EFFECT OF HEAT TREATMENTS AND SEASONS OF THE YEAR ON THE PROTECTIVE PROTEINS IN MILK OF DIFFERENT ANIMALS

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Abstract

The aim of the present work was to study the effect of heat treatments and seasons of year on *immunoglobulin*, *lactoferrin* and *lysozyme* contents in camel's, cow's and buffalo's milk. The milk samples were heat treated at 63, 72, and 95°C for 30 min., 15 sec. and 15 min. respectively. Camel's milk contained significantly higher amounts of *immunoglobulin* (IG), *lactoferrin* (LF) and *lysozyme* (LZ) than cow's and buffalo's milk. Heating milk at 63°C /30 min. had significant effect on *lysozyme* and *lactoferrin.* While the *immunoglobulin* was more affected in the three kinds of milk. The amounts of *immunoglobuli*n of cow's and buffalo's milk were observed at 72°C /15 sec. compared with camel's *immunoglobulin*. The amounts of *lactoferrin* were lost at 95°C /15 min. in all kinds of milk. However, at this level of heat treatment, the losses of *lysozyme* at 95°C /15 min. were 0.12 ± 0.18, 0.06 ± 0.17 and 0.02 ± 0.14 ug/ml for camel's, cow's and buffalo's milk, respectively. When milk was heated at 95°C /15 min. camel's milk protective proteins were relatively more heat resistant than cow's and buffalo's milk proteins. It was found that the heat resistance for *lysozyme* > *lactoferrin* > *immunoglobulin.* The amounts of milk protective proteins were higher in winter for all kinds of milk, compared with summer milk.

Keywords **:** *immunoglobulin, lactoferrin*, *lysozyme*, milk, heat treatments

INTRODUCTION

Although protective proteins including *lysozyme, lactoferrin and immunoglobulins* represent only a minor fraction of milk proteins, they play an important role as first line defence due to their direct and indirect antimicrobial activity and for other important physiological and health promoting functions (Gorbenko, et al.,2007). It is suggested that colostral Igs and *lysozyme* would provide as one of the considerable prospects for consumers health promotion in the future (Benkerroum,2008). *The immunoglobulins*, are a family of proteins with a range of protective bioactivities. They are divided into several classes, the major *immunoglobulin* classes in mammary secretions are IgG, IgA and IgM (Mix, et al.,

2006). IgM is the class that appears initially when an organism is exposed to an antigen for the first time (primary infection). IgM has a low specificity and hence a lower potency in defeating the infection. IgA is the major immunoglobulin class found in mucosal secretions and prevents mucosal infections by agglutination of microbes, whereas IgG is the primary *immunoglobuli*n class found in bovine colostrum and milk. Several subclasses of IgG being, with IgG1 and IgG2 are the major *immunoglobulins* in serum (Walter and Theil 2011). *Immunoglobulins* (*Igs*), together with *lactoferrin* and *lysozyme* form important antimicrobial system of bovine lacteal secretions. The concentration of the various bovine Igs in serum and in lacteal secretions varies according to the breed, age, health status, and stage of lactation of the animal (Benkerroum, 2008).

In literature there is contradictory information about thermo stability of antimicrobial proteins. According to Chen, et al., (2000) results *immunoglobulins* are thermolabile. Exposure to temperatures of 75 °C can reduce detectable isolated bovine IgG by 40% in 5 min, and by 100% at 95 °C for 15 s. The explanation of it is conformational changes in the IgG molecule causes by heat exposure. Antigen-binding activity of bovine IgG also is reduced after heat treatment (Dominguez, et al., 2001). The studies suggesting that the antigen-binding region of the *immunoglobulin* molecule is more thermo labile than the other regions of the molecule, thermal protect ants such as sugars or glycerol can increase the stability of isolated IgG to heat treatment (Chen, et al., 2000). However, it was reported that IgG is the most thermostable and IgM is the least thermostable *Lysozyme* is an antimicrobial enzyme that is found in a wide variety of organisms and ranged from 0 to 3 mg /L in cow's milk to 790 mg /L in mare's milk (Farkey 2002). The enzyme is often used for lysing of peptidoglycan present in the bacterial cell walls. Gram-positive cells are quite susceptible to this hydrolysis as their cell walls have a high proportion of peptidoglycan. Gram-negative bacteria are less susceptible due to the presence of an outer membrane and a lower proportion of peptidoglycan. *Lysozyme* protects against bacterial infection by breaking down the carbohydrates in bacterial cell walls, killing them. *Lysozyme* also has fungicidal properties, protecting mucosal areas from invasion by pathogenic yeast or fungi. *Lysozyme* has been shown to inhibit viral replication and infection such HIV (Samaranayake, et al., 2001). In addition, the concentration of soluble *lysozyme* in milk varies considerably from one species to another and within the same species depending on various factors such as the breed, stage of lactation, parturition, nutrition, udder health and season of the year (Priyadarshini and Kansal 2003). *Lysozyme* is termostable, 75% of lysozyme activity mantains after milk heat treatment 75 °C 15 min or 80 °C 15 s (Farkey 2002). On the

