

Bovine whey proteins – Overview on their main biological properties

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Keywords: Bioactive protein; Functional food; Health benefits

Abstract

Whey, a liquid by-product, is widely accepted to contain many valuable constituents. These include especially proteins that possess important nutritional and biological properties – particularly with regard to promotion of health, as well as prevention of diseases and health conditions. Antimicrobial and antiviral actions, immune system stimulation, anticarcinogenic activity and other metabolic features have indeed been associated with such whey proteins, as α -lactalbumin, β -lactoglobulin, lactoferrin, lactoperoxidase, and bovine serum albumin. The most important advances reported to date pertaining to biological properties of whey proteins are reviewed in this communication.

Introduction

Drinking milk is a practice that dates back to the domestication of animals in prehistoric times, and has taken advantage of the extensive nutritional value of that natural product – not only to the young born, but also to the child, the adult and the elderly. However, one major part of milk – whey, has traditionally not been paid as much attention as happened with source milk, probably because it is a by-product of cheese making, viewed for a long time as of little value (besides additive to animal feed). Interestingly, Hippocrates already applauded the health properties of whey in Ancient Greece; and during the Middle Age, whey was considered not only as a medicine, but also even as an aphrodisiac and a skin balm: it was in fact a regular component of salves and potions to soothe burns, to inspire vitality and to cure various illnesses (Kosikowski, 1982). Recent decades have witnessed an increased interest in whey protein products, owing to their nutritional role, and more and more to their active role upon human health. A multitude of bibliographic material covering that topic has accordingly been published; this review will focus on the most recent research advances pertaining to the biological properties of whey proteins.

Whey protein system

Milk possesses a protein system constituted by two major families of proteins: caseins (insoluble) and whey proteins (soluble). Caseins account for 80% (w/w) of the whole protein inventory, and can easily be recovered from skim milk via isoelectric precipitation (by addition of, or *in situ* production of acid) or rennet-driven coagulation – both of which release whey as by-product (Fig. 1). Whey proteins are globular molecules with a substantial content of α -helix motifs, in which the acidic/basic and hydrophobic/hydrophilic amino acids are distributed in a fairly balanced way along their polypeptide chains (Evans, 1982). The whey protein profile, including general chemical and physicochemical properties, is depicted in Table 1. Whey proteins include β -lactoglobulin (β -LG, for short), α -lactalbumin (α -LA), immunoglobulins (IG), bovine

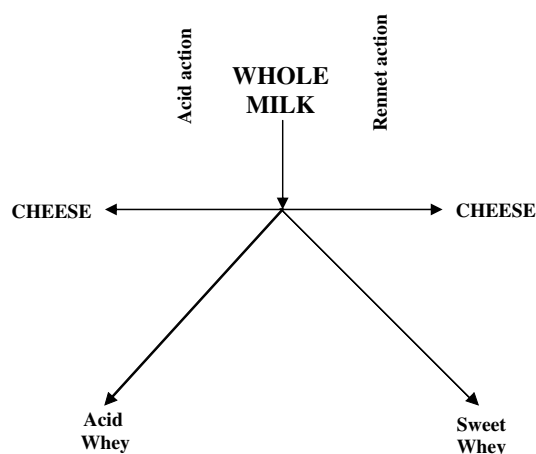


Fig. 1. Schematic diagram of acid- and rennet-mediated cheese making.

Table 1
Protein profile of whey and primary structure basic properties

Protein	Concentration (g/l) ^{a,d}	Molecular weight (kDa) ^{b,c}	Number of amino acids residues ^b
β -Lactoglobulin	1.3	18,277	162
α -Lactalbumin	1.2	14,175	123
Bovine serum albumin	0.4	66,267	582
Immunoglobulins (A, M and C)	0.7	25,000 (light chain) + 50,000–70,000 (heavy chain)	–
Lactoferrin	0.1	80,000	700
Lactoperoxidase	0.03	70,000	612
Glycomacropetide	1.2	6700	64

^a de Wit (1998).

^b Eigel et al. (1984).

^c Brew et al. (1970).

^d Korhonen (1995).

serum albumin (BSA), bovine lactoferrin (BLF) and lactoperoxidase (LP), together with other minor components. Their main biological activities are summarized in Table 2.

Table 2
Biological functions of whey proteins

Protein	Biological function	References
Whole whey proteins	Prevention of cancer	Gill and Cross (2000)
	<ul style="list-style-type: none"> • Breast and intestinal cancer • Chemically induced cancer 	Badger et al. (2001), MacIntosh et al. (1995) Hakkak et al. (2000), Rowlands et al. (2001)
	Increment of glutathione levels	Parodi (1998)
	<ul style="list-style-type: none"> • Increase of tumour cell vulnerability • Treatment of HIV patients 	Micke et al. (2001), Micke et al. (2002)
	Antimicrobial activities	Clare et al. (2003)
β -Lactoglobulin	Increment of satiety response	Hall et al. (2003)
	<ul style="list-style-type: none"> • Increment in plasma amino acids, cholecystokinin and glucagon-like peptide 	
	Transporter	Puyol et al. (1991)
	<ul style="list-style-type: none"> • Retinol • Palmitate • Fatty acids • Vitamin D and cholesterol 	Wu et al. (1999) Puyol et al. (1991) Wang et al. (1997)
	Enhancement of pregrastic esterase activity	Perez et al. (1992)
α -Lactalbumin	Transfer of passive immunity	Warme et al. (1974)
	Regulation of mammary gland phosphorus metabolism	Farrell et al. (1987)
	Prevention of cancer	de Wit (1998)
Bovine serum albumin	Lactose synthesis	Markus et al. (2002)
	Treatment of chronic stress-induced disease	Ganjam et al. (1997)
	Fatty acid binding	Walzem, Dillard, and German (2002)
Immunomodulation	Anti-mutagenic function	Bosselaers, Caessens, Van Boekel, and Alink (1994)
	Prevention of cancer	Laursen et al. (1990)
	Immunomodulation	Ormrod and Miller (1991)
Immunomodulation	<ul style="list-style-type: none"> • Disease protection through passive immunity 	Mitra et al. (1995), Tomita et al. (1995), Loimaranta et al. (1999)
	Antibacterial activity	Oona et al. (1997), Freedman et al. (1998)
	<ul style="list-style-type: none"> • HIV 	
Immunomodulation	Antifungal activity	Okhuysen et al. (1998)
	Opioid activity	Sharpe et al. (1994)

