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Chapter 8

Bioactive Lactoferrin-Derived Peptides

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Abstract

Lactoferrin (LF) is a member of the transferrin family that is a cationic iron-binding protein. It is an 80-kDa glycoprotein that is found in many secretions in the body and is highly present in milk and colostrums. It exerts antibacterial effects and has a wide range of biological activities. Moreover, it is considered as a precursor of different peptides that have multifunctional bioactivities. During the last decade, several applications of LF and its peptides have been discovered, which has led to its commercial production. Therefore, LF and its peptides can offer a variety of specialized ingredients that can be tailored to meet the needs of natural food preservatives and functional food ingredients.

Keywords: lactoferrin, structure, purification, food preservation, therapeutic activities

1. Introduction

Milk is the first complete functional food devised by nature for the protection and development of newborn mammals. The antimicrobial and bioactive components in milk have received strong attention in recent years. These components have been isolated from a variety of sources including cheese whey and colostrums from hyperimmunized cows [1]. Lactoferrin (LF), lactoperoxidase system, lactoglobulin, and lactolipids are the most common antimicrobial preservatives in dairy industry [2].

The biodefensive properties of mammalian milk proteins have been well described and widely acknowledged for many years. As early as 1930, it was reported that milk possessed active inhibitors [3]. LF was originally discovered in cow's milk in 1939, which was identified as a red iron-binding protein, so it was called LF (lacto=milk; ferrin= iron) [4]. Later, Schade and Caroline [5] isolated an iron-binding protein from human serum "serotransferrin," which was later named transferrin.

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Milk is the major source of LF. It is abundantly excreted in colostrums of human milk in a concentration up to 7 g/L and the LF concentration in mature milk declines approximately 7-fold in time during lactation to 1 g/L [6]. LF concentration in milk varies among different mammals. In bovine milk, LF occurs in relatively low amounts from 0.02 to 0.03 g/L, although, in colostrums, this amount is much higher from 2 to 5 g/L [7], whereas, in rats and dogs, no LF has been detected so far [8].

LF, a member of the transferrin family, is a cationic iron-binding protein that is found in many exocrine secretions, including milk, tears, saliva, and serum [9]. It exerts antibacterial effects by limiting iron availability for microorganisms [10]. In addition, it shows some biological activities as immunomodulatory effect and chemoprevention of carcinogenesis [11].

It has been demonstrated that peptides derived from LF by pepsin digestion showed higher antimicrobial activity against both Gram-positive and Gram-negative bacteria than intact LF. However, other proteases such as trypsin, chymotrypsin, and plant and microbial proteases do not generate significant antimicrobial peptides from LF [12]. Antimicrobial peptides purified by high-performance liquid chromatography (HPLC) have been extensively used for the fractionation and identification of peptides on a laboratory scale [13]. This system allows the high resolution of peptides in a short time. Therefore, these peptides are potential food preservatives that can suppress microbial growth. However, liquid chromatography is a relatively expensive system for the large-scale preparation of peptides; moreover, some solvents used in chromatography are harmful to humans and not suitable for food processing [14]. Therefore, there is a large-scale, low-cost, and biocompatible approach to peptide fractionation based on the amphoteric nature of the sample peptides dissolved in water [14]. This approach is referred to as autofocusing that is considered as a potential industrial peptide fractionation process [15]. In the sight of these facts, the concerns of this chapter were

- Structure of LF;
- Methods of production, isolation and purification of peptides derived from LF;
- Stability of LF and its derived peptides; and
- Bioactive properties and applications of LF and its derived peptides.

2. Structure

LF is an iron-binding monomeric glycoprotein with a molecular weight of approximately 80 kDa [7]. Moore et al. [16] demonstrated some physicochemical characteristics of bovine LF. Its carbohydrate content is 11.2%, maximal iron content is 1.4 mg/g, isoelectric point is 8, and absorbance at 465 nm of 1% solution (iron saturation) is 0.58.

LF consists of 692 amino acids. The protein contains a single polypeptide chain folded into two distinct lobes called N- and C-lobe, referring to the N- and C-terminal parts of the molecule, respectively [17]. Both lobes have the same fold, consistent with their high level of sequence identity; in each case, the lobe is subdivided into two sublobes or domains called N1, N2, C1,

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and C2, respectively. It is separated by a deep interdomain cleft that surfaces an iron-binding site (Fe^{3+}), which is tightly bound in synergistic cooperation with a bicarbonate anion [18]. The use of this anion can affect the metal binding capacity, giving pH control of iron release [19]. Furthermore, LF retains iron to as low as pH 3.5. This gives LF a more potent iron withholding capacity than other transferrins [7]. There is just a single covalent link between the two lobes, which is called a hinge region that forms a short helix conformation with three turns and represent the sequences 334 to 344. In the N-terminal lobe of LF, the sequences start in one domain, move into the second domain, and then back into the first domain before running through a short helix to the C-lobe where the folding pattern is repeated [17].