other hand, the increased pool of antimicrobial components can be enriched further through concentration techniques, leading to production of products containing high *immunoglobulin* and *lysozyme* concentration. Such preparation may find beneficial application as in human healthcare and wellbeing by preventing infection and controlling microorganisms grow and diseases, as in a new functional food development (Mehra, et al., 2006). Through such processing, *immunoglobulins* and *lysozyme* are exposed to a number of conditions that may alter the structure and function of the proteins. Some of methods used for concentration or isolation of *immunoglobulins* and *lysozyme* include steps that involve exposing the protein to *heat*, *acid or pressure* which may affect the conformation of the protein, and ultimately the immunological activity of it. Independent on method, which is used for concentration *immunoglobulin* and *lysozyme,* thermal treatment, is obligatory step. The combination of temperature and time used in processing can affect also the structure of the proteins and involve unfolding and aggregation (Elfstrand, et al., 2002).

The research objective was to the protective proteins content, i.e. *immunoglobulin, lactoferrin and lysozyme* in milk obtained from camel's, cow's and buffalo's as affected by heat treatment and seasons of the year.

MATERIALS AND METHODS

Bulk samples of each type of milk were used in the present study. Samples were taken during winter and summer seasons. Camel's milk samples were obtained from the El-Alamin area around Alexandria. Cow's and buffalo's milk samples were collected from Animal Production Research Station(Sakha and Mehalet Mosa). Milk samples were defatted by using the separator. Skim milk was then divided into four equal portions, one portion was used as a control (raw), the others were heated at 65, 72 and 95°C for 30 min, 15 sec. and 15 min. respectively in a water bath. Samples were rapidly cooled to 40°C, renneted and centrifuged at 3000 g for 20 min at 4°C. The concentrations of IgG was determined in the separated whey by turbodimetric method (Gray et al., 1969) using pH-meter "Jenway 3520" and spectrophotometer "Jenway 6705 UV/VIS" (UK).

Lactoferrin and lysozyme contents were determined using the reversed-phase highperformance liquid chromatography (RP-HPLC) with UV-Vis detector according to Maynard, et al., (1989) From each sample of raw milk 50 ml was taken and adjusted to pH 4.6 with 0.1 mol/L HCl, and allowed to stand at room temperature for about one hour for to precipite the caseins. The whey (7 ml) was taken and centrifuged at

10,000 rpm for 15 min. Finally, whey solutions were filtered through paper quality filter discs (diameter: 125 mm, density: 65 g/m2, grade: 3 hours (Munktell, Germany)) and 0.20-µm disposable sterile filters (Millipore type GSTF, USA). The supernatants were kept in refrigerator until analysis, and were injected into the chromatograph at the suitable time (in the amount of 20 µl). Protein separation was performed on liquid chromatography ProStar 210 model and UV-Vis ProStar 325 detector (Varian, USA). The measurements were carried out using the water/acetonitrile mobile phase at gradient elution and column NUCLEOSIL 300-5 C18 (Varian, USA) of 250 mm length and 4.6 mm diameter. The mobile phase was solvent A (90% water, 10% acetonitryle) and solvent B (90% acetonitryle, 10% water), purchased from Sigma (Germany). The solvents were filtered through 0.45-µm filters (Millipore, USA) and degassed by using ultrasounds. The total analysis time for a single sample was 35 min at 205 nm wavelength with column temperature of 37°C. The analyses of reference substances were conducted under the same conditions. On the grounds of the obtained chromatograms, using program Star 6.2 Chromatography Workstation (Varian, USA), the qualitative and quantitative identification of each substance were performed followed by their concentration determination. Calibration of the chromatographic system for whey proteins determination was carried out by the external standard method. For this purpose, each protein was calibrated individually by injecting solutions of the standards (20 µl). The standards were purified proteins, i.e. *lactoferrin* (90 %) from bovine milk and *lysozyme* (95 %) from hen egg whites, which were purchased from Sigma (Germany). All chemicals were of HPLC analytical grade. Concentrations of *lactoferrin and lysozyme* solutions ranged from 0 to 200 mg/l and from 0 to 20 µg/l respectively, and were prepared to create the calibration curves. The limits of quantification LOQ (for *lactoferrin* – 40 mg/l and for *lysozyme* – 2.8 µg/l) and detection LOD (for *lactoferrin* – 8.7 mg/l and *lysozyme* – 0.9 µg/l) were determined.

