International Archives of Medical Research 15:2 (2023) 27-34

ORIGINAL RESEARCH

Determination of Antibacterial Efficacy of Lactoferrin Glycoprotein Obtained from Cow Colostrum

Received Date: 12.07.2023, Accepted Date: 18.10.2023 **DOI: 10.56484/iamr.1326284**

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Abstract

Objective: *It was aimed to investigate the antibacterial effect levels of Lactoferrin protein in cow colostrum. The aim of the study was to evaluate the effect of lactoferrin protein obtained from a natural product on the growth of Escherichia coli, Pseudomonas aerugenosa and Staphylococcus aureus.*

Method: *Lactoferrin at 4 different concentrations (100 mg/ml, 50 mg/ml, 25mg/ml and 12.5mg/ml) was impregnated on 6 mm discs prepared from filter paper and placed on the MHA plate and after incubation at 37˚C for 24 hours, inhibition was evaluated. The antibacterial effect levels of naturally obtained lactoferrin at different concentrations were compared.*

Results: *It was determined that the antibacterial activity of lactoferrin against Gram-negative bacteria Pseudomonas aeruginosa was higher than the other bacterial species in the study. It was determined that Lactoferrin at a concentration of 12.5mg/ml had the least inhibitory effect in the studied bacteria. It was concluded that E.coli and S. aureus were bacteria resistant to the antibacterial activity of lactoferrin.*

Conclusion: *It was observed that this effect was limited in E.coli bacteria. In the light of these results, it was concluded that Lactoferrin, an antibacterial agent, has different antibacterial effects depending on the bacterial species and dose.*

Keywords: Cow colostrum, lactoferrin, antibacterial effect and whey protein.

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Introduction

Lactoferrin (Lf) was identified in bovine milk in 1939 and isolated from both human and bovine milk in 1960[1]. Lactoferrin, a multifunctional iron-binding glycoprotein, plays an important role in immune regulation and defense mechanisms against bacteria, fungi and viruses[2]. Colostrum is the first milk produced by mammals after birth and has a high nutritional profile[3]. It is a complex biological fluid that provides the baby with all the necessary nutrients, immunological and developmental factors and is significantly different from mature milk[4]. Colostrum contains proteins, lipids, essential fatty acids and amino acids in greater concentration than mature milk. It also contains oligosaccharides, lactoferrin, lysozyme, lactoperoxidase, proline-rich polypeptides, insulin, growth hormone, cytokines and nucleosides, all of which act as natural anti-microbials[5,6]. The fact that lactoferrin has a high iron binding ability and is the only protein in the transferrin family that can show this ability in a wide pH range, especially at very low pH values, is highly resistant to proteolysis, has a net positive charge and is found in many tissues[7]. Colostrum has the feature of preventing infections and diseases in humans and provides passive immunity to human and bovine newborns[8]. Bovine colostrum is very often used as a dietary supplement. Clinical studies have revealed that colostrum is taken by humans as supplements; exhibits antioxidant, immunomodulatory and anti-inflammatory activity[9]. Lf is 80-kD, and the protein consisting of 692 amino acids consists of a single polypeptide chain with 2 symmetrical folds to which it is glycosylated[10]. In most cases, enzymes activate the milk's natural antibacterial system and kill target microbes. The antimicrobial systems in natural milk are distinguished by their simultaneous attack on the oxidative and lytic mechanisms of microorganisms[11]. The second mechanism of lactoferrin and lactoferrin-derived peptides is in direct interaction with microorganisms through positively charged amino acids that interact with anionic molecules on certain bacterial, viral, fungal and parasitic surfaces, causing cell lysis[12]. Lactoferrin has high levels of amylase, DNase, RNase and ATPase activity. Therefore, Lf can damage the nucleic acids of bacteria through hydrolysis and inhibit the metabolism of the organism[13]. The antibacterial activity of lactoferrin varies depending on its concentration, interaction with other minerals in the environment, and the degree of saturation with iron. The antimicrobial activity of lactoferrin decreases when other macromolecules are bound and saturated with iron, but increases when it is found together with other antimicrobial proteins (lysozyme)[14]. It was determined that the main reason for the increase in antimicrobial activity was lactoferricin, a bioactive peptide released during enzymatic degradation. Lactoferricin is the 25 amino acid portion containing amino acids 18-42 at the N-terminus of cow's milk lactoferrin. The lactoferricin molecule folds into two globular units and each can bind one iron ion (Fe+3)[15].

