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Development of New Strategy for Non-Antibiotic Therapy: Bovine Lactoferrin Has a Potent Antimicrobial and Immunomodulator Effects*

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ABSTRACT

The antibiotic therapy has many problems, such as antibiotics resistance, hypersensitivity, direct toxicity, antibiotic-induced immunosuppresion and super-infections. This is highlighting the need for a new strategy for non-antibiotic therapy through the use of novel immunomodulators as naturally released ones (Lactoferrin). The present study investigates the potential of bovine lactoferrin (bLf), isolated from bovine milk whey, to prevent *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Streptococcus agalactiae* (*S. agalactiae*) and *Pseudomonas aeruginosa* (*P. aerogenosa*) growth and then evaluates its immunomodulator effect. First, bLf isolation was attempted from bovine milk whey using a cation exchange chromatography by SP-Sepharose. Second, the antimicrobial activity assays were trailed to study the antimicrobial activity of bLf. Finally, the immune effect of bLf was studied by lymphocyte transformation test. It was found that bLf was separated around molecular weight of 80 kDa and showed significant inhibitory effect against *E. coli* followed by *P. aeruginosa*, *S. agalactiae* and *S. aureus*. bLf increased lymphocyte transformations mean values in a dose dependant manner. The highest transformations mean value was determined at 50 µg/mL. In conclusion, these results suggest that bLf is a potent natural antimicrobials and immunomodulator agent.

Keywords: Bovine Lactoferrin; Isolation; Antimicrobial and Immunomodulator Effects

1. Introduction

Antibiotics are commonly used for both prophylaxis and treatment of various bacterial infections in human and farm animals. In recent years, antibiotics resistance in bacteria of animal origin and its impact on human health have drawn much attention worldwide [1]. Bovine mastitis is the most common cause for the use of antibiotics agents in lactating dairy cattle [2] and the detection of antibiotics residues in milk poses health hazards to consumers and of high economic importance because such milk unfit for processing and subsequent consumption [3]. Moreover, the antibiotic therapy has many complications, such as hypersensitivity, direct toxicity, antibiotic-induced immunosuppresion and super-infections. This is highlighting the need for a new strategy for non antibiotic therapy through the use of novel immunomodulators as naturally released immunomodulators (Lactoferrin and cathelicidins) or bacterial products (Periplasmic proteins and lipopolysaccharides).

Lactoferrin (Lf), is an iron-binding glycoprotein found in a variety of body secretions including tears, bronchial mucus, and saliva and it is found in high concentrations in the mammary secretions of nonlactating dairy animals and it is important in regulation of iron metabolism [4,5]. Lf has been reported to have important nutraceutical and biological properties such as antimicrobial activities [6] and regulating the immune system [7]. It is known to act as a growth factor by stimulating mucosal growth of the small intestine [8] and to increase hepatic protein synthesis in newborn [9]. It modulates the inflammatory response [10]. Lf has the ability to change in immunity

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level (e.g., blood phagocytic activities, serum IL-18, interferon) and disease resistance (e.g., hepatitis C virus, influenza virus) in fish [11] and humans [12]. Furthermore, Lf has been shown to have inhibitory effects on tumourigenesis [13] and anti-metastatic activity [14]. The large potential applications of Lf have led scientists to develop this nutraceutical protein for use in feed, food and pharmaceutical applications.

More than twenty-five years of research and development were passed on bovine lactoferrin (bLf) and large-scale manufacturing of bLf was established worldwide that was assumed to be over 60 t/year. Using this commercially available material, research for bLf applications has advanced from basic studies to clinical studies, and bLf has been applied to commercial food products [15]. Research demonstrated the beneficial effects of oral bLf in other animal infection models, including herpes virus skin infection, oral candidiasis, and influenza virus pneumonia [16-18]. It was also found that bLf facilitated the cure of dermatophytosis and decreases fungal abundance in the skin [19]. Other research reported the anti-infective effects of oral bLf in animals with *H. pylori* gastric infection, *Staphylococcus aureus* systemic infection, and *Escherichia coli* urinary tract infection [20]. Recently, the beneficial effects of bLf on rotaviral gastroenteritis were shown [21].

Many processing technologies have been developed to isolate the high purity fraction of Lf, and most of them are focused on column-based chromatography [22,23].

The aim of this investigation was, mainly, to isolate bovine lactoferrin from bovine milk whey and evaluate its efficacy including antimicrobial activity and immunomodulator effects.

