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Molecular Identification of Camel, Goat and Sheep Milk Lactoferrin and Determination of it's Minimal Inhibitory Concentration and Bactericidal Effect on *Staphylococcus Aureus* and *Escherichia Coli*

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Abstract:- Lacto ferrin is an iron chelating glycoprotein of the transferrin family present in several biological fluids. The study did a molecular identification, quantitative analysis of LF from Camel, Goat and Sheep milk andalso investigated the effect of the purified LF on the growth inhibition of *Staphylococcus aureus* and *Escherichiacoli*. Lactoferrin, was isolated from camel, goat and sheep milk. Both isolation and subsequent purification of the Lactoferrin from these sources using CM Sephadex C-50, showed that camel milk contained appreciable quantities of LF (2.60mg/ml).While Goat milk and Sheep milk contained less quantities of LF as compared to Camel milk (2.10mg/ml and 1.70mg/ml) respectively. The result of bactericidal activity demonstrate that Camel LF is bacteriocidal towards *S.aureus* and *E.coli*. Minimal Inhibitory Concentrations were seen even at higher concentrations and the least concentration was at 0.60mg/ml of camel LF against *E.coli* and 0.2mg/ml of camel LF against *S.aureus*. The findings of this study Suggest that lactoferrin has inhibitory potential against *S. aureus* and *E. coli*.

I. INTRODUCTION

Lacto ferrin (LF), also known as lactotransferrin, is an 80-kDa glycoprotein that belongs to the transferrin family, which includes proteins that can bind and transfer Fe3+ ions. LF can be found in milk, saliva, tears, and nasal secretions, among other things (Brock, 2002). It's most prevalent in human colostrum, then human milk, and finally cow milk, from which it may be readily and safely separated. Lactoferrin is classified as an acute-phase protein by numerous authors since its concentration rises during most inflammatory reactions and some viral infections. Its content rises in all bodily fluids, although the largest concentrations have been found in the nidus of inflammation (Karav*et al.,* 2017).

The fact that LF's surface is positively charged is one of its most notable characteristics. This makes it easier for LF to bind to anionic bio-compounds. The positively charged LF moieties are mostly located on the outside area of the N1 domain's first helix and near the end of the C terminal (Fig. 1). In the interlobe region, where two lobes are joined by a helix, there is another, much smaller, but extremely positively charged point. DNA, heparin, and lipopolysaccharide are all found to attach to the basic area around the N-terminus (Lizzi*et al.,* 2016). Through N-linked glycosylation, sugars (mostly high mannose and N-Acetyleglucosamine) are linked to LF (Moore*et al.,*1997).