Because of the bioactive components of colostrum, it may be useful in this study, reinforcing the notion that Lactoferrin is a significant barrier in the mucosal wall that is effective against bacterial infection attacks in terms of its capacity to exert a potent antibacterial activity through binding to host cells or viral particles. It can be applied as a new strategy in the treatment of pathogenic bacterial infections.

Materials and Method

This study was carried out in Dicle University Hospital Central Laboratory. In our study, after separating cow colostrum into whey protein fractions, lactoferrin protein was obtained as concentrated by using ultrafiltration membrane filter at 30kD. Total protein concentrations were measured according to the method of bicinchoninic acid colorimetric assay (BCA, Bio-Rad, USA). After lyophilization, the protein was ground into powder. Protein concentrations were measured according to the method of bicinchoninic acid colorimetric assay (BCA, Bio-Rad, USA). 10 µl of sample was taken and 40 µl of PBS was added and diluted 1/5. It was homogenized and 7 standards were added to 96 well wells and then two replicates of 20 µl samples were added respectively. After the auxiliary solutions were prepared in a sterile falcon tube, 160 µl was added to all wells, including the standard wells. The plate was closed and incubated in an oven at 37 ^{0}C for 30 minutes. Then, absorbance was measured at a wavelength of 562 nm in the Elisa Reader Thermo Scientific device.

For antibacterial analysis; Escherichia coli ATCC 25922 and Pseudomonas aerugenosa ATCC 27853 were used as Gram negative organisms, Staphylococcus aureus ATCC 29213 as Gram positive organisms. Tested bacterial strains were proliferated by incubating at 350°C in tubes containing Tryptic Soy Broth (TSB). 0.5 McFarland standard bacterial suspension was prepared from the bacterial culture obtained after 24 hours of incubation. Bacteria taken from the bacterial suspension using sterile cotton swabs were spread on the Mueller-Hinton Agar (MHA) surface and inoculated. Whatman No. Lactoferrin at 4 different concentrations (100 mg/ml, 50 mg/ml, 25mg/ml and 12.5mg/ml) was impregnated on 6 mm discs prepared from 1 filter paper and placed on the MHA plate. Inhibition was evaluated after the Petri dishes were incubated at 37˚C for 24 hours. A 1 mg/ml Ceftriaxone disc was used as a positive control. The antibacterial effect levels of naturally obtained lactoferrin at different concentrations were compared.

Statistical analyses

All analyzes were performed in duplicate and the data obtained as a result of the analyzes were subjected to analysis of variance (ANOVA) using the SPSS 22.0.0 package program. To compare the effects of statistically different concentrations, Duncan's multiple range test was analyzed at a significance level of $P < 0.05$.

Results

The antibacterial activity of lactoferrin was conducted on Gram-positive and Gram-negative bacterial strains. Total protein concentrations were measured according to the method of bicinchoninic acid colorimetric assay (BCA, Bio-Rad, USA). In these measurements, mean and standard deviation values at 100 mg/ml concentration were: 6894.5±21.99; 2513±36.77 at 50 mg/ml concentration; The values of 1154±35.35 at 25 mg/ml concentration and 923.5±0.70 at 12.5mg/ml concentration were determined statistically (Figures 1 and 2). Different concentrations of lactoferrin were prepared as the samples were 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5mg/ml. 1 mg/ml Ceftriaxone was used as a positive control. It was determined that the antibacterial activity of lactoferrin against Gram-negative bacteria Pseudomonas aeruginosa was higher than the other bacterial species in the study. It was observed that its antibacterial activity was limited in E.coli bacteria. It was determined that Lactoferrin at a concentration of 12.5mg/ml had the least inhibitory effect in the studied bacteria. It was concluded that E.coli and S. aureus were bacteria resistant to the antibacterial activity of lactoferrin. (Pictures 1,2 and 3). It was determined that lactoferrin applied in Pseudomonas aeruginosa bacterial species inhibited bacterial proliferation at high concentration. Matijasic et al., (2020) investigated the antimicrobial effects of lactoferrin isolated from whey and produced in the pilot plant by disc diffusion method. The bacteria examined were Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Salmonella enterica, Clostridium difficile, Klebsiella oxytoca and Clostridium perfringens. As a result of the study, partial or complete inhibitory properties were observed on all bacteria except lactic acid bacteria and Clostridium bacteria[16]. In this study, lactoferrin isolated from whey showed an inhibitory effect against Pseudomonas aeruginosa bacteria. The antibacterial activity of lactoferrin varies depending on the protein concentration and the degree of saturation with iron. The activity level of these antimicrobial molecules decreases when they are saturated with iron, and increases when they are found together with other antimicrobial proteins (lysozyme)[17]. Lactoferrin has serine protease activity and can degrade the arginine-rich portion of proteins. It can show antibacterial activity by degrading lactoferrin with many serine protease activities[18]. Lactoferrin; It also causes inhibition by triggering the fragmentation of intracellular invasive plasmid antigens such as Escherichia coli, Listeria monocytogenes and Shigella flexneri[19]. In fact, it has been demonstrated that the candidacidal activity of lactoferrampin is higher than that of lactoferrin and that it has antibacterial activity against many bacteria such as Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa[20]. The inhibition effect of lactoferrin obtained from the cow colostrum fraction against Escherichia coli and Pseudomonas aeruginosa bacteria is in parallel with the above study.