Statistical Analysis:-

The obtained data were statistically analyzed for variance average and Duncan's test according to SPSS computer program (SPSS, 1998).

RESULTS AND DISCUSSION

The mean quantity of total *immunoglobulins* in raw camel's milk was 1.86 ± 0.11 mg/ml (Table 1). The decrease of *immunoglobulin* quantity was recorded by heat treatment at 63°C 30 min. (Fig 1) Quantity of *immunoglobulin* was reduced to 1.58 ± 0.11. Increasing heat treatment up to 72 °C and 95°C with holding time of

15sec. and 15 min. respectively had similar influence on quantity of *immunoglobulin*, since it reduced to 0.52 ± 0.12 and 0.04 ± 0.13 mg/ml respectively. Quantity of *immunoglobulin* from camel milk was the highest and more resistant to heat treatment compared to cow's and buffalo's milk. Some studies on total cow milk *immunoglobulin*s and their heat stability indicated that heating skim milk at 70°C for 30 min resulted 89% loss in *immunoglobulin*. Li-Chan, et al., (1995) found that no change in bovine IgG after heating cow milk at 62.7°C for 30 min but Dhar, et al., (1996) reported that pasteurization of cow milk at 71°C for 9 s resulted retention of 75% of IgG. Vetter et al., (2013) found that HTST pasteurization (72°C/15 s) led to 25 to 40% loss of IgG concentration. On the other hand, quantity of *immunoglobulin* as affected by season was studied. The highest quantity of *immunoglobulin* was recorded in raw camel's milk(2.23 ± 0.12 mg/ml) in winter season, but the lowest quantity was recorded in raw buffalo's milk in winter season (0.36 ± 0.16 mg/ml) compared to summer season. Milk of camel's, cow's and buffalo's grazing the pasture was characterized by a higher content of IgG by 39.6 mg/L (Król, et al., (2011).

Table 1. Effect of heat treatments and seasons of the year on the quantity of *Immunoglobulin* (mg/ml) in camel's, cow's and buffalo's milk(Average ± SE of three replicates).

Treatments	Summer			Winter		
	Camel's milk	Cow's milk	Buffalo's milk	Camel's milk	Cow's milk	Buffalo's milk
Control	1.86±0.11 [a]	0.39±0.12 [a]	0.28±0.14 [a]	2.23±0.12 [a]	0.52±0.15 [a]	0.36±0.16 [a]
63°C /30 min.	1.58±0.11 [b]	0.18±0.14 [b]	0.12±0.16 [b]	2.02±0.14 [b]	0.35±0.14 [b]	0.18±0.17 [b]
72°C /15 Sec.	0.52±0.12 [c]	0.09±0.16 [c]	0.03±0.17 [c]	0.70±0.13 [c]	0.06±0.16 [c]	0.02±0.15 [c]
95°C/15 min.	0.04±0.13 [d]	ND	ND	0.05±0.14 [d]	ND	ND

Means ±standard error

a, b, c Means within the same column with different letters are significantly different (P≤ 0.05).