The two domains fold with a ligand-binding site that is exploited by many binding proteins, including the large family of bacterial periplasmic binding proteins [20]. Moreover, LF is also highly basic due to a unique basic region in the N-terminal of the molecule. One important consequence of this property is that LF can bind with many acid molecules, including heparin and various cell surface molecules [21]. Also, there is a positive charge concentrated in the interlobe region associated with the short helix. This appeals as a likely DNA and many cell types binding region [22].

3. Methods of production, isolation, and purification of peptides derived from LF

3.1. Enzymatic hydrolysis

The antimicrobial sequence of LF was found mainly near its N-terminus, which has an important role in iron chelation. The high proportion of basic residues in the identified domain is allowing its interaction with surface components of microbial cells [23]. Cationic peptide generated upon gastric pepsin cleavage of bovine LF with porcine pepsin, cod pepsin, or acid protease from *Penicillium duporzti* showed strong activity against *Escherichia coli* O111, whereas hydrolysates produced by trypsin, papain, or other neutral proteases were much less active [12]. Bellamy et al. [24] reported that a 25-residue peptide could be released from the N-terminal region of bovine LF with potent bactericidal activity. Interestingly, this reaction is catalyzed at acidic pH by pepsin. After cleavage by this enzyme, the region from amino acids 17 to 41 (FKCRRWQWRMKKLGAPSITCVRRAF) is released as lactoferricin B (Lf-cin B) from bovine LF. Lf-cin B showed broad-spectrum antibacterial activity against both Gram-negative and Gram-positive species, even strains that were resistant to native LF. This indicated that the antibacterial activity of hydrolysate was greater than that of undigested LF with all strains tested approximately 8-fold [24].

3.2. Rennet enzyme and autofocusing technique

LF-cin (RRWQWRMKKLG) as well as other three peptides (KLLSKAQEKFGKNKSRSFQL, APRKNVRWCTISQPEWFKCR, and TRVVWCAVG) was also isolated in a single step from calf rennet hydrolyzed LF by autofocusing technique and were purified by reverse-phase

HPLC. They were characterized by N-terminal Edman sequencing, mass spectrometry, and antibacterial activity determination [25]. The autofocusing is conducted based on the amphoteric nature of the sample using 5 L autofocusing apparatus without using any chemical substances [26]. The basic autofocusing fractions exhibited potent antibacterial activity against *E. coli* and *Bacillus subtilis* in comparison to acidic ones and much higher than the crude LF. In addition, they had greater antibacterial activities than those resulted from autofocusing fractions of LF hydrolyzed by both porcine pepsin and fungal rennet. The most active autofocusing fraction was purified by standard chromatography techniques and assessed for antibacterial activity. The synthetic peptides were found to inhibit the growth of *E. coli* and *B. subtilis* with minimum inhibitory concentration (MIC) ranging from 25 to 100 μg/ml and from 6.25 to 50 μg/ml, respectively. Moreover, the autofocusing can produce stronger antimicrobials than those in crude ones [25].

3.3. Chromatographic techniques

LF has a cationic nature according to its amino acid composition. This gives it suitability to be purified by cation-exchange chromatography such as carboxymethyl-Sephadex [27]. It is considered the most popular procedure for LF peptide purification in most companies. For example, skim milk or cheese-whey is applied to a cation-exchange chromatography column without pH adjustment after its filtration. First, the column is washed with a low concentration (1.6%) NaCl solution in which lactoperoxidase is eluted. Then, LF is eluted with a high concentration (5%) NaCl solution. Ultrafiltration is applied to concentrate LF and is separated from NaCl by dia-filtration. The LF is then freeze- or spray-dried to make a powder [27]. In the same manner, LF can bind transition metals and anionic compounds such as heparin and DNA. These materials have been used to purify LF. Metal (copper)-chelate affinity chromatography followed by gel filtration was reported for the purification of LF from human whey [28]. Heparin-agarose affinity chromatography was used to purify bovine LF and its peptides from the secretions of the involuting mammary gland [29].

