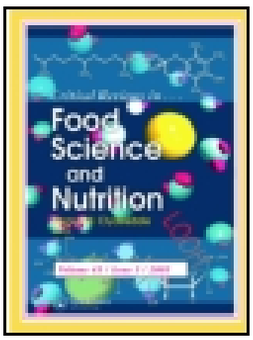

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Lactoferrin: Structure, Function, Denaturation and Digestion

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Abstract

Lactoferrin (LF) is a multifunctional protein occurring in many biological secretions including milk. It possesses iron binding/transferring, antibacterial, antiviral, antifungal, anti-inflammatory and anti-carcinogenic properties. These functional properties intimately depend on the structural integrity of LF especially its higher order conformation. LF is primarily extracted from bovine milk and it is subsequently added into many commercial products such as nutritional supplements, infant formula, cosmetics and toothpaste. LF is sensitive to denaturation induced by temperature and other physicochemical stresses. Hence, the extraction, powder formation processes of LF and processing parameters of LF-containing products have to be optimized to minimise its undesired denaturation. This review documents the advances made on structure-function relationships and discusses the effectiveness of methods used to preserve the structure of LF during thermal processing. Oral delivery, as the most convenient way for administering LF, is also discussed focusing on digestion of LF in oral, gastric and intestinal stages. The effectiveness of methods used to deliver LF to intestinal digestion stage in structurally intact

form is also compared. Altogether, this work comprehensively reviews the fate of LF during thermal processing and digestion, and suggests suitable means to preserve its structural integrity and functional properties.

Scope of review

The manuscript aims at providing a comprehensive review of the latest publications on four aspects of LF: structural features, functional properties, nature and extent of denaturation and gastrointestinal digestion. It also analyses how these publications benefit food and pharmaceutical industries.

Key words:

Lactoferrin, Structure, Function, Drying, Denaturation, Digestion and Application

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1. Introduction

Lactoferrin (LF) was first identified in 1939 as a red protein in whey (Sorensen & Sorensen, 1940). In the year of 1960, it was isolated and purified from human and bovine milk (Groves, 1960; Johanson, 1960). The isolated protein was structurally similar to serum transferrin with ~60% sequence identity and can reversibly bind ferric (Fe^{3+}) ion (Baker, 1994; Johanson, 1960). Due to this reason, LF is classified as a member of transferrin family along with serum transferrin, melanotransferrin and ovotransferrin (Lambert, Perri & Meehan, 2005).

LF is present in biological fluids including milk, saliva and seminal fluid (Cheng et al., 2008). It is also present in mucosal surfaces and in some granules of polymorphonuclear leukocytes. The most abundant source of LF is human and bovine milk. The concentration of LF in milk varies widely with lactation stages and across species. Human colostrum contains higher than 5 g/L of LF as compared to 2--3 g/L in mature breast milk. LF content in bovine colostrum is approximately 0.8 g/L; whereas bovine milk contains only 0.03-0.49 g/L (Table 1). The higher amount of LF in colostrum is believed to provide protections to breast-fed infants against bacterial infection and inflammation (Artym & Zimecki, 2005).

LF promotes iron absorption in the human body (Paesano et al., 2010). It modulates cell growth, scavenges harmful free radicals and inhibits the formation of several toxic compounds (Baveye et al., 1999). Due to these reasons, LF is added in many commercial products including infant formula powders, therapeutic drinks, fermented milk, cosmetics and toothpaste (Tomita et al., 2009). The multiple health promoting functions of LF and its wide real-life applications has stimulated increased research interest.

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2. Structure of Lactoferrin

LF is a single polypeptide chain glycoprotein with a molecular weight of around 78 kDa. Detailed structural studies have reported that there are 691 and 696 amino acids in human and bovine LF, respectively (Baker et al., 2000; Moore et al., 1997). LF from mammalian species has similar amino acid sequence. Human and bovine LF share approximately 70% sequence identity whereas human and chimpanzee LF share almost 97% sequence identity (Yount et al., 2007). This pronounced similarity in primary structure of LF in various mammalian species indicates that it performs identical biological functions in different species. LF has ordered secondary structural features comprising 33--34% helices and 17--18% strands (Table 2). The tertiary structures of LF, as determined by X-ray crystallography (Fig. 1A), consists of two symmetric lobes (N-lobe and C- lobe) joined by a short α-helix. These two lobes are globular and can be further divided into two similarly sized sub-domains: N_1 and N_2; C_1 and C_2, respectively. In bovine LF, sub lobe N_1 is composed of residues 1--90 & 251--333; N_2 is made up of residues 91--250. Similarly, C_1 sub lobe is comprised of 345--431 & 593--676 residues and the C2 sub lobe is comprised of 432--592 residues. Residues 334--344 make a small 3-turn helix that connects the N and C lobes. This small (3-turn) helix behaves as a flexible hinge during opening and closing of the domains when LF releases/binds iron (Baker & Baker, 2009; Steijns & van Hooijdonk, 2000). Bovine LF contains 17 disulfide bonds (Moore et al., 1997).

One of the most noteworthy features of LF's structure is that its surface is positively charged. This facilitates the binding of LF with anionic bio-compounds. The positively charged moieties of LF are mainly concentrated on the outer region of first helix of the N_1 domain and towards the

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end of C terminal (Fig. 1B). Another, much smaller, but intensely positively charged spot exists in the inter lobe region where two lobes are connected by a helix. The basic region surrounding the N-terminus is shown to be responsible for binding DNA, heparin, and lipopolysaccharide (He & Furmanski, 1995; Lizzi et al., 2016; van Berkel et al. 1997). Sugars (mainly high mannose and N-Acetyleglucosamine) are associated with LF through N-linked glycosylation (Moore et al., 1997). In human LF, approximately 5% of the molecules are glycosylated at one site (Asn 479), 85% of the molecules are glycosylated at two sites (Asn138 and 479) and 9% of the molecules are glycosylated at three sites (Asn138, 479 and 624) (van Berkel et al., 1996). In bovine LF, 5 potential glycosylation sites have been reported. Most bovine LF molecules are glycosylated with sugars at Asn 233, 281, 368, 476, while only 15--30% of LF molecules make use of the Asn281 glycosylation site (Spik et al., 1994; Wei et al., 2000; Yoshida el al., 2000). The number of N-linked glycosylation sites and the types of sugar attached to LF are precisely regulated by gene expression. These variations in glycosylation sites affect the susceptibility of LF to proteolysis and thermal denaturation (Moore et al., 1997; van Veen et al., 2004). The glyco-variation is also involved in modulating the association of pathogens with LF (Barboza et al., 2012).

3. Function and Applications of Lactoferrin

The unique structural characteristics of LF provide a variety of nutritional and medicinal values. In terms of nutritional function, LF transport iron and detoxify free radicals in biological fluids. Since iron deficiency is one of the most common nutritional deficiencies in the world (Gupta et al., 2016; Lopez et al., 2016), LF is increasingly considered to be a safe and effective ingredient

to deliver iron to deficient people. Earlier study conducted by Kawakami et al. (1988) compared the iron absorption from bovine LF and ferrous sulphate in iron deficient anaemic rats. It was found that the iron from bovine LF was more easily absorbed across the intestinal mucosa compared to the iron from ferrous salts. A recently reported clinical research on pregnant women showed that oral administration of bovine LF increased iron absorption whereas the iron from ferrous salts was not absorbed in the same level (Paesano et al., 2010). The more efficient iron absorption from LF than that from iron salts might be due to the presence of specific LF receptors on human mucosa cells, which generate a receptor-mechanism pathway (Cox et al., 1979; Iyer & Lönnerdal, 1993; Lönnerdal et al., 2015). Nevertheless, conflicting results have been presented in infants. Some researches demonstrated that oral administration of LF promotes iron absorption of newborns whereas other studies did not observe any significant effect (Davidsson et al., 1994; Hernell & Lönnerdal, 2002; Ke et al., 2015). One of the possible reasons for these contradictory results is that LF might not be recognized by mucosal LF receptors due to the difference in state of iron in individuals (Hernell & Lönnerdal, 2002; Lönnerdal, 2015). Another hypothesis put forward by Sánchez. (1992a) is that LF regulates rather than promoting the absorption of iron in infants. This hypothesis was further tested and verified by Davidsson et al. (1994), Scarino (2007) and Lönnerdal et al. (2015).

LF also possesses antimicrobial, anti-inflammatory and anti-carcinogenic activities, highlighting the therapeutic values of this multifunctional protein. Many vaccines, therapeutic drugs and oral health care products are developed making use of the medicinal functions of LF. These functions and applications are further discussed in section 3.2-3.3.