The actual concentrations of whey proteins depend on the type of whey (acid or sweet), the source of milk (bovine, caprine or ovine), the time of the year, the type of feed, the stage of lactation and the quality of processing. Acid whey – the pH of which is equal to or smaller than 5.1, is obtained by direct acidification of milk, as happens in cottage cheese manufacture. Sweet whey – the pH of which is equal to or higher than 5.6, is obtained after rennet-coagulation, as happens in most cheese making processes worldwide (Pintado, Macedo, & Malcata, 2001).

Whey protein concentrates and whey protein isolates

In the latest two decades, the evolution of separation technologies, *viz.* those relying on selective, porous membranes, have permitted a number of protein whey components to become widespread additives in food. They may indeed be subject to several treatments, thus originating whey products with specific qualitative and quantitative profiles of proteins, minerals, lipids and sugars; the aforementioned membrane-based separation technologies include ultrafiltration (UF) to concentrate proteins, or diafiltration (DF) to remove most lactose, minerals and low

molecular weight components – and thus produce whey protein concentrates (WPC). Depending on their concentration, there are WPC containing 35%, 50%, 65% and 80% (w/w) protein. When the threshold of 90% (w/w) protein is reached, a whey protein isolate (WPI) is accordingly obtained – which is a protein concentrate bearing high-quality and purity. Both those products are used as vectors for the promotion of many biological properties upon addition to foods. If a thermal process is applied to whey then α -LA denatures easily, so it fraction can be separated via precipitation (Huffman & Harper, 1999).

Antimicrobial and antiviral properties

The biological feature of whey proteins that has been more thoroughly reviewed is their antimicrobial function (Clare, Catignani, & Swaisgood, 2003). WPC enriched with *Helicobacter pylori*-specific antibodies produced by lactating cows prevented infections thereby (Early, Hardy, Forde, & Kane, 2001).

On the other hand, proteins chemically modified by 3-hydroxyphthalic anhydride (3-HP), *viz.* BSA, α -LA and β -LG, were tested for their antiviral action against such

enveloped viruses as human herpes simplex virus type 1 (HSV-1), bovine parainfluenza virus type 3 and porcine respiratory corona virus (Oevermann, Engels, Thomas, & Pellegrini, 2003). Of those three viruses, only HSV-1 was sensitive to 3-HP-proteins. All said chemically modified proteins exhibited antiviral activity against HSV-1 when assayed before, during or after infection; however, significantly higher concentrations thereof were required to produce HSV-1 inhibition if present before infection, than during or afterwards.

Immune system modulation

Whey proteins suppressed *in vitro* lymphocyte mitogenesis and alloantigen-induced proliferation, when included in mature murine lymphocytes solutions (Barta, Barta, Crisman, & Akers, 1991). Modified WPC can also suppress the mitogen-stimulated secretion of γ -interferon, as well as the surface expression of interleukin-2 receptor, when added to T and B lymphocyte cultures (Cross & Gill, 1999). On the other hand, Mercier, Gauthier, and Fliss (2004) claimed that addition of whey proteins from micro-filtered-WPI to cell culture media, at a concentration of 100 $\mu\text{g/ml}$, stimulates *in vitro* proliferation of murine spleen lymphocytes.

Glutathione (GSH) is naturally found in all cells of mammals, where it provides the main intracellular defense against oxidative stresses – when illness occurs, GSH is depleted because of said stress (Fig. 2). The amino acids cysteine, glutamate and glycine are part of the primary structure of this peptide; cysteine incorporation is the rate-limiting step for its synthesis; and both cysteine and glutamine are major players in the coordinated T-cell response of macrophages and lymphocytes. Recall that whey proteins are rich in cysteine and glutamate residues; this suggests that their ingestion may contribute to increase the level of free cysteine, and consequent production of GSH. WPC is indeed an effective cysteine donor for GSH replenishment, during immune deficiency states; note that GSH is important in immune regulation and cancer prevention in animals, in improvement of immune and liver functions, and in helping overcome GSH-deficiency in seropositive and Alzheimer's disease patients.

Cell culture studies and *in vivo* experiments (Gomez, Ochoa, Herrera-Insua, Carlin, & Cleary, 2002) have demonstrated that whey proteins may enhance non-specific and specific immune responses. Dietary supplementation

with a whey-based product increased lymphocyte GSH levels in patients suffering from lung inflammation associated with cystic fibrosis (Grey, Mohammed, Smountas, Bahlool, & Lands, 2003).

The already mentioned high concentration of amino acid precursors in the synthesis of GSH – i.e. cysteine and glutamate in whey proteins, is believed to contribute to said immuno-enhancing effects (Wong & Watson, 1995).

Furthermore, the (long-term) Immunocal™ supplement is a WPC that has proven effective toward improvement of liver disfunctions in patients exhibiting chronic hepatitis B. Serum alanine aminotransferase (ALT) activity decrease, and plasma GSH levels increased in most patients suffering from that condition, 12 weeks after provision of the aforementioned supplement started; on the other hand, serum lipid peroxide levels decreased significantly, and interleukin (IL)-2 levels and natural killer (NK) activity increased significantly (Watanabe et al., 2000). WPC has also been reported (Rosaneli, Bighetti, António, Carvalho, & Sgarbieri, 2002) to protect the gastric mucosa from ethanol damage; this protection depends on the sulfhydryl compounds present therein. In addition, WPC in moderate concentrations (10 mg/ml) can promote GSH production, which in turn enhances the antioxidant activity in a pheochromocytoma (PC12) cell line after acute ethanol exposure (Tseng et al., 2006).