4. Stability and solubility of LF and its derived peptides

4.1. Temperature

It is well known that heat treatment of milk and milk protein solutions affect the functional properties of the native proteins. A consideration of heat stability is very important if LF is to be used as a bioactive component of foods [30]. Several studies were recorded and varied about the heat stability of LF. Heating of milk at 65°C for 30 min had no significant effect on LF. However, the entire activity of LF was lost at 85°C/30 min in all kinds of milk. However, camel's milk antimicrobial factors were significantly more heat resistant than cow's and buffalo's milk proteins because it contains a higher concentration of LF [31]. Similarly, Paulsson et al. [32] found that milk LF was unaffected by pasteurization but completely denatured by ultra-high temperature (UHT) treatment and decrease the interaction capacity of LF with bacteria. Luf and Rosner [33] found that heat treatment at 63°C/30 min reduced the native LF content by

40%, whereas high temperature short time treatment of milk had no significant effect on LF denaturation.

LF is denatured more rapidly in its apo-form than in the iron-saturated form. Both apo- and iron-saturated LF are more heat sensitive when treated in milk than in phosphate buffer [34]. Abe et al. [35] reported that LF resisted heating at 80°C/5 min without any significant loss of the iron-binding capability. After heating at 100°C/5 min, LF was still able to bind approximately 85% of the amount of iron bound by the unheated LF. All iron-binding capability was lost when LF was heated to 120°C in phosphate buffer.

The thermostability of LF was dependent on both pH and ionic strength. LF was thermostable at acidic pH than neutral pH [35]. However, under more extreme conditions (120°C, pH 2), LF is acid hydrolyzed; however, it retained antimicrobial activity independent of iron binding [36]. Kawakami et al. [37] reported that, at pH 3.5, LF was resistant to heating at ionic strength 0.37 or below, but turbidity and precipitation occurred at ionic strength above 0.47. LF and its derived peptides' antibacterial activity are varied with the growth temperature. Decreased temperature resulted in a corresponding decrease in MIC and minimum bactericidal concentration (MBC). This was most pronounced for *E. coli*, which increased its sensitivity for LF with decreased growth temperature [38].

4.2. pH

pH is an important factor affecting the denaturation of LF. The rate of LF denaturation was highest at pH above 5.2 in which it mainly affects the rate of the unfolding and aggregation stages of denaturation [39]. In the same aspect, Saito et al. [40] reported that LF and its hydrolysates were denatured to an insoluble state by heat treatment under neutral or alkaline conditions above pH 6. In contrast, they remain soluble after heat treatment under acidic conditions at pH 2 to 5.

LF retained their functional activities at pH ranging from 2 to 7.4 [41]. LF is very stable at pH 4 and high temperature. It resisted heating at 90°C for 5 min at pH 4 without any loss of iron-binding capacity, antigenic activity, or antibacterial activity. LF treated at pH 2 or 3 and 100°C or 120°C for 5 min was apparently degraded, but antibacterial activity was equal to or stronger than that of unheated one [35].

LF was demonstrated to bind to the surface of some pathogenic microorganisms associated with intramammary infections. This binding is optimal at acidic pH with time-dependent binding surface varied according to bacterial species [42]. Griffith and Humphreys [43] concluded that LF was active near neutral pH only in the presence of bicarbonate ions. Because bicarbonate is secreted in the lumen of the intestine, the conditions are favorable for the antimicrobial activity of LF [44].

In the combination between pH and temperature, Murdock and Mathews [45] reported that, under low pH and refrigeration conditions, LF and its pepsin-digested peptides could limit the growth or reduce the population of pathogenic bacteria in dairy products. Moreover, Troost et al. [46] concluded that LF resisted stomach pH and survive gastric transit after oral administration.

4.3. Solubility

The iron-binding capability of bovine LF affects mainly its solubility. Ferrous iron was not stable in solution and was easily changed to insoluble ferric state, but the solubility of the ferrous iron was stabilized in solution in the presence of LF [47].

At acidic pH 2 to 5, LF hydrolysate in the heated samples at 80°C to 120°C remained soluble and the solutions were clear. On the other aspect, at neutral and alkaline pH 6 to 10, turbidity and gel formation occurred, which is markedly increased with temperature rise. At pH 11, the LF hydrolysate in the heated samples remained soluble, but the color of the samples became dark [35].

4.4. Salts

Reiter et al. [48] suggested that citrate reduces the antibacterial activity of LF by competing with the iron-binding proteins. In contrast, bicarbonate enhances the iron-binding properties of LF.