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3.1. Iron transferring function

LF has very high affinity to iron with an equilibrium dissociation constant value (K_D) of ~10^{-20} M (Baker & Baker, 2004; Steijns & van Hooijdonk, 2000). Two ferric ions (Fe^{3+}) can covalently bind to a LF molecule in the presence of 2 carbonate ions (CO_3^{2-}). Based on the iron-saturation levels, LF has been found to exist in three different forms: iron depleted (apo) form (both C lobe and N lobe are free from iron), iron saturated (holo) form (1 iron atom is attached to both C and N lobes) and partially iron saturated (mono) form (1 iron atom is attached to either C or N lobe). In practice, if the iron saturation level of LF lies in between 0--6%, it is considered as apo form whereas the holo forms of LF have an iron saturation level between 76--100% (Bokkhim et al., 2013; Sui et al., 2010; Voswinkel et al., 2016). The apo and holo forms of LF are prepared in small scale for research purpose. Commercially available LF (native-LF) is primarily extracted from bovine milk, which has an iron saturation level of 10--20%.

3.1.1. Iron binding and iron release mechanisms

The iron binding mechanism of human and bovine LF has been thoroughly studied (Baker & Baker, 2009; Moore et al., 1997; Rastogi et al., 2016). The metal-ligand bonds and hydrogen bonds in the iron binding domains were found to be identical for bovine and human LF. Four different amino acid residues are involved in binding Fe^{3+} ion. Asp60, Tyr92, Tyr192, His253 covalently bind with Fe^{3+} ion in N lobe (Fig. 2). The two oxygen atoms of carbonate ion also covalently attach with Fe^{3+}. In the C lobe, Asp395, Tyr433, Tyr526, His595 and carbonate ion bind with Fe^{3+} in the similar fashion. Both the N and C lobes of LF bind to carbonate ion (CO_3^{2-}) first and then they bind the Fe^{3+}. The CO_3^{2-} binding sites are located in a positively charged

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pocket in N_2 and C_2 domain. These pockets are supported by the α-helix structure in the vicinity of the iron binding cleft (121-131 residues in N lobe and 395--407 in C lobe). The side-chains of arginine and threonine residues (Arg121 and Thr117 in the N lobe, Arg463 and Thr459 in the C lobe) are also involved in the CO_3^{2-} binding pocket. This positively charged pocket satisfies the full hydrogen bonding potential of the carbonate ion, and allows it to fit perfectly between the Fe^{3+} atom and anion binding sites (Moore et al., 1997). The iron binding by LF is a cooperative type of binding where C-lobe first binds with Fe^{3+} and then stimulates the N-lobe for iron binding (Abdallah & Chahine, 2000). It has been reported that after iron binding, the inter domain hydrogen bonding occurs at the lips of the iron binding cleft. In this way the Fe^{3+} molecule is enclosed within the iron binding domains. The inclusion of Fe^{3+} atoms in both two lobes makes the structure of iron saturated LF more compact compared to that of the iron-depleted LF. The structurally compact iron saturated (holo) form of LF is, thus, more stable against external stressors (Abdallah & Chahine, 2000).

Iron release from LF follows a reverse path of iron binding. In this case, structural changes at the iron binding site, i.e., the opening of the closed iron binding domains occurs first followed by the release of iron. There are three factors that cause the structural changes essential for the release of iron: firstly, presence of specific receptors similar to that in serum transferrin; secondly, reduction of Fe^{3+} to Fe^{2+} (LF has much lower affinity to Fe^{2+}); and thirdly, decrease of the pH in the environment (Baker & Baker, 2009; Baker et al., 2002). The pH trigged release of iron has been studied to a considerable detail using crystallographic and iron release kinetics (Abdallah & Chahine, 2000; Baker & Baker 2009; Rastogi et al., 2016). The carbonate ion, tyrosine and/or histidine ligands present in the iron binding domain are protonated at certain low pH values

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depending on the source of LF. This lowering of pH weakens the iron coordination to a point at which it cannot hold the two LF domains together. When this happens, the iron binding domains will open and interact with positive charges prevailing in the environment and ultimately result in the release of iron. Rastogi et al. (2016) reported that the N-lobe of bovine LF started to lose iron at pH 6.5 while C-lobe began to lose iron at pH 5.5. N-lobe and C-lobe showed identical iron release behaviour below pH 4.0. In the case of human LF, the N lobe started to release iron at ~pH 5.0 while the C lobe retained iron at pH as low as 3.5 (Baker, 1994; Baker & Baker, 2004). Therefore, the iron binding ability of LF is fully retained at prevailing pH values of fresh human and bovine milk (pH 6.5-6.8). Interestingly, the iron binding ability or affinity to hold iron of other transferrins (e.g. serum transferrin and ovotransferrin) already starts to lose at these pH values; thus, their bacteriostatic ability is greatly reduced (Abdallah & Chahine, 2000; Erickson et al., 2013; Tsioulpas et al., 2007). The receptor-driven iron release pathway of LF is involved in the iron metabolism and regulation iron content in human body. Human LF is absorbed through the apical membrane of the intestinal cell by a specific LF receptor and internalized with its bound iron (Lönnerdal et al., 2015). The absorbed LF is then transported by microsomes and ultimately participates in the redox reactions of the iron cycle (Lönnerdal et al., 2015).

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3.1.2. Iron delivery function

Iron is an essential mineral for growth and survival of all living organisms because it is required as a cofactor for essential enzymes that are involved in many basic cellular functions and metabolic pathways. Iron deficiency causes fatigue and decreased immunity. Excess of iron is

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also toxic as free irons catalyses reactions that produce free oxygen radicals, which are highly toxic to cells (Bokare, & Choi, 2014; Phaniendra et al., 2015). Most of the required iron in human body can be obtained from the recycling metabolism, thus, absorption of 1--2 mg of iron is sufficient in balancing the iron recycling cycle (Camaschella, 2015). Despite of this highly efficient system, iron deficiency is one of the most common nutritional deficiencies in the world (Gupta et al., 2016; Lopez et al., 2016). As a transporter of iron in iron recycling cycle, LF is expected to maintain the balance of iron within the normal range, and help avoid both iron deficiency and iron overload. This is the reason why a large number of studies have been conducted to investigate the nutritional and therapeutic benefits of LF.

It has been shown that oral delivery of bovine LF is effective in preventing iron deficiency anaemia in pregnant women (Paesano et al., 2010; Rezk et al., 2015). Hence, it is expected that LF can be used as an effective iron source to people suffering from iron deficiency. LF has also been added into functional beverages to compensate the loss of iron during professional sport training and excise (sweating) (Özer & Kirmaci, 2010; Tang et al., 2016). In order to use LF as an effective vehicle of iron delivery, it has to be stabilised both in liquid and solid forms. Cao & Maas (2015) have reported that when the LF-containing functional beverages were heated (80-100^{o}C, 15--60 s) under acidic conditions (pH 2.0-5.0) with adequate amount of stabilizers (35-70% mannitol, w/w) and sugars (>30% sucrose, w/w), the shelf life of LF was extended to >4 weeks as indicated by the preservation of primary structure and iron binding capacity. This means that when the formulation and the processing condition are carefully chosen LF can be delivered through beverages. Although the question of whether or not the LF promotes absorption of iron in infants is still not conclusively answered, it is commonly used to fortify

infant formula products. The increased incorporation of LF in infant formula products also stems from its additional benefits such as promotion of bone growth (Cornish et al., 2004), modulation of immune functions (Legrand, 2016) and anti-pathogenic effects (Chen et al., 2016). Furthermore, LF also helps better absorption of other nutrients such as calcium and magnesium (Lönnerdal, 2016; Liao et al., 2012).

3.2. Antibacterial and antiviral activities

3.2.1. Antibacterial property of intact LF

LF demonstrated bacteriostatic effects against a variety of Gram-positive (Francesca et al., 2004; Lee et al., 2005; Rodriguez-Franco et al., 2004) and Gram-negative (Beeckman et al., 2007;Ostan et al., 2017; Rogan et al., 2004) bacteria. In earlier researches, it was believed that the antibacterial activity of LF is due to its high affinity to iron. The iron-free form of LF (apo-LF) is expected to impede iron utilization and cause iron deficiency in iron requiring microorganisms. This finally results into a slower bacterial growth rate (Law & Reiter, 1977). However, later studies found that even iron-saturated form of LF (holo-LF) inhibits the growth of many bacterial strains (Ellison 3rd & Giehl, 1991; Ochoa & Cleary, 2009; Ostan et al., 2017). This means that antibacterial property of LF is not fully dependent on iron binding or scavenging.

