THE AGGLUTININS OF COW AND BUFFALO MILK

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THE AGGLUTININS OF COW AND BUFFALO MILK

THESIS

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Freeze dried ampoules
of euglobulin F and
cryoglobulins

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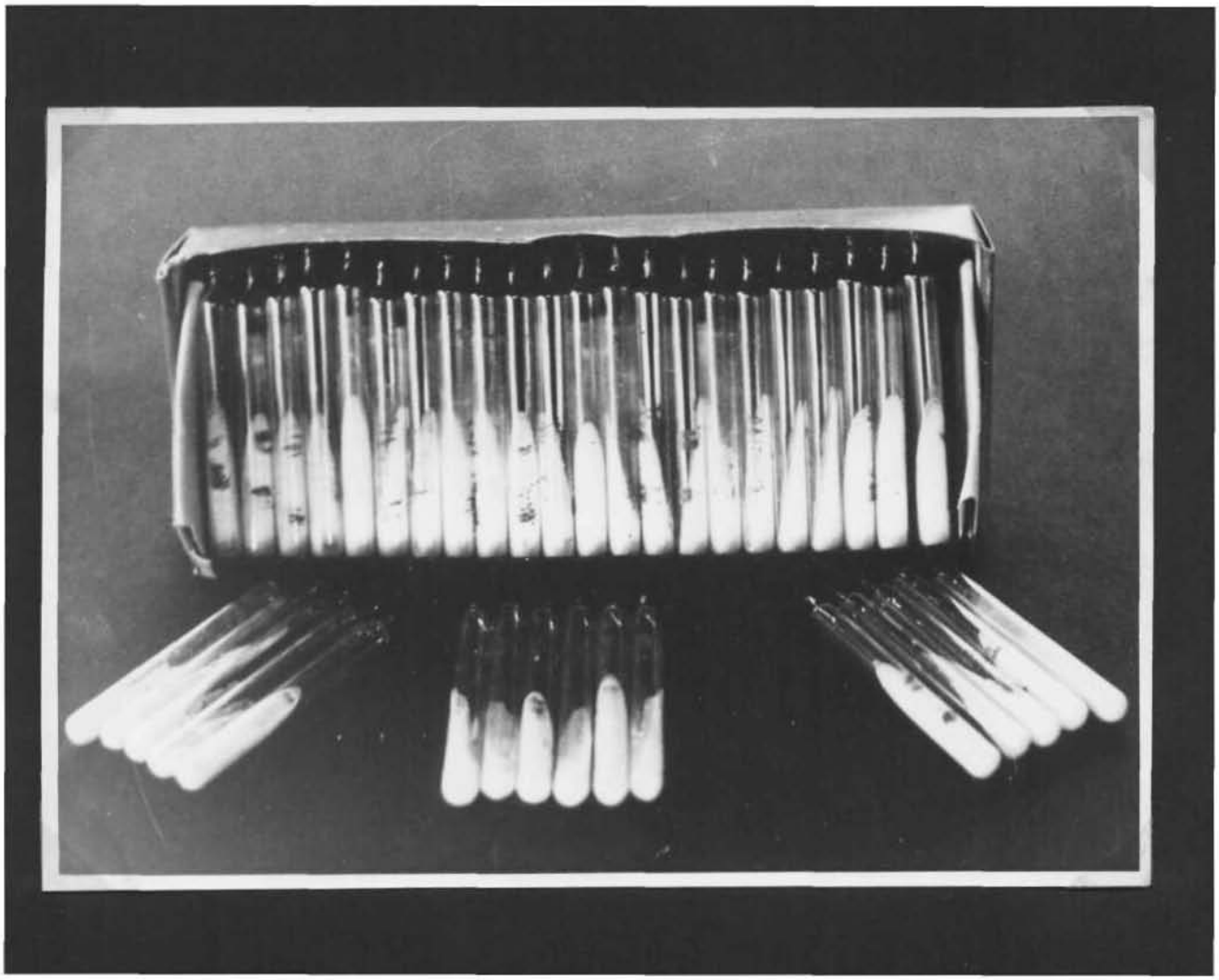

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INTRODUCTION

I N T R O D U C T I O N

The bactericidal activity of raw milk was first observed by Fokker (1890) in raw goat's milk. Since then interest has increased greatly in recent years with the realisation that, the antibacterial property of milk was due to a group of heterogeneous substances probably unrelated to each other, with differing physico-chemical properties. These substances exerted a total or partial inhibition on the growth of many micro organisms including strains of lactic streptococci.

This group of heterogeneous substances with anti-bacterial activity in milk can be recognised as the agglutinins in the whey proteins of milk, as well as that associated with the fat globules of milk and the inhibitory substances in the whey proteins of milk.

In recent years a number of investigations have been conducted on the agglutinins in milk which are specific antibodies, that have been shown to occur in the immune globulin fraction of milk which are preformed in the blood of cow. Hence, the recent advances in the elucidation of the structure and properties of the immuno-globulins of mammalian sera is also applicable to the immune globulins of milk and colostrum since, as in blood serum, three main classes of immunoglobulins, IgG, IgM and IgA, in milk and colostrum, which appear to carry

antibody activity have been recognised (Mach et al, 1969).

Yet another group of agglutinins has been shown to occur on the surface of the fat globule membrane called the fat globule agglutinins (Kenyon et al, 1966).

The observation, that the phenomenon of agglutination interfered seriously with the manufacture of cottage cheese (Emmons et al, 1963), and the role of fat globules in the inhibition of starters (Jago, 1954 and Gillies, 1960) were the focus of interest to the dairy microbiologists in recent years.

The published reports in the past decade or two on the agglutinins of the whey proteins and the fat globules of milk are inadequate to provide conclusive evidence to prove whether these two antibodies are identical or only antigenically related, in which case they may be expected to have both common and distinctive structural features.

Although extensive studies have been undertaken on the agglutinins of cow milk, there is paucity of similar studies on the agglutinins of buffalo milk.

The importance of the buffalo for milk production in India need hardly be emphasised. Hence, it was thought that a study of agglutinins in buffalo and cow milk would

be of value for the dairy industry due to the important role of agglutinins in the general immune response as well as in the passive transfer of immunity from mother to offspring.

This investigation is undertaken to study: a) the nature and properties of the agglutinins of cow and buffalo milk with a view to elucidate the inter relationship between the whey protein and the fat globule agglutinins of the two species, b) the behaviour of different bacterial strains towards the agglutinins of cow and buffalo milk, c) the agglutinin activity in the milk from mastitic udder and the response of udder towards invading pathogens, d) the influence of colostral antibodies on the agglutinin activity in the blood of cow and buffalo calves and e) the effect of susceptible and resistant bacteria on the fermentation of milk.

The present findings of the investigation have been discussed in relation to their implications on such parameters as differential susceptibility to diseases as well as its possible role in calf mortality and the importance of selecting suitable cultures for preparing fermented milk products.

Further, a study on the agglutinating antibodies in the milk of different breeds of animals, and their role in the control of udder infections, may provide interest-

ing information and valuable solution to combat the losses due to mastitis. The annual loss incurred due to mastitis alone in India, is estimated to be about Rs.50 to 60 crores (Anantaramiah, 1969) and in Tamil-Nadu about Rs.2.6 to 3 crores (Krishnaswamy, 1964).

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Raw milk has the property of killing or inhibiting the growth of many microorganisms (pathogenic and saprophytic) to some extent. There are evidences for the presence of inhibitors in raw milk which differ in their antibacterial specificities. This property is referred to as the germicidal property of milk.

1. The germicidal property of milk:

The presence in milk of a germicidal activity was first demonstrated by Fokker (1890) in raw goat milk. Hunziker (1901) studied the action of milk of the individual cows on their own milk flora, and observed a marked decrease in bacteria in the milk of some cows and none in others. He also investigated the possible variation in the germicidal power in the milk from different cows. Stocking (1904) explained that the reduction in the number of bacteria in the mixed milk from a herd of 30 cows was due to the death of those organisms which found milk an unfavourable medium. Using pure cultures Coplans (1907), Evans and Cope (1908) and Roseneau and McCoy (1909) established definite germicidal action in the milk in varying degrees on quite a number of different species of bacteria. With the aid of microscopic examination and plate count, Chambers (1920) demonstrated the germicidal property in raw milk which was specific,

depending on both the individual cow and the species of bacteria. Sherman and Curran (1924) observed that Streptococcus lactis was definitely inhibited when inoculated into fresh milk. Jones and Little (1927) found that milk of cows possessed bactericidal properties which inhibited the growth of microorganisms in milk. The substance responsible for the inhibition of organisms was referred by Jones and Simms (1930) as lactenins. The relatively quick death of Streptococcus pyogenes in milk stored at room temperature was attributed to the natural inhibitory properties of milk by Pullinger and Kemp (1937). Wilson and Rosenblum (1952) reported that human and goat milk contained an antistreptococcal principle similar to the lactenin of cow milk but their attempts and those of the previous workers to purify and identify the lactenin failed. They also observed, that milk was inhibitory only under aerobic conditions and that there was no inhibitory action invive. Auclair and Hirsch (1953) demonstrated the presence of two inhibitory substances in milk which according to them were identical with the lactenins of Jones. Wolin and Kosikowski (1955) recognised the presence of natural bacterial inhibitory properties of milk in some raw milk samples, known not to contain antibodies, sulpha drugs or quarternary ammonium compounds. Using Streptococcus pyogenes as the test organisms to study the germicidal

property of milk Henningson and Kosikowski (1957) reported that the lethal effect of raw milk and whey for Streptococcus pyogenes was probably due to deficiency of free sulphydryl groups and denatured albumins. Wright and Tramer (1957) attributed the inhibition of acid production by certain starters in raw milk to the action of agglutinins and cream rising. In the following year (1958) the authors, on further investigations, suggested that, the agglutinin was lactenin 1 and lactoperoxidase was lactenin 2. Sipka (1959) reported the existence of two inhibitory substances in sheep milk. Sasaki and Aibara (1959) studied the effect of acetone fractions of milk whey on Bacillus subtilis, Corynebacterium xerosis and Escherichia coli, and reported that the inhibitory substances in milk remained in the whey and was associated with the beta lactoglobulin of whey proteins. Reiter et al (1963) showed that the inhibition of certain strains of Streptococcus cremoris in milk was due to the effect of lactoperoxidase. Kosikowski and O'Leary (1963) observed that during May and June about 56 percent of Irish milks showed thermolabile inhibition, attributable to natural inhibitory sources. Reiter and Moller Madsen (1963) reported that the inhibitory substances of milk are numerous, they being, lactenins, lactoperoxidase, agglutinins and the inhibitor associated with the fat. They further reported that raw milk was

bactericidal against group A Streptococci and some strains of groups F, G, H, K, and L but all the strains of groups B, C, D and E were resistant. A heat stable inhibitory factor in milk against Bacillus Stearothermophilus was observed by Cheesman and Jayne-Williams (1964). They found that this factor was nondialysable separated with the milk proteins on filtration on sephadex - G-25, lost its activity on treatment with trypsin and was probably associated with the electrophoretically slower moving component. Randolph and Gould (1966) observed that milk from individual cows and herds inhibited the acid production of single and mixed strain cultures. Oram and Reiter (1966) who studied the inhibition of certain strains of group N Streptococci and the chemical nature of the inhibitory compounds, postulated that the inhibitory system was composed of lactoperoxidase /thiocyanate/Hydrogen peroxide, whose effect on streptococci was highly strain specific. Reiter and Oram (1967) found that some of the inhibitory systems in milk operated in other body fluids also. The inhibitory systems reported by them were lactoperoxidase/thiocyanate/Hydrogen peroxide, Xanthine oxidase, lactotransferrin and complements and antibodies. Vedanayakam et al (1968) studied the germicidal property of milk and reported that the germicidal action of milk was only bacteriostatic and not bactericidal.

2. Agglutinins in milk:

a) Agglutinins associated with whey proteins: The observation of agglutination of certain bacteria in milk serum lead Hinemann and Glenn (1908) to conclude that the germicidal action of milk was due to agglutinins. Using three different pure cultures Chambers (1920) observed a best agglutination of Culture R and a good agglutination of Bacterium lactis acidi in raw milk and a weak agglutination of Bacterium coli. He further reported that there was no common relation between agglutination and bacterial inhibition. Hobbs (1939) examined a variety of milk organisms and attributed the clumping effect of milk to nonspecific agglutinins. The suggestion that agglutination and creaming were mainly responsible for the inhibitory action of milk was proposed by Wright and Tramer (1957). McPhillips (1958) examined a number of fast and slow acid producing streptococcal cultures for agglutination in raw whole milk and found that only slow acid producing cultures were agglutinated. Keogh (1958) reported that agglutination did not appear to be the direct cause of inhibition, and creaming resulted in physical removal of organisms from the bulk of the milk. He reported significant inhibition occurred due to agglutination, only when there was a substantially complete removal of organisms into the cream layer. Auclair and Portmann (1959) named the

agglutinins as inhibitory principles in milk 'Lactenin Lg'. Portmann and Auclair (1959) studied the inhibitory and agglutinating power of raw milk and suggested that because of the close relationship, the substances responsible for these two phenomena were identical. Agglutination was caused by relatively specific antibodies, was shown by McPhillips (1958) and Portmann and Auclair (1959). Randolph (1963) and Stadhouders (1963) stated that these antibodies occurred in the immune-globulin fraction of milk. Emmons et al (1963) associated the sludge formation at the bottom of cottage cheese vats and slow acid development, with the agglutination and settling of certain strains of starter bacteria. They also found that the starter bacteria were agglutinated in the rennet whey from the pasteurized skim milk. However, Stadhouders (1963) studied the inhibitory effect of Lactenin L_3 on acid production in milk and found that the inhibition in whole milk and skim milk was the same and that rennet addition prevented the agglutinins from inhibiting acid production and concluded that agglutinins were of no importance for the activity of cheese starters. He also further stated that the agglutinins were probably present in the euglobulin fraction and the actual agglutination was not the cause of inhibition, and the inhibition of acid production was brought about by the removal of the bacteria

from the greater part of the milk. Reiter and Moller Madsen (1963) said that agglutinins occurred at low titres in raw milk throughout the lactation. They also stated that the physical removal and subsequent starvation of the bacteria were unlikely to be the only reason for the inhibition since some starters were inhibited even when creaming was prevented. Emmons et al (1966) stated that acid production in the supernatant skim milk was retarded through displacement of agglutinated bacteria to the bottom indicating that agglutinating antibodies in skim milk were not inhibitory perse. Reiter and Oram (1967) attributed the bactericidal activity of raw milk against Gram negative organisms to the killing effect of complement and antibodies and also said that agglutination reduced the count of Gram positive organisms. Vedanayakam et al (1968) confirmed the views of earlier workers that the agglutinins of milk were associated with the Lactenin L_1 fraction.

b) Agglutinins associated with fat globules: Jago (1954) found that the inhibitory substance in milk was closely associated with the fat globules in milk which was bacteriostatic and probably enzymic in nature. He also stated that the substances were adsorbed to the surface of the fat globules, since after churning only the buttermilk was found to be inhibitory while the fat globules were inert in their effect upon susceptible

organisms. Gillies (1960) demonstrated a correlation between the degree of inhibition and the amount of fat present in milk and observed that inhibition was manifested only with the formation of a cream layer, when the cells were taken up by the rising fat globules, the inhibitory factor was associated with the cream layer, the inhibitory substances could be washed out of the cream layer and that the inhibitory factors from skim milk could be adsorbed on to the washed cream. He concluded that agglutination was not necessarily an integral part of inhibition, but accompanied the same and that, skim milk appeared to donate a factor which combined with another factor on the fat globule membrane, resulted in inhibition, if creaming was permitted, and both were necessary for maximum inhibition. Reiter and Moller Madsen (1963) reported that agglutinated organisms as well as individual chains were observed to aggregate around the fat globules and the hypothesis of inhibition by starvation of bacteria in the cream layer was not convincing. Since the difference in the acid production in whole milk and skim milk was very small Stadhouders (1963) concluded that a smaller part of the inhibitory principle was associated with the fat globules while a greater part was present in the skim milk fraction. Narasimham (1968) observed that the cream washings of cow and buffalo milk contained

agglutinins. He found that the concentration of agglutinins on the fat globular membrane of cow milk was higher than that of buffalo milk and that the agglutinins were adsorbed on the fat globular membrane of cow and buffalo milk. Vedanayakam et al (1968) reported the presence of agglutinins in the cream washings of cow milk.

3. Factors influencing the properties of agglutinating antibodies in milk:

a) Nature of euglobulin antibodies: Kenyon and Jennes (1958) confirmed that the euglobulin of milk was responsible for fat globule agglutination, since addition of colostral euglobulin restored the creaming ability of skim milk. Randolph (1963) showed that the immune globulin fraction of milk carried the inhibitory factor and that the inhibition was due to specific antibodies. Stadhouders (1963) reported that the euglobulin of milk carried the agglutinin activity for the inhibition of acid production by lactic acid bacteria. Kenyon et al (1968) indicated that the immune globulins were able to bring about fat globule clustering and creaming in milk. Fat globule agglutinins isolated from the surface of fat globules were also shown by them, to contain immune globulins, and the demonstrated that the immune globulins were responsible for the clustering of fat

globules and the adherence and the agglutination of Brucella cells on the fat globule surface with Brucella ring test Stadhouders and Hup (1970) clearly demonstrated the presence of three distinct classes of antibodies in the euglobulin of milk, one causing the agglutination of bacteria (which was euglobulin F, after the removal of cryoglobulins), another one causing the agglutination of fat globules and a third one causing the attachment of bacteria to fat globules (these two properties were attributed to cryoglobulins obtained by cryoprecipitation of euglobulin).

b) Specificity of agglutinins: McPhillips (1968) by acidity and bacterial ring test established that the agglutinins in milk showed a high degree of specificity in regard to various susceptible cultures. Portmann and Auclair (1959) demonstrated by absorption tests, that the milk absorbed by a given sensitive strain lost almost completely its inhibitory activity and its agglutinating power in relation to the same strain or strain of the same group. From the behaviour of different strains they were able to recognise five groups of strains reacting in identical way, With the help of bacterial ring test, and employing bacterial strains of Streptococcus lactis 57 and Streptococcus cremoris S_2, Narasimhan (1968) demonstrated the specificity of agglu-

tinins in milk. Stadhouders and Hup (1970) showed that while the euglobulin bacterium complex was specific, the euglobulin fat globule complex and the antibodies attaching bacteria to fat globules were non-specific.

c) pH: Thompson (1940) found the inhibitory substances most stable at pH 6.5. Wilson and Rosenblum (1952) showed that Lactenin L_1 was not stable to heat at pH 6.0 to 6.5. Auclair and Hirsch (1953) reported that the maximum stability of Lactenin L_1 was pH 6.0 to 6.5. Kosikowski and O'Leary (1963) observed that most of the milks inhibitory for all test bacteria were lower than normal in pH and all milks having a pH 6.4 or less inhibited atleast one of the test organism.

d) Temperature: Thompson (1940) reported that the inhibitory substances of milk were destroyed by heating for 20 minutes at 71° to 74°C. Morris (1945) demonstrated that a factor active against certain strains of coliform bacteria being destroyed at 53°C for 30 minutes, while another factor like Lactenin was partially destroyed at 65°C for 30 minutes and completely inactivated at 70°C for 30 minutes. RoadHouse and Henderson (1950) reported that high temperature i.e. 140° to 176°F for 30 minutes destroyed the germicidal property of milk. This property was more marked at 100°F for a shorter

duration than at 40°F. Wilson and Rosenblum (1952) found that about 90% of Lactenin L_1 was destroyed at 68°C for 20 minutes. Foster (1952) reported the cause for the improved growth of organisms in milk subjected to heat treatments between pasteurization and boiling, was the destruction of one or more inhibitory components naturally present in raw milk. Auclair and Hirsh (1953) showed that the inhibitory substances of milk were destroyed by heating for 20 minutes at 71°C to 74°C. At 71°C Lactenin L_1 was completely destroyed and heating to 70°C at pH 7.0 for 20 minutes also destroyed Lactenin L_1. Jago (1954) stated that heating the milk to boiling point for variable periods, or autoclaving the milk, destroyed the inhibitory effects of milk on susceptible cultures. Sasaki and Aibara (1955) found that the natural germicidal action of milk, increased by heating the milk to 58°C for 20 minutes and was destroyed by heating for more than 20 minutes, at 70°C. Kosikowski and Mocquot (1958) stated that Lactenin L_1 was inactivated at 70°C for 20 minutes. Randolph (1963) reported that pasteurisation reduced inhibition by approximately 15%. According to Kosikowski and O'Leary (1963) a minimum pasteurisation temperature was ineffective in eliminating the natural inhibitory effects and heat treating the milk to 180°F for 5 minutes removed the natural inhibitory effects. Emmons et al (1966), observed that skim milk heated to

71°C for 30 minutes eliminated the defects due to agglutination and that pasteurization (72°C for 16 seconds) reduced the agglutinating activity of skim milk by 50 to 75%. Randolph and Gould (1966) reported that al though pasteurization produced slight reduction in the inhibitory property of milk, it failed to alter greatly the acid production of the culture, indicating that the inhibitory substances involved were heat stables. Narasimhan (1968) found that pasteurization did not destroy the agglutinins in milk and that the agglutinins were destroyed when milk was heated to 70°C for 20 minutes.

Dunkley and Sommer (1944) reported that at lower temperature there were more agglutinins in the surface layers of fat globules. Wilson and Rosenblum (1952) found reduction in the Lactenin activity at 22°C rather than at 37°C. Randolph (1963) observed that the inhibitory activity of milk was not affected by storage at 40°F for 48 hours. Stadhouders (1963) suggested an increased adsorption of the agglutinins on the fat globules at low temperature (6°C) and conluded that the distribution of the agglutinins between the cream and the skim milk fractions was dependant on the temperature. Randolph and Gould (1966) reported that storage of individual cow samples at 4.5°C for 48 hours, had no significant effect on the inhibitory capacity of the milk. Narasimhan

(1968), observed that storage of cow milk for 24 hours at 4.5°C did not affect the agglutinin activity. Stadhouders and Hup (1970) showed that the euglobulin which carry the agglutinating antibodies were able to aggregate forming cryoglobulins at low temperature (2°C).

4. Factors affecting the agglutinin activity of milk:

a) Species: Fokker (1890) reported the germicidal action in goat milk. Wilson and Rosenblum (1952) found that human and goat milk contained antistreptococcal principle similar to that of cow milk. Kosikowski and Mocquot (1958) reported that the concentration of Lactenin L_1 and L_2 in milk from individual cows and bulk milk were relatively constant. Sipka (1959) observed the presence of two inhibitory substances in sheep milk. Natarajan et al (1964) stated that the agglutinin content was more in cow milk than in buffalo milk. Emmons et al (1966) studied the agglutinin concentration in the blood and milk of ruminants and nonruminants and observed that the agglutinin concentrations of milk from the beef cattle and sheep were within the range found in milk from dairy cows. Thomas et al (1966) reported that the rate of acid production by Streptococcus cremoris, Streptococcus diacetilactis and Streptococcus thermophilus was higher in buffalo milk than in cow milk. Narasimhan (1968) studied the agglutinin content in the milk of different species of animals

by means of bacterial ring test and reported that cow and sheep milk gave positive ring test while the milk of buffalo, goat, ass and human were negative for the ring test. He observed that the agglutinin content varied from species to species. Vedanayakam et al (1968) observed that the Lactenin L_1 fraction of cow had a greater inhibitory effect than that of buffalo.

b) Breed: Highest agglutinin content in the cross bred cows followed by pure bred Thari and Sindhi animals was observed by Natarajan et al (1964). Emmons et al (1966) studied the concentration of agglutinins for three different strains of organisms, in the blood and milk of Holstein, Ayrshire, Guernsey and Jersey breeds and found that concentrations varied between breeds and concluded that the concentration of agglutinins in milk were independent of breed of the cows. Randolph and Gould (1966) found variation in the average inhibition of acid production by the milk from different breeds: Ayrshire - 70%; Guernsey - 69%; Jersey - 68%; Holstein - 52%; Brown Swiss - 43%. Narasimham (1968) analysed the milk of Gir, Sindhi, Jersey, Cross bred Singhi, Cross bred Gir, Cross bred Jersey and Nondescript cows for agglutinin content by means of milk ring test and found that cross bred cows contained more agglutinins in milk than pure bred cows.

c) Stage of lactation: McEwen & White (1950) showed that the antibacterial activity decreased during the drying off period. Auclair and Hirsch (1953) demonstrated that Lactenin L_1, was the substance mainly occurring in colostrum giving partial inhibition to high dilutions of the samples, and observed little variations in the titres with various samples of bulk milk and milk from cows in mid lactation. Auclair (1954) however, found, that the agglutinin content of 60 samples of mid lactation milk varied between 70-220 units and that colostrum was rich in Lactenin L_1. Kosikowski and Mocquot (1958) stated that large variations in the L_1 occurred only between colostrum milk and normal milk, and that Lactenin L_1 was present in large concentration in colostrum and in lesser quantities in mid lactation milk.

Auclair and Portmann (1959) found high agglutinin titre in colostrum as compared to mid lactation milk. Portmann and Auclair (1959) stated that Lactenin L_1 was found in high concentration in colostrum. In addition they found another factor Lactenin L_3 which inhibited the growth of Streptococcus cremoris 760 occurring in milk and especially in colostrum. Campbell and Norcross (1964) showed the presence of antibodies against Streptococcus agalactiae in the colostrum of first calf heifers.

Emmons et al (1966) studied the lactic streptococci agglutinins in milk and blood of cows throughout the stage of lactation and observed, that the agglutinin titres were relatively high in the lacteal secretion obtained the day prior to parturition, titres dropped slightly in the first colostrum collected, and in milk titres decreased during the first two weeks to levels that remained relatively constant until normal lactation ceased, and rose again just before the end of lactation. They suggested that late lactation milk might be a source of high titres in bulked milk. Variation in the agglutinin content of milk at different stages of lactation was reported by Narasimhan (1968). Using the technique of bacterial ring test, he observed maximum agglutinin content in colostrum, which decreased slowly from the 5th day of lactation, remained more or less constant till about the 9th month of lactation and increased at the 10th month of lactation.

d) Miscellaneous factors: Variation in the agglutinin content of milk from different quarters of the same cow was noted by Jones and Simms (1930). McEwen and White (1950) showed that the inhibitory activity in milk did not vary either with season or the diet. Czulak and Meanwell (1951) attributed the 'winter slowness' of cheese starters in pasteurized to an inhibitory substance and a growth factor and in summer when grass

and green fodder were fed to the animals, the growth factor was sufficiently high to overcome inhibition. Jago (1954) said that the inhibitory substance was normally present in all milk supplies throughout the year. Wolin and Kosikowski (1958) observed seasonal variations in the initial zone size of bacterial inhibitory zones in whey agar by raw milk and stated that zone size decreased or disappeared during the winter months. Wide variations were observed from day to day in the ability of milk to support acid production by Keogh (1958) and they said that none of these variations were associated with weather conditions. Kosikowski and O'Leary (1963) observed thermolabile inhibition attributable to natural inhibitory sources in about 56% of Irish milks tested during the warm months of May and June. Seasonal variation of the inhibition of acid production by the agglutinins in milk was recognised by Stadhouders (1963) who observed that from March to June the inhibition was rather small. Emmons et al (1966) found that the concentration of agglutinins in milk were independent of the age of the cows.

5. Methods of estimation for agglutinin activity:

a) pH assay: Using Streptococcus pyogenes 'Richards' strain as the test organism Auclair and Hirsch (1953) estimated the inhibitory activity of milk by pH response

assays. A sample of milk from a normal cow in mid-lactation which was inhibitory to the test organism at 1/100 dilution was chosen as a standard. The standard was defined as containing 100 units of inhibitory substance (Lactenins) per ml. of milk. Dilutions of unknown samples were prepared in sterile separated milk adjusted to pH 7. To 1 ml. quantities of these dilutions was added 1 ml. of glucose peptone broth containing 5% V/V of an 18 hour serum broth culture of Streptococcus pyogenes. The tubes were incubated at 37°C for 6 hours, then 10 ml. of distilled water was added to each tube and the pH determined electrometrically. The pH response assay of Auclair and Hirsch (1953) was followed by Sasaki and Aibara (1959) using Streptococcus pyogenes 'Yugo' as the test organism selected from more than 20 strains of animal and human type. Natarajan and Dudani (1961) determined the Lactenin content of cow and buffalo milk using 21 different lactic as well as other organisms including a few pathogenic cultures by pH response assay. Srinivasan (1968) estimated the natural inhibitory property of cow, buffalo, sheep and goat milks by pH response assays.

b) Activity test: Keogh (1958) assessed the variations in acid production by starter cultures in milk by activity tests. Tests were performed in raw and heat treated milk.

