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Prospects of lactoferrin as potential natural antibiotic

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Abstract

Lactoferrin's iron-binding capacity makes it undoubtedly advantageous to immune system modulation and different bacterial strains. The aim of this study was to explore the possibilities of using the milk protein as an antimicrobial agent. For the current study, four raw and seventeen commercially available milk samples were collected from different farms and supermarkets in Bangladesh. The presence of lactoferrin was confirmed using SDS-PAGE gel electrophoresis. Lactoferrin from Goat milk and Milk Vita commercial milk was recovered from SDS-PAGE gel and purified using the dialysis method. The concentration of purified protein was determined by NanoDrop technology. Then, the purified lactoferrin was tested for its antimicrobial activity against 18 bacterial strains. Interestingly, *Vibrio cholerae, Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Enterococcus faecalis* and *Streptococcus pneumoniae* significantly displayed sensitivity against lactoferrin extracted from the aforementioned milk samples. This study suggested that lactoferrin has the potential to be used as an alternative to antibiotics for many diseases and also can be used to reduce microbial deterioration in the food industry.

1. Introduction

Many new and promising treatments for reducing or diminishing the adverse effects of microorganisms are being discovered day by day. On the other hand, the dairy industry is accelerating the economic wheel of Bangladesh. Considering all these facts, new thoughts were developed to isolate milk proteins by the present experiment for opening a new era of developing natural antibiotics from milk. The main sources of lactoferrin are mammalian secretions such as milk, tears, saliva, seminal fluids, vaginal fluids, nasal mucosa, bronchial mucosa as well as some white blood cells and secondary granules of neutrophils (Rodrigues *et al.*, 2009; Iigo *et al.*, 2009). However, milk is by far the most abundant source of lactoferrin, for instance, early milk contains up to 7 g/L while tears and blood have lactoferrin concentrations as high as 2 g/L, and 1 g/L respectively (Farnaud and Evans, 2003).

Having a molecular weight of 77-80 kDa, it contains around 690-702 amino acid residues and belongs to the transferrin family (Legrand *et al.*, 2008; Berlutti *et al.*, 2011). Moreover, at least 60 gene sequences of lactoferrin have been characterized in 11 mammal species including humans where the stop codon is TAA and TGA in most of the species (Baker *et al.*, 2002). Interestingly, lactoferrin is found to have an affinity not only for iron but also for other metals such as Cu^{2+}, Mn^{2+}, and Zn^{2+}; regardless of the possibility that with a lower affinity (Farnaud and Evans, 2003). Such property of lactoferrin plays a significant role in retiring microorganisms as the iron is removed by the proteins. It even inhibits the microorganism's infectivity and is also used as an antioxidant.

Scientists do not have an explicit idea of the biological functions of this protein yet. However, numerous potential properties have been discovered over the past years. For example, it has been proposed that lactoferrin may have antimicrobial, antitumor, anti-inflammatory, antiparasitic, protease inhibitor and many more activities (Brock, 2002; Jahani *et al.*, 2015; Fernandes and Carter, 2017; Superti, 2020). Among all the proposed significances, the antimicrobial property

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seems to show higher acceptance from the scientific community because of its inherent iron-restricting ability (Orsi, 2004). Besides, various prospective applications have also been identified. Some of these are, for instance, rice expressing Lactoferrin + Lysozyme may prevent acute diarrhoea, Oral Recombinant Human Lactoferrin (rhLf) may facilitate the enhancement of Interleukine-18 (IL-18) in gut cells; Suppression of tumour cell growth, Topical Bovine Lactoferrin (bLf) may promote bone repair, Oral apo-Lactoferrin + Probiotic may suppress the overgrowth of enteric pathogens, Bovine Lactoferrin (bLf) sprayed on meat products may prevent the bacterial growth during storage (Wakabayashi *et al.*, 2006; Tomita *et al.*, 2009). Apart from these uses, lactoferrin was observed to exhibit a high success rate in treating Hepatitis C patients (Tanaka *et al.*, 1999; Okada *et al.*, 2002).