Table 1. *Data Obtained by BCA Method of Concentrated Lactoferrin from Cow Whey Protein*

S_1	S_2	S_3	S_4	S_5	S_6	S_7	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
2000	1500	1000	750	500	250	125	6739	2487	1129	924.8
2000	1500	1000	750	500	250	125	7050	2539	1179	923

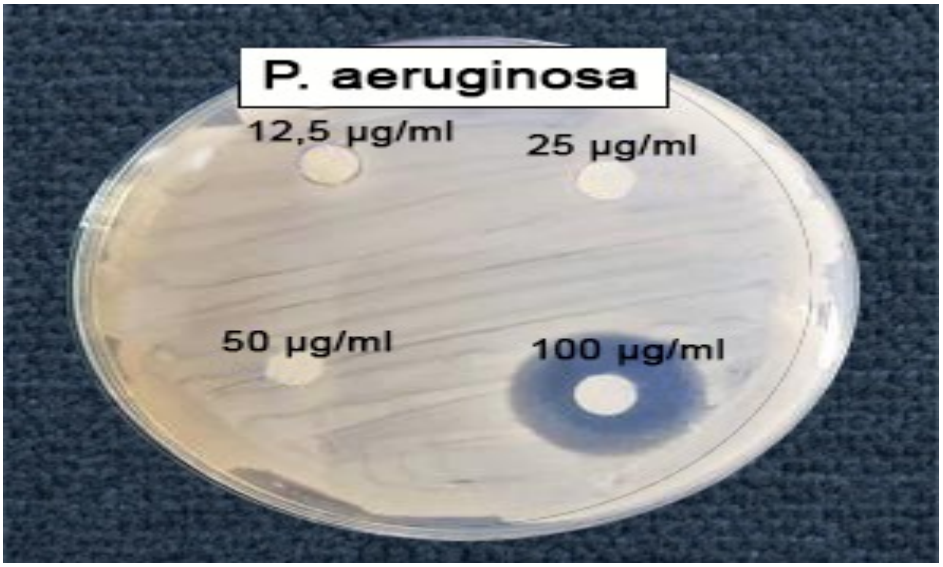
S_1, S_2, S_3, S_4, S_5, S_6, S_7 : BCA Yönteminde Kullanılan Standartlar