Recent research findings show that the bactericidal effect is directly related to the interaction between LF and bacteria (Jenssen & Hancock, 2009; Orsi, 2004; Ostan et al., 2017). Ostan et al. 2017 reported that C-lobe of LF associates with a bi-lobed outer membrane-bound lipoprotein in two different sites of Gram-negative bacterial cells and forms receptor complexes. The binding

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of LF in these two sites either inhibits the iron intake of bacteria or removes the protective effect membrane bound lipoprotein against antimicrobial cationic peptides. Other researches (Coughlin et al., 1983; González-Chávez et al., 2009) suggested that the highly positively charged N-lobe of LF could prevent the interaction between lipopolysaccharide and the cations (Ca^{2+} and Mg^{2+}) required for bacterial growth. LF causes release of lipopolysaccharide from cell wall, increases permeability of membrane and finally obliterates the Gram-negative bacteria. LF binds the anionic molecules (e.g. lipoteichoic acid) on the cell surface of Gram-positive bacteria. This electrostatic binding reduces the overall negative charge of cell wall and facilitates effectiveness of antibacterial compounds such as lysozyme and antibiotics (Barbiroli et al., 2012; González-Chávez et al., 2009; Leitch & Willcox, 1999).

LF prevents the interaction between bacteria and host cells. It inhibits bacterial adhesion to host cells by occupying the surface of bacterial cells (Francesca et al., 2004; Oho et al., 2002; Valenti & Antonini, 2005). Although the detailed mechanism of this effect has not been adequately understood, it has been shown that the (oligo) mannoside glycan of LF is involved in binding with bacterial cells through adhesion (Barboza et al., 2012; da Motta Willer et al., 2004; Gomez et al., 2003).

3.2.2. Antibacterial peptides of LF

Bacteriostatic effect of LF is not restricted to its structurally intact form. In fact, the peptides obtained from LF have higher bacteriostatic efficiency. Lactoferricin is a small bactericidal peptide obtained from cleavage of human and bovine LF structure by gastric pepsin (Bellamy et al., 1992). Lactoferricin derived from human LF (lactoferricin H) contains the 1--47 amino acid

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residue sequence of N- terminus and it has a molecular weight of ~5.6 kDa. Similarly, the peptide derived from bovine LF (lactoferricin B) is comprised of 17--41 amino acid sequence and it has a molecular weight of ~3.2 kDa (Table 3). It was found that both lactoferricins (H and B) had 9--25 fold higher efficiency in inhibiting the growth of common pathogenic bacteria such as *Escherichia coil, Klebsiella pneumoniae,* and *Listeria monocytogenes* compared to intact LF (Bellamy et al., 1992; Lizzi et al., 2016). Interestingly, lactoferricin B was found to possess stronger antimicrobial activity than lactoferricin H which might be due to differences in their primary and secondary structural features. It has also been reported that the residues 1 to 17 in lactoferricin H are not responsible for antibacterial activity while the 25--30 amino acid residues (sequence in intact LF) had strong bacteriostatic activity (Bellamy et al., 1992).

Another antibacterial peptide, called lactoferampin, has also been derived from the N-terminal lobe of LF. It is comprised of the amino acids residues of 269--285 in human LF and amino acids residues of 265--284 in bovine LF. These lactoferampins have slightly weaker bactericidal activity compared to lactoferricin (Haney et al., 2009; van der Kraan et al., 2004). Similar to the case of lactoferricin, lactoferampin obtained from bovine LF is found to have stronger antibacterial activity than the one obtained from human LF. This might be due to structural differences and higher net positive surface charge in bovine lactoferampin than in human lactoferampin (van der Kraan et al., 2004). Both properties play important roles in membrane-mediated activities (van der Kraan et al., 2004). It has been shown that the bactericidal effect of both peptides can be correlated to their ability to disturb membrane integrity (permeabilization and depolarization) of bacteria (Sijbrandij et al., 2017).

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3.2.3. Antiviral activity of LF

The antiviral effect of LF lies in the early phase of infection. LF not only prevents the infection of host cells by viruses (Beljaars et al., 2004; Hasegawa et al., 1994; Marchetti et al., 1999) but also inhibits the growth of viruses after the host cells have been infected (Ikeda et al., 2000; Superti et al., 1997). The domain in LF which acts against viruses seems to be different from the domain that acts against bacteria. This is the reason why the bacteriostatic peptide lactoferricin was not found to be effective against viruses (van der Strate et al., 2001). Earlier studies indicated that LF only inhibits the infection of enveloped viruses; however, recent studies have found that it also inhibits non-enveloped species (Lin et al., 2002; Seganti et al., 2004). The most widely reported hypothesis for the antiviral activity of LF is that it binds to and blocks glycosaminoglycan viral receptors, especially the heparan sulphate. The binding of LF with viral receptors prevents the first contact between virus and host cell, thus preventing the infection (González-Chávez et al., 2009; Shimizu et al., 1996).

3.2.4. LF in antibacterial and antiviral drugs

The bacteriostatic and other antimicrobial effects of LF and LF derived peptides are studied to considerable details. These studies have led to the development of vaccines, antimicrobial drugs and oral health care products using LF as the main agent.

LF is used in oral care products (e.g. toothpaste, mouth-rinses and chewing gums) to inhibit the growth of oral pathogens (e.g. *Streptococcus mutans*) and supress oral malodour (Francesca et al., 2004; Shin et al., 2011). LF is also used in vaccines and antimicrobial drugs. It has been shown that injection of bovine LF is a safe and efficient method in producing antibody against

H1N1 influenza virus (Sherman et al., 2015). The administration of bovine and human LF also generated a stronger T-cell helper 1 response and boosted the effectiveness of vaccines against *Mycobacterium tuberculosis* infection (Hwang et al., 2005; Hwang et al., 2015). No detrimental effects have been reported when LF is in above mentioned and other vaccines (Nevison, 2014). Regarding its application as a drug, the therapeutic potential of LF has demonstrated against a number of diseases caused by bacteria and viruses. For example, it has been reported that LF could reduce the number of pathogens in *Helicobacter pylori* induced gastric infection (Wada et al., 1999). LF has also shown to be effective against a number of human and animal viruses such as *hepatitis* B (Hara et al., 2002), *hepatitis* C (Kaito et al., 2007), *cytomegalovirus* (van der Strate et al., 2001) and human immunodeficiency viruses (Wang et al., 2016).

3.3. Other Functional properties

Besides the intensively studied iron transferring, antibacterial and antiviral properties, LF also possesses other functional values such as antifungal, anti-inflammatory and anti-carcinogenic activities. These functional properties are briefly discussed.

3.3.1. Antifungal activity

LF and LF derived peptides can effectively act on a broad spectrum of fungal species due to their strong iron (Fe^{3+}) scavenging property. It has been observed that LF kills *Candida albicans* and *Candida krusei* (Kirkpatrick et al., 1971; Al-Sheikh, 2009). It has been reported that the sequestration of iron by apo-LF was essential for the host defence against *Aspergillus fumigatus* (Zarember et al., 2007). Apart from the iron depriving effect, LF directly binds on the surface of fungal cells, disrupts the surface and causes increased membrane permeability leading to their

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death. Thus, LF is incorporated in a number of antifungal drugs including those used to cure oral candidiasis (Takakura et al., 2003). The combination of LF with other antifungal compounds significantly enhanced the inhibitory activity against Candida species (Wakabayashi et al., 1998; Venkatesh & Rong, 2008; Kobayashi et al., 2011) and *Cryptococcus neoformans* (Lai et al., 2016).

3.3.2. Anti-inflammatory activity

LF is secreted in mucosa environment of human and animal body. Since many pathogens tend to enter the body via the mucosa, LF plays a key role in the host defence system (Wiesner & Vilcinskas, 2010). Its anti-inflammatory effect can be evidenced by the sharp increase of LF content in body fluids during inflammation (Sagel et al., 2009; Pfefferkorn et al., 2010). Clinical trials have shown that LF can help cure or prevent the inflammation of lung (Hwang et al., 2016; Valenti et al., 2016), gut (Brimelow et al., 2017; Drago-Serrano et al., 2017), intestine (MacManus et al., 2017; Nguyen et al., 2016) etc. The anti-inflammatory activity of LF can be attributed to its positively charged surface. LF interacts with negatively charged moieties (e.g. proteoglycans) on the surface of immune cells. This association can trigger signalling pathways that lead to physiological anti-inflammatory response (González-Chávez et al., 2009; Legrand, 2016). The incorporation of LF in anti-inflamation drugs can minimise their side effects. For example, indomethacin (a non-steroidal anti-inflammatory drug) inhibits human tenocyte growth and proliferation at high concentration (100 μM) whereas LF helps the survival and growth of human tenocytes. The side effect of indomethacin was reversed when LF was administered together with indomechacin (Zhang et al., 2014).