The above findings have provided a basis for prevention (or treatment) of HIV-infected patients, which possess systemic GSH deficiency – that permits multiplication of that virus. Inclusion of whey protein formulae in their diet is accordingly an effective and well-tolerated route to increase their GSH levels (Micke, Beeh, Schlaak, & Buhl, 2001; Micke, Beeh, & Buhl, 2002). Moreover, whey proteins possess inhibitory activity against HIV-1 enzymes themselves – which is then crucial in attempts to constrain the life cycles of said virus (Ng, Lam, Au, Ye, & Wan, 2001).

Anticarcinogenic activity

Whey proteins have been claimed by Tsuda et al. (2000) to prevent cancer; examples include breast and intestinal cancers in female rats, when included in their diet (MacIntosh, Regester, le Leu, Royle, & Smithers, 1995; Badger, Ronis, & Hakkak, 2001). The impact of whey proteins upon cancer prevention has been thoroughly reviewed by Gill and Cross (2000). The whole whey protein system apparently protects against colon and mammary tumours

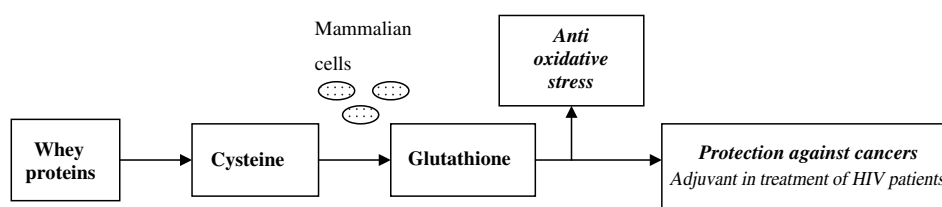


Fig. 2. Schematic diagram of glutathione (GSH) synthesis following whey protein intake, and most important functions associated therewith.

that had been chemically induced *in vivo* (Hakkak et al., 2000; Rowlands, He, Hakkak, Ronis, & Badger, 2001).

In vivo experiments have also unfolded the anticarcinogenic and anticancer activities ascertained to WPC, via their effect upon increase of GSH concentration in relevant tissues; stimulation of immunity via the GSH pathway actually originates anti-tumour effects in low-volume tumours (Bounous, 2000). On the other hand, published evidence (Parodi, 1998) suggests that WPC can deplete tumour cells which possess a concentration of GSH higher than normal cells, and in addition render them more vulnerable to chemotherapy; Immunocal™ actually caused depletion of GSH and inhibition of proliferation of human breast cancer cells in *in vitro* assays, at concentrations that induce GSH synthesis in normal human cells.

Hydrolysed WPI protected against oxidant-induced cell death in a human prostate epithelial cell line (RWPE-1), again because of the increment of GSH synthesis effected thereby (Kent, Harper, & Bomser, 2003). WPI may also protect from cancer by acting as a coadjuvant of baicalein – an anticancer drug; the cytotoxicity of this molecule is enhanced by inducing more apoptosis in the human hepatoma cell line Hep G2, which is in turn associated with depletion of GSH (Tsai, Chang, Chen, & Lu, 2000).

Nutrition system effects

Whey proteins apparently facilitate attainment of favourable weight and composition. Whey proteins were found (Hall, Millward, Long, & Morgan, 2003; Roberts, McMcCrory, & Saltzman, 2002) to be more effective in satiation than caseins, when both these proteins were studied in terms of food intake – based on ratings of hunger and fullness by selected subjects, and on postprandial metabolite and gastrointestinal hormone responses. These results imply post-absorptive increases in plasma amino acids, and suggest that both plasma cholecystokinin (CCK) and glucagon-like peptide (GLP-1) are potential mediators towards increased satiety response to whey.

Furthermore, whey proteins lower plasma and liver cholesterol, as well as plasma triacylglycerol levels in model animals fed with cholesterol-containing diets (Beena & Prasad, 1997; Zhang & Beynen, 1993).

Other metabolic features

Whey proteins contribute to improve muscle strength and reduce the tendency for bone breaking; these features are directly related to their amino acid profiles. In fact, their overall amino acid composition is rather similar to that of skeletal muscle; hence, whey protein may be a good anabolic supplement. The advantages of whey proteins in terms of muscle anabolism rely on their fast absorption, coupled with the leucine abundance required to initiate synthesis – as well as on their amino acid composition, that provides substrates for protein synthesis (Ha & Zemel, 2003). In women infected with HIV, muscle strength was

improved following a diet rich in whey proteins (Agin et al., 2001).

The loss of bone mass during menopause is a well documented realisation; whey proteins may provide help toward bone formation and activation of osteoblasts (bone cells) – especially in what concerns proliferation and differentiation of osteoblastic MC3T3-E1 cells (Takada, Aoe, & Kumegawa, 1996). Bone metabolism is promoted by increments in the amount of such bone protein as collagen. Rats fed with either plain whey protein or fractioned whey protein showed a significant increase in resistance to bone breaking, and in the amounts of hydroxyproline and proline (i.e. structural amino acids of collagen) in the femur (Takada et al., 1997). Recently, acidic protein fraction prepared from mineral acid WPC reduced bone loss due to ovariectomy, maintaining bone density above ovariectomy levels at week 16 of feeding of mature female rats (Kruger et al., 2006).

β-Lactoglobulin

β-LG is quantitatively the dominant whey protein (58% (w/w)), and was first discovered in 1934. When in isolated form, it exhibits a low solubility (despite its globular nature) and a low ionic strength. Synthesized in the mammary gland of ruminants (and other species) and designed to be included in milk, this protein has several genetic variants – of which β-LG A is the most common. It is composed mainly of β-sheet motifs, and consists of 162 amino acid residues – which lead to a molecular weight of ca. 18,277 kDa (Eigel et al., 1984). Its quaternary structure depends on the medium pH: it occurs mainly as a stable dimer, with a molecular weight of 36,700 kDa, at pH values between 7 and 5.2; as an octamer, with a molecular weight of ca. 140,000 kDa, at pH values between 5.2 and 3.5; and as a monomer, with two-cysteine residues per monomer, at pH 3.0 and above 8.0 (de Wit, 1989).

