

# Detection and characterisation of Complement protein activity in bovine milk by bactericidal sequestration assay

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While the Complement protein system in human milk is well characterised, there is little information on its presence and activity in bovine milk. Complement forms part of the innate immune system, hence the importance of its contribution during milk ingestion to the overall defences of the neonate. A bactericidal sequestration assay, featuring a Complement sensitive strain, *Escherichia coli* 0111, originally used to characterise Complement activity in human milk was successfully applied to freshly drawn bovine milk samples, thus, providing an opportunity to compare Complement activities in both human and bovine milks. Although not identical in response, the levels of Complement activity in bovine milk were found to be closely comparable with that of human milk. Differential counts of *Esch. coli* 0111 after 2 h incubation were 6.20 and 6.06 log CFU/ml, for raw bovine and human milks, respectively – the lower value representing a stronger Complement response. Exposing bovine milk to a range of thermal treatments e.g. 42, 45, 65, 72, 85 or 95 °C for 10 min, progressively inhibited Complement activity by increasing temperature, thus confirming the heat labile nature of this immune protein system. Low level Complement activity was found, however, in 65 and 72 °C heat treated samples and in retailed pasteurised milk which highlights the outer limit to which high temperature, short time (HTST) industrial thermal processes should be applied if retention of activity is a priority. Concentration of Complement in the fat phase was evident following cream separation, and this was also reflected in the further loss of activity recorded in low fat variants of retailed pasteurised milk. Laboratory-based churning of the cream during simulated buttermaking generated an aqueous (buttermilk) phase with higher levels of Complement activity than the fat phase, thus pointing to a likely association with the milk fat globule membrane (MFGM) layer.

**Keywords:** Complement, bactericidal activity, milk fat globule membrane, bovine, milk.

**Abbreviations:** MFGM, Milk fat globule membrane, HTST, high temperature, short time, CFU, colony forming units, SCC, somatic cell count.

As well as being a source of essential nutrients for optimum growth and development, milk provides the neonate with protective elements to support survival of the new-born (Hanssen, 1924; Van Hooijdonk et al. 2000). While the bacteriostatic qualities of milk were recognised as far back as 1924 (Hanssen, 1924) research over the years has focussed on the characterisation of its antimicrobial components such as lactoferrin, lactoperoxidase, lysozyme, peptides derived in vivo during digestion and sialyl-containing oligosaccharides. However, less consideration appears to have been given to the role of another line of defence i.e. the extent

to which the innate immune system of the lactating mammal is manifested in its milk secretions. Innate immunity with its physical barriers and cellular components provides non-specific protection as a first line of defence (Ricklin & Lambris, 2007) which is distinguishable from that of the adaptive immune system that typically responds to specific pathogenic challenge (Galyean et al. 1999). Within innate immunity, Complement consists of serum and membrane proteins which circulate as inactive precursors in the blood system. The triggering of these precursors initiates a cascade of protein cleavages which culminates in the amplification of a cell-killing membrane attack complex (Oviedo-Boyso et al. 2007). Though initiated by different mechanisms, all three established Complement pathways i.e. classical, alternative and mannan-binding

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lectin (MBL) converge at the point when complement component C3 becomes active (Abbas et al. 1994). The classical pathway commences with immune complexation by IgG or IgM to pathogens or foreign antigens, the alternative pathway is triggered by either carbohydrates, lipids or proteins found on the surface of foreign microbes or cells in the body, and the MBL pathway is activated when either MBL or Ficolin binds to the surface of pathogens due to carbohydrate moieties (Sarma & Ward, 2011).

Efforts to date at addressing the presence and role of Complement in human milk (Ogunde, 1998) also lend support to the view that such innate immune components migrate from the circulatory system in the mammary into the secreted milk. However, little is known about its activity in bovine milk and its presence is not even recognised in the recent revision (6th) of the nomenclature of the proteins in cows' milk (Farrell et al. 2004). Lack of suitable assays may have been a contributing factor to this paucity of information. Gradually, the bactericidal sequestration assay-based analytical approach was adapted initially to help identify bactericidal and haemolytic activity of bovine colostrum (Reiter & Brock, 1975), bovine mastitic milk (Rainard & Caffin, 1984) and human milk (Ogunde, 1998). The uniqueness of the bactericidal sequestration assay in this case is that growth of a specific pathogenic bacterial strain i.e. *Escherichia coli* 0111 is sensitive to the presence of Complement, thus exploiting the extent of growth inactivation as an indirect measure of Complement activity present.