Considering all these aspects of Lactoferrin, the present study was designed to develop an operative isolation protocol for the milk protein as well as to evaluate the antimicrobial prospective of it. Here, only Gram-positive and Gram-negative bacteria were considered to evaluate the antimicrobial property of lactoferrin. Briefly, in terms of Gram-positive bacteria, lactoferrin binds to the negatively charged molecules of the cell membrane such as lipoteichoic acid, neutralizing wall charge and thus allows the action of other antibacterial compounds (e.g., Lysozyme) (Jahani *et al.*, 2015). On the other hand, in Gram-negative bacteria, it binds to lipid A of lipopolysaccharide, causing consequent damage to the cell membrane due to the liberation of this lipid (Jahani *et al.*, 2015).

This study was also conducted to establish an effective extraction method for lactoferrin from several milk sources. Thus, not only the raw milk samples but also the commercial ones were taken into account. In previous years, researchers explored a few different experimental conditions to isolate lactoferrin. All these conditions were set on the basis of pH adjustments and the extraction method consisted of two phases (Masson and Heremans, 1971; Moradian *et al.*, 2014; Rachman *et al.*, 2015; Parkar *et al.*, 2016). After analysing the isolation methods and experimental conditions used by Masson *et al.* (1971), Moradian *et al.* (2014), Rachman *et al.* (2015), and Parkar *et al.* (2016) in their respective researches, a potential method was developed and evaluated in the present study.

2. Materials and methods

2.1 Materials

2.1.1 Utilized milk samples

A total of twenty-one milk samples were collected from local markets in Bangladesh where seventeen of those were commercial milk and the other four were raw milk. The collected raw milk samples were preserved at 0°C to prevent deterioration. On the contrary, commercial milk samples were preserved at 4°C.

2.1.2 Bacterial pathogens used

A total of eighteen bacterial strains (*Vibrio cholerae, Shigella flexneri, Shigella dysenteriae, Salmonella enterica* serovar Typhi*, Bacillus subtilis, Bacillus cereus, Enterococcus faecalis,* STEC-Shiga toxin-producing *Escherichia coli,* EAEC-Enteroaggregative *Escherichia coli,* ETEC-Enterotoxigenic *Escherichia coli,* EPEC [typical and atypical]-Enteropathogenic *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Proteus vulgaris, Streptococcus pyogenes, Streptococcus pneumoniae*) were used in this experiment to identify the antibacterial activity of the protein. Twelve of these strains were Gram-negative bacteria whereas the other 6 were Gram-positive. These strains were collected from the stock of Biotechnology and Microbiology Laboratory, Brac University, Bangladesh. The main source of these pathogenic strains is from International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B).

2.1.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE, Biometra Eco-Maxi were used in order to identify lactoferrin. All the reagents utilized in the experiment were collected from the Biotechnology and Microbiology Laboratory, Brac University, Bangladesh. 12.5% resolving gel and 5% stacking gel, 1x TGS running buffer (pH 8.3), 0.25% Coomassie Brilliant Blue -R250-staining solution and a de-staining solution containing de-ionized water, Methanol, Acetic acid, were prepared accordingly and used to run the SDS-PAGE (Roy and Kumar, 2014; Parkar *et al.*, 2016).

2.1.4 Dialysis bag

The dialysis bag used in this experiment was a kind gift from Protein Biochemistry Laboratory, Chosun University, Gwangju, South Korea.

2.2 Method

The main two parts of this study were to extract lactoferrin from milk samples and then investigate its antibacterial property. The study design followed the pathway as-sample collection, fat separation, casein separation, lactoferrin extraction, protein concentration determination, protein separation, recovery and purification of lactoferrin and lastly antibacterial assessment.

2.2.1 Casein separation

Both the raw and commercial milk samples were centrifuged at 4000 rpm for 10 mins, followed by the removal of the fat layer (topmost). Then, an equal volume of distilled water was added to the defatted milk and the initial pH was measured and noted down. As all the samples were added with distilled water, so the initial pH was lower than 7.0. Following this, the pH was adjusted to 4.6 which is the optimal pH for casein precipitation. The process involved the addition of 1N HCl and 1N NaOH when needed. Once the pH reached 4.6, the samples were again centrifuged at 2000 rpm for 10 mins at 4°C. Finally, supernatants were collected from each sample and stored in McCartney bottles at 4° C.

