



## Research Article

# Antimicrobial evaluation of Bovine Lactoferrin: A bioactive protein isolated from milk of crossbred and Indigenous cow breeds

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## Abstract

A multifunctional iron-binding glycoprotein called lactoferrin (Lf) acts as a broad-spectrum first line of defense against fungi, protozoa, bacteria, and viruses. Bovine Lactoferrin (BLf), which was extracted from the milk of crossbred and native breeds of cattle, was investigated for its antibacterial properties. The BLf protein was purified from the milk of HF crossbred and indigenous breeds like Sahiwal and *Poda thurpu* cows using the cation-exchange chromatography method and Broth microdilution and disc diffusion techniques were used to investigate the antibacterial activity. The minimum inhibitory concentration (MIC) of isolated BLf from the milk of HF cross-bred cow was found at 8000 µg/mL against *Staphylococcus aureus* and BLf isolated from the milk of Sahiwal and *Poda thurpu* cattle breeds respectively showed MIC of 4000 µg/mL against *S. aureus*. The MIC against *Bacillus spp.* was at 4000 µg/mL for all the experimental cow breeds. The MIC value of isolated BLf from Holstein-Friesian (HF) cross bred against *Escherichia coli* and *Proteus spp.* was 4000 µg/mL. However, the MIC for isolated BLf from Sahiwal and *Poda thurpu* breeds against *E. coli* and *Proteus spp.* in the present study was 2000 µg/mL. The antimicrobial activity was also studied by disc diffusion studies against selected gram-positive and gram-negative bacteria. The BLf isolated from the milk of all the three experimental breeds showed a smaller zone of inhibition at 2000 µg/mL concentration and a larger inhibition zone was observed at 4000µg/mL against the selected gram-positive and gram-negative bacteria. However, more potency was shown by the BLf isolated from the indigenous breed viz. *Poda thurpu* followed by Sahiwal.

**Keywords** bovine lactoferrin, fast protein liquid chromatography, Poda thurpu, zone of inhibition

## Introduction

Nasal, Tears, vaginal fluids, saliva, bronchial secretions and semen, bile, urine, and gastrointestinal fluids are all mucosal secretions that include the iron-binding glycoprotein known as lactoferrin (Lf) [1-2]. Additionally, colostrum and milk both contain large concentrations of it [3]. This biomolecule has a wide range of functionalities, including antibacterial action against gram-positive and gram-negative bacteria, parasites, fungi, and viruses [4], immunomodulatory capabilities in

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connection to innate and adaptive immune responses, and more [5]. Anti-inflammatory and Antioxidant interaction endorses its capacity for tissue redevelopment [6], and anti-carcinogenic activity that directly affects cancerous cells or indirectly affects them through the immune system [7]. Lf is a single polypeptide chain glycoprotein with a molecular weight of around 75–80 kDa [8]. Bovine lactoferrin (BLf), according to its amino acid sequence, is made up of a single polypeptide chain of 689 residues [9-10]. Two lobes make up its structure, and each one can reversibly chelate two  $Fe^{3+}$  ions per molecule. Initially, it was believed that Lf had antibacterial effects based on its capacity to trap iron [4]. Lf antibacterial efficacy for gram-positive and gram-negative bacteria has been thoroughly investigated both in vitro and in vivo. The bacteriostatic properties of Lf were attributed to the fact that the protein was present in milk in a highly unsaturated form to its iron binding capacity [11]. Because of its capacity to absorb the  $Fe^{3+}$  ion, Lf has bacteriostatic properties that prevent bacteria from growing and from expressing their virulence factors at the site of infection [12]. There have been suggestions that Lf's direct contact with bacterial surfaces is what causes it to have a bactericidal effect [13]. Specific interactions between Lf and gram-positive bacteria's lipoteichoic acid (LTA) and gram-negative bacteria's lipopolysaccharide (LPS) have been described [14]. According to various studies, the milk of native cow breeds is superior in terms of protein profile [15]. Though there are many experimental studies regarding antimicrobial activity but a detailed and comparative study on the anti-microbial activity of BLf between cattle breeds lacking so far. Therefore the present studies focus on the evaluation and comparison of BLf isolated from the milk of cross-bred (HF) and indigenous breeds like Sahiwal and *Poda thurpu* [recently recognized cattle breed by National Bureau of Animal Genetic Resources (NBAGR) and is native to Telangana state ]