Thus, an objective of this study was to adapt and apply the bactericidal sequestration assay used by Ogunde (1998) to establish the level of Complement activity in fresh bovine milk and its comparability with that of human breast milk. A further objective was to evaluate the extent to how such activity may be affected by typical milk processes such as heating and cream separation. This interest was taking place against a backdrop of where advances in the 'humanisation' of bovine milk for use as infant feed formula requires a deeper understanding of the biological comparability between both bovine and human breast milk Complement, so that appropriate strategies may be designed for its protection in freshly secreted bovine milk during the course of dairy manufacturing processes.

## Material and methods

### Sample preparation

**Milk samples.** Fresh whole bovine milk was collected from the Moorepark Dairy Production Centre (MDPC) farm on the day of testing. The somatic cell counts (SCC) of the freshly collected milk was determined using Bentley Somacount 300<sup>®</sup> (Bentley Instruments Inc., Chaska MN, USA), SCC of <200 000 cells/ml was required for use of the milk.

Fresh commercial products including cream, full fat milk and low fat milk were purchased from local supermarkets on the day of testing.

Human milk was collected from the Western Health and Social Care Trust (Glenshane Road, Londonderry BT47 6SB, United Kingdom) and was stored in aliquots at  $-80^{\circ}\text{C}$  until required.

**Control preparation.** Freshly collected raw milk was heat treated at  $56^{\circ}\text{C}$  for 30 min to ensure complete inactivation of the Complement protein (Korhonen et al. 2000a). All samples were screened using the International Dairy Federation (IDF) methods (IDF, 2004, 2013) and also the milks were streaked on Nutrient and Luria Bertani agar.

**Effect of heat treatment.** Individual milk samples were exposed to a range of temperatures: 42, 45, 65, 72, 85 and  $95^{\circ}\text{C}$  with 10 min holding time before bactericidal sequestration assay in order to determine the effect of heating on Complement activity (Korhonen et al. 2000b). Milk samples were heat-treated using Hettich Elbanton special products oil bath (Hettich Benelux B.V., De Aaldor 9-4191 PC Geldermalsen, Netherlands) with rocking arm to ensure uniform heat treatment throughout the samples. A control sample generated by heating at  $56^{\circ}\text{C}$  for 30 min was used as reference for complete Complement inactivation.

**Milk samples separation and washing.** An Armfield disc bowl centrifuge (Armfield, Ringwood, UK) was used to generate cream samples (approximately 40% fat) and skimmed milk (approximately 1% fat) from the fresh raw milk. The cream was then stored at  $4^{\circ}\text{C}$  overnight and the following morning a Kenwood Chef food mixer (Model KM220, Harvart, Hants, UK) was used to disrupt the cream emulsion resulting in the production of butter. From this buttermilk was released and was filtered through glass wool to remove butter grains. Human milk was thawed overnight at  $4^{\circ}\text{C}$ . This was then separated by centrifugation at 2000 g for 15 min at  $4^{\circ}\text{C}$  (Patton & Huston, 1986) in order to separate the fat fraction.

Washed buttermilk was prepared using the Armfield disc bowl centrifuge however, an additional step was incorporated whereby the cream was suspended in sterile distilled water and held at  $37^{\circ}\text{C}$  for 1 h to wash the buttermilk. The separation procedure was repeated (Armfield disc bowl centrifuge) and cream collected. This step was repeated twice more.

### Bacterial strain

*Escherichia coli* 0111 (*Esch. coli* NCTC 8007, serotype 0111 K58(B4)) a pathogenic Complement sensitive strain was purchased from Health Protection Agency Culture Collections (Health Protection Agency Culture Collections Porton Down Salisbury Wiltshire, SP4 0JG UK). This strain was routinely grown in Luria-Bertani (LB) medium at  $37^{\circ}\text{C}$  with shaking. Standard LB broth and agar was prepared as described Sambrook et al. (2001).