ND = Not detected

Raw camel's milk contained a significantly (P≤ 0.05) higher level of *lactoferrin* compared to cow's and buffalo's milk (Table 2). The effect of heat treatments on *lactoferrin* content was shown in Fig. 2. Heating milk at 63°C for 30 min. had significant effect on *lactoferrin* quantity in the milk for all species. However, increasing the temperature to 72°C for 15 sec. resulted signicant loss of *lactoferrin*. Luf and Rosner (1997) found that HTST treatment of cow milk has no significant effect on

lactoferrin denaturation, whereas, heat treatment at 63°C for 30 min reduced the native *lactoferrin* content by 40%. In the present study, heating of milk at 95°C for 15 min. resulted a complete loss of *lactoferrin* in cow's and buffalo's milk versus 96.5% of denaturation of camel *lactoferrin.* Generally, on the basis of these findings, it could be concluded that camel milk *lactoferrin* was more resistant to heat than that of cow's and buffalo's milk. On the other hand, season of the year affected the quantity of *lactoferrin*. The highest quantity of *lactoferrin* was recorded in raw camel's milk(0.72 ± 0.17 mg/ml) in winter season, but the lowest one was recorded in raw buffalo's milk in winter season (0.31 ± 0.23 mg/ml) compared to summer season. Some authors found lower levels of *lactoferrin* in the milk of cows kept on the pasture (145.66-148.83 mg/l) in comparison with milk of cows fed in barns (174.63-204.89 mg/l). Turner, et al., (2003) also reported higher levels of *lactoferrin* in milk of cows fed system in relation to milk of cows grazing the pasture.

Table 2. Effect of heat treatments and seasons of the year the on quantity of *lactoferrin* content (mg/ml) in camel's, cow's and buffalo's milk (Average ± SE of three replicates).

Treatments	Summar			Winter		
	Camel's milk	Cow's milk	Buffalo's milk	Camel's milk	Cow's milk	Buffalo's milk
Control	0.55±0.21 [a]	0.38±0.19 [a]	0.22±0.22 [a]	0.72±0.17 [a]	0.49±0.13 [a]	0.31±0.23 [a]
63°C /30 min.	0.46±0.16 [b]	0.19±0.17 [b]	0.12±0.18 [b]	0.65±0.18 [b]	0.25±0.16 [b]	0.15±0.15 [b]
72°C /15 Sec.	0.11±0.18 [c]	0.08±0.15 [c]	0.08±0.16 [c]	0.21±0.16 [c]	0.12±0.19 [c]	0.10±0.18 [c]
95°C/15 min.	0.06±0.13 [d]	0.05±0.14 [d]	ND	0.08±0.14 [d]	ND	ND

Means ±standard error

a, b, c Means within the same column with different letters are significantly different (P≤ 0.05).

ND=Not detected

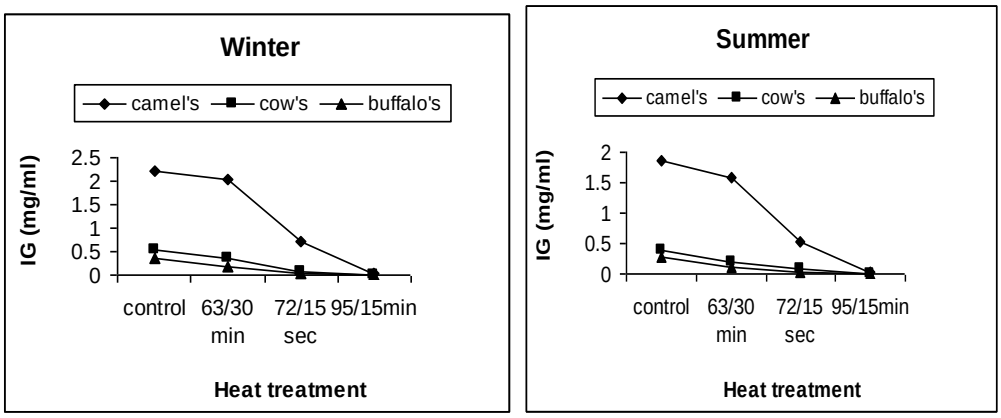

Fig. 1. Effect of heat treatment and season on camel's, cow's and buffalo's milk immunoglobulin.