The milk samples were inoculated with 1 ml. of 10% starter cultures, incubated for 5½ hours at 30°C and titrated with N/10 Sodium hydroxide to phenolphthalein end point and the results reported as net acidity. Gillies (1960) studied the role of fat globules in the inhibition of certain strains of Streptococcus cremoris in pasteurised milk by an examination of the acidity production by these starters. Stadhouders (1963) determined the inhibitory effect of Lactenin Ly on acid production by the starter Streptococcus cremoris 803 by activity tests.

c) Milk ring test: To study the effect of agglutinins in raw milk on the various starter cultures Keogh (1958) employed the technique of ring test. To a drop of stained antigen 2 ml. of whole raw milk was added, mixed and incubated at 30°C for 3 hours. Tests with heat treated whole milk were performed in parallel. In positive tests a deep purple ring appeared rapidly in the cream layer, the lower milk clearing to white. In the negative tests the milk remained mauve throughout. Narasimhan (1968) studied the agglutinin content of cow, buffalo, sheep, goat, ass and human milks by the milk ring test.

d) Microscopical observation of agglutination: Cahmbers (1920) employed the microscopical observation method to determine the degree of agglutination for three

different strains of bacteria in raw milk by making smears of raw milk. Keogh (1956) studied the role of agglutination and creaming in inhibition by raw milk by using microscopic observation of agglutination as one of the techniques. For the test he made smears of milks after incubation in the activity tests, stained with Loeffler's Methylene blue and examined microscopically. Measurement of the agglutinating power of the milk was performed by Portmann and Auclair (1959) by the technique of microscopical observation of agglutination. Two fold dilutions of milk samples were prepared. On a glass slide a drop of each dilutions was mixed with one drop of the bacterial suspension and the agglutination of the bacteria were observed under the microscope. Emmons et al (1965) reported a sensitive test for lactic streptococcal agglutinins, wherein serial dilutions of whey were prepared. A volume of 0.2 ml. of each dilution was mixed with 0.2 ml. of the cell suspensions in Kahn tubes. A 0.04 ml. aliquot of each mixture was transferred to the well of a Kline slide and the slide was shaken for 60 minutes and the wells on the slide were observed for agglutination, under a stereo microscope (30x). The last dilution showing agglutination was recorded as the titre of the whey. Narasimhan (1968) employed the technique of microscopic observation of agglutination to examine the whey, lactenins, and cream

washings of cow and buffalo. Stadhouders and Hup (1970) estimated the agglutination titre for the bacteria by the microscopic observation method for agglutination and suggested that if aggregates of 10 or more cells were observed under the microscope within 20 minutes the result was considered to be positive.

6. Agglutinin activity on different bacterial strains:

Chambers (1920) observed that a brilliant red chromogen isolated from a creamery can, called culture R showed best agglutination in raw milk, Bacterium lactis acidi gave a good agglutination and Bacterium coli a weaker agglutination. Sherman and Curran (1924) found that Streptococcous lactis was definitely inhibited when inoculated into fresh milk. Curran (1931) noted that rapid growing invasive type of streptococci were most affected. Jones et al (1937) reported that agglutinins of milk were very active against Streptococcus pyogenes. Auclair and Hirsch (1953) employed Streptococcus pyogenes 'Richards' strain for estimating the Lactenins L_1 and L_2 in milk and colostrum and reported that Streptococcus pyogenes was very sensitive to inhibition by raw milk, when grown aerotically, and Streptococcus agalactiae which was also sensitive to inhibition was able to overcome the inhibition in the presence of peptone. Jago (1954) observed that many single strain

lactic streptococci exhibited a difference in susceptibility to an inhibitory substance present in raw and H.T.S.T. pasteurized milk, some being markedly affected while others were completely resistant. He further noticed that when cream was removed from raw whole milk susceptible culture Streptococcus cremoris ML_1 showed a marked increase in lactic acid production. Keogh (1958) investigated the effect of agglutinins in raw milk on the following starter cultures by ring test:

Streptococcus cremoris (C_1, C_3, K, DR_7, R_1, US_3, HP, E_8 and KH),

Streptococcus lactis (C_2 and C_{10}),

Streptococcus diacetilactis (DRC_1, DRC_2 and DRC_3).

He found the following cultures were positive for the ring test.

Streptococcus cremoris (C_1, DR_7, R_1, US_3, HP, E_8 and KH),

Streptococcus lactis (C_{10}) and

Streptococcus diacetilactis (DRC_2).

McPhillips (1958) who demonstrated the specificity of agglutinins in milk used as test organisms Streptococcus lactis C_{10} a fast acid producing culture and Streptococcus cremoris ML_1, KH and DR_7 all slow acid producing cultures. He established that in all cases the slow cultures were agglutinated. With a view to investigate the relationship between inhibition and agglutination of

different strains of lactic streptococci by raw milk Portmann and Auclair (1959) selected the following 15 strains of lactic streptococci.

Streptococcus cremoris (972, E_8, 760, DR_7, K, 803 R_1; US_3, KH, ML_1, C_3 and HP),

Streptococcus lactis (C_{10} and T_7) and

Streptococcus diacetilactis (DRC_3).

They found out of these 15 strains, 11 were agglutinated except strains C_3, C_{10}, T_7 and DRC_3. Gillies (1960) examined susceptible strains of Streptococcus cremoris HP and R_8 and resistant strains of Streptococcus cremoris C_{13}, and C_3 for acid production and found inhibition of the susceptible strains occurred only when cream layer was allowed to form on top of the milk. Smears of susceptible strain HP was found to be agglutinated while resistant strain C_3 showed no evidence of agglutination. Natarajan and Dudani (1961) studied the natural inhibitory action of cow and buffalo milk on 21 cultures consisting of lactic acid bacteria, and other milk spoilage organisms as well as a few pathogens encountered in milk, and found that except four cultures the rest were inhibited with varying titres. Sharpe et al (1962) observed a reduction in the growth of a strain of Staphylococcus aureus in raw or pasteurized milk than in steamed or autoclaved milk due to agglutination of the bacteria which interfered with the counts on agar plates. Randolph (1963) examined the susceptibility of 2 strains of

Streptococcus lactis, 9 strains of Streptococcus cremoris and 7 mixed strain commercial cultures to natural inhibitors in milk and found that susceptibility varied from 7 to 85% and that single strain cultures were generally more susceptible than mixed culture. Emmons et al (1963) found certain strains of starter bacteria for cheese manufacture - Streptococcus cremoris and Streptococcus lactis were agglutinated resulting in slow acid production and sludge formation. They also observed that agglutination was evident with 14 strains in rennet whey from pasteurised skim milk and that the agglutinin titres for each strain was not directly related to the extent of settling, which was greater with some strains. Auclair and Yvonnevassal (1963) using strains of Streptococcus lactis C_{10} and C_2 and Streptococcus cremoris 760 and 972 showed that a resistant strain of Streptococcus lactis C_{10} became sensitive to lactoperoxidase as well as to both agglutinins and lactoperoxidase on repeated subculturing in autoclaved milk. Variants sensitive to both agglutinins and lactoperoxidase appeared after a number of subcultures. In none of the cultures of C_{10} any cells sensitive only to agglutinins and not to lactoperoxidase were found. The 100% resistant strain of Streptococcus lactis C_2 did not show any sign of sensitivity to lactenins even after 12 months of subculturings in autoclaved milk. Strain Streptococcus cremoris 760 sensitive to

agglutinins on subculturing contained cells sensitive to both agglutinins and peroxidase, while Streptococcus cremoris 972 sensitive to both agglutinins and to peroxidase on subculturing contained 100% cells sensitive to both the inhibitors. Stadhouders (1963) showed the inhibitory effect of Lactenin L_3 on acid production in milk by Streptococcus cremoris 803, a strain sensitive to agglutinins in milk and insensitive to lactoperoxidase, and used this as the test organism in the investigations. Reiter and Moller Madsen (1963) reported that the natural non specific antibodies of milk agglutinated the non-pathogenic group N streptococci. An agglutinin sensitive organism Streptococcus cremoris DR_7 was used by Emmons et al (1965) to develop a sensitive test for lactic streptococcal agglutinins and they found the organism was positive for agglutination with 1/4 and 1/8 dilutions of whey and negative for 1/16 dilution. Randolph and Gould (1966) showed that Lactenin L_3 was active on many strains of lactic streptococci, while the other Lactenins L_1 and L_2 were active only on a few strains. They examined the following single and mixed strains cultures for their susceptibility.

Single strains: Streptococcus cremoris (K, KH, ML_1, R_1, US_3, HP, E_8, C_3 and C_7).
Streptococcus lactis C_2 and C_6.

Mixed strains: Streptococcus lactis C_2 (resistant) with any one of Streptococcus cremoris R, HP and KH (all susceptible) in different proportions.

They observed that the cultures varied widely in their acid producing capacities, that the inhibition of acid production were higher for the single strain cultures than for the mixed strain cultures, and that even 0.1% of resistant C_2 and 0.9% of any one susceptible cultures produced significantly beneficial results in markedly reducing the inhibitory property of both milk and skim milk. Emmons et al (1966a) studied the agglutinin titres for Streptococcus lactis C_2, Streptococcus cremoris R_1, and Streptococcus cremoris DR_7 in blood and milk throughout the lactation of cows and found that strain C_2 did not agglutinate in normal milk and strains DR_7 and R_1 agglutinated in blood and milk of all mature lactating animals but showed wide variations in titres. The authors (1966b) also reported the results of their study on the agglutination of a number of cultures of Streptococcus cremoris and Streptococcus lactis. They found the strains of Streptococcus cremoris K, ML_4, HP, ML_1, KH, R_1, Z_8, TR, R_6, E_8, DR_7, US_3 and C_1 and the strains of Streptococcus lactis C_6 were agglutinated with varying titres and the strains of Streptococcus cremoris C_3, C_{11}, C_7, and C_{13} and Streptococcus lactis C_2 were not agglutinated. Reiter and Oram (1967) attributed the inhibition of gram negative

organisms by raw milk to the killing effect of compliment and antibodies and the reduction in the count of gram positive organisms was due to agglutination. They also detected agglutinins against Streptococcus pyogenes, Streptococcus dysgalactiae, Streptococcus agalactiae, Escherichia coli and Corynebacterium ulcerans in the blood, milk and secretions of different animals. Narasimhan (1968) studied the response of bacterial cultures to ring test and found that Streptococcus lactis 57 and Streptococcus cremoris Sc gave positive ring test while Streptococcus thermophilus, Streptococcus lactis A (isolate) and B (isolate) and Streptococcus faecalis were negative for the test.

7. Antibacterial activity in the milk from infected udders:

a) Natural inhibitory action: Jones and Simms (1929, 1930) showed that cows resistant to mastitis produced milk with a higher inhibitory titre than susceptible cows. Singh and Laxminarayana (1948) reported that mastitis milk reduced the growth of starter organisms, when compared with normal milk. Murphy et al (1952) stated that the bactericidal or bacteriostatic properties of normal milk, would be unable to prevent the growth of even small numbers of Streptococcus agalactiae, but found that in two quarters which were already infected, one with micrococci and

one with non-haemolytic corynebacteria, infection was prevented. No correlation between resistance to infection and the inhibitory power of raw milk was found by Auclair and Hirsh (1953). Brown (1962) reported that the antibody titres in blood might be influenced by the number of infected quarters. Derbyshire (1964) proposed that severe mastitis seemed to occur when the milk became less bacteriostatic and also demonstrated that milk from a cow with mild mastitis had an increased inhibitory effect on the multiplication of Staphylococcus aureus. Narasimhan (1968) by means of bacterial ring test found that mastitis milk was positive for agglutination in both cow and buffalo milk and that the agglutinin content was also high.

b) Antibody production in the udder: Smith et al (1923) concluded that the udder distinctly participated in the production of agglutinin when the gland was invaded by living or dead Brucella organisms and they recorded a tissue reaction following the infusion of the udder by these bacteria. Kerr et al (1959) demonstrated by infusing bacterial and protozoal antigens into lactating and nonlactating udders, that a series of infusions could stimulate the production of specific antibodies within the gland. Porterfield et al (1959) made similar observations and showed that udder was site of antibody produc-

tion and responded to stimulation, and that intra mammary infusions of an antigen made during the dry period resulted in a higher antibody content in the colostrum than in the blood serum. They also found that antibodies appeared in the milk within two hours after lactating cows were infused with the antigens.

c) Leucocytes: The geometric average cell count of milk from healthy cows was found to be about 70,000 per ml. by MacLeod and Anderson (1952). MacLeod et al (1953) showed a relationship between the number of leucocytes in the herd milk and the percentage of mastitis animals in the herd. They found if the percentage of the infected animals within a herd was 40% or more, the average leucocyte count might be 1,000,000 or more per ml. of milk, and the milk from herds affected with Streptococcus agalactiae infection had higher leucocyte counts than with organisms other than Streptococcus agalactiae. Newbould (1964) stated that in any bacterial invasion of the human or animal body, leucocytes constituted the major natural defence mechanism and that in the invasion of the bovine mammary gland, milk from normal glands contained few leucocytes and they could not probably play any role in the very early stages of infection. Derbyshire (1964) investigated the part played by cellular and humoral components of mastitis

milk in inhibiting Staphylococcal multiplication and observed that the milk became relatively inhibitory to the growth of Staphylococci in proportion to the number of leucocytes added to the normal milk. They also found the supernatant fraction of mastitis milk after the removal of leucocytes was also inhibitory, and that cellular fraction was no longer bacteriostatic when the cells were killed by heat and the humoral inhibitory component of mastitis milk which might originate from blood serum was also heat labile. Singh and Marshall (1965) observed reduction in plate counts with increasing concentrations of leucocytes in sterile skim milk.

d) Whey proteins: Lecce and Legates (1959) reported a change in the whey protein pattern of milk from mastitic quarters, where the appearance of blood serum albumins and an increase in the amount of immune globulins were evident. They concluded that the presence of blood serum albumin in whey was a reliable indicator of mastitis and the appearance of blood serum albumin and the increase in immune globulin were due to the increased capillary permeability of the tissue as a result of infection which lead to the out pouring of plasma proteins from the blood circulation.

8. Influence of agglutinins on the production of fermented milk products:

Singh and Laxminarayana (1948) studied the growth of starter organisms in milk and observed that pasteurisation and boiling improved the growth and activity of the starters in ordinary milk, especially with early and late lactation milks. Keogh (1958) reported that depression of acid production at times took place in the cheese vat, and suggested that the cultures not affected by creaming were most useful in cheese manufacture when the milk was seasonally inhibitory. Natarajan and Dudani (1961) stated that the natural inhibitory substances in milk were of practical importance particularly in cheese making and maintenance of starter cultures and also on the keeping quality of milk. Emmons et al (1963) attributed the sludge formation on the bottom of cottage cheese vats and slow acid development, to the agglutination and settling of certain strains of starter bacteria. They found that the rate of acid development in the rest of the vat was reduced, and if serious, resulted in the failure of manufacture and that the curd formed lacked uniformity of firmness and particle size. However Stadhouders (1963) was of the view that allutinins were of no importance for the activity of cheese starters, since they found that rennet addition prevented the agglutinins from inhibiting acid production. Reiter and Moller Madsen (1963) supported the above view and stated that since rennetting of milk prevented the creaming up, the

agglutinins associated with the inhibition was of no concern in cheese making. Emmons et al (1966a) further investigated the lactic streptococcal agglutinins in milk and blood of different cows with susceptible and resistant strains and concluded that for the selection of strains for cottage cheese starters, it must be assumed that strains agglutinating in milk from several herds agglutinated in most milk supplies and that strains with low agglutinin titres in blood and first colostrum were satisfactory for cottage cheese manufacture. The authors in the same year (1966b) after a detailed study on several susceptible and resistant strains recommended selection of agglutination resistant strains for cottage cheese cultures. Randolph and Gould (1966) recommended the use of sufficient amounts of resistant strains in mixed strain cultures, to reduce the possibility of starter failures or slow acid production due to the natural inhibitory effect of milk and permitting at the same time the use of susceptible strains which might have beneficial effects on the quality of the products.

9. The importance of colostral antibodies to the new born calves:

Mason et al (1930) observed that in cows placental transmission of antitoxin produced actively or passively did not take place and the calves obtained antitoxin through the lactoserum. Smith (1948) stated that colos-

trum served a special function to enhance the resistance of the new born to infectious disease and that the colostrum contained extremely high concentrations of immune lacto globulins which were passively transferred to the offspring, where they might persist in the blood stream for many months, until the new born were able to make antibodies of their own. Comline et al (1951) reported that the appearance of certain of colostrum proteins in the blood serum occurred following the feeding of colostrum to the new born of many species and that the capacity to absorb large amounts of unchanged proteins disappeared within 24 to 48 hours of birth. They further observed that increase in agglutination titre was either greatly reduced or absent in three animals in which whey was not administered until 63, 64 and 65 hours after birth and there was an interval of 60 to 120 minutes between the introduction of the whey into the duodenum and the appearance of colostral proteins in the lymph of young animals and suggested that after absorption the globulins might be carried by the lymph to the systemic circulation. Deutsch and Smith (1957) found the gut of the new born herbivore lost its permeability to large molecules during the first 24 to 30 hours of post partum life. Larson (1958) showed that the immunoglobulins were not altered in passing from the blood stream to the secretions of the mammary gland and the increased transfer of

the immune globulins was timed with the approaching parturition and although the rate of transfer started to increase about a month before parturition, it did not reach a maximum until just before parturition. Pierce (1962) stated that the ungulates at birth were immunologically unstable, and stability was established by suckling the mother, and recorded over 50 percent of immune lactoglobulin in the serum of suckling calves 48 hours after birth. Pierce and Feinstein (1965) indicated that the mammary gland showed a highly selective preference for, and hence ability to concentrate in colostrum, the electrophoretically fastest serum immune globulins and they found in contrast to the mammary gland the intestine of the new born calf (permeable to the undegraded protein during the first 24 hours of life) showed no selectivity and that the immune globulins showing three electrophoretic mobilities were absorbed readily equally. They showed that the calf received into its circulation from ingested colostrum selected maternal serum immunoglobulins and this selection occurred within the mammary gland during the formation of colostrum. Klaus et al (1969) reported that the colostrum IgG was significantly higher than the level in maternal serum and the IgM and IgG in calf serum reached a peak at 48 hours which was much higher than the maternal level. They also observed that both IgG and IgM were absorbed from the gut of the calves with equal efficiency.

Kruse (1970) found highly significant differences between breeds in the yield of colostrum at first milking after calving and in the concentration of immune globulin in colostrum. He reported that the Red Danish Breed animals had the highest colostrum yield, but the lowest immune globulins percent; the individual variation in the colostrum yield, immune globulins percent and immune globulin yield was very marked; heifers had a lower colostrum and immune globulin yield than cows in second and later lactations. He concluded that, some of the case of hypogammaglobulinaemia in calves was not due to failures in the calf's ability to ingest colostrum and to absorb the immunoglobulin, but, rather to an insufficiency in the immunoglobulins transfer from the mother. Kruse (1970a) reported that the increase in serum immunoglobulin concentration during the first 24 hours after colostrum feeding, was a function of the mass of immunoglobulin fed to the calf, the age at colostrum feeding, and the birth weight of the calf; and the first two were predominant factors. He observed that the delayed feeding of colostrum from 2 to 20 hours, reduced the absorption coefficient to about half. The absorption coefficient appeared to be unaffected by the immunoglobulin percent in the colostrum, and also by the immune globulin given to the calf. The author (1970b) further observed that the probability of an individual

getting hypogammaglobulinaemia could be minimised by early feeding of a large quantity of colostrum at the first feeding after birth.

MATERIALS AND METHODS

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1. Samples:

The first colostrum from 68 cows and 53 buffaloes and mid lactation milk from 371 cows and 268 buffaloes were collected from Sindhi, Jersey, Nondescript, Sindhi Jersey cross and Nondescript Jersey grades, cows and Murrah and Nondescript buffaloes.

Blood and milk samples from 9 Sindhi, 8 Jersey, 18 Nondescript, 11 Sindhi Jersey cross and 7 Nondescript Jersey grades, cows and 12 Murrah and 16 Nondescript buffaloes were collected throughout the lactation from about a day prior to parturition upto the end of lactation. The samples were taken daily upto the seventh day of lactation, then on the fourteenth day and subsequently at fortnightly intervals.

The calf blood from the above mentioned breeds of animals was collected immediately after birth, before feeding colostrum and thereafter at different intervals upto the age of six months.

A total number of 129 samples of milk from infected udders were collected of which 82 were from cows and 47 from buffaloes.

All samples were collected aseptically in sterile milk bottles or test tubes and subjected to necessary

treatments immediately after collection.

2. Bacterial cultures:

The following cultures of bacteria, isolated from milk and curd samples in this laboratory, as well as type cultures obtained from other sources were used for the study.

a) Isolated from milk and curd:

Streptococcus lactis (A, B and C)

Lactobacillus bulgaricus

Bacillus cereus

Escherichia coli

b) Isolated from mastitis udders of cows:

Staphylococcus species

Streptococcus species

Pseudomonas species

Escherichia coli

c) Type cultures:

Name of the organisms:	Source
Streptococcus lactis (SR and 57)	National Dairy Research Institute, Bangalore.
Streptococcus lactis (18, C_2, C_{10}, ML_8 and 69)	National Dairy Research Institute, Karnal.
Streptococcus cremoris (HP, 495, C_1, K, KH, ML_1, R_1, C_3, C_7, S_2, DR_7, 972 and 760)	

Streptococcus citrovorus (209)	National Dairy Research Institute, Karnal
Streptococcus diacetilactis (DRC_1, DRC_2, DRC_3)	
Streptococcus thermophilus (489 and ST8)	
Lactobacillus bulgaricus (Hansen's and 1373)	
Lactobacillus acidophilus (L_1 and BA + ES)	
Lactobacillus plantarum (89)	
Streptococcus faecalis (190 and 30)	
Streptococcus agalactiae (865)	
Streptococcus pyogenes (B - 49 - 3)	
Staphylococcus aureus (KIGM)	
Escherichia coli (745 and 555)	
Pseudomonas aerogenosa	Institute of Veterinary Preventive Medicine, Ranipet.
Pseudomonas fluorescens	
Pasteurella boviseptieum	
Clostridium welchii	
Clostridium chauveii	
Salmonella typhosa	
Salmonella paratyphi	
Streptococcus cremoris (803 - assay culture)	Netherlands Institute for Dairy Research, the Netherlands

3. Preparation of samples for analysis:

a) Colostral and milk whey: Whey from colostrum and milk was prepared by the addition of rennet. Whey was concentrated when necessary to 1/3rd or 1/5th its original volume by freezing, and sterlized by filtration through a sintered glass bacterial filter.

b) Blood serum: Blood from the jugular vein of the animal was collected aseptically and serum was obtained by centrifugation of the clotted blood.

c) Milk ultrafiltrate: Milk ultrafiltrate was prepared by adopting the method of Stadhouders and Hup (1970).

d) Fat globules: The method outlined by Stadhouders and Hup (1970) was followed for the preparation of the two types of fat globules, one from raw milk and another from heat treated milk (80°C for 5 min.) for studying their agglutination and the bacterial attachment to the fat globules respectively.

e) Fat globule washings: The method of Kenyon et al (1966) was followed for the preparation of fat globule washings which constitute the fat globule agglutinins. Concentration was effected by drying from the frozen state under vacuum over calcium chloride and sterilized by filtration through a sintered glass bacterial

filter. The proteins in the fat globule washings were recovered by cold acetone precipitation (Askonas, 1951) using 100% acetone concentration, redissolved in appropriate quantities of phosphate or tris citrate or tris hydrochloric acid buffers and used in the analysis.

f) Fat globule membrane: The method of Herald and Brunner (1957) was used for the preparation of fat globule membrane materials.

g) Inhibitory fractions of milk: Lactenin fractions L and L were precipitated by cold acetone from the concentrated colostral whey as per the method of Auclair and Berridge (1953) employing 35% acetone concentration for L_1 fraction and 25% for L_2 fraction (Kosikowski and Mocquot, 1958). The precipitates were dried from the frozen state under vacuum over calcium chloride.

The concentrated colostral whey was also fractionated by cold acetone precipitation using 10, 20, 30, 40 and 50 percent concentrations of acetone as was done by Sasaki and Aibara (1959). These fractions were dried as mentioned earlier.

h) Whey proteins: Euglobulin and pseudoglobulin were prepared from colostral whey according to the method of Smith (1946) and the solutions were concentrated by the method of Kohn (1959). The method of Aschaffenburg

and Drewry (1957) was followed for the preparation of beta lactoglobulin and alpha lactalbumin.

Samples of beta lactoglobulin and alpha lactalbumin were also obtained from Dr. McMeekin, U.S.A.

i) Cryoglobulin: The cryoglobulin was separated from the euglobulin F fraction at low temperature (0 to 2°C) as per the method of Stadhouders and Hup (1970) with minor alterations in the procedure to suit the purpose of this investigation.

Euglobulin about 2 mg. per ml., was dissolved in 0.06 M sodium chloride solution at 37°C, distributed in small tubes 8 x 0.4 cm., cooled to 2°C and stored at that temperature for 15 to 27 hours in seven batches. All the seven batches were examined in the following manner at two hourly intervals, starting from the 15th hour of storage upto the 27th hour. The tubes were centrifuged at 20,000 rpm for 30 minutes and the sediment was dissolved in half the original volume of 0.06 M sodium chloride solution. The protein concentration was determined by estimating the optical density at 280 Mu in a Beckman DU 2 ultraviolet spectrophotometer. Then the volume of the solution was made upto that of the original euglobulin solution and the whole procedure of cold aggregation and centrifugation repeated. Finally the sediment was dissolved in half the original volume

of 0.06 M sodium chloride solution and the concentration determined as before. The time taken for the maximum aggregation of euglobulin to form cryoglobulin at 2°C for the two species cow and buffalo was determined.

The supernatant and the sediment obtained after the second cold aggregation were retained and used in the agglutination and attachment tests. Cryoglobulin and euglobulin F from cow and buffalo samples, were freeze dried in an Edward lyophiliser and stored for further studies.

4. Preparation of bacterial antigens:

All the lactic cultures except a few were grown in yeast dextrose broth containing 0.3% yeast extract, 0.5% peptone and 0.1% dextrose. Streptococcus cremoris organisms were grown in TGV medium (Stadhouders, 1963) containing 1% tryptone, 1% beef extract, 0.5% yeast extract, 4% filtrated tomato extract, 2% glucose, 0.1% Tween 80, and 0.2% potassium hydrogen phosphate. Streptococcus thermophilus was grown in yeast dextrose broth containing 10% papain digested milk. Streptococcus progenes was grown in serum broth. The rest of the organisms were grown in nutrient broth (Standard methods of examination of dairy products, 1953), except the culture of clostridium, which was grown under anaerobic conditions.

The test cultures were inoculated in appropriate broth and incubated at 37°C for 24 hours. The tubes were centrifuged and packed cells transferred to bulk medium (100 ml.) and incubated for 18 hours at 37°C. At the second hour of incubation 10 mg. of 2, 3, 5, triphenyl tetrazolium chloride was added (concentration 0.01%) to the inoculated broth. The cultures were inactivated by keeping in a waterbath at 60°C for one hour. The broth was centrifuged and the cells were washed six times with sterile phosphate buffered saline and finally suspended in 5 ml. of normal saline (0.85 gm. % sodium chloride) containing 1% phenol and 1% glycerol. This constituted the stained, inactivated antigen.

5. Detection of agglutinin activity in different fractions of milk and colostrum:

The inhibitory fractions of milk prepared by cold acetone precipitation of colostral whey (section 3.g), the whey proteins of milk (3.h) and the fat globule washings (3.e) were tested for agglutinin activity, by the tube agglutination test and the microscopical observation method, using Streptococcus oremoris 803 organisms.

a) Tube agglutination test (Milk ring test): This test was based on the principle of Wood (1950) for Brucella. For the ring test, samples of cow or buffalo milk were adjusted to contain 4% fat, since upto a level of 4.5% fat, the milk ring test gave distinct ring and

the results could be read easily (Mohanlingam, 1964). All the protein fractions were dissolved in the heat treated milk (80°C for 5 minutes) containing 4% fat, at a concentration of 1 mg. per ml. To about 5 ml. of fat globule washings, enough of fat globules prepared according to section 3.3. were added to give a final concentration of 4%. To 2 ml. of the test solutions in Khan test tubes, 0.1 ml. of stained antigen of Streptococcus cremoris 803 was added, mixed and incubated at 40°C ± 0.5°C until the ring formation in the tubes were evident. The raw and heat treated (80°C for 5 minutes) cow and buffalo milk were employed as controls.

b) Microscopic observation of agglutination: The method was similar to that of Emmons et al (1965). All the protein fractions mentioned above were dissolved in milk ultrafiltrate at the rate of 1 mg. per ml. To 0.2 ml. of these solutions and fat globule washings and fat globule membrane materials (section 3.f) was added 0.2 ml. of the stained antigen of Streptococcus cremoris 803, in a Khan test tube. A 0.04 ml. aliquot of each mixture was transferred to a cavity slide, incubated at 37°C for 15 minutes and examined under a microscope for agglutination. The result was considered to be positive if aggregates of ten or more cells were observed under the microscope.

6. Estimation of agglutinin activity:

The agglutinin activity was estimated by following the changes in pH, and acidity of test samples and also agglutination pehomena, using Streptococcous cremoris 803 organisms.

a) Determination of pH: The colostral whey, milk (skim milk), fat globule washings and euglobulin dissolved in milk ultrafiltrate (2 mg. per ml.) were subjected to the lactenin assay method of Auclair and Hirsch (1953).

Test samples were diluted to different levels, starting from 1/5th dilution and making 1/10th, 1/15th, 1/20th and so on upto 1/100th dilution for milk and euglobulin and starting from 1/100th dilution and making 1/200, 1/400, 1/600 and so on upto 1/2000th dilution for the colostral whey and starting from the undiluted samples and making two fold dilutions for the fat globule washings. Dilutions in between were made when the lower dilution was strongly positive and the next higher dilution was negative. The conventional two fold serial dilution was not made since an accurate estimate of the titre at higher dilution was not possible. These dilutions were made using sterile reconstituted skim milk adjusted to pH 7.0 with N/10 Sodium hydroxide. The duplicate dilutions of the samples were heated in a waterbath at 80°C

for 20 minutes to destroy the agglutinins. The highest dilution of the test samples, at which inhibition of acid development was evident as compared to the corresponding control was taken as the maximum inhibitory titre.

b) Determination of acidity: As described above, similar dilutions of the test samples were prepared along with the controls, using sterile reconstituted skim milk adjusted to pH 7.0 with N/10 Sodium hydroxide. After inoculation with an 18 hour old culture of Streptococcus cremoris 803 at 1% level the tubes were incubated for 6 hours at 37°C. The acidity was determined as percent lactic acid by titrating against N/10 sodium hydroxide to phenolphthalein end point, and the maximum titre determined as before.

c) Tube agglutination test (Milk ring test): The test solutions and the controls were diluted as before with milk ultrafiltrate. Fat globules prepared according to section 3.d. were added to the diluted test solutions to a final concentration of 4%. The test was performed as described under section 5.a. The highest dilution in which the appearance of pink ring was observed was taken as the titre.

d) Microscopic observation of agglutination: The test solutions and the controls were diluted to different

levels with phosphate buffered saline, and the test was performed as described under section 5.b. The highest dilution of the samples giving positive tests were taken as the titre.