Immune system modulation

β-LG plays a role in transfer of passive immunity to the newborn, and in regulation of phosphorus metabolism at the mammary gland (Farrell, Bede, & Enyeart, 1987).

The amino acid content of this protein is rather important, because – besides fueling muscle growth, it is a source rich in the essential amino acid cysteine, which is important for synthesis of GSH (de Wit, 1998).

Other metabolic features

The actual biological function of β-LG is still unclear; however it often binds to small hydrophobic ligands, such as retinol, fatty acids, protoporphyrin IX, triacylglycerols, alkanes, aliphatic ketones, aromatic compounds, vitamin D, cholesterol, palmitic acid and calcium (at pH 5.0) (Brown, 1984; Cho, Batt, & Sawyer, 1994; Farrell et al., 1987; Futterman & Heller, 1972; O'Neil & Kinsella, 1987;

Patocka & Jelen, 1991; Puyol, Pérez, Ena, & Calvo, 1991; Said, Ong, & Shingleton, 1989; Smith, Fantozzi, & Creveling, 1983; Wang, Allen, & Swaisgood, 1997; Wu, Pérez, Puyol, & Sawyer, 1999). Specifically, this protein binds to free fatty acids as they are released by pregastric lipases, in order to facilitate digestion of milk fat (Perez & Calvo, 1995). Because of its high stability at low pH, β -LG protects hydrophobic molecules during passage through the stomach, in order to deliver such ligands to a specific receptor located in the intestine of the suckling neonate (Cho et al., 1994).

α -Lactalbumin

α -LA is quantitatively the second most important protein in whey – representing ca. 20% (w/w) of the total whey protein inventory, and is fully synthesized in the mammary gland. Here α -LA acts as coenzyme for biosynthesis of lactose – an important source of energy for the newborn (de Wit, 1998). It contains 123 amino acid residues (its sequence is quite homologous to that of lysozyme), which lead to a molecular weight of 14,175 kDa (Brew, Castellino, Vanaman, & Hill, 1970). Three genetic variants have already been identified – A, B and C (Fox, 1989). Its globular structure is stabilized by four disulphide bonds, at pH values in the range 5.4–9.0 (Evans, 1982).

Anticarcinogenic activity

α -LA contributes to reduce the risk of incidence of some cancers – as it constrains cell division, when incubated in distinct mammalian intestinal cell lines (Ganjam, Thornton, Marshall, & MacDonald, 1997). It can also be a potent Ca^{2+} -elevating and apoptosis-inducing agent (Hakansson, Zhivotovski, Orrenius, Sabharwal, & Svanborg, 1995).

This protein also was demonstrated to possess anti-proliferative effects in colon adenocarcinoma cell lines (Caco-2 or HT-29 monolayers), delaying initiation of cell apoptosis, after 4 days of growth with low concentrations (10–25 $\mu\text{g/ml}$) of such protein (Sternhagen & Allen, 2001).

Other metabolic features

This whey protein has been associated to treatment of chronic stress-induced cognitive decline. In fact, an imbalance in brain serotonin (5-hydroxytryptamine) function was claimed to be a factor mediating the negative effect of chronic stress on cognitive performance; serotonin release decreases under exposure to chronic stress, thus decreasing the available concentrations of brain serotonin and tryptophan (a precursor of serotonin) both of which cause serotonin activity to fall below functional needs. Due to its high tryptophan content (ca. 6% (w/w)), a diet based on α -LA increases the plasma tryptophan-large neutral amino acids (Trp-LNAA) ratio (Markus, Olivier, & de Haan, 2002).

Bovine serum albumin

BSA is not synthesized in the mammary gland, but appears instead in milk following passive leakage from the blood stream. It contains 582 amino acid residues, which lead to a molecular weight of 66,267 kDa; it also possesses 17 intermolecular disulphide bridges and one thiol group at residue 34 (Fox, 1989). Because of its size and higher levels of structure, BSA can bind to free fatty acids and other lipids, as well as flavour compounds (Kinsella, Whitehead, Brady, & Bringe, 1989) – a feature that is severely hampered upon denaturation. Its heat-induced gelation at pH 6.5 is initiated by an intermolecular thiol–disulphide interchange, similar to what happens with β -LG (de Wit, 1989).

Anticarcinogenic activity

One important property that has been associated to BSA is the ability to inhibit tumour growth; *in vitro* incubation with human breast cancer cell line MCF-7 has provided adequate evidence thereof, which lies on modulation of activities of the autocrine growth regulatory factors (Laursen, Briand, & Lykkesfeldt, 1990).

Other metabolic features

The aforementioned binding properties of BSA depend on the fatty acid (or other small molecules) in stake (Brown & Shockley, 1982). Binding to fatty acids, that are stored in the human body as fat, allow it to participate in synthesis of lipids – which are a part of all outer and inner cell membranes, and which provide energy; this issue has been reviewed at some length by Choi et al. (2002).

The antioxidant activities of this protein have been tackled by Tong, Sasaki, McClements, and Decker (2000). BSA has been shown to *in vitro* protect lipids against phenolic-induced oxidation (Smith, Halliwell, & Aruoma, 1992).

Immunoglobulins

IG constitute a complex group, the elements of which are produced by B-lymphocytes; they make a significant contribution to the whey protein content – besides exerting an important immunological function (especially in colostrums). These proteins are present in the serum and physiological fluids of all mammals; some of them attach to surfaces, where they behave as receptors, whereas others function as antibodies, which are released in the blood and lymph. IG are subject to postnatal transfer via colostrum – as the placenta does not permit passage of macromolecules (Butler, 1994). The structure and general function of bovine IG have been reviewed by Korhonen, Marnila, and Gill (2000).