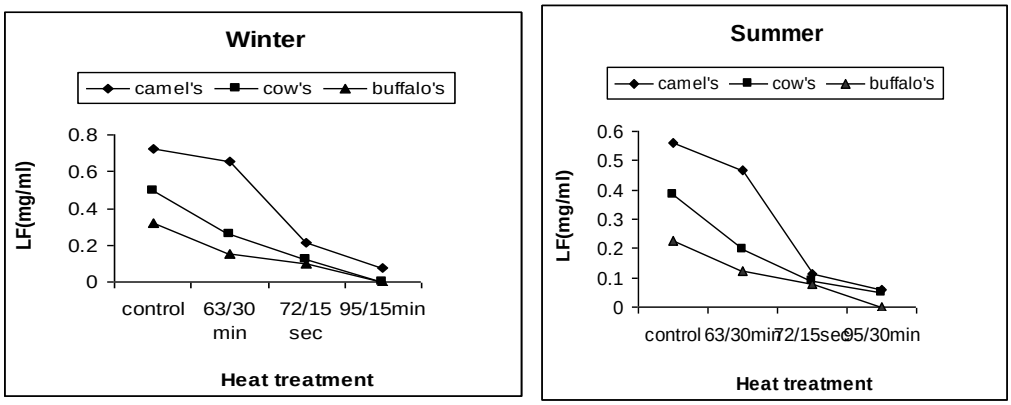

Fig. 2. Effect of heat treatment and season on camel's, cow's and buffalo's milk lactoferrin.

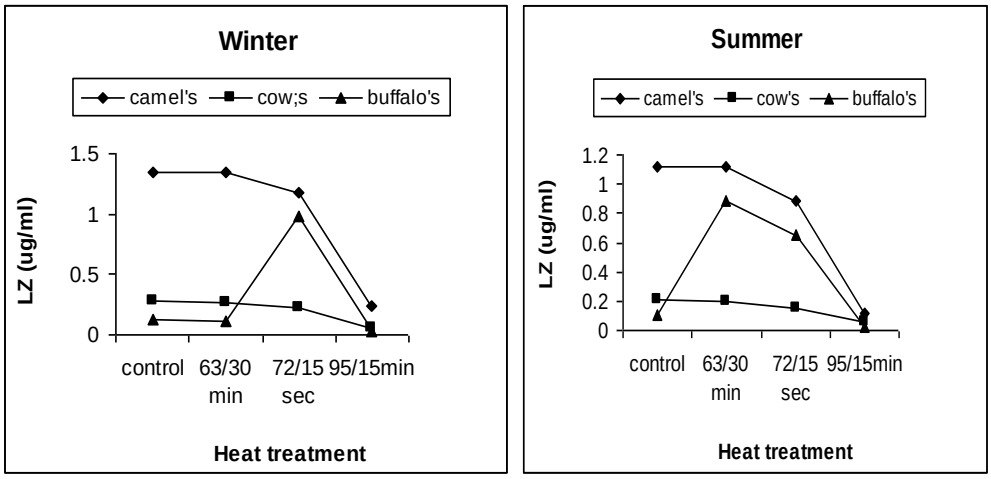

Fig. 3. Effect of heat treatment and season on camel's, cow's and buffalo's milk lysozyme.

Significant differences were observed in *lysozyme* (LZ) from three kinds of milk. Camel's milk contained 1.12±0.14 ug /ml and 1.35±0.21 ug /ml in summer and winter respectively. Cow's milk 0.21±0.14 and 0.27±0.17, buffalo's milk 0.10±0.11 and 0.12 ±0.18 in summer and winter respectively (Table 3). Fig. 3 showed the effect of various heat treatment on *lysozyme* in camel's, cow's and buffalo's milk. Heating milk at 63 and 72°C for 30 min. and 15 sec. respectively had significant effect on lysozyme in the three kinds of milk (Table 3). However, highly significant differences between the effect of 72°C and 95°C were observed especially in camel's and cow's milk. Increasing the temperature to 95°C for 15 min resulted in a significant greater loss of *lysozyme* in milk. Buffalo's milk *lysozyme* was more affected by heat treatment than camel's and cow's milk *lysozyme.* Although at 95°C /30 min., the entire activity of buffalo's and cow's milk *lysozyme* was lost versus 94% of activity loss of camel's milk *lysozyme*, there was significant differences among them. Different results were given by some other authors, they mentioned, that only 75 % of *lysozyme* maintains after milk pasteurization at 80°C 15 s (Farkey, 2002). In current research *lysozyme* showed higher results and was more stable during heat treatment. On the other hand, season of the year affected the quantity of *lysozyme* since the highest quantity was recorded in raw camel's milk (1.35±0.21 ug/ml) in winter season, but the lowest one was recorded in raw buffalo's milk in winter season (0.12±0.18 ug/ml) compared to summer season.

