Mesopotamia Journal of Agriculture

https://magrj.uomosul.edu.iq/

ESTIMATION OF THE PERCENTAGE OF LACTOFERRIN AND IMMUNE PROTEINS IgG IN BUFFALO COLOSTRUM WITHIN FIVE DAYS OF BIRTH

Saja M. Qasim [1], Taha M. T. Mohammed [2], Sumyia K. Badawi [3]
Department of Food Science, College of Agriculture and Forestry, University of Mosul, Iraq 1,2,3

Article information
Article history:
Received: 7/5/2024
Accepted: 14/7/2024
Published: 31/12/2024

Keywords:
Biologically active proteins, immune globulin IgG, lactoferrin, milk proteins.

DOI:
https://doi.org/10.33899/mja.2024.149536.1430

Correspondence Email:
Saja.20agp72@student.uomosul.edu.iq

ABSTRACT

The study aimed to estimate the proportions of lactoferrin and IgG immunoglobulins, and to separate lactoferrin from buffalo colostrum. Buffalo colostrum samples were collected from the first hour to the five days after birth. Then, the percentages of lactoferrin were estimated using HPLC technology. The percentage of IgG using an ELISA device in colostrum, after that the lactoferrin protein was separated from the colostrum by the sodium dodecyl sulfate-polyacrylamide Gel electrophoresis (SDS-PAGE) method, and the protein was extracted from the Gel (SDS-PAGE). The results showed that the lactoferrin level was higher on the first day, after and decrease within days of milk production. The IgG level was higher in the first hour; afterward, it decreased and then increased on the fourth day, reaching 30.46 mg/ml. Lactoferrin was separated by electrophoresis based on the molecular weight of the standard lactoferrin sample. Clear lactoferrin separation areas appeared on the acrylamide Gel. Lactoferrin was then extracted by SDS-PAGE and eluted from the Gel.

College of Agriculture and Forestry, University of Mosul.

INTRODUCTION

Buffalo production varies depending on many factors, including the sequence of births and the season of birth, which affect the productive characteristics represented by the total amount of milk, the length of the milking season, as well as the drought period (Mahmoud *et al*., 2005). Al-Mallah *et al* (2018) mentioned that animal nutrition has an effect on colostrum productivity.

Colostrum was a biological food for new-borns in all types of mammalians during the first 5 to 7 days after birth it was known as regular food source for initial micro and macro-nutrients-Peptides have an antimicrobial activity and factors growth, Moreover, colostrum contains more protective proteins than natural milk, due to presence of both lactoferrin and IgG, these proteins considered as milk preservative materials (Steijns *et al*., 2001). Lactoferrin is one of the non-specialized immune proteins in colostrum; it belongs to the essential glycoproteins of the central transferrin family and can efficiently bind with iron (Talukder, 2021). Furthermore, it has an inhibitory effect on various types of negative and positive Gram- stain bacteria (Ochoa and Cleary, 2009). Jahani (2015) stated that lactoferrin affected bacteria positive for the Gram-stain more than negative bacteria. Yassin (2023) reported that it has an inhibitory effect on E. coli and S. aureus bacteria. It has a major role in inhibiting the oxidation of fats and oils (Satué-Gracia, 2000). It can produce

several peptides (Piacentini *et al*., 2024). These derived peptides have an essential role as a natural addition to combat large range of diseases, including cancer, inflammation, and allergies, due to their highly biological effectiveness hindering growth of the living organisms and inhibiting peroxides (Kanwar *et al*., 2015., and Kim *et al*., 2010). It can be a natural antioxidant (Hanoush., 2023). Qader and Tayeb, (2024) stated that the action of natural antioxidants is to reduce the enzyme that catalyzes oxidation and raise the antioxidant enzyme. Fadhil (2022) mentioned the possibility of using nutritional supplements using nanotechnology, including lactoferrin.

IgG immune proteins are present in colostrum and milk, their percentage in colostrum is often higher than milk; these proteins have a crucial role in transferring immunity against microbes to the newborn and, supporting maturation of the newborn's immune system. It also shows anti-bacterial, anti-fungal, anti-parasitic, and anti-tumor activities (Superti, 2020). There are several methods to separate and extract lactoferrin protein from colostrum; some methods have disadvantages, including loss of activity, low yield, cumbersome procedures, high economic cost, and environmental negative impact (Neelam *et al*., 2022). As lactoferrin is a significant type of biologically active protein, it has a relatively high isoelectric point (pI) compared to other milk proteins. Thus, it's suitable to isolate lactoferrin using this method. The SDS-PAGE electrophoresis method is considered a method for separating proteins with high accuracy and complex mixtures of proteins (Nowakowski, 2014). High-Performance Liquid Chromatography (HPLC) has become a vital technology used to separate and characterize proteins and peptides. Based on the differences in polarity and non-polarity in the stationary and mobile phases (Pochet *et al*., 2018). The primary objective was to estimate the lactoferrin content in the colostrum using High-Performance Liquid Chromatography (HPLC).

MATERIALS AND METHODS

Colostrum samples were collected from the Hawi al-Jawsaq area in the Mosul--Iraq, spanning from the first hour of birth to the fifth day after birth. These samples were kept frozen until they underwent quantitative analysis to ensure the preservation of their biochemical properties. Colostrum sample blanks were prepared, and a centrifugation process was employed to remove any fat content. Samples were centrifuged at 3000-4000 rpm for 30 minutes at 4four °C to achieve a clear supernatant for subsequent testing. Following centrifugation, the samples underwent a dialysis process using membrane osmosis to separate lactose and salts from the colostrum over a 24-hour, ensuring that only the desired components were retained for analysis.