### Bactericidal sequestration assay

*Esch. coli* 0111 was prepared for overnight growth at 37 °C on LB agar. An isolated colony from replicate plates was inoculated into 3 tubes of LB broth (Merck KGaA, Darmstadt, Germany) and grown overnight at 37 °C. The overnight cultures were centrifuged at 392 rcf, a pellet was formed, the supernatant was removed and the pellet was re-suspended in phosphate buffer saline solution (1 × PBS solution) this step was repeated twice. On the final step the pellet was suspended in LB broth and adjusted to  $3 \times 10^8$  colony forming units (CFU) per ml using the McFarland Method (Goldman et al. 1986). Using 96-well plates  $3 \times 20$  µl of each of the three replicate cultures was added to each round bottomed well (SARSTEDT Ltd, Wexford, Ireland) and 80 µl of the sample to be tested was then added. The plate was incubated at 37 °C for 2 h shaking at 200 rpm (Model Mini 4450 SHKA4450-1CE, Fischer Scientific, Ballycoolin, Dublin). A 20 µl sample was taken from each well after the incubation time and total viable counts enumerated on LB agar using the pour plate method.

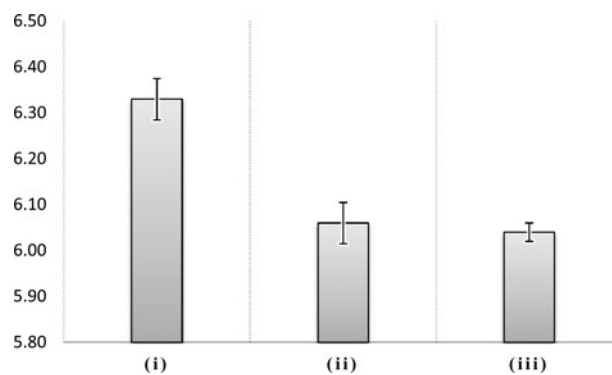
### Serum susceptibility assay

The blood serum susceptibility assay developed by Hogan et al. (1989) was adapted by substituting blood with milk. Freshly collected bovine milk (125 µl) and heat treated milk (control; 125 µl) were added to individual wells of a 96-well plate, subsequently 100 µl Tris-NaCl<sub>2</sub> buffer was added (1 × Sigma Chemical Company, St. Louis, MO). The next step was the addition of 25 µl of the *Esch. coli* 0111 inoculum which had been grown to a level of  $10^6$  CFU/ml. The 96-well plate was incubated at 37 °C and viability of the bacteria evaluated at 0 and 4 h. Viable bacteria were plated on nutrient agar (Merck KGaA, Darmstadt, Germany) for 15 h at 37 °C and results were expressed in terms of differential growth between the serum susceptibility assay and nutrient agar. This was accomplished by subtracting the initial amount of colonies (0 h) from the final number of viable colonies (4 h).

## Results

### Comparison of Complement-associated bactericidal activity in bovine and human milks

The sequestration assay relies on the specific sensitivity of *Esch. coli* 0111 to the presence of Complement, thus, an active Complement system in milk would be expected to retard growth of the strain. Furthermore, since Complement is known to be inactivated at temperatures >56 °C for 30 min (Korhonen et al. 2000a) it is, thus, possible to compare growth of *Esch. coli* 0111 in heated v. raw milk – the differential in growth rates between the heated (control) and raw milks being attributable to Complement activity. This was evident when comparing the growth of *Esch. coli* in heat-treated (56 °C, 30 min) milk, freshly collected raw bovine milk and human milk (Fig. 1) where differential



**Fig. 1.** Differential reduction in *Esch. coli* 0111 (log<sub>10</sub> CFU/ml) during bactericidal sequestration assay of (i) heated bovine milk (control), (ii) raw bovine milk and (iii) human milk, after inoculation and after 2 h incubation.

counts after 2 h incubation at 37 °C were 6.32, 6.20 and 6.06 viable colonies of *Esch. coli* 0111 (log CFU/ml), respectively – the lower value indicating a more effective Complement response. This corresponded to cell reduction factors of 0.12 and 0.26 ( $P < 0.01$ ) following incubation with raw bovine milk and human milk, respectively. Following analysis for normality it was determined that a Kruskal–Wallis test should be applied to the differential data, the results of which confirmed that there was a statistical difference ( $P < 0.01$ ) between all three samples, which was supported further by a Mann–Whitney test to show that both raw bovine milk and human milk were significantly different ( $P < 0.01$ ) from the control (heat-treated milk).