Table 3. Effect of heat treatments and seasons of the year on the quantity of lysozyme (ug /ml) in camel's, cow's and buffalo's milk (Average ± SE of three replicates).

Treatments	Summer			Winter		
	Camel's milk	Cow's milk	Buffalo's milk	Camel's milk	Cow's milk	Buffalo's milk
Control	1.12±0.14 [a]	0.21±0.21[a]	0.10±0.11 [a]	1.35±0.21 [a]	0.27±0.17 [a]	0.12±0.18 [a]
63°C /30 min.	1.12±0.21 [b]	0.19±0.15[b]	0.89±0.21 [b]	1.35±0.13 [b]	0.26±0.18 [b]	0.11±0.16 [b]
72°C /15 Sec.	0.89±0.16 [c]	0.14±0.18[c]	0.65±0.13 [c]	1.18±0.16 [c]	0.22±0.15 [c]	0.98±0.14 [c]
95°C/15 min.	0.12±0.18 [d]	0.06±0.17[d]	0.02±0.14 [d]	0.23±0.19 [d]	0.05±0.12 [d]	0.03±0.11 [d]

Means ±standard error

a, b, c Means within the same column with different letters are significantly different ($P<0.05$).

CONCLUSION

From the results obtained it can be concluded that (a) Camel's milk was more heat stable than cow's and buffalo's milk. (b) Antimicrobial factors are significantly present in higher concentration in camel milk than in cow's or buffalo's milk and they are more heat resistant than their counterparts in cow's and buffalo's milk. This means that the biological activity of protective proteins in heat-treated camel milk at 95°C /30 min. was higher than that of cow's and buffalo's milk proteins.(c) On the other hand, the quantity of antimicrobial factors were significantly present in higher concentration in winter milk compared to summer milk.

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تأثير المعاملات الحرارية المختلفة و فصول السنه على البروتينات المناعيه فى انواع الألبان المختلفة

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تهدف هذه الدراسه الى تقييم الثبات الحرارى و فصول السنه للبروتينات المناعيه مثل الامينوجلوبيولين و اللاكتوفيرين و الليزوزيم للبن الابل والابقار و الجاموس. حيث تم اخذ عينات اللبن و معاملاتها حراريا ٦٣° م/٣٠ق و ٧٢° م/١٥ ثانيه و ٩٥° م/١٥ق. واظهرت النتائج ان لبن الابل كان اعلى معنويه فى المحتوى من البروتينات المناعيه الليزوزيم و اللاكتوفيرين و الامينوجلوبيولين. فى حين اظهرت المعامله الحراريه ٦٣°م/٣٠ق و جود فروق معنويه فى لبن الابل و الابقار و الجاموس للامينوجلوبيولين. و كان الفقد فى كميه الامينوجلوبيولين اعلى للابقار ثم الجاموس مقارنتا بلبن الابل عند المعامله الحراريه ٧٢° م/١٥ ثانيه. فى حين كان الفقد معنويا فى اللاكتوفيرين فى كل من لبن الابل و الابقار و الجاموس عند المعامله الحراريه ٩٥م/١٥ق وقد حدث فقد ملحوظ فى الليزوزيم عند المعامله الحراريه ٩٥ °م/١٥ق حيث وصل الى٠,١٢±٠,١٨ و ٠,٠٦±٠,١٧ و٠,٠٢±٠,١٤ ميكرو جرام /مل للبن الابل و الابقار و الجاموس على التوالى٠من ذلك يتضح ان لبن الابل اكثر مقاومه للمعاملات الحراريه يليه لبن الابقار ثم لبن الجاموس٠ كذلك فان الليزوزيم اكثر مقاومه للمعاملات الحراريه يليه اللاكتوفيرين ثم الامينوجلوبيولين٠ من ناحيه اخرى فان البيئه و نظم التغذيه تلعب دور كبير ومهم و تؤثر على كميه البروتينات المناعيه حيث كانت اعلى كميه فى لبن فصل الشتاء نظرا لتوافر البيئه المناسبه و التغذيه والرعى مقارنه بلبن الصيف حيث التغذيه الجافه.