Buffalo colostrum samples were stored according to the procedures recommended by Shimadzu Co., which included specific protocols for maintaining sample integrity. The preparation process involved centrifugation but also filtration to eliminate any particulate matter that could interfere with the HPLC analysis. The HPLC system used for this analysis was equipped with a C18 column, maintained at room temperature to ensure optimal separation conditions. The mobile phase comprised 5% acetonitrile and 95% water, with the addition of 0.1% trifluoroacetic acid (TFA) to aid in the proper elution of lactoferrin. Detection of lactoferrin was

performed at a wavelength of 280 nm, allowing for precise measurement of its concentration. Colostrum samples were introduced into the HPLC system at a 1 ml/min flow rate, with lactoferrin peaks identified at a retention time of 2.592 minutes. The accuracy of these measurements was confirmed by comparing them to a standard lactoferrin sample. In addition to lactoferrin, the percentage of IgG immune proteins in the colostrum was estimated using a commercially available test kit, following the method specified in the kit's instructions. This estimation was carried out using an Enzyme-Linked Immunosorbent Assay (ELISA) device, which provided a reliable quantification of IgG levels, crucial for assessing the immunological quality of the colostrum.

Protein isolation from the colostrum samples was also performed using the sodium dodecyl sulfate-polyacrylamide Gel electrophoresis (SDS-PAGE) method, as described by (Neelam *et al*., 2022, Laemmli 1970, and Thermo Fisher, 2019). This method involved the denaturation of proteins, followed by their separation based on molecular weight, allowing for detailed analysis of the protein composition of the colostrum. SDS-PAGE provided a clear profile of the various proteins, contributing to a comprehensive understanding of the colostrum's biochemical properties. This detailed characterization is essential for evaluating buffalo colostrum's nutritional and therapeutic potential, particularly in the context of enhancing neonatal health and immunity.

RESULTS AND DISCUSSION

Estimation of the percentage of lactoferrin in buffalo colostrum

Table (1) displays the results of quantitative estimation of lactoferrin isolated from buffalo colostrum from the first hour 5 days after birth. It was noted that lactoferrin percentage in the first hour after birth reached about 1.907 mg/ml and then increased during the first day after birth to 7.972 mg/ml. Then, from the second, the percentage level decreases gradually to around 1.230 mg/ml. while the third day showed a slight increase on the third day to 1.972 mg/ml, then returned and decreased on the fourth day to 0.443 mg/ml. Thus, the highest lactoferrin percentage was recorded on the first day after birth. In contrast, the lowest lactoferrin percentage was observed on the fourth day after birth when its composition approaches that of natural milk. These results were consistent with previous research (Abd EL-Fattah *et al*., 2012), confirmed in their study that the concentration of lactoferrin in buffalo colostrum was highest on the first day of birth, reaching 1.085 mg/ml, and it decreased significantly to get the lowest value on the 14th day after birth, to 0.123 mg/ml It is less than what was found in Mahran (2007) research study, which showed that the lactoferrin concentration in buffalo colostrum on the first day of birth was 0.591 mg/ml. Then, it decreased on the fourth day to 0.225 mg/ml. Then, it continued decreasing until it reached 0.152 mg/ml 14 days after birth. The study also concluded that lactoferrin levels do not differ much in natural milk compared to the difference in lactoferrin levels in colostrum (Guiseppina *et al*., 2013).

The reason for the increase in lactoferrin in colostrum after birth to give the newborn passive immunity is through its effect on a wide range of pathogens. Therefore, it has been proven effective in supporting resistance to viruses, bacteria and in modifying the immune system in infants (Li *et al*., 2019). Lactoferrin levels

are highest in the first days after birth due to the incomplete development of their immune system at birth (Ronayne *et al*., 2000). There are many factors responsible for the variation in lactoferrin concentration in colostrum and milk, including season, breed, stage of milk production, nutrition, biological conditions, and method of estimation (Levieux *et al*., 2006). Figure (1) shows the proportions of lactoferrin, and Figures (2) and (3) show the Determination of lactoferrin and retention time at 2.592 minutes and measured at a wavelength (280nm).

Table (1): The ratio of lactoferrin in Buffalo colostrum (mg/ml).

Period	HPLC Heights	Final Con. mg /ml
1st hour	422.118	1.907
1st day	1764.414	7.977
2nd day	272.125	1.229
3th day	436.617	1.973
4th day	98.006	0.443