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3.3.3. Anti-carcinogenic activity

LF and peptides derived from it have demonstrated notable therapeutic potential in treatment of cancer. Studies have shown that LF and its peptides possess inhibitory effect against lung (A549), gastric (AGS), intestinal (HT-29) and breast (MDA-MB-231, MCF-7) cancer cell lines (Amiri et al., 2015; Gibbons et al., 2015; Jiang & Lönnerdal, 2016; Tung et al., 2013). Bovine LF inhibited the expression of survivin (a protein enhances survival rate of cancer cell) and modulated the apoptosis of cancer cells both *in-vitro* and *in-vivo* (Gibbons et al., 2015; Kanwar et al., 2015). In a rat model, vascular endothelial growth factor, a major angiogenic factor in tumours, was significantly reduced by oral administration of bovine LF (Norrby et al., 2001). It was found that orally administrated recombinant human LF inhibited the growth of cancer (head and neck carcinoma) cells (60-80% inhibition) through direct cellular inhibition and systemic immunomodulation (Wolf et al., 2007). The carcinoma inhibition effect of LF and its peptides is that they trigger a variety of signalling pathways (apoptosis, and angiopoietin signalling) in human body (Jiang & Lönnerdal, 2016; Tomita et al., 2002) and increase the expression of tumour suppression proteins (Jiang & Lönnerdal, 2016). Lactoferricin obtained during its *in-vitro* gastrointestinal digestion also show anti-carcinogenic effect (Khan et al., 2015; Jiang & Lönnerdal, 2016). Thus, oral administration of LF could be used as a chemotherapeutic agent together with other anticancer drugs.

4. Thermal Denaturation of Lactoferrin

Denaturation occurs when proteins are subjected to harsh environmental conditions such as strong acid/base, elevated or sub-zero temperatures and concentrated organic/inorganic salt.

These conditions alter the conformation of the protein and cause the breakdown of forces (e.g. hydrogen and disulphide bonds) that give rise and stabilise higher order (secondary, tertiary and quaternary) structural features. In general, denaturation of LF is not desired as it alters its 3D structure and compromises functional properties (e.g. iron binding and antibacterial activities). Thus, during the extraction and production processes, the extent of denaturation of LF has to be carefully considered and the processing parameters have to be optimized to preserve its biological activity. Thus, the effect of pH, temperature and drying (dehydration) conditions on the denaturation characteristics of LF are discussed in the ensuing sections.

4.1. Effect of temperature and pH on the denaturation of lactoferrin

LF is present in milk and other physiological fluids in dissolved state. In aqueous form, it is stabilized by hydrogen bonding, hydrophobic/hydrophilic interactions, disulphide bonds and ligand binding. These structure stabilising forces could be affected at a certain pH and temperature conditions. The pH affects salt bridges and hydrogen bonding whereas temperature changes the kinetic energy of hydrogen bonds, non-polar hydrophobic and intermolecular thiol/disulphide interactions (Brisson et al., 2007; Hendsch & Tidor, 1994; Privalov & Khechinashvili, 1974).

The isoelectric point (pI) of LF falls within 8.0-9.0 pH range (Hirai et al., 1990; Bokkhim et al., 2013; Wang et al., 2017a). LF is soluble in water at any pH other than the pI range. It is shown that solubility of LF was >92% at pH 7.0 at ambient temperature (25^{o}C) (Wang et al., 2017b). The structural and functional properties of LF in between 2.0 and 8.0 pH values have been documented. Baker & Baker (2004) and Rastogi et al. (2016) reported that LF started losing iron

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at pH 5.0-6.5 and >90% of its iron was released at pH 2.0. The loss of iron was found to depend on the alteration in tertiary structure. As the depletion of iron was complete when the tertiary or higher order structures were altered, there is no need for the secondary structural features to be altered for complete depletion of iron (Bokkhim et al., 2013; Wang et al., 2017b). It was found that the thermal stability of apo-LF was severely affected and it gelled at neutral and alkaline pH when it was heated at 80--100 ^{o}C for 5min (Abe et al., 1991). However, the apo-LF was relatively stable at pH 4.0 under the same heating condition (Abe et al., 1991). Thus, the pH of LF solution has to be maintained > 6.5 in order to retain its iron content even though its thermal stability is highest at pH 4.0.

In addition to the temperature, thermal denaturation of LF depends on the environment factors such as pH, ionic strength, and the presence of other proteins and polysaccharides (Bengoechea et al., 2011; Li & Zhao 2017; Sreedhara et al., 2010). A number of studies have been carried out to determine the denaturation temperature of LF by using differential scanning calorimeter. It was found that the pure apo-LF denatured at ~70^{o}C whereas the pure holo-LF denatured at ~90^{o}C at pH 6.0-7.0 (Bokkhim et al., 2013; Sreedhara et al., 2010; Wang et al., 2017c). The higher denaturation temperature of holo-LF is attributed to its more compact structure formed by the binding of iron (Rastogi et al., 2016; Stănciuc et al., 2013). Therefore, iron saturation increases its resistance to thermally induced denaturation. This finding was verified by a kinetic study (72–85°C, pH 7.4) that apo-LF denatured faster than holo-LF (Sánchez, 1992b). It has also been reported that thermal stability of LF in milk is lower than its stability in buffered aqueous solutions (Sánchez, 1992b). Temperatures higher than ambient are commonly used during extraction and other processes such as pasteurization, sterilization and ultra-heat treatment

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(UHT). Thus, it is essential to understand the nature and degree of denaturation of LF during these thermal processes. A study conducted by Elagamy, (2000) reported that heating bovine, camel and buffalo milk at 65^{o}C for 30 min at their natural pH did not denature LF in significant degree. However, heating of above milks at 85^{o}C for 30 min significantly denatured LF and substantially reduced its biological activity. Abe et al. (1991) preheated bovine LF at 70^{o}C for 3 min and then subjected it to a UHT process at 130^{o}C for 2 s (pH 4.0). These authors reported that the iron binding ability of bovine LF was almost fully retained, indicating the fact the denaturation of LF in commonly applied UHT process is negligible. By contrast, Saito et al. (1991) reported that bovine LF was thermally degraded and its iron biding ability was irreversibly lost when it was heated at 120^{o}C for 15min (pH 2.0). These two observations indicate that exposure time is critical factor causing denaturation of LF. The effect of commonly used temperature-time combination of pasteurization (72^{o}C, 15 s) and UHT treatment (135^{o}C, 4 s) on denaturation of LF has also been investigated. It was found that bacteriostatic activity of bovine and human LF was retained after pasteurization (72^{o}C, 15 s); however, this functional property was lost after the UHT (135^{o}C, 4 s) treatment (Conesa et al., 2009; Paulsson et al., 1993). It is essential to enhance the thermal stability of LF in many food processing operations which can be achieved forming complexes or complex coacervates with other proteins or polysaccharides (Bengoechea et al., 2011; Li & Zhao 2017).

It is also important to understand the denaturation and loss of functional properties of LF at sub zero temperatures. Rollo et al. (2014) reported that human LF was stable at −18 to −20^{o}C for 5 days; however, loss due to denaturation of the order of 37% was observed when stored for 3 months. Wang et al. (2017b) carried out freezing of bovine LF at -30^{o}C for 24 h before freeze

drying and reported that the loss of intact LF due to denaturation was < 2% at the end of combined freezing-freeze drying process. These studies implied that LF adequately resists chilling/freezing induced denaturation at short storage period (< 5 days).

4.2. Effect of powder formation process on the denaturation of lactoferrin

LF in aqueous form is very sensitive to chemical and microbial degradation when it is stored at ambient temperature. Therefore, it is generally converted into powder form to extend its shelf-life and to preserve its functional properties. Freeze drying is overwhelmingly preferred in industry to transfer LF into powder form in order to preserve its functional properties to the best possible degree (Sharbafi et al., 2011; Tomita et al., 2002). However, long drying time, batch mode of production and higher capital and operational costs are major drawbacks of this process (Haque & Adhikari, 2015; Peters et al., 2016). Alternatively, spray drying can be used industrially to produce LF powders (Wang et al., 2017b; Wang et al., 2017c). The challenge of this drying method is that LF encounters thermal, interfacial and rapid evaporation-related stresses. These stresses can lead to conformation changes (denaturation) and also affect the functional attributes of the dried LF powders if the process parameters are not carefully chosen. Therefore, the understanding of the denaturation characteristics of LF in drying processes (especially freeze drying and spray drying) is important. The understanding of extent and nature of denaturation of LF in spray and freeze drying processes helps optimisation of the process parameters and minimise the extent of denaturation of LF powders. This will ultimately benefits the application of LF in many food and pharmaceutical formulations.