In terms of quaternary structure, IG are either monomers or polymers of a four-chain molecule, consisting of two light polypeptide chains (with a molecular weight in

the range 25,000 kDa) and two heavy chains (with molecular weight of 50,000–70,000 kDa) (Mulvihill & Donovan, 1987). The nomenclature of the elements of this family is based on their immunological cross-reaction with reference proteins, preferably of human origin, as proposed by WHO (Butler, 1971). There are, however, three basic classes of IG: IG_G, IG_A and IG_M, although IG_G is often sub-divided into two subclasses – IG_{G1} and IG_{G2}. Up to 80% (w/w) of all IG in milk or whey is accounted for by IG_G (de Wit, 1989) but qualitatively, the family of IG found in bovine whey and colostrum include IG_A and secretory IG_A, IG_{G1}, IG_{G2} and IG_G fragments, IG_M, IG_E, J-chain or component, and free secretor component.

Antimicrobial and antiviral properties

Antibody concentrates derived from immune colostrum and milk collected from cows immunised with inactivated human rotavirus (HRV) serotypes 1 (Wa) and 2 (S2), as well as simian rotavirus serotype 3 (SA11) were shown to possess preventive (or treatment) features in enteric disease caused by said viruses in piglets (Schaller et al., 1992), and in therapeutics of child infections caused thereby (Sarker et al., 1998). Infant gastritis originated by *H. pylori* is well fought via a diet including immune milk containing specific anti-*H. pylori* antibodies (Oona et al., 1997). Other studies were performed against infections in calves caused by enterotoxigenic *Escherichia coli* (Moon & Bunn, 1993), and even infections in humans caused by enteropathogenic (Freedman, Tacket, & Delehanty, 1998).

Okhuysen et al. (1998) tackled the use of immune bovine colostrum on treatment of HIV-patients bearing infections caused by *Cryptosporidium parvum*. There is also evidence of protection via bovine antibodies against dental caries caused by cariogenic streptococci (Loimaranta et al., 1999). In a study involving two groups of mice – one fed with immune milk obtained from cows immunised with a mixed bacterial flora, and a control group fed regularly, they were exposed to lethal doses of irradiation, and their survival rate was compared; the survival rate of the test group was higher than that of the control group, and the concentration of IG_A in cell culture derived from Peyer's patches, mesenteric lymph node and spleen of the former group was also higher (Kobayashi et al., 1991).

Immune system modulation

IG are recognised to provide protection against diseases in the newborn through passive immunity. Most studies have focussed on newborn ruminants, but more recent research has started exploring the effects on non-ruminants and adults.

Feeding of pregnant cows with immune milk, or systemic immunisation thereof has been a common technique applied in several research efforts; the latter procedure increases the levels of antibodies against immunising bacteria, and also reduces susceptibility to disease (Ormrod &

Miller, 1991). Vaccination of pregnant cows originates colostrum characterized by high concentrations of specific antibodies against the antigens of the vaccine used (Korhonen et al., 1995). Tomita, Todhunter, Hogan, and Smith (1995) studied the effect on cows of a vaccine containing a lipopolysaccharide–protein conjugate derived from *E. coli* J5; immunisation enhanced the serum antibody titer to J5, but not the whey titer to IG_G.

Other metabolic features

Immune milk was also suggested to lower blood pressure (Sharpe, Gamble, & Sharpe, 1994). In a double-blind, clinical-trial study, the effects on reduction of plasma cholesterol and blood pressure of immune milk produced by dairy cows previously hyper-immunised with a multivalent bacterial vaccine were assessed, involving human hypercholesterolemic subjects – who consumed 90 g of immune milk daily, versus regular milk; the former was a useful adjunct in the dietary management of hypercholesterolemia (Sharpe et al., 1994).

Lactoferrin

BLF is an iron-chelating, monomeric glycoprotein, characterized by a molecular weight of 80,000 kDa, to which two carbohydrate groups are attached (de Wit, 1989; Lonnerdal & Iyer, 1995). It is present in the human body as a secretor protein – synthesized by glandular epithelial cells and mature neutrophils, and can be found in milk, saliva, tears, nasal and intestinal secretions, pancreatic juice and seminal fluid, as well as in secondary granules of neutrophils (Lonnerdal & Iyer, 1995). Bovine milk contains between 0.02 and 0.35 mg/ml of BLF, depending on the period of lactation – whereas human milk contains ca. 4.00 mg/ml. Two other proteins with iron-chelating properties are transferrin and ovotransferrin; these proteins – as happens with BLF, possess two metal-binding sites, designed to attach to ferric ion (Fe³⁺) and bicarbonate ion (HCO₃⁻), respectively. The iron-free protein is usually termed apo-BLF.

Antimicrobial and antiviral properties

A review on the antimicrobial properties of lactoferrin is available elsewhere (Fernaund & Evans, 2003). Its antibacterial activity has been attributed to the aforementioned iron-binding property – which makes it unavailable the (otherwise active) microorganisms, or by direct binding to their microbial membrane. Nevertheless, the action of this protein is not restricted to bacteria with iron requirements (e.g. coliforms), because apo-BLF was also shown to possess bactericidal power against a wide range of microorganisms (Dionysius, Grieve, & Milne, 1993). BLF can damage the outer membrane of Gram-negative bacteria via binding to Lipid A lipopolysaccharides (LPS) – which compose their asymmetric lipid bilayer membrane

(Appelmek et al., 1994); release thereof causes structural changes, which include loss of membrane potential and integrity. The antibacterial activity of LF also depends on its concentration or the degree of iron saturation of the molecule, and on its interaction with mineral medium constituents (Payne, Davidson, Oliver, & Christen, 1990).

The antimicrobial effect of BLF can be taken advantage of in treatment of enteric infections (as depicted in Table 3).