### Growth characteristics of *Esch. coli* 0111

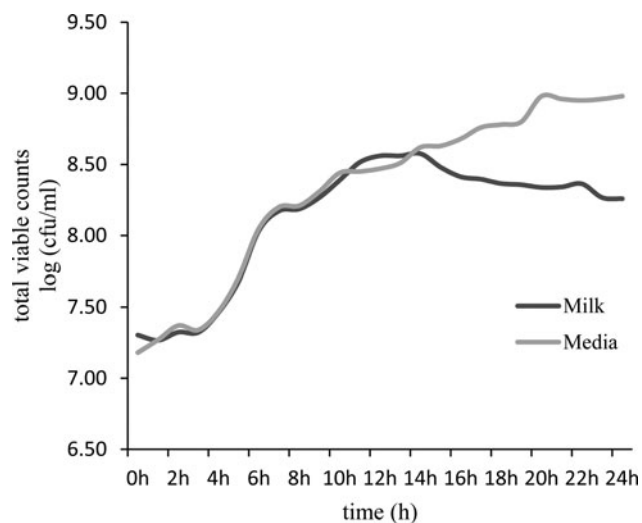
The growth characteristics of *Esch. coli* 0111 was compared by incubating the strain separately in nutrient broth or bovine milk overnight at 37 °C in order to have an improved understanding of its vitality during deployment in the bactericidal sequestration assay.

The *Esch. coli* 0111 strain grew steadily on nutrient agar starting from an initial viable colony count of 7.18 to reach ~8.98 log CFU/ml after 24 h incubation (Fig. 2). Growth of the strain in bovine milk peaked at 8.56 log CFU/ml at 13 h during the same period after which it declined slightly.

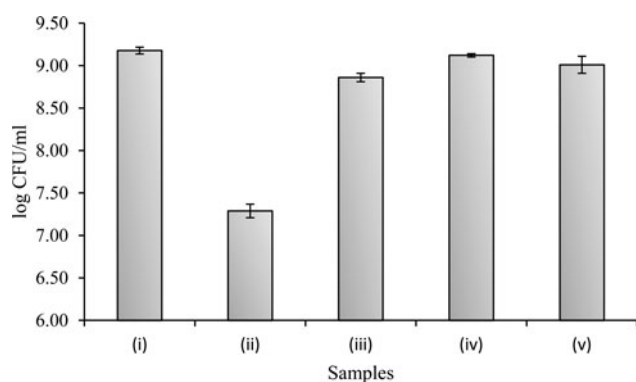
Complement activity was also evident when *Esch. coli* was inhibited in untreated wells during a serum susceptibility assay of forewarmed (56 °C, 30 min) and non-heated bovine milk. The plated viable colonies reached peak growth of 8.0 and 8.95 log cells/ml after incubation with the raw bovine and heated bovine milks (control), respectively ( $P < 0.05$ ).

### Investigation of Complement activity in cream separated bovine milk

Preliminary laboratory work carried out on the variation between freshly collected bovine milk and in-store

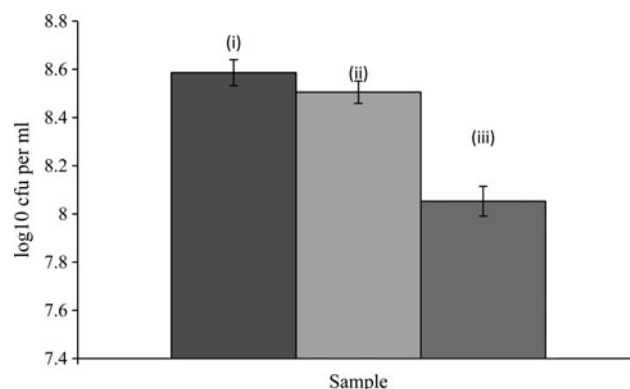


**Fig. 2.** Total viable counts of *Esch. coli* 0111 (log<sub>10</sub> CFU/ml) during incubation at 37 °C for 24 h in nutrient broth and bovine milk.

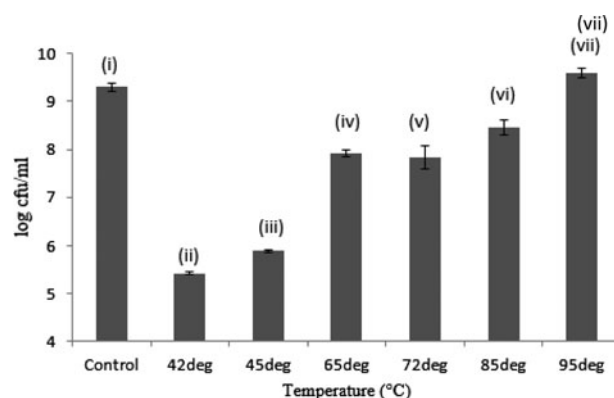


**Fig. 3.** Total viable counts of *Esch. coli* 0111 (log<sub>10</sub> CFU/ml) after 2 h following incubation in (i) heated bovine milk (control), (ii) raw bovine milk and (iii) retail pasteurised milk, (iv) raw bovine skim milk, (v) retail pasteurised low fat milk during the bactericidal sequestration assay for 2 h at 37 °C.