Salanlah and al-Obaidi [49] studied the effect of pH, temperature, magnesium, and calcium on the bactericidal activity of LF against *Yersinia pseudotuberculosis*. The bactericidal activity of LF was higher at acid pH. However, it was not efficient at 4, 15, and 25°C, but it was effective at 37°C. The activity of LF-derived peptides was time and concentration dependent. Calcium did not affect their activity up to 60 mM, whereas magnesium reduced the activity of LF only. Furthermore, Branen and Davidson [50] reported that the addition of EDTA enhanced the activity of hydrolyzed LF in tryptic soy broth against Enterohemorrahgic *E. coli*, *Listeria monocytogenes*, and *Salmonella enteritidis*. The EDTA chelate the excess of cations in broth that reduces the activity of hydrolyzed LF.

4.5. Proteases

Holo-LF is more resistant to proteolysis than apo-LF. The five different fragments with molecular weight that ranged from 25 to 53 kDa were generated from trypsin digest of LF. However, β-lactoglobulin has the ability to reduce the susceptibility of LF to tryptic digestion, suggesting that complex formation is not a mechanism for protecting LF against intestinal degradation [51].

4.6. Interaction with other antimicrobial agents

The anti-*Candida* activity of LF and its peptides or Lf-cin in combination with clotrimazole was shown synergistic by checkerboard analysis. These results indicate that LF-related substances function cooperatively with azole antifungal agents against *Candida albicans*. The concentration of Lf-cin required for inhibiting the growth of *C. albicans* decreased in the presence of relatively low concentrations of clotrimazole [52].

The concomitant use of LF with antibiotic cefodoxime proxetil resulted in a synergistic activity against *Staphylococcus aureus*, *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* and an

additive activity against *E. coli* strain. The antibiotic MIC in the presence of LF was reduced to <1/64 with an efficacy rate of 53/57 (92.9%) in a patient group with infections [53].

Soukka et al. [54] reported that both LF or its peptides and lactoperoxidase system showed bactericidal activity against *Streptococcus mutans* at low pH. LF hydrolysate enhanced lactoperoxidase enzyme activity against *S. mutans* but decreased the yield of antimicrobial components. Denisova et al. [55] reported that the combined administration of LF, lactoperoxidase, and lactoglobulin obtained from cow milk's as well as LF obtained from human milk contributed to the elimination of *Shigella sonnei* from the lungs of infected mice. They prevented the death of the animals. On the contrary, each one has no protective action. López-Expósito and Recio [56] reported that there is a synergistic effect when Lf-cin B was combined with bovine LF against both *E. coli* and *Staphylococcus epidermidis*. Bovine LF increased its antimicrobial activity when it was assayed with bovine αs2-casein f (183-207).

5. Bioactive properties and applications of LF and its derived peptides

5.1. Antimicrobial activity

The role of LF in innate defense enhances the broad spectrum of antimicrobial activity. Accordingly, various modes of antimicrobial effects have been reported for LF [2].

5.1.1. Stasis effect

The bacteriostatic mechanism is related to the high iron-binding affinity of the protein that deprives iron-necessary for microbial growth. Because the bacteriostatic properties of LF are due to its iron-binding ability, the protein is capable of retarding the growth of a broad range of Gram-negative and Gram-positive bacteria (*E. coli*, *Salmonella typhimurium*, *L. monocytogenes*, *B. subtilis*, *Bacillus stearothermophilus*, and *Shigella dysenteriae*) and certain yeasts [57]. However, bacteriostasis is often temporary because some Gram-negative bacteria adapt to iron-restrictive conditions by synthesizing low molecular weight iron chelators (siderophores) that can remove iron from LF [58]. In the same way, Bishop et al. [59] stated that 0.02 mg/ml apo-LF showed marked inhibition of growth of coliforms. In addition, it can inhibit the growth of *Klebsiella* spp. and *Aerobucter aerogenes* at concentrations of 0.2 and 2 mg/ml, respectively.

5.1.2. Cidal effect

The bactericidal effect of Lf-cin against some Gram-negative and Gram-positive bacteria has not any relation simple iron deprivation. It causes a rapid loss of bacterial viability [60]. It was observed that Lf-cin could bind to the outer membrane of Gram-negative bacteria causing alterations in microbial permeation that lead to bacterial death [61]. Moreover, it has the ability to interfere with the carbohydrate metabolism of invading bacteria or LF's ribonuclease activity, which may interfere with microbial protein synthesis [10].

