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Title: LACTIC STREPTOCOCCAL AGGLUTININS: A RAPID TESTING PROCEDURE DETECTING PRESENCE OF THE AGGLUTININS AND CORRELATION STUDIES BETWEEN IMMUNOFLUORESCENT ASSAYS, ROSE BENGAL PLATE TEST AND AGGLUTINATION BEHAVIOUR OF STARTER BACTERIA

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William E. Sandine

Lactic streptococcal agglutinins are known to promote settling of starter bacteria in milk, resulting in spongy, mealy, shattered curd and sludge formation defects in cottage cheese. These agglutinins are natural antibodies secreted by lactating cows in mature milk in response to infections (mastitis) and in colostrum milk as protective antibodies for newborn calves. The starter bacteria act as particulate antigens and form lattice-like complexes with the agglutinins and sediment to the bottom of milk vats, depriving upper milk layers from lactic acid production. A simple rapid screening test (Rose-bengal-stained antigen plate test) was developed to detect the presence of these natural antibodies in milk. Trismaleate buffer at pH 7.0 with a high molarity (0.5 M) was selected as the most appropriate antigen-suspending solution. Two percent packed streptococcal cell volumes (PCV) were found to yield best results with all tested lactic streptococcal stained antigens. Each of 3%, 4% and 5% stain concentrations gave results of easy color

readability. No totally agglutinin-resistant lactic streptococcal strains were found in testing colostrum whey samples. However, less agglutinin-susceptible strains such as S. lactis C_2 were detected by the test. The specificity of the agglutinins was studied by immunofluorescent assays, revealing a high specificity of starter bacteria for the IgG immunoglobulins. Moreover less specificity was seen with IgM immunoglobulins. The agglutination behaviour of starter bacteria in milk was followed through a pH measurement profile at three different vertical locations. Agitation of milk with high agglutination titers for 75 minutes did not alter settling of starter bacteria. Nevertheless, the pattern of settling was disturbed with lower agglutinin titers. Autoclaving the milk before mixing with unheated agglutinin-containing whey samples, dramatically reduced the agglutination reaction. The Rose bengal-stained plate antigen test results proved to be comparable with the immunofluorescent studies and the studied starter agglutination reactions. Some evidence for seasonal occurrence of cheese starter agglutation was acquired from the results of the different studies. Studies also were made to explore the possible usefulness of the milk ring test for detecting the presence of lactic streptococcal agglutinins in milk.

Lactic Streptococcal Agglutinins

by

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Typed by Carlene Pelroy for Mohammed Ali Salih

To Assya whose love, care, patience and understanding has been of great importance to me. To our loved son "Hanni" we dedicate this too.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF PHOTOGRAPHS

LIST OF PHOTOGRAPHS (continued)

Chapter 1

LACTIC STREPTOCOCCAL AGGLUTININS: A REVIEW[1]

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ABSTRACT

Agglutinins that cause clumping and settling of lactic streptococci are reviewed. Evidence defining the role played by agglutinins in sedimentation defects occurring during cottage cheese manufacture is presented. The needs for a fast, simple and reliable agglutination test, and additional research on the influence of agglutinins on other fermented milk products, are noted.

INTRODUCTION

Generally, agglutination is the reaction of a soluble antigen with its homologous antibody to cause aggregation and clumping of cells (41). These aggregated clumps pose a problem in lactic streptococcal cheese starters either by promoting an uneven distribution of the bacteria in milk or by complete removal of settling of bacteria. This causes uneven production of acid and may adversely affect curd formation (9,11,36). In cottage cheese manufacture, spongy curd formation on the bottom of the vat may occur or floating flecks with a light cream of tan to almost brown color (9,11,24,26,32) may be evident. The possibility that milk agglutinins may be an important cause for slow acid production by starters during milk and cream fermentations has also been suggested (31). Cottage cheese is the only dairy product which has been studied in relation to starter cell agglutination, but it likely is not the only product affected adversely by the phenomenon (18,28).

EARLY STUDIES

The ability of cow's milk to inhibit bacterial species has been known since early reports in 1894 and 1904 (15.37), as well as from more recent studies (30.38). One of the first observations of anti-streptococcal activity was noted in 1927 by Jones and his collaborators (20) when certain strains inoculated into raw milk died while other streptococcal strains grew well. They assumed that the milk contained a heat-labile antistreptococcal substance and named that substance lactenin (21). It was characterized as a whey fraction, non-dialyzable, digested by trypsin, inactivated by exposure to 80 C or higher, and surviving pasterurization (40). Attempts by Gough et al. (14) and Wilson and Rosenblum (40) to purify and identify lactenin were not successful. Jones and Little (20) suggested that lactinin was enzymatic in nature, having a protective function for the udder.