purchased full-fat milk was extended to include low-fat (1% fat) retail milks and skim milk prepared by cream separation in the laboratory from freshly collected raw bovine milk (Fig. 3). Subsequent to assessment for normality, a Kruskal–Wallis test revealed that there was a statistically significant difference ( $P < 0.001$ ) in colony forming units across the five groups [control (heat inactivated milk), raw milk, retained full-fat milk, skim milk, retained low-fat milk]. The raw untreated bovine milk was more effective in inhibiting growth of *Esch. coli* 0111 than the pasteurised sample. The lower-fat milk variants (in-store purchased and lab-prepared) were also less effective in reducing growth of the Complement-sensitive strain. Further statistical analysis, which examined individual groups (Mann–Whitney test), confirmed that full fat raw milk was significantly different from all other samples ( $P < 0.005$ ).



**Fig. 4.** Total viable counts of *Esch. coli* 0111 (log<sub>10</sub> CFU/ml) after 2 h incubation in (i) heat treated bovine milk (control), (ii) lab prepared skim human breast milk, (iii) human breast milk in accordance with the bactericidal sequestration assay.



**Fig. 5** Total viable counts of *Esch. coli* 0111 following 2 h incubation in bovine milk heated at (i) 56 °C, 30 min (control), (ii) 42 °C, 10 min, (iii) 45 °C, 10 min, (iv) 65 °C, 10 min, (v) 72 °C, 10 min, (vi) 85 °C, 10 min, (vii) 95 °C, 10 min, in accordance with bactericidal sequestration assay for 2 h, 37 °C.

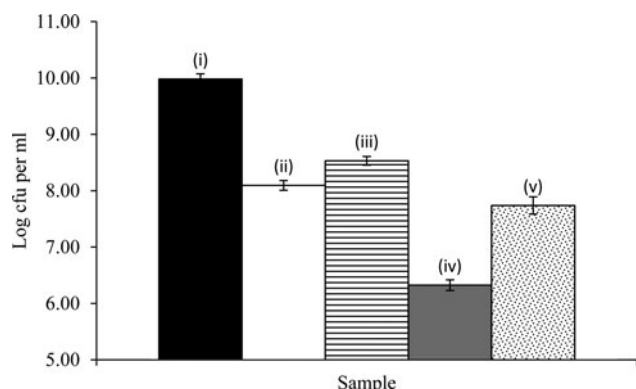
#### Effect of fat reduction in human milk

Laboratory centrifugation (Patton & Huston, 1986) was used to defat (remove the upper cream layer) human milk since the available volume was too small for treatment by conventional dairy cream separation technology.

Similar to bovine milk, the full-fat form of human milk was significantly ( $P < 0.05$ ) more effective than the defatted/skimmed human milk (Fig. 4), thus confirming the association between Complement and fat phase of the milks of both species at a microstructure level.

#### Effect of heat treatment on Complement activity

The bactericidal sequestration assay was performed on bovine milks which had been heated to each of the following temperatures: 42, 45, 65, 72, 85 or 95 °C with holding for 10 min and compared with that of bovine milk heated to 56 °C for 30 min as control (Fig. 5). Maximum Complement activity



**Fig. 6.** Total viable counts of *Esch. coli* 0111 (log<sub>10</sub> CFU/ml) following 2 h incubation in (i) heated bovine milk, 56 °C, 30 min (control), (ii) cream (~40% fat) prepared from freshly collected raw bovine milk (iii) retail pasteurised cream, (iv) lab prepared buttermilk from cream of raw milk (v) lab prepared buttermilk made from the cream (washed twice at 37 °C for 1 h) of raw milk, after 2 h incubation at 37 °C according to the a bactericidal sequestration assay.

(5.41 log CFU/ml) was observed at 42 °C followed by a slight loss (5.89 log CFU/ml) as the temperature increased to 45 °C. Based on assay counts of 7.82–7.92 log CFU/ml, a sizeable loss in Complement activity occurred when these milks were heated to either 65 or 72 °C for 10 min (Fig. 5). At the same time, the residual Complement activity associated with the latter two temperatures falling within batch pasteurisation (65 °C, 30 min) and continuous high temperature, short time (HTST) holding conditions (72 °C, 15 s) concurs with its low level presence in retailed, commercially pasteurised milks referred to earlier. Further increases in temperature (85 or 95 °C for 10 min) continued to inactivate Complement. All samples were significantly different from one another  $\chi^2$  (6,  $n = 21$ ) = 28.566,  $P < 0.001$  (Fig. 5). Further post-hoc comparisons using the Mann–Whitney test to look at individual samples identified that there was no significant difference between 65 and 72 °C ( $P < 0.05$ ).