In vivo studies, encompassing animal models, have proven that BLF helps eliminate *E. coli* endotoxins, when bovine colostrum is administered into the gut under conditions of septic shock (Dohler & Nebermann, 2002). BLF may act synergistically with lysozyme or antibodies (Ellison & Giehl, 1991), immunoglobulins and LP, and can potentiate the activity of such antibiotics as novobiocin against Gram-negative bacteria (Sanchez & Watts, 1999). Alone or in

Table 3
Antiviral and antimicrobial properties of whey proteins, and mechanisms of action thereof

Protein	Protection against	Protection mechanisms	References
Immunoglobulin (antibodies)	<i>Helicobacter pylori</i>	Agglutination	Oona et al. (1997)
	Enteropathogenic <i>Escherichia coli</i>	Bacteriolysis	Freedman et al. (1998)
	Cariogenic streptococci	Bacteriostasis	Loimaranta et al. (1999)
	<i>E. coli</i> J5	Oposonization	Tomita et al. (1995)
	<i>Cryptosporidium parvum</i>		Okhuysen et al. (1998)
Lactoferrin	Human and simian rotavirus	Neutralization of virus and toxins	Schaller et al. (1992)
	Enterotoxigenic <i>E. coli</i> (ETEC)	Binding of iron	Yamaguchi et al. (1993)
	Enterohemorrhagic <i>E. coli</i> (EHEC)	Destabilization of outer membranes	Shin et al. (2000)
	<i>Shigella flexneri</i> -induced inflammatory enteritis	Synergetic action with antibiotics, lysozyme or antibodies	Gomez et al. (2002)
	<i>Haemophilus influenzae</i>	Modulation of mononuclear cell functions or peripheral	Hoerr and Bostwick (2000)
	<i>Vibrio cholerae</i>	Blood neutrophil polymorphonuclear leucocytes	Mitoma et al. (2001), Oho et al. (2002)
	<i>Streptococcus mutans</i>		
	<i>Trichophyton mentagrophytes</i> , <i>T. rubrum</i>		Wakabayashi et al. (2000)
	<i>Carnobacterium viridans</i>		al-Nabulsi and Holley (2005)
	HIV-1	Prevention of virus binding	Berkhout et al. (2002), Ng et al. (2001), Wang et al. (2000)
		Inhibition of HIV-1 enzyme (reverse transcriptase)	Lubashevsky et al. (2004)
		Inhibition of viral entrance	
	Seoul type hantavirus		
	Cytomegalovirus		
	Poliovirus-1		
Simian rotavirus SA11		Superti and Donelli, 1995	
Enterovirus 71	Binding to VP-1 protein and host cells	Lin et al. (2005), Weng et al. (2005)	
Human papillomavirus	Inhibition of viral entrance	Drobni et al. (2004)	
Hepatitis C, G and B		Interaction with E1 and E2 envelope proteins	Ikeda et al. (1998), Isawa et al. (2002), Ishii et al. (2003), Yi et al. (1997)
		Inhibition of viral entrance by neutralisation of virion	
		Increase of Th-1-cytokine in peripheral blood	
		Interaction with host cell surface proteins	
		Binding to host cells and to receptor co-receptor of virus (HSPG)	Laquerre et al. (1998), Marchetti et al. (2004)
Lactoperoxidase	Herpes simplex virus (HSV-1, HSV-2)		
	Gram-positive bacteria	Oxidation of SH-groups of cell membranes	Santos et al. (1994), Zapico et al. (1995)
	Gram-negative bacteria	Bacteriostasis Bacteriolysis	
Glycomacropeptide	Dental plaque	Inhibition of adhesion of bacteria and viruses to epithelial cells/dental plaque	Isoda, Kawasaki, Tanimoto, Dosako, & Idota (1999), Kawasaki et al. (1992), Schupbach, Neeser, Golliard, Rouvet, & Guggenheim (1996)
	Dental caries	Inhibition of binding of cholera toxin to its receptor and heat-labile enterotoxins LT-I and LT-II	
	Enterotoxigenic <i>E. coli</i> Influenza virus haemagglutinin		Kawasaki et al. (1993)

combination with penicillin G, BLF enhances *Staphylococcus aureus* susceptibility to immune-defense mechanisms; this can be a beneficial trait in the treatment of infections caused thereby (Diarra et al., 2003). Another bacterium inhibited by that protein is *Streptococcus mutans* – the prime cause of dental caries; BLF inhibits aggregation of those saliva induced-bacteria to salivary components, or to salivary films (Mitoma, Oho, Shimazaki, & Koga, 2001; Oho, Mitoma, & Koga, 2002). In addition, BLF has been demonstrated (Hoerr & Bostwick, 2000) to protect against such pathogens as *Haemophilus influenzae*, which causes otitis media (ear infections) in children. *In vivo* and *in vitro* tests carried out by Dial et al. (1998) confirmed that BLF possesses antimicrobial activity against *H. pylori* infections at pH 6, at concentrations above 0.5 mg/ml, and is able to clear *H. felis* infection in mouse; and that a non-iron-dependent component of such a protein displays antimicrobial activity. Thereof, use of bovine BLF as therapeutic agent to assist antimicrobial agents towards eradication of such harmful bacterium was suggested (Di Mário et al., 2003). Recently, BLF was found to exhibit antibacterial activity against *Carnobacterium viridans*, at neutral pH and refrigeration temperatures (al-Nabulsi & Holley, 2005).

Recent studies encompassing guinea pigs have demonstrated that iron-unsaturated BLF is active against the fungi *Trichophyton mentagrophytes* and *Trichophyton rubrum* – which are responsible for dermatophytosis (Wakabayashi et al., 2000; Wakabayashi et al., 2002). Diets containing BLF fed to guinea-pigs previously infected (or immunised) with *T. mentagrophytes* potentiated the host antifungal defense systems, by modulating the mononuclear cell functions encompassing antifungal activity, or the release of peripheral blood neutrophil polymorphonuclear leucocytes in plasma.

Incidentally, BLF was the first whey protein which was ascribed an anti-viral activity; this subject was comprehensively reviewed by van der Strate, Beljaars, Molema, Harmsen, and Meijer (2001). Said activity was suggested against several species of virus, as depicted in Table 3. BLF also plays a preventive role of AIDS-like disease (ALD) in mice, at least for a restricted period of time – when administrated 20 days prior to viral infection (Lubashevsky et al., 2004).