5.1.3. Cationic effect

Current opinion on the mechanism of action of basic antibacterial peptides is focused on their interaction with the negatively charged elements in the membranes of susceptible bacteria causing an increase in cell permeability [62, 63]. These elements are lipopolysaccharide in Gram-negative bacteria and lipotechoic acid in Gram-positive bacteria. This is in agreement with the study that demonstrates that only basic autofocusing fractions have antibacterial activity [25]. Notably, 6 of the 20 amino acid residues in peptide (KLLSKAQEKFGKNKSRSFQL) and 5 of 11 amino acid residues of peptide (RRWQWRMKKLG) (Lf-cin B fragment) revealed from rennet digestion of LF are basic residues and showed antibacterial activity and this feature probably has an important role in determining their potent bactericidal properties [25]. In addition, Hoek et al. [64] reported that the first 10-amino acid residues of Lf-cin B had more potent antibacterial activity than the rest of 25-amino acid residues) FKCRRWQWRMKKLGAPSITCVRRAF)against *E. coli L361*. In the same way, antimicrobial domain was identified in the N1-domain of LF, designated as lactoferrampin (WKLLSKAQEKFGKNKSR), corresponding to the amino acids 268 to 284 of LF. This peptide exhibited candidacidal activity, which was substantially higher than the activity of LF. Furthermore, lactoferrampin was active against *B. subtilis*, *E. coli*, and *P. aeruginosa* [65].

Morten et al. [66] reported that the activity increases with an increased amount of tryptophan (Trp) residues, especially against *B. subtilis*. Trp is thought to function as an anchor in membrane proteins. The aromaticity of Trp has been suggested to interact with both hydrophobic and hydrophilic constituents of the membrane due to its amphipathic nature [67]. Trp is also suggested to act as a needle that pulls α-helices across phospholipid membranes. In cationic peptides, the insertion of Trp residues into bacterial cell membranes disturbs the packing of the phospholipids and causes a weakening of the membrane [68]. The presence of several Trp residues in the 11-residue Lf-cin B peptide (RRWQWRMKKLG) clearly leads to a more effective disruption of the membrane [69]. Moreover, the presence of Trp in Lf-cin B fragment revealed from rennet digest may be responsible for the antibacterial activity by this mechanism [25].

The hydrophobicity, amphipathicity, and net positive charge have been described as important characteristics of antimicrobial peptides [70]. The penetrating ability of antimicrobial peptides to the surface membrane is known as the carpet model [71, 72]. In this model, the peptides first bind to the phospholipid head groups at the surface of the membrane. The hydrophobic core portion of the peptide is then able to interact with the lipid bilayer when hydrophobicity threshold concentration is above 3, causing a disruption of membrane permeability [72]. However, some basic and hydrophobic peptides generated from rennet digested LF have no antibacterial activity [25, 73]. These facts suggest that not only the basic and hydrophobic nature but also other factors may contribute to the antibacterial activity.

5.1.4. Adhesion-blockade effect

Bacterial adherence to target epithelia is an important step in the pathogenesis of the disease. There are many forms of adhesion sites according to the nature of microorganisms.

5.1.4.1. Adhesion-blockade of enteric pathogens

Several carbohydrates, such as 0.1% fructose or 0.5% glucose, strongly inhibit the adherence of shigellae to guinea pig colonic cells. Fructose-containing peptides from LF also inhibit the adhesion of *Shigella flexneri* to colonic epithelial cells [74]. LF-derived peptides have the ability to inhibit the hemagglutination activity of type 1 fimbriated *E. coli* [75]. The agglutination reaction was specifically inhibited by glycopeptides derived from LF or α-methyl-D-mannoside. These observations indicate that the glycans of LF could serve as receptors for type 1 fimbrial lectin of *E. coli*. Furthermore, more than 3 log CFU/g *E. coli* reduction in feces resulted from the oral administration of LF (20 mg/ml in 20% sucrose solution) [75]. Bovine LF inhibited the binding of fibronectin, fibrinogen, collagen type I, collagen type IV, and laminin to bacteria. Electron microscopy revealed the loss of type 1, CFA-I and CFA-I1 fimbria of *E. coli* grown in broth containing 10 μM LF. These data suggest a strong influence of LF on adhesion-colonization properties of *E. coli* [2].

5.1.4.2. Adhesion-blockade of oral pathogens

LF-derived peptides have the ability to adsorb hydroxyapatite, which resulted in interfering with the attachment of *S. mutans* to hydroxyapatite [76]. Also, LF-derived peptides inhibit the growth of *Prevotella intermedia* by interfering with the binding of this bacteria to fibronectin, collagen type I and type IV, and laminin [77]. LF-dependent adhesion-inhibition of *Actinomyces comitans* and *P. intermedia* to fibroblasts and Matrigel could involve the binding of LF to both the bacteria and substrata. The decreased adhesion may be due to the blocking of both specific adhesin-ligand and nonspecific charge-dependent interactions [78].