In addition, the estimation of agglutinin activity was done in the blood serum, colostrum and milk and the fat globule washings throughout the lactation in different breeds of cows and buffaloes using Streptococcus cremoris 803 and in the midlactation milk using Streptococcus cremoris 803 and Streptococcus lactis 57 organisms, and in the blood serum of the calves, from birth upto the age of six months at regular intervals, using Streptococcus cremoris 803 and Escheriohia coli organisms.

7. Analysis of different fractions showing agglutinin activity:

The fractions that were tested for agglutinins activity were analysed by the methods of electrophoresis, gel filtration and paper chromatography.

a) Electrophoretic analysis: The acetone fractions, Lactenin L_1, and L_2 fractions, euglobulin, fat globule washings and cryoglobulins were subjected to horizontal starch gel electrophoresis of Smithies (1953). Hydrolysed starch (Connaught research laboratories, Toronto, Canada) was used at 11.5% concentration for electrophoresis. Poulik's discontinuous buffer was used (Poulik, 1957). The composition and concentration of the buffer are:

Gel buffer:

0.076 M Tris (hydroxymethyl) aminomethane	... 9.21 gms./lit.
0.005 M Citric acid	... 1.05 gms./lit.

Bridge buffer:

0.3 M Boric acid	...18.55 gms./lit.
0.05 Sodium Hydroxide	... 2.0 gms./lit.

The pH of the buffers was adjusted to 8.65.

Starch at 11.5% level was weighed and transferred to a side armed conical flask. The method of preparation of starch gel for electrophoresis was similar to that of Kristjansson (1963, 1966) with minor alterations. Two thirds quantity of the required gel buffer was heated to boiling. Starch was mixed with the remaining buffer and the heated buffer was added to the starch - buffer mixture swirling the flask all the while. The air bubbles were removed by degassing under vacuum for about half a minute and the gel was poured into perspex trays fitted with false bottom and allowed to set.

The materials were absorbed in whatman No.3 MM filter paper and inserted into the gel. The electrophoresis was carried out for 5 hours at 500 volts. After electrophoresis the gels were sliced horizontally, stained with 1% amidoblack (Sargent 1964) in methanol, acetic acid, water (5 : 2 : 5) for 20 minutes and cleared in methanol, acetic acid, and water (5 : 2 : 5).

b) Sephadex gel filtration: The colostral whey, milk whey, mastitis milk whey, fat globule washings and cryoglobulin were fractionated in a column of Sephadex gel. Sephadex G 200 (40 - 120 u, Pharmacia, Uppsala, Sweden) was used for fractionation employing methods suggested by Porath and Flodin (1959).

The Sephadex was swollen in excess of solvent in a boiling waterbath for six hours and deaerated under vacuum. The slurry in the buffer was packed in a special jacketed Sephadex column (K25/45 jacketed, Pharmacia, Uppsala, Sweden) 45 cms. long and 2.5 cms. internal diameter, mounted vertically. Extra care was taken to maintain the steady rise of the horizontal zone of the gel bed while packing. After the entire slurry was added and a required gel bed height had been obtained, the column was connected to a reservoir containing the eluant buffer and the gel bed was stabilised by running the eluant overnight.

The samples were dialysed against the eluant 0.1 M Tris - Hydrochloric acid, buffer pH 8.0 and 1 M sodium-chloride for 24 hours at 5°C. A quantity of 2 ml. of the dialysed sample was applied to the sample applicator kept in position over the gel bed and the eluant was run through the column. The flow rate was adjusted to 12 - 15 ml. per hour and 2 ml. fractions were collected in

test tubes. The fractions were analysed for the protein content at 280 mu in a Beckman D U 2 ultraviolet Spectrophotometer, and the elution curves were drawn for each of the samples. The fractionation of cryoglobulin was carried out at 4°C by circulating ice cold water in the jacketed column.

Fractions exhibiting agglutinin activity were pooled, concentrated by dehydration under vacuum over calcium chloride and refractionated on Sephadex G 200 under the conditions given above.

c) Paper Chromatography of hydrolysed proteins: Solutions of euglobulin and cryoglobulin and the active fraction in the fat globule agglutinin were hydrolysed in 2 N Hydrochloric acid for 10 hours at 15 lbs. pressure (Hansen et al, 1947) except for tryptophan determination for which the procedure of Hirs et al, (1954) was employed, for hydrolysis.

The hydrolysed proteins were concentrated by drying under vacuum over calcium chloride and spotted in a whatman No.1 MM Chromatographic paper (22.5" x 18.5") and developed in a Chromatographic chamber following two dimensional technique, using saturated phenol water (500 gms. of phenol dissolved in 125 ml. of distilled water), (Ivor Smith, 1958) for 24 hours and n - butanol-acetic acid water (250 : 60 : 250), Woiwood, 1949) for

18 hours. After drying, the amino acids were developed with ninhydrin (0.5 gms. % ninhydrin in acetone).

The separated spots were evaluated quantitatively by eluting the stained portions with 3 ml. of 2% sodium bicarbonate in 50% methanol. The colorimetric readings were taken in a Klett Summerson photoelectric colorimeter employing a No.52 filter. The analysis were done in duplicate under identical conditions.

Pure known amino acids of 0.03 M concentration were also chromatographed simultaneously and used as standards to determine the concentration of the amino-acids in the protein hydrolysates.

8. Influence of pH and temperature on agglutinin activity:

a) pH: The euglobulin was dissolved at the rate of 2 mg. per ml. in a buffer containing different proportions of 0.2 M solution of monobasic sodium phosphate and 0.2 M solution of dibasic sodium phosphate, (Cruickshank, 1965) with pH values of 5.6 to 7.4. The fat globule washings was also adjusted to the same pH values.

Different dilutions of the test solutions were made and the agglutination titre determined, by direct microscopic observation method. The maximum titre and the corresponding pH values were determined.

b) Temperature: The euglobulin solution in milk ultrafiltrate and the fat globule washings were kept in waterbaths at different temperature ranging from 60°C to 80°C and examined at frequent intervals, for activity, and the time taken for inactivation at each temperature was determined.

9. Mechanism of agglutination:

a) Agglutination of bacteria: Different dilutions of the euglobulin (2 mg. per ml.) in milk ultrafiltrate and the supernatant and the sediment after the second cold aggregation were made, and subjected to microscopical observation of agglutination using Streptococcus cremoris 803 antigen, as described earlier and the titres determined.

b) Agglutination of fat globules: Test samples of euglobulin, supernatant and the sediment after the second cold aggregation were diluted to different levels starting from 1/5th and making 1/10th, 1/15th and so on upto 1/100th dilution. To 0.2 ml. of the test solution 0.02 ml. of the fat globules were added and held for 10 minutes at 37°C. The solution was taken on a glass slide and examined under the microscope for the clustered fat globules. The fat globules suspended in 0.2 ml. of normal saline was used as the control.

c) Attachment of bacteria to fat globules:

(i) Centrifuge test: The method of Stadhouders and Hup (1970) was followed.

Different dilutions of the euglobulin, the supernatant and the sediment after the second cold aggregation, were subjected to the centrifuge test and the percentage of organisms attached to the cream layer was determined by the plate count estimation.

In addition the centrifuge test was also performed using 50 ml. of cow and buffalo whole milk adjusted to contain 4% fat, to which a bacterial suspension of Streptococcus cremoris 803, Streptococcus lactis 57 and Streptococcus lactis C_2 , containing 5 x 10^6 organisms per ml. was added. The percentage of organisms in the cream layer, skim milk and sediment fractions were determined.

(ii) Cream rising experiments: The method followed was similar to that of Stadhouders (1963), employing pasteurized cow and buffalo whole milk and skim milk.

(d) Saturation tests: The saturation tests were done to find out whether the three groups of antibodies present in euglobulin are different.

(i) Adsorption of antibody to bacterial cells: The method of Stadhouders and Hup (1970) was followed. After the prescribed treatment the supernatant was with-

drawn and subjected to agglutination tests with Streptococcus cremoris 803 antigen, fat globules and bacterial attachment to fat globules.

(ii) Adsorption of antibody to fat globules: The procedure described by Stadhouders and Hup (1970) was adopted and the fat globules treated solution was tested for bacterial agglutination using Streptococcus cremoris 803antigen, fat globule agglutination and attachment of bacteria to fat globules.

e) Specificity of agglutinins:

(i) Antibody - bacteria complex: This test was based on the principle of Portmann and Auclair (1959) for the investigation on the specificity of the antibodies which agglutinate the bacteria.

Saturation tests were carried out using the different bacterial strains and the treated solutions were again tested with all the bacterial strains and their immunological relationships determined.

(ii) Antibody - fat globule complex: The test was performed according to the method of Stadhouders and Hup (1970) using fat globules from Sindhi, Jersey, and Nondescript cows and Murrah and Nondescript buffaloes.

(iii) Antibody attaching bacterial cells to fat globules: The euglobulin solution divided into two

portions was subjected to the centrifuge test using the fat globules of cow and buffalo milk separately. The centrifuge test was repeated again with the treated solution and untreated bacteria and fat globules, and the percentage of bacterial attachment was determined in each case.

10. Agglutinin activity in milk from infected udders:

a) Concentration of agglutinins: From the onset of infection, milk from the infected udder was subjected to agglutination test at every six hours by the microscopical observation method and the agglutination titre was determined using Streptococcus cremoris 803 organisms. Simultaneously the leucocyte count of these samples were determined by the direct microscopic count method (Standard methods for the examination of dairy products, 1953).

b) Influence of leucocyte cells on bacterial agglutination: The leucocyte suspension required for the tests was prepared by the following method: About 30 to 50 ml. of 0.85% sterile sodium chloride solution was infused into each quarter of the udder and the milk was collected asceptically 8 hours after the injection. The milk was centrifuged at 6000 rpm for 30 minutes and the supernatant discarded. The leucocytes were suspended in sterile milk ultrafiltrate, in which serial dilutions were prepared, to contain from 100,000 leucocytes per ml.

upto 4,000,000 per ml. using milk ultrafiltrate. The tubes were centrifuged and the sedimented leucocytes were added to milk with normal agglutination titre.

A sample of milk from infected udder having a high leucocyte count was centrifuged at 4000 rpm. for 30 minutes. The agglutination titre of the mastitic milk sample before and after centrifugation was determined.

c) Agglutinin activity against causative organisms: Until antigens of isolated organisms were prepared the samples of milk and blood were stored at -5°C. The causative organisms from mastitis milk samples were isolated, and the organisms typed as per the methods outlined in Bergey's Manual of Determinative Bacteriology (1948).

The agglutination titres of infected milk and blood were determined in respect of the organisms isolated from the infected udder, by the microscopical examination method.

d) Agglutinin inhibitor: In a few cases of chronic infection the milk and blood serum of the animal showed negative agglutinin activity against the organisms isolated from the infected samples. In such cases the

following procedure was adopted.

The isolated organisms were tested with normal milk and blood serum for agglutination.

Cultures of other bacteria viz. Streptococcus cremoris 803, Streptococcus lactis 57, Escherichia coli were inoculated into the milk and blood serum from the infected animals and the samples tested for agglutination.

The milk and blood serum from infected animal were also subjected to the following treatments and then tested for agglutination using the isolated organisms.

(i) The milk whey from infected animal was dialysed against normal milk whey at 4°C for 24 hours.

(ii) The milk and blood serum from infected animals were mixed with an equal quantity of normal milk and blood serum.

(iii) The milk and blood serum from the infected animal were diluted and then mixed with the undiluted normal milk and blood serum.

(iv) The milk and blood serum from infected animal were heated at 60°C for 20 minutes.

(v) The milk and blood serum from infected animal

were subjected to saturation tests with the organisms isolated from the infected milk and then retested with the same untreated organisms for agglutination.

11. The effect of agglutinins on the fermentation of milk:

In the course of the study it was observed that certain strains of organisms which were initially resistant to agglutinins in milk became sensitive on subculturing them a number of times. Hence, it was thought necessary to study the behaviour of agglutinin resistant and sensitive strains of organisms individually as well as in the form of mixed cultures towards curd formation in pasteurized skim milk of cow and buffalo.

a) Isolation of agglutinating and non agglutinating variants: The method outlined by Stadhouders and Hup (1970) was followed for the isolation of agglutinating and non agglutinating variants of Streptococcus lactis C10.

b) Bacterial cultures: The following cultures were selected for this study: Streptococcus lactis 57, a culture sensitive to both agglutinins and lactoperoxidase in milk, three different strains of Streptococcus lactis C10, one, sensitive to both agglutinins and lactoperoxidase, second, sensitive only to lactoperoxidase and resistant to agglutinins, and third, resistant

to both types of inhibitors in milk, and strains of Streptococcus lactis C2 which were 100% resistant to both the types of inhibitors in milk.

c) Culture suitability test: The above mentioned single strain cultures as well as mixed cultures containing two of these strains of organisms in different proportions were inoculated into pasteurized skim milk (72°C for 10 seconds) of cow and buffalo at 1% level and incubated at 37°C. The time taken for clotting and the nature of the curd formed were then determined in respect of these cultures.

R E S U L T S

R E S U L T S

1. Isolation of agglutinin active protein fraction from cow and buffalo milk:

a) Agglutination of bacteria: Tables 1, 2 and 3 present the influence of acetone fractions, whey protein fractions and fat globule washings of cow and buffalo samples on the agglutination of bacteria, by the milk ring test (tube agglutination test). Raw cow and buffalo milk with 4% fat gave positive tests in 20 45 minutes respectively, while the heat treated milk was negative.

The ring formation in heat treated milk could be restored by the addition of whey protein fractions and fat globule washings of milk. Except 50% acetone fraction, the rest of the fractions were positive with varying lengths of incubation time, the shortest for Lactenin L_1 extract - 45 and 55 minutes for cow and buffalo samples respectively and the longest for the 10% acetone fraction - 100 and 120 minutes for cow and buffalo samples respectively. All the fractions from buffalo colostral whey required a longer incubation time than that of the corresponding fractions of cow to give a positive test. All the fractions except Lactenin L_1 extract of cow and buffalo colostrum and 30% fraction of cow colostrum, required a longer incubation time in heat treated buffalo milk than in heat

TABLE 1

THE AGGLUTINATION OF BACTERIA WITH ACETONE FRACTIONS OF COLOSTRAL WHEY

(Test organism - *Streptococcus cremoris* 803)

Variety of milk	Acetone fractions (img. per ml.)	Time taken for ring formation in minutes	
		Cow	Buffalo
Cow milk	None	20 (control)	
Heated cow milk*	None	-	
" " "	L extract (35% fraction)	45	55
" " "	L extract (25% fraction)	95	110
" " "	10% fraction	100	120
" " "	20% fraction	100	115
" " "	30% fraction	55	65
" " "	40% fraction	65	75
" " "	50% fraction	-	-
Buffalo milk	None	45 (control)	
Heated buffalo milk*	None	-	
" " "	L extract (35% fraction)	45	55
" " "	L extract (25% fraction)	100	115
" " "	10% fraction	105	125
" " "	20% fraction	105	120
" " "	30% fraction	55	70
" " "	40% fraction	70	75
" " "	50% fraction	-	-

Note: * Heat treatment at 80°C for 15 minutes.

\- No ring formation.

TABLE 2

THE AGGLUTINATION OF BACTERIA WITH WHEY PROTEINS

(Test organism - *Streptococcus cremoris* 803)

Variety of milk	Whey protein fractions (1mg. per ml.)	Time taken for ring formation in minutes	
		Cow	Buffalo
Cow milk	None	20 (control)	
Heated cow milk*	None	-	
" " "	Euglobulin	25	40
" " "	Pseudoglobulin	-	-
" " "	Beta Lactoglobulin	-	-
" " "	Alpha Lactalbumin	-	-
Buffalo milk	None	45 (control)	
Heated buffalo milk*	None	-	
" " "	Euglobulin	28	45
" " "	Pseudoglobulin	-	-
" " "	Beta Lactoglobulin	-	-
" " "	Alpha Lactalbumin	-	-

Note: * Heat treatment at 80°C for 15 minutes.

\- No ring formation.

TABLE 3

THE AGGLUTINATION OF BACTERIA WITH FAT GLOBULE WASHINGS

(Test organism - *Streptococcus cremoris* 803)

Variety of milk	Additions	Time taken for ring formation in minutes
Cow milk	None	20 (control)
Heated cow milk*	None	-
" " "	Cow fat globule washings	140
" " "	Buffalo fat globule washings	-
Buffalo milk	None	45 (control)
Heated buffalo milk*	None	-
" " "	Cow fat globule washings	160
" " "	Buffalo fat globule washings	-

Note: * Heat treatment at 80°C for 15 minutes.

\- No ring formation.

treated cow milk for a positive test.

The euglobulin of the whey proteins gave a positive milk ring test. The euglobulin of buffalo milk required a longer incubation time for a positive test than that of cow milk, the time taken being 40 and 45 minutes in heat treated cow and buffalo milk respectively, while the time taken by the euglobulin of cow milk was 25 and 28 minutes only in heat treated cow and buffalo milk respectively. The euglobulin of both cow and buffalo milk in heat treated buffalo milk took a longer time for a positive test than in heat treated cow milk.

The fat globule washings of cow alone was positive for the milk ring test, requiring 140 minutes in heat treated cow milk and 160 minutes in heat treated buffalo milk.

The results of the microscopical observation of the bacterial agglutination by milk, colostral whey, acetone fractions, whey proteins and fat globule washings, from the milk and colostrum of cow and buffalo are shown in table 4. The milk, colostral, whey all the acetone fractions except 50% fraction, euglobulin, pseudoglobulin and the fat globule washings of cow and buffalo samples were positive for agglutination. The agglutination of bacteria in cow and buffalo samples showed variations in intensity. The agglutination in milk, colostral whey,

Microphotographs of the agglutination of bacteria by colostral whey, milk, euglobulin and fat globule washings of cow and buffalo samples.

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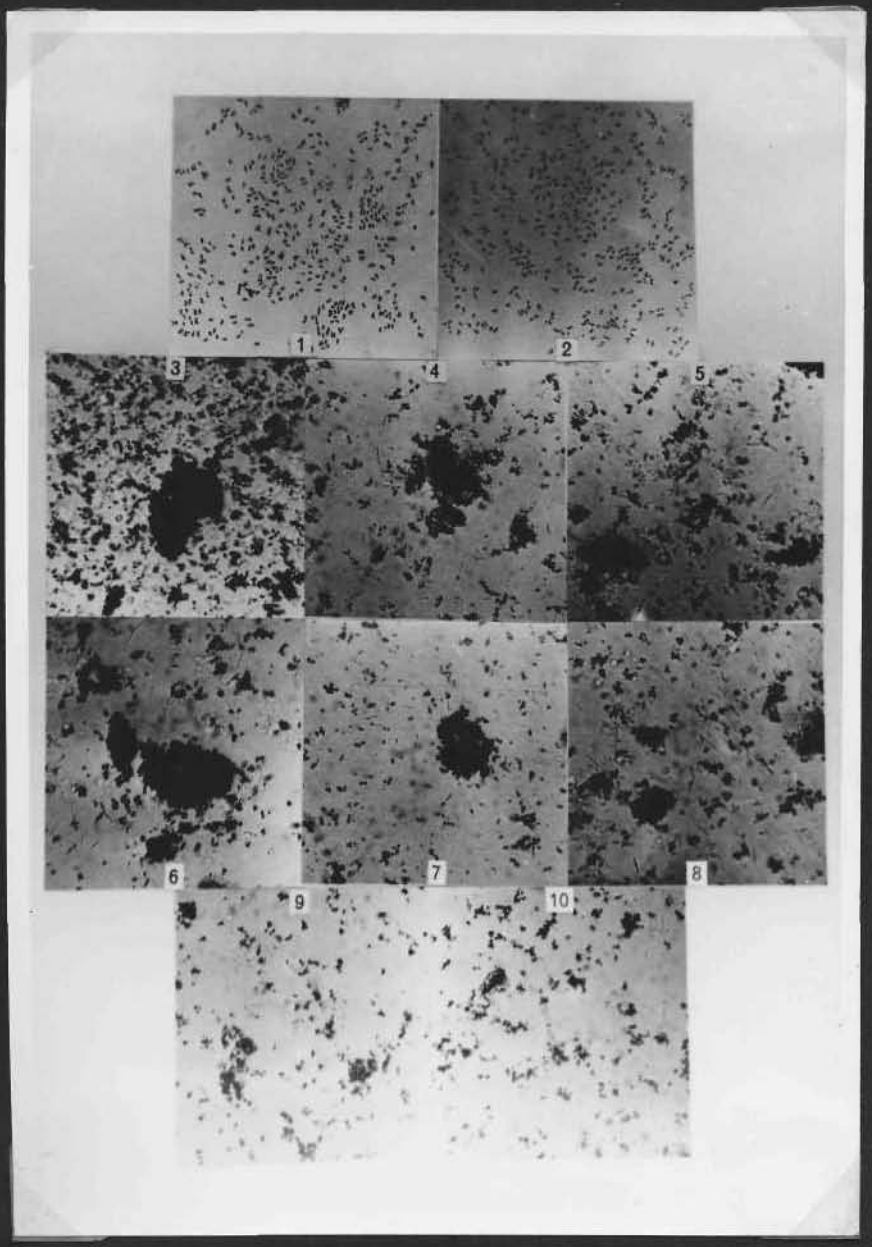
PLATE 1

Bacterial Agglutination (*Streptococcus cremoris* 803)



1 and 2 controls.

3 - Cow, 6 - Buffalo } Colostral whey

4 - Cow, 7 - Buffalo } Milk

5 - Cow, 8 - Buffalo } Euglobulin

9 - Cow, 10 - Buffalo } Fat globule washings

TABLE 4

MICROSCOPICAL OBSERVATION OF AGGLUTINATION WITH VARIOUS PROTEIN FRACTIONS OF MILK

(Test organism - Streptococcus cremoris 803)

Fractions	Microscopical observation of bacterial agglutination	
	Cow	Buffalo
Antigen + Normalsaline	- (control)	
Milk	+++	++
Colostral whey	++++	+++
Acetone fractions:		
L_1 extract	+++	++
L_2 extract	+	+
10% fraction	+	+
20% fraction	+	+
30% fraction	++	++
40% fraction	+	+
50% fraction	-	-
Whey proteins:		
Euglobulin	+++	++
Pseudoglobulin	+	+
Beta lactoglobulin	-	-
Alpha lactalbumin	-	-
Fat globules:		
Fat globule washings	+	+

Note: - No agglutination.
+ Visible agglutination.
++ Clear visible agglutination.
+++ Strong agglutination.
++++ Very strong agglutination.

Lactenin L1 extract and euglobulin was stronger in cow samples than the corresponding fractions of buffalo samples.

Plate 1 shows the agglutination of bacteria by colostral whey, milk, euglobulin and fat globule washings of cow and buffalo samples.

b) Agglutination of fat globules: Table 5 shows the effect of whey protein fractions and fat globule washings on the agglutination of fat globules. The euglobulin of cow and buffalo milk strongly favoured clumping of fat globules. The fat globule washings of cow and buffalo samples also were positive, but the effect was not as strong as that of euglobulin. The rest of the fractions tested were negative. Among positive fractions no differences were observed between cow and buffalo fractions in the degree of clumping.

Plate 2 presents the agglutination of fat globules by the euglobulin solution of cow and buffalo.

Table 6 gives the effect of fat globule membrane materials of cow and buffalo milk, on the bacteria and fat globules by the direct microscopic observation method for agglutination. The membrane materials of fat globules of both cow and buffalo milk did not have any effect on the bacteria as well as on the fat globules of cow and

TABLE 5

THE AGGLUTINATION OF FAT GLOBULES WITH WHEY PROTEINS

Test solution	Microscopical observation of fat globule agglutination	
	Cow	Buffalo
Normal saline (control)	-	-
Cow euglobulin (2mg. per ml.)	++	++
Cow pseudoglobulin (2mg. per ml.)	-	-
Cow beta lactoglobulin (2mg. per ml.)	-	-
Cow alpha lactalbumin (2mg. per ml.)	-	-
Buffalo euglobulin (2mg. per ml.)	++	++
Buffalo pseudoglobulin (2mg. per ml.)	-	-
Buffalo beta lactoglobulin (2mg. per ml.)	-	-
Buffalo alpha lactalbumin (2mg. per ml.)	-	-
Cow fat globule washings	+	+
Buffalo fat globule washings	+	+

Note: - No Agglutination.
+ Visible agglutination.
++ Strong agglutination.

Microphotographs of the agglutination of fat globules by the euglobulin solution of cow and buffalo milk.

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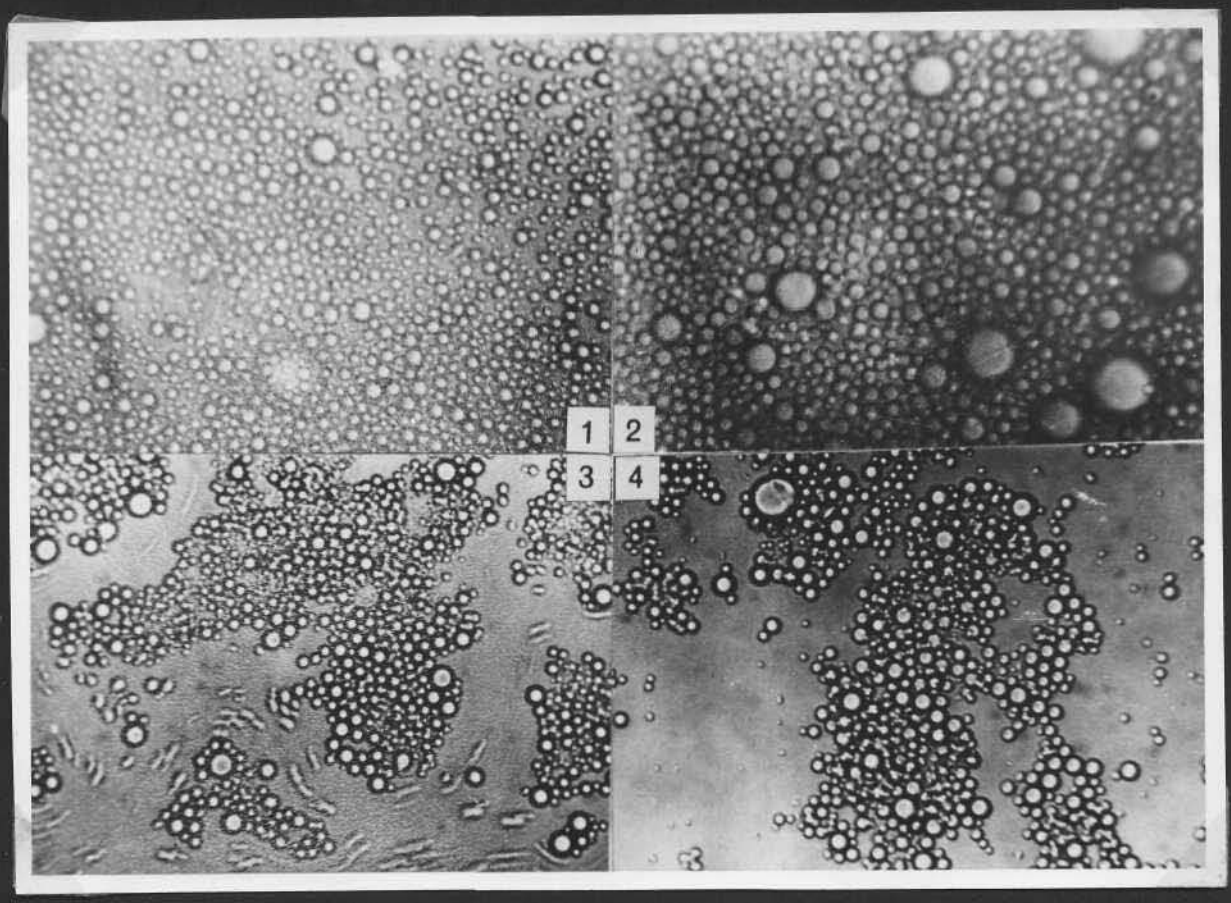
PLATE 2

Fat globule agglutination



1 - Cow
2 - Buffalo } Controls

3 - Cow
4 - Buffalo } Euglobulin

TABLE 6

EFFECT OF FAT GLOBULE MEMBRANE MATERIALS ON THE BACTERIA AND FAT GLOBULES

(Test organism - *Streptococcus cremoris* 803)

Test solution	Microscopical observation for agglutination of:	
	Bacteria	Fat globules
Antigen + Normal Saline	-	
Fat globules + Normal Saline		-
Fat globule membrane material of cow + Antigen	-	
Fat globule membrane material of buffalo + Antigen	-	
Fat globule membrane material of cow + Cow fat globules		-
Fat globule membrane material of cow + Buffalo fat globules		-
Fat globule membrane material of buffalo + Cow fat globules		-
Fat globule membrane material of buffalo + Buffalo fat globules		-

Note: - No agglutination.

buffalo milk.

