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Original Article

Purified Lactoferrin from *Bubalus bubalis* and *Capra hircus* Milk from Mankarwadi, Baramati, India exhibits Antimicrobial Effects against *Pseudomonas* spp., *Bacillus* spp., *Escherichia coli*, and *Staphylococcus* spp.

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Abstract

Lactoferrin is an 80 kDa (Kilodalton) multifunctional glycoprotein present in the milk of most mammals. Beyond its well-established iron-binding capacity, lactoferrin exhibits several important biological activities, including promoting cell proliferation and differentiation, as well as notable antibacterial, antiviral, and antiparasitic properties. This study aimed to isolate and characterize lactoferrin from *Bubalus bubalis* and *Capra hircus* milk and to evaluate its antimicrobial activity. Commercial milk samples were collected locally from Mankarwadi, Baramati, India. The samples were defatted, casein was removed, and lactoferrin was extracted and quantified using a NanoDrop spectrophotometer. SDS-PAGE confirmed the presence of lactoferrin. The antimicrobial activity of the purified protein was assessed against four bacterial strains. The results demonstrated that the extracted lactoferrin effectively inhibited the growth of *Pseudomonas* spp., *Bacillus* spp., *Escherichia coli*, and *Staphylococcus* spp. Lactoferrin shows significant antimicrobial potential. These findings suggest that lactoferrin could be produced on a commercial scale and may serve as a natural alternative to conventional antimicrobial agents for preventing bacterial infections.

1. Introduction:

Milk is a highly nutritious white fluid synthesised in the mammary glands of mammals. It contains essential nutrients, including fats, carbohydrates, proteins, vitamins, growth factors, and various bioactive compounds [1]. Among these, milk proteins have numerous physiological and immunological functions crucial for maintaining overall health. After birth, Colostrum serves as the primary source of proteins for infants. This first milk is rich in bioactive compounds that support growth and enhance the newborn's immune system [2]. Lactoferrin is a non-heme, iron-binding glycoprotein with a molecular weight of 78–80 kDa, composed of approximately 690–702 amino acid residues. It belongs to the transferrin family [3]. The strong iron-binding capability of lactoferrin plays a crucial role in inhibiting microbial growth by

sequestering accessible iron. In addition, it reduces microbial infectivity and functions as an antioxidant. It is active against both Gram-negative and Gram-positive bacteria [3].

Lactoferrin is currently used in supportive treatment for patients with Hepatitis C. Although bioactive compounds in milk are present in small quantities relative to other food nutrients, ongoing research aims to extract them on a large scale and at low cost. Colostrum contains significantly higher concentrations of bioactive compounds, particularly lactoferrin and immunoglobulin G, compared to mature milk [4]. Lactoferrin is found in various mammalian secretions such as milk, tears, saliva, seminal fluid, vaginal fluid, nasal and bronchial mucosa, and also in the secondary granules of neutrophils [5]. It is considered a key host defence molecule and exhibits numerous physiological properties, including antibacterial, antiprotozoal, antifungal, antiviral, anticancer, antioxidant, anti-inflammatory, and immunomodulatory activities [5,6]. As a natural protein, lactoferrin is emerging as a promising biotherapeutic agent in antibacterial research [7]. The growing threat of drug-resistant bacterial infections has increased interest in combining lactoferrin supplements with chemotherapeutic drugs to enhance treatment effectiveness [6]. The X-ray structure of human lactoferrin provided foundational insight into the iron-binding properties of the transferrin family. Currently, extensive high-resolution data are available for lactoferrin in both the metal-free and metal-bound states. Although its antimicrobial activity was initially attributed solely to iron sequestration, further research has demonstrated that lactoferrin also exerts iron-independent antimicrobial effects [8].

This study involves the extraction and characterisation of lactoferrin from commercially available milk samples from *Bubalus bubalis* and *Capra hircus*. All analyses were performed in triplicate. The extraction procedure consisted of two main stages. Initially, the samples were centrifuged to remove the fat layer, and casein was precipitated by adjusting the pH. Further purification involved adding varying concentrations of ammonium sulfate, then centrifugation. Casein was removed under acidic conditions, and excess proteins were selectively precipitated using ammonium sulfate [1]. After isolation and purification, the antimicrobial activity of lactoferrin extracted from *Bubalus bubalis* and *Capra hircus* milk was evaluated against different microorganisms [9]. Opportunities include industrial-scale purification of nutraceutical products, synergistic combinations with local plant extracts, and veterinary use to treat mastitis in livestock in Maharashtra. This ties into mechanical engineering via process optimization for industrial extraction, such as CNC-integrated bioreactors or quality control in dairy fabrication[10].

While most research centres on bovine lactoferrin, studies comparing *Bubalus bubalis* and *Capra hircus* variants from Indian regions such as Baramati ignore local factors, including diet, lactation stage, and somatic cell counts, that influence milk composition. The mechanisms driving their distinct antimicrobial effects beyond iron binding, with gaps in vivo trials, biofilm assays, processing stability, therapeutic dosing, efficacy against local dairy pathogens such as *Salmonella*, and genetic influences.