2.2.2 Lactoferrin extraction

Firstly, the pH of the supernatants was adjusted to 6.0 by adding 1N NaOH. After that, an equal volume of 45% Ammonium sulphate was added and the stirring was fixed gradually from 100 rpm to 420 rpm for 1 hour at room temperature. The samples were then subjected to the addition of 1N HCl slowly until the pH reaches 4.0 and then again 1N NaOH was added until the pH reached 8.0. At pH 8.0, an equal volume of 80% Ammonium Sulphate was added, followed by constant magnetic stirring at 420 rpm (increased gradually) for 1 hr. Further, the treated samples were incubated at 4°C overnight for precipitation. Lastly, 500 µL 1X PBS (Phosphate-Buffered Saline) buffer (pH 7.4) was incorporated into the obtained precipitates (presumed to contain lactoferrin) to dissolve and re-suspend after completing centrifugation at 4000 rpm for 10 mins at 4° C.

2.2.3 Protein concentration determination

Nanodrop assay at 260/280 nm wavelength was used to quantify the protein concentration. TE buffer was mixed with the samples before measuring the concentrations while Bovine Serum Albumin (BSA) was considered as standard protein.

2.2.4 Protein separation

SDS-PAGE method was used in this perspective. Fifteen µL of the sample added with 15 µL of 4× loading dye was kept in the hot water bath at 95°C for 2 mins prior to conducting the gel run. Here, 1× TGS running buffer was used along with an electricity supply of 100 V for 2 hrs. After electrophoresis, the gel is subjected to the staining solution and kept in the hot water bath for 20 mins at 55°C. After that, the stained gel was kept overnight in the de-staining solution once finishing the hot water-bath treatment for 20 mins at 55°C again.

2.2.5 Recovery of lactoferrin from SDS-PAGE

To elute desired protein lactoferrin, the SDS-PAGE gel containing protein bands was washed extensively with distilled water and then the optimum band of lactoferrin was sliced into 0.5 cm section. Furthermore, a 3 mL syringe was filled with the gel slice, and it was again transferred into another one by forcing through the opening (without the use of a needle). Following the addition of 1 mL distilled water, the mixture was vortexed for 30 secs and centrifuged at 12000 *g* for 1 min. Additionally, the mixture was incubated at room temperature for 5 mins in between the vortex and centrifugation. Lastly, the supernatant was collected and then analyzed again in SDS-PAGE to reconfirm the protein's presence.

2.2.6 Protein purification using dialysis

A 9 kDa semi-permeable membrane was used to remove different salts and dye pigments which may interfere with the antibacterial assay from the proteins of the stored supernatants. Firstly, 15 mL of protein extract was poured inside that dialysis membrane having sealed both sides by the use of cotton thread. Then, the bag was stirred with a magnetic stirrer for 2 hrs and 4°C was maintained throughout the process. After completing the dialysis, the solution from the dialysis bag was again syringe micro-filtered (Pore size, 22µm).

2.2.7 Antibacterial assessment

Two types of media, Nutrient Agar (NA) and Mueller Hinton Agar (MHA) were used in this part of the experiment. NA media was prepared for bacterial sub -culture and MHA media was prepared to test bacterial susceptibility. Firstly, bacterial strains were transferred into 0.9% saline solution and vortexed until it becomes turbid. Then, optical density (OD) was adjusted by McFarland standard method as 0.1. The culture suspension of selected bacterial strains was lawn cultured with a sterile cotton swab on MHA media and then wells were made using a cork borer. After that, the milk protein, lactoferrin was transferred into the wells. Lastly, the cultured MHA plates were kept in the incubator overnight at 37°C. All the steps were done inside the laminar hood and aseptic conditions were maintained.

3. Results

3.1 Protein concentration of different milk samples

Commercially packed milk samples are believed to have lower protein concentrations than raw milk. Among all the four raw milk samples analyzed, Goat milk showed the highest amount of protein present is 28.06

mg/mL and the Buffalo milk showed the lowest amount of all is 22.72 mg/mL. On the other hand, higher concentrations of protein have been observed in terms of commercially packed milk- Aarong, Milk Vita, Olympic and Ama which were 26.33 mg/mL, 22.06 mg/mL, 21.29 mg/mL and 19.72 mg/mL, respectively. Overall protein concentration ranges from 7.18 mg/mL to 28.06 mg/mL and the protein concentration of standard sample used was 0.43 mg/mL. All the above information is summarized in Table 1.