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The drying and denaturation characteristics of three forms of bovine LF (apo-, native- and holo-LF) during convective air drying has been determined by Wang et al. (2017c) using a single droplet drying (SDD) apparatus. SDD produces and dries a single LF droplet under a controlled convective drying environment. This method mimics the spray drying process. It allows measurement of drying and denaturation kinetics of bioactive compound contained in the droplet at chosen time interval (Che et al., 2012; Fu et al., 2012; Mezhericher et al., 2007). Wang et al. (2017c) reported the drying and denaturation kinetics of three forms of LF measured using single droplet drying (Fig. 3). Their work has shown that 10 (holo-LF) to 30% (apo-LF) of LF was denatured during convective air drying of 70 and 95^{o}C when dried for 10 min. The alteration of secondary structural features (α-helix, β-sheet, β-turn and random coil) of all the three forms of LF in the above study was of the order of 10%. This study also showed that iron-saturated holo form of LF is more suitable than native or apo forms to produce LF powders through convective air drying (spray drying) system. Wang et al. (2017b) also studied the extent of denaturation of native bovine LF during spray and freeze drying processes. The denaturation profiles of spray and freeze dried LF powders are reported by these authors which are presented in Fig. 4. The spray drying was carried out at 180^{o}C inlet temperature and 70 and 95^{o}C outlet temperatures. The freeze drying process was comprised of pre-frozen (-30^{o}C, 24 h), primary (0^{o}C, 12 h) and secondary (20^{o}C, 6 h) drying stages. Interestingly, the results of this study showed both the spray- and freeze-dried LF powders had negligible denaturation (< 2%) and conformation changes when compared to the fresh membrane-filtered LF solution (Fig. 4). The antioxidant activity of spray-and freeze-dried powders was similar to that of fresh membrane-filtered LF sample. Although the inlet and outlet temperatures of the spray drying process were within the

denaturation temperature of native-LF, the very short drying and exposure time (<2 s) helped to avoid the denaturation during spray drying. The combination of low temperature and reasonably short freezing and drying time of the freeze drying process also avoided the denaturation. Thus, denaturation of LF was avoided in both spray- and freeze-drying conditions. The result of this study implied that industries could produce LF powders without losing its structural integrity and functional properties by selecting suitably optimised spray- and freeze-drying process parameters.

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5. Digestion of Lactoferrin

Dietary proteins including LF are digested into small molecules in human body. It ensures nutrients (e.g. essential amino acids and small molecular weight peptides) are absorbed by the intestine and then transported in the blood. Human digestion system is comprised of three digestion phases: salivary, gastric and intestinal digestion. The salivary phase plays a role in mastication and, to some extent digestion of starch whereas gastric and intestinal phases are the stages where digestion of protein occurs.

It is expected that the functional properties of LF (e.g. iron transporting, antibacterial and antioxidant activity) could be affected at gastric and intestinal stage of digestion. Since the functional domains of LF (e.g. iron binding) are highly dependent on its unique 3D structural conformation, the gastrointestinal breakdown of LF causes undesirable loss of its functional properties. Nevertheless, the degradation of LF during gastrointestinal tract also could be beneficial. As mentioned earlier, it has been reported that strong antibacterial peptides such as lactoferricin and lactoferrampin are produced by pepsin hydrolysis (Bellamy et al., 1992; Lizzi et

al., 2016). Therefore, the understanding of the digestion behaviour of LF is important which provides essential information (e.g. degradation patterns and changes in functional properties) of LF and peptides derived from LF at different digestion stages (gastric and intestinal digestion). This information further benefits the utilization of LF in high value food products such as infant formula, nutritional supplements and other formulations that aims at delivering LF through oral administration.

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5.1. Fate of lactoferrin during digestion

The *in-vivo* digestion of LF in human digestive system is poorly understood and there are many mutually contradictory claims. Troost et al. (2002) investigated the digestion behaviour of recombinant human LF in 43--57 year-old female volunteers. The recombinant human LF was in iron depleted (apo) form which had the identical 3-D structure as that of human LF. It was found that the recombinant human LF was completely degraded at upper gastrointestinal tract. Another *in-vivo* digestion study carried out on 20--22 year-old volunteers used 20% and 100% iron saturated bovine LF as the test specimen (Troost et al., 2001). It reported that >60% of orally administered bovine LF passed through the gastric (stomach) stage in structurally intact form. In the above mentioned *in-vivo* digestion study, the gastric emptying time was ~30 min and the intragastric pH was decreased from 7.0 to 4.0 during the digestion period. The pH range (4.0-7.0) is substantially higher than the optimum pH (1.5-2.0) required for pepsin digestion. In fasting stage, the intragastric pH of adults is in the range of 5.0-6.0 and it takes up to 100 minutes to generate enough hydrochloric acid to reach the optimum pH of pepsin digestion (Kong & Singh, 2010). Hence, the gastric emptying rate and the buffering capacity of food play an important role

in the *in-vivo* digestion of LF. LF in liquid products has faster gastric emptying time than that in solid products. The nature of glycosylation and source of LFs also affect their stability in gastrointestinal environment. For example, glycosylation at Asn281 protects bovine LF against cleavage by trypsin at Lys282 while the glycosylation of human LF did not show any protection (van Veen et al., 2004). The information regarding the digestion of LF in human infant and newborns is less controversial. As the digestive system in infants and newborns is not mature enough (e.g. the intragastric pH and the gastric emptying rate is higher than adults), LF could not be completely digested in this condition. This hypothesis is confirmed by measuring the unhydrolysed LF in faecal extracts of babies (Spik et al., 1982; Gisbert et al., 2009). However, to date, there is no *in-vivo* study which conclusively shows the extent and nature of digestion of LF at different stages of digestion and the chemical structure and functional properties of the resultant digesta.

In-vitro studies of LF simulating the digestion of LF in adult/infant gastrointestinal systems are quite abundant as these studies are faster and are also easier to carry out. The parameters including types of enzymes and their activities, enzyme to substrate ratio, time and pH must confirm to the age, the fasting/ feeding stage and other physicochemical conditions in human digestive system. The most commonly used digestion conditions chosen in *in-vitro* digestion studies of LF and the percentage of structurally intact or undigested LF in different stages of gastrointestinal tract are given in Table 4. These *in-vitro* digestion studies of LF can be classified into two types: a) study on the digestion behaviour of LF to assess the effects of heat treatment and encapsulation and b) generation of new peptides and determine their sequence and functional properties. Currently, most of the *in-vitro* digestion studies indicate that LF gets completely

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degraded during gastric stage of digestion (Table 4) and hence it cannot reach the absorption sites of intestine. Due to the proteolysis and pH induced degradation, the iron binding ability of LF was lost and its antioxidant capacity was also decreased before it reached intestinal stage (Wang et al., 2017a). Moreover, it has been reported that mild thermal treatment (70^{o}C, 10 min) of bovine LF before subjecting it to the *in-vitro* digestion did not have any significant effect on its digestion behaviour compared to the unheated sample as shown by the degradation pattern of LF(SDS-PAGE, Fig. 5). Furlund et al. 2013 showed that small-molecular-weight peptides (<10 kDa) are generated when bovine LF is subjected to *in-vivo* and *in-vitro* digestion for 30 min. The *in-vivo* digestion produced more than 40 peptides with 6--22 amino acid residues with molecular weight of less than 2.5 kDa whereas the *in-vitro* digestion generated 4--33 peptides of similar molecular weight depending on the digestion conditions used. The bioactive peptide-lactoferricin was not produced from bovine LF during *in-vivo* and *in-vitro* digestions by human gastric and duodenal juices (Furlund et al., 2013). In contrast, Kuwata et al. (1998) reported the detection of high concentration ($5.7\pm0.7 \times 10^{-5}$ mol/L) of lactoferricin in the gastric fluids after 10 min of gastric digestion of bovine LF. Therefore, although the bioactive peptides are produced from the digestion of LF, their production depends greatly on the prevailing digestion conditions such as intragastric pH, gastric emptying times and type and activity of enzymes. For example, lactoferricin can be degraded at longer gastric emptying time and its health benefit may not be achieved.

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5.2. Protection of LF from premature digestion

The important receptors of LF are located at the intestinal mucosa and lymphatic tissue cells in the gut (Jiang et al., 2011; Takeuchi et al., 2006; Yamano et al., 2010; Yao et al., 2015). Hence, the delivery of LF through oral administration requires that it is protected so that it passes through stomach and is delivered to the absorption sites in functionally active form. The most commonly used methods to protect LF during its passage through the gastrointestinal tract are: a) iron saturation b) microencapsulation and c) PEGylation. The various methods used to protect the LF during oral and gastric (stomach) stages of gastrointestinal tract and their effectiveness are presented in Table 5.