Table 2. *Statistical Analysis of Lactoferrin BCA Total Protein*

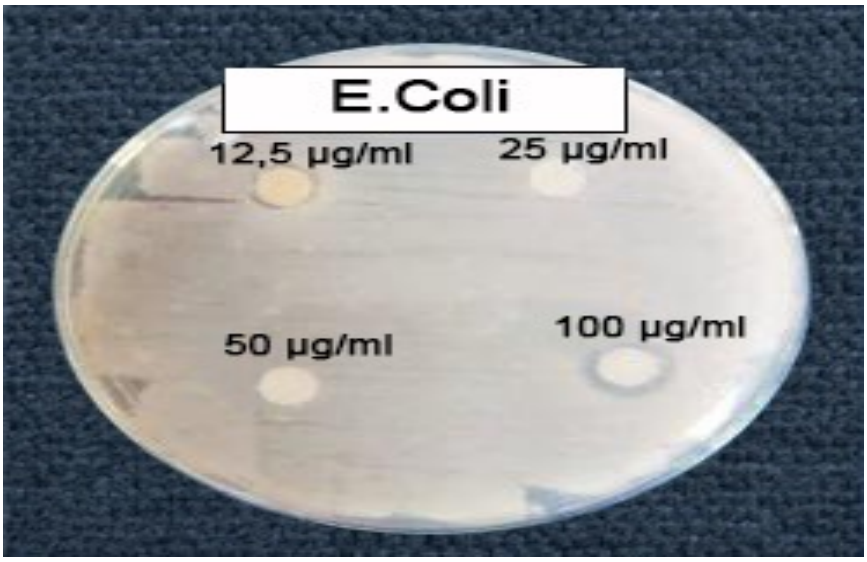
S_1 Mean±Sd	S_2	S_3	S_4	S_5	S_6	S_7
2000[a]	1500[a]	1000[a]	750[a]	500[a]	250[a]	125[a]
P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05	P<0.05

100mg/ml	50mg/ml	25mg/ml	12.5mg/ml
6894.5±21.99[a]	2513±36.77[a]	1154±35.35[a]	923.5±0.7[b]
P<0.05	P<0.05	P<0.05	P<0.05

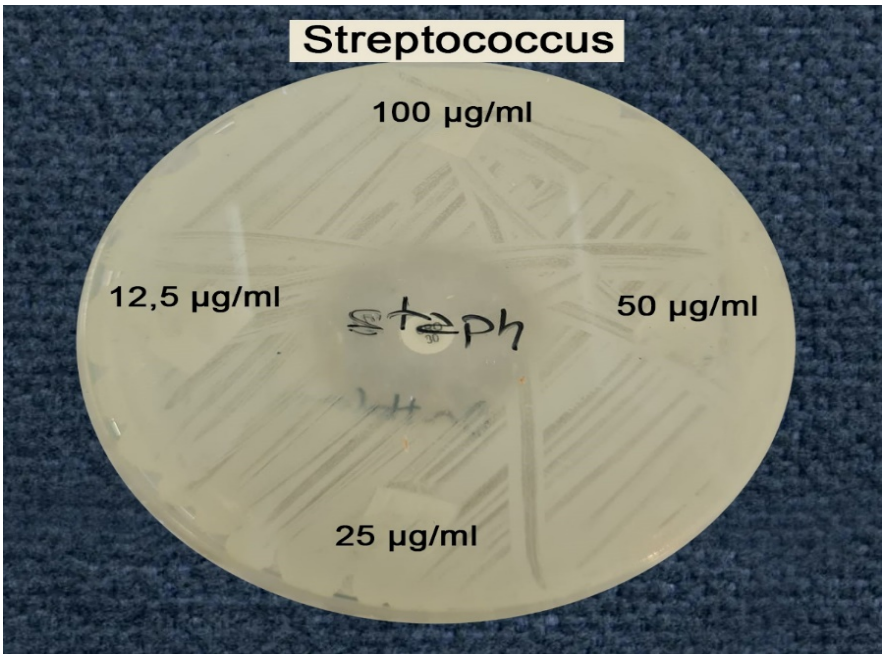
[a,b]Absorbanslar arasında anlamlılık düzeyi faklı olanlar aynı harflerle ifade edilmiştir.



Picture 1. *Effect of Lactoferrin on P.aeruginosa Bacteria at Different Doses*



Picture 2. *Effect of Lactoferrin on E.coli Bacteria at Different Doses*



Picture 3. *Staphylococcus aureus Effect level of Lactoferrin at different doses*

Conclusion:

Lactoferrin glycoprotein obtained from bovine colostrum has a significant and dose-dependent antibacterial effect on the growth of P. aeruginosa. In this study, we think that the inhibitory effect against Pseudomonas aeruginosa bacteria may be due to the serine protease activity of this protein. In our study, we determined that the inhibition effect of Lf on E. coli bacteria was limited. Lactoferrampin, determined from the N1 domain of cow's milk lactoferrin, was also found to have antimicrobial activity. S. aureus bacteria were found to be resistant to all doses of lactoferrin

protein. In the light of these results, it was concluded that Lactoferrin, an antibacterial agent, has different antibacterial effects depending on the bacterial species and dose.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

Financial Disclosure: This study was supported by Dicle University Scientific Research Project Coordinator, TIP 20.029.