Table 1. Protein concentration of the raw and commercial milk samples

	Sample's Name	Protein Concentration
Raw Milk	Goat Milk	28.06
	Cow milk	25.43
	Human Breast Milk	23.22
	Buffalo Milk	22.72
Commercial Milk	Fresh	13.37
	Red Cow	11.42
	King	13.97
	Dutch Lady	18.57
	Nido	12.79
	Diploma	11.82
	Olympic	21.29
	Aarong	26.33
	PRAN	10.12
	Milk Vita	22.06
	DANO	13.35
	Farmland Gold	7.18
	AMA	19.72
	Farm Fresh	15.29
	Super Pure	17.57
	Marks	13.13
	No. 1	9.64

3.2 Lactoferrin identification using SDS-PAGE

The ladder (10-120 kDa) was used on the right well of the gel to compare the bands. However, only 5 samples showed bands in the range of 77-80 kDa which is the molecular weight of lactoferrin (Legrand *et al.*, 2008). The milk samples that were concluded to contain the protein are Goat milk, Cow milk, Human Breast milk, Buffalo milk and Milk vita commercial milk (Figure 1).

3.3 Lactoferrin recovery from SDS-PAGE

Goat milk and Milk Vita commercial milk were observed to have higher protein concentration as well as showed bands for lactoferrin in the SDS-PAGE. Thus, the protein was recovered and purified from both these samples for antibacterial analysis. Then, the purified lactoferrin showed a single band at 77-80 kDa in SDS-PAGE which reconfirmed its presence (Figure 2). Furthermore, the concentrations of eluted lactoferrin were 3.73 mg/mL (Goat milk) and 1.91 mg/mL (Milk Vita), quantified using NanoDrop technology. The protein concentration of BSA, 0.43 mg/mL was again considered standard in this experiment.

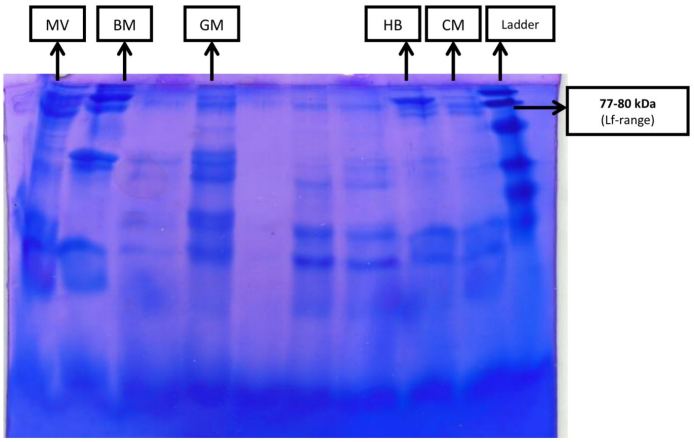

Figure 1. Milk samples show bands in the ladder's molecular weight range of 77-80 kDa on the SDS-PAGE. MV: Milk Vita, BM: Buffalo Milk, GM: Goat Milk, HBM: Human Breast Milk, CM: Cow Milk, Lf: Lactoferrin