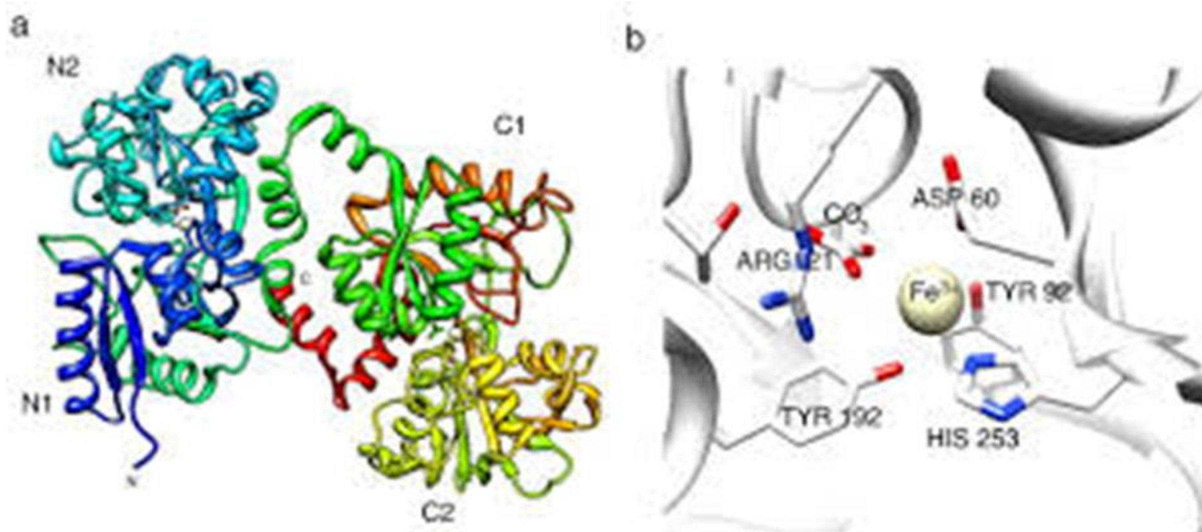

Fig. 1: Structure of Lactoferrin (Yount*et. al.,* 2007)

Sheep and goat milk proteins are important sources of bioactive Angiotensin Converting Enzyme (ACE) inhibitor and antihypertensive peptides. They can provide a defense for diseases of non-immune system and control of microbial infections. Important minor milk proteins include immunoglobulins, lactoferrin, transferrin, ferritin, proteose, peptone, calmodulin (calcium binding protein), prolactin, and folate-binding protein (Atanasovaand Ivanova, 2010).

It has been found that camel milk has antidiabetic properties, anti-hepatitis properties (Agrawal*et al.,* 2009) and bactericidal properties (El-Fakharay*et al.,* 2012). Camel's milk is reported to have a stronger inhibitory capability than that of cow's milk (El-Agamy*et al.,* 1992). In particular, the levels of lysozyme and lactoferrin are reported to be two and three times higher than those of cow's milk, respectively.

Many studies have shown that LF from various sources (human, bovine, porcine, caprine, camelid, and buffalo) can successfully inhibit the growth of a variety of infections. The majority of findings suggest that LF from various sources can be bacteriostatic due to its iron-chelating activity, but it can also be bactericidal due to interactions with LPS and porins in Gram-negative bacteria, or teichoic acids in Gram-positive bacteria. Membrane damage and bacterial death result from these interactions. The capacity of bovine and human lactoferrin (hLF), two natural antimicrobial proteins found in milk, to suppress *E. coliO157*:H7 growth and adhesion to a human epithelial colorectal cancer cell line was investigated by Yekta*et al.,* (2010). (Caco-2). On *E. coli* O157:H7, bovine lactoferrin (bLF) had a greater direct antibacterial activity than human lactoferrin (hLF).Nonetheless, even at high doses (10 mg/ml), both lactoferrins displayed bacteriostatic effects, implying that LF action is blocked by an unknown bacterial defense mechanism.

S. aureus has been a prominent cause of healthcare-associated infections as hospital-based medicine has grown in popularity. The emergence of multidrug-resistant, highly transmissible strains, as well as rising morbidity and mortality rates associated with *S. aureus*and *E. coli*infections, have heightened interest in antibiotic alternatives. As a result, this study emphasizes the efficacy and utility of naturally produced lactoferrin from milk as a new non-antibiotic therapeutic technique.

II. MATERIALS AND METHODS

A. Sample Collection and Preparation

Goat and sheep milk samples were collected from Rayuwa Farms in Abuja while camel milk was collected from a local herder in Katsina State. The milk were collected from healthy animals by hand milking in sterile screw bottles and kept in cool boxes until transported to the laboratory.

The clinical bacterial isolates of Methicillin-resistant *Staphylococcus aureus (*MRSA) and *Escherichia coli* were collected from the Department of Laboratory services (Federal Medical Center, Abuja).

The bacterial culture of *S. aureus* and*E.coli*were grown in tryptic broth and incubated overnight. Using Gram-staining, the cultures were tested for purity. Bacteria were then harvested by centrifuging at 4,000rpm for 15 min and the pellet re-suspended and washed with sterile phosphate buffered saline (PBS). A suspension containing approximately 10^9 colony forming units (CFU) in 0.9% NaCl was prepared according to the McFarland standard.

