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SURVELLIANCE OF BOVINE MILK SAMPLES AND DETERMINED ITS IMMUNOLOGICAL ACTIVITY OF LACTOFERRIN

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ABSTRACT

Milk is a very complex product and reported more than hundred separate chemical compounds. The major components of milk are water, casein, whey proteins, fat, lactose and minerals. These components should be varied according to the species of various animals. Milk also contains trace amounts of other substances such as pigments, enzymes, vitamins, phospholipids including gases. Once the residue is left only when water and gases are removed is called the dry matter (DM) or total solids content of the milk. In this study, we observed lactoferrin content in milk samples of different bovine animals and also studied about its proliferation and estimation of Th1 (IFN-gamma and TNF alpha) cytokines against non-specific protein antigen (Concanavalin, Con A) in human whole blood samples and also studied about its antibacterial properties using *Bacillus subtilis* and *Pseudomonas aeruginosa*. The results of these studies showed that lactoferrin at higher doses inhibit proliferation rate and Th1 cytokines against non-specific protein antigen. In addition, lactoferrin also showed antibacterial properties as well. Overall conclusion of these studies which represents its anti-inflammatory and antibacterial property of lactoferrin.

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INTRODUCTION

As per the literature, milk provides some of the essential nutrients that are required and showed vital source of dietary energy including high quality proteins and fats. In other words, milk from animals showed significant contribution with respect to human health nutrition [1, 2]. So, animal milk played an important role in the diets of children populations with very low fat intakes and limited access to other animal source foods. In general, several species of animals are needed for dairy milk products and it depends on its breed, age, diet, stage of lactation, physical environment and season influence the colour, flavour etc. [1-3] According to the literature, gross composition of cow's milk (87.7% water, 4.9% lactose (carbohydrate), 3.4% fat, 3.3% protein, and 0.7% minerals) is reported especially in U.S. As per the literature, milk composition varies from species to species e.g. cow, goat, sheep etc. but there is minor variation that are reported in milk composition as shown in Table 1. Normally, collection of milk samples from different animals i.e. cow/goat/sheep are stored together in big tankers and provides a relatively consistent source of dairy milk products [1-4].

In milk, two major types of proteins are reported i.e. casein (0.04 to 0.3 µm in diameter; porous structures) and whey protein (individual units dissolved in the water phase of milk). One of the proteins i.e. Casein micelles that are stable but dynamic structures that do not settle out of solution. They can be heated to boiling or cooled, and they can be dried and reconstituted without showing any adverse effects. ß-casein, protein along with some calcium phosphate, will migrate in and out of the micelle with changes in temperature, but this does not affect the nutritional properties of the protein and minerals [5, 6]. On the other hand, whey protein extracted from whey (liquid material) used as by-product for cheese production [7, 8]. These whey proteins are available commercially as shown in Table 2.

In this study, SDS PAGE was performed in order to analyse its KDa protein in bovine milk samples. First of all, milk samples diluted in similar volume of PBS and then centrifuged (2500 rpm; 10 min, 4 C) two to three times with PBS. Pellet containing cells were taken and diluted in PBS and then centrifuged at 10000 rpm for 10 minutes at 4 ºC. Collect the supernatant for sample loading in SDS PAGE. In this experiment, resolving (15 %) and stacking (4%) gels were used for isolation of protein bands of bovine milk samples. About 10 µl of milk supernatant was loaded into the wells and voltage (80 Volts) was required to run the gel. After the separation of protein bands of different bovine animals through electrophoresis, staining solution was utilized to stain the gel in order to make bands visible. Afterwards the gel was placed in to a destaining solution for 24 hours on shaker and was changed frequently until clear gel was obtained. The results showed various protein bands i.e. cow (≅ 14-80 KDa); goat (≅ 14-45 kDa) and sheep (≅16 - 48 kDa)