2. Materials and Methods

2.1. Isolation of Lactoferrin from Bovine Milk Whey

Lactoferrin (Lf) isolation was attempted from bovine milk whey. Lf was purified using a cation exchange chromatography on SP-Sepharose following the procedure of [24]. Milk whey was obtained from bovine milk using ultra speed centrifuge, 15000×g at 4˚C for 30 min. Skim milk was then diluted 1:1 with the dilution buffer (0.04 M NaH_2PO_4, 0.8 M NaCl, 0.04% (v/v) Tween 20, pH 7.4) and it was incubated with SP-Sepharose at 4˚C overnight. Afterwards, the SP-Sepharose was washed with the washing buffer (0.02 M NaH_2PO_4, 0.4 M NaCl, 0.02% (v/v) Tween 20, pH 7.4) to elute the unbound proteins. The gel then packed into a column (5 × 30 cm or 3 × 30 cm, depending on the milk volume) and lactoferrin was eluted with the elution buffer (0.02 M NaH_2PO_4, 1MNaCl, pH 7.4). The column was run at a flow rate of 3 mL/min.

2.2. Electrophoresis of Milk and Fractions Containing Lactoferrin

Purity control and characterization of bovine Lf (bLf) was done using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Collected fractions of bovine milk whey and broad range protein ladder (Fermentra SM1841) were resolved in 12% polyacrylamide minigel-protein II electrophoresis cell (Bio-Rad). Samples were diluted in sample buffer 2-mercaptoethanol (Sigma Chemical Co.) , boiled for 5 minutes before being loaded in the gels and run at 70 volts for 3 hours. Gels were stained with 1% Coomassie blue R-250 (Sigma Chemical Co.), then destained at room temperature in 5% methanol and 7.5% acetic acid with shaking for 30 minutes. The different fractions were quantified using Bio-Rad GS 700 imaging densitometer molecular analysis software [25].

2.3. Antimicrobial Activity Assays

Staphylococcus aureus (*S. aureus*), *Escherichia coli* (*E. coli*), *Streptococcus agalactiae* (*S. agalactiae*) and *Pseudomonas aeruginosa* (*P. aerogenosa*) isolates were used to study the antimicrobial activity of bLf. The tested microorganisms were kept in their specific soft agar. Working cultures were obtained by growing the tested isolates on their specific media. After an overnight incubation, an isolated colony was transferred to 10 mL of Mueller-Hinton broth (MHB, Difco Laboratories, Detroit, MI) and incubated at 37˚C for 16 - 20 h. Final concentration of 1×10^6 CFU /mL was used. A volume of 1mL of bLf solution in different concentrations (1 and 3 mg/mL) was added to 4 wells of tissue culture plates (NUNC, A/S, Roskidle, Denmark) for each of tested microorganisms as previously described [26]. The control consisted of the tested microorganisms in phosphate buffer saline (PBS, 10 mM, pH 7.4). Plates were incubated at 37°C. Aliquots were removed after 1, 3, 6, 12, 24 hours and ten serially diluted, then plated at 37˚C on Mueller Hinton agar (MHA, Difco Laboratories, Detroit, MI) to be counted after 48 h incubation. Total aerobic bacterial count (TBC) of tested microorganisms was done in which viable aerobic mesophlic bacteria was determined as described by [27]. All equipments used were either sterile new glass or plastic to avoid iron contamination. All experiments were repeated at least two times.

2.4. *In Vitro* Lymphocyte Proliferation Studies

Lymphocyte proliferation test using MTT (3-(4, 5-dimethyl thiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) was performed [28] with modification. Briefly, heparinized calf blood samples were aseptically collected in sterile tubes. The separation of lymphocytes was done by

layering of blood in Ficol (2:1) and centrifuged at 400×g at 4˚C for 30 minutes to give packed blood cells with granulocyte, interface layer (which contain lymphocytes) and upper plasma layer. The interface layer was carefully aspirated using sterile glass Pasteur pipette, then placed in sterile tubes containing 2 mL RPMI 1640 medium. Cells were washed 3 times with RPMI 1640 medium by centrifugation at 400×g for 10 min at 4˚C. After the last wash, the sediment lymphocytes were resuspended in 1mL of RPMI 1640 medium containing 10% fetal calf serum (FCS). RBCs contamination, if any, was removed by the distilled water lysis method. Lymphocytes were seeded in triplicate in flat-bottom 96-well micro titer plates (Costar) at 1×10^6 cells per well in 150 µL of culture medium either alone or with various concentrations of bLf (10 µg/mL, 20 µg/mL and 50 µg/mL) or 15 µg of Phytohemagglutinin (PHA) control per mL. Another 100 µL of cell suspension was added to three sets of triplicate wells of a RPMI-1640 containing different concentration of bLf (10 µg/mL, 20 µg/mL and 50 µg/mL) plus 50 µL PHA in conc. of 15 µg/mL. The plates were incubated for 3 days under 5% CO_2 at 37˚C. Then 100 µL of supernatant was removed from the wells and 10 µL of MTT solution was added to all the wells. The plate was incubated further for 4 h at 37˚C. The MTT formazon was extracted from the cells using dimethyl-sulphoxide (100 µL/well). Then the OD was taken using an ELISA reader at a test wave length of 570 nm. All experiments were repeated at least two times.