## Methodology

At the Livestock Farm Complex of the College of Veterinary Science in Rajendranagar, Hyderabad, Telangana State, milk samples of HF crossbred and Sahiwal cattle were obtained and milk samples of *Poda thurpu* were procured from Achampet mandal of Nagarkurnool district, Telangana state. The collected milk samples were processed for the isolation of BLf [16-17]. The neutralized whey was obtained from skimmed milk and was subjected to ammonium sulfate precipitation initially by half saturation (0-45%) by adding ammonium sulfate and was brought to 45-80 percent saturation later. The sample fractionated was then dialyzed using Himedia dialysis membrane 50. The clear solution was then used for column chromatography. The ion exchange column was prepared using CM-Sephadex C-50 (Sigma Aldrich). The  $OD_{280}$  value of each fraction collected was measured in a spectrophotometer (Labomed INC, UVD 3200). The fractions with a minimum OD of 0.065 were recovered from the eluted samples. The samples with peak OD values were pooled and used for further confirmation of the protein by SDS-PAGE [18].

## Antibacterial assay

The minimum inhibitory concentration (MIC) was estimated using the Broth microdilution technique. Inoculums were prepared from respective cultures in MH broth. 5 $\mu$ L inoculum of gram positive (*Staphylococcus aureus*, *Bacillus* spp.) and gram negative (*E. coli*, *Proteus* spp.) on a 96-well microtiter plate, bacteria were put to each well with 150  $\mu$ L of HF crossbred lactoferrin containing concentrations, 8000  $\mu$ g- 125  $\mu$ g. The same procedure was repeated with Sahiwal lactoferrin and *Poda thurpu* lactoferrin. Antibiotic and culture controls were also used to compare antibacterial activity. The plate was incubated at 37 °C for 16-20 hours. The minimum concentration at which growth was completely inhibited was referred to as the Minimum Inhibitory Concentration (MIC). The disc diffusion assay with Muller-Hinton (MH) agar and in compliance with CLSI guidelines was used to determine the isolates' antimicrobial susceptibility. A sterile swab was used to remove any excess liquid from the tube well after turbidity adjustment to 0.5 on the Mac Farland scale. The



surface of a petri dish containing MH agar was then seeded with a variety of gram negative (*E. coli*, *Proteus* spp.) and gram positive (*S. aureus*, *Bacillus* spp.) cultures while rotating the dish at least 2 times. Using decontaminated forceps 5 discs infused with antimicrobials (chloramphenicol, cephotaxime, gentamicin, pencillin-G, and tetracycline) and two sterile discs impregnated with isolated BLf from HF cross-bred and native breeds *i.e.* Sahiwal and *Poda thurpu* with concentrations 2000µg/mL and 4000µg/mL. The plate was then inverted and incubated for 24 hours at 37 °C. Following incubation, disc readings were taken, and a ruler was used to estimate the diameter of the inhibition zones.

## Results and Discussion

BLf was isolated from all the three experimental cow breeds by using cation exchange chromatography. The eluted protein fraction formed a single band on the polymerized acrylamide gel at the same position as the position of molecular weight of the BLf *i.e.* approximately 80 kDa. This confirmed the identity and purity of BLf of all the three experimental cow breeds. The results were in strong resemblance with various studies where a single band was formed at approximately 80 kDa [16, 19-23]. Table 1 shows the MIC of isolated BLf from the milk of HF cross bred was 8000 µg/mL against *S. aureus* and isolated BLf from the milk of Sahiwal and *Poda thurpu* breed were 4000 µg/mL against *S. aureus*.

**Table 1. MIC of isolated BLf from HF cross-bred and indigenous breeds against selected gram positive and gram negative bacteria**

Bacteria	HF cross bred	Sahiwal	Poda thurpu
<i>Staphylococcus aureus</i>	8000 µg/mL	4000 µg/mL	4000 µg/mL
<i>Bacillus</i> spp.	4000 µg/mL	4000 µg/mL	4000 µg/mL
<i>E. coli</i>	4000 µg/mL	2000 µg/mL	2000 µg/mL
<i>Proteus</i> spp.	4000 µg/mL	2000 µg/mL	2000 µg/mL