Crystallographic studies have shown that the binding of iron to LF made the structural conformation of LF more compact (Baker et al., 2000; Baker & Baker, 2012; Moore et al., 1997; Rastogi et al., 2016). This change in LF structure offers higher resistance to pepsin induced proteolysis (Kuwata et al., 2001; Troost et al., 2001; Bokkhim et al., 2016). However, the protection of LF by iron saturation alone is not sufficient. It has been reported that iron saturation is able to protect about 80% LF (increased from 60% to 80%) in the gastric fluid without encapsulation (Troost et al., 2001; Bokkhim et al., 2016). In contrast, other studies have shown that unprotected or unencapsulated LFs, whether they are iron saturated or depleted, cannot pass through stomach (gastric stage) in their structurally intact form (Chan & Li-Chan, 2007; Wang et al., 2017a). Therefore, although iron saturation could be considered as an optional method to slow down the enzymatic hydrolysis of LF, it is not adequately effective in delivering LF to small intestine in its structurally intact form through oral administration.

A more commonly acceptable method to protect LF during digestion is microencapsulation. In this method, a protective (shell) matrix is created around the LF core. Food grade proteins (e.g. bovine serum albumin, β- lactoglobulin) and polysaccharides (e.g. pectin, carrageenan, sodium alginate, gum Arabic) are commonly used as the shell materials. This core-shell structure excellently protects LF from the harsh environment prevailing in human digestive system. Microencapsulation also helps achieve targeted and controlled release of LF by simply using shell materials with suitable properties. For example, sodium alginate is resistant to pepsin attack but breaks down during intestinal digestion and releases the LF which is desirable (Chater et al., 2015). Kilic et al. (2017) prepared a bilayer (bovine serum albumin-tannic acids) capsules loaded with bovine LF. It achieved >76% protection efficiency during gastric digestion. The shell of this capsule was degraded and the LF was released during intestinal digestion. The released LF was further absorbed by intestine. Consequently, the level of LF in the blood stream of subjects who were administered with these capsules was 2--4 times higher than those who were administered with unencapsulated native bovine LF. It was found that the percentage of structurally intact (protected) bovine LF encapsulated in sodium alginate (during gastric digestion) increased from 57% to 79% (Bokkhim et al., 2016). Liu et al. (2013) encapsulated bovine LF in liposomes prepared from milk fat globule membrane-derived phospholipids and reported that liposomes could prevent gastric degradation of bovine LF. The liposomes also reduced the rate of hydrolysis of bovine LF under intestinal conditions. Therefore, encapsulation could be used to modulate the digestion behaviour of LF and hence achieve sufficient bioavailability through oral administration.

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The covalent attachment of polyethylene glycol (PEG) has been reported in protecting LF against harsh gastric environment. This technique, known as PEGylation, is expected to increase the resistance of LF against proteolysis by steric hindrance. It also inhibits the renal clearance due to the increased molecular mass after PEGylation (Yao et al., 2013). Nojima et al. (2008) synthesized a bovine LF-PEG (20 kDa) conjugate and determined its digestion behaviour. It was found that the proteolytic half-life (*in-vitro* gastric stage) of LF was prolonged by ~2-fold and the amount of absorption of LF through the intestinal mucosa increased by ~10-fold compared to the unPEGylated LF. At a further study, Nojima et al. (2009) prepared a covalently linked conjugate of LF with a branched 40 kDa PEG and reported that this conjugate fully retained the iron binding and anti-inflammatory activity of LF during gastric stage of digestion and delivered almost 100% structurally intact LF to the intestine. Furthermore, the 40kDa PEGylated LF had 8-fold longer plasma half-life than that of the unPEGylated bovine LF in rat model. Hence, PEGylation is also efficient in protecting LF in oral delivery route.

The electrostatic complexation (complex coacervation) of LF with acid-stable gums and proteins also increases its stability and hence this system has good potential in delivering LF through oral route. The complex coacervation process involves at least two oppositely charged biopolymers such as proteins and polysaccharides in aqueous medium. Positively charged proteins undergo complex coacervation with negatively charged polysaccharides and form insoluble complex coacervates at some specific pH, ionic strength and protein-to-polysaccharide mixing ratios (De Kruif et al., 2004; Eratte et al., 2014). The complex coacervates are more surface active and are considered as better encapsulants compared to individual protein or polysaccharide gum. In addition, simple operation and easy to scale-up features are some of the advantages of complex

coacervation process. Due to these reasons, complex coacervates are widely used in food and pharmaceutical industries for encapsulation of unstable bioactive ingredients such as vitamins, flavour oils and polyunsaturated fatty acids (Junyaprasert et al., 2001; Timilsena et al., 2017; Yeo et al., 2005). Since LF is positively charged at large pH range (<8.0) it can easily undergo complex coacervation with many polysaccharides (including gums). As sodium alginate is negatively charged over a large pH range (>2.0) it is expected that it can form stable complex coacervates with relative ease. Research is needed to ascertain the efficiency of LF-sodium alginate complex coacervates in avoiding premature digestion of LF in gastric digestion stage and delivering it to the intestinal stage.

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6. Concluding Remarks

LF is a multifunctional protein with well-documented conformational features. The iron transferring/binding and antibacterial properties of LF and peptides obtained from it have been studied to a considerable detail. This has led to a range of nutritional and pharmaceutical applications of LF such as infant formula, functional beverages and toothpaste. Other functional properties of LF (e.g. antiviral and anti-carcinogenic activity) have also raised research attentions, showing potential in developing new drugs and vaccines. The functional properties of LF depend on its higher order (secondary and tertiary) structural conformation. Therefore, denaturation of the native structure must be taken into consideration during its processing. The pH, temperature and drying conditions involved in extraction and production of LF and processing of LF-containing products have to be optimized to avoid or minimise the denaturation of LF. Although oral administration of LF is the most widely adopted method of its delivery into

human body, it still possesses some challenges that must be addressed to get highest benefit from its intake. To date, most of literature indicates that LF cannot pass through the stomach (gastric stage) in its structurally intact form and hence protection is required. Microencapsulation and PEGylation are the most effective methods used to deliver LF to the intestinal absorption sites. Formation of complex coacervates of LF with oppositely charged biopolymers such as polysaccharide gums is technologically convenient and useful method for oral delivery of LF.

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Table 1. Major sources and concentration of lactoferrin (LF).

Biological Fluids	Concentration (mg/mL)	References
Human colostrum	5.80 ± 4.30	Montagne, 2001
Bovine colostrum	0.82 ± 0.54	Kehoe, 2007
Camel colostrum	0.81 ± 0.31	Konuspayeva, 2007
Goat colostrum	0.39 ± 0.07	Hiss, 2008
Human milk	2.00 – 3.30	Montagne, 2001
Bovine milk	0.03 – 0.49	Cheng, 2008
Camel milk	0.06 – 0.89	Konuspayeva, 2007
Goat milk	0.17- 0.59	Chen, 2004
Human tears	1.13 ± 0.29	Balasubramanian, 2012

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Table 2. Amino acids composition and secondary structural features of human and bovine lactoferrin (LF) (Baker et al., 2000, Moore et al., 1991).

Amino acids residues			**Secondary structural features (%)**		
	Bovine LF	Human LF		Bovine LF	Human LF
Ala (A)	67	63	Alpha helix	30.6	29.4
Arg (R)	37	45	3-10 helix	2.6	4.6
Asn (N)	29	33	Strand	17.4	18.1
Asp (D)	36	38	Others	49.3	47.9
Cys (C)	34	32			
Glu (E)	40	41			
Gln (Q)	29	28			
Gly (G)	49	54			
His (H)	10	9			
Ile (I)	16	16			
Leu (L)	66	58			
Lys (K)	54	45			
Met (M)	4	5			
Phe (F)	27	30			
Pro (P)	30	35			

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Ser (S)	45	50			
Thr (T)	36	31			
Trp (W)	13	10			
Tyr (Y)	21	21			
Val (V)	46	48			
Total	689	691			

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Table 3. Amino acids sequence of antibacterial peptides derived from human and bovine lactoferrin (LF). The primary structure of human and bovine LF is determined by Baker et al. (2000) and Moore et al. (1997), respectively. The sequence of amino acid residues is based on the intact form of human or bovine lactoferrin.