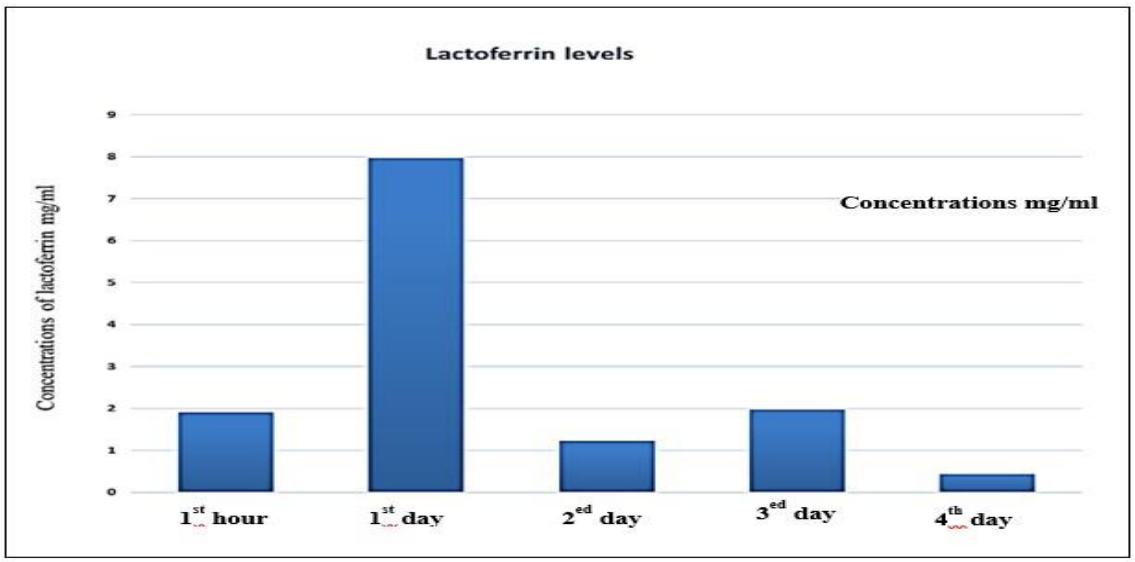


Figure (1): The ratio of lactoferrin in Buffalo colostrum

Percentage of IgG immune proteins

As can be seen in Figure (4), the percentage of immune proteins in the first hour was 22.15 mg/ml. Then starts to decrease on the first day 10.71 mg/ml then it was increased again to reach 30.46 mg/ml on the fourth day which recorded the highest value of 30.46 mg/ml. Rise in IgG as it considered as an important element in the immune activity present in the infant's first food because it is obtained directly from the mother (Wheeler *et al*., 2007). As it is known, the percentage of IgG in ruminants is the highest because the newborn receives IgG from colostrum within 2-3 days after birth, while in colostrum and human milk, the percentage of IgG is found to be lower. The reason is that the fetus receives a high percentage of IgG involuntary from the placenta before birth. Additionally, it obtains more immunity from colostrum after birth (Dingess *et al.*, 2022).

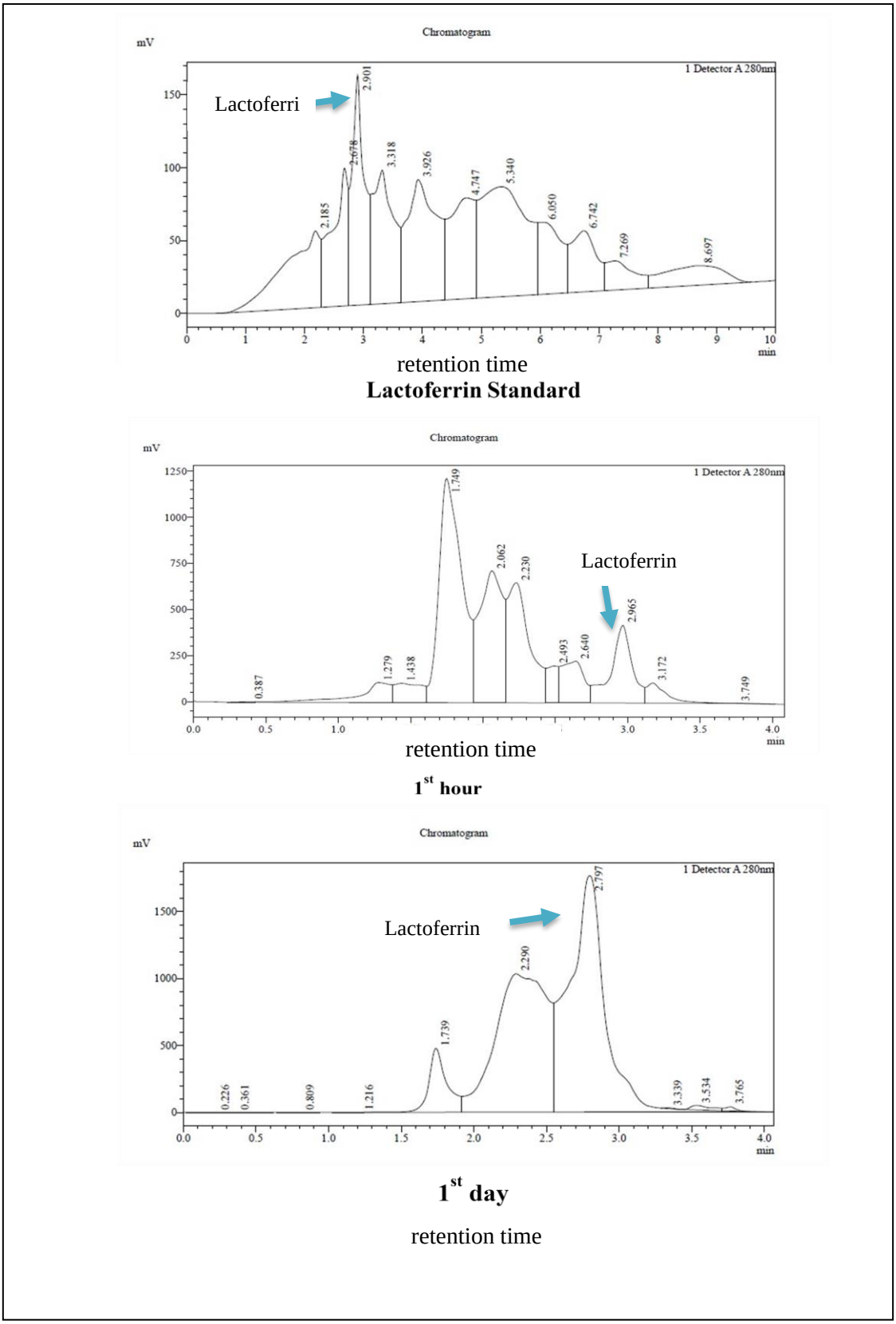
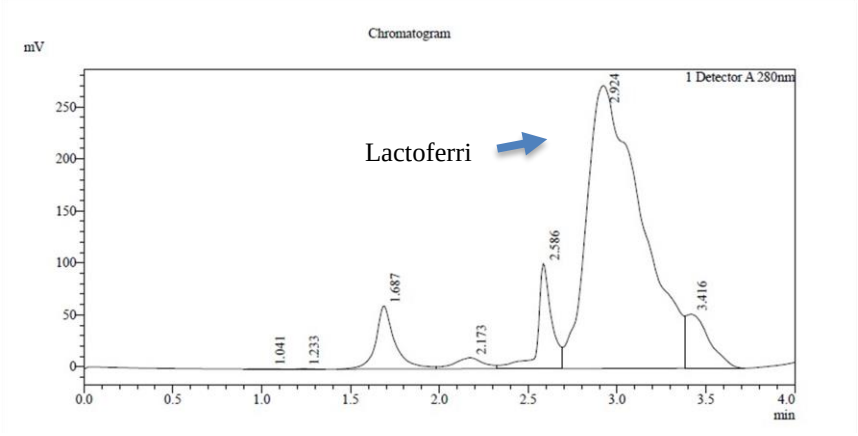
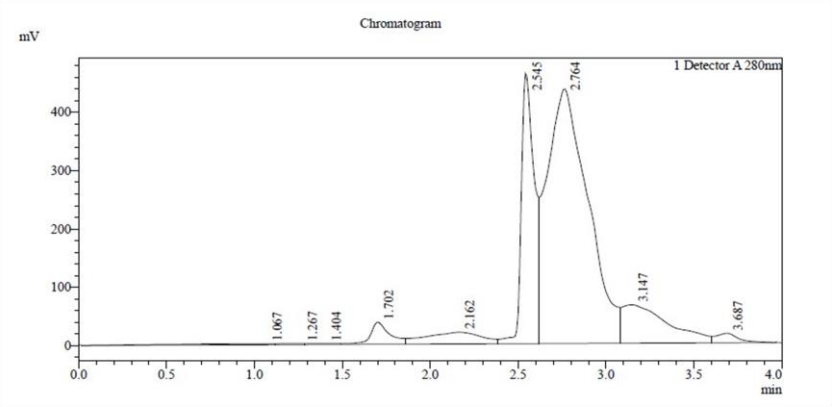