2. Materials and Methods

2.1 Collection of Sample

Milk samples were collected from Mankarwadi, Baramati. A total of two milk samples were collected, both in liquid form. Samples were stored at 4°C before experiments [11].

2.2 Casein Separation

2.2.1 Fat Layer Separation

A 40 mL volume of each sample was centrifuged for 10 minutes at 4000 rpm at 4°C. After transferring the samples to the respective centrifuge tubes, they were defatted. The centrifuge tubes were placed into the centrifuge's chambers. The rotation was set to 4000 rpm for 10 minutes. Because some of the milk samples contained a minimal amount of fat, they were centrifuged twice. After centrifugation, the fat layer on top of the sample was removed with a spatula and discarded.

The volume of the defatted milk samples was noted, and an equal volume of distilled water was added. The initial pH of each sample was recorded. The initial pH of the *Capra hircus* sample was 6.54, and the *Bubalus bubalis* was 6.51. After measuring the initial pH, all samples were placed in a stand. A 1N HCl solution was added drop-wise to each milk sample with constant stirring until the pH was adjusted to 4.6 to precipitate casein.

Figure 1: Tube containing supernatant of *Bubalus bubalis* and *Capra hircus* milk

2.3 Lactoferrin Extraction

Three millilitres of supernatant were aliquoted into tubes and adjusted to pH 6.0 by drop-wise addition of 1N NaOH. An equal volume of 45% (w/v) ammonium sulfate was added slowly under magnetic stirring, initially at 100 rpm and then increased to 420 rpm after addition. Mixtures were stirred for 1 hour at room temperature [11]. Samples were acidified to pH 4.0 with 1N HCl, then neutralized to pH 8.0 with 1N NaOH. An equal volume of 80% (w/v) ammonium sulfate was added. The mixture of the samples and reagents was again transferred to a beaker containing a magnetic bead. The samples were stirred at 420 rpm for approximately 1 hour after the addition of the entire Ammonium Sulphate solution [11]. All the samples were then transferred to Centrifuge tubes, incubated at 4°C overnight for the lactoferrin to precipitate. After the overnight incubation, the samples were centrifuged at 4000 rpm for 10 minutes at 4°C and re-suspended in 500 µL of 1X PBS (Phosphate-Buffer Saline) (pH 7.4). Re-suspended lactoferrin stocks were stored at 4°C.

2.4. Protein Quantification and Purification

Samples were frozen at -20°C overnight. 20µl of each was mixed with Tris-EDTA buffer (pH 7.5), loaded onto a NanoDrop, and analysed at 260/280 nm for concentration determination [11].

Crude lactoferrin extracts were loaded into dialysis tubing (MWCO 9-14 kDa) and dialysed overnight at 4°C against Tris-HCl buffer (50 mM, pH 6.8 ± 0.2) with three buffer changes to remove salts and impurities. [12].

2.5 Antibacterial Assessment

Antibacterial activity of purified lactoferrin from buffalo and goat milk was evaluated using the agar well diffusion method against four clinically relevant bacterial strains, *Pseudomonas* spp. *Bacillus* spp., *Escherichia coli*, *Staphylococcus* spp. [13].

3. Results

3.1 Protein quantification by nanodrop technology

Both samples showed protein concentration in the test. The *Capra hircus* milk sample showed a high protein accuracy, whereas the *Bubalus bubalis* sample showed a low protein accuracy. Measurements were taken more than one time

Table 1: Protein quantification by Nanodrop UV-VIS spectroscopy

Sr. no	Sample name	A280	Protein Concentration (µg/mL)
1	Blank	0.43	0.43
2	*Capra hircus milk*	11.067	11.067
3	*Bubalus bubalis* milk	3.725	3.725

3.2 Antimicrobial activity

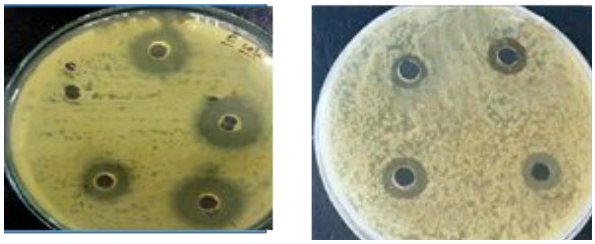
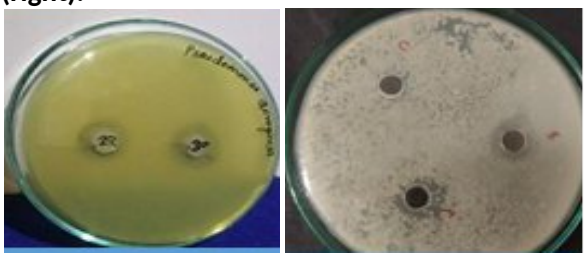


Figure 1: Antimicrobial activity of *Capra hircus* milk against *Escherichia coli* (left) and *Staphylococcus* spp. (right).



***Pseudomonas* spp.** **Bacillus spp**

Figure 2: Antimicrobial activity of *Capra hircus* milk against *Pseudomonas* spp. (left) *Bacillus* spp., (right) respectively.