One of the most familiar example which is reported in denatured whey concentrate i.e. Lactoferrin (80 kDa, Molecular weight; glycoprotein) is reported in milk samples of different bovine animals and showed its higher affinity for iron content. This glycoprotein based component i.e. lactoferrin is composed of single polypeptide chain (703 amino acids) which is further folded into two globular lobes as mentioned in the literature [9, 10]. Two globular lobes i.e. Carboxy and amino terminal regions are connected with alpha helix. In addition, carboxy and amino terminal lobes are further divided into two domains i.e. Carboxy1 and Carboxy2; amino1 and amino2. Both these domains create one iron binding site. As per the literature, lactoferrin isoforms have been reported i.e. Lactoferrin-α (iron binding form with no ribonuclease activity); lactoferrin-β and lactoferrin-γ (ribonuclease activity not able to bind iron) [9, 10].

In addition, Lactoferrin (basic proteins, 8.7 isoelectric point) exists in two forms i.e. iron-rich hololactoferrin and iron-free apolactoferrin but its tertiary structures are totally different. Apolactoferrin means open conformation of N-lobe and closed conformation of the C-lobe whereas both these lobes are closed in the hololactoferrin. Normally, lactoferrin reversibly bind with two ions of iron, copper, zinc and other metals but its affinity in case of iron is still 300 times higher than other metals. If other metals is present, it does not affect the ability of iron binding site of lactoferrin content [9, 10].

The molecular structure and amino acid sequence of human lactoferrin were reported in 1984. Lactoferrin was then classified as a member of the transferrin family, due to its 60% sequence identity with serum transferrin [11, 12]. This protein is reported in mucosal secretions which is synthesized by epithelial cells and also showed its presence in neutrophilic granules. In addition, lactoferrin considered as first line of defence against various microbial infections. In addition, lactoferrin showed antimicrobial activities as mentioned in the literature. These activity could be due to large quantities of iron content in order to provide protection against various pathogens and its metabolites enhancing the phagocytosis activity and also helpful in order to controlling the level of pro-inflammatory cytokines [12, 13].

For these studies, fresh milk samples of different animals were collected from local dairy farm, Baramati, District Pune, Maharashtra, India. All milk samples (n =10 ml) were diluted in equal quantity of PBS and centrifuging at 5000 rpm and 4 ^{o}C for 10 minutes. In order to prevent the microbial growth in milk samples, add minute amount of sodium azide solution. The samples were stored at 4 ^{o}C in refrigerator. For identification of lactoferrin content, capsules containing lactoferrin of bovine animals were collected.

For quantification of lactoferrin content in bovine samples using bovine serum albumin (1 mg/ml) as coating antigen. Milk samples of different bovine animals (1:10 dilution) were used for the estimation of IgG antibody titre [14]. For these studies, Lactoferrin capsule used as standard for these studies and diluted in PBS of different concentration. Horse anti-serum (1:20000) used as secondary antibody and optical density measured at 450 nm. All these readings were compared with lactoferrin and readings should be expressed in microgram (µg). The results of these studies showed that colostrum showed higher amount of lactoferrin content as compared to goat followed by sheep and bovine animals as shown in **Fig.1**

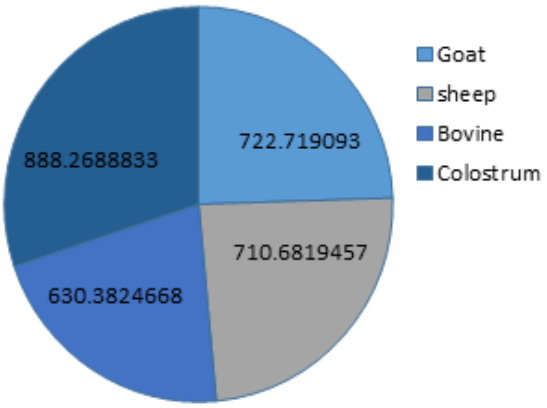

Fig.1. ELISA assay. Indirect ELISA was performed using bovine serum albumin (1 mg/ml) as coating antigen. Milk samples from different animals were used for the estimation of lactoferrin antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm.