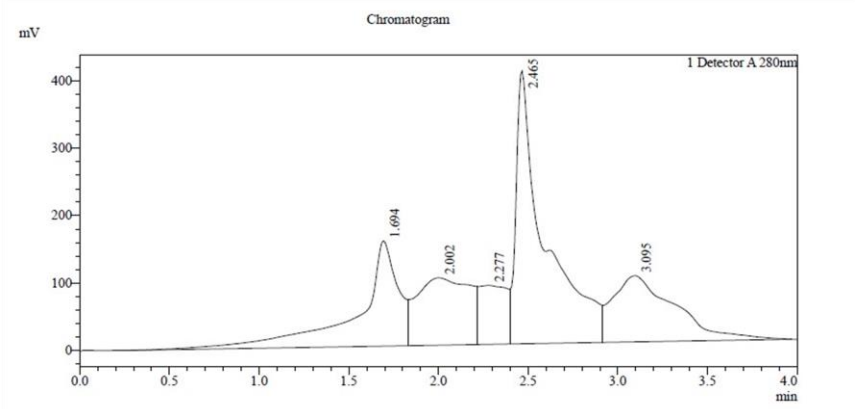
Figure (2): Determination of lactoferrin and retention time at 2.592 minutes and measured at a wavelength (280 nm).



2ed day retention time



3ed day retention time



4th day retention time

Figure (3): Determination of lactoferrin and retention time at 2.592 minutes and measured at a wavelength (280 nm).

The percentage of IgG is high in colostrum in the first days of birth due to calves' need for immunity and the acquisition of immune bodies due to their lack of immunity before birth from the mother through the placenta because it does not allow the penetration of substances into calves, unlike other mammals (Godden 2008), and therefore IgG shows antigenic activity. It contains microbes and protects the newborn from infection until his immune system develops (Baumrucker, 2014). Furthermore, the reported results in this study are consistent with research by Abd El-Fattah *et al*. (2012). They noted that IgG percentage can be high after birth when it reaches up to 34 mg/ml. Then, it decreased on the second day to 11.22 mg/ml. Then, it continued decreasing to reach 0.12 mg/ml. Intestinal permeability in newborn calves is high in the first hours, as the percentage of IgG is high in the colostrum at the beginning of birth, after which it begins to decrease as the lactation stage advances (Bush and Stanley, 1980).

The different reasons for IgG percentage behavior belong to differences between one animal and the other and the season of animal breeding. For example, Konuspayeva *et al*. (2017) mentioned that the difference in IgG percentage in camel milk could be affected by region, season, and type. Seasonal effects were the most prominent difference factor, as the winter IgG value was the highest. Ali and Amin (2008) showed that the percentage of IgG immunoglobulins in sheep colostrum decreased during the milking stage; it was 61.4 mg/ml 6 hours after birth, then reduced to 48 mg/ml after 240 hours of milking. The percentage of immunoglobulin was found to be high in colostrum and began to decline after that until reaching the average percentage in natural milk. It was reported that the percentage of IgG in goat colostrum was 63.3 mg/ml 6 hours after birth. The percentage decreased to 44.3 mg/ml after 240 hours. The percentage of sheep colostrum was lower, as it was 61.4 mg/ ml a few hours after birth, and there was also a decrease, reaching 41.0 mg/ml 240 hours after birth. Giammarco *et al*. (2021) found the percentage of IgG in buffalo colostrum to average 29.3-64.9 mg/ml. Kessler *et al*. (2020) mentioned a wide variation in IgG concentration between breeds, ranging between 12.7-204.1 mg/ml, and that the reason for this variation is not only due to the difference in breed; there is also variation in the individuality of the animals and other external factors, including the drought period, the number of previous births, the length of the gestation period, seasonal influence, etc.