Table 7 presents evidence that the euglobulin antibodies are adsorbed on the surface of the fat globules, capable of being recovered from the fat globules by suitable treatments. The washings from the already washed fat globules of both cow and buffalo samples were found to be negative for both bacterial and fat globule agglutination by microscopical observation. Fat globule washings from the washed fat globules of cow milk, resuspended in cow milk were positive for agglutination of bacteria, and fat globules with a titre of 1/2 and 1/4 respectively. Similarly the fat globule washings from the washed fat globules of buffalo milk, resuspended in buffalo skim milk was positive with a titre of 1 (undiluted sample) for bacterial agglutination and 1/4 for the agglutination of fat globules.

When the washed fat globules of cow milk were adsorbed in the skim milk of buffalo, the washings from the resuspended fat globules of cow milk gave the same titre as was obtained with adsorption treatments with cow skim milk, the titres being 1/2 for bacterial agglutination and 1/4 for fat globule agglutination. When the washed fat globules of buffalo milk were adsorbed in cow skim milk, the washings from the

TABLE 7

EVIDENCE FOR THE ADSORPTION OF EUGLOBULIN ANTIBODIES ON THE SURFACE OF FAT GLOBULES

Material	Treatment	Agglutination titre by microscopical observation for the agglutination of:	
		Bacteria	Fat globules
Washed fat globules of cow	Washings from the washed fat globules	-	-
Washed fat globules of cow resuspended in cow skim milk	Washings from the resuspended washed fat globules	1/2	1/4
Washed fat globules of buffalo	Washings from the washed fat globules	-	-
Washed fat globules of buffalo resuspended in buffalo skim milk	Washings from the resuspended washed fat globules	1	1/4
Washed fat globules of cow resuspended in buffalo skim milk	Washings from the resuspended washed cow fat globules	1/2	1/4
Washed fat globules of buffalo resuspended in cow skim milk	Washings from the resuspended washed buffalo fat globules	1	1/4

Note: - No agglutination.

resuspended fat globules of buffalo milk gave titres similar to that obtained with adsorption treatments in buffalo skim milk, the titres being 1 (undiluted sample) and 1/4 for bacterial and fat globule agglutination respectively.

2. Analysis of fractions carrying agglutinin activity:

a) Starch gel electrophoresis: Plates 3, 4 and 5 present the protein patterns on starch gel electrophoresis of the acetone fractions of colostral whey, Lactenin L_1 and L_2 fractions and euglobulin, fat globule agglutinins and cryoglobulins of cow and buffalo samples. The 10% acetone fractions contained only the immune globulins. The 20% fraction in addition carried beta lactoglobulin. The 30 and 40% fractions showed the presence of immune globulins, alpha lactalbumin and beta lactoglobulin in varying intensities. The concentration of the immune globulins in the 30% fraction was the highest. The patterns were similar in both cow and buffalo samples except for the lower concentration of the immune globulins in all the fractions of buffalo samples.

The Lactenin L_1 carried the immune globulins, alpha lactalbumin and beta lactoglobulin in different concentrations, while the Lactenin L_2 showed the presence of immune globulins and beta lactoglobulin only. The

Starch gel electrophoretic patterns of acetone fraction of colostral whey of cow and buffalo.

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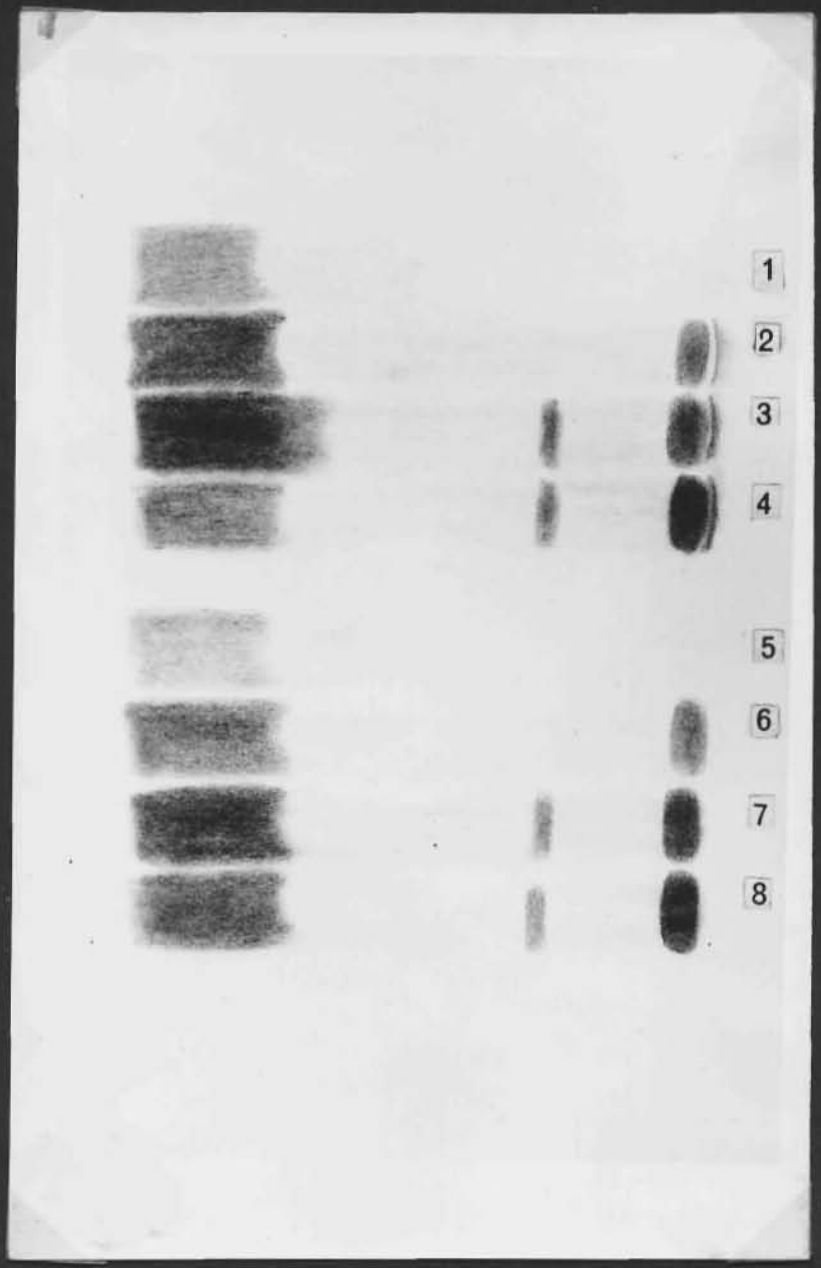
PLATE 3

Starch gel electrophoresis



1 - Cow, 5 - Buffalo } 10% acetone fraction

2 - Cow, 6 - Buffalo } 20% acetone fraction

3 - Cow, 7 - Buffalo } 30% acetone fraction

4 - Cow, 8 - Buffalo } 40% acetone fraction

Starch gel electrophoretic patterns of Lactenin L_1 and L_2 of cow and buffalo milk

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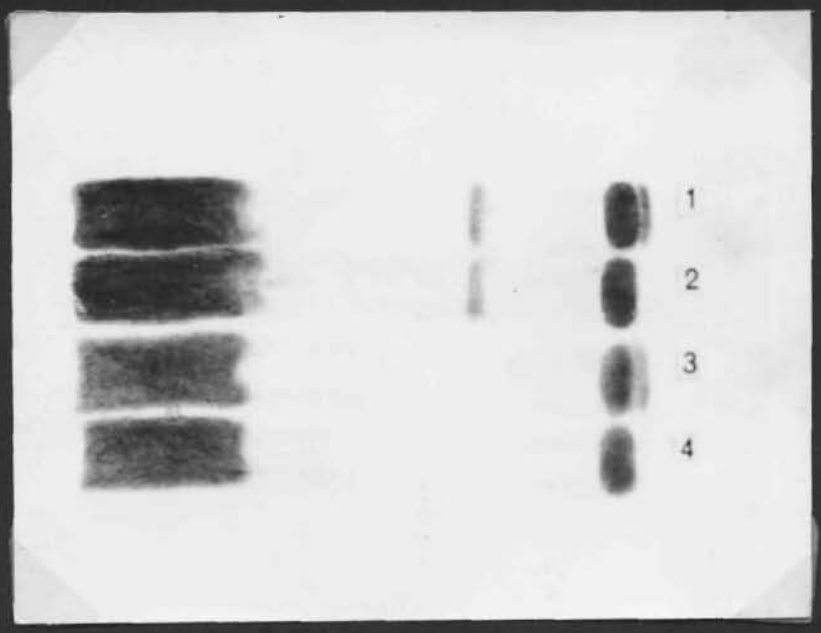
PLATE 4

Starch gel electrophoresis



1 - Cow
2 - Buffalo } Lactenin L_1

3 - Cow
4 - Buffalo } Lactenin L_2

Starch gel electrophoretic patterns of euglobulin, fat globule agglutinin and cryoglobulins of cow and buffalo samples.

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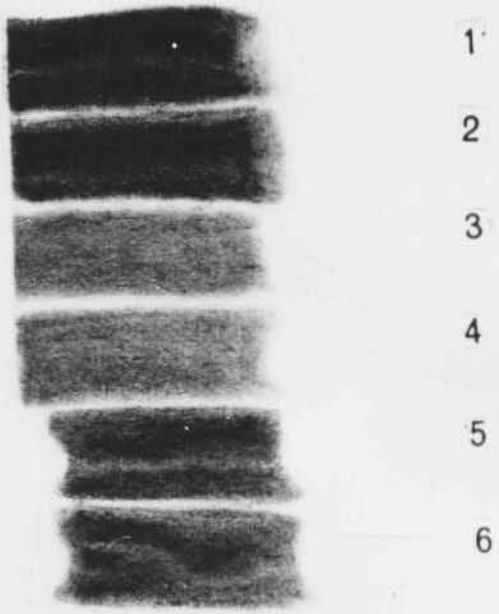
PLATE 5

Starch gel electrophoresis



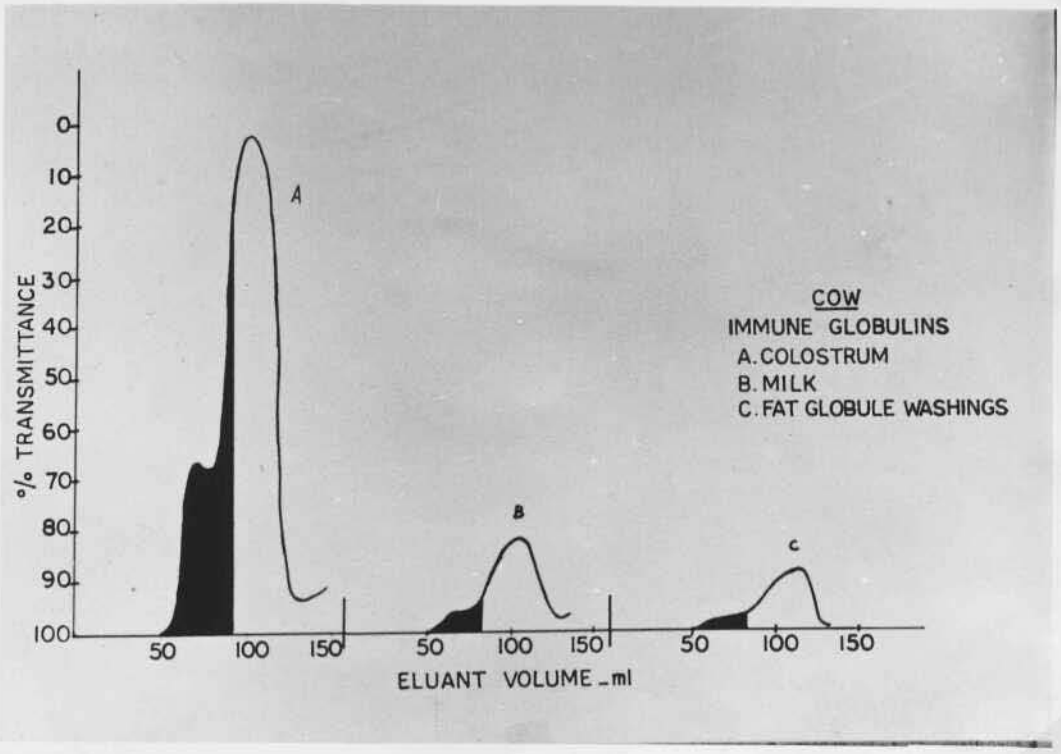
1 - Cow, 2 - Buffalo } Euglobulin

3 - Cow, 4 - Buffalo } Fat globule agglutinins

5 - Cow, 6 - Buffalo } Cryoglobulins

Protein elution pattern on Sephadex G 200, of the immune globulins of colostrum, milk and fat globule membrane of cow.

GRAPH 1



Shaded area represents natural antibodies (agglutinins) in milk

Protein elution pattern
on Sephadex G 200, of
the immune globulins of
colostrum, milk and fat
globule washings of buffalo.

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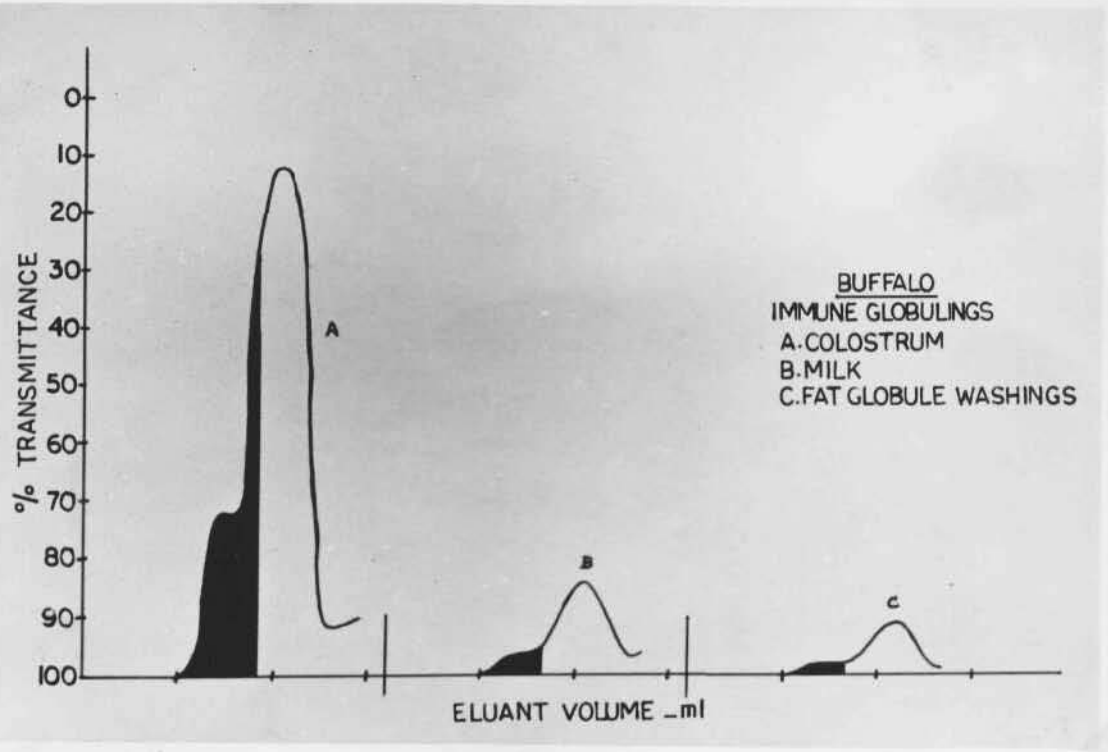
GRAPH 2



Shaded area represents natural antibodies (agglutinins) in milk

Protein elution patterns on Sephadex G 200, of the cryoglobulins of cow and buffalo samples.

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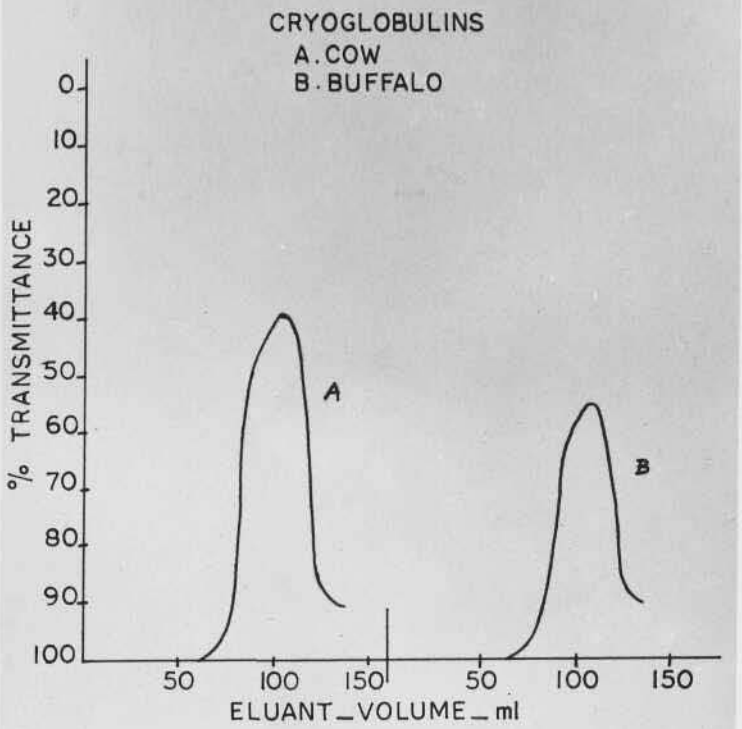
GRAPH 3


CRYOGLOBULINS
A. COW
B. BUFFALO
% TRANSMITTANCE
0
10
20
30
40
50
60
70
80
90
100
A
B
50
100
150
50
100
150
ELUANT_VOLUME_ml

buffalo samples showed a single peak in the immune globulin position of the elution pattern. The concentration of cryoglobulins obtained from cow samples were 0.69% and that from buffalo samples were 0.42%.

c) Paper Chromatography: The amino acid composition of the protein hydrolysates of the euglobulin, cryoglobulin and fat globule agglutinins of cow and buffalo samples is shown in table 8 and the chromatograms in plates 6 and 7. The following eleven amino acids have been spotted in the three protein fractions of cow and buffalo samples: valine, leucine, isoleucine, phenylalanine, cystine, methionine, tryptophan, arginine, histidine, lysine and threonine. On a quantitative basis, within species wide variations were not evident, although between samples of cow and buffalo some amount of variation was seen in respect of individual amino acids.

In the euglobulin and the fat globule agglutinins of the buffalo samples, in addition to the above mentioned eleven amino acids, tyrosine was present. But this was absent in the cryoglobulin of buffalo samples. The amino acid composition of the cryoglobulin of buffalo samples showed very little deviations from the composition of the cryoglobulin of cow samples.

TABLE 8

AMINO ACID COMPOSITION OF THE WHEY PROTEIN AND FAT GLOBULE AGGLUTININS

Amino acids	Cow			Buffalo		
	Eu globulin percent	Cryo globulin percent	Fat globule agglutinins percent	Eu globulin percent	Cryo globulin percent	Fat globule agglutinins percent
Valine	9.2 ± 0.51	9.6 ± 0.47	8.9 ± 0.55	10.1 ± 0.87	9.9 ± 0.55	10.2 ± 0.65
Leucine	9.7 ± 0.80	10.1 ± 0.65	9.8 ± 0.55	10.3 ± 0.40	10.1 ± 0.46	9.7 ± 0.38
Iso leucine	3.3 ± 0.03	3.1 ± 0.10	2.8 ± 0.09	3.7 ± 0.10	3.6 ± 0.12	3.1 ± 0.09
Phenyl alanine	2.9 ± 0.10	3.0 ± 0.10	2.4 ± 0.08	2.7 ± 0.08	2.9 ± 0.10	3.0 ± 0.09
Cystine	4.1 ± 0.09	3.9 ± 0.09	3.1 ± 0.05	3.6 ± 0.06	4.1 ± 0.10	3.2 ± 0.08
Methionine	0.7 ± 0.01	0.7 ± 0.01	0.8 ± 0.03	0.6 ± 0.01	0.5 ± 0.02	0.7 ± 0.04
Tryptophan	1.9 ± 0.02	1.7 ± 0.01	1.6 ± 0.03	1.8 ± 0.04	1.6 ± 0.02	1.9 ± 0.03
Arginine	5.0 ± 0.10	5.2 ± 0.09	4.6 ± 0.12	4.4 ± 0.09	4.2 ± 0.11	4.5 ± 0.10
Histidine	2.1 ± 0.15	1.9 ± 0.11	1.8 ± 0.13	2.3 ± 0.12	2.0 ± 0.14	2.1 ± 0.10
Lysine	5.9 ± 0.10	6.1 ± 0.10	6.2 ± 0.09	6.2 ± 0.11	5.9 ± 0.10	6.1 ± 0.12
Threonine	10.3 ± 0.50	9.9 ± 0.70	10.4 ± 0.55	10.6 ± 0.60	10.1 ± 0.71	9.9 ± 0.59
Tyrosine	- -	- -	- -	2.5 ± 0.03	- -	1.2 ± 0.01

Paper chromatogram of the protein hydrolysates of euglobulin, cryoglobulin and fat globule agglutinins of cow samples.

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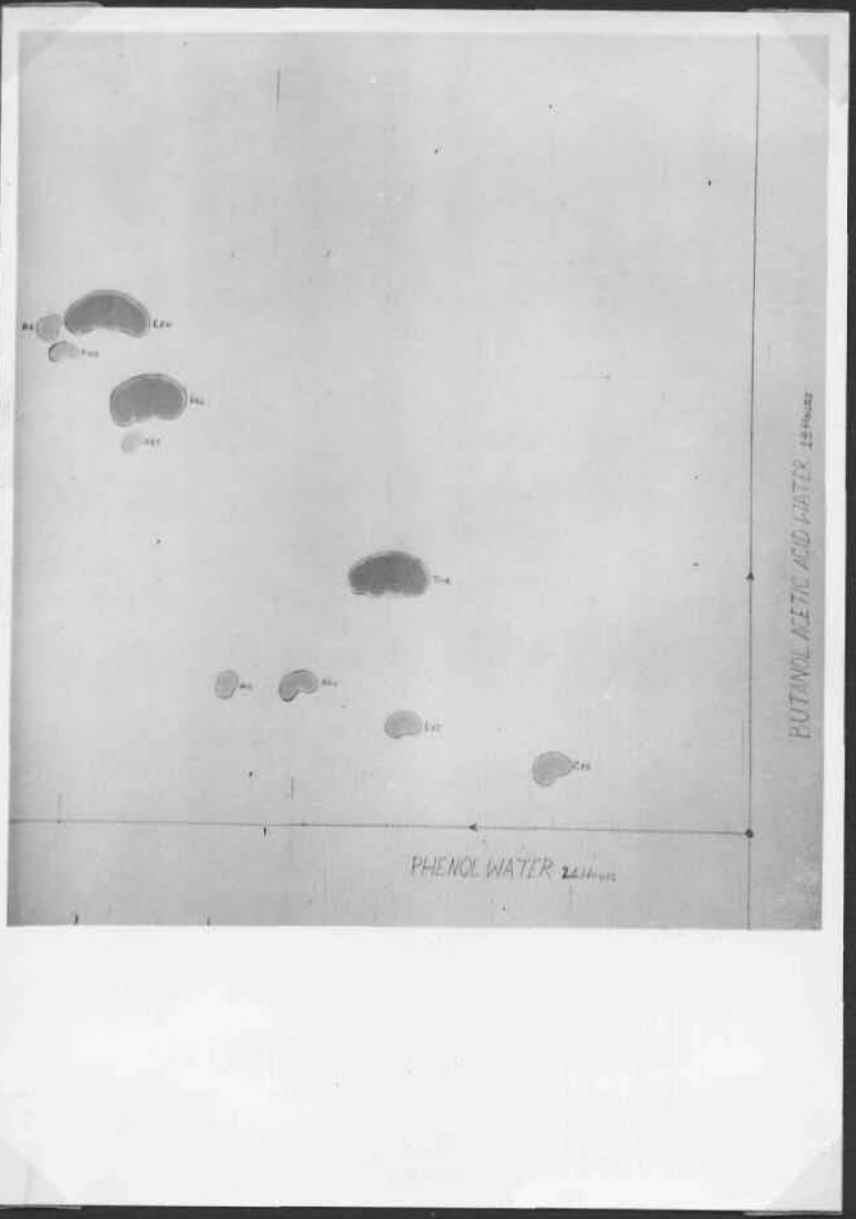
PLATE 6

Paper chromatogram of cow samples



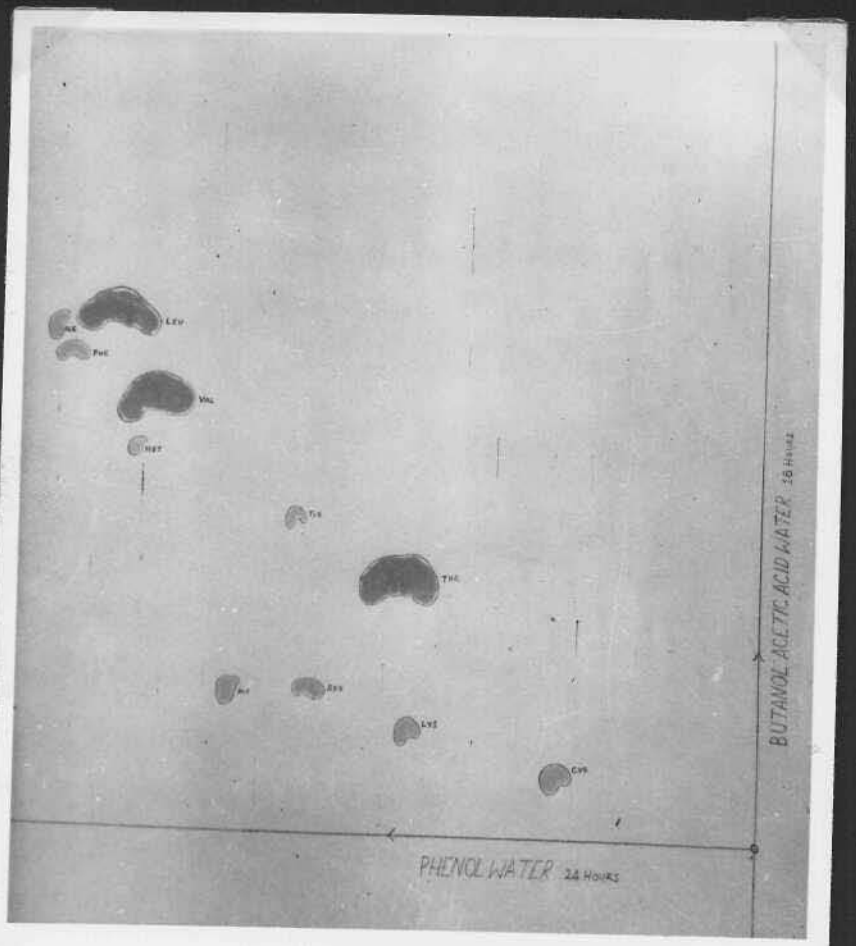
VAL - Valine; LEU - Leucine; ILE - Isoleucine;
PHE - Phenylalanine; CYS - Cystine; MET - Methionine;
ARG - Arginine; HIS - Histidine; LYS - Lysine;
THR - Threonine;

Paper chromatogram of the protein hydrolysates of euglobulin, cryoglobulin and fat globule agglutinins of buffalo samples.

-o-O-o-

PLATE 7

Paper chromatogram of buffalo samples



VAL - Valine; LEU - Leucine; ILE - Isoleucine;
PHE - Phenylalanine; CYS - Cystine; MET - Methionine;
ARG - Arginine; HIS - Histidine; LYS - Lysine;
THR - Threonine; TYR - Tyrosine.

3. Properties of euglobulin antibodies:

a) Effect of pH: In the table 9 the pH range at which the agglutinins of cow and buffalo milk exhibited optimal activity is presented. No differences in the pH range were evident between the whey protein and the fat globule agglutinins of the samples from the same species. The pH range for maximum activity for the agglutinins of cow and buffalo samples were slightly different. The maximum activity for samples from cow milk was between pH 6.1 and 6.5 whereas the maximum activity for samples from buffalo milk was between pH 6.3 and 6.6.

b) Effect of temperature: Table 10 provides the results regarding the inactivation temperature for the agglutinins. The temperature of inactivation did not very much between the whey protein and the fat globule agglutinins of cow and buffalo milk.

Table 11 shows the effect of holding euglobulin solution from cow and buffalo milk for prolonged periods at $2^{o}C$. Storage of euglobulin solution at low temperature for extended length of time resulted in the aggregation of euglobulin forming cryoglobulin in both cow and buffalo samples. For the formation of cryoglobulins to a maximum level, euglobulin of cow milk

TABLE 9

THE pH OF OPTIMAL ACTIVITY FOR WHEY PROTEIN AND FAT GLOBULE AGGLUTININS

(Test organism - *Streptococcus cremoris* 803)

pH	*Concentration of whey protein agglutinins (Euglobulin solution 1mg. per ml.)		*Concentration of fat globule agglutinins**	
	Cow	Buffalo	Cow	Buffalo
5.6	15	10	1.0	0.5
5.8	20	15	1.0	0.5
6.0	20	15	1.5	0.5
6.1	25	15	2.0	0.5
6.2	25	15	2.0	0.5
6.3	25	20	2.0	1.0
6.4	25	20	2.0	1.0
6.5	25	20	2.0	1.0
6.6	20	20	1.5	1.0
6.7	20	15	1.5	1.0
6.8	20	15	1.0	0.5
6.9	15	15	1.0	0.5
7.0	15	15	1.0	0.5
7.2	15	10	1.0	0.5
7.4	15	10	1.0	0.5

Note: * Concentration is the reciprocal of the titre.

** 0.5: Positive at two fold concentration of the solution and the actual solution itself was negative. 1.0: Original solution itself was positive. 1.5: Positive at one and half times dilution. 2.0: Positive at two fold dilution.

