakabe *et al.,* 1994] would favour saccharides containing sialic acid.

Modulation of immune system responses

Splenocyte (spleen lymphocyte) proliferation is a step in the inflammatory response. Inhibition of splenocyte proliferation can be used to demonstrate suppression of an immune response such as an allergic reaction. Research by Otani *et al.* [1992] demonstrated that casein inhibits mouse splenocyte proliferation induced by the mitogen *Salmonella typhimurium* lipopolysaccharide (LPS). Inhibitory activity was due to κ-casein, which upon rennet hydrolysis, results in inhibitory activity being found in the GMP fraction. Para-κ-casein had no inhibitory activity. Upon sialidase digestion, GMP lost its inhibitory activity, indicating that sialic acid is critical to the phenomenon [Otani & Monnai, 1993]. Inhibitory activity was reduced after GMP digestion with chymotrypsin but inhibitory activity increased after GMP digestion with trypsin or pronase so the peptide chain must also participate. Inhibition of splenocyte proliferation by GMP was also observed against concanavalin A (Con A), phytohaemagglutinin-P (PHA) and pokeweed mitogen in addition to LPS [Otani *et al.,* 1992; Otani & Hata, 1995]. Otani *et al.* [1995] were also able to separate GMP into seven distinct fractions with up to five sialic acid groups containing one or two of the di-, tri- and tetrasaccharides shown above. The fractions inhibited both mouse splenocyte and rabbit Peyer's patch cell proliferation as follows: (1) three fractions inhibited LPS-induced proliferation; (2) five fractions, with activity in proportion to the number of sialic acids present, inhibited PHA induced proliferation; (3) none of the fractions inhibited Con A-induced proliferation. Because of reduced inhibitory activity after chymotrypsin digestion, the researchers suggest that the Ser-149 phosphate plays a part in GMP binding to the mitogen receptor. The investigation then turned to the mechanism by which GMP inhibits mitogens from inducing splenocyte proliferation. Cells were incubated with and without GMP. The cells were then immunostained with anti-κ-casein antibody. Only the cells incubated with GMP retained the anti-κ-casein antibody thus demonstrating that GMP adheres directly to the cell surface [Otani & Monnai, 1995]. Monnai & Otani [1997] found that when cells were incubated with GMP, one of the cytokines in the interleukin-1 (IL-1) family, IL-1ra in particular, is synthesized. The IL-1ra blocks the action of IL-1 by binding to IL-1 receptors. Since IL-1 cannot bind to its receptors, it cannot trigger splenocyte proliferation and, in turn, an inflammatory response. In addition to the inhibition of LPS-induced binding of IL-1, they also showed that GMP binds to CD4+T cells and suppresses PHA-stimulated expression of interleukin-2 (IL-2) receptor and inflammatory response [Otani *et al.,* 1996] and whereas GMP binds to CD4+T cells, it does not bind to CD8+T cells. Yun *et al.* [1996] studied the effect of GMP on immunoglobulins produced by LPS-stimulated splenocytes. They found that only the IgA concentration was increased by GMP and that only the population of surface IgA positive cells was increased by GMP. Snow Brand Milk Products were granted a patent on the use of bovine GMP for accelerating human B lymphocyte growth. In a culture test they showed that GMP accelerates proliferation of normal human B lymphocytes, but not T lymphocytes. This finding substantiates up-regulation of the humoral immune system [Brody, 2000].

The specific antibody response to GMP was evaluated as well as the antigen-specific T-cell response. The results demonstrated that immunization or feeding with κ-casein induced GMP-specific antibodies, whereas GMP *per se* lacked immunogenicity independently of the mode of presentation. The size of the presented form of GMP did not influence its immunogenicity. Because the results showed that GMP did not induce a specific T-cell response, it was postulated that GMP lacks the ability to stimulate antigen-specific T cells [Mikkelsen *et al.,* 2006].