Solation and extraction of lactoferrin from buffalo colostrum by Gel electrophoresis method

Figure (5) shows the method of separating lactoferrin protein by sodium dodecyl sulfate-polyacrylamide Gel electrophoresis (SDS-PAGE) of buffalo colostrum from first hour of birth to fifth day after birth. Figure (5) shows the Gel obtained by electrophoresis of lactoferrin with standard lactoferrin. According to the molecular weight path, a band of greater intensity of about 84 kDa can be observed. Neelam *et al*. (2022) isolated lactoferrin from camel milk using the same method in presence of bovine lactoferrin as a standard sample to determine the molecular weight. Then, lactoferrin protein was extracted from the Gel pieces after identifying lactoferrin bundle areas in the Gel. The process of recovering the protein requires liberating it from the Gel. This process often accrued after identify the lactoferrin area in Gel. The lactoferrin Gel pieces were cut and placed in special solutions (the rinsing

process). Centrifuge step was performed to extract the protein from the Gel, as shown in Figure (6). Biji and Hal, (2012) mentioned the possibility of extracting several types of proteins and enzymes from the Gel in this way. (Zrehen *et al.*, 2020) stated that among the advantages of separation by the SDS-PAGE method is separation based on protein size, short analysis time, low sample consumption, and most importantly, identifying a single protein separation technique.

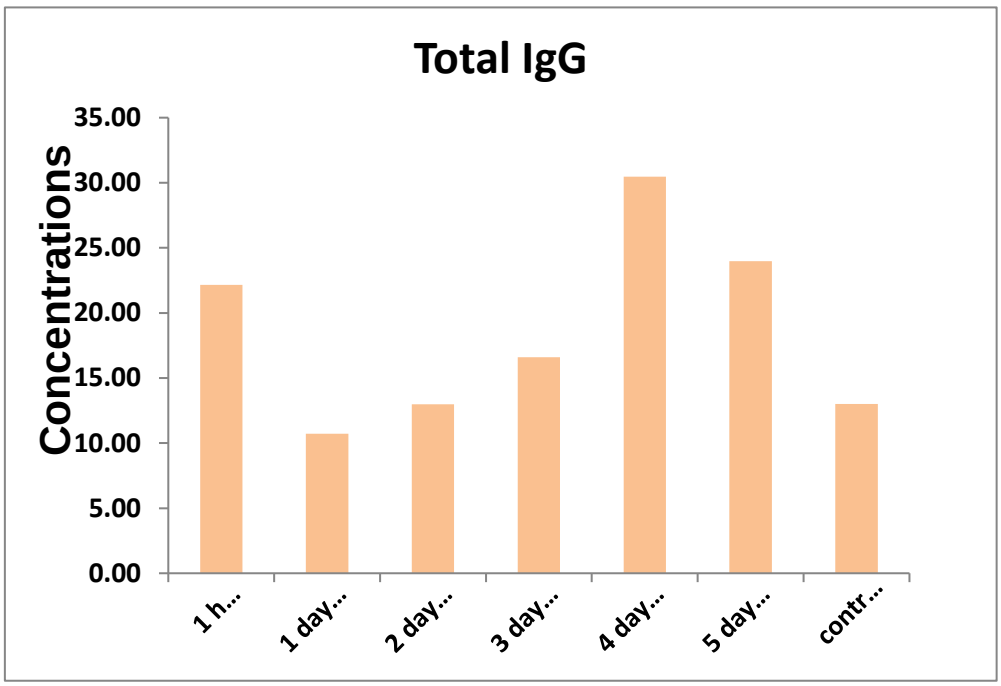


Figure (4): The ratio of IgG immune proteins in buffalo colostrum.