2.5. Statistical Analysis

The Statistical Products and Service Solutions (SPSS) program was used for all analysis according to [29]. Data were expressed as mean ± standard error (SE). Comparisons were tested using an analysis of variance (ANOVA) test. A difference was considered to be significant at $P < 0.05$.

3. Results

3.1. Isolation and Characterization of Bovine Lactoferrin

Lactoferrin (Lf) was isolated and purified from bovine milk whey using a cation exchange chromatography on SP-Sepharose. Characterization of bovine Lf (bLf) was done using reduced polyacrylamide gel electrophoresis (SDS-PAGE). The results revealed that the bLf was separated around molecular weight of 80 kDa (**Figure 1**).

3.2. Antimicrobial Effect of Bovine Lactoferrin

The antimicrobial activity of the isolated bLf was investigated against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Streptococcus agalactiae* (*S. agalactiae*) and *Pseudomonas aeruginosa* (*P. aerogenosa*) strains. The bLf showed significant inhibitory effect against *E. coli* followed by *P. aeruginosa*, *S. agalactiae* and *S. aureus* (**Table 1**). The inhibition of growth by bLf was concentration-dependent in which a significant inhibitory effect of *E. coli* was observed in a conc. of 1 mg/mL of bLf after 3 h and at conc. of 3 mg/mL after 1 h. Severe inhibition of growth was observed against *P. aerogenosa* and *S. agalactiae* at conc. of 3 mg/mL after 6 h and 12 h respectively. *S. aureus* showed slight inhibition of growth at conc. of 3 mg/mL in compared to control.

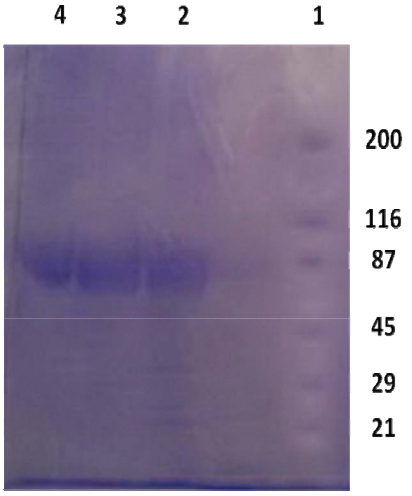

Figure 1. SDS-PAGE of various fractions of Lf purification from bovine milk whey. Lane 1, Molecular weight marker; lane 2, Lf standard; lane 3 and 4, bovine Lf fractions eluted from SP-Sepharose.

3.3. Immunomodulator Effect of Bovine Lactoferrin

The immune effect of bLf was studied by lymphocyte transformation test (LTT). Phytohemagglutinin (PHA) was used as a control. The obtained results showed that the lymphocyte transformation mean value of PHA was 2.36 ± 0.07 (**Table 2**). While the lymphocyte transformation mean values of bLf alone at concentrations of 10 µg/mL, 20 µg/mL and 50 µg/mL were 1.788 ± 0.037, 1.891 ± 0.065 and 2.37 ± 0.057 respectively (**Table 2**). The bLf increased lymphocyte transformations mean values in a dose dependant manner. The highest transformations mean value was at concentration of 50 µg/mL. On the other side, the lymphocyte transformation mean values of bLf with PHA, at concentrations of 10 µg/mL, 20 µg/mL and 50 µg/mL were 2.15 ± 0.041, 1.896 ± 0.033 and 1.798 ± 0.21 respectively (**Table 2**). This means bLf decreased lymphocyte transformations mean

Table 1. Antimicrobial effect of bovine lactoferrin (bLf) on *E. coli, S. aureus, S. agalactiae* and *P. aeruginosa* counts after 1, 3, 6, 12, 24 hours of incubation.