5.2. Food preservation

LF possesses an intrinsic bactericidal activity that is unrelated to its capacity to bind iron [62]. From this point, there has been an increased interest in the food industry for using LF and its peptides as a preservative in a wide variety of food products. Furthermore, LF has been used as a functional food ingredient in some products such as in infant formula, supplemental tablets, yoghurt, skim milk, drinks, and pet foods. In addition, these products have many therapeutic effects, including anti-infection, improvement of gastrointestinal microflora, immunomodulation, anti-inflammation, and antioxidation [52].

A diverse range of Gram-positive and Gram-negative bacteria was found to be susceptible to the inhibition and inactivation by Lf-cin B. It had the ability to reduce the bacterial population by approximately 3 $\log_{10}$ CFU/ml for both *E. coli* and *B. subtilis* at concentration 31 μg/ml [64]. In a study reported by Shin et al. [79] about the activity of bovine LF and its peptides, they demonstrated that MIC against *E. coli* O157:H7 was 3000 μg/ml for LF, 100 to 200 μg/ml for LF hydrolysate, and 8 to 10 μg/ml for Lf-cin B in 1% bactopeptone broth. In addition, Lf-cin B killed these bacteria at a concentration above 10 μg/ml.

In the meat industry, LF-cin B was primarily tested in ground beef at concentrations of 50 or 100 μg/ml, where it was found to cause a maximum of 2 log CFU/g reductions at 4°C or 10°C [80]. The LF-derived peptides can also be sprayed onto carcasses at a concentration of

3.26 ml spray/kg beef or 65.2 mg/kg beef for preventing bacterial contamination during processing or can be applied to finished beef surface before final packaging to extend the shelf life [81]. In addition, it has been used in the preservation of poultry, pork, fish, and other seafood [82].

The addition of EDTA has shown a positive effect on the antimicrobial activity of Lf-cin. Lf-cin B was unable to inhibit the growth of *E. coli* at 37°C at a concentration of 1600 μg/ml, whereas the addition of 100 or 400 μg/ml EDTA, depending on the strain of *E. coli*, totally prevented the growth [50]. However, Murdock and Mathews [45] reported that there was no effect for EDTA on the antimicrobial action of Lf-cin B in UHT milk even at 4000 μg/ml Lf-cin and 10 mg/ml EDTA.

On the contrary, LF and its peptides have been used in the preservation of raw, pasteurized, and UHT milk, butter, and cheese due to its antimicrobial activity against pathogenic and spoilage bacteria [83]. Moreover, Hugunin [84] reported that LF should be added after pasteurization and fermentation of yoghurt to extend the shelf life of yoghurt and stimulate the growth of its starter cultures. Chen and Allen [85] reported that LF preserves milk with a concentration of 2 mg/ml. Suzuki et al. [41] recorded that LF at a concentration of 1 mg/ml inhibited the formation of hydroperioxides by 48%, whereas the application of 11 mg/ml completely inhibited both the formation of hydroperioxides and several microorganisms. In 2009, Elbarbary reported that the 11-residue Lf-cin B fragment (RRWQWRMKKLG) generated from calf rennet digest of LF has a significant activity in reduction of approximately 3 $\log_{10}$ CFU/ml of *E. coli* and *B. subtilis* populations in milk.

Nowadays, the surface of vegetables, fruit, and carrot juice was coated with Lf-cin B to extend their shelf life. It is more significant when it was added to juice filtrate after its dialysis. As the concentration of cations in filtrate decreases, the antimicrobial efficacy of LF-cin B increases [9].

Moreover, LF can be incorporated into edible films. This is applied for dual purposes that enhance food safety and preservation, as this film acts as a physical barrier and prevents lipid oxidation as well as an antimicrobial substance [86].

Emerging preservation technologies, such as the combination of high hydrostatic pressure with LF, have proven promising in the effort to overcome the limitations in LF and its peptide application [120]. The use of LF and LF-cin B in combination with high pressure (155–400 mPa) enhanced the bactericidal activity against *E. coli*, *S. enteritidis*, *S. typhimurium*, *S. sonnei*, *S. flexneri*, *Pseudomonas fluorescens*, and *S. aureus* in potassium phosphate buffer at 20°C and showed more marked bacterial reduction than the application of LF or Lf-cin B alone [87].