Table 2: Antimicrobial activity of *Capra hircus and Bubalus bubalis* milk against *Escherichia coli* and *Staphylococcus* spp., *Pseudomonas* spp,. *Bacillus* spp.

Sr. No.	Bacterial strains	*Bubalus bubalis*	*Capra hircus*
1	*Pseudomonas* spp.	7mm	13mm
2	*Bacillus* spp.	5mm	12 mm
3	*Escherichia coli*	15mm	17mm
4	*Staphylococcus* spp.	9mm	15mm

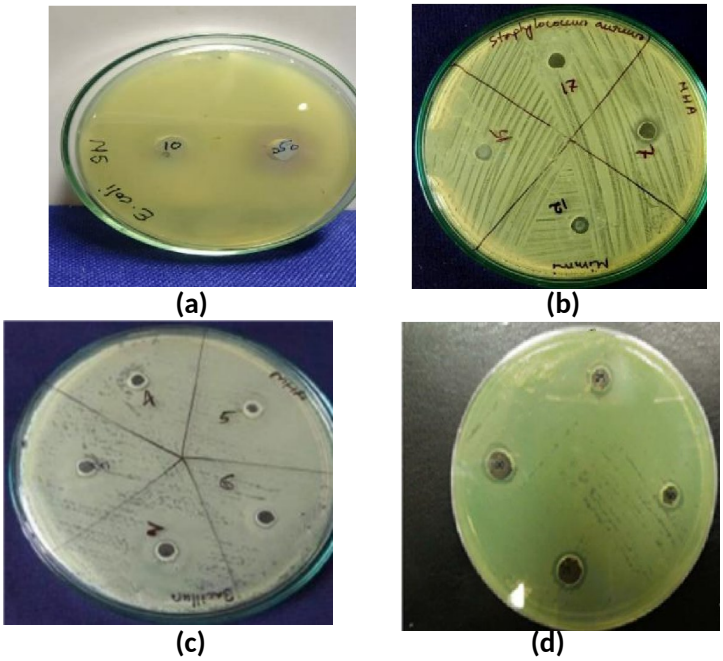


Figure 3: Antimicrobial activity of *Bubalus bubalis* milk against *Escherichia coli* (a), *Staphylococcus* spp. (b) *Pseudomonas* spp. (c) *Bacillus* spp. (d).

1. Discussion

The current research compared the extraction and antimicrobial properties of lactoferrin extracted from *Capra hircus* and *Bubalus bubalis* milk. The results indicate that purified lactoferrin has measurable antibacterial effects. It is typically characterised by a molecular mass of about 77-80 kDa and is abundant in mammalian secretions such as milk, saliva, and mucosal fluids [3,7]. It has biological implications beyond nutrient transport, as it is a major component of host defence mechanisms with antibacterial, antiviral, antifungal, antioxidant, and immunomodulatory actions [14,15].

Nano Drop analysis of the protein content in the milk extracts revealed that there was a significant increase in the concentration of protein in the *Capra hircus* milk extract (11.067mg/mL) over that of *Bubalus bubalis* milk extract (3.725mg/mL). Although spectrophotometric analysis could not sufficiently quantify individual protein identities, SDS-PAGE identified bands in the anticipated molecular weight range that could be related to lactoferrin, indicating successful extraction of the target protein.

The agar well diffusion tests conducted in this research further demonstrated the antibacterial activity of the extracted lactoferrin. Interestingly, compared with *Bubalus bubalis* milk lactoferrin, *Capra hircus* lactoferrin showed larger zones of inhibition against all bacterial strains tested, including *Escherichia coli*, *Staphylococcus* spp., *Pseudomonas* spp., and *Bacillus* spp. There was the highest level of inhibition against *E. coli* and *Staphylococcus* spp., which indicates that the purified protein is a broad-spectrum antimicrobial. These results align with previous literature indicating that lactoferrin and its peptide derivatives, especially lactoferricin, have strong bactericidal effects on Gram-positive and Gram-negative microorganisms by destabilising membranes and disrupting bacterial metabolic processes [16,17].

5. Conclusion:

The *Capra hircus* milk from Mankarwadi, Baramati, India shows better antimicrobial activity than *Bubalus bubalis* milk from Mankarwadi, Baramati, India, against *Escherichia coli* spp. *Staphylococcus* spp. *Pseudomonas* spp. *Bacillus* spp.

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Competing interest: The authors declare that no conflict of interest exists.

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Abbreviation

c: Celsius

HCl: Hydrochloric acid

kDa: Kilodalton

NaOH: Sodium hydroxide

w/v: Weight per volume

µL: Microliter

PBS: Phosphate-buffered saline

Tris-EDTA: Tris (hydroxymethyl) aminomethane-ethylenediaminetetraacetic acid

mM: Millimolar

µg/mL: Micrograms per milliliter

nm: Nanometer

mg/mL: Milligrams per milliliter

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CNC-integrated bioreactors: Computer Numerical Control Integrated bioreactors

Rpm-Revolutions Per Minute