B. Isolation and Purification of Lactoferrin

- Isolation of Lactoferrin
  Forty milliliters (40 ml) of each milk sample was centrifuged in a Sigma MA3-18 centrifuge for 10 minutes at 4000 rpm at 4° C. This was repeated twice. The fat layer (top most) obtained was separated using a spatula and discarded. The volume of all defatted milk samples was noted and an equal volume of distilled water was added. Then to precipitate casein, 1N HCl was added slowly to each sample with constant stirring until pH of 4.6 was attained., followed by centrifugation at 2000 rpm for 10 minutes at 4° C. Supernatants from each sample were stored in a refrigerator at 4^{oc} for further analysis (Moradian*et al.,* 2014).
- LF extraction from stored supernatants
  Moradian *et al.* (2014)'s technique was applied for this. In a nutshell, 1N NaOH was slowly added to all of the supernatants obtained from the preceding treatment, stirring constantly, until pH 6.0 was attained. With steady magnetic swirling at 100 rpm, an equal volume of 45 percent ammonium sulphate solution was applied to all samples. After adding the remaining 45 percent ammonium sulphate solution, stirring was gradually increased to 420 rpm, and the sample was left at room temperature for 1 hour. Furthermore, 1N HCL was progressively added to all samples with continual stirring until pH 4.0 was attained, then 1N NaOH was slowly added until pH 8.0 was reached. At pH 8.0, an equal amount of 80 percent ammonium sulphate solution was added with steady stirring at 100 rpm for 1 hour after the remainder of the ammonium sulphate solution was added.After overnight incubation at 40 C to precipitate LF, all of the samples were centrifuged at 4000 rpm for 10 minutes at 40 C. The obtained LF precipitate was then dissolved and re-suspended in 1mL 1x PBS buffer (pH 7.4) before being kept at 40 C for further analysis (Moradian et al., 2014).
- Purification by Chromatography
  Using 0.2 M phosphate buffer (pH 6.7) and a linear gradient NaCl from 0.0 to 0.5 M at a flow rate of 3mL/min, crude Lactoferrin from each source was purified using carboxymethyl Sephadex-C50 chromatography (FPLC, Bio-RAD, USA) (the LF had been previously equilibrated with 50mM phosphate buffer pH 6.7). The protein-loaded column was then washed in 500 mL of the same buffer to eliminate any loose or unbound sample components. In the phosphate buffer, bound protein was eluted with the same linear gradient of NaCl (total volume

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of 200 mL). At a flow rate of 4 ml/min, 2 mL fractions were collected. The purity of the protein-containing fractions was verified, and the active fractions were pooled, dialyzed against the same phosphate buffer, and stored at 40°C (Moradian*et al.,* 2014).

Protein in the eluents was measured using UV absorption with cation exchange chromatography during the chromatography.

- LF identification by SDS-PAGE

Both the stacking (1 mol/L Tris-HCL pH 6.8) and resolving gel (1.5 mol/L Tris-HCL pH 8.8) were prepared and the gel was cast.The protein sample was prepared by mixing 15 µL of sample to 15 µL of 5x protein loading dye (10% SDS, 500Mm DTT, 50% Glycerol, 500mM Tris-HCL and 0.5% bromophenol blue dye.) which was kept in the water-bath for 2min at 95°C.

The wells were loaded with 20µl µl of protein sample and super signal molecular weight protein ladder (MAKE) and were run at 100 V for 2 hours in a reservoir of 1 x TGS protein running buffer (0.19M glycine, 25mM Tris base, 1% (w/v) SDS). The gel was then placed on Coomassie Brilliant Blue staining solution (0.1% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) and kept in water-bath for 20min at 55°C. After that the gel was kept in the destaining solution (40% methanol and 10% glacial acetic acid) followed by 20min water-bath treatment at 55°C. The gel was then kept overnight in the destaining solution and observed next day.

C. *Antibacterial activity*

- Inhibitory and Bactericidal Effect

Nine tubes were used to determine the minimal inhibitory concentration.