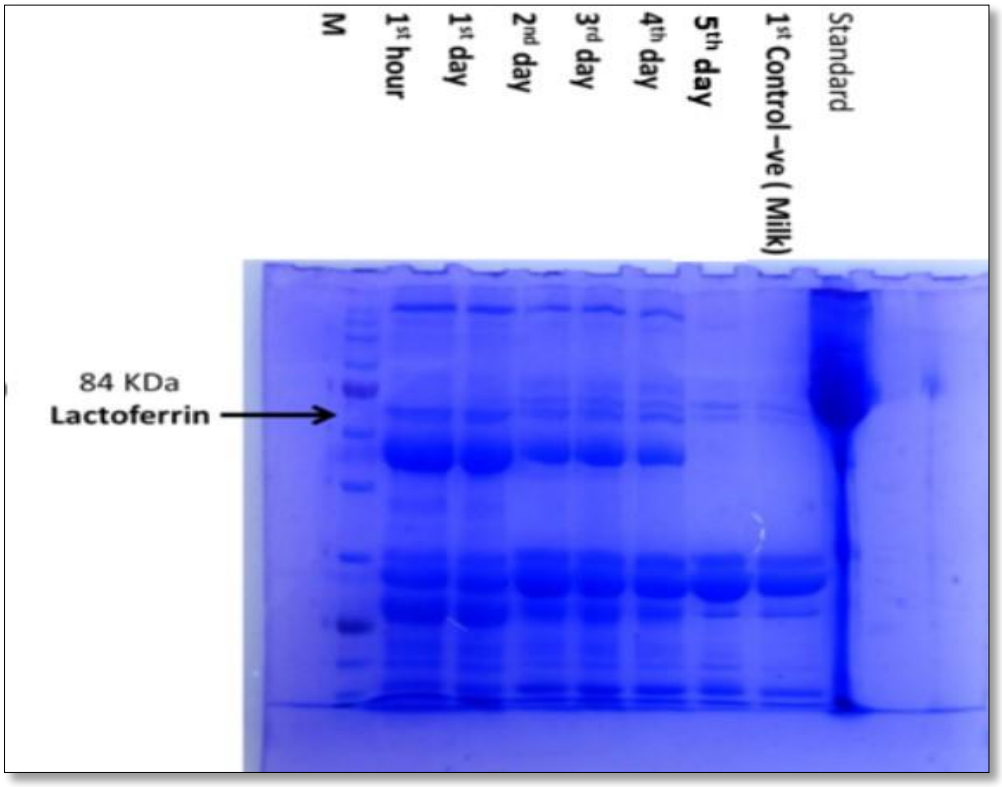


Figure (5): Isolation of lactoferrin by SDS-PAGE electrophoresis

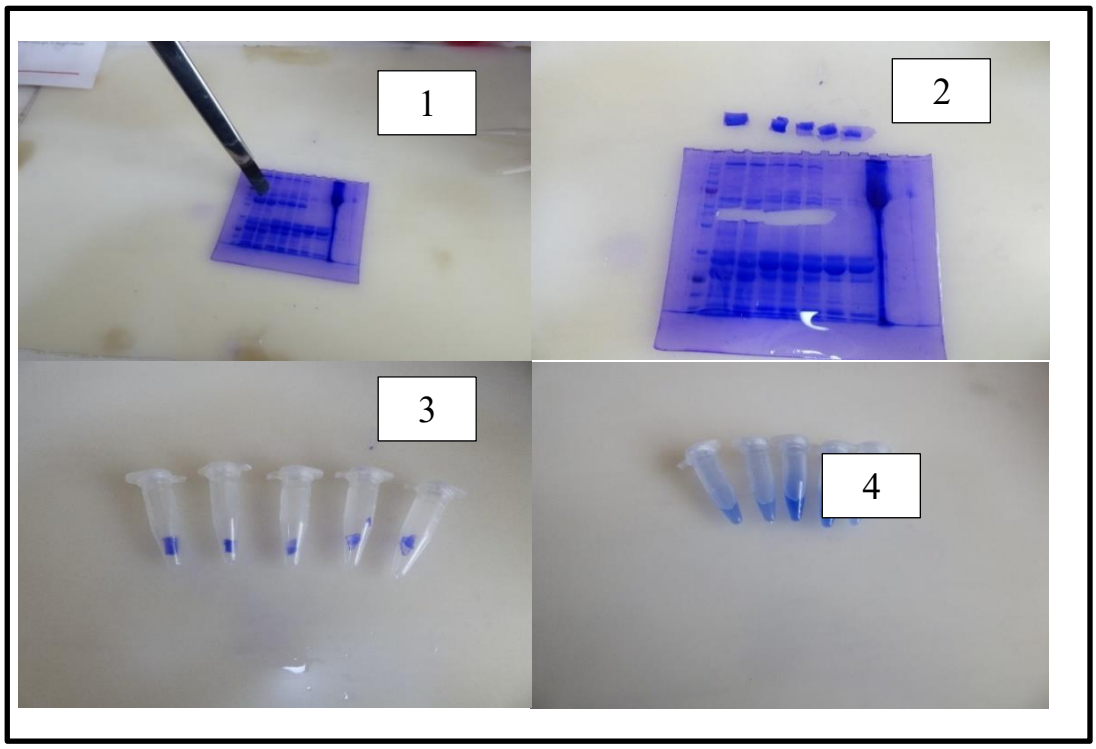


Figure (6): Stages of protein extraction from SDS-PAGE Gel.

CONCLUSIONS

From this study, we conclude that levels of lactoferrin and IgG are high in the first days after birth, and then begin to decrease as colostrum turns into natural milk several days after birth. The study also showed the possibility of separating lactoferrin by electrophoresis. The study recommends estimating the level of lactoferrin from other sources and the level of IgG immunoglobulin at different stages of breastfeeding, and using simpler lactoferrin isolation methods due to the possibility of using lactoferrin in many fields.

ACKNOWLEDGMENT

The researcher extends thanks and appreciation to the Department of Food Sciences / College of Agriculture and Forestry / University of Mosul for the opportunity to complete the research and assistance in providing materials and laboratories.

CONFLICT OF INTEREST

The authors report no conflicts of interest and are responsible for the content and writing of the paper.

تقدير نسبة البروتينات المناعية اللاكتوفيرين وIgG في لبأ الجاموس خلال خمسة أيام من الولادة

سجى مروان قاسم [1]، طه محمد تقي محمد [2]، سمية خلف بدوي [3]
قسم علوم الاغذية / كلية الزراعة والغابات / جامعة الموصل / الموصل / العراق[1، 2، 3]

الخلاصة

أجريت الدراسة لتقدير نسب البروتينات النشطة بايولوجياً اللاكتوفيرين والكلوبيولينات المناعي IgG، وفصل اللاكتوفيرين من لبأ الجاموس. تم جمع عينات اللبأ الجاموسي من الساعة الأولى الى اليوم الخامس بعد الولادة. ثم تم تقدير نسب اللاكتوفيرين باستخدام تقنية HPLC ونسبة IgG بجهاز ELISA في اللبأ الأيام الخمسة بعد الولادة، بعدها تم فصل بروتين اللاكتوفيرين من اللبأ بواسطة طريقة الترحيل الكهربي للهلام بولي أكريلامايد كبريتات دوديسيل الصوديوم (SDS–PAGE)، وتم استخلاص البروتين من هلام (SDS–PAGE) . أظهرت النتائج أن نسبة اللاكتوفيرين كانت مرتفعة في اليوم الأول، بعد ذلك بدأت بالانخفاض خلال أيام من مرور إنتاج الحليب، نسبة الكلوبيولين المناعي IgGكانت مرتفعة في الساعة الأولى، بعدها حصل انخفاض ثم الارتفاع في اليوم الرابع وصل إلى 30.46 ملغم/مل. تم فصل اللاكتوفيرين بطريقة الترحيل الكهربائي بالاعتماد على الوزن الجزيئي لعينة اللاكتوفيرين القياسية، ظهرت مناطق فصل اللاكتوفيرين واضحة على الهلام الاكريلامايد، بعدها تم استخلاص اللاكتوفيرين من الهلام وباستخدام محاليل والحصول على اللاكتوفيرين المعزول.