On the contrary, a novel LF derivative [activated LF (ALF)] can be applied as alternative to LF. Naidu [82] reported that ALF has more potent bacteriostatic efficacy against *E. coli* in contaminated beef steak, and the MIC of ALF and LF was 62 and >1000 μg/ml, respectively. In the same manner, Ransom et al. [88] recorded that ALF inhibited the growth of *L. monocytogenes*, *S. typhimurium*, and *E. coli* O157:H7 in beef carcass adipose tissue after its dipping into ALF followed by dipping into 2% lactic acid in comparison to LF, which had no effect on the growth of bacterial population.

5.3. Antimicrobial effect in human therapy

5.3.1. Antibacterial effect

The antibacterial function of LF has been substantiated by both *in vitro* [89] and *in vivo* [90]. It appears that two different mechanisms involving two separate domains of the protein contribute to the antibacterial function of LF, which are its bacteriostatic and bactericidal mechanisms [89].

The ingestion of milk containing LF at 40 mg/ml will result in the formation of LF-cin B at a molar concentration corresponding to 4.5% of ingested LF. The highest concentration of Lf-cin B was found mainly in the stomach then in the upper small intestine then the lower one. Not all ingested LF was digested and some LF were detected in lower gastrointestinal tract [91].

Feeding the infants with LF or its peptides-enriched formula at a concentration of 1 mg/ml for 2 weeks leads to the increase and establishment of bifidobacterium-predominant flora in infant [92], whereas the ratios of Enterobacteriaceae, *Streptococcus*, and *Clostridium* showed a tendency to decrease [93].

The oral administration of LF hydrolysate prepared by porcine pepsin in mice showed an inhibitory effect on the proliferation of *Clostridium ramosum* [94]. Furthermore, supplementation of the milk diet with LF or a pepsin-generated hydrolysate of LF resulted in a significant suppression of bacterial translocation from the intestines to the mesenteric lymph nodes, and the bacteria involved were mainly members of the family Enterobacteriaceae. This ability of LF to inhibit bacterial translocation may be due to its suppression of bacterial overgrowth in the guts of milk-fed mice [95].

Lf-cin B was identified as the most active antimicrobial peptide generated from pepsin digest. It has significant bacteriostatic and bactericidal activities against multiple strains of *Helicobacter pylori* [96], which is mainly associated with chronic gastritis, peptic ulcer, and gastric cancer. There are numerous possible mechanisms by which Lf-cin B alone may suppress *H. pylori* infection. First, it possesses a direct antimicrobial activity against *Helicobacter* species [97]. Second, Lf-cin B may interfere with *H. pylori* adhesion to the gastric surface [98]. Third and last, Lf-cin B may suppress the production of proinflammatory cytokines, tumor necrotic factor, as a part of a regulatory role in the host immune response [99]. There is also evidence that Lf-cin B can exert a direct bactericidal effect on certain *S. mutans* and *Vibrio cholerae* [100].

5.3.2. Antiviral activity

In addition to the antibacterial activity of LF and its peptides, they display antiviral activity against a wide range of human and animal viruses, both DNA and RNA viruses, including rotavirus, respiratory syncytial virus, herpes virus, HIV, hepatitis C virus (HCV), and poliovirus. The antiviral effect of LF lies in the early phase of infection [101]. The antiviral mechanism of LF against viral infection depends on preventing the entry of virus in the host cell either by interfering with adsorption of the virus to the target cell, as LF is capable of binding to viral particles or its envelope protein [102], or by interfering with the viral-

coreceptor interaction [103]. The N-terminal region of LF proved to be essential for its antiviral activity that is responsible for binding to the receptor specific to viral infection, which in turn act as a binding site for the initial interaction of virus with host cells [104] .

Another mechanism for the antiviral effect of LF is the ability to block viral replication in target cells [105]. Further studies indicated that the protective effect of LF was due to the up-regulation of natural killer (NK) cells, monocytes, and granulocytes, which eliminated the infection [104]. Tanaka et al. [106] reported that patients with chronic viral infection, such as HCV, when given LF orally at a dose 1.8 or 3.6 mg/day for 8 weeks showed a decrease in their serum alanine transaminase and viral concentration. In another study, 25 patients with chronic hepatitis C genotype 1b received a 6-month course of LF. The serum level of HCV RNA significantly decreased in patients given LF during the 6 months of treatment [107].

Fujihara and Hayashi [108] reported that LF and its peptides could inhibit the infection in mouse with herpes simplex virus type-1 (HSV-1) by preventing HSV-1 plaque formation after a topical application of 1% LF. However, it did not inhibit the propagation of the virus.

The administration of LF (1 mg/g body weight) protected mice from death due to infection with murine cytomegalovirus (MCMV) through the augmentation of NK cell activity. LF-treated mice showed a significant increase in the NK cell activity but not of the cytolytic T lymphocytes that recognize an MCMV-derived peptide [109].