Table 1. Comparative chart of milk samples collected from different animals.

Cow milk	Buffalo milk	Camel milk	Sheep milk	Goat milk	Yak milk	Equine milk
Fat (3- 4 %), protein (3.5%) and lactose (5 %). Most familiar example i.e. fat content is usually higher in breed i.e. Bos indicus than B. taurus cattle. The fat content of milk from B. indicus cattle can be as much as 5.5 percent.	High fat content (2 times higher than cow milk). Fat-to-protein ratio in buffalo milk is about 2:1. Compared with cow milk, buffalo milk also showed higher casein-to-protein ratio. The high calcium content of casein facilitates cheese making.	Slightly saltier, similar composition to cow milk; Three times rich in vitamin C as compared to cow milk; rich in unsaturated fatty acids and B vitamins. Milk from Bactrian camels has a higher percentage of fat than milk from dromedaries, but levels of proteins and lactose are similar.	Higher fat and protein contents than goat and cow milk; higher lactose content than milk from cows, buffaloes and goats. The high protein and overall solid contents of sheep milk make it particularly appropriate for cheese and yoghurt making.	Similar composition to cow milk.	Tastes sweet and has a fragrant, sweetish smell. Yak milk showed 15 and 18 % solid content, 5.5 to 9 % fat and 4 to 5.9 % protein. It therefore has higher solid, fat and protein contents than cow and goat milk, and resembles buffalo milk. Yak milk can be processed into a variety of milk products including butter, cheese and fermented milk products.	Horse and donkey milk showed similar compositions. Compared with that of other dairy species, equine milk contains low levels of fat and protein. Most equine milk is consumed fermented and it is not suitable for cheese making.

Table 2. Commercially available whey protein products.

Product description	Protein concentration	Fat, lactose, mineral content etc.
Whey protein isolate	90-95 %	Minute quantity
Whey protein concentrate	< 80 %	Minute quantity
Hydrolysed whey protein	Cleaves peptide bonds and creating smaller peptide fragments	Varies with protein concentration
Undenatured whey concentrate	25-89%; higher amount of lactoferrin and immunoglobulins	Decrease these contents when protein concentration increases.

In this regard, our group focused on lactoferrin capsule and determined its immunological activity in human whole blood against non-specific protein antigen i.e. Concanavalin (Con) A, T cell mitogen. For these studies, lysed human whole blood were cultured in 96 well plate along with variable doses of lactoferrin (1 – 10 mg/ml; 50 µl) in presence of Con A (5 µg/ml; 50 µl) and determined its proliferation assay using MTT. Incubate these samples which is exposed with lactoferrin and Con A for 24 h at 37°C, 5% carbon dioxide incubator. After incubation, centrifuging the plate and collected the supernatant for the estimation of Th1 (IFN-gamma and TNF alpha) cytokines and then add fresh media containing 10 % FBS. Add MTT solution (2.5 mg/ml; 10 µl) and then incubate the plate for another 4 h at carbon dioxide incubator. Afterwards, formazan crystals will appear and settled at the bottom and dissolved in dimethyl sulphoxide (DMSO) solution after centrifuging and discarding the supernatant. The optical density (OD) was

measured at 570 nm [15]. In this study, our results showed that lactoferrin at higher doses showed drastic declined in Con A proliferation as compared to control (**Fig.2**).