Antibacterial peptides	Sequences in lactoferrin	Primary structure
Human lactoferricin	1-47	GRRRRSVQWCAVSNPEATKCFQWQR NMRKVRGPPVSCIKRDSPIQCI
Human lactoferampin	269–285	WKLLSKAQEKFGKNKSR
Bovine lactoferricin	17-41	FKCRRWQWRMKKLGAPSITCVRRAF
Bovine lactoferampin	265-284	DLIWKLLSKAQEKFGKNKSR
Bovine lactoferrin chimera	-	DLIWKLLSKAQEKFGKNKSR | FKCRRWQWRMKKLG − K

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Table 4. Digestion conditions and percentage of structurally intact lactoferrin (LF) during gastrointestinal digestion. Apo-LF = iron depleted LF, holo-LF = iron saturated LF, native-LF = natural form of LF with an iron saturation level of 10--20%. GI = gastrointestinal tract, G = Gastric tract, I = Intestinal tract.

Material	Study Model	Volunteers	pH	Emptying time in GI	Percentage of structurally intact LF in stomach	Percentage of structurally intact LF in intestine	References
Recombinant human apo-LF	*In-vivo, GI*	Adult	-	24 h	-	0	Troost, 2002
Bovine native-LF	*In-vivo, GI*	Rats	-	< 0.4 h in stomach < 1 h in upper small intestine < 12 h in lower small intestine	0	0	Kuwata, 2001
Bovine native-LF	*In-vivo, G*	Adult	7.0→4.0	< 0.5 h	60-80%	-	Troost, 2001
Bovine holo-LF	*In-vivo, G*	Adult	7.0→4.0	< 0.5 h	80-100%	-	Troost, 2001
Bovine LF	*In-vivo, GI*	-	2.3 in stomach 6.5→7.5 in intestine	0.5 h in stomach 0.5 h in intestine	0	0	Furlund, 2013
Bovine LF	*In-vitro, I*	-	7.4	6 h	-	0	Yao, 2014
Bovine apo-, native- and holo-LF	*In-vitro, GI*	-	7.0 in mouth 2.0 in stomach 7.0 in intestine	2 min in oral 2 h in stomach 2 h in intestine	0	0	Wang, 2017a
Bovine apo-, native- and holo-LF	*In-vitro, GI*	-	2.0→3.5 in stomach 7.5 in intestine	2 h in stomach 2 h in intestine	54% (apo), 57%, (native) and 96% (holo)	0	Bokkhim, 2016
Bovine LF	*In-vitro, G*	-	6.8 in mouth 4.5→1.7 in stomach	2 s in oral 2 h in stomach	0	-	Shimoni, 2013

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Bovine LF	*In-vitro, G*	-	3.2	100 min	0	-	Nojima, 2008; Nojima, 2009
Bovine LF	*In-vitro, GI*	-	2.5 in stomach	1 h in stomach	0	0	David-Birman, 2013
			6.5 in intestine	0.5 h in intestine			
Bovine LF	*In-vitro, GI*	-	2.5 in stomach	0.5 h in stomach	0	0	Furlund, 2013
			7.0 in intestine	0.5 h in intestine			
Bovine LF	*In-vitro, GI*	-	2.0 in stomach	0.5 h in stomach	< 6%, porcine enzyme digestion	< 6%	Eriksen, 2010
			8.0 in intestine	0.5 h in intestine	<19%, human juices digestion		
Bovine, caprine, human and equine LF	*In-vitro, GI*	-	2.5 in stomach	0.5 h in stomach	-	~0	Inglingstad, 2010
			8.0 in intestine	0.5 h in intestine			

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Table 5. Methods applied to protect the orally administrated LF during digestion.

Methods	Source of LF	Protecting materials	Protection in stomach	References
Iron saturation	Bovine	-	Increased from 57% to 79%	Bokkhim, 2016
Iron saturation	Bovine	-	Increased from 60% to 80%	Troost, 2001
Encapsulation	Bovine	Bovine serum albumin, tannic acids	≥ 76%	Kilic, 2017
Encapsulation	Bovine	Sodium alginate	Increased from 57% to ≥ 76%	Bokkhim, 2016
Encapsulation	Bovine	Milk fat globule membrane-derived phospholipids	100%	Liu, 2013
Encapsulation	Bovine	High-methoxypectin, Low- methoxy pectin,Sodium alginate, Iota-carrageenan	0 (slower degradation)	David-Birman, 2013
Oil-LF-polysaccharide emulsion	Bovine	I-carrageenan, alginate	~0 (slower degradation)	Shimoni, 2013
Polyethylene glycol (PEG) conjunction	Bovine	PEG molecule (2 × 10 kDa)	>0 (proteolytic half-life prolonged from 17 min to 35 min)	Nojima, 2008
Polyethylene glycol (PEG) conjunction	Bovine	PEG molecule (40 kDa)	100%	Nojima, 2009

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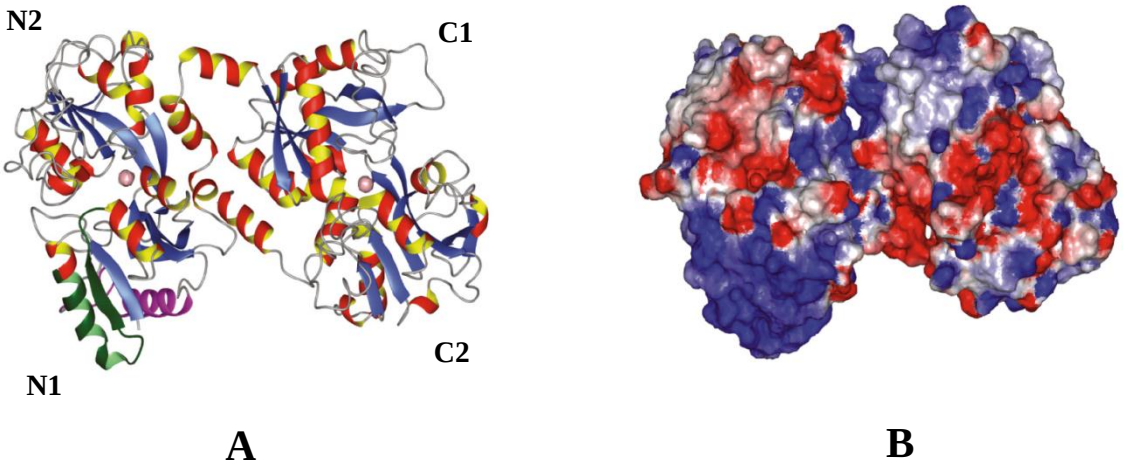

Figure 1. (A) 3D crystal structure and **(B)** surface charge distribution of iron saturated bovine LF (protein databank code: 1BLF) in the same orientation. N1, N2, C1 and C2 represent the four subdomains of LF. The blue, white and red colors correspond to net positive, neutral and negative charges, respectively (Vogel, 2012). This figure is reproduced with permission of the publisher.

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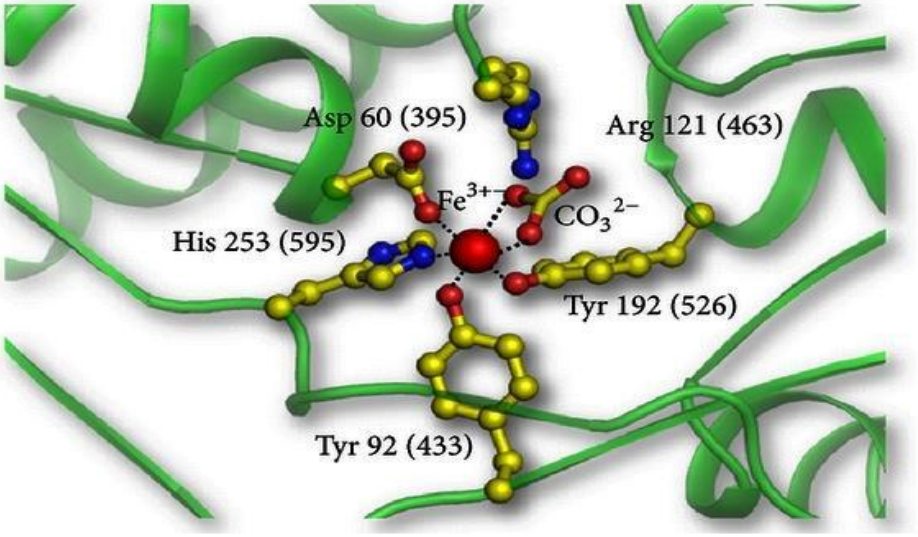

Figure 2. Schematic figure of the iron-binding site of bovine lactoferrin (Sharma et al., 2013). The iron atom is shown as a red sphere, while the interacting amino acid residues of lactoferrin are in yellow. The residue numbers correspond to N-lobe, while the corresponding residues of C-lobe are in brackets (with permission of the publisher).