The MIC of isolated BLf from the milk of all three experimental cattle breeds against *Bacillus* spp. was 4000 µg/mL. These results were contradictory to the MIC values obtained by Kutila et al., [24] where MIC of BLf was observed at 2.67 mg/mL against *S. aureus*, and also to results obtained by Shahidi et al., [25] which was found to be greater than 8mg/ml. The MIC value of isolated BLf from HF cross bred against *E. coli* and *Proteus* spp. was 4000 µg/mL. This result was contradictory to the value obtained by Conesa et al., [26] as well as Shahidi et al where the MIC of BLf was 2 mg/mL and greater than 16mg/mL. However, the MIC of *E. coli* and *Proteus* spp. for isolated BLf from the milk of Sahiwal and Poda thurpu breeds in the present study was 2000 µg/mL. The results of indigenous breeds were in close resemblance with the values obtained from various studies against *E. coli* where the inhibition was seen at 1.67 mg/mL [24] and 2 mg/mL [21]. However, El baky et al., [27] reported MIC values of BLf at 0.25 mg/ml against *S. aureus* and 0.5 mg/ml against *B. cereus* while MIC was found to be 1 mg/ml against *E. coli* and *P. vulgaris* which were contradictory to the result obtained in the present study. Tomita et al., [28] found the MIC value of BLf as 2mg/ml against *E. coli* which are in consistent with the MIC values of BLf isolated from the milk of indigenous breeds *i.e.* Sahiwal and *Poda thurpu* cattle against *E. coli*. Conesa et al., [26] observed that the MIC values of BLf against *E. coli* were 0.5 mg/ml which to the values obtained. The antimicrobial activity was also studied by disc dispersal studies contrary to selected gram-positive and gram-negative bacteria. The zone of inhibition was observed at 2000 µg/mL concentration and a larger inhibition zone was observed at 4000µg/mL by the isolated BLf from all the three experimental breeds of cattle as shown in Figures 1 and 2.

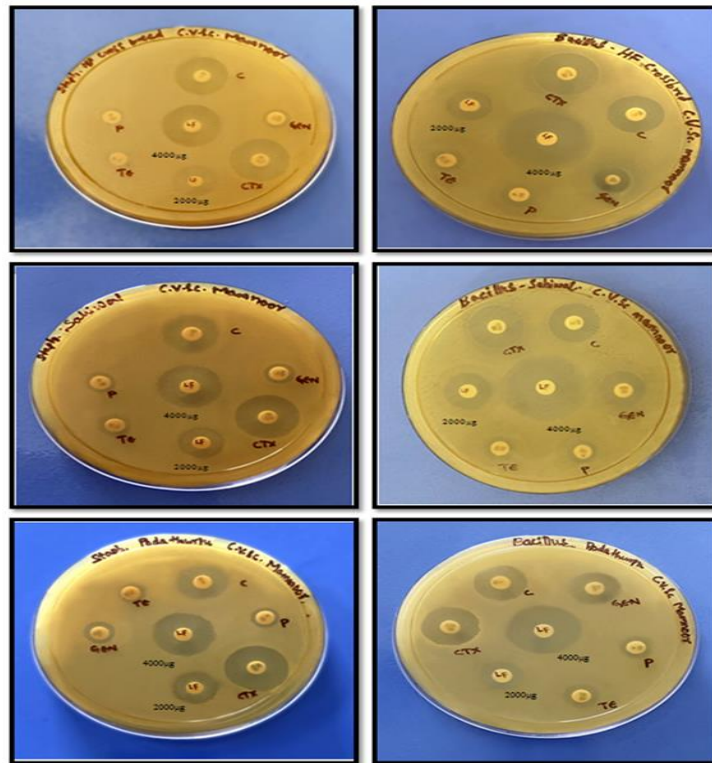


Figure 1. Evaluation of antimicrobial activity of isolated BLF from HF cross bred and Indigenous breeds like Sahiwal and *Poda thurpu* cows against selected gram-positive bacteria