Items		Microbial count (CFU/mL) after				
		1 hour	3 hours	6 hours	12 hours	24 hours
E. coli count	Control	50,000	364,000	2.8×10^6	3.3×10^7	2.8×10^7
	bLf (1 mg/mL)	37,000	18,000	10,000	700	CIG
	bLf (3 mg/mL)	CIG	CIG	CIG	CIG	CIG
P. aeruginosa count	Control	1.76×10^4	2.35×10^4	1.6×10^5	2.6×10^6	2.85×10^7
	bLf (1 mg/mL)	153.000	107.000	56.000	27,000	18,000
	bLf (3 mg/mL)	112.000	89.000	19.000	1100	10.300
S. aureus count	Control	89,000	2.5×10^6	2.8×10^7	2.7×10^8	2.3×10^8
	bLf (1 mg/mL)	76,000	2.4×10^6	2.6×10^7	2.8×10^8	2.3×10^8
	bLf (3 mg/mL)	52,000	1.1×10^6	2.15×10^7	1.94×10^8	1.86×10^8
S. agalactiae count	Control	0.9×10^6	2.8×10^6	3.7×10^7	2.8×10^8	3.5×10^8
	bLf (1 mg/mL)	0.5×10^6	1.73×10^6	2.4×10^5	1.9×10^4	2.2×10^5
	bLf (3 mg/mL)	2.3×10^5	1.89×10^5	1.12×10^4	1400	3400

bLf: Bovine lactoferrin; **CIG:** Complete inhibition of growth; **N.B.:** *S. agalactiae* was more diluted to be easily counted.

Table 2. Immunomodulator effect of bovine lactoferrin (bLf) using lymphocyte transformation test (LTT).

Items	PHA alone	Bovine lactoferrin alone			Bovine lactoferrin with PHA		
		10 µg/mL	20 µg/mL	50 µg/mL	10 µg/mL	20 µg/mL	50 µg/mL
LTT means ± SE	2.36 ± 0.07	$1.788 \pm 0.037^{*}$	$1.891 \pm 0.065^{*}$	$2.37 \pm 0.057^{n.s}$	$2.15 \pm 0.041^{**}$	$1.896 \pm 0.033^{***}$	$1.798 \pm 0.21^{***}$

PHA: Phytohemagglutinin; ***Significant ($P < 0.001$); **Significant ($P < 0.01$); *Significant ($P < 0.05$); n.s = non-significant.

values in a dose dependant manner.

4. Discussion

In the present study, lactoferrin (Lf) was isolated and purified from bovine milk whey using a cation exchange chromatography on SP-Sepharose. Characterization of bovine Lf (bLf) was done using reduced polyacrylamide gel electrophoresis (SDS-PAGE). bLf was separated around molecular weight of 80 kDa. Due to Lf large potential applications, many processing technologies have been developed to isolate high purity fractions [22,23]. Cation-exchange chromatography is already used for the production of Lf at industrial scale [24]. This technology has the advantage of producing Lf with a high degree of purity (>90% dry basis).

The limitation of this technology for large scale applications lies with its high cost and its relatively low yield [30]. However, affinity membranes with immobilized triazinic dyes have not achieved yet good acceptance in the biotechnological industry, mainly because of their low capacity for proteins in comparison with the same ligands immobilized on soft gels [31,32] and the dye leaching in the elution and regeneration steps [33]. Although, under equilibrium conditions, membranes show an acceptable chromatographic performance for Lf purification from bovine colostrum, better than the obtained with d-Sepharose, as a model of soft gels [34], the main problems affecting industrial utilization of adsorptive dye membranes, such as low capacity, dye leaching and pressure drop along the fiber axis need to be overcome. On the other side, the recovery of Lf from whey is a relatively difficult task, not only because the huge volume of whey needs to be dealt with, but also the major proteins complicate the separation process [22,35].

The antimicrobial activity of the isolated bLf was investigated against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Streptococcus agalactiae* (*S. agalactiae*) and *Pseudomonas aeruginosa* (*P. aerogenosa*) strains. The bLf showed significant inhibitory effect against *E. coli* followed by *P. aeruginosa*, *S. agalactiae* and *S. aureus*. One of the first antimicrobial properties discovered for Lf was its role in sequestering iron from bacterial pathogens as in case of *S. aureus* [36] which is known to be resistant to antimicrobials. This was believed to be the sole antimicrobial action of lactoferrin because apo-lactoferrin possessed antibacterial activity [37]. It was later demonstrated that Lf's bactericidal function has been attributed to its direct interaction with bacterial surfaces [38] and through an iron-independent mechanism [39] as in case of *E. coli* [40]. Biofilm formation, which was proposed as a colonial organization adhesion method for *P. aeruginosa*, is a

well-studied phenomenon. Through biofilm formation, bacteria become highly resistant to host cell defense mechanisms and antibiotic treatment [41]. It is well known that some bacterial strains require high levels of iron to form biofilms. Thus, Lf's function as an iron chelator has been hypothesized to effectively inhibit biofilm formation through iron sequestration [42]. Structural characteristics and spatial orientation of the molecule are critical factors in the functionality of an antimicrobial compound. Occurrence in various milieus strongly emphasizes the significance of the structure-function relationship in the multifunctionality of the Lf [43].