Czulak and Meanwell (7), investigating "winter-slowness" in high temperature short time pasteurized milk in England, postulated that a growth factor and an inhibitory substance were involved in the problem. Auclair and co-workers (1), working with strains of Streptococcus pyogenes, Streptococcus agalactiae, Staphylococcus aureus and Streptococcus lactis, suggested that these organisms were inhibited by two heat-labile substances present in milk which they called lactenin 2. They also confirmed earlier findings that milk contained both inhibitory substances and growth factors affecting bacterial growth (16).

Jago (18) studied inhibition of starter bacteria by milk in Australia, and found that the effect was associated with the fat

globules which could exert a marked effect, owing to the large surface area exposed. He also suggested that the inhibitory substance, present in appreciable amounts in skim milk, was enzymatic in nature. Wright and Tramer (42) suggested that lactenin 1 was probably the agglutinin of the fat globules but its nature was not further studied. The strain specificity of agglutinins was established by McPhilips (27) who concluded that they are antibodies naturally occurring in milk. Wright and Tramer (43) associated lactenin 2 with lactoperoxidase because of their similar sensitivity to heat and reversion of inhibition by reducing agents. Keogh (2.3) confirmed that milk contains both inhibitory and stimulatory substances, some of which are destroyed or produced by heating, and that cultures differed widely in their relative sensitivity to these factors, which vary from day to day. She emphasized that with certain cultures, creaming, but not agglutination per se, played a great role in the inhibitory nature of raw whole milk, as determined by activity tests. She extracted by acetone a preparation from colostrum that contained agglutinin and peroxidase. These extracts caused inhibition of the same cultures which were inhibited by raw milk. Gillies (13) found that whey was an unsatisfactory growth medium for all sensitive and resistant starter strains tested. He observed that addition of nitrogen stimulated starter strains despite the inhibitory tendencies in milk, while oxygen tended to accentuate the inhibition.

During the 1950s, intensive studies established the existence of both agglutination and lactoperoxidase inhibitors for bacteria in milk. The most definitive contributions regarding the streptococcal agglutina-

tion problem, however were made from 1962. Portman (28) studied the influence of lactoperoxidase and agglutinins of milk and colostrum on the acid production of several strains of Streptococcus thermophilus and lactobacilli. Two strains of S. thermophilus that were resistant to lactoperoxidase were agglutinated by raw whey and colostrum proteins without any reduction in their acid production. The various strains of lactobacilli studied were generally inhibited both by lactoperoxidase and by agglutinins.

In the Netherlands, Stadhouders and Veringa (36) published observations on the inhibitory action of milk peroxidase on lactic acid streptococci. On addition of cysteine and hydrogen peroxide to the milk, the enzyme peroxidase was rendered inactive and streptococcal inhibition was prevented. They concluded that differences in the sensitivity of lactic streptococci to milk peroxidase corresponded to differences in their capacity for inactivating peroxidase.

Stadhouders (34) further investigated the inhibitory effect of milk agglutinins which he named lactenin 3. In whole and skim milk, agglutinins were present, the greater concentration being associated with the cream layer. In skim milk, bacteria deposited on the bottom of the container only when a sufficient number were present to effect agglutination. On addition of rennet, agglutinins failed to inhibit acid production. His findings supported previous results (13,42) that agitation and homogenization inactivate agglutinins. Stadhouders also concluded that agglutinins are of no importance for the activity of cheese starters.

In the same year (1963), Emmons extended Stadhouders observations. He noticed granular sludge formation on the bottom of cottage cheese vats when certain starter bacteria were used. These sludges were similar to those noted by Lucas (26), spongy forms having a low pH while milk in the rest of the vat showed a high pH and low acid development. The sedimentations showed a high bacterial concentration, resulting in casein precipitation due to high acid production in the area. During cooking the sludge broke readily into grits or fines, reducing quality and yield (24). These studies were carried out with 14 starter strains which also agglutinated in renneted whey obtained from pasteurized skim milk used in cottage cheese manufacture.

Emmons et al. (11) contributed further on this subject with a study on the effect of lactic streptococcal agglutinins in milk on curd formation during manufacture of cottage cheese. Contrary to the findings of Stadhouders (34), Emmons observed that prolonged agitation of inoculated skim milk promoted starter strain agglutination and retarded acid development. He confirmed that agglutinating antibodies in skim milk were not inhibitory per se (13,23,34), but noted that settling of bacteria was the actual cause, thus nullifying pH as a direct indicator of the time to cut the curd. Removal of the agglutinins and elimination of the defect was accomplished by adsorption of antibodies on heat-killed cells or by heating skim milk at 70 C for 30 min. Variability between agglutinin-sensitive strains also has been noted and even some strains, i.e., S. lactis C10, have given rise to agglutination variants (2,11).

A study by Emmons (12) on agglutination titers in blood and milk (B:M) from four lactating cows revealed considerable variability in B:M ratios between samples and between strains within samples. Mastitic conditions had a marked bearing on the B:M ratios. Probably possession of similar somatic antigens by bacteria other than group N-streptococci, as shown by Briggs (4) and Hucker (17), caused the variability.