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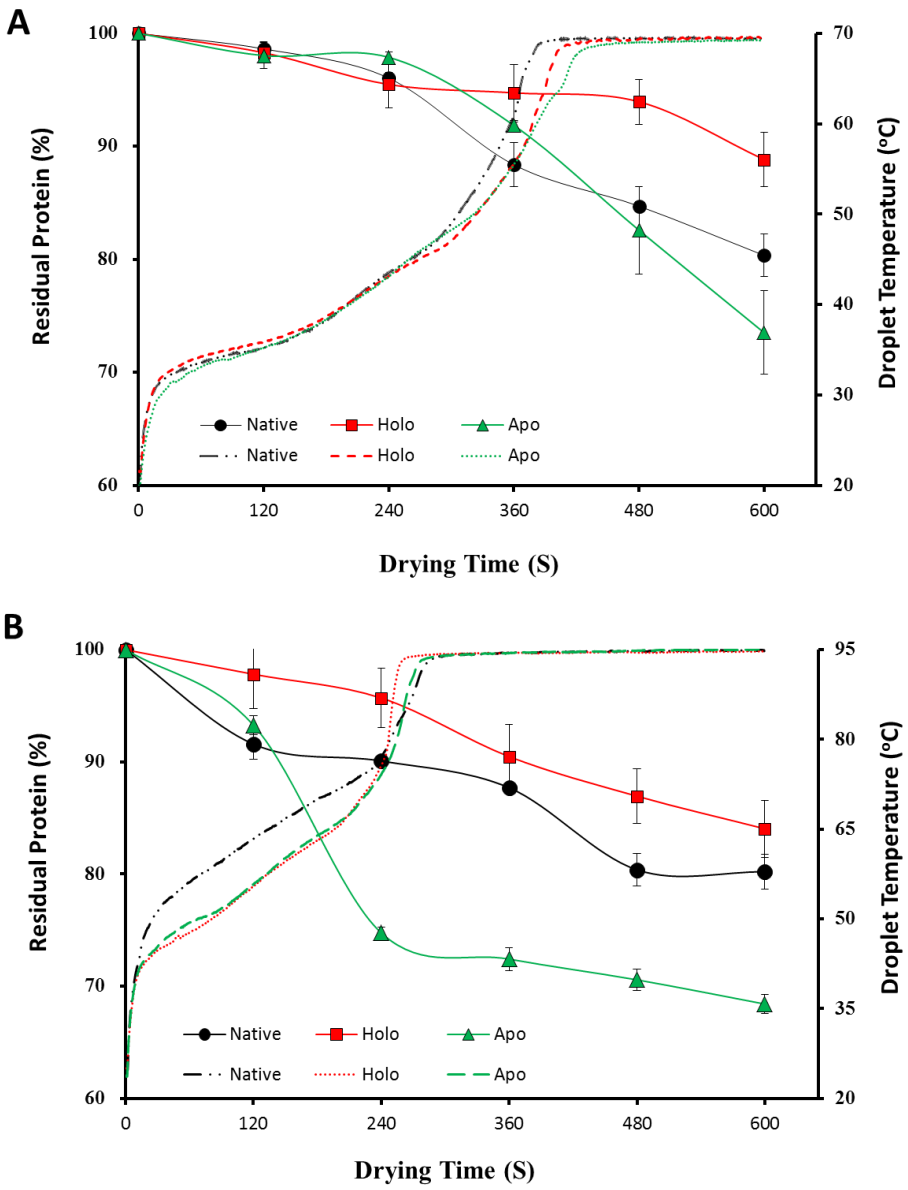

Figure 3. Denaturation profiles of three forms of bovine lactoferrin (LF) at **(A)** 70°C and **(B)** 95°C convective drying temperature. The black, red and green colors represent native (native-), iron saturated (holo-) and iron depleted (apo-) LF forms, respectively (Wang et al., 2017c).

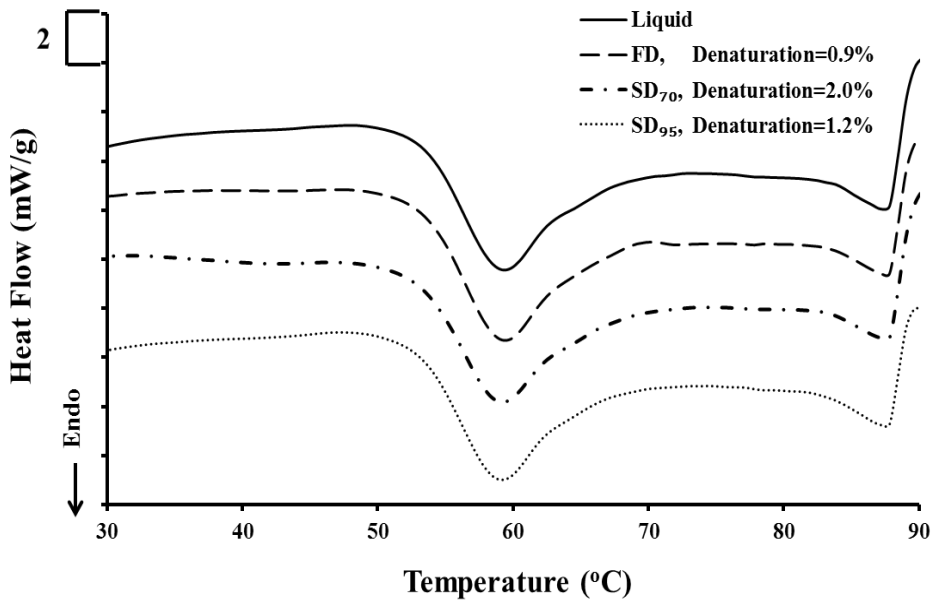

Figure 4. Differential scanning calorimetric thermograms of liquid, spray-dried (SD) and freeze-dried (FD) bovine LF powders. SD_{70} = spray-dried LF powder at outlet drying temperature of 70ºC, SD_{95} = spray-dried LF powder at outlet drying temperature of 95ºC. The inlet drying temperature was 180^{o}C. The extent of denaturation was determined using decrease in the enthalpy (ΔH) due to denaturation (Wang et al., 2017b).

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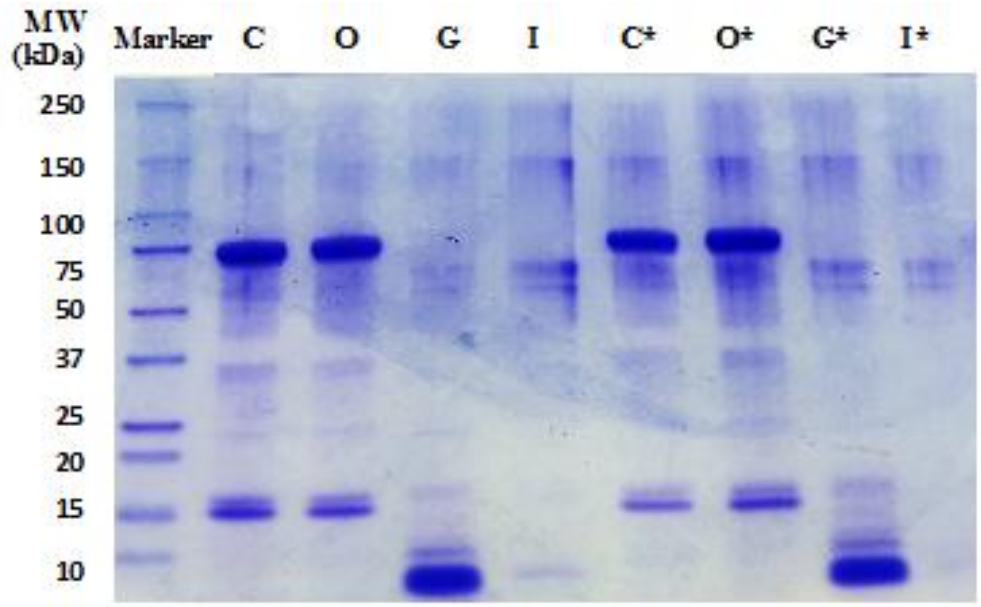

Figure 5. Degradation patterns of native bovine LF at different stages of adult human digestion (*in vitro*) determined using SDS-PAGE. "**C**" indicates the control sample without any treatment; "O" represents the sample after oral digestion. "**G**" and "**I**" are the samples after successive oral+gastric and oral+gastic+intestinal digestion stages, respectively. The samples subjected to isothermal heat treatment (70^{o}C for 10min) prior to digestion are labeled with * (Wang et al., 2017a). The band at ~75 kDa corresponds to the intact form of (non-degraded) LF.

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