Studying the immune effect of bLf through lymphocyte transformation test (LTT) was done. Phytohemagglutinin (PHA) was used as a control. The obtained results showed that the lymphocyte transformation mean value of PHA was 2.36 ± 0.07 while the lymphocyte transformation mean values of bLf alone at concentrations of 10 µg/mL, 20 µg/mL and 50 µg/mL were 1.788 ± 0.037, 1.891 ± 0.065 and 2.37 ± 0.057 respectively. The bLf increased lymphocyte transformations mean values in a dose dependant manner. The highest transformations mean value was of lactoferrin in conc. of 50 µg/mL. This finding was agreed with [44] who reported that the addition of recombinant human lactoferrin (Talactoferrin Alfa (TLf)) to human peripheral blood or monocyte-derived dendritic cell cultures resulted in cell maturation, as evidenced by up-regulated expression of CD80, CD83, and CD86, production of proinflammatory cytokines, and increased capacity to stimulate the proliferation of allogeneic lymphocytes. Also, this finding was agreed to some extend with [45] who found that the effects of Lf in experimental models were differential and dependent on an individual PBMC reactivity, mitogen or alloantigen and Lf concentration. Generally, lymphocytes from donors responsive to Lf exhibited higher proliferation indices to PHA when compared with non-responsive individuals, suggest that the differential action of Lf might be due to its ability to sense the activation status of lymphocytes, although he mentioned that data on Lf effects on mitogen-induced proliferation are scarce, through fairly consistent both in the mouse and human systems. It has been demonstrated that human and bovine lactoferrin inhibit proliferative responses *in vitro*.

On the other side, the lymphocyte transformation mean values of bLf with PHA, at concentrations of 10 µg/mL, 20 µg/mL and 50 µg/mL were 2.15 ± 0.041, 1.896 ± 0.033 and 1.798 ± 0.21 respectively. This means bLf decreased lymphocyte transformations mean values in a dose dependant manner. This opinion goes hand in hand with [46] who reported that purified lactoferrin, isolated from human milk, was tested for its effect on human T-lymphocyte proliferative responses to Phytohaemaglutinin (PHA) and to alloantigen in mixed lymphocyte culture. Lf inhibited proliferation in both assays in a dose-dependent manner. The suppressive effect was not due to Lf mediated cytotoxicity since washing cells that had been pre-incubated with Lf restored their proliferative activity. Lf was most effective in suppressing the PHA response when added within 24 h of culture initiation. Iron saturated Lf failed to inhibit PHA-induced proliferation, suggesting that the mechanisms of suppression involve the chelating property of Lf. The suppressive effect of Lf on T-lymphocyte proliferative response *in vitro* supports the notion that Lf has significant immunoregulatory potential *in vivo*. The same agreement was concluded by [45] who reported that the effects of Lf on the proliferative response of lymphocytes to PHA were generally stimulatory at lower and inhibitory at higher concentrations. This might be through the increased production of cytokines that play a significant role in the down-regulation of mitogen-induced lymphocyte proliferation in the presence of Lf. Other research data suggest that ingested bLf is generally not absorbed in the blood [47], but acts on the intestinal immune system and influences the systemic host protective system [48]. Orally administered bLf increased the numbers of $CD4^+$ cells, $CD8^+$ cells, and natural killer cells in the intestinal mucosa of mice [49], and enhanced production of interleukin (IL)-18 in intestinal epithelial cells and IL-10 and interferon (IFN)-γ in intestinal intraepithelial lymphocytes and mesenteric lymph node cells [50].

5. Conclusion and Recommendation

In conclusion, these results suggest that bLf is a potent natural antimicrobials and immunomodulator agent. These results are approximately similar to that of commercially produced bLf, in addition to new immunoantimicrobial effects. The extensive use of Lf in the treatment of various infectious diseases in animals and humans has been the driving force in Lf research however, a lot of work is still required to obtain a better understanding of its activity. Moreover, lactoferrin administration methods need to be evaluated.

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