RECENT STUDIES

The chief reason for the sedimentation defects encountered in cottage cheese manufacture was proven to be the antigen-antibody agglutination reactions (11,22). It is not attributed to overheating and insolubility of nonfat dry milk (26) nor to Ca^{++} ion accumulation, as suggested by Sandine et al. (32). Formation of long bacterial chains (11) also does not seem to be a cause because the agglutination problem is seasonal (7,23,27,34) and no defects were measured in milk during a rainy season, for example.

Emmons et al. (10) and Ellerman (8) designed tests to detect streptococci which are sensitive to agglutinins in skim milk. Ellerman used formalized-killed cells as an antigen, whereas the method of Emmons depends on whole cell suspensions as an antigen source. The latter test is overwhelmed by many difficulties, including antigen stability and autoagglutinations. In addition to its complexity, the test is costly, time consuming and inaccurate, since there is no absolute relationship between the measured agglutination titers and the degree of the bacterial setting or the experienced sedimentation

defects (10,11). Ellerman overcame some of these shortcomings but inconsistent results and the 18-20 h needed to clear the results hinder the value of the test.

Three classes of immunoglobulins have been identified (5,6) in bovine milk: IgG, IgA and IgM. IgG, which comprises 85-90% of the whey proteins (25), is further subdivided into two classes: IgG_1 and IgG_2. IgG_1 is the principal immunoglobulin transported by the blood circulation for passive immunization of the calf. IgG_2 is more homogenous and appears in high concentration in bovine serum. Bovine IgM occurs in serum, colostrum and milk. It is a distinct macroglobulin, comprising less than 10% of serum and colostral immunoglobulins. It is easily reduced by 2-mercaptoethanol, which eliminates its antibody activity. It appears that IgM is important in the primary immune response, complement fixation and as an agglutinating antibody (14,19). Several unsuccessful trials have been made to separate a purified agglutination factor for lactic streptococci identifiable with an immunoglobulin class (21,43,41).

The observations made by Stadhouders and Hup (35) that agglutinins are associated with the euglobulin fraction (33) of milk alerted scientists to consider the euglobulin fraction as a source of the inhibitory factor. Randolph and Gould (29) and Balakrishnan and Vedanayakam (3) confirmed these findings.

In 1976, Kanno et al. (22) successfully isolated a purified agglutinating factor identical to the bovine IgM from milk. The factor showed a high agglutination potency towards lactic streptococci. The crude euglobulin was fractionated by gel filtration on Bio-gel A-50 m,

followed by three filtrations on A-15 m. The agglutination activity for S. cremoris strain HP increased greatly during the purification. Activity was lost after heating at 70 C for 10 min or after reduction with 2-mercaptoethanol. Physical-chemical studies showed that the purified agglutination factor was the same as IgM from bovine serum and colostrum. Therefore it was concluded that IgM immunoglobulin is the component in normal milk causing agglutination of lactic streptococci.

Further research is needed to explain the seasonal variations of IgM in milk as well as the variability in responses of lactic streptococci towards it. Additional research also is required to develop a reliable test to quickly assess the sensitivity of lactic streptococcal starter strains to agglutinins present in milk intended for fermentation.

REFERENCES

1. Auclair, J. E., and A. Hirsch. 1953. The inhibition of microorganisms by raw milk. The occurrence of inhibitory and stimulatory phenomenae. J. Dairy Res. 20:45.
2. Auclair, J. E., and Y. Vassal. 1963. Occurrence of variants sensitive to agglutinins and to lactoperoxidase in a lactenin resistant strain of S. lactis. Dairy Res. 30:345.
3. Balakrishnan, R., and A. R. Vedanayakam. 1973. The agglutinins of cow and buffalo milk - The nature and properties of the agglutinins. Indian Vet. J. 50:111-124.
4. Briggs, C. A. E., and L. G. M. Newland. 1953. The serological classification of S. cremoris. J. Dairy Res. 19:160.
5. Butler, J. E. 1969. Bovine immunoglobulins: A review. J. Dairy Sci. 52:1895.
6. Butler, J. E., and C. F. Mascwell. 1972. Preparation of bovine immunoglobulins and free secretory components and their specific antisera. J. Dairy Sci. 55:151.
7. Czulak, J., and L. J. Meanwell. 1951. Seasonal variation in cheese starter activity. Proc. Soc. Appl. Bacteriol. 14:1.
8. Ellerman, J. A. G. 1975. Testing the lactic streptococci for agglutinin titers in cows milk. Austral. J. Dairy Technol. 30:3, 111-113.
9. Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1963. Agglutination of starter bacteria, sludge formation and slow acid development in cottage cheese manufacture. J. Dairy Sci. 46:600.

10. Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1965. Sensitive test for lactic streptococcal agglutinins. J. Dairy Sci. 48:1245.

11. Emmons, D. B., J. A. ELliott, and D. C. Beckett. 1966. Effect of lactic streptococcal agglutinins in milk on curd formation and manufacture of cottage cheese. J. Dairy Sci. 49:1357.

12. Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1966. Lactic streptococcal agglutinins in milk and blood. Inter. Dairy Congr. XVII:D2:499.

13. Gillies, A. J. 1959. Inhibitory factors in cheese milk. XVth Inter. Dairy Congr. 2:523.

14. Gough, P. M., R. Jenness, and R. K. Anderson. 1966. Characterization of immunoglobulins. J. Dairy Sci. 49:713 (Abst.).

15. Hesse, W. 1894. Z. Hyg. Infektkr. 17:238.

16. Hobbs, B. C. 1939. The part played by the bacteria in the reduction of methylene blue in milk. J. Dairy Res. 10:35.

17. Hucker, G. J. 1932. Agglutination as a means of differentiating the species of Streptococcus and Leuconostoc. New York Agr. Expt. Sta. Tech. Bull. 190.

18. Jago, G. R. 1954. Factors influencing the lactic acid producing properties of streptococci used in the manufacture of Cheddar cheese. J. Dairy Res. 21:111.

19. Jenness, R., R. K. Anderson, and P. M. Cough. 1965. Fractionation of bovine milk and blood agglutinins for Brucella by gel-filtration and specific absorption techniques. Fred. Proc. 24:503.

20. Jones, F. S., and R. B. Little. 1927. The bacteriocidal property of cows' milk. J. Exptl. Med. 45:319.

21. Jones, F. S., and H. S. Simms. 1930. Bacterial growth inhibitory (lactenin) of milk. J. Exptl. Med. 51:327.

22. Kanno, C., D. B. Emmons, V. R. Harwalker, and J. A. Elliott. 1976. Purification and characterization of the agglutinating factor for lactic streptococci from bovine milk: IgM immunoglobulin. J. Dairy Sci. 59:2036-2045.

23. Keogh, B. P. 1958. Variations in lactic acid production in milk. Austral. J. Dairy Technol. 13:132.

24. Klaus, G. G. B., A. Bennett, and E. W. Jones. 1969. A quantitative study of the transfer of colostral immunoglobulins to the newborn calf. Immunology 16:293.

25. Kosikowski, F. V. 1963. Some distribution patterns of cottage cheese particles and conditions to contributing to curd shattering. J. Dairy Sci. 46:391.

26. Lucas, P. S. 1962. What causes spongy formation in cottage cheese? Am. Milk Rev. 24(10):74 .

27. McPhilips, J. 1958. Specificity of agglutinins in milk. Nature 182:869.

28. Portman, A., Y. Gate, and J. Auclair. 1962. Influence of lacto-peroxidase and agglutinins of milk on the activity of thermophilic starter bacteria. XVI Int. Dairy Congr. (B) 729.

29. Randolph, H. E., and I. A. Gould. 1968. Characterization of the natural inhibitors in skim milk affecting lactic acid bacteria. J. Dairy Sci. 51:8.

30. Reiter, B., and A. M. Madsen. 1963. Reviews of the progress of dairy science. Section B. Cheese and butter starters. J. Dairy Res. 30:419.

31. Sandine, W. E. 1977. New techniques in handling lactic cultures to enhance their performance. J. Dairy Sci. 60:822-828.

32. Sandine, W. E., A. W. Anderson, P. R. Elliker, and R. W. Stein. 1963. Observations on citrate utilization by mixed-strain starter cultures and protein stability of cheese milk. J. Dairy Sci. 46:610.

33. Smith, E. L. 1946. Isolation and properties of immune lactoglobulins from bovine whey. J. Biol. Chem. 165:665.

34. Stadhouders, J. 1963. Inhibitory effect of lactenin L3 on acid production in milk by S. cremoris 803. Neth. Milk Dairy J. 17:96.

35. Stadhouders, J. and G. Hup. 1970. Complexity and specificity of euglobulins in relation to inhibition or bacteria and to cream rising. Neth. Milk Dairy J. 24:79.

36. Stadhouders, J. and H. A. Veringa. 1962. Some experiments related to the inhibitory action of milk peroxidase on lactic acid streptococci. Neth. Milk Dairy J. 16:96.

37. Stocking, W. A., Jr. 1904. The so-called germicidal property of milk. 16th annual report of Storrs Conn. Agr. Expt. Sta. 16:89.

38. Vedamuthu, E. R., G. W. Reinbold, and E. G. Hammond. 1967. Inhibitory activity of acid and rennet whey on propionibacteria. J. Dairy Sci. 51:4, 503-510.

39. White, C. H., and B. W. Ray. 1977. Influence of heat treatment and methods of acidification on manufacture and properties of cottage cheese. J. Dairy Sci. 60:1236.

40. Wilson, A. T., and H. Rosenblum. 1952. The antistreptococcal property of milk. J. Expt. Med. 95:25.

41. Wilson, C. S., and A. A. Miles. 1975. Topley and Wilson's principles of bacteriology and immunity, 6th ed., Vol. 1, Williams & Wilkins Co., Baltimore.