الكلمات المفتاحية: البروتينات النشطة بيولوجيا، الكلوبيولين المناعي IgG، اللاكتوفيرين، بروتينات الحليب.

REFERENCES

Abd El-Fattah, A. M., Abd Rabo, F. H., El-Dieb, S. M., & El-Kashef, H. A. (2012). Changes in composition of colostrum of Egyptian buffaloes and Holstein cows. *BMC veterinary research*, *8*, 1-7. https://doi.org/10.1186/1746-6148-8-19

Ali, M. M, Amin, N. M. (2008). Immune globulins in colostrum, milk, and blood serum of sheep and goats. *Mesopotamia Agriculture Journal*,36(3),74-87. https://doi.org/10.33899/magrj.2008.26808

AlMallah, O. D., M. N. Abdullah, N.Y. Abbo, and G. K. Khattab, (2018). Effect of feeding formaldehyde treated barley on producing colostrum and milk and their components and some blood parameters in Damascus goats. *Mesopotamia J. Agri*. 46 (2): 148-157. http://doi.org/10.33899/magrj.2018.161453

Artym, J., & Zimecki, M. (2005). The role of lactoferrin in the proper development of newborns. *Advances in Hygiene and Experimental Medicine*, *59*.https://doi.org/10.3390/pharmaceutics15041056

Baumrucker, C. R., & Bruckmaier, R. M. (2014). Colostrogenesis: IgG 1 transcytosis mechanisms. *Journal of Mammary Gland Biology and Neoplasia*, *19*, 103-117. https://doi.org/10.1007/s10911-013-9313-5

Bush, L. J., & Staley, T. E. (1980). Absorption of colostral immunoglobulins in newborn calves. *Journal of Dairy Science*, *63*(4), 672-680. https://doi.org/10.3168/jds.S0022-0302(80)82989-4

Campanella, L., Martini, E., Pintore, M., & Tomassetti, M. (2009). Determination of lactoferrin and immunoglobulin G in animal milks by new immunosensors. *Sensors*, *9*(3),2202-2221. https://doi.org/10.3390/s90302202

Dingess, K. A., Hoek, M., van Rijswijk, D. M., Tamara, S., den Boer, M. A., Veth, T., ... & Heck, A. J. (2023). Identification of common and distinct origins of human serum and breastmilk IgA1 by mass spectrometry-based clonal profiling. *Cellular & Molecular Immunology*, *20*(1), 26-37. https://doi.org/10.1038/s41423-022-00954-2

Elfstrand, L., Lindmark-Månsson, H., Paulsson, M., Nyberg, L., & Åkesson, B. (2002). Immunoglobulins, growth factors and growth hormone in bovine colostrum and the effects of processing. *International Dairy Journal*, *12*(11), 879-887. https://doi.org/10.1016/S0958-6946(02)00089-4

Fadhil, Y. S. (2022). Applications of Nanotechnology in food processing and packaging. *Mesopotamia Journal of Agriculture*, 50(3):26-36. https://doi.org/10.33899/MAGRJ.2022.134238.1178

Giacinti, G., Basiricò, L., Ronchi, B., & Bernabucci, U. (2013). Lactoferrin concentration in buffalo milk. *Italian Journal of Animal Science*, *12*(1), e23. https://doi.org/10.4081/ijas.2013.e23

Giammarco, M., Chincarini, M., Fusaro, I., Manetta, A. C., Contri, A., Gloria, A., ... & Vignola, G. (2021). Evaluation of brix refractometry to estimate immunoglobulin G content in buffalo colostrum and neonatal calf serum. *Animals*, *11*(9), 2616. https://doi.org/10.3390/ani11092616

Godden, S. (2008). Colostrum management for dairy calves. *Veterinary Clinics of North America: Food Animal Practice*, *24*(1), 19-39. https://doi.org/10.1016/j.cvfa.2007.10.005

Hanoush, N. H. (2023). Honey as antibacterial agent used against bacillus spp. Isolated from locally produced juice. *Mesopotamia Journal of Agriculture*, 51(4), 72-85. https://doi.org/10.33899/MJA.2023.143201.1273

Jahani, S., Shakiba, A., & Jahani, L. (2015). The Antimicrobial effect of lactoferrin on Gram-negative and Gram-positive bacteria. *International Journal of Infection*, *2*(3). https://doi.org/10.17795/iji27594

Jenssen, H., & Hancock, R. E. (2009). Antimicrobial properties of lactoferrin. *Biochimie*. *91*(1),19-29. https://doi.org/10.1016/j.biochi.2008.05.015

Kai, K., Komine, Y., Komine, K. I., Asai, K. I., Kuroishi, T., Kozutsumi, T., ... & Kumagai, K. (2002). Effects of bovine lactoferrin by the intramammary infusion in cows with staphylococcal mastitis during the early non-lactating period. *Journal of veterinary medical science*, *64*(10), 873-878. https://doi.org/10.1292/jvms.64.873.

Kanwar, J. R., Roy, K., Patel, Y., Zhou, S. F., Singh, M. R., Singh, D., ... & Kanwar, R. K. (2015). Multifunctional iron bound lactoferrin and nanomedicinal approaches to enhance its bioactive functions. *Molecules*, *20*(6), 9703-9731 https://doi.org/10.3390/molecules20069703.