TABLE 10

THE TEMPERATURE OF INACTIVATION FOR WHEY PROTEIN AND FAT GLOBULE AGGLUTININS

(Test organism - Streptococcus cremoris 803)

Temperature (°C)	Time in minutes			
	Whey protein agglutinins		Fat globule agglutinins	
	Cow	Buffalo	Cow	Buffalo
60	40	40	40	40
64	33	32	32	32
68	19	19	18	19
70	12	12	12	12
72	10	10	10	9
74	4	4	4	4
76	2	2	2	2
80	1/2	1/2	1/2	1/2

TABLE 11

THE EFFECT OF LOW TEMPERATURE ON THE EUGLOBULIN SOLUTION FOR PROLONGED PERIODS

Time (hours)	Concentration of euglobulin solution (mg. per 100ml.)		Concentration of the sediment after first aggregation (mg. per 100ml.)		Concentration of the sediment after second aggregation (mg. per 100ml.)		Aggregated euglobulin after second aggregation (cryoglobulin %)	
	Cow	Buffalo	Cow	Buffalo	Cow	Buffalo	Cow	Buffalo
15	300	300	16.2	15.3	12.9	9.4	4.30	3.11
17	300	300	21.3	18.6	13.6	10.2	4.53	3.40
19	300	300	21.6	19.2	18.3	12.8	6.10	4.26
21	300	300	27.1	21.9	25.4	13.2	8.46	4.40
23	300	300	27.1	22.8	25.4	14.2	8.46	4.73
25	300	300	27.1	24.3	25.4	15.3	8.46	5.10
27	300	300	27.1	24.3	25.4	15.3	8.46	5.10

required 21 hours at 2°C, while the euglobulin of buffalo milk required 25 hours. In the case of cow samples at the end of the second cold aggregation 8.46% of the euglobulin aggregated at low temperature while in buffalo samples only 5.1% of the euglobulin aggregated to form cryoglobulin.

o) Action of euglobulin antibodies on the bacteria and fat globules: Table 12 indicates that the euglobulin antibodies are capable of attaching bacteria to the fat globules in both cow and buffalo samples. The attachment of bacteria to the fat globules, as measured by the percentage of bacteria in the cream layer was higher in euglobulin of cow milk which was 89%, than in the euglobulin of buffalo milk, where it was only 49.5%. When the fat globules of buffalo milk were used in the test with the euglobulin of cow milk, the bacterial attachment was 75.4%, which was higher than the attachment of bacteria with the fat globules and the euglobulin of buffalo milk. With the fat globules of cow milk and euglobulin of buffalo milk, the bacterial attachment was only 68.5%, which was lower than the attachment obtained with the fat globules and euglobulin of cow milk, but higher than the bacterial attachment with the fat globules and euglobulin of buffalo milk.

The results of the centrifuge test on whole milk

TABLE 12

EUGLOBULIN ACTIVITY IN THE ATTACHMENT OF BACTERIA TO FAT GLOBULES

(Test organism - *Streptococcus cremoris* 803)

Source of euglobulin (2mg. per ml.)	Number of bacteria added per ml.	Attachment of bacteria (% bacteria in the cream layer) to the fat globules of	
		Cow	Buffalo
Cow	5×10^6	89.0	75.4
Buffalo	5×10^6	68.5	49.5

and the cream rising experiments with whole milk and skim milk of cow and buffalo, with two agglutinin sensitive and one agglutinin resistant strains of organisms are presented in tables 13 and 14. In the centrifuge test with the agglutinin sensitive strains Streptococcus cremoris 803 and Streptococcus lactis 57, the percentage of bacteria in the cream layer of cow milk was highest, while the sediment contained a much lower percentage of bacteria, and the middle portion - the skim milk had the lowest percentage of organisms. The percentage of bacteria in the cream, skim milk and sediment for Streptococcus cremoris 803 were 86.6, 2.3 and 11.1 respectively and for Streptococcus lactis 57 were 75.8, 4.3 and 19.9 respectively. In the buffalo milk the percentage of bacteria in the cream layer and the sediment did not show wide differences as in the cow samples, while the skim milk in the middle portion contained very low percentage of organisms; the values for Streptococcus cremoris 803 in the cream, skim milk and sediment being 48.7%, 5.9%, and 45.4% respectively and for Streptococcus lactis 57, the values were 41.4%, 9.4% and 49.2% respectively.

The results of the centrifuge test with an agglutinin resistant strain Streptococcus lactis C_2 in both cow and buffalo samples were far different from those of the agglutinin sensitive strains. The skim milk in

TABLE 13

DISTRIBUTION OF AGGLUTININ SENSITIVE AND RESISTANT BACTERIA IN DIFFERENT LAYERS OF WHOLE MILK (CENTRIFUGE TEST)

Variety of milk	Number of bacteria added per ml.	Percentage of bacteria in different layers								
		Streptococcus cremoris 803 (sensitive)			Streptococcus lactis 57 (sensitive)			Streptococcus lactis C2 (resistant)		
		Cream	Skim-milk	Sedi-ment	Cream	Skim-milk	Sedi-ment	Cream	Skim-milk	Sedi-ment
Cow milk with 4% fat	5×10^6	86.6	2.3	11.1	75.8	4.3	19.9	4.8	88.5	6.7
Buffalo milk with 4% fat	5×10^6	48.7	5.9	45.4	41.4	9.4	49.2	2.1	93.3	4.6

TABLE 14

DISTRIBUTION OF AGGLUTININ SENSITIVE AND RESISTANT BACTERIA IN DIFFERENT LAYERS OF WHOLEMILK AND SKIM MILK (CREAM RISING EXPERIMENT)

Variety of milk	*Bacterial counts in different layers								
	Streptococcus cremoris 803 (sensitive)			Streptococcus lactis 57 (sensitive)			Streptococcus lactis C_2 (resistant)		
	Cream (upper part)	Middle portion	Bottom layer	Cream (upper part)	Middle portion	Bottom layer	Cream (upper part)	Middle portion	Bottom layer
	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7	x 10^7
Cow milk with 4% fat	272	10.5	47.0	227.4	14.9	69.0	24.2	341.7	31.8
Buffalo milk with 4% fat	146	24.0	139.5	121.2	28.1	140.0	7.0	310.2	18.7
	Upper portion	Bottom layer		Upper portion	Bottom layer		Upper portion	Bottom layer	
Cow skim milk	26.8	290.0		18.1	284.3		338.4	11.2	
Buffalo skim milk	61.0	247.5		47.2	283.7		327.0	7.1	

Note: * A 24 hours old culture of all the organisms were inoculated and incubated for six hours at 37°C.

the middle portion contained the highest percentage of organisms - 88.5% in cow milk and 93.3% in buffalo milk, while the cream layer and the sediment contained 4.8% and 6.7% respectively in cow milk and 2.1% and 4.8% respectively in buffalo milk.

In the cream rising experiments with the same agglutinin sensitive and resistant strains of organisms, a similar result as in the case of centrifuge test was obtained in the cow and buffalo whole milk. In cow and buffalo skim milk most of the agglutinin sensitive organisms settled to the bottom leaving a fewer number of organisms in the upper layer. The number of organisms in the upper portion of buffalo skim milk was much higher than the bacterial count in the upper layer of cow skim milk. The reverse was the case with respect to the distribution of agglutinin resistant strains in cow and buffalo skim milk. The upper layer of both cow and buffalo skim milk contained the largest number of organisms.

Table 15 shows the influence of different levels of euglobulin antibodies on the agglutination of bacteria, fat globules and the attachment of bacteria to fat globules. With increasing concentration of euglobulin from 0.2 mg. per ml. to 2.0 mg. per ml. a progressive increase was observed in the titres for bacterial and

TABLE 15

THE EFFECT OF DIFFERENT LEVELS OF EUGLOBULIN ON THE AGGLUTINATION OF BACTERIA, FAT GLOBULES AND THE ATTACHMENT OF BACTERIA TO FAT GLOBULES

(Test organism - *Streptococcus cremoris* 803)

Test solution	Concentration of euglobulin added (mg. per ml.)	Agglutination titre (bacteria)		Agglutination titre (fat globules)		Attachment of bacteria to fat globules (% bacteria in cream layer)	
		Cow	Buffalo	Cow	Buffalo	Cow	Buffalo
Milk ultra filtrate + fat globules of cow or buffalo to a level of 4% fat	None	-	-	-	-	0.4	0.2
	0.2	1/2	1	1/4	1/4	68.0	19.3
	0.5	1/5	1/2	1/15	1/15	85.2	84.7
	1.0	1/20	1/15	1/40	1/40	86.8	48.4
	1.5	1/25	1/15	1/45	1/45	87.4	49.2
	2.0	1/30	1/20	1/50	1/50	89.0	49.5

fat globule agglutination and the percentage of bacteria in the cream layer of both cow and buffalo samples. In the absence of euglobulin in the test solution of the two species, the bacterial and fat globule agglutination were negative and a very small or negligible percentage of bacteria were only present in the cream layer.

d) Differentiation of three classes of antibodies: The effect of different fractions obtained after the cold aggregation of euglobulin for the formation of *cryoglobulin, on the bacteria and fat globules are* presented in table 16. The control solution of euglobulin at a concentration of 2 mg. per ml. gave titres of 1/30 and 1/20 for bacterial agglutination in cow and buffalo samples respectively and 1/50 for the agglutination of fat globules of both cow and buffalo milk and bacterial attachment of 89% to the fat globules of cow milk and 49.5% to the fat globules of buffalo milk. The supernatant after the second cold aggregation of the euglobulin solution agglutinated only the bacteria with titres of 1/30 and 1/20 for cow and buffalo samples respectively, and the fat globule agglutination and the bacterial attachment to the fat globules were extremely feeble and negligible when compared with the values for the control. In fact, the attachment of the bacteria to the fat globules in the case of buffalo samples was

TABLE 16

THE AGGLUTINATION OF BACTERIA, FAT GLOBULES AND THE ATTACHMENT OF BACTERIA TO FAT GLOBULES WITH FRACTIONS OBTAINED AFTER COLD AGGREGATION

Test solution	Agglutination titre (bacteria*)		Agglutination titre (fat globules)		Attachment of bacteria* to fat globules (% bacteria in cream layer)	
	Cow	Buffalo	Cow	Buffalo	Cow	Buffalo
Euglobulin (2mg. per ml.) control	1/30	1/20	1/50	1/50	89.0	49.5
Supernatant after second cold aggregation	1/30	1/20	1/2	1/2	0.3	-
Sediment after second cold aggregation	-	-	1/40	1/40	83.0	47.0

Note: * Bacteria - Streptococcus cremoris 803

not demonstrable. The titre for fat globule agglutination was only 1/2 in both the cases. The sediment obtained after the second cold aggregation of the euglobulin solution of cow and buffalo samples was negative for the agglutination of bacteria and contained the factors responsible for the agglutination of fat globules and the attachment of bacteria to the fat globules. The titre for fat globule agglutination was 1/40 in both cow and buffalo samples and the percentage of bacteria in the cream layer was 83.0 in cow samples and 47.0 in buffalo samples.

Table 17 presents the results of the saturation tests in the euglobulin solution of cow and buffalo samples with bacteria and fat globules. The euglobulin solution of cow and buffalo samples (control) gave the titres of 1/30 and 1/20 for the bacterial agglutination in cow and buffalo samples respectively and 1/50 for the agglutination of fat globules of both cow and buffalo samples and bacterial attachment of 89% to the fat globules of cow milk and 49.5% to the fat globules of buffalo milk. After absorbing the antibodies in the euglobulin solution with bacteria (Streptococcus cremoris 803), by conducting the saturation test, the treated euglobulin solution contained only the antibodies agglutinating the fat globules whose titre was unchanged, and the antibodies for the agglutination of

TABLE 17

THE AGGLUTINATION OF BACTERIA, FAT GLOBULES AND THE ATTACHMENT OF BACTERIA TO FAT GLOBULES BY THE EUGLOBULIN SOLUTION AFTER TREATMENT WITH BACTERIA AND FAT GLOBULES (SATURATION TESTS)

Source of euglobulin (2mg. per ml.)	Treatment of euglobulin	Agglutination titre (bacteria*)	Agglutination titre (fat globules)	Attachment of bacteria* to fat globules (% bacteria in cream layer)
Cow	Control	1/30	1/50	89.0
	Adsorption by bacteria*	-	1/50	9.0
	Adsorption by cow fat globules	1/30	1/2	14.5
Buffalo	Control	1/20	1/50	49.5
	Adsorption by bacteria*	-	1/50	5.2
	Adsorption by buffalo fat globules	1/20	1/2	8.3

Note: * Bacteria - Streptococcus cremoris 803

bacteria and for the attachment of bacteria to the fat globules were absent which was indicated by a negative result for the agglutination of bacteria and a very low percentage of bacteria - 9.0 and 5.2 only in cow and buffalo samples respectively in the cream layer. Similar absorption of euglobulin solution with fat globules was performed. In the treated euglobulin solution, the antibody which agglutinated the bacteria only were present in the same concentration as in the control, and the antibodies for fat globule agglutination and bacterial attachment to the fat globules were absent in both cow and buffalo samples. The titre for the agglutination of fat globules in the treated euglobulin solution was only 1/2 for both cow and buffalo samples and the bacterial attachment was only 14.5% to the fat globules of cow milk and 8.3% to the fat globules of buffalo milk.

Plate 8 shows the agglutination of bacteria, fat globules and the attachment of agglutinated bacteria to the fat globules, by the euglobulin solution, the agglutination of fat globules and the attachment of bacteria (unagglutinated) to the fat globules by the cryoglobulins. The results were similar in both cow and buffalo samples.

Microphotograph of the agglutination of bacteria, fat globules and the attachment of bacteria to fat globules by the euglobulin and cryoglobulin of cow and buffalo samples.

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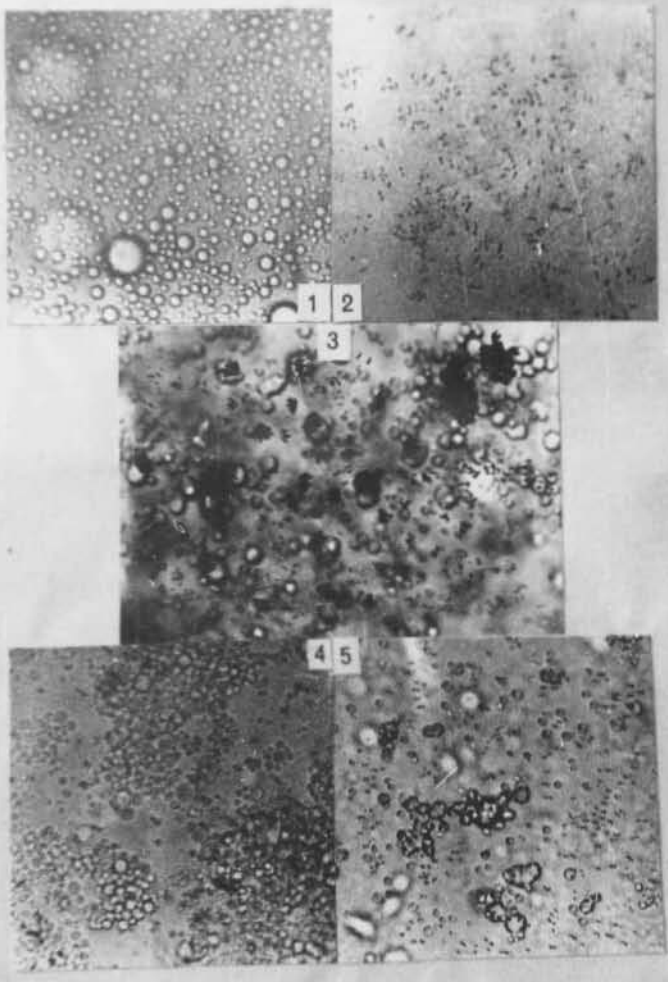
PLATE 8

Bacterial and fat globule agglutination



1 - Control (fat globules)

2 - Control (Bacteria - Str. cremoris 803)

3 - Euglobulin F (Attachment of agglutinated bacteria to agglutinated fat globules)

4 - Cryoglobulin (agglutination of fat globules)

5 - Cryoglobulin (Attachment of unagglutinated cells to the agglutinated fat globules)

4. Estimation of agglutinin activity:

a) Bacterial agglutination: Tables 18 and 21 give the results of the estimation of agglutinin activity by pH assay, acidity tests, tube agglutination test (milk ring test) and microscopical observation method, in colostral whey, milk, fat globule washings and euglobulin of cow and buffalo.

The cow samples of colostral whey, milk, fat globule washings and euglobulin solution inhibited the test organism - Streptococcus cremoris 803 at a titre of 1/1200, 1/25, 1/2 and 1/30 respectively, whereas the same samples of buffalo gave inhibition titres of 1/800, 1/20, 1 (undiluted sample) and 1/20 respectively in the methods of estimation of agglutinins by pH assay and acidity tests. The pH values of cow samples of colostral whey, milk, fat globule washings and euglobulin at inhibition were, 6.45, 6.3, 6.3 and 6.4 respectively and the pH values of the corresponding samples of buffalo at inhibition were, 6.35, 6.5, 6.4 and 6.45 respectively. The controls for all the above four samples from cow, developed pH values of 5.3, 5.2, 5.6 and 5.25 respectively and from buffalo developed pH values of 5.35, 5.3, 5.8 and 5.3 respectively.

In the acidity tests the cow samples of colostral whey, milk, fat globule washings and euglobulin solu-

TABLE 18

ESTIMATION OF AGGLUTININS BY pH ASSAY

(Test organism 1% inoculum of *Streptococcus cremoris* 803)

Test solution		pH at half dilution with skim milk	pH of the control after incubation	pH at which inhibited	Dilution at which inhibited
Colostral whey	Cow	6.7	5.3	6.45	1/1200
	Buffalo	6.8	5.35	6.35	1/800
Milk	Cow	6.85	5.2	6.3	1/25
	Buffalo	6.9	5.3	6.5	1/20
Fat globule washings	Cow	6.9	5.6	6.3	1/2
	Buffalo	6.9	5.8	6.4	1
Euglobulin (2mg. per ml.)	Cow	6.55	5.25	6.4	1/30
	Buffalo	6.4	5.3	6.45	1/20

TABLE 19

ESTIMATION OF AGGLUTININS BY ACIDITY DETERMINATION

(Test organism – 1% inoculum of *Streptococcus cremoris* 803)

Test solution		Net % acidity of the control after incubation	Net % acidity of inhibition	Dilution at which inhibited
Colostral whey	Cow	0.42	0.11	1/1200
	Buffalo	0.40	0.16	1/800
Milk	Cow	0.39	0.17	1/25
	Buffalo	0.36	0.10	1/20
Fat globule washings	Cow	0.31	0.18	1/2
	Buffalo	0.28	0.15	1*
Euglobulin (2mg. per ml.)	Cow	0.41	0.15	1/30
	Buffalo	0.37	0.13	1/20

Note: * 1 indicates that the undiluted solution was positive and a two fold dilution was negative.

tion developed acidity percentage of 0.11, 0.17, 0.18 and 0.15 at inhibition as against the acidity percentages of 0.42, 0.39, 0.31 and 0.41 respectively in the corresponding controls. The buffalo samples of colostral whey, milk, fat globule washings and euglobulin solution at inhibition developed acidity percentages of 0.16, 0.10, 0.15 and 0.13 respectively while in the corresponding controls, acidity percentages of 0.40, 0.36, 0.28 and 0.37 respectively.

In the method of estimation by milk ring test the agglutination titres for the respective fractions of cow and buffalo samples were much lower than the inhibitory titres obtained by the previous two methods for the same test organism Streptococcus cremoris 803. In cow samples of colostra whey, milk, fat globule washings and euglobulin solution the maximum titres upto which ring formation was evident, were 1/600, 1/10, 1 (undiluted sample) and 1/10 respectively and the time taken in minutes for the ring formation for the respective fractions were 20, 20, 140 and 20 respectively. In the buffalo samples of the corresponding fractions the maximum titres were 1/200, 1/5, 2/1 (two fold concentration of the solution) and 1/5 respectively and the time taken in minutes for the ring formation for these fractions were 40, 45, 125 and 40 respectively. No ring formation

TABLE 20

AGGLUTININ ESTIMATION BY MILK RING TEST

(Test organism - *Streptococcus cremoris* 803)

Test solution		Reaction of control	Incubation time in minutes	Titres for positive reaction
Colostral whey	Cow	-	20	1/600
	Buffalo	-	40	1/200
Milk	Cow	-	20	1/10
	Buffalo	-	45	1/5
Fat globule washings	Cow	-	140	1*
	Buffalo	-	125	2/1**
Euglobulin (2mg. per ml.)	Cow	-	20	1/10
	Buffalo	-	40	1/5

Note: - indicates no ring formation.

* 1 indicates that the undiluted solution was positive and a two fold dilution was negative.

** 2/1 indicates that a two fold concentration of the solution was positive and that the solution itself was negative.

TABLE 21

AGGLUTININ ESTIMATION BY MICROSCOPICAL OBSERVATION OF AGGLUTINATION

(Test organism - *Streptococcus cremoris* 803)

Test solution		Control	Microscopical observation	Titres for positive reaction
Colostral whey	Cow	-	+	1/1200
	Buffalo	-	+	1/800
Milk	Cow	-	+	1/25
	Buffalo	-	+	1/20
Fat globule washings	Cow	-	+	1/2
	Buffalo	-	+	1*
Euglobulin (2mg. per ml.)	Cow	-	+	1/30
	Buffalo	-	+	1/20

Note: + indicates visible agglutination.

* 1 indicates that the undiluted solution was positive and a two fold dilution was negative.

was evident in the controls of the respective fractions.

The agglutination titres obtained by the microscopical observation method for agglutinin estimation for the same four fractions of cow and buffalo samples were the same as that of the inhibitory titres give by the first two methods - pH assay and acidity tests. All the samples were positive for agglutination, while the corresponding controls were negative.

b) Fat globule agglutination: Table 22 presents the antibody titre of the euglobulin solution and fat globule washings of cow and buffalo samples for the agglutination of fat globules of cow and buffalo milk.

The titres of the euglobulin of cow and buffalo samples for the agglutination of the fat globules of the milk of the two species were found to be the same, the value being, 1/40. The titres also remained the same when the euglobulin solution of cow and buffalo samples were each tested on the fat globules of milk of both the species. The behaviour of fat globule washings of cow and buffalo samples on the agglutination of the fat globules of the two species was similar to that of the euglobulin solution, but the titre was 1/4.

c) Attachment of bacteria to fat globules: In

TABLE 22

THE ANTIBODY TITRE FOR FAT GLOBULE AGGLUTINATION

Test solution and species of animal	Fat globules and species of animal	Agglutination titre for fat globules
Euglobulin (1mg. per ml.)		
Cow	Cow fat globules	1/40
	Buffalo fat globules	1/40
Buffalo	Cow fat globules	1/40
	Buffalo fat globules	1/40
Fat globule washings		
Cow	Cow fat globules	1/4
	Buffalo fat globules	1/4
Buffalo	Cow fat globules	1/4
	Buffalo fat globules	1/4

table 23 the titre of milk and euglobulin solution for the attachment of bacteria to the fat globules is presented.

For the undiluted whole milk and euglobulin solution (concentration 2 mg. per ml.) of cow and buffalo, the bacterial attachment of the fat globules, as evidenced by the percentage of bacteria in the cream layer, was maximum. The bacterial attachment in the cow and buffalo whole milk was 86.6% and 48.7% respectively, and the bacterial attachment in the euglobulin solution of cow and buffalo was 89.0% and 49.5% respectively. With an increase in the dilution of whole milk and the euglobulin solution, a progressive decrease in the bacterial attachment to the fat globules was observed in both cow and buffalo samples. But, even at a titre of 1/10, in the whole milk and euglobulin solution of cow samples, the bacterial attachment was considerably higher, the values being 65.0% and 70.3% respectively, whereas, in the buffalo samples even at a titre of 1/5 there was considerable decrease in the bacterial attachment to the fat globules, the percentage of bacteria in the cream layer, in whole milk was 32.5 and in euglobulin solution was 33.0.

5. Factors influencing the variations in the agglutinin activity of milk:

a) Stage of lactation: Table 24 and 24a the concen-

TABLE 23

THE ANTIBODY TITRE FOR THE ATTACHMENT OF BACTERIA TO THE FAT GLOBULES

(Test organism - *Streptococcus cremoris* 803)

Test solution	Dilutions (titres)	Bacterial attachment to fat globules (% bacteria in cream layer)	
		Cow	Buffalo
Whole milk	Undiluted	86.6	48.7
	1/2	85.5	45.0
	1/5	81.0	32.5
	1/10	65.0	21.8
	1/15	10.5	1.2
Euglobulin (2mg. per ml.)	Undiluted	89.0	49.5
	1/2	87.0	48.8
	1/5	84.5	33.0
	1/10	70.3	20.9
	1/15	12.6	1.4

tration of agglutinins in the blood, milk and fat globule washings of different breeds of cows and buffaloes, throughout the lactation period, commencing from about 24 hours before calving. In general the agglutinin activity in blood, milk and fat globule washings of cow was found to be higher than in the corresponding fractions of buffalo. In both cow and buffalo samples, the prepartum lacteal secretion and the colostrum exhibited the highest agglutinin activity and the activity was found to be in the following decreasing order in the different breeds of cow and buffalo: Nondescript, Nondescript Jersey Grades, Sindhi Jersey cross, Sindhi and Jersey among cows and Nondescript and Murrah among buffaloes.

While following the agglutinin activity throughout the lactation, it was evident that there was a very sharp fall in the activity in milk, after 24 hours following calving and the concentration of agglutinin on the fifth day of lactation itself was very low when compared to the agglutinin concentration in the colostrum or the prepartum lacteal secretion.

The agglutinin activity in the blood serum about 24 hours prior to calving was also high with a gradual and progressive decrease with advancing lactation after calving.

TABLE 24

CONCENTRATION OF AGGLUTININS IN BLOOD, MILK AND FAT GLOBULE WASHINGS

DURING THE LACTATION CYCLE OF COWS

Stage of lactation	Nondescript		Nondescript Jersey grades		Sindhi Jersey cross		Sindhi		Jersey	
	Milk	Blood	Milk	Blood	Milk	Blood	Milk	Blood	Milk	Blood
About 24 hours before calving	1311.1	754	1303.2	712	1298.4	647	1244.0	641	1212.6	620
Immediately after calving	1178.6	631	1170.9	634	1103.7	601	1102.3	354	1058.5	512
1 day	732.3	512	646.8	502	629.2	493	601.1	474	527.2	470
2 days	441.7	414	304.6	429	259.5	487	219.5	403	202.3	391
3 days	204.5	427	169.7	421	148.1	479	109.8	407	103.4	427
4 days	109.7	453	101.3	407	97.4	421	78.2	449	51.7	431
5 days	74.3	451	71.0	371	70.3	334	56.6	313	47.2	354
6 days	61.8	379	56.4	329	56.2	307	43.4	327	38.1	336
7 days	45.2	443	41.1	304	40.3	372	35.6	305	31.2	293
14 days	35.0	316	31.2	271	25.6	367	25.0	231	21.6	204
1 month	30.4	272	27.3	243	21.2	231	20.1	207	16.9	229
1½ months	26.8	215	24.2	206	19.3	220	19.8	189	20.1	191
2 months	21.7	219	21.4	197	38.9	207	15.7	231	14.6	212
2½ months	45.1	298	29.1	199	15.7	214	20.1	217	40.3	256
3 months	26.4	341	37.7	278	16.9	309	15.1	279	13.6	251
3½ months	21.7	316	18.9	269	15.3	344	34.4	296	10.8	128
4 months	28.1	374	24.7	214	20.4	311	17.8	194	15.6	131
4½ months	32.7	327	27.1	251	18.2	214	17.0	157	13.9	128
5 months	22.4	249	20.3	237	18.4	187	15.9	132	11.7	159
5½ months	25.8	278	24.9	269	21.4	228	15.7	199	14.1	214
6 months	29.4	294	28.1	286	23.2	263	19.9	251	16.3	237
6½ months	25.7	321	23.6	273	21.1	235	18.7	223	16.1	211
7 months	28.3	334	23.9	269	24.8	249	19.1	237	20.3	219
7½ months	32.1	307	26.7	310	25.1	274	22.1	259	20.8	251
8 months	24.3	265	19.9	271	19.1	257	18.6	216	15.3	256
8½ months	27.4	253	20.1	224	21.3	198	20.4	191	15.8	223
9 months	47.4	374	29.7	245	26.9	226	21.7	223	16.3	204
9½ months	170.6	543	34.4	307	32.1	272	26.4	264	24.7	252
10 months	347.2	722	41.6	371	40.3	370	40.0	339	34.4	327
10½ months			161.3	603	160.7	561	151.4	482	143.6	516
11 months			300.7	748	291.4	624	280.8	679	251.3	687

Note: The concentration of agglutinins in the fat globule washings in all the breeds of cows from 3 days after calving upto the end of lactation was 2.

TABLE 24a

CONCENTRATION OF AGGLUTININS IN BLOOD, MILK AND FAT GLOBULE WASHINGS DURING THE LACTATION CYCLE OF BUFFALOES

Stage of lactation	Nondescript		Murrah		Both the breeds of buffaloes
	Milk	Blood	Milk	Blood	Fat globule washings
About 24 hours before calving	1210.7	645	1184.1	632	-
Immediately after calving	1050.1	597	981.9	604	- *
1 day	509.2	521	503.0	518	-
2 days	211.4	463	200.2	429	-
3 days	90.3	394	93.5	386	1
4 days	41.6	396	41.1	391	1
5 days	33.1	387	29.7	347	1
6 days	25.2	362	26.4	311	1
7 days	22.4	310	20.8	209	1
14 days	20.7	228	20.1	186	1
1 month	17.9	274	15.2	212	1
1½ months	15.4	211	12.4	172	1
2 months	20.1	173	8.3	137	1
2½ months	22.3	214	16.5	199	1
3 months	15.3	148	18.7	244	1
3½ months	17.8	193	11.9	165	1
4 months	29.4	218	17.4	121	1
4½ months	18.4	257	21.3	102	1
5 months	16.3	316	14.6	194	1
5½ months	15.7	335	11.2	271	1
6 months	21.4	361	10.8	283	1
6½ months	15.6	288	15.4	316	1
7 months	13.5	226	24.7	327	1
7½ months	19.7	201	18.6	254	1
8 months	21.4	184	15.1	214	1
8½ months	43.4	373	16.2	193	1
9 months	107.9	441	16.7	267	1
9½ months	220.8	584	30.3	243	1
10 months			39.7	291	1
10½ months			147.8	403	1
11 months			234.5	592	1

Note: * not determined.