The major mechanisms by which BLF is believed to exert its antiviral activity are summarized in Table 3. BLF interacts with viruses, but not with cells infected thereby; however, it can interact with the host cells before they are infected – like happens with hepatitis B virus (HBV) for which interaction with the cell surface proteins blocks viral adhesion to the cells themselves (Hara et al., 2002). This issue was confirmed with regard to E1 and E2 HVC envelope proteins *in vitro* (Yi, Kaneko, Yu, & Murakumi, 1997), and in the case of HIV-1 reverse transcriptase (Ng et al., 2001). In what concerns hepatitis C and G viruses (HVC and HGV, respectively), BLF inhibits the viral entry into hepatocyte and lymphocyte cells – via neutralising the virion, and blocking the invasion of the cell

thereby (Ikeda et al., 1998). In the case of HIV-1, succinylation of that protein slightly increases the extent of inhibition of the HIV-1 reverse transcriptase (Wang, Ye, & Ng, 2000). *In vivo* experiments involving HIV-infected individuals have indicated that BLF inhibits strongly that enzyme, but only slightly protease and integrase (Ng et al., 2001). Recently, LF was found to inhibit *in vitro* enterovirus 71 (EV 71), via binding to one of their four capsid proteins, VP1; furthermore, BLF can induce IFN- α expression of neuronal (SK-N-SH) cells and inhibit EV 7-induced IL-6 production (Weng et al., 2005).

Except in the case of simian rotavirus (Superti & Donelli, 1995), another mechanism hypothesized for binding of BLF to host cells is via a receptor (or co-receptor) – such as heparin sulphate proteoglycans (HSPG), which prevent binding of the virus thereto, and subsequent infection of the host cell (Laquerre et al., 1998). Recently, Marchetti, Trybala, Superti, Johansson, and Bergström (2004) elucidated the mechanism of BLF antiviral activity against HSV-1 infection initiates infection of cells via binding to it glycosaminoglycans (GAG) (WuDunn & Spear, 1989), which are composed of repeating (specifically sulphated) disaccharide units. BLF binds specifically to the two common classes, heparan sulfate (HS) and/or chondroitin sulfate (CS), on cell surfaces – hence occupying moieties that would function as initial receptors for said virus. This mechanism was promote (Drobni, Näslund, & Evander, 2004) to be similar to human papillomavirus inhibition. Bovine BLF was also shown (Ishii et al., 2003) to reduce serum HVC-RNA levels in patients suffering from chronic hepatitis C; a dominant environment of Th1-cytokine is produced in the peripheral blood that favours eradication of HVC via interferon (IFN) therapy.

Immune system modulation

BLF plays an important role upon stimulation of the immune system, probably owing to increase of macrophage activity, as well as induction of inflammatory cytokines, including IL-8, TNF- α and nitric oxide (McCormick, Markey, Morris, Auld, & Alexander, 1991; Sorimachi, Akimoto, Hattori, Ieiri, & Niwa, 1997), stimulation of proliferation of lymphocytes (Potjewijd, 1999), and activation of monocytes, natural killer (NK) cells (Ambruso & Johnson, 1981; Nishiya & Horwitz, 1982) and neutrophils (Gahr, Speer, Damerau, & Sawatzki, 1991). Kuhara et al. (2000) administrated LF orally, and reported that the induction of IL-8 secretion by epithelial cells acts so as to enhance Th1 cell functions, and augment NK cells and cytotoxic lymphocyte activities.

BLF has also been reported to stimulate humoral immune response to sheep red blood cells, to promote appropriate intestinal and peripheral specific antibody response, and to modulate production of lymphokines (German, Dillard, & Walzem, 2000). When of bovine origin, both BLF and hydrolysed forms thereof enhance mucosal immunity – which is dependent on the ability of

BLF to bind to the intestinal mucosa (Debbabi, Dubarry, Rautureau, & Tome, 1998).

The aforementioned activities depend on the target cells, and arise from the ability of BLF to bind to specific molecules. As suggested before, BLF possesses an LPS-binding property, and this property is a part of the immunomodulatory function of BLF. LPS, as a highly cationic glycoprotein, binds to lipid A portion of LPS via charge–charge interaction. Recently, Na et al. (2004) claimed that, upon mixing LPS and purified LF, they subsequently formed a complex LPS-LF, which induced inflammatory mediators in macrophages rather than LPS activity. The latter work proposed that lipid A – a toxic portion of LPS, can be recognized even after LF-LPS has been formed, and that this complex induces tolerance to LPS-stimulation.

Anticarcinogenic activity

Several studies, based on data from such animals as mice and rats, have provided stronger and stronger evidence for the therapeutic value of BLF in treating distinct types of cancer (Gill & Cross, 2000), *viz.* colon cancer (Masuda et al., 2000). The iron-binding capacity of BLF has been put forward as the molecular rationale behind such a biological activity; free iron may act as a mutagenic promoter, by inducing oxidative damage to the nucleic acid structure; hence, when BLF binds iron in tissues, it reduces the risk of oxidant-induced carcinomas (Weinberg, 1996) and colon adenocarcinomas (Tsuda et al., 1998). Studies are available (Masuda et al., 2000; Tanaka et al., 2000) that pertain to such other cancers as lung, bladder, tongue and oesophagus, and which convey similar results.

Other metabolic features

Supplementing the diet of dairy calves with BLF has been tried, along with monitoring its effect on their performance (Joslin et al., 2002); BLF was able to improve the average daily gain, and decrease weaning in dairy calves.

An *in vivo* research effort conducted by Kajikawa et al. (1994) led them to hypothesize that BLF may act as an antiatherogenic agent, by inhibiting accumulation of cholesterol esters in macrophages – which are ‘precursors of the foam cells found in early atherosclerotic lesions. This protein can furthermore survive gastric digestion in adults (Troost, Steijns, Saris, & Brummer, 2001), and bears no toxicity when orally administered to rats (Yamauchi et al., 2000); these two reasons account for its regular use as bioactive ingredient for therapeutic administration.