Kessler, E. C., Bruckmaier, R. M., & Gross, J. J. (2020). Colostrum composition and immunoglobulin G content in dairy and dual-purpose cattle breeds. *Journal of animal science*, *98*(8), skaa237. https://doi.org/10.1093/jas/skaa237

Kim, N. I., Ha, W. K., & Cho, Y. (2010). Dietary effect of lactoferrin-enriched fermented milk on skin surface lipid and clinical improvement of acne vulgaris. *Nutrition*, *26*(9),902-909. https://doi.org/10.1016/j.nut.2010.05.011

Konuspayeva, G., Faye, B., Loiseau, G., & Levieux, D. (2007). Lactoferrin and immunoglobulin contents in camel's milk (Camelus bactrianus, Camelus dromedarius, and Hybrids) from Kazakhstan. *Journal of Dairy Science*, *90*(1), 38-46. https://doi.org/10.3168/jds.S0022-0302(07)72606-1

Levieux, D., Levieux, A., El-Hatmi, H., & Rigaudière, J. P. (2006). Immunochemical quantification of heat denaturation of camel (Camelus dromedarius) whey proteins. *Journal of Dairy Research*, *73*(1), 1-9. https://doi.org/10.1017/S0022029905001391

Li, F., Wu, S. S., Berseth, C. L., Harris, C. L., Richards, J. D., Wampler, J. L., ... & Colombo, J. (2019). Improved neurodevelopmental outcomes associated with bovine milk fat globule membrane and lactoferrin in infant formula: a randomized, controlled trial. *The Journal of pediatrics*, *215*, 24-31. https://doi.org/10.1016/j.jpeds.2019.08.030

Mahmoud, b. A., Abdullah, R.K., Taha, M. B. (2005). studied some factors that affect the productive traits of buffalo raised in Nineveh Governorate. *Mesopotamia Agriculture Journal*, 33(3),94-98. https://doi.org/10.33899/MJA.2005.35820

Mahran, G. A., Asker, A. A., Hamza, A. S., & Ali, J. B. (2007). Lactoferrin in buffalo colostrum and milk. *Egyptian Journal of Dairy Science*, 35(1):21-29. https://doi.org/10.1186/1746-6148-8-19

Nowakowski, A. B., Wobig, W. J., & Petering, D. H. (2014). Native SDS-PAGE: high resolution electrophoretic separation of proteins with retention of native properties including bound metal ions. *Metallomics*, *6*(5), 1068-1078. https://doi.org/10.1039/c4mt00033a

Oberčkal, J., Liaqat, H., Matijašić, B. B., Rozman, V., & Treven, P. (2023). Quantification of lactoferrin in human milk using monolithic cation exchange HPLC. *Journal of ChromatographyB*, *1214*,123548. https://doi.org/10.1016/j.jchromb.2022.123548

Ochoa, T. J., & Cleary, T. G. (2009). Effect of lactoferrin on enteric pathogens. *Biochimie*, *91*(1), 30-34. https://doi.org/10.1016/j.biochi.2008.04.006

Pochet, S., Arnould, C., Debournoux, P., Flament, J., Rolet-Répécaud, O., & Beuvier, E. (2018). A simple micro-batch ion-exchange resin extraction method coupled with reverse-phase HPLC (MBRE-HPLC) to quantify lactoferrin in raw and heat-treated bovine milk. *Food chemistry*, *259*,36-45. https://doi.org/10.1016/j.foodchem.2018.03.058

Qader, G. K., & Tayeb, I. T. (2024). Effect of medicinal plants and vitamin e on productive performance, some physiological, immunological parameters and antioxidant status of broiler under cold stress. *Mesopotamia Journal of Agriculture*, *52*(1), 60-78. https://doi.org/10.33899/MJA.2024.143936.1282

Ronayne de Ferrer, P. A., Baroni, A., Sambucetti, M. E., López, N. E., & Ceriani Cernadas, J. M. (2000). Lactoferrin levels in term and preterm milk. *Journal of the American College of Nutrition*, *19*(3), 370-373. https://doi.org/10.1080/07315724.2000.10718933

Satué-Gracia, M. T., Frankel, E. N., Rangavajhyala, N., & German, J. B. (2000). Lactoferrin in infant formulas: effect on oxidation. *Journal of Agricultural and Food Chemistry*, *48*(10), 4984-4990. https://doi.org/10.1021/jf0002490

Steijns, J. M. (2001). Milk ingredients as nutraceuticals. *International Journal of Dairy Technology*, *54*(3),81-88. https://doi.org/10.1046/j.1364-727x.2001.00019.x

Superti, F. (2020). Lactoferrin from bovine milk: a protective companion for life. *Nutrients*, *12*(9), 2562. https://doi.org/10.3390/nu12092562

Talukder, J. (2021). Role of transferrin: an iron-binding protein in health and diseases. In *Nutraceuticals* (pp. 1011-1025). Academic Press. https://doi.org/10.1016/B978-0-12-821038-3.00060-4

Thermo Fisher Scientific Inc (2019). Extract proteins from polyacrylamide Gels. TECH TIP., COL23449 0719. Protein Gel and Membrane Stains | Thermo Fisher Scientific - IQ. https://assets.thermofisher.com/TFS-Assets/BID/Technical-Notes/extract-proteins-polyacrylamide-Gels-tech-tip.pdf.

Wheeler, T. T., Hodgkinson, A. J., Prosser, C. G., & Davis, S. R. (2007). Immune components of colostrum and milk—a historical perspective. *Journal of mammary gland biology and neoplasia*, *12*, 237-247. https://doi.org/.10.1007/s10911-007-9051-7

Yassin, S. A. (2023). Utilization of lactoferrin to inhibit *E. coli* and *S. aureus* isolates from milk and kariesh cheese. *Iraqi Journal of Veterinary Sciences*, 37(4), 853-863. https://doi.org/10.33899/ijvs.2023.137384.2677

Zrehen, A., Ohayon, S., Huttner, D., & Meller, A. (2020). On-chip protein separation with single-molecule resolution. *Scientific RepoRtS*, *10*(1),15313. https://doi.org/10.1038/s41598-020-72463-z