Further it was seen that the concentration of agglutinin was several times higher in the colostrum and prepartum lacteal secretion than in blood. The agglutinin activity appeared to reach a normal level in both blood and milk by about the 14 day or one month after parturition, and the activity showed limited fluctuations in both blood and milk throughout the mid lactation period. There seemed to be no correlation between the concentration of agglutinins in blood and that in milk, because an increase in activity in blood serum did not necessarily give rise to an increase in the activity in milk and a decrease in blood level also did not always reduced the activity in milk, as is evident from the table.

At the end of lactation, even about a fortnight prior to the cessation of milking a considerable increase in agglutinin activity in both milk and blood was observed.

The above mentioned observations were made in respect of all the breeds of cows and buffaloes studied.

The estimation of agglutinin activity in the fat globule washings of cow and buffalo could not be made in the prepartum lacteal secretion and in the milk upto the second day after calving. The concentration of

Effect of stage of
lactation on the
agglutinin activity
of milk and blood in
different breeds of cows.

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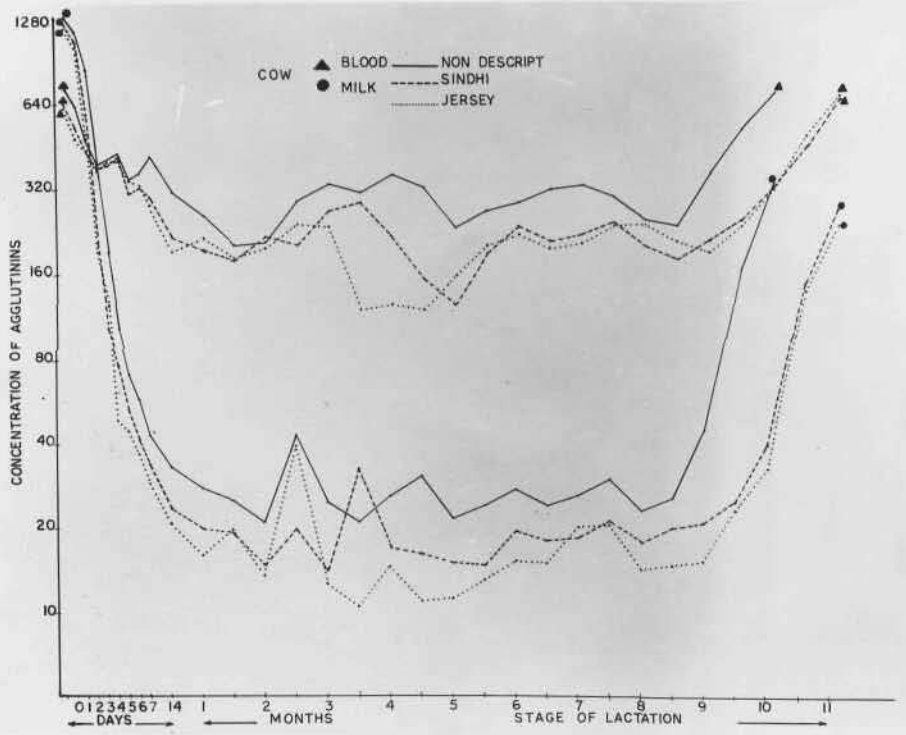
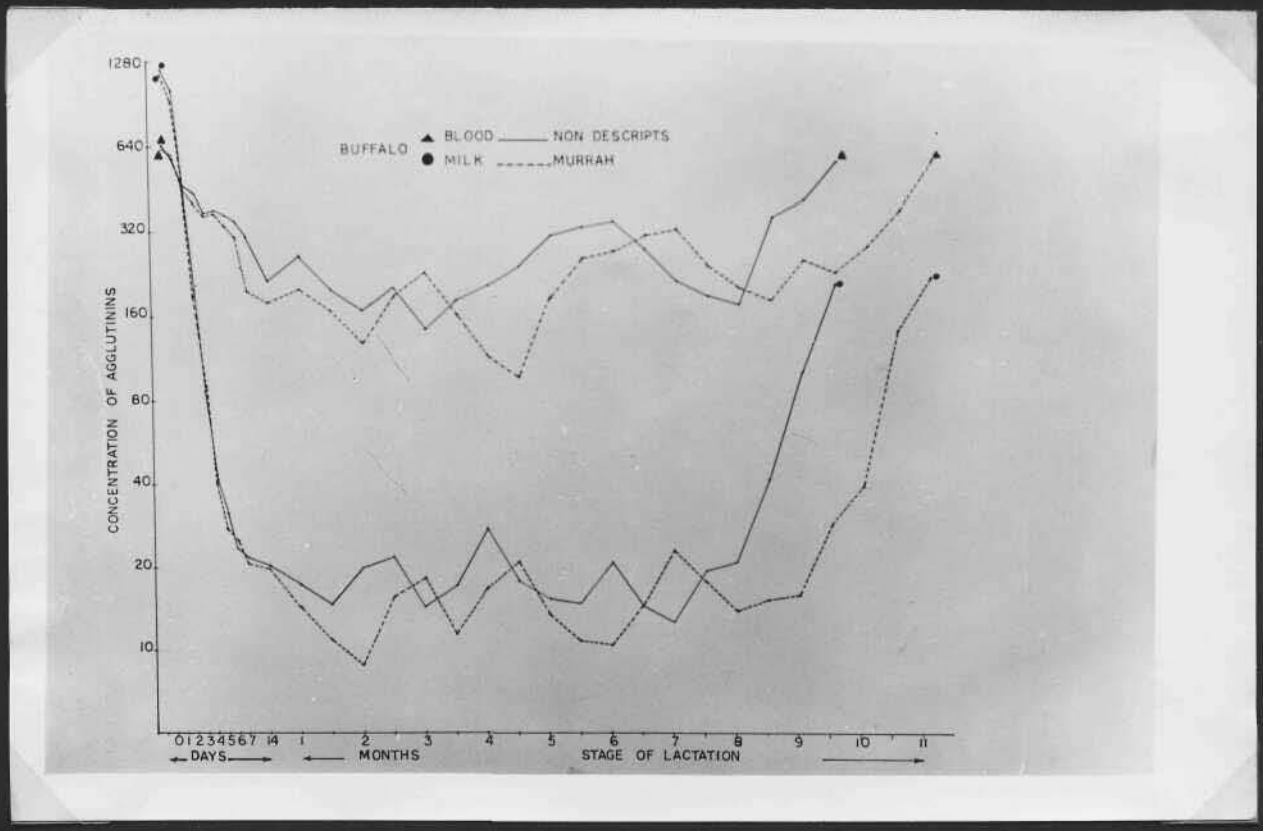
GRAPH 4


COW
BLOOD
MILK
NON DESCRIPT
SINDHI
JERSEY
CONCENTRATION OF AGGLUTININS
1280
640
320
160
80
40
20
10
0 1 2 3 4 5 6 7 14
DAYS
1
2
3
4
5
6
7
8
9
10
11
MONTHS
STAGE OF LACTATION

Effect of stage of lactation on the agglutinin activity of milk and blood in different breeds of buffaloes

-o-o-o-

GRAPH 5


1280
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CONCENTRATION OF AGGLUTININS
BUFFALO
BLOOD
MILK
NON DESCRIPTS
MURRAH
0 1 2 3 4 5 6 7 14
DAYS
1
2
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4
5
6
7
8
9
10
11
MONTHS
STAGE OF LACTATION

agglutinin in the fat globule washings of cow and buffalo did not show any variation throughout the lactation period, but the agglutinin concentration in the fat globule washings from the milk of all the breeds of cows studied was higher, the value being, 2, than in the two breeds of buffaloes studied, where the concentration was 1 (undiluted samples only were positive).

Graphs 4 and 5 show the variation in the agglutinin activity in the blood and milk of Sindhi, Jersey, Nondescript, cows and Murrah and Nondescript, buffaloes throughout the stage of lactation.

b) Species and breeds: The concentration of agglutinin in the milk and fat globule washings of different breeds of cows and buffaloes, in respect of two different agglutinin sensitive strains of organisms are presented in table 25. The breeds studied were, Jersey, Sindhi, Nondescript, Sindhi Jersey cross and Nondescript Jersey Grades in cows and Murrah and Nondescript in buffaloes. The agglutinin concentration was determined in the mid lactation milk from normal and healthy animals. In general, in milk, the average concentration of agglutinin was higher for Streptococcus cremoris 803 organisms than Streptococcus lactis 57 organisms, in all the breeds of cow and buffalo.

TABLE 25

CONCENTRATION OF AGGLUTININS IN THE MILK AND FAT GLOBULE WASHINGS OF DIFFERENT BREEDS OF COW AND BUFFALO

(Test organisms - *Streptococcus cremoris* 803 and *Streptococcus lactis* 57)

Species and breeds of animals		Concentration of agglutinins					
		Milk				Fat globule washings	
		S.cremoris 803		S.lactis 57			
		Range	Mean	Range	Mean	S.cremoris 803	S.lactis 57
Cow	Jersey	10-30	15.3	8-20	10.8	2	2
	Sindhi	10-40	18.9	8-30	13.1	2	2
	Non descript	15-50	27.3	10-40	18.5	2	2
	Sindhi Jersey cross	10-45	19.2	10-30	14.7	2	2
	Non descript Jersey cross	15-45	20.1	10-35	15.4	2	2
Buffalo	Murrah	10-30	15.1	6-20	9.9	1	1
	Non descript	10-35	17.6	6-25	12.4	1	1

Among the species, the agglutinin concentration was low in buffaloes. Among cows the milk of Nondescript animals showed highest activity, 27.3 for Streptococcus lactis 57; Jerseys exhibited lowest activity, 15.3 for Streptococcus cremoris 803 and 10.8 for Streptococcus lactis 57; Sindhi animals had an agglutinin concentration of 18.9 for Streptococcus cremoris 803 and 13.1 for Streptococcus lactis 57. The milk from the crosses between Sindhi and Jersey showed higher agglutinin activity than either of the pure bred animals, while the milk from Nondescript Jersey grades had an activity in between the two species.

Among buffaloes, the milk of Nondescript animals had a higher agglutinin concentration, 17.6 for Streptococcus cremoris 803 and 12.4 for Streptococcus lactis 57 than the milk of Murrah buffaloes which had 15.1 for Streptococcus cremoris 803 and 9.9 for Streptococcus lactis 57.

With regard to fat globule washings, all the breeds of cows had the same agglutinin concentration of 2 in respect of both the organisms Streptococcus cremoris 803 and Streptococcus lactis 57, which was higher than the agglutinin concentration in the two breeds of buffaloes, which was 1 (undiluted samples only were positive) in respect of the organisms Strepto-

Variations in the agglutinin activity in the milk of different breeds of cows and buffaloes.

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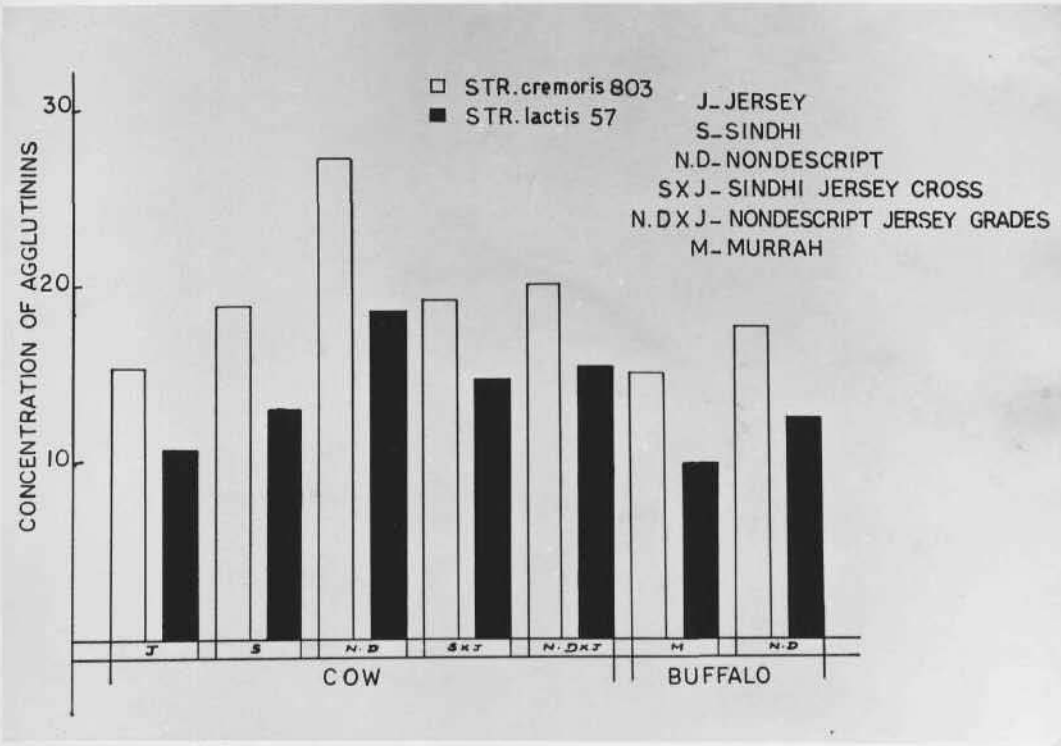
HISTOGRAM


STR.cremoris 803
STR.lactis 57
J-JERSEY
S-SINDHI
N.D-NONDESCRIPT
SXJ-SINDHI JERSEY CROSS
N.DXJ-NONDESCRIPT JERSEY GRADES
M-MURRAH
CONCENTRATION OF AGGLUTININS
30
20
10
J
S
N.D
SXJ
N.DXJ
M
N.D
COW
BUFFALO

coccus cremoris 803 and Streptococcus lactis 57.

The histogram clearly shows the variations in agglutinin activity in different breeds of cows and buffaloes in respect of Streptococcus cremoris 803 and Streptococcus lactis 57 organisms.

6. Behaviour of euglobulin antibodies in agglutination reactions:

a) Euglobulin bacteria complex: Table 26 and 27 describe the nature of the euglobulin bacteria complex of cow and buffalo samples. A total number of 31 selected, agglutinin sensitive bacterial strains, including 22 lactic bacteria were tested for specificity of the antibody bacterium complex. The results indicated that all the organisms that were sensitive to agglutinins in milk, showed high degree of specificity. The euglobulin solution treated with a particular sensitive strain, when tested again with the same untreated strain for agglutination was found to have lost all its agglutinating capacity in relation to the same strain, the titres obtained being 0 (no agglutination). It was further observed, that the euglobulin solution treated with a particular sensitive strain of organism, not only lost its agglutinating power in respect of that particular organism, but also was found to have lost the agglutinating capacity towards one or more other

sensitive strains of the same species, in the same treatment. This type of reaction was observed between the strains of the same species, and not between organisms of one species and the other. But in respect of the bacteria belonging to different species, it was found, that the euglobulin solution absorbed by a given strain also lost a part of its agglutinating activity towards strains of different species as evidenced by a reduction in the titre towards other organisms, when compared with the control.

Based on the behaviour of different strains of Streptococcus lactis, Streptococcus cremoris, and Escherichia coli organisms, it could be recognised, that the six sensitive strains of Streptococcus lactis fall in three groups, the eleven sensitive strains of Streptococcus cremoris in six groups and the two sensitive of Escherichia coli in one group, strains in each group reacting in identical way. In Streptococcus lactis, the first group comprised of strains C (isolate), 18 and 69, the second SR and 57 and the third, ML_2. In Streptococcus cremoris the first group consisted of strains K, DR_7 and 760, the second 803 and R_1, the third ML_1 and KH, the fourth 495 and S_2, the fifth 972 and the sixth HP. In Escherichia coli the single group contained strains 745 and A (isolate).

All these observations were recorded for both cow

and buffalo samples, and except for the differences in the titres for the individual organisms in the samples of these two species, there were no differences regarding the specificity of the antibody - bacteria complex and the existence of groups in Streptococcus lactis, Streptococcus cremoris and Escherichia coli.

b) Euglobulin fat globule complex: Table 28 explains the nature of the euglobulin fat globule complex. The fat globules of Sindhi, Nondescript and Jersey cows and Murrah and Nondescript buffaloes were tested for the nature of the euglobulin fat globule complex. The titres of the controls for the agglutination of fat globules from the milk of the three breeds of cows and two breeds of buffaloes were 1/50. The euglobulin solution absorbed by the fat globules from the milk of a particular breed of cow or buffalo, lost the agglutinating activity towards the fat globules from the milk of the same as well as the other breeds of cows and buffaloes, and the titres were either 0 (negative for agglutination) or very negligible when compared to the titre of the controls.

c) Nature of antibody attaching bacteria to fat globules: The nature of the antibody attaching bacteria to fat globules is brought out in table 29. The fat globules of cow and buffalo milk were employed to

TABLE 28

NATURE OF ANTIBODY FAT GLOBULE COMPLEX

			Sources of fat globules used for adsorption in euglobulin solution				
			Cow			Buffalo	
			Sindhi	Non descript	Jersey	Murrah	Non descript
Agglutination titre in euglobulin (2mg. per ml.) solution (control)			1/50	1/50	1/50	1/50	1/50
Agglutination titre after adsorption with fat globules of	Cow	Sindhi	0	0	1	1/2	1/2
		Non descript	0	0	1	1/2	1/2
	Buffalo	Jersey	1	1	0	1/2	1/2
		Murrah	1/2	1/2	1/2	0	0
		Non descript	1/2	1/2	1/2	0	0

TABLE 29

NATURE OF ANTIBODY ATTACHING

BACTERIA TO FAT GLOBULES

Source of euglobulin (2mg. per ml.)	Treatment of euglobulin	Attachment of bacteria (% bacteria in cream layer) to fat globules of	
		Cow	Buffalo
Cow	Control	89.0	75.4
	Adsorption by cow fat globule	16.1	10.8
	Adsorption by buffalo fat globule	16.8	9.9
Buffalo	Control	66.5	49.5
	Adsorption by cow fat globule	14.4	9.6
	Adsorption by buffalo fat globule	13.8	8.7

absorb the antibodies from the euglobulin solution of cow and buffalo samples by saturation tests. The treated euglobulin solution of cow and buffalo samples, each when tested with fat globules from the milk of both the species, for the percentage of bacteria in the cream layer by centrifuge test, it was found that the bacterial attachment to the fat globules was very low when compared to the values of the control samples. The fat globules from cow and buffalo milk reacted similarly towards each one of the euglobulin solution of cow and buffalo samples except for the differences in the percentage of bacteria in the cream layer of cow and buffalo samples.

7. Agglutinin activity on different bacterial strains:

The behaviour of a number of bacterial strains towards the agglutinins of blood serum, milk and fat globulin washings of cow and buffalo samples is presented in table 30. Milk consists of both whey protein and fat globule agglutinins. To represent these two agglutinins separately, euglobulin solution was taken for the whey protein agglutinins and fat globule washings for the fat globule agglutinins.

Out of the 56 strains of organisms tested for agglutination, 36 were lactic cultures, 15 pathogenic bacteria and 5 nonpathogenic but undesirable in milk

TABLE 30

THE AGGLUTININ ACTIVITY IN BLOOD, MILK AND FAT GLOBULE WASHINGS OF COW AND BUFFALO ON DIFFERENT STRAINS OF BACTERIA

Bacterial strains	Agglutination titre					
	Cow			Buffalo		
	Blood serum	Milk*	Fat globule washings	Blood serum	Milk*	Fat globule washings
1. Streptococcus lactis A (iso)	-	-	-	-	-	-
2. Streptococcus lactis B (iso)	-	-	-	-	-	-
3. Streptococcus lactis C (iso)	1/220	1/20	1/2	1/200	1/20	1
4. Streptococcus lactis S.R.	1/190	1/20	1/2	1/150	1/15	1
5. Streptococcus lactis 57	1/240	1/25	1/2	1/180	1/15	1
6. Streptococcus lactis 18	1/200	1/25	1/2	1/170	1/20	1
7. Streptococcus lactis C_2	-	-	-	-	-	-
8. Streptococcus lactis C_{10}	-	-	-	-	-	-
9. Streptococcus lactis ML_2	1/150	1/15	1/2	1/150	1/10	1
10. Streptococcus lactis 69	1/160	1/15	1/2	1/130	1/15	1
11. Streptococcus lactis HP	1/220	1/20	1/2	1/130	1/10	1
12. Streptococcus lactis 495	1/200	1/20	1/2	1/140	1/15	1
13. Streptococcus lactis 803	1/300	1/30	1/2	1/220	1/20	1

contd..

Bacterial strains	Agglutination titre					
	Cow			Buffalo		
	Blood serum	Milk*	Fat globule washings	Blood serum	Milk*	Fat globule washings
14. Streptococcus cremoris C1	-	-	-	-	-	-
15. Streptococcus cremoris K	1/220	1/25	1/2	1/150	1/15	1
16. Streptococcus cremoris KH	1/260	1/30	1/2	1/200	1/20	1
17. Streptococcus cremoris ML1	1/220	1/30	1/2	1/160	1/20	1
18. Streptococcus cremoris R1	1/150	1/20	1/2	1/150	1/15	1
19. Streptococcus cremoris C3	-	-	-	-	-	-
20. Streptococcus cremoris C7	-	-	-	-	-	-
21. Streptococcus cremoris S2	1/250	1/20	1/2	1/150	1/10	1
22. Streptococcus cremoris DR7	1/300	1/30	1/2	1/170	1/20	1
23. Streptococcus cremoris 972	1/250	1/25	1/2	1/280	1/25	1
24. Streptococcus cremoris 760	1/150	1/20	1/2	1/250	1/25	1
25. Streptococcus citrovorus 209	1/200	1/20	1/2	1/250	1/25	1
26. Streptococcus diacetilactis DRC1	-	-	-	-	-	-
27. Streptococcus diacetilactis DRC2	1/180	1/20	1/2	1/220	1/25	1
28. Streptococcus diacetilactis DRC3	-	-	-	-	-	-
29. Streptococcus thermophilus 489	-	-	-	-	-	-
30. Streptococcus thermophilus STS	-	-	-	-	-	-

contd..

Bacterial strains	Agglutination titre					
	Cow			Buffalo		
	Blood serum	Milk*	Fat globule washings	Blood serum	Milk*	Fat globule washings
31. Lactobacillus bulgaricus 1373	-	-	-	-	-	-
32. Lactobacillus bulgaricus Hansen's	-	-	-	-	-	-
33. Lactobacillus bulgaricus A (iso)	1/100	1/10	1/2	1/100	1/10	1
34. Lactobacillus acidophilus L_1	1/150	1/10	1/2	1/100	1/5	1
35. Lactobacillus acidophilus BA - EE	-	-	-	-	-	-
36. Lactobacillus plantarum 89	1/140	1/15	1/2	1/150	1/10	1
37. Streptococcus faecalis 190	1/90	1/10	1/2	1/130	1/15	1
38. Streptococcus faecalis 30	-	-	-	-	-	-
39. Streptococcus agalactiae 865	1/100	1/20	1/2	1/100	1/15	1
40. Streptococcus pyogenes B - 49 - 3	1/120	1/20	1/2	1/75	1/15	1
41. Staphylococcus aureus KIGM	1/100	1/15	1/2	1/90	1/15	1
42. Bacillus cereus (iso)	-	-	-	-	-	-
43. Escherichia coli 745	1/120	1/15	1/2	1/70	1/15	1
44. Escherichia coli 555	-	-	-	-	-	-
45. Escherichia coli (iso)	1/120	1/20	1/2	1/90	1/10	1
46. Pseudomonas aerogenosa	1/100	1/10	1/2	1/100	1/5	1
47. Pseudomonas fluorescens	-	-	-	-	-	-

contd..

Bacterial strains	Agglutination titre					
	Cow			Buffalo		
	Blood serum	Milk*	Fat globule washings	Blood serum	Milk*	Fat globule washings
48. Pasteurella bovisepticum	1/70	1/5	1	1/50	-	-
49. Salmonella typhosa	-	-	-	-	-	-
50. Salmonella paratyphi	-	-	-	-	-	-
51. Clostridium chauveii	-	-	-	-	-	-
52. Clostridium welchii	1/110	1/5	1	1/75	1	-
Organisms isolated from mastitis milk:						
53. Streptococci (iso)	1/100	1/25	1/2	1/100	1/15	1
54. Staphylococci (iso)	1/70	1/20	1/2	1/50	1/10	1
55. Escherichia coli (iso)	1/90	1/15	1/2	1/50	1/15	1
56. Pseudomonas (iso)	1/70	1/2	-	-	-	-

Note: *Milk represents titre for euglobulin solution (2mg. per ml.)

'(iso)' indicates organisms isolated from curd, normal milk and mastitis milk samples.

and milk products. A total number of 35 strains were found to be sensitive to agglutinins in the milk and blood serum of cows and 34 strains in the milk and blood serum of buffaloes. All the strains of organisms that were sensitive to agglutinins, were agglutinated by the agglutinins of blood serum, whey protein and fat globules and the titres were higher in blood, lower in milk (whey protein) and lowest in fat globule washings. If a bacterial strain was positive for agglutinin in blood, it was also positive for agglutinin in milk and fat globule washings. But there were two exceptions in the case of buffalo samples, where the blood serum was positive for Pasteurella bovisepticum, with a low titre (1/50) and the whey protein and fat globule washings were positive for Clostridium welchii with low titres (1/75 and 1 respectively), but the fat globule washings were negative. In all positive cases, there were considerable variations in the agglutination titres of blood serum and whey protein, from one bacteria to the other. In general, the blood serum and whey protein titres towards majority of lactic cultures were higher than the titres for the pathogenic bacteria. The titre in the fat globule washings remained unchanged, toward all sensitive organisms in both cow and buffalo samples (1/2 and 1 respectively), except towards Pasteurella bovisepticum where the titre was 1 for cow samples and

Clostridium welchii, where the titre was 0 for buffalo samples, and towards Pseudomonas (isolated from mastitis milk) where the titre was 0 for both cow and buffalo samples. Further in most of the strains including pathogenic ones, the agglutination titre of whey proteins was higher in cow samples than in buffalo samples. The agglutination titre of blood serum although variable, did not show significant differences between cow and buffalo samples. In very few cases like Streptococcus cremoris 760, Streptococcus citrovorus 209, Streptococcus diacetilactis DRC_2 and Streptococcus faecalis 190, the whey proteins of buffalo samples gave a higher agglutination titres than the cow samples.

8. Agglutinin activity in the milk from infected udders:

a) Nonpathogenic organisms and leucocytes: Tables 31, 32 and 33 present the agglutinin activity and the leucocyte count in the milk from infected udder the agglutinin activity in relation to the concentration of leucocytes and the agglutination titre in mastitis milk after removal of leucocytes. The organism Streptococcus cremoris 803, that was employed for the assay methods for agglutinins, was used in these experiements also for testing agglutination.

It was found that at the beginning of the infection in the udder, when very mild clinical symptoms only

TABLE 31

AGGLUTININ ACTIVITY AND THE LEUCOCYTE COUNT IN THE MILK FROM INFECTED UDDER

(Test organism - *Streptococcus cremoris* 803)

Stage of infection	Agglutination titre		Number of leucocytes per ml.	
	Cow	Buffalo	Cow	Buffalo
(control) Normal milk	1/30	1/20	0.78×10^4	0.93×10^4
At the onset of infection	1/40	1/35	1.50×10^4	2.10×10^4
After 6 hours	1/45	1/35	4.60×10^4	3.80×10^4
After 12 hours	1/50	1/40	8.10×10^4	9.20×10^4
After 18 hours	1/60	1/40	1.13×10^5	1.06×10^5
After 24 hours	1/60	1/45	1.67×10^5	1.78×10^5
After 36 hours	1/25	1/25	2.60×10^5	3.20×10^5
After 48 hours	1/15	1/10	7.20×10^5	8.60×10^5
After 60 hours	1/2	1/2	1.60×10^6	1.72×10^6
After 72 hours	-	-	2.21×10^6	1.94×10^5
After 84 hours	-	-	2.63×10^6	2.10×10^6

TABLE 32

AGGLUTININ ACTIVITY IN RELATION TO THE

CONCENTRATION OF LEUCOCYTES

(Test organism - *Streptococcus cremoris* 803)

Concentration of leucocytes per ml.	Agglutination titre in normal milk	
	Cow	Buffalo
Control (raw milk)	1/30	1/20
1.0×10^5	1/30	1/20
2.0×10^5	1/25	1/20
4.0×10^5	1/20	1/15
6.0×10^5	1/15	1/10
8.0×10^5	1/10	1/10
1.0×10^6	1/10	1/5
1.2×10^6	1/5	1/5
1.6×10^6	1/2	1/2
1.8×10^6	1	1
2.0×10^6	-	-
2.5×10^6	-	-
3.0×10^6	-	-
3.5×10^6	-	-
4.0×10^6	-	-

TABLE 33

THE AGGLUTINATION TITRE IN MASTITIS MILK

AFTER REMOVAL OF LEUCOCYTES

(Test organism - *Streptococcus cremoris* 803)

Test sample	Agglutination titre		Leucocyte count per ml.	
	Cow	Buffalo	Cow	Buffalo
Mastitis milk before centrifugation	-	1	2.1×10^6	1.82×10^6
Mastitis milk after centrifugation	1/35	1/25	-	-

were evident, without the accompaniment of even the slightest visible changes in milk, the agglutination titre for the organism Streptococcus cremoris 803, was slightly higher in both cow and buffalo samples, for the same organism, which was employed as the control for comparison of titres. The agglutination titre at the onset of infection was 1/40 for cow milk and 1/35 for buffalo milk, while in the controls the titres were 1/30 and 1/20 for cow and buffalo samples respectively. This increase in titres continued upto the 24th hour of infection, the titres at this stage being 1/60 and 1/45 for cow and buffalo samples respectively. At the 36th hour of infection there was a sharp fall in the titre to a level of 1/25 in the infected milk of both the species. The titres reached very low levels (1/2) at the 60th hour of infection and became undetectable from 72nd hour of infection onwards. A progressive increase in the leucocyte count in the mastitis milk, with advancing stages of infection was observed. This increase in leucocyte count was not much upto the sixth hour of infection and was well within the normal levels, the average normal leucocyte count from healthy cows being upto 70,000 per ml. (MacLeod and Anderson, 1952). From the 12th hour of infection, the leucocyte count showed a much higher increase and at the 60th hour of infection when the agglutination titres of both the

samples were only 1/2, the leucocyte count was 1.6 and 1.72 millions per ml. in cow and buffalo samples respectively. After 72 hours and 84 hours of infection when the agglutination titre was negative, the leucocyte count was 2.21 and 2.63 millions per ml. in cow samples and 1.94 and 2.1 millions per ml. in buffalo samples.