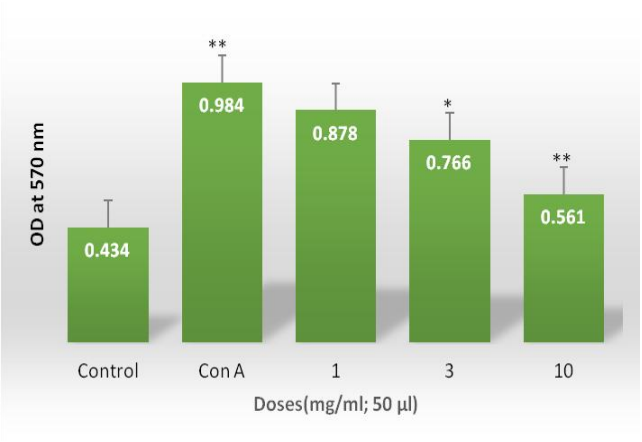

Fig.2. Proliferation assay. Lysed human whole blood samples were cultured with variable doses of lactoferrin (1-10 mg/ml; 50 µl) along with fixed concentration of Con A. Incubate these samples and determined its proliferation through MTT assay. Values are expressed as Mean ± S.E. The difference between control and variable doses of lactoferrin is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001

Further confirmation of these studies, Th1 cytokines (IFN-gamma and TNF alpha) were estimated in cell culture supernatant which is exposed with variable doses of lactoferrin content along with fixed concentration of Con A in human whole blood. In this assay, diluted capture antibodies (IFN-gamma and TNF alpha; 2 µg/ml) were added in two different Elisa plates and allowed to adhere overnight for 4 °C. After incubation, washed properly with PBS (pH 7.4) and blocked with bovine serum albumin (BSA, 1%) for 1 h at room temperature. After blocking, washing Elisa plates 2-3 times with PBS and then add serial dilutions of standard samples of cytokines (IFN-gamma and TNF alpha) along with cell culture supernatant samples of lactoferrin containing Con A. These samples were incubated for 4 h at carbon dioxide incubator. Afterwards, Elisa plates were washed (2-3 times) and then add working detector solution (including detector antibody and avidin–horse radish peroxidase reagent) was added. Elisa plates were sealed and incubated for 1 h at room temperature. After washing, tri-methyl benzidine (TMB, 100 µl) substrate was added into each well. Stop solution (2 N H2SO4) was finally added after incubation in the dark for 30 min at room temperature. The absorbance was read at 450 nm [15, 16]. The result was analysed using softmax program and values determined against the standard provided by the manufacturer. In this study, the results showed that lactoferrin showed rapid declined in Th1 cytokines (IFN-gamma and TNF alpha) at higher doses in cell culture supernatant which is exposed with Con A stimulated human whole blood samples (**Fig.3**). In other words, lactoferrin showed anti-inflammatory effect at higher doses. This effect could be due to decline in Con A proliferation (T cell population) along with pro-inflammatory cytokines (IFN-gamma and TNF alpha) at higher doses. In short, proinflammatory cytokine response of IFN-gamma and TNFα following Con A stimulation of human whole blood is profoundly inhibited by lactoferrin. So it may have a therapeutic potential as a modulator of cytokine effects in inflammatory disease.

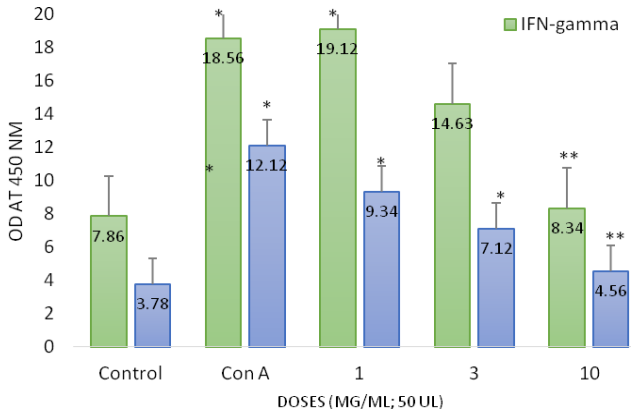

Fig.3. Th1 cytokines estimation. Lysed human whole blood samples were cultured with variable doses of lactoferrin (1-10 mg/ml; 50 µl) along with fixed concentration of Con A. After incubation, centrifuging these samples and collect cell culture supernatant for estimation of Th1 cytokines. Values are expressed as Mean ± S.E. The difference between control and variable doses of lactoferrin is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001