42. Wright, R. C., and J. Trammer. 1957. The influence of cream rising upon the activity of bacteria in heat-treated milk. J. Dairy Res. 24:124.

43. Wright, R. C., and J. Trammer. 1959. Factors influencing the activity of cheese starters. The role of milk peroxidase. J. Dairy Res. 25:104.

Chapter 2

LACTIC STREPTOTOCCAL AGGLUTININS:

I. DEVELOPMENT OF A RAPID SCREENING TEST[a]

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[a]Technical Paper No. _____ Oregon Agricultural Experiment Station.

ABSTRACT

A Rose bengal-stained antigen plate testing procedure was developed for detecting lactic streptococcal agglutinins in milk. Colostrum samples were used as sources for both IgG and IgM immunoglobulins. Strong positive results were obtained from 2% packed cell volumes Rose bengal-stained cells suspended in 0.5 molar tris maleate buffer (pH 7.0). Lactic streptococcal antigens were susceptible to IgG and IgM immunoglobulins. Streptotoccus lactis strain C_2 was not an agglutination resistant organism but, among 15 strains tested, was the least susceptible under low levels of agglutinins.

INTRODUCTION

Lactic streptococcal agglutinins are known to cause settling of starter bacteria or uneven distribution of lactic acid production in milk as has been noted during cottage cheese manufacture (9,11,12, 16,21,24). Development of a rapid testing procedure to aid in detecting the presence of these agglutinins in milk before addition of starter bacteria would save the cheese industry from these defects as well as the loss of vats of milk due to the problem. Furthermore, starter bacteria which are agglutinin resistant could be selected for use in cheese manufacture. Agglutination testing procedures should consider (a) simplicity and reliability, (b) sensitivity, (c) antigen shelf life and (d) end points or titers that reflect the agglutination results in the cheese milk. Existing testing procedures for lactic streptococcal agglutinins (8,10) are inadequate from these considerations, though agglutination test procedures are finding increased use in diagnostic microbiology. For example Wampole Laboratories announced (ASM News 47(7):3, 1981) a rapid slide agglutination test for Hemophilus influenzae type B antigen which causes bacterial meningitis. Also, Scott Laboratories has just released information on a new latex slide agglutination test for the rapid identification of Staphylococcus aureus.

Utilization of Rose bengal-stained antigens for rapid screening of Brucella agglutinins has been practiced for some time (1,2,7,14,17, 18,20). Utilizing this rapid testing procedure, an antigen was developed from lactic streptococci for screening the presence of lactic streptococcal agglutinins. The technique provides an antigen shelf-life that

exceeds 3 years at 4 C (19). In addition end points of the test can be correlated to the agglutination reaction of cheese milk.

MATERIALS AND METHODS

Antigen Preparation

Fifteen lactic streptoccccal strains (5 fast and 10 slow) were grown on Fast-Slow Differential Agar (15) incubated anaerobically at 30 C for 48 h. One loopful from each strain was inoculated into 50 ml of M17 broth (25) and incubated at 30 C (slow) or at 22 C (fast) for 18 h. Fifteen flasks (500-ml capacity) containing 250 ml of M17 broth were inoculated with the 50-ml cultures (16% inoculum) and incubated as before. Cultures in flasks were transferred aseptically to 250 ml centrifuge bottles and culture pellets harvested by centrifugation at 10,000 rpm for 10 min. The collected pellets were washed twice with phosphate buffer solution (0.1 M, pH 7.0). Then cultures were heat inactivated at 60 C for one h in a water bath. Cultures were centrifuged again, suspended in phosphate buffer and filtered through loosely-packed glass wool-loaded funnels into preweighed 30 ml centrifuge tubes. Centrifugation was carried out once again to determine the cell wet weight in g. Phosphate buffer was added to cell pellets to make a final concentration of 0.125 g/ml to prepare the cells for staining.

Stock solution of freshly prepared Rose bengal stain (5% aqueous concentration) was filtered through Whatman No. 1 filter paper. The methods described by George et al. (14,15) were followed for staining the plate test antigen. The stained cells were transferred to 12-ml

graduated conical glass centrifuge tubes and the volumes of the pellets adjusted to a final concentration of 6% packed cell volumes (PCV) with phosphate buffer (0.1 M, pH 7.0).

Standardization of the Antigen

Three test parameters were considered for the antigen standardization: type of buffer, cell concentration and actual test procedure.

Three different kinds of buffers were chosen and prepared as follows:

(a) Tris-maleate buffer (pH 7.0), prepared in concentrations ranging from 0.1 to 1.0 molar.

(b) Phenol-saline buffer (pH 7.0) prepared from 0.85 NaCl and 0.5% phenol adjusted by citric acid solution and dibasic sodium phosphate to pH 7.0 (2).

(c) Sodium glycerophosphate buffer prepared in two concentrations, 1.9% and 3.8%, and then adjusted to pH 7.0 with citric acid solution.