When leucocytes were added to cow and buffalo milk obtained from healthy udder, with normal agglutination titre of 1/30 and 1/20 respectively for the organism Streptococcus cremoris 803, it was observed, that with progressive increases in leucocytes concentration in the milk, the agglutination titre decreased in both the samples. When the leucocyte count was upto 100,000 per ml. in cow milk and 200,000 per ml. in buffalo milk the titre was not affected. A decrease in the titre of milk started when the leucocyte count was 200,000 per ml. in cow milk and 400,000 per ml. in buffalo milk. When the leucocyte count was 2.0 millions per ml. in both cow and buffalo milks, no agglutination of bacteria was evident.

Mastitis milk samples from cow and buffalo, obtained from established cases of infection, had high leucocyte counts with undetectable or negligible levels of agglutinins. After centrifugation for the removal of the leucocytes, when the supernatant containing no leucocytes

was examined for agglutination, the titres for the same samples were found to be 1/35 and 1/25 for the cow and buffalo samples respectively.

b) Causative organisms: Table 34 presents the agglutination titre in the blood serum and the milk of mastitis and healthy animals for the causative organisms, isolated from the infected milk. From the milk of cows suffering from mastitis, four organisms, namely, Streptococci, Staphylococci, Escherichia coli and Pseudomonas were isolated. These organisms were tested for agglutination in the blood serum and milk of the infected animals and also in the blood serum and milk of other healthy animals. It was observed that the milk of the infected animals had very high titres towards the organisms isolated from the infected milk of the same animals, while the titres for these organisms in the milk from healthy animals were much lower. The blood serum of the infected animals did not show considerable increase in the agglutination titres towards these organisms, when compared with the serum titres obtained in healthy animals towards the same organisms.

Further the agglutination titres in the infected milk of cows showed much higher increase than the titres in the infected milk of buffaloes, toward the respective organisms. The blood serum titres of the infected cows

TABLE 34

AGGLUTINATION TITRE FOR THE CAUSATING ORGANISMS IN THE BLOOD AND MILK OF THE INFECTED AND HEALTHY ANIMALS

Species of animal	Type of organisms	Agglutination titre in infected animals		Agglutination titre in healthy animals	
		Blood	Milk	Blood	Milk
Cow	Streptococci	1/100	1/80	1/100	1/25
	Staphylococci	1/90	1/90	1/70	1/20
	Escherichia coli	1/100	1/95	1/90	1/15
	Pseudomonas	1/20	1/70	1/70	1/2
Buffalo	Streptococci	1/100	1/45	1/100	1/15
	Staphylococci	1/80	1/40	1/50	1/10
	Escherichia coli	1/70	1/45	1/50	1/15

and buffaloes did not show wide variations.

c) Agglutinin inhibitors in infected milk: Tables 35, 36 and 37 show the etiological agents isolated from mastitic milk samples which exhibited inhibition of agglutination, the behaviour of these organisms in the blood and milk of the same infected animals and other healthy animals, and the behaviour of three other agglutinin sensitive strains in the blood and milk of infected animals exhibiting inhibition of agglutination and in other healthy animals.

Out of 82 cows and 47 buffaloes suffering from mastitis, milk from 5 cows and one buffalo did not agglutinate the organisms isolated from the infected milk samples of these animals. Such a phenomenon was observed in 6.1% of the cases in cows and 2.1% of the cases in buffaloes. From such cow milk samples Escherichia coli (from 3 animals), Pseudomonas (from one animal) and Streptococci (from one animal) organisms and buffalo milk samples, Escherichia coli (from one animal) organisms were isolated. These animals were found to be suffering from chronic mastitis. All the isolated organisms except one in which Streptococci was involved were gram negative organisms.

No agglutination was observed in these bacterial strains when they were tested with the blood serum and

TABLE 35

THE ETIOLOGICAL AGENTS IN MASTITIS CASES EXHIBITING INHIBITION OF AGGLUTINATION

Species of animal	Total number of animals with mastitis	Number of animals in which inhibition of agglutination was observed	Percentage	Etiological agents isolated
Cow	82	5	6.1	Escherichia coli (3 animals) Pseudomonas (1 animal) Streptococci (1 animal)
Buffalo	47	1	2.1	Escherichia coli (1 animal)

TABLE 36

THE BEHAVIOUR OF ORGANISMS ISOLATED FROM MASTITIS MILK SHOWING INHIBITION OF AGGLUTINATION TOWARDS MILK AND BLOOD SERUM OF THE SAME INFECTED ANIMALS AND OTHER HEALTHY ANIMALS

Species of animal	Organisms isolated from the infected animals	Agglutination titre in infected animals		Agglutination titre in healthy animals	
		Blood	Milk	Blood	Milk
Cow	Escherichia coli	-	-	1/85	1/15
	Pseudomonas	-	-	1/70	1/2
	Streptococci	-	-	1/100	1/25
Buffalo	Escherichia coli	-	-	1/50	1/15

TABLE 37

THE BEHAVIOUR OF THREE OTHER AGGLUTININ SENSITIVE ORGANISMS TOWARDS MILK AND BLOOD SERUM OF INFECTED ANIMALS EXHIBITING INHIBITION OF AGGLUTINATION AND, OF HEALTHY ANIMALS

Species of animal	Organisms	Agglutination titre in infected animals		Agglutination titre in healthy animals	
		Blood	Milk	Blood	Milk
Cow	S.cremoris 803	1/350	1/50	1/300	1/30
	S.lactis 57	1/300	1/45	1/300	1/25
	E.coli 745	1/150	1/30	1/120	1/15
Buffalo	S.cremoris 803	1/250	1/35	1/250	1/20
	S.lactis 57	1/250	1/30	1/200	1/20
	E.coli 745	1/100	1/20	1/70	1/15

milk of the infected animals from which they were isolated. But they all showed agglutination when tested with the blood serum and milk from other healthy animal with varying titres.

Three other strains of organisms - Streptococcus cremoris 803, Streptococcus lactis 57 and Escherichia coli 745, known to be sensitive to agglutinins in milk, were tested for agglutination, with the blood serum and milk of those infected animals which did not agglutinate the organisms isolated from their milk, and also with the blood serum and milk of other healthy animals. It was found that all these organisms were agglutinated by the blood and milk of the infected as well as the healthy animals. The agglutination titres obtained for the milk of these infected animals towards all the three organisms tested were higher than the titres obtained with the milk of normal healthy animals. These observations were made for both cow and buffalo samples.

Table 38 presents the nature of agglutinin inhibitors. The milk whey from the infected animal which did not agglutinate the organisms isolated from the same infected milk, was dialysed against the milk whey obtained from healthy animals showing normal agglutination titre. Even after dialysis the sample was negative for agglutination. The infected milk and blood

TABLE 38

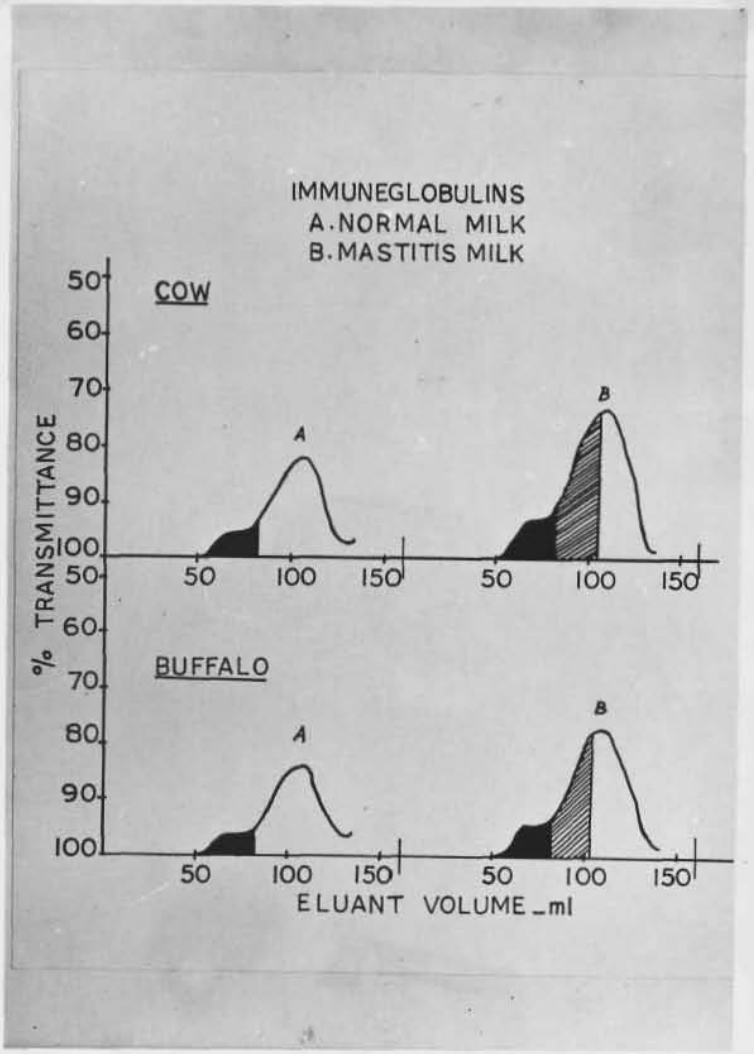
NATURE OF THE AGGLUTININ INHIBITORS

Nature of treatments	Microscopical observation of agglutination
1. Milk whey from infected animal, dialysed against normal whey.	- (no agglutination)
2. Infected milk and blood serum, mixed with an equal quantity of normal milk and blood serum.	- (no agglutination)
3. Infected milk and blood serum was diluted and mixed with undiluted normal milk and blood serum.	+ (Agglutination was seen in two fold dilution for milk and six times dilution for blood)
4. Heated to 60°C for 20 minutes.	- (no agglutination)
5. Adsorbed with the isolated organism and retested with the same untreated organism	+ (All the isolated organisms were agglutinated)

Protein elution patterns on Sephadex G 200, of the immune globulins from mastitic milk whey of cow and buffalo.

-o-O-o-

GRAPH 6



Shaded area represents natural antibodies in milk

Striped area represents induced antibodies in mastitic milk

serum when mixed with normal milk and blood serum, the normal milk and blood serum lost their ability to agglutinate the bacterial cells. Two fold dilution of the infected milk and a six fold dilution of the blood serum from the infected animals, when mixed with equal quantity of undiluted normal milk and blood serum, a positive reaction for agglutination was obtained. This factor present in the infected milk was not inactivated at 60°C for 20 minutes. Infeoted milk and blood serum from such animals when tested with the isolated organisms and retested with the same untreated organisms were found to be positive for agglutination towards these organisms. All these observations were recorded for both cow and buffalo samples.

Graph 6 shows the protein elution pattern of the immune globulins from mastitic milk whey of cow and buffalo. An increase in the total immune globulins is evident in both cow and buffalo samples. But the increase in buffalo sample was lesser than that of cow.

9. Transfer of immune globulins from colostrum to the new born calf:

Table 39 presents the colostral influence on the agglutinins in the blood serum of calves from birth, upto the age of six months. The bacterial strains

employed were Streptococcus cremoris 803 and Escherichia coli. The precolostral calf blood serum had the lowest agglutinin concentration of 15 and 5 for Streptococcus cremoris 803 and 8 and 2 for Escherichia coli (isolate) in cow and buffalo samples respectively. After colostral feeding a very rapid increase in the agglutinin activity was observed in the blood serum within 6 hours of suckling and the activity reached the dam's level at 24 hours after birth in calves of white cattle. The agglutinin activity continued to increase still further to reach a maximum level at the age of 3 days, the concentration being 414 and 189 for Streptococcus cremoris 803 and Escherichia coli (isolate) respectively. Thereafter, although a slight fall in the activity was observed with fluctuations upto the age of six months, the concentration was higher than or almost equal to that of the dam's level.

The increase in agglutinin activity in the blood serum of buffalo calves after colostral feeding was very slow and the maximum concentration of 193 and 87 for Streptococcus cremoris 803 and Escherichia coli (isolate) respectively was reached on the 4th day after birth which itself was much lower than the concentration in the dam's blood serum. As in the case of calves of white cattle, here too, the activity decreased with

TABLE 39

COLOSTRAL INFLUENCE ON THE AGGLUTININS IN THE BLOOD SERUM OF CALVES FROM BIRTH TO SIX MONTHS OF AGE, IN RESPECT OF TWO DIFFERENT AGGLUTININ SENSITIVE ORGANISMS

Age of calf	Concentration of agglutinin in the calf blood serum			
	Cow		Buffalo	
	Streptococcus cremoris 803	Escherichia coli (iso)	Streptococcus cremoris 803	Escherichia coli (iso)
Precolostral period:				
0 hour*	15	8	5	2
Post colostral period:				
1 hour	24	14	12	6
4 hours	57	27	23	14
6 hours	121	49	43	19
12 hours	147	74	68	27
18 hours	198	104	87	36
1 day	304	147	103	44
2 days	414	189	127	51
3 days	362	162	193	87
4 days	358	168	167	76
5 days	327	147	181	74
6 days	304	144	189	78
7 days	358	177	173	81
14 days	329	126	161	75
1 month	298	135	172	79
2 months	319	133	134	69
3 months	308	121	125	61
4 months	321	145	158	76
5 months	305	129	139	77
6 months	311	131	159	82

Note: *0 hour indicates immediately after birth but before feeding colostrum.

The concentration of agglutinins in the maternal blood serum in the case of white cattle was 297 for Streptococcus cremoris 803 and 138 for Escherichia coli (iso) and in the case of buffaloes was 272 for Streptococcus cremoris 803 and 114 for Escherichia coli (iso).

advancing age, showing fluctuations, but even upto the age of six months upto which tested the mother's level was not reached.

Further it was observed that, throughout the period from birth to six months of age, the concentration of agglutinins in respect of the two organisms studied, was much higher in the blood serum of the calves of white cattle than in the blood serum of the buffalo calves at every stage.

10. Agglutinin resistant and sensitive strains on the fermentation of milk:

Table 40 and 41 present the behaviour of single strain and mixed strain cultures of agglutinin sensitive and resistant organisms in the pasteurized skim milk of cow and buffalo in respect of their curd forming activity.

The organisms chosen were Streptococcus lactis 57, a strain sensitive to both agglutinins and lactoperoxidase in milk, three variant strains of the organisms Streptococcus lactis C_{10}, in which one was sensitive to both agglutinins and lactoperoxidase in milk, a second strain sensitive only to lactoperoxidase, but resistant to agglutinins and a third strain resistant to both lactoperoxidase and agglutinins, and Streptococcus lactis C_2, a hundred percent resistant strain. These organisms

TABLE 40

THE EFFECT OF AGGLUTININ SENSITIVE AND RESISTANT SINGLE STRAIN CULTURES ON THE CURD FORMATION IN COW AND BUFFALO MILK (PASTURIZED SKIM MILK)

Type and nature of organisms	Sensitivity to agglutinins	Number of organisms added per ml.	Time taken for clotting in hours		Texture (consistency) of the curd formed	
			Cow	Buffalo	Cow	Buffalo
Streptococcus lactis 57 (sensitive)	+	15.0×10^7	16½	17½	Semi solid curd with slight whey separation	Fairly firm curd with slight whey separation
Streptococcus lactis C_{10} (sensitive)	+	19.0×10^7	32	29	Loose flaky clots with considerable whey separation	Loose flaky clots with considerable whey separation
Streptococcus lactis C_{10} (sensitive to lactoperoxidase only)	-	18.5×10^7	14	17	Fairly firm curd with few small whey pockets	Fairly firm curd with large whey pockets
Streptococcus lactis C_{10} (resistant)	-	14.0×10^7	5½	7	Firm curd with no whey separation or whey pockets	Firm curd with no whey separation or whey pockets
Streptococcus lactis C_2 (resistant)	-	16.0×10^7	6	6½	Firm curd with no whey separation or whey pockets	Firm curd with no whey separation or whey pockets

were inoculated in approximately equal concentrations in the pasteurized skim milk and incubated at 37°C.

It was seen that the two resistant strains, C_2 and C_{10} took a very short incubation time for clotting the cow and buffalo milk samples, while the clotting time was very much prolonged for other strains. The strain C_{10}, which was sensitive only to lactoperoxidase, took 14 and 17 hours for clotting the cow and buffalo milk respectively. The strains C_{10} and 57, sensitive to both the inhibitory substances in milk took 32, 29 and 16½ and 19½ hours respectively for clotting cow and buffalo milk. Even after the determination of clotting time for the respective cultures, the incubation was prolonged further to study the nature of the curd formed. Differences were observed in the consistency of the curd formed. Resistant strains that had shorter clotting time, produced curds, with a firm clot and no whey separation was observed. The agglutinin resistant but lactoperoxidase sensitive strains of C_{10} produced a fairly firm curd with isolated small whey pockets trapped in the curd. The sensitive strain, 57, produced a semisolid curd with slight whey separation in cow milk and a fairly firm curd with slight whey separation in buffalo milk. The curd formed by the sensitive organisms of strain C_{10} was very loose in consistency with flaky clots and considerable amount

TABLE 41

THE EFFECT OF MIXED CULTURES OF AGGLUTININ SENSITIVE AND RESISTANT STRAINS ON THE CURD FORMATION IN COW AND BUFFALO MILK (PASTEURIZED SKIM MILK)

Type and nature of organisms	Proportion in mixed culture	Time taken for clotting in hours		Texture (consistency) of the curd formed	
	C_2 : C_{10}	Cow	Buffalo	Cow	Buffalo
	0 : 10	32	29	Loose flaky clots with considerable whey separation	Loose flaky clots with considerable whey separation
	1 : 9	19½	19	Loose clot with whey separation	Loose clot with whey separation
	2 : 8	13	14½	Almost firm curd with few whey pockets	Fairly firm curd with large whey pockets.
Streptococcus lactis C_2 (resistant)	3 : 7	11	12	Firm curd with few small whey pockets	Firm curd with few small whey pockets
Streptococcus lactis C_{10} (sensitive)	4 : 6	8	10	Firm curd and no whey separation or whey pockets	Firm curd with two or three small whey pockets
	5 : 5	7	8½	Firm curd	Firm curd and no whey separation or whey pockets
	6 : 4	6½	7½	Firm curd	Firm curd
	7 : 3	6½	7	Firm curd	Firm curd
	8 : 2	6	7	Firm curd	Firm curd
	9 : 1	6	6½	Firm curd	Firm curd
	10 : 0	6	6½	Firm curd	Firm curd

of whey separation. All the organisms except Streptococcus lactis C_{10} which was sensitive to both the inhibitors in milk, took a slightly longer incubation time for clotting buffalo milk samples.

To study the effect of mixed culture consisting of agglutinin sensitive and resistant strains on the curd formation in milk, sensitive strains of Streptococcus lactis C_{10} and resistant strains of Streptococcus lactis C_2 were mixed in different proportions and the clotting time and the nature of curd formed in respect of these mixed cultures were determined.

The sensitive strain of Streptococcus lactis C_{10} required 32 and 29 hours for clotting the cow and buffalo milk respectively and the curd formed was very loose with flaky clots and considerable whey separations was evident in both the samples. The resistant strain Streptococcus lactis C_2 took only 6 and 6½ hours for clotting the cow and buffalo milk respectively and also produced a firm curd and no whey separation was evident.

With progressive increase in the proportion of resistant strain in a mixed culture, the clotting time in the cow and buffalo milk samples were shortened and the consistency of the curd formed also was improved. Even the presence of 10% of the resistant strain C_2

in the mixed culture, was able to bring about a marked reduction in the clotting time for both cow and buffalo milk. A level of 30 to 40% of the resistant strain in the mixed culture, besides shortening the clotting time considerably, was able to produce curd of good quality. In general the clotting time for buffalo milk was found to be slightly higher than the clotting time for cow milk.

D I S C U S S I O N

D I S C U S S I O N

1. Isolation of agglutinin active protein fraction from cow and buffalo milk:

a) Agglutination of bacteria: The results of the milk ring test with different protein fractions of cow and buffalo milk (Tables 1, 2 and 3) indicate that the level of the immune globulin content within and between the species in the various acetone fractions of colostral whey, is responsible for the variation noticed in the incubation time required for a positive test.

The euglobulins of the whey proteins of cow and buffalo samples only carry the agglutinins. The level of euglobulin alone is not responsible for the differential behaviour of cow and buffalo samples towards the milk ring test, but other factors too are involved.

The fat globule washings also contain the agglutinins for bacterial agglutination. The fat globule washings of buffalo contain a low concentration of agglutinins and hence is negative for the milk ring test.

In the microscopical observation method for agglutination (Table 4), the variations observed in the degree of agglutination with milk, colostral whey and Lactenin L_1 extract of cow and buffalo samples is

due to the differences in the immune globulin content within and between the species. Variations in the degree of agglutination with equal concentration of euglobulin in cow and buffalo samples is due to the differences in the agglutinin content of the respective euglobulins. Fat globule washings of cow and buffalo milk also carry agglutinin activity.

The agglutination reaction observed with the whey protein fractions of cow are in agreement with the results of Stadhouders (1963).

b) Agglutination of fat globules: Among the whey proteins of milk, euglobulin alone carries the factors responsible for the agglutination of fat globules(Table 5). Since the degree of clumping between the cow and buffalo samples is same, the concentration of the agglutinins for the fat globule agglutination may be equal in the samples of the two species. Variations in the degree of clumping by the fat globule washings and the euglobulin is obviously due to the differences in the concentrations of this particular factor in these two fractions. The results of the effect of euglobulin and fat globule washings of cow milk samples on the fat globules of cow milk agree with those of Stadhouders and Hup (1970) and Kenyon et al (1966) respectively.

The fat globule membrane materials do not carry

any antibodies for the agglutination of either bacteria or the fat globules (Table 6). The agglutinin activity present in the fat globule washings is due to the substances adsorbed on the surface of the fat globules (Table 7). The adsorption experiments indicate that the euglobulin of cow as well as buffalo samples can be adsorbed on the fat globules of cow and buffalo milk. Although the agglutinin activity of cow milk towards bacteria is higher than that of buffalo milk, adsorption of euglobulin antibodies by the fat globules of cow and buffalo milk, from the cow and buffalo skim milk, is constant for the respective species. This means, irrespective of the concentration of the euglobulin antibodies in milk, the adsorption on the fat globules remains constant for the particular species. This may be due to the differences in the surface area of the fat globules of cow and buffalo milk.

From the foregoing discussion it is clear that the euglobulin of the whey proteins of cow and buffalo milk carry the agglutinins. The agglutinin activity in the buffalo milk is less than that of cow milk. The agglutinin activity of the fat globule washings is due to the agglutinins adsorbed on the surface of fat globules. Euglobulin carry factors responsible for the agglutination of fat globules.

2. <u>Analysis of fractions carrying agglutinin activity</u>:

a) Starch gel electrophoresis: The electrophoretogram (Plates 3, 4 and 5) reveal that the proteins carrying the agglutinin activity cannot be fractionated in the pure form by the acetone fractionation technique adopted. The Ammonium sulphate fractionation (Smith, 1946) gives pure euglobulin which carries all the agglutinin activity in milk. The presence of a protein fraction in the immune globulin position in the fat globule washings, indicate that probably the euglobulin is responsible for the agglutinin activity of the fat globule washings and hence it can be construed that the fat globule agglutinins may be identical with the whey protein agglutinins. This has been confirmed by other criteria also. The cryoglobulins of cow and buffalo milk appear to be the same.

b) Gel filtration on Sephadex: The protein elution pattern on sephadex (Graphs 1, 2 and 3) reveal that the technique of gel filtration on sephadex G 200 is suited for the separation of the protein fraction associated with the agglutinin activity. The lower agglutinin activity of buffalo milk is due to the lower concentration of the agglutinin active fraction of the immune globulin of buffalo milk than that of cow milk. The elution pattern also reveal that the fat globule agglutinins are identical with the whey protein agglutinins in both cow and buffalo samples. The position of the agglutinin active fraction in the elution pattern indicate that these are natural

antibodies in milk probably belonging to the IgM class of immune globulins (Mach et al, 1969)

The protein elution pattern of cryoglobulins of cow and buffalo milk indicates that this protein of the two species are similar except for the differences in the concentration.

c) Paper chromatography: From the chromatograms (Plates 6 and 7) and the results (Table 8), it is seen that the amino acid composition of the euglobulin, cryoglobulin and the fat globule agglutinins of cow and buffalo samples are qualitatively same except for the presence of tyrosine in the buffalo samples of euglobulin and fat globule agglutinins. The great similarity in the composition of the different fractions of the immune globulins of cow and buffalo milk indicate their similar functions. Since different globulin fractions concerned with immunity do not possess the same amino acid composition (Smith and Greene, 1947) it is reasonable to expect differences in the amino acid make up of the immune globulins of cow and buffalo milk.

The above discussions clearly indicate that a portion of immune globulins of milk of cow and buffalo carry the natural antibodies (agglutinins). The fat globule agglutinins and the whey protein agglutinins are identical.

3. Properties of euglobulin antibodies:

a) Effect of pH: The agglutinins in the milk and fat globule washings manifest their maximum activity at a particular pH range of 6.1 to 6.5 and 6.3 to 6.6 in cow and buffalo samples respectively (Table 9). These pH ranges seem to be ideal for the maximum activity of the euglobulin antibodies.

b) Effect of temperature: From the results (Table 10), it is seen that the agglutinins are inactivated in 12 minutes at 70°C. Kosikowski and Mocquot (1958) have observed that the Lactenin L_1 fraction was destroyed at 70°C for 20 minutes. Since the Lactenin L_1 fraction which carries the agglutinin activity is heterogeneous with a mixture of three proteins, immune globulins, alpha lactalbumin and beta lactoglobulin, probably it was able to withstand the heat treatment at 70°C for a longer duration as observed by the authors mentioned above.

When euglobulin solution is held at low temperatures, a minor portion of it appears to aggregate or precipitate which is referred as cryoglobulin. Probably, the differences observed between cow and buffalo samples of euglobulin in the time taken for maximum aggregation and the percentage of aggregated euglobulin (Table 11), is due to species differences that might exist in the

intricate structure of the proteins. Stadhouders and Hup (1970) observed that only 5 percent of the euglobulin aggrega ed in a holding time of 20 hours at 2°C at the second cold aggregation in cow milk samples, although in the present experiments 8.46 percent of the euglobulin solution of cow milk samples aggregated after a holding time of 21 hours.

c) Action of euglobulin antibodies on the bacteria and fat globules: The euglobulin antibodies are able to attach the bacteria to the fat globules. The greater bacterial attachment (Table 12), in the case of cow samples than that of buffalo samples, is evidently due to higher antibody activity of cow milk for bacterial attachment.

In the centrifuge tests and cream rising experiments (Tables 13 and 14) the number of organisms in the cream layer are the agglutinated cells which are also attached to the fat globules, the sedimented cells are the agglutinated bacteria and the cells that remained in the middle portion - skim milk, must be the unagglutinated cells. The greater number of agglutinin sensitive bacteria in the cream layer and the fewer number of cells in the skim milk portion in cow milk is an indication of the higher antibody activity for the attachment of bacteria to fat globules and for the agglutination of bacteria,

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than the antibody activity in buffalo milk, for these two phenomena.

The reason for the large number of cells in the sediment or the bottom layer in buffalo milk samples is due to the attachment of a lesser number of the agglutinated cells to the fat globules, since the antibody activity for the attachment of the bacteria to the fat globules is much less in buffalo milk.

In the case of agglutinin resistant strains, the majority of cells remain in the skim milk portion and a very few cells in the cream layer indicated, that the organisms resistant to the agglutinin activity for bacterial agglutination will not get attached to the fat globules which means that they will also be resistant to the antibody activity to the bacterial attachment. The results are in agreement with those of Stadhouders and Hup (1970).

In the two agglutinin sensitive strains, the number or percentage of organisms in the cream layer representing the bacterial attachment shows considerable variations, because, for different bacteria, the biding sites are different in relation to the number and distribution of the determinants. The results of cream rising experiments showing the distribution of bacteria throughout the milk and the centrifuge tests with cow milk are in

agreement with those of Stadhouders (1963) and Stadhouders and Hup (1970).

d) Differentiation of three classes of antibodies: From the behaviour of the two fractions, supernatant and the sediment obtained after treatment of the euglobulin for cold aggregation (Table 16), it is seen that the entire agglutinin activity towards bacterial agglutination is confined to the supernatant fraction, while the antibody activity for fat globule agglutination and the bacterial attachment to the fat globules are present only in the sediment fraction - the cryoglobulins. It can be construed then, that these two antibodies, one for bacterial agglutination and the other for fat globule agglutination and the attachment of bacteria to fat globules are different from each other.