0.1 ml of sterile physiological saline was added to each tube, followed with 0.1 ml, 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml, 1.0 ml, 1.2 ml, and 1.4 ml of camel LF, with the 9th tube serving as a control. Ten milliliters of tryptic broth were added to each tube, followed by 0.1 milliliters of log-phase organisms (1.1 x 103 colony-forming units). The antibacterial concentration that inhibited the development of the test microorganisms was defined as the least inhibitory concentration after the 9 tubes were incubated overnight at 37°C. This procedure was carried out on all LF samples.

100µl of tryptic soy broth, 50µl of bacterial suspension, and 200µg of LF concentrate were inoculated into separate wells in an automated bioscreen apparatus (Bioscreen C Reader System, OY growth curves AB Limited) for 20 hours to investigate the bactericidal effect of Camel, Goat, and Sheep LF. To make the final capacity of 250µl, physiological saline was added (the final LF concentration in the well was 0.67 mg/ml). As controls, one clinical bacterial isolate of Methicillin-resistant *Staphylococcus aureus* (MRSA) and one clinical bacterial isolate of Escherichia coli were employed. The bactericidal effect of Camel, Goat, and Sheep LF in the wells was confirmed after a 20-hour incubation period in the bioscreen device by culturing aliquots of 1.0 ml on blood agar plates and incubating the plates overnight at 37°C.

D. *Statistical Analysis*

The results from the bacteria assay using the bioscreen C instrument are reported as CSV formatted OD numbers. Using the Nodern Logic Professional Software, the reported OD numbers were then exported to MS Excel for graph generation. The effect of different LF concentrations on lag time, slope, and maximum absorbance were then tested by repeated measures analysis of variance with concentration as a within factor. The significance of concentration was evaluated by Greenhouse-Geisser adjusted p-values. A $p \leq 0.05$ was considered statistically significant.

III. RESULTS

A. *Lactoferrin Purification using CM Sephadex C-50*

The LF isolated from camel milk and purified using CM Sephadex C-50 was eluted at higher molarity than Goat and Sheep LF (0.6ml/min, 0.9ml/min and 2.4ml/min) respectively. The results reflected that camel milk contained appreciable quantities of LF (2.6mg/ml) (Fig. 2). However, goat milk and sheep milk contained less quantities of LF as compared to camel LF (2.1mg/ml and 1.7mg/ml) respectively.

B. *SDS-PAGE of Purified Lactoferrin*

Plate 1 showed thatCamel LF, Goat LF, and Sheep LF all have a molecular weight of 80 kDa, and the migration pattern of Camel LF was slightly slower than that of Goat and Sheep LF.

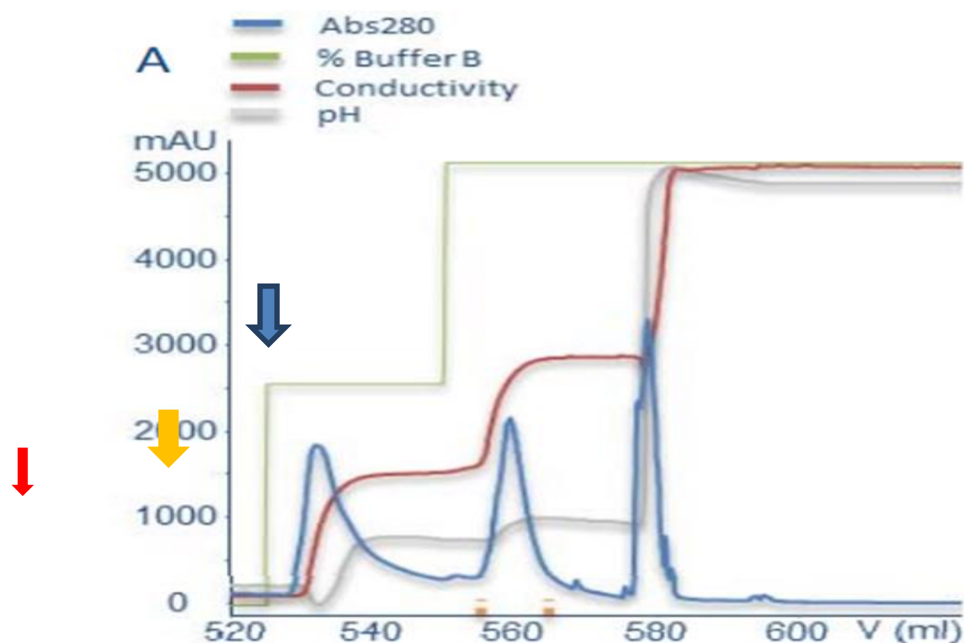

Fig. 2: Elution by step gradient purification of lactoferrin from camel, goat, and sheep milk.
The goat LF peak (red) is 1.7mg/ml, the sheep LF peak (yellow) is 2.1mg, and the camel LF peak (blue) is 2.6mg/ml.