#### Comparison of cream and buttermilk samples

Buttermilk is known to be rich in milk fat globule membrane (MFGM) components (El-Loly, 2011). The Complement activity of buttermilks was significantly ( $P < 0.05$ ) greater than that of the creams from which they were generated according to the Kruskal Wallis test (Fig. 6). The buttermilk prepared from the cream of raw milk was significantly different ( $P < 0.01$ ) from the laboratory-prepared cream, retail cream and the washed buttermilk samples based on the Mann–Whitney test of comparing each group individually. A loss of bactericidal sequestration activity in the buttermilks generated from washed cream would suggest that Complement components were removed to some extent into the aqueous phase.

#### Discussion

This study confirms that Ogundele's (1998) bactericidal sequestration assay which uses a specific strain of *Esch. coli* to detect the presence of Complement activity in human milk may now be extended for similar assay detection in bovine milk. The bactericidal sequestration assay confirmed the presence and activity of Complement in bovine milk, this also suggests that both human and bovine milks are broadly analogous in terms of their sequestration response (Figs 1 & 4).

This is an interesting finding given the complexity of Complement with its 30+ proteins and protein fragments that there should be considerable homology between human and bovine milks. Thus, the milks of both species would appear to share the same amplifying Complement cascade that builds up to the activation of a cell-killing membrane attack complex (Ogundele, 2001). The results in Fig. 1 – also highlight that the bactericidal sequestration assay was adaptable to allow for the direct comparison of human and bovine milk, thus making it possible to further investigate various bovine milk by-product streams and also milks subjected to different process treatments.

Exposure to a range of thermal treatments 42, 45, 65, 72, 85 and 95 °C for 10 min progressively inhibited Complement activity (Fig. 5). The initial loss in Complement activity over the course of a 3 °C rise during heating of bovine milk from 42 to 45 °C highlights how heat labile these immune proteins are. Fortunately, some residual Complement activity is still detectable in and around typical milk pasteurisation temperatures whether by batch heating or HTST methods. This concurred also with assay results generated with samples of commercially retailed pasteurised milks. Testing of fresh untreated and retail pasteurised bovine milks in both full and low fat variants provided the first indication that Complement activity appeared to be greater in milks containing a higher fat content (Fig. 3). This was confirmed in the course of a follow-up study involving cream separation of milk by either gravity-induced or centrifugally-employed methods which showed that greater Complement activity was detectable in the milk fat enriched phase of both bovine and human milk. Attempts at cream dilution to ascertain if there was a relationship between Complement response and fat content were not conclusive. This may have been due to limitations with the microbiological assay as a prozone-based inhibition effect has previously been described (Rainard & Caffin, 1984; Ogundele, 2001). In any case, Complement-based sequestration of *Esch. coli* 0111 was no longer observed in samples where milk fat was depleted or substantially reduced in the case of raw and retail low-fat milks. Simulated buttermaking of the prepared creams enabled Complement activity to be tracked during further partitioning between the fat and aqueous (buttermilk) phases. Potent Complement activity evident in buttermilk samples suggests that it most likely associates with the MFGM components present. MFGM has already been shown to exhibit some antibacterial properties

(Singh, 2006). MFGM as a tri-layer of both proteins and lipids from the endoplasmic reticulum membrane and mammary epithelium cells that envelopes the lipids in milk (Mather, 1999) is also a carrier of biologically active molecules like phospholipids, sphingolipids, cholesterol and other bio-functional proteins (Lopez, 2011; Lopez et al. 2011).

Overall, it can be seen that the Complement system is present in bovine milk and while heat labile, a certain amount of activity is detectable after milk pasteurisation. Although not identical in response, the levels of Complement activity in bovine milk are closely comparable with that of human milk. Complement's association with the fat phase of milk has implications for the development of low fat variants of fresh dairy products, and indeed where milk fat is replaced by non-dairy fat sources in infant formula. Future studies should aim to further investigate the factors that influence Complement activity in bovine milk and methods to retain its activity during processing.

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