From the previously-stained stock cultures (6% suspended in 0.1 M phosphate buffer), 13 ml were taken from the fast strains and one ml aliquots deposited in each of 13 small centrifuge tubes. Centrifugation was carried out for washing and suspension in 1 ml of each buffer type at the concentration mentioned above. Whey sample preparations were made from colostrum, mastitic and mature bovine milk. Colostrum milk (first and second day colostrum), mastitic milk (with >500,000/ml leucocytic counts) and normal milk samples were collected from the Oregon State University Dairy Center. Whey samples were separated on the same day by the Emmons method (10) and kept at -20 C for later use.

Six strains (3 fast and 3 slow) were chosen for determination of proper cell concentration. The method used was as shown in Tables 1 and 2. Strain concentrations less than 2% were found inappropriate due to autoagglutination. At 3, 4 and 5% strain concentrations, no difference in easing the readibility of the test results was noted. The 2% cell concentration was selected as the best for the standardization of the antigen.

Testing Procedure

Brewer's diagnostic cards (Brucellosis card test; Hynson, Westcott and Dunning, Inc. Baltimore, Maryland 31301) with 10 wells were used. Micro-dose pippetes with plastic tips were employed to drop 25 µl at a time. One drop of antigen was placed at one end of the well and one drop of whey placed at the other end. With rounded toothpicks (sterilized) the two drops were mixed together. The test was read after rocking for 4 min. (Figures 1 and 2).

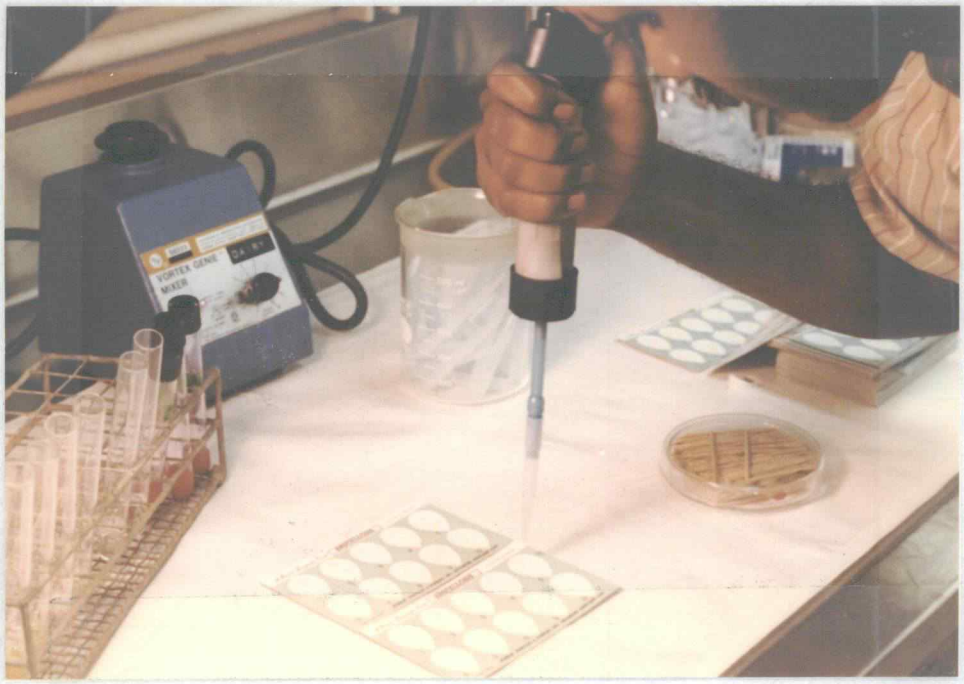
Figure 1. Methodology of Antigen Plate Testing.

A. Deposition of 25 µl of undiluted and 1/2 serially-diluted whey samples on one end of the wells using the Micro-doser and a Brewer Diagnostic Card.