The saturation tests in euglobulin solution with bacteria and fat globules provide further evidence (Table 17), that these three classes of antibodies are different. Adsorption of euglobulin solution by bacteria, removes the antibodies for bacterial agglutination and the attachment of bacteria to fat globules. The titre for fat globule agglutination remains unchanged indicating that the antibodies for fat globule agglutination are different from each of the other two groups of antibodies. Adsorption of euglobulin solution by fat globules, removes

the antibodies for fat globule agglutination as well as those attaching bacteria to fat globules, leaving the antibodies for bacterial agglutination unaltered, which means that the antibodies for bacterial agglutination are different from each of the other two groups of antibodies. These observations imply that these three groups of antibodies present in the euglobulin of cow and buffalo milk are different from each other. These results are in accordance with the observation of Stadhouders and Hup (1970).

The discussions bring out the following facts: The euglobulin antibodies have an optimum pH range for activity with species differences. They are inactivated at 70°C for 12 minutes. At low temperature (2°C) the euglobulin antibodies aggregate forming cryoglobulin. The euglobulin antibodies are responsible for the attachment of bacteria to fat globules showing higher bacterial attachments with cow samples than with buffalo samples. The euglobulin antibodies are composed of three different antibodies, 1. for agglutination of bacteria, 2. for agglutination of fat globules and 3. for attachment of bacteria to fat globules.

4. Estimation of agglutinin activity:

a) Bacterial agglutination: The results indicate

that the antibodies responsible for the agglutination of bacteria are higher in the cow samples than in buffalo samples (Tables 18 to 21). Higher agglutinin activity in the cow samples of colostral whey, milk and fat globule washings is due to a higher concentration of euglobulin in these fractions, than in the corresponding fractions of buffalo samples. In the euglobulin solution from cow milk the higher agglutinin activity is due to a higher concentration of the fraction carrying antibody activity, which inturn indicates the high level of antibody content in cow samples.

The three methods, pH assay, acidity determination and microscopical observation of agglutination give an accurate estimate of the agglutinin activity towards bacterial agglutination. The methods of pH assay and acidity tests, for estimating agglutinin activity can be employed, only by making use of an organism which is sensitive to the agglutinins in milk and insensitive to other inhibitory substances. Hence, the direct microscopic observation of agglutination is the best method for the estimation of agglutinin activity in milk for the agglutination of bacteria.

The agglutination titres for colostrum and milk samples of cow agree with the values of Emmons et al (1966) and the titre for the euglobulin solution from

cow milk is comparable to the values of Stadhouders and Hup (1970). Natarajan and Dudani (1961) obtained higher titres for the inhibitory action of cow and buffalo milk. Further, they reported that the titre value for buffalo milk were higher than for cow milk, and that the lactenin content of buffalo milk was higher, compared to that of cow milk. But they have determined the total inhibitory action of cow and buffalo milk which includes in addition to the agglutinins, the other inhibitory substances as well. In this investigation only the agglutinins of cow and buffalo milk have determined and this may be the reason for the contradictory observations recorded.

b) Fat globule agglutination: The euglobulin antibody for fat globule agglutination of cow and buffalo samples are equal (Table 22), which may mean that the binding sites of the euglobulin antibodies of the cow and buffalo samples towards the fat globules of cow and buffalo milk are equal and similar. Since the fat globules of cow and buffalo milk behave identically towards the euglobulin from the milk of either species, it is also probable that the determinants of the fat globules of the milks of the two species are same or similar. Quantitative differences in the euglobulin concentration must be responsible for the low titre in the fat globule washings towards the

fat globule agglutination. The results in respect of euglobulin of cow agrees with those of Stadhouders and Hup (1970).

c) Attachment of bacteria to fat globules: From the results (Table 23), it is evident that the antibodies for the attachment of bacteria to fat globules are higher in the cow samples than in buffalo.

From the above discussion it is evident that the microscopical observation of agglutination method is the best for the estimation of agglutination titre for bacterial agglutination; the agglutination titre for bacterial agglutination in cow milk is higher than that of buffalo; the antibodies for the agglutination of fat globules are equal in both cow and buffalo samples, and the antibodies for the attachment of bacteria to fat globules is higher in cow milk than in buffalo.

5. Factors influencing the variation in the agglutinin activity of milk:

a) Stage of lactation: It is quite apparent from the results (Tables 24 and 24a), that the agglutinin activity is considerably influenced by the stage of lactation. Very high increase in the agglutinin activity in the prepartum lacteal secretion, colostrum, in the milk obtained for a few days following parturition

and in the milk obtained at the end of lactation, could be due to a high concentration of immune globulins in them. Although the immune lactoglobulins of bovine colostrum are qualitatively similar to those in serum, marked differences between their relative concentrations in serum and colostrum are evident as seen from the wide differences in the agglutinin activity. Since the mammary gland shows a highly selective preference for absorption of the electrophoretically faster serum immune globulins and has the ability to concentrate this protein in colostrum (Pierce and Feinstein, 1965) such wide differences in the concentration of immune globulins in the blood serum and milk during colostral stage and end of lactation are obvious. The variations in the concentration of agglutinins in blood does not directly influence the agglutinin concentration in milk, probably because of this property of high selectivity towards the absorption of serum immunoglobulins. Emmons et al (1966) have concluded that agglutinating antibodies do not filter into milk from blood at a constant rate. The udder itself is suggested to be one of the site of antibody production by Kerr et al (1959) and Porterfield et al (1959), in which case a part of the variation in the agglutinating antibodies in milk especially during mid lactation period may be, a result of its own response to necessities like bacterial stimulation or other cause.

Hence, the variations in the agglutinin activity in the milk at different stages of lactation from a day prior to calving upto the end of lactation could be due to the combined effect of the selective preference of the mammary glands towards serum immuno globulins and the response of the udder for antibody production.

The reason for a constant level of agglutinins in the fat globule washings at different stages of lactation could be due to the fact that the surface area available for adsorption of euglobulin is more or less constant and could be expected to alter only with variations in the size of the fat globules and in the fat content. But these variations in the level of fat and size of the fat globules are only limited, and hence is unable to manifest a pronounced effect in the agglutinin content to any detectable levels.

b) Species and breeds: The results showing the agglutinin activity in different breeds of cow and buffaloes indicate that breed and species variations do exist in the concentration of agglutinins in milk. The agglutinin activity in milk or blood serum is a contribution of that particular class of proteins, the immune globulins of milk and blood serum, the level or concentration of which is controlled by genetic variations. In as much as the degree of agglutinin

activity is due to the level of immune globulins which is controlled by genetic variations, differences in the agglutinin activity in the milk of different breeds and species are bound to occur. Emmons et al (1966) have stated that the concentration of agglutinating antibodies in milk were independent of the breed and age of the cows. One interesting observation is that the milk of cross breds (50% exotic blood) exhibited a higher agglutinin activity than that in the milk of either of the pure bred animals, namely Jersey or Sindhi.

The agglutinin activity towards the two organisms Streptococcus cremoris 803 and Streptococcus lactis 57, are different in all the breeds of cows and buffaloes examined, because, for the different bacteria the binding sites as well as the number and distribution of determinants are different, (Stadhouders and Hup, 1970).

The agglutinin activity varies with the stage of lactation, species and breeds of animals and strain of test organisms.

6. Behaviour of euglobulin antibodies in agglutination reaction:

a) Euglobulin bacteria complex: The antibody bacteria complex is found to be highly specific, because,

the treatment of euglobulin solution with one particular sensitive strain is able to remove completely the homogeneous antibodies only, but not those antibodies of a different sensitive strain of organism (Table 26 and 27). This means that the binding sites in the antibodies for any one organism is different from the other. But this specificity appears to be limited, because the euglobulin solution after treatment with a particular sensitive bacteria apart from losing its agglutinating capacity towards that organism, also loses its agglutinating power towards one or several other sensitive strains of the same species, probably due to the sharing of the antibodies by closely related strains of the same species. This is possible, because a cell surface possesses a great diversity of antigenic determinants and many of these determinants are identical or similar in groups of different, but related cells (Davis et al, 1969). The observation that certain strains of sensitive bacteria of one species, are capable of absorbing partially the antibodies corresponding to sensitive bacteria of different species, is an indication of the existence of cross reactions between strains of different groups. The six strains of Streptococcus lactis, eleven strains of Streptococcus cremoris and two strains of Escherichia coli, have three, six and one immunological group or groups respectively,

determined on the basis of the immunological relationship between strains within a group. Strains of different groups are unrelated as to their determinants.

These observations relating to the results obtained from the samples of cow milk, agree with the results of McPhillips (1958), Portmann and Auclair (1959), and Stadhouders and Hup (1970).

b) Euglobulin - fat globule complex: From the results it is evident that the treatment of the euglobulin solution with fat globules from the milk of any one breed of cows or buffaloes is able to remove the antibodies agglutinating the fat globules from the milk of the same as well as the other breeds of cows or buffaloes examined (Table 28). If the euglobulin fat globule complex is specific, then the treatment of euglobulin solution with one type of fat globules will remove only the corresponding homogeneous antibodies, and when the fat globules from the other sources are tested, they will be agglutinated, since the agglutinating antibodies for them will be present in the same euglobulin solution. Hence, the results indicate that the euglobulin-fat globule complex is not specific, as reported by Stadhouders and Hup (1970). Probably the binding sites in the antibody molecules in the euglobulin solution from cow or buffalo milk

for the fat globules from the milk of different breeds of cows or buffaloes are either identical or similar.

c) Nature of antibody attaching bacteria to fat globules: The euglobulin antibodies attaching bacteria to fat globules (Table 29)bring out the fact that these antibodies for the attachment of bacteria to fat globules, also lack specificity, since the treatment of euglobulin solution with the fat globules from the milk of either cows or buffaloes removes this class of antibodies towards the fat globules from the milk of either of the two species, rendering the euglobulin solution incapable of attaching the bacteria to fat globules. But it appears from the results in table 29, that the treatment in the saturation test with the fat globules, which has been repeated three times, does not remove the entire antibody, wherein, in all the cases after treatment, the property of bacterial attachment to the fat globules is not completely lost. The reason must be that the treatment repeated thrice, for the adsorption of antibodies from the euglobulin solution by the fat globules, is either insufficient to remove all the antibodies, or that this group of antibodies cannot be completely removed by adsorption. The first explanation seems to be more appropriate, because in the experiments for the aggregation of the euglobulin, it has been possible to separate these antibodies completely from

the supernatant of the euglobulin solution at low temperature. To test this possibility, the adsorption treatments were further carried out and it was found that atleast two more treatments were necessary to reduce this group of antibodies to negligible levels. The results obtained with the fat globules of cow milk are in agreement with the results of Stadhouders and Hup (1970).

In the agglutination reaction it is found that the euglobulin - bacteria complex is specific, the euglobulin - fat globule complex and the antibody attaching bacteria to fat globules are non specific.

7. Agglutinin activity on different bacterial strains:

A large number of organisms are agglutinated by the antibodies in milk and blood serum (Table 30), indicates that these are natural antibodies. They have not formed due to any specific stimulus, but might have arisen as a result of invasion of the oral cavity and the intestinal tract by a large number of microorganisms through feed and other means (Evans et al, 1966). Further, it appears that it is not necessary for all these organisms to be present in the alimentary tract of the animal to stimulate antibody production. The presence of few strains of organisms are sufficient to induce production of anti-

bodies, which could be shared by other strains of organisms as well, since immunological relationship between different strains of same groups within a species has been demonstrated in this study as well as by Portmann and Auclair (1959).

If the blood serum is positive for agglutination towards any organism, milk and the fat globule washings also are positive, since the immunoglobulins of blood which carry these natural antibodies, are the source of the immune globulins of milk.

The differences in the agglutination titres of blood serum, whey proteins and fat globule washings, is due to differences in the concentration of the immune-globulins in them. In the few exceptional case observed in the cow and buffalo samples towards Pasteurella boviseptieum, Clostridium welchii and Pseudomonas, where the milk or fat globule washings or both giving negative results when blood serum was positive, might be due to the very low titre in blood, resulting in such a low antibody content in milk towards those particular organisms. There is no relationship between the agglutinin activity in blood serum and in milk, because of the highly selective preference shown by the mammary gland towards adsorption of serum immunoglobulins (Pierce and Feinstein, 1965) and due to the fact that agglutinating antibodies

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do not filter into milk from blood at a constant rate (Emmons et al, 1966).

Variations in titres between one bacteria, and the other could arise probably as a result of the extent of invasion of the alimentary tract of the animals, by these organisms, and also in the differences in the determinants of these organisms as well as the number of binding sites in the antibodies for these organisms.

The reason for a high titre for most of the organisms in cow milk than in buffalo milk, could be explained on the basis of the higher euglobulin content. But, the behaviour of the few strains which have given a higher titre in buffalo samples than in cow samples, could be either possibly due to differences in bacterial strains and their capacity to react with antibodies or, probably the number of binding sites in the antibodies, for these organisms, in buffalo milk are more than in the case of cow milk. As per Davis et al (1965) agglutination titres obtained with different bacteria, are not necessarily at all comparable.

It is seen that majority of the organisms (non - pathogenic and pathogenic), tested are sensitive to agglutinins with varying titres. The agglutination titres for all the sensitive organisms except four, is higher in cow than in buffalo samples.

8. Agglutinin activity in the milk from mastitic udders:

a) Nonpathogenic organisms and Leucocytes: The agglutination titre in mastitic milk towards the non-pathogenic organisms increased (Table 31), due to the increased permeability of the capillaries of the infected udder leading to an increased amount of immune globulins in milk, from the blood, (Lecce and Legates, 1959). This is very clearly seen from the protein elution pattern - plate 14 of the immune globulins from mastitic milk whey from cow and buffalo. In the elution pattern the second striped area of the first peak which is positive for the causative organisms alone seems to represent the specific antibodies produced by the udder tissues in response to the infection. The lower specific antibody activity in buffalo samples is an indication of its poor response to the infection or inefficient antibody production in the tissues against invading pathogens.

The results clearly show that whenever there is greater increase in the leucocytic count the agglutination titres decrease (Table 32), until it reaches a negligible level. In mastitic milk showing negative agglutination titre the removal of leucocytes restored most of the agglutinin activity (Table 33). This indicates that the leucocytes have a neutralising effect on

the inhibitory factors in the milk (Jones and Little 1934).

b) Causative organisms: The results show that the causative organisms are agglutinated by the milk and blood of other healthy animals (Table 34) indicating that they are sensitive to the natural agglutinins in them, although the titre is found to be low. The agglutination titre of the infected milk towards causative organisms is high, which indicates that it is a specific induced antibody. The increase in the agglutinin activity, in blood serum due to infection is very little and negligible while the agglutinin activity in the milk from infected animals is very high, when compared to the titre of the milk from healthy animals towards the causative organisms. This confirms the view of Kerr et al (1959) and Porterfield et al (1959) that udder is a site of antibody production. From the results it is seen that the immune response of the buffaloes towards infection is very poor resulting in smaller antibody activity in the milk.

c) Agglutinin inhibitors: The milk of infected animals should normally give a higher titre towards the causative organisms isolated. In some cases negative results were obtained (Table 35), towards causative organisms. The causative organisms isolated from the

mastitic milk showing inhibition of agglutination , are sensitive to agglutinins in milk, since they are agglutinated by the blood and milk of healthy animals (Table 36). Such mastitic milk showing inhibition of agglutination towards causative organisms, carry agglutinin activity is shown by the agglutination of three other agglutinin sensitive organisms (Table 37). Hence, the inhibition of agglutination towards causative organisms may be due to the presence of some inhibitory factor suppressing the agglutinin activity. This factor is nondialysable, heat stable and also specific to the particular organism (Table 38). It appears to be present in certain concentration in milk and blood and its effect of inhibition of agglutination can be removed by appropriate dilutions. These inhibitors seem to possess the properties of antibodies, or they may be a modified antibody e.g. modified by a proteinase excreted into the milk in the abnormal udders interfering with the agglutins. Although the nature of this factor is not known at present, it is worthwhile to further study in this regard to combat such factors to arrest the course of infection for ultimate increase of milk production.

From the foregoing it is seen that mastitic milk contains a higher agglutinin activity; leucocytes exert a neutralizing effect on the agglutinin activity; there

are certain substances which inhibit the agglutinin activity; and the immune response of the buffaloes towards infection is poor when compared to cows.

9. Transfer of immune globulin from colostrum to the new born calf:

The results indicate (Table 39), the occurrence of agglutinin activity in the precolostral calf serum, taken immediately after birth due to (i) stimulation by substances related to the organisms used for the assay or (ii) the passage of small amounts of maternal antibody to the foetal side or (iii) the exertion of antibacterial activity by the complement in the absence of specific antibody (Evans et al 1966).

The rapidity with which the agglutination titre in the calf serum increases, is an indication of higher permeability of the intestinal mucous membrane to absorb the colostral antibodies. The absorption of colostral antibodies disappears within 24 to 48 hours of birth (Comline et al, 1951). The level of maternal antibodies is reached in about 24 hours after birth in the calves of white cattle and the maximum titre at 48 hours after birth which is in accordance with Klaus et al (1969). The maximum antibody titre in the blood serum of buffalo calves appears at about 72 hours after birth and does not reach that of the maternal level even upto the age

of six months. Hence, it is evident that the buffalo calves seem to have a limited capacity for absorption of colostral antibodies. Due to the above reasons the buffalo calves appear to be more prone to several infections diseases in the early calfhood.

The results also show that even after the maternal level is reached in the blood serum of calves the titre continues to increase further in the case of white cattle. This is because, the level of IgG in the maternal colostrum is significantly higher than the level in maternal serum (Klause et al, 1969) and the further increase in the titre in the serum of calves even after the intestinal permeability is lost, is due to the transport of unchanged immune globulins of colostrum after absorption from the small intestine of young calves through the lymphatics to the systemic circulation (Comline et al, 1951). The gut of the new born calf has a high absorption capacity to utilize large volumes of colostrum (Kruse, 1970).

The antibody absorption from the colostrum in the case of buffalo calves is lower than that of the calves of white cattle. The maternal antibody level is reached in about 24 hours in the calves of white cattle whereas in the buffalo calves even upto the age of 6 months it is not reached.

10. Agglutinin resistant and sensitive strains on the fermentation of milk:

The clotting time for the sensitive strains of Streptococcus lactis 57, and Streptococcus lactis C_{10} are much prolonged as they are sensitive to lactoperoxidase and the agglutinins in milk (Table 40). A comparison of the two strains C_{10}, one sensitive to both agglutinins and lactoperoxidase and the other only to lactoperoxidase gives an idea as to the extent of inhibition of acid production by the agglutinins. In the presence of the pronounced effect of both the inhibitory substances a loose flaky curd is formed, due to very low levels of acid production. These observations indicate the importance of the strains of organisms chosen for the preparation of fermented milk products. All the cultures tested for curd formation are observed to take a slightly longer incubation time in buffalo milk than in cow milk. Probably this is due to the higher lactenin (both the inhibitory substance together) content of buffalo milk than that of cow milk as reported by Natarajan and Dudani (1961). The results (Table 41) indicate that the inclusion of a resistant strain in a mixed culture helps the susceptible strain to overcome the inhibition due to agglutination to some extent and produces beneficial effects. The observations are in accordance with those of Randolph and Gould (1966)

A level of atleast 30% of the resistant strain is required in the mixed culture to produce a good quality curd.

The sensitivity of the starter organism influence the quality of the fermented milk product.

C O N C L U S I O N

C O N C L U S I O N

The immune globulins of milk, present a wide range of antibodies capable of agglutinating multivarious species of organisms that might invade the udder irrespective of their pathogenicity. These agglutinating antibodies are present in the euglobulin fraction of the immune globulins of milk.

The fat globule agglutinins are identical with the whey protein agglutinins and it means that the euglobulin adsorbed on the surface of the fat globules are responsible for the agglutinin activity of the fat globule washings. Hence, the fat globules, for exerting inhibitory action have to depend on the euglobulin antibodies of the whey or milk. The role of fat globules in the inhibition of acid production by carrying majority of susceptible cells into the cream layer, is due to an altogether different type of antibodies in the euglobulin of milk or whey which favours the attachment of bacteria to fat globules and hence, the fat globules by themselves may not play a major role in the inhibitory action of milk.

The agglutinating antibodies in milk are shown to exist as three distinct classes of antibodies exhibiting different biological activities: (i) the agglutination of bacterial strains, (ii) the agglutination of fat

globules and (iii) the attachment of bacteria to fat globules.

The foregoing facts apply to the milk of the two species, cow and buffalo. The present study regarding these three classes of antibodies in the milk of both cow and buffalo yielded interesting results. These observations have helped to throw some light on the differential behaviour of the cow and buffalo milk towards the milk ring test.

It was found that the antibodies for bacterial agglutination and the antibodies for the attachment of bacteria to fat globules were higher in cow milk than in buffalo milk, while antibodies for the fat globule agglutination was about the same in the milk of both the species. The higher antibody titre for the attachment of bacteria to the fat globules appears to be responsible for giving a positive milk ring test at a shorter incubation time in cow milk, than in buffalo milk. Incidentally, these antibodies provide a clearer method for differentiation of the milk of these two species. A study of these antibodies in the milk of other species of animals might provide further information in this regard.

It has been shown that the agglutination titres

vary between breeds and species - Nondescript animals had high titres, pure bred have low titres while the crosses had either intermediate or higher titres than either of the pure bred animals. Hence, it seems probable that the agglutination titres may give an indication as to the extent of blood of a known or exotic breed in an individual animal, provided the titres in all the breeds of animals are known. Hence, further studies can be taken up to determine the level of agglutinins in the blood and milk of various other breeds.

The observation of this study, that the euglobulin antibodies from the skim milk or whey of cow or buffalo can be adsorbed on the membranes of the fat globules of either of the species seems to indicate that probably the membranes of milk fat globules of cow and buffalo belong to the same immmnological group. Further study is needed to determine the immunological relationship between the milk fat globule membranes of different species of animals.

These agglutinating antibodies in milk may play a significant role in the control of udder infections, Although these naturally occurring antibodies are present in much lower titres, they can control the infection to a certain extent, until the udder respond to the infection by an increased synthesis of antibodies.

Hence, it follows that the greater the antibody activity towards the pathogens, the greater will be the resistance to the infection.

In India, where all probable measures are being taken to augment milk production by introducing high proportion of exotic blood it is necessary to take cognizance of the fact that there would be deleterious effects on the capacity of such animals to disease resistance, since the present study reveal that the exotic bred have the lowest agglutinin content. Thus it appears, a more rational basis is required in introducing exotic blood in the efforts for augmenting milk production. The present studies present a complementary factors towards that. The crosses between Sindhi and Jersey prove to be better than either of the two pure bred stock, concerning the antibody activity of the milk. Therefore, it appears reasonable to favour such crosses.

The observation of the present study, that there is an initial increase in the agglutination titre in mastitis milk may be useful in detecting subclinical cases of mastitis and the increase in the specific antibody may be useful to identify the causative organism by employing several antigens. Further work is to be taken up in this aspect.

The present study affords explanation for the

observation that the maternal antibody level is reached in the calves of white cattle within 24 hours after suckling colostrum, whereas even after six months of age the maternal antibody level is not reached in the buffalo calves. This implies that the rate of absorption of colostral antibodies is very slow and inefficient in buffalo calves, while it is at a very rapid and higher rate in the calves of white cattle. The slow absorption of colostral antibodies, coupled with a considerably low antibody titres in blood serum observed in buffalo calves could have serious repurcussions on their resistance to disease resulting in early calf mortality. Early calf mortality in cows is rather negligible when compared to the mortality in buffalo calves, and it appears that the above reason may be one of the possible explanation for such a calamity in buffalo calves. In India, where buffaloes do play a major role in milk production, the problem of calf mortality is a great handicap and this problem remain unsolved due to the natural causes.

The present study serves to emphasize a more judicious selection of starter cultures in dairy industry. The higher antibody activity of cow milk towards a large number of lactic streptococci presents a problem to select the suitable type of cultures for the preparation of fermented milk products, cheese etc. Therefore starter cultures must be screened, for their susceptibility to

be agglutinated by the antibodies present in milk. However, buffalo milk appears to be less exacting in this respect. Anyway, in both cases care must be taken in selecting cultures. Resistant cultures will be more useful when single cultures are used, but when mixed cultures are used, where use of a sensitive culture is essential to bring about desirable changes in milk, a resistant culture also must be used to overcome the effect of agglutinins.

S U M M A R Y

SUMMARY

1. A total number of 1132 samples of colostrum, milk and blood from cows and buffaloes were analysed for the agglutinin activity. Of the 1132 samples, 680 were from cows, which comprised of 68 samples of colostrum, 371 samples of mid lactation milk, 53 samples of blood and 53 samples of milk, both collected throughout the stage of lactation from a day prior to parturition upto the end of lactation, 53 samples of calf blood from birth upto the age of six months and 82 samples of milk from infected udders. A total of 452 samples were from buffaloes which consisted of 53 samples of colostrum, 268 samples of mid lactation milk, 28 samples of blood and 28 samples of milk both collected throughout the stage of lactation from a day prior to parturition upto the end of lactation, 28 samples of calf blood from birth upto the age of six months and 47 samples of milk from infected udders.

2. The technique of gel filtration on Sephadex G - 200 can be used for isolating the pure fraction of the protein carrying agglutinin activity.

3. The agglutinin activity was carried by the euglobulin fraction of colostrum and milk of cow and buffalo.

4. The fat globule washings of cow and buffalo milk also exhibited agglutinin activity.

5. The euglobulin and fat globule washings of cow and buffalo milk agglutinated the fat globules of both cow and buffalo milk.

6. The fat globule membrane materials from the fat globules of the milk of cow and buffalo, did not carry and agglutinin activity.

7. Euglobulin antibodies adsorbed on the surface of the fat globules were responsible for the agglutinin activity of the fat globule washings of the milk of cow and buffalo.

8. The concentration of the immune globulins and the fraction of immune globulin carrying agglutinin activity was lower in buffalo than in the cow samples.

9. Amino acid composition of the agglutinin active fractions of the euglobulin, fat globule washings and the cryoglobulins was qualitatively the same with minor quantitative variations. The euglobulin and fat globule washings of buffalo samples showed one additional amino acid - tyrosine.

10. The whey protein agglutinins and the fat globule agglutinins appeared to be identical, in both cow and buffalo samples.

11. The agglutinins of cow milk samples showed maximum activity at pH range of 6.1 to 6.5 and buffalo milk samples at pH range of 6.3 to 6.6.

12. The agglutinins of cow and buffalo milk, were inactivated at the temperature, time combinations of 70°C for 12 minutes, 72°C for 10 minutes and 74°C for 4 minutes.

13. The euglobulin solution of cow and buffalo samples aggregated to form cryoglobulins at 2°C for 21 and 25 hours respectively.

14. The euglobulin solution of cow and buffalo samples were able to attach the bacterial cells to the fat globules.

15. The euglobulin antibodies in cow and buffalo milk comprised of: a) the antibodies for the bacterial agglutination, b) the antibodies for the attachment of bacteria to the fat globules and c) the antibodies for the fat globule agglutination.

16. The cryoglobulin, a fraction of the euglobulin was responsible for the fat globule agglutination and for attaching the bacteria to the fat globules in cow and buffalo samples.

17. The agglutinin activity for bacterial aggluti-

nin activity for bacterial agglutination was higher in cow samples than in buffalo.

18. The agglutination titre of euglobulin and fat globule washings towards fat globule agglutination was the same in cow and buffalo samples.

19. The antibody titre of milk and euglobulin solution towards the attachment of bacteria to the fat globules was higher in cow samples than in buffalo.

20. The whey protein agglutinin activity was influenced by the species, breed and stage of lactation.

21. Only species variation influenced the fat globule agglutinin activity.

22. The euglobulin bacteria complex showed a high degree of specificity, while the euglobulin fat globule complex and the antibody attaching bacteria to the fat globules were non specific.

23. Majority of the organisms tested (pathogenic, nonpathogenic, desirable and undesirable), were sensitive to the agglutinins of milk and blood serum of cow and buffalo.

24. The agglutinin activity in the mastitis milk of cow and buffalo increased in the early stages of

the disease, but decreased with the progress of the disease.

25. The increase in the leucocyte concentration in the infected milk, decreased the agglutination titre in milk.

26. The infected milk showed a much higher titre towards the causative organisms for mastitis, while the titre of normal milk towards these organisms was very low. This increase in the titre shown by the infected milk of cows, was much higher than that of buffaloes.

27. The agglutination titre in the blood serum of the infected cows and buffaloes did not show significant increase as that obtained in the infected milk of these animals.

28. The milk and blood serum of 6.1 percent of the infected cows and 2.1 percent of the infected buffaloes did not agglutinate the causative organisms, but showed agglutinin activity towards other nonpathogenic organisms.

29. The factor responsible for the inhibition of agglutination was nondialysable, heat stable and specific to the particular organism.

30. The agglutinin activity in the blood serum of new born calves exhibited a rapid increase following the ingestion of colostrum. This rate of increase in the blood serum of the calves of white cattle was very rapid reaching the dam's level at 24 hours after birth, while in buffalo calves the rate of increase was very slow, and the dam's level was never reached even upto the age of six months.

31. Agglutinin resistant strains clotted the milk at a much shorter time, besides producing curd of good consistency, whereas, sensitive strains took a long incubation time, but produced loose flaky curd with whey separation.

32. Addition of resistant strains to a level of 30 or 40 percent, to a culture of desirable sensitive strain produced curd of good quality.

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