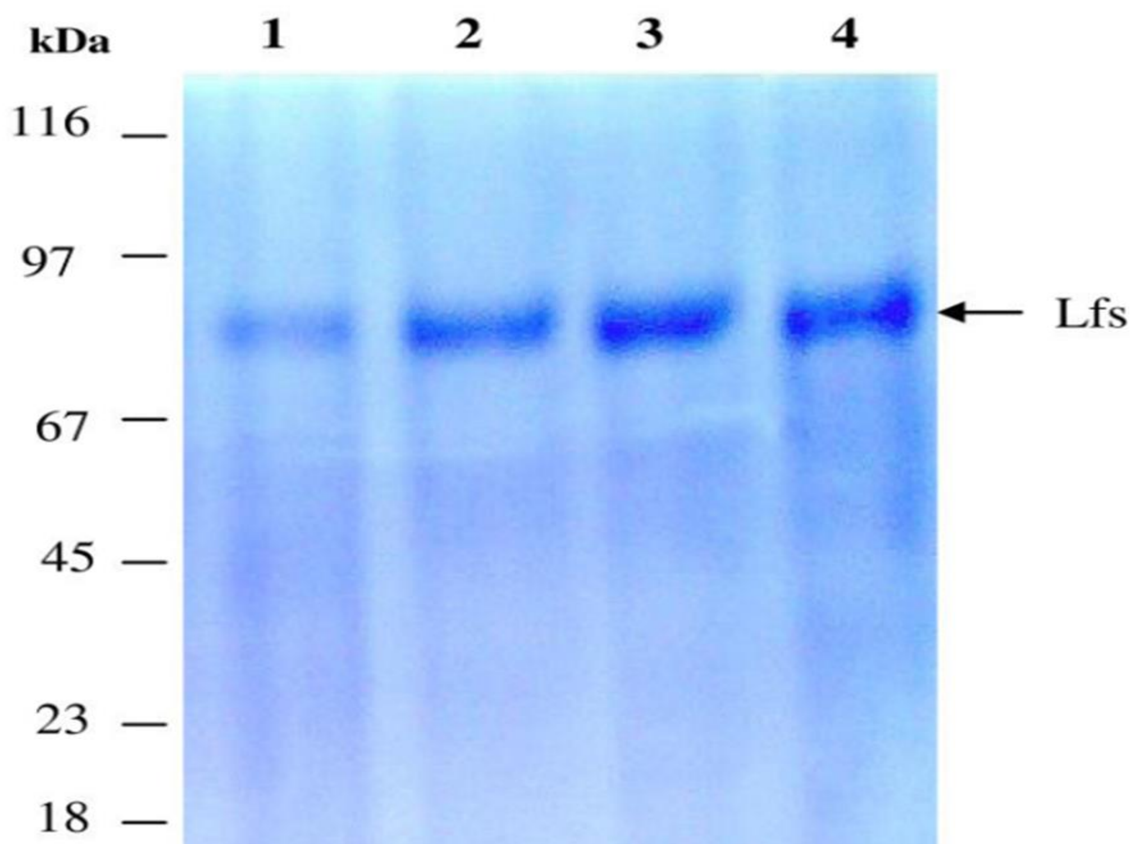

Plate 1: SDS-PAGE analysis of different types of purified LFs (camel LF, sheep LF and goat LF). Lanes 1–4 are protein marker, purified Sheep, Goat and Camel lactoferrin, respectively.