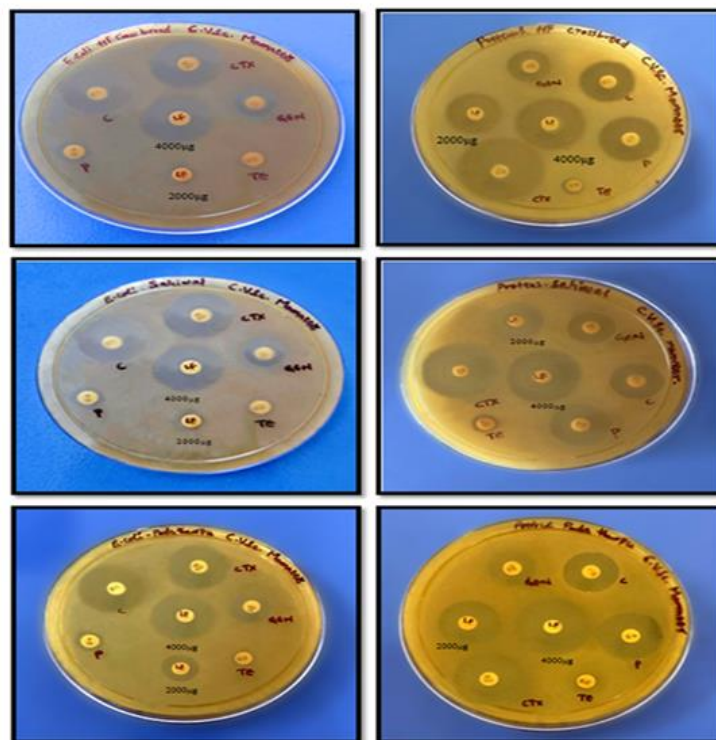


Figure 2. Evaluation of antimicrobial activity of isolated Bovine Lactoferrin from HF cross bred and Indigenous breeds like Sahiwal and *Poda thurpu* cows against selected gram negative bacteria



However, more potency was shown by the BLf isolated from the indigenous breed viz. Poda thurpu followed by Sahiwal (Table 2). The results were contradictory lower when associated with the study of Bhimani et al., [29] where the zone of inhibition was seen at 5 mg/mL and extreme inhibition was at 20 mg/mL. Meanwhile, Shahidi et al., [25] reported a zone of inhibition at 16mg/ml of BLf concentration against *S. aureus* and a zone of inhibition at greater than 16mg/ml against *E. coli*. At a dosage of 1 mg/ml BLf, El Baky et al., [27] reported a zone of inhibition of 34.3 mm against *S. aureus* and 20.3 mm against *B. cereus*.

**Table 2. Isolated BLf from HF cross-bred and native breeds creates a zone of inhibition around some gram-positive and gram-negative bacteria**

Antimicrobial/ Antibiotic discs	Concentration in each disc	Zone of inhibition			
		<i>S. aureus</i>	<i>Bacillus spp</i>	<i>E. coli</i>	<i>Proteus spp.</i>
Chloramphenicol	30 mcg	17	18	22	18
Cephotaxime	30 mcg	20	21	22	25
Gentamicin	10 mcg	11	14	14	15
Pencillin-G	10 units	10	11	11	22
Tetracycline	30 mcg	10	10	10	8
HF cross bred BLf	2000 µg/mL	13	14	10	17
	4000 µg/mL	17	25	19	23
Sahiwal BLf	2000 µg/mL	14	15	10	18
	4000 µg/mL	20	26	22	25
Poda thurpu BLf	2000 µg/mL	15	17	11	19
	4000 µg/mL	21	27	23	26

However, the zone of inhibition at 1 mg/ml BLf against *E. coli* and *P. Vulgaris* was 31.0 mm and 30.7 mm respectively. These results were contradictory to the results obtained in the current study. The results obtained in this study differed from some of the studies as the BLf activity depends upon the genetic makeup of the individual cattle breed, type of feeding, and also lactoferrin used in various studies may have been obtained commercially or the purification technique may be different.

## Conclusion

Gram-negative and gram-positive bacteria are both susceptible to the antibacterial properties of BLf. This antibacterial property of BLf is helpful in its application as a potent antibiotic as well as probiotic. In the present study, the MIC results indicated that the higher antibacterial activity of isolated BLf was evidenced by lower MIC values and higher zone of inhibition from indigenous cow milk (Poda thurpu and Sahiwal) when compared to HF cross-bred milk against gram-negative bacteria like *E. coli* and gram-positive bacteria i. e. *S. aureus* and *Proteus spp.* However, there was no difference in MIC value of all three experimental breeds against gram positive i. e. *Bacillus spp.* bacteria. It can be noticed the native breeds of cattle showed greater antibacterial activity than the exotic crossbred. The higher genetic makeup and superior adaptability of local environmental conditions of indigenous cattle over the exotic cross-bred may have contributed to such higher antibacterial activity. However further research and experimental studies are required for evaluating the antimicrobial activity of BLf in the milk of different cattle breeds.

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