References

1- **Soerensen M, Soerensen SPL.** Proteins in whey. CR Trav. Lab. Carlsberg. 1940; 23 (7): 55-99.

2- **Johansson B.** Isolation of iron-containing red protein from human milk. Açta Chem. Scand. 1960; 14:510-512. doi: 10.3891 / acta.chem.scand.14-0510.

3- **Christiansen S, Guo M, Kjelden D.** Chemical composition and nutrient profile of low molecular weight fraction of bovine colostrum. Int. Dairy J 2010; 20, 630–636. DOI:10.1016/j.idairyj.2009.12.005

4- **Godhia ML and Patel N.** Colostrum İts composition, benefits as a nutraceutical: a review. Current Res. Nutr. Food Sci. 2013; 1:37-47. DOI:10.12944/crnfsj.1.1.04

5- **Kelly GS.** Bovine colostrum: a review of clinical uses. Altern. Med. Rev. 2003; 8:378-394.

6- **McGrath BA, Fox PF, McSweeney PLH, Kelly AL.** Composition and properties of bovine colostrum: a review. Dairy Sci. And Technol. 2016; 96:133-158. DOI 10.1007/s13594-015-0258-x.

7- **Gonzalez-Chavez SA, Arevalo-Gallego S, Rascon-Cruz Q.** Lactoferrin: structure, function and applications. International Journal of Antimicrobial Agents 2009; 33:301-309.

8- **Sahana N, Ramasamy D, Pugazhenthi TR, Manikkavasagan I.** Studies on physical and microbial parameters of bovine colostrum powder. Int. J. Curr. Microbiol. App. Sci.7, 2018: 2766- 2771. DOI:10.20546/ijcmas.2018.708.290

9- **Korohnen H.** Colostrum immunoglobulins andthe complement system-potential ingredients offunctional foods. Bulletin Inter. Dairy Federation, Brussels. 1998: 36-40.

10- **Baveye S, Elass E, Mazurier J, Spik G, Legrand D.** (Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. Clin Chem Lab Med. 1999; 37:281-286. doi: 10.1515 / CCLM.1999.049

11- **Seifu E, Buys EM, Donkin EF.** Signifcance of the lactoperoxidase system in the dairy industry and its potential applications: a review. Trends Food Sci. Technol. 16, 2005: 137- 154 https://doi.org/10.1016/j.tifs.2004.11.002

12- **Ammons M and Copié V.** Mini-review: lactoferrin: a bioinspired, anti-bioflm therapeutic. Biofouling. 2013; 29:443-455.

13- **Gonzalez-Chavez SA, Arévalo-Gallegos S, Rascón-Cruz Q.** Lactoferrin: structure, function and applications. Int. J. of Antimicrob. Agents, 2009; 33:301-308. https://doi.org/10.1016/j.ijantimicag.2008.07.02

14- **Farnaud S, Evans RW.** Lactoferrin: a multifunctional protein with antimicrobial properties. Molecular Immunology. 2003; 40:395–405.

15- **Jones EM, Smart A, Bloomberg G, Burgess L, Millar MR.** Lactoferricin, a new antimicrobial peptide. Journal of Applied Microbiology. 1994; 77:208-214.

16- **Matijasic BB, Oberckal J, Lorbeg PM et al.** Characterisation of lactoferrin isolated from acidwhey using pilot-scale monolithic ion-exchangechromatography Processes. 2020; 8(7):804-811. https://doi.org/10.3390/pr8070804.

17- **Farnaud S, Evans RW.** Lactoferrin: amultifunctional protein with antimicrobialproperties. Molecular Immunology. 2003; 40:395–405.

18- **Plaut AG, Qiu J, St Geme JW.** Humanlactoferrin proteolitic activity: analysis of thecleaved region in the IgA protease of Haemophilusinfluenzae. Vaccine 2000; 8:148–152.

19- **Longhi C, Conte MP, Seganti L et al.** Influence of lactoferrin on the entry process ofEscherichia coli HB101 (pRI203) in HeLa cells.Medical Microbiology and Immunology (Berl). 1993; 182:25–35.

20- **Van der Kraan, MIA, Groenink J et al.** Lactoferrampin: a novelantimicrobial peptide in the N1-domain of bovinelactoferrin. Peptides. 2004; 25:177–183.