In contrast, antibacterial activity was also observed using variable concentration of lactoferrin on gram positive bacteria i.e. *bacillus subtilis* and gram negative bacteria i.e. *Pseudomonas aeruginosa* on human whole blood. In this study, serially diluted sample of lactoferrin content (1 – 10 mg/ml; 50 µl) along with fixed concentration of bacterial colony forming units (10^6 CFU/ml; 50 µl) on lysed human whole blood in flat bottom 96 well plate. Incubate these samples for 4-6 h at BOD. After incubation, centrifuging these samples at 10,000 rpm and then finally analysing the supernatant through UV visible spectrophotometer. The results of these studies showed that lactoferrin showed sudden decline in bacterial growth at higher concentration (**Fig.4**).

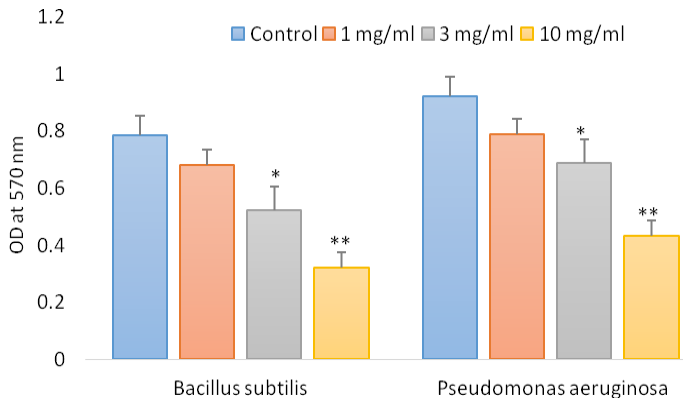

Fig.4. Antibacterial activity. Lysed human whole blood samples were cultured with variable doses of lactoferrin (1-10 mg/ml; 50 µl) along with fixed concentration of bacterial colony (CFU/ml). After incubation, centrifuging these samples and collect cell culture supernatant for estimated its antibacterial activity. Values are expressed as Mean ± S.E. The difference between control and variable doses of lactoferrin is controlled by one way ANOVA test (Bonferroni multiple comparison test). *P<0.05; **P<0.01 and ***P<0.001

Overall, the results indicates that lactoferrin showed its antibacterial activity. In short, lactoferrin showed many activities i.e. anti-inflammatory; antibacterial etc. In short, milk contained a wide variety of proteins which showed several immunobiologically activities ranging from antimicrobial effects to immunostimulatory functions. In addition, proteins in milk samples that provides adequate amount of essential amino acids to growing infants. This suggests a highly adapted digestive system, which allows the survival of some proteins and peptides in the upper gastrointestinal tract and amino acid utilization from them further down in the gut. Further studies should be taken for consideration related to immunopathological studies and these studies are still in progress.

CONCLUSION

Lactoferrin binding iron content that played a vital role in cell metabolism and also helpful in transporting oxygen. Actually, sports person normally use iron supplements to boost oxygenation and also help in order to improve its performance rate. In short, those sports person who take lactoferrin containing iron supplements that reduce the risk of iron related sickness and other problems. Generally, lactoferrin can bind and sequester lipopolysaccharides, thus preventing pro-inflammatory cytokines activation, sepsis and tissue damages. In other words, lactoferrin is also considered a cell-secreted mediator that bridges the innate and adaptive immune responses.

AUTHORS CONTRIBUTION

This work was carried out in collaboration between four authors. AG designed the study, wrote the protocol and interpreted the data where VP anchored the field study, gathered the initial data related to her M.Sc Microbiology dissertation work under AG guidance and performed preliminary data analysis. AG, VP, SK and BS managed the literature searches whereas AG produced the initial draft. The final manuscript has been read and approved by all authors.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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