B. Dropping 25 µl of stained lactic streptococcal antigens at the other end of the well.

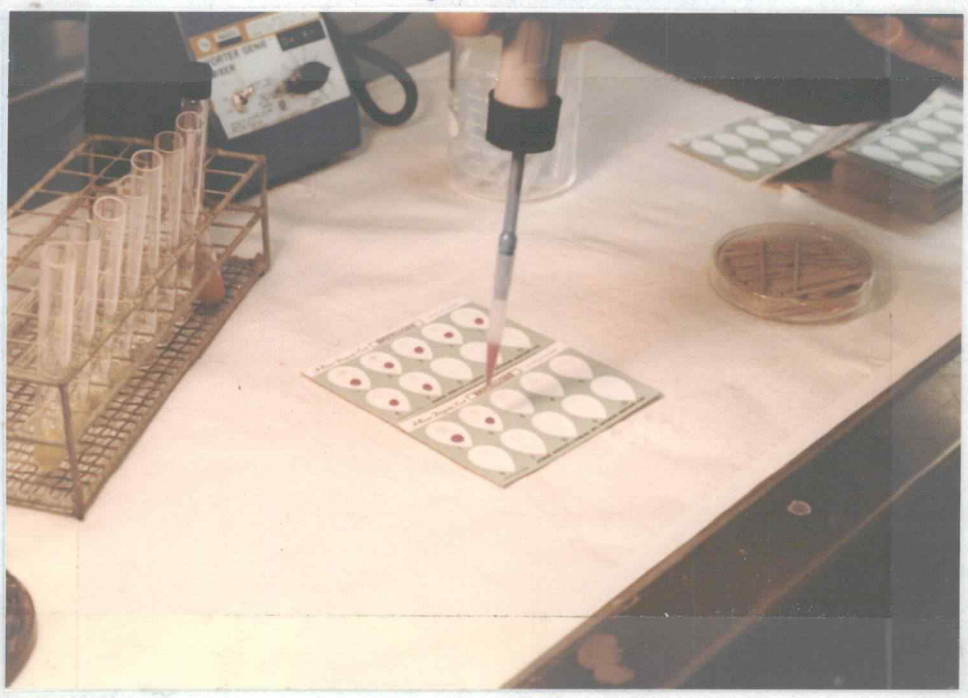
C. Using sterile rounded toothpicks; the two drops were thoroughly mixed together.

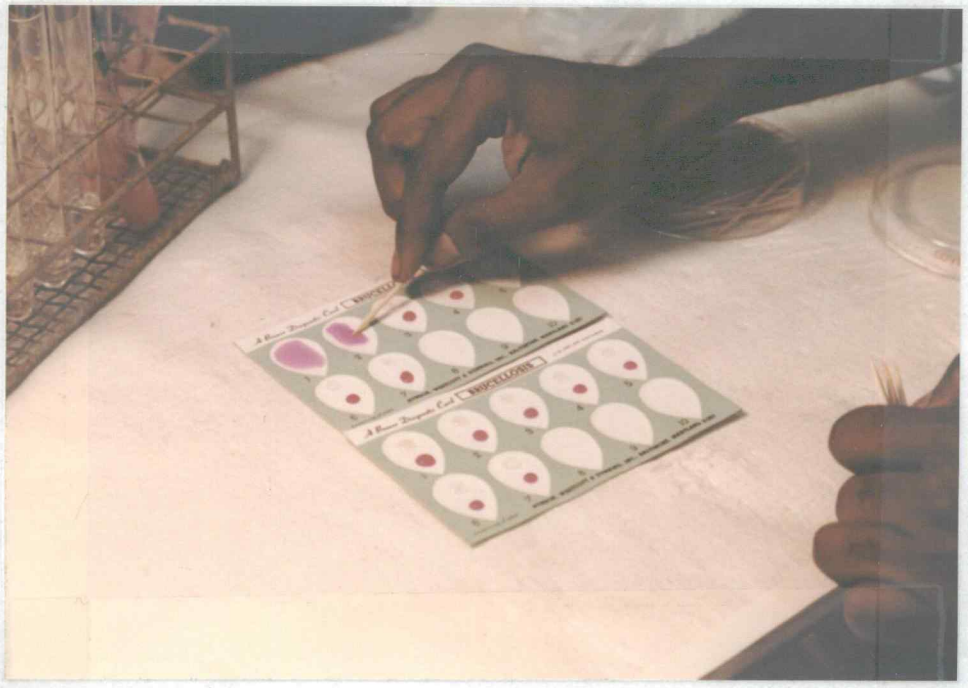
D. Hand rocking of the plate for 4 minutes to allow for maximum cellular clumping and agglutination.



Photograph 1-A

Photograph 1-B





Photograph 1-C

Photograph 1-D

RESULTS AND DISCUSSION

Positive samples (see Figure 2) showed clear large aggregates of clumping cells, while the negative samples showed no clumping of cells.

Fifteen different strains of lactic streptococci were tested against colostrum whey samples; eight of these strains (5 fast and 3 slow) were titrated to their end points as shown in Table 3. The agglutination response of the slow strains was not much different from the fast ones, except that sometimes slow strains showed higher end points.

The testing procedure does not measure titers *per se*, only end points that could be determined as relative intensities of agglutination. Difficulties in differential of color intensities was the main reason behind difficulties in actually measuring titers. Only for approximate purposes were relative intensities in Tables 1, 2 and 3 made. Strong agglutination was given a 4, and weak agglutinating cells given 1; non-agglutinating samples were given 0 (Figure 2).