Organism/LF sample	MIC (mg/ml)
E.coli	
Camel LF	1.0
Goat LF	0.6
Sheep LF	0.8
S.aureus	
Camel LF	0.2
Goat LF	0.6
Sheep LF	none

Table 1: Minimal Inhibitory Concentration (MIC) of camel, goat and sheep LF on *S.aureus* and *E.coli*

LF sample	*S. aureus*	*E.coli*
Camel	✓	✓
Goat	✓	✓
Sheep	X	✓

Table 2 : Bactericidal activity of camel, goat and sheep LF against *S. aureus* and *E.coli*

✓ = LF sample killed bacteria

X = LF sample did not kill bacteria (it was bacteriostatic)

IV. DISCUSSION

Data obtained from this study showed that the content of Lactoferrin in camel milk was most appreciable in quantity (2.6mg/ml) compared to LF from Goat milk (2.1mg/ml) and LF from sheep milk (1.7mg/ml). Although several reports have illustrated different levels of this protein, as different workers have adopted different units, methods and antagonistic activity of Camel Lactoferrin approaches for quantitative analyses. However, the findings of the present study were quite in line with those of El-Hatmi*et al.*(2006), who adopted the same method as was performed in the present study.

The SDS-PAGE analysis of the protein isolated from camel, goat and sheep confirmed the presence of a 80kDa protein which agrees with work of El-Hatmi*et al.* (2006). Lactoferrin is an iron binding glycoprotein of the transferrin family with a molecular weight of 80kDa (ref).

In this study, the result of bactericidal activity demonstrate that Camel LF and Goat LF is bacteriostatic towards *S.aureus* and *E.coli*, while Sheep LF showed a bacteriostatic activity against*S.aureus*only. The most interesting finding was the clear inhibitory activity of Camel LF against *E. coli,* which is in agreement with previous studies (Del Olmo*et al.,* 2010;Atanasova and Ivanova. 2010).

The least Minimal Inhibitory Concentrations of LF (0.60mg/ml) against *E.coli* was observed in camel LF. This is in contrast with the work of Sanchez and Watts (1999) as they did not see effect of LF alone at concentrations from 0.5 to 3 mg/ml on three *E. coli* strains isolated from bovine mastitis. This may be due to the pathogen strain tested. Bhimani*et al.*(2003) pointed out that forms of Bovine LF, Camel LF and Sheep LF showed an average MIC of 1.0mg/ml against experimental *S. aureus* in in-vivo infections in mice. Similarly, Magdy*et al.* (2015) showed that Camel milk decreased the total bacterial count of *S. aureus* and *E. coli* in all tissues of rats injected with pathogen compared to pathogens alone injected rats. They attributed their findings to high amounts of antimicrobial peptides such as Lysozyme (LZ), lactoferrin (LF), lactoperoxidase (LP), short peptidoglycan recognition protein (PGRP) present in camel milk.

V. CONCLUSION

Lactoferrin was isolated satisfactorily from camel, goat, and sheep milk in this study. The molecular weight of Camel LF, Goat LF, and Sheep LF was determined to be 80 kDa by SDS-PAGE, and the migration pattern of Camel LF was found to be slightly slower than that of Goat and Sheep LF. Camel LF is bacteriostatic against *S.aureus* and *E.coli*, according to the results of bactericidal activity. Even at greater concentrations, Minimal Inhibitory Values were observed, with the lowest concentrations being 0.60mg/ml of camel LF against *E.coli* and 0.2mg/ml of camel LF against *S.aureus*. Overall, these findings show that lactoferrin could be used to treat S. *aureus* and *E. coli* isolates, particularly Camel LF against Methicillin-resistant *S. aureus.*

VI. RECOMMENDATIONS

- Therapeutic techniques based on ferro-chelating substances, such as lactoferrin, may assist to enhance current antibiotics' shockingly poor efficiency.
- Research into the molecular mechanisms behind lactoferrin's antibacterial activity could provide further information about its mode of action. As a result, lactoferrin could be used as an alternative antibacterial

agent against S. aureus and E. coli that targets molecular sites not linked to antibiotic resistance.

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