Lactoperoxidase

Lactoperoxidase (LP) is present in a variety of animal secretions, *viz.* tears, saliva and milk. A member of the family of mammalian peroxidases, it is one of the most abundant enzymes in plain milk – it represents ca. 1% (w/w) of the total protein pool in whey (Reiter & Perrau-

din, 1991). The complete LP system (i.e. enzyme plus substrate) was originally characterized in milk by Reiter, Pickering, Oram, and Pope (1963); its activity depends on many factors, e.g. animal species (Pruitt & Reiter, 1985), breed and lactation cycle (Zapico, Gaya, de Paz, Nunez, & Medina, 1991).

Other members of that group of oxidoreductases include myeloperoxidase (present in neutrophils and monocytes), eosinophil peroxidase and thyroid peroxidase. Chemical sequencing unfolded a great degree of homology between them, which suggests a close evolutionary relationship among those enzymes (Petrides, 1998). Peroxidases utilise hydrogen peroxide to oxidise thiocyanate to hypothiocyanate, and are active in a variety of anatomic locations (Thomas, Bozeman, & Learn, 1991).

Antimicrobial and antiviral properties

LP is an important part of the natural host defense system in mammals, which provides protection against invading microorganisms (de Wit & van Hooydonk, 1996). The antibacterial ability of peroxidases was first described (Agner, 1941) in verdoperoxidase (later renamed myeloperoxidase) from leukocytes – which is able to inactivate diphtheria toxin; such an antimicrobial activity has been proven against Gram-positive (Zapico, Gaya, Nunez, & Medina, 1995) and negative (Zapico et al., 1995) microorganisms.

The mechanism of action of LP has been explained in detail elsewhere (de Wit & van Hooydonk, 1996); such an enzyme is more active at acidic pH (Wever, Kast, Kasinodien, & Boelend, 1982). The antimicrobial activity of peroxidases depends on the ion acting as electron donor. The LP system is completed when LP, thiocyanate ion (SCN^-) and hydrogen peroxide (H_2O_2) are present together (Reiter & Harnulv, 1984). The thiocyanate anion – which is necessary for the antibacterial activity of LP be expressed, appears to significant extents in saliva, milk and airway secretions (Reiter & Perraudin, 1991), and originates from glucosinolates via detoxification of the cyanogenic glucosides present in the feed. The amount of SCN^- in cow's milk ranges from 0.1 to 15 mg/kg (Perraudin, 1991); its concentration varies according to animal species (de Wit & van Hooydonk, 1996), breed, lactation cycle (Zapico et al., 1991), season (Dabur, Srivastava, & Kapoor, 1996) and composition of feed (Thomas, 1981).

LP-catalysed reactions yield short-life, intermediary oxidation products of SCN^- , which are responsible for its antibacterial activity. The major intermediary oxidation product, at physiological pH, is hypothiocyanate (OSCN^-), which is produced at the stoichiometric ratio of 1 mol/mol of hydrogen peroxide (Pruitt & Tenovuo, 1982). Other products possessing antimicrobial activity are also formed in this reaction, *viz.* cyanosulphurous acid (HO_2SCN) and cyanosulphuric acid (HO_3SCN) (Bjorck, 1979); these compounds are able to oxidise sulphidril groups of bacterial proteins (Reiter & Harnulv, 1984). The anion OSCN^- is thought to mediate bacterial killing,

as it is cell-permeable and can inhibit glycolysis, as well as nicotinamide adenine dinucleotide (NADH)/nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reactions in bacteria (Reiter & Perraudin, 1991).

Hydrogen peroxide is produced in endogenous form by polymorphonuclear leukocytes (de Wit & van Hooydonk, 1996), and by numerous other microorganisms (Pruitt, Kamau, Miller, Mansson-Rahemtulla, & Rahemtulla, 1990), viz. resident bacteria in such sites as the oral cavity, or generated *in situ* by cells the respiratory tract (Reiter & Perraudin, 1991). It can also be produced by specific generator systems, such as via oxidation of ascorbic acid, oxidation of glucose-oxidase, oxidation of hypoxanthine by xanthine oxidase, and manganese-dependent aerobic oxidation of reduced pyridine nucleotides by peroxidase (Wolason & Sumner, 1993). The H₂O₂ content is rapidly reduced by catalases and peroxidases that are adventitious in milk; hence, it is normally present to very small levels (Bjorck, Rosen, Marshall, & Reiter, 1975). While H₂O₂ can behave as bactericidal or bacteriostatic, experimental evidence indicates that inhibition of bacterial growth is far more efficient in the presence of the complete LP system (Reiter & Perraudin, 1991).

Studies pertaining to the antimicrobial effects of LP have focused on its role upon thermal resistance of *Salmonella* spp. (Doyle & Mazzotta, 2000), in both raw (Heuvelink et al., 1998) and mature milk (Shin, Tomita, & Lonnerdal, 2000). Peroxidases are important in inhibiting dental caries, in controlling microorganism growth in milk from lactating animals, and in cell-mediated pathogen killing (Reiter & Perraudin, 1991). Poulsson, Hegg, and Castberg (1986) patented a process that uses an LP system for dental (and other wound) treatment.

Bacterial resistance to LP-mediated killing may occur via enzymes which act in a reverse way – and which inactivate OSCN⁻, prevent pigment generation and constrain anaerobic metabolism (Reiter & Perraudin, 1991).

Acknowledgements

Partial funding for this research work was provided via project PROBIOSORO, administrated by Agência de Inovação – POCTI: Programa Operacional de Ciência, Tecnologia e Inovação. Funding for authors A.R. Madureira and C.I. Pereira was via Ph.D. fellowships, administered by Fundação para a Ciência e a Tecnologia (SFRH/BD/18500/2004 and SFRH/BD/18000/2004, respectively).

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