Anti-inflammatory activity *versus* hapten-induced colitis

The intestinal anti-inflammatory activity of bovine GMP was assessed by Daddaoua *et al.* [2005] in trinitrobenzenesulfonic acid-induced colitis in rats. Rats were administered GMP daily starting either 2 d before (pretreatment) or 3 h after (post-treat-

ment) colitis induction. Pretreatment with GMP had a dose-dependent anti-inflammatory effect, characterized by lower body weight loss, decreased anorexia (57%), colonic damage (65%), and weight to length ratio (32%), as well as a reduction in colonic alkaline phosphatase activity (42%) and interleukin 1, trefoil factor 3, and inducible nitric oxide synthase mRNA levels. The mechanism of action of GMP is unknown but is consistent with an inhibition of the activation of immune cells. The magnitude of the anti-inflammatory effect was generally comparable to that of sulfasalazine, an established drug used in the treatment of inflammatory bowel disease. Bovine GMP may play a role in the management of patients with inflammatory bowel disease.

CONCLUSIONS: BIOLOGICAL COMPARISON BETWEEN LACTOFERRIN AND GLYCOMACROPEPTIDE

Recently, LF was biologically compared with GMP added at the level of nil, 0.025, 0.050 or 0.100% to milk whether unfermented on cultured either with conventional yoghurt starter culture (BSC) YC XII or ABT-2 starter culture containing *Lb. acidophilus, Bifido.* sp. and *Str. thermophilus*. The blood picture of rats fed on dried yoghurt treatments for 3 weeks indicated that the feeding 0.025%-fortified yoghurt, regardless the type of BSC used, led to lowering the values of white blood cells (WBC) count, hemoglobin (HB), haematocrit (HCT), platelet (PLT) count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) as well as mean corpuscular hemoglobin concentration (MCHC), and did not affect red blood cells (RBC) count. The RBC, HB and HCT values of rats blood fed on LF yoghurts did not vary from those fed on GMP ones. While WBC decreased and PLT, HVC, MCH as well as MCHC increased in the former *versus* the latter. The just fermentation, regardless the type of BSC used, led to increase in the values of WBC, RBC and HB in the rats blood. The milk fermentation with ABT-2 starter culture caused significant increases in both HCT and PLT; and decreases in MCH and consequently in MCHC in the blood of rats fed thereon. Without any exception, the fortification with LF or GMP whether of milk or fermented product resulted significantly in heightening the levels of all parameters measured namely total protein (TP), albumin (Alb), globulin (Glb), calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), ferrous (Fe), copper (Cu), manganese (Mn) and zinc (Zn) of blood serum of rats fed thereon. The blood serum of rats fed on LF fortified milk, whether unfermented or fermented with YC XII or ABT-2, was significantly distinguished with higher level of TP, Alb, Ca, P, Mg, Na, Fe, Mn and Zn compared with those fed on GMP fortified milk or yoghurt. While both of Glb and Cu of blood serum did not exhibit any response toward the type of fortifier added. Rats feeding on fermented milk were associated with increment in the levels of TP, Alb, Glb and Cu in their blood serum. In comparison with conventional yoghurt, the feeding on yoghurts cultured with ABT-2 led to increased levels of Alb, Ca, P, and Zn; and to lower levels of TP, Glb, Mg, Na, Fe, Cu and Mn in the blood serum of rats. The profile of feces flora was improved significantly by rats feeding on yoghurt especially fermented with ABT-2 and / or fortified with LF as indicated from the count enumerated for *Str. thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus, Lb. acidophilus* and *Bifidobacterium* sp., those have considerably predominated and *Escherichia coli*, which was significantly harmed either by GP and / or the fermentation regardless the type whether of GP or BSC [Fayed *et al,* 2011a,b]. They suggested consequently that the carbohydratic parts bound to them (LF and GMP) may act as prebiotics in the last portion of the digestive apparatus, essentially, the intestine and colon.

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