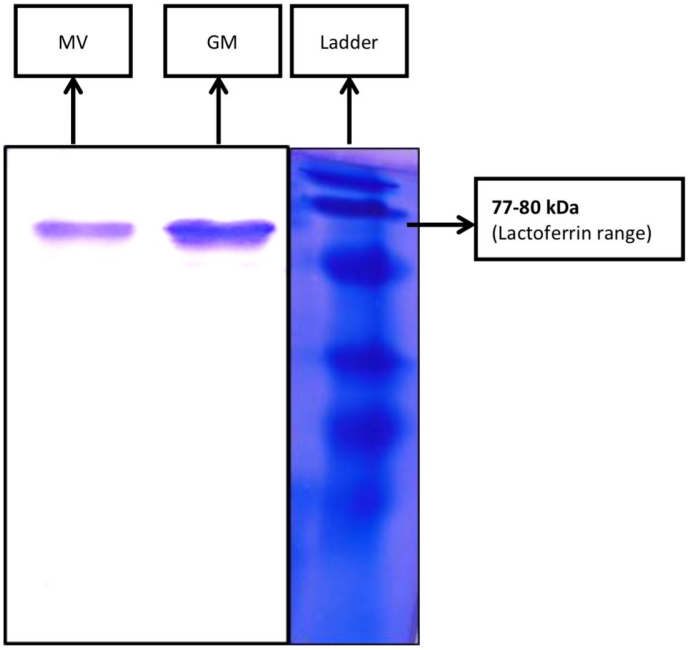

Figure 2. Recovered lactoferrin from Goat milk (GM) and Milk Vita (MV). This SDS-PAGE gel electrophoresis was done in the Protein Biochemistry Lab of Chosun University.

3.4 Antibacterial activity

The zone of inhibition (ZOI) was the main priority in this experiment. The protein samples were considered susceptible to the bacterial strains that showed ZOI greater than 1 mm and denoted as Y (Kazemipoor *et al.*, 2012). The 20 µL (concentration 0.5 mg/mL, 0.6 mg/mL, and 0.7 mg/mL) of lactoferrin extract from both the Goat milk and Milk Vita was used to evaluate the antibacterial activity. Lactoferrin from both the milk samples showed

Table 2. Antibacterial inhibitions of extracted lactoferrin from milk samples

Samples \ Bacteria strains	*Vibrio cholerae*	*Shigella flexneri*	*Shigella dysenteriae*	*Salmonella enterica serovar Typhi*	*Bacillus subtilis*	*Bacillus cereus*	*Enterococcus faecalis*	STEC	EAEC	ETEC	EPEC (typical)	EPEC (atypical)	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Proteus vulgaris*	*Streptococcus pyogenes*	*Streptococcus pneumoniae*
Goat Milk	Y	N	N	N	N	Y	N	N	N	N	N	N	Y	Y	N	N	N	N
Milk Vita	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	Y

STEC: Shiga toxin-producing *Escherichia coli,* EAEC: Enteroaggregative *Escherichia coli,* ETEC: Enterotoxigenic *Escherichia coli,* EPEC: Enteropathogenic *Escherichia coli,* Y: ZOI observed, N: no ZOI observed.

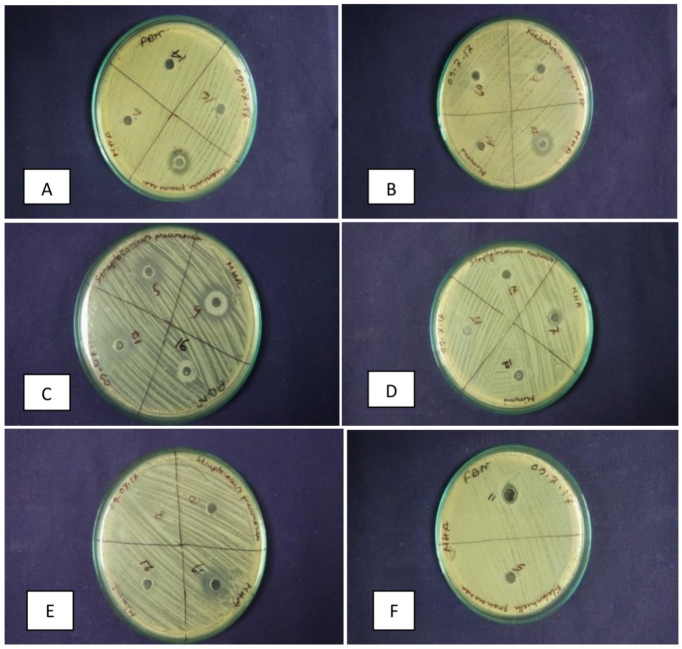

Figure 3. Antibacterial activity (ZOI observed) against different bacterial strains. A: *Klebsiella pneumoniae,* B: *Klebsiella pneumoniae*, C: *Streptococcus pneumoniae*, D: *Staphylococcus aureus*, E: *Streptococcus pneumoniae*, and F: *Klebsiella pneumoniae*

better susceptibly at a concentration of 0.7 mg/mL (data was shown only for this concentration). Interestingly, lactoferrin from the Goat milk was susceptible to *Vibrio cholerae, Bacillus cereus, Pseudomonas aeruginosa, and Staphylococcus aureus* (Table 2, Figure 3). On the contrary, the extracted lactoferrin from Milk Vita commercial milk showed ZOI against three bacterial strains- *Klebsiella pneumoniae, Enterococcus faecalis* and *Streptococcus pneumoniae* (Table 2, Figure 3).