Bovine LF and its peptides could also inhibit the integrin-mediated internalization of adenovirus into host cells through its binding to viral polypeptides III and IIIa [110].

5.3.3. Antifungal activity

The antifungal activity of LF has been studied. It is mainly through the ability of LF to change the structure of the cell wall of fungi, inactivate the enzyme, and deprive fungi from iron [111].

C. albicans and other *Candida* species are common pathogens that frequently cause oral infections in immunocompromised individuals due to the suppression of local as well as systemic defense mechanisms [112]. LF with concentrations ranging from 0.5 to 100 mg/ml is able to inhibit the growth of several *Candida* species [113]. Moreover, Takakura et al. [114] showed the protective activities of LF administered orally against oral candidiasis. LF at a dose of more than 0.5 g/kg/day exerts therapeutic activity and facilitated the recovery from oral candidiasis within 5 to 7 days. Furthermore, Masci [115] reported that a mouthwash containing LF and lysozyme was effective against oral candidiasis in immunocompromised patients rather than oral administration.

LF has also been shown to improve inflammatory diseases such as dermatophytosis caused by the fungus *Trichophyton mentagrophytes* and intractable stomatitis [116].

5.4. Antiparasitic activity

The role of LF in parasitic diseases is not well defined and may involve multiple mechanisms. Preincubation of *Toxoplasma gondii* and *Eimeria stiedae* sporozoites with LF peptides

reduced their infectivity in animal models [117]. Mice challenged with *T. gondii* survived for 35 days as a result of oral administration of 5.0 mg Lf-cin. Furthermore, mice challenged with *T. gondii* cysts were still alive after an intraperitoneal injection of 0.1 mg Lf-cin. In contrast, 80% of untreated mice died due to acute toxoplasmosis within 14 days [118]. This suggests an effect of basic peptides on parasite membrane integrity and/or interaction with host tissues. Moreover, LF inhibits some parasites via the stimulation of the process of phagocytosis where immune cells engulf and digest foreign organisms [118]. Also, LF has the ability to reduce the binding of *Plasmodium berghei* to the surface of the target cells [119]. Other reported antiparasitic activities appear to involve interference with parasite iron acquisition, such as *Pneumocystis carinii* [120]. In other parasites, such as *Trichomonas foetus*, LF appears to act as a specific iron donor and could thus be expected to enhance infection [121].

5.5. Enhancer of iron absorption

The role of LF as an enhancer of iron absorption was proposed because LF binds a majority of iron in breast milk and therefore could facilitate the uptake of iron in the small intestine of infants [122]. The notion that LF is involved in iron absorption was supported by studies that showed the presence of specific LF receptor on brush membranes from human intestine [123]. Lönnerdal and Bryant [124] showed that iron is equally well absorbed from LF (whether heat-treated or untreated) and ferrous sulfate. Thus, iron provided by dietary LF is likely to be well utilized in human adults. The ability of LF to bind iron naturally support its function in intestinal iron absorption [123].

5.6. Anticancer activity

LF has shown anticancer activity in experimental lung, bladder, tongue, colon, and liver carcinogenesis on rats [125]. Also, in another study, LF suppressed the incidence of oral cancer by 50% [126]. *In vivo* studies have indicated that LF may induce apoptosis in cancer cells; in addition, the immunomodulatory activity of LF is critical in preventing cancer growth by enhancing the activity of NK cells [127]. Therefore, LF offers promise as a potential chemopreventive agent for oral cancer. Currently, LF is used as an ingredient in yogurt, chewing gums, infant formulas, and cosmetics [126].

5.7. Immunomodulatory activity

The immunomodulatory activity of LF is attributed to its ability to up-regulate T-cell proliferation, boost NK cell number, promote lymphocyte maturation, and also play a role in the myelopoietic process [128]. Moreover, LF released from neutrophils forms an integral part of the innate immunity protecting against infection through its antimicrobial activity [129].

6. Conclusion

Several studies proved that LF and its peptides have great potential for use as natural antimicrobials for food preservation and other health benefits in addition to the well-known

iron-binding activity. Because LF is commercially available from bovine milk (~90 MT/year worldwide) and considered as a highly safe food additive, it may be an excellent agent for administration to humans. The enzymatic hydrolysis of LF results in the elaboration of functional peptides that exert, in some cases, greater functionality than the native LF. These peptides could serve as an attractive model for the development of new natural food preservatives, functional food ingredients, food supplements, and drug discovery.

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