4. Discussion

In developing countries like Bangladesh, millions of people are consuming milk throughout the year as it is a great source of protein and nutrition. Milk contains certain bioactive components (e.g., minerals, lipids, vitamins, casein, whey proteins and also lactoferrin) that strengthen the immune system of infants. As such bioactive compounds in milk facilitate the immune system, so its absence can cause deleterious effects on human health which is undesirable. In this study, the specific protein lactoferrin was isolated from milk samples, both the raw and commercial ones to identify its concentration and its impact on various pathogens. In addition to this, unwanted salts were removed by incorporating the dialysis method because the salt particles pass from higher concentration to lower concentration by passive diffusion (Harcum, 2008).

The data presented indicate that the protein concentrations in raw and commercial milk samples vary from each other. According to the findings, the amount of protein concentration is higher in raw milk (e.g., raw cow milk = 25.43 mg/mL) in contrast to commercially available milk samples except Aarong (26.33 mg/mL). It again supports that usually commercial milk contains less amount of protein than raw ones. However, in the case of commercial milk, Aarong (26.33 mg/mL), Milk Vita (22.06 mg/mL), Olympic (21.29 mg/mL), AMA (19.72 mg/mL), samples were found to pertaining high concentration of protein. On the contrary, other commercial milk, Farmland Gold, No. 1, PRAN and Red Cow had lower protein concentration 7.18 mg/mL, 9.64 mg/mL, 10.12 mg/mL, and 11.42 mg/mL, respectively. Milk usually contains 30-35 mg/mL protein but the milk samples used in this study were observed to have a lower concentration of protein. The findings point out the possibility of adulteration in milk and thus mass people of Bangladesh are being deprived of getting an adequate amount of protein from milk.

In the next step, lactoferrin was confirmed by performing SDS-PAGE. The purity of the extracted lactoferrin was further affirmed by recovering bands in the region of 77-80 kDa, confirming that the extraction procedure gives pure lactoferrin. The results of the present study are similar to other studies conducted by Abbas *et al.* (2015) where SDS-PAGE was used to confirm the purity of the protein (Abbas *et al.*, 2015). Yafei *et al.* (2011) also conducted a study based on a single band in the gel of SDS-PAGE in order to confirm the purity of the lactoferrin from defatted bovine colostrum (Liang *et al.*, 2011). In this experiment, all the raw milk samples were observed to show bands in the lactoferrin range whereas commercial milk samples had no bands except only for Milk Vita. Thus, it can be

assumed that in spite of having a high amount of protein concentration, the milk samples can lack lactoferrin and raw milk has higher chances of containing lactoferrin than the commercially available ones.

The iron-binding is considered to be the major mechanism responsible for the bacteriostatic activity of lactoferrin (Roseanu *et al.*, 2010). As we know, lactoferrin has a high affinity for iron and together with its presence in an iron-free form in body secretions allows lactoferrin to produce an iron-deficient environment that limits bacterial growth (Arnold *et al.*, 1980; Kalmar and Arnold, 1988; Yamauchi *et al.*, 1993). It was found from the results that lactoferrin extracted from both the Goat milk and Milk Vita commercially available milk showed bacterial inhibition against 7 strains at a concentration of 0.7 mg/mL. As a result, it indicates that lactoferrin might be a potential natural alternative to treat pathogenic bacterial strains upon a more framed future research with these 7 bacterial strains.

5. Conclusion

The objective of this study was to find natural alternatives to antibiotics. Moreover, the iron-binding nature of lactoferrin in milk makes the investigation more promising. Finally, lactoferrin was isolated from milk samples and tested for its sensitivity against bacterial strains. The data presented in the current study concludes that not all the milk variants have the protein lactoferrin, however, consumption of some milk can also eradicate bacterial growth. Further research with lactoferrin can open a new era for the treatment of many diseases and the food industry most importantly.

Conflict of interest

The authors declare that they have no known conflict financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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