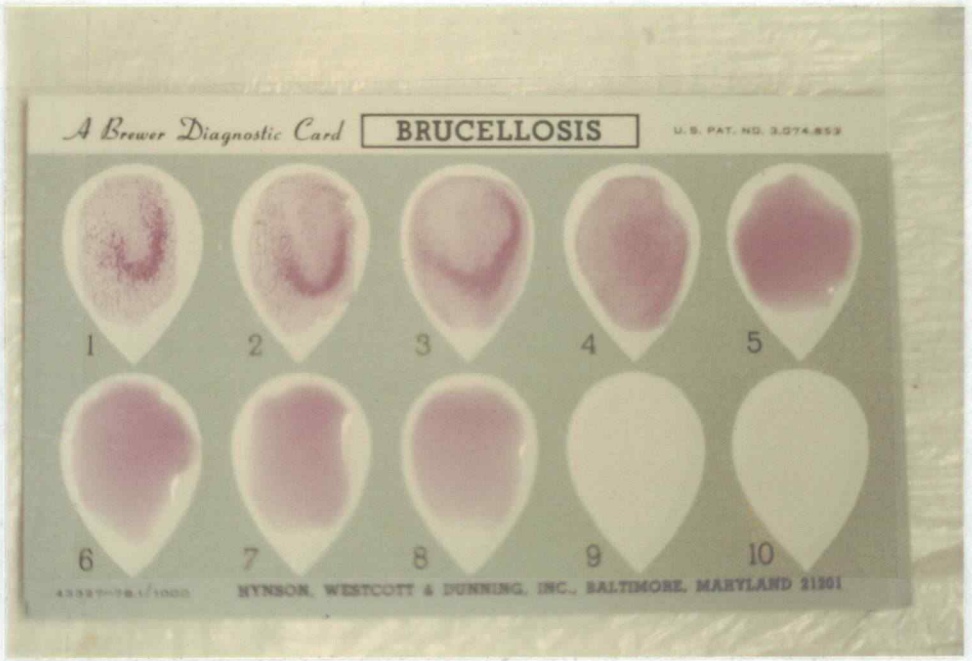
Several mastitic whey samples (with high leucocytic counts) were tested, yielding only very few positive cases even in the undiluted form. All the normal milk samples that were taken from individual cows were negative.

Once lactic streptococcal Rose bengal-stained antigens had been suspended in a high molarity buffer with a neutral pH, autoagglutination was eliminated and non-specific agglutinins were promoted to agglutinate, as in George et al. (14). According to the shelf-life studies carried out on Rose bengal-stained brucella antigens suspended in 0.4 molar tris maleate buffer (pH 7.0), keeping qualities for the antigens of

Figure 2. Results of the agglutination reactions of 2 different lactic lactic streptococcal strains.

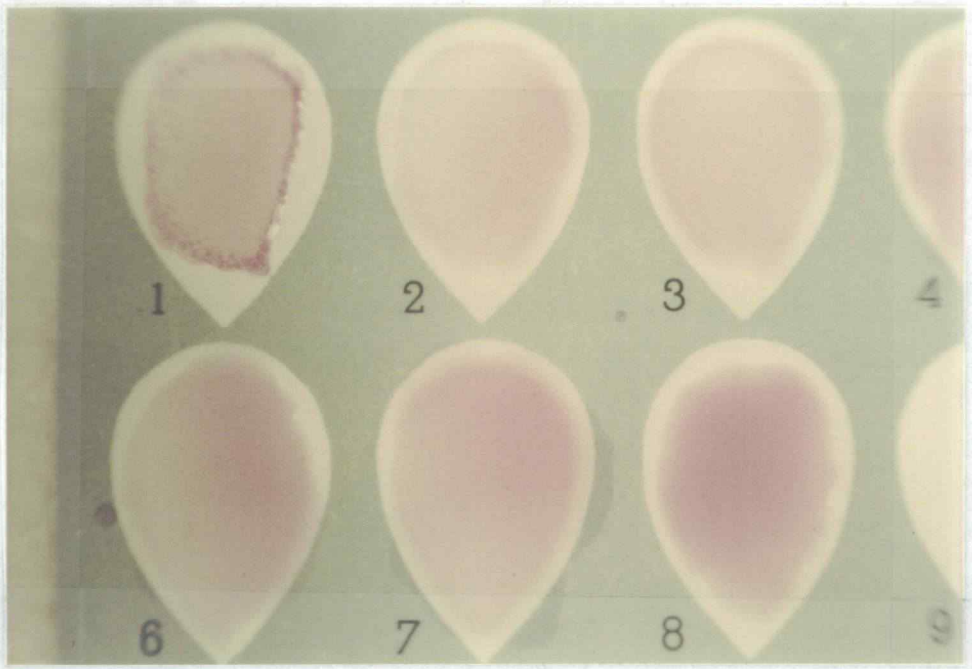
A. Streptococcus cremoris strain HP (agglutinin sensitive): Eminent agglutination at well 1 given +4 and weak agglutination at well 4 given +1. Well 8 is a control (tris-maleate buffer and antigen) does not show any cellular clumping given 0 as a negative.

B. Streptococcus lactis strain C_2 (agglutinin resistant): It shows agglutination at wells 1 and 2 given 3 and 1 as positives. Well 8 is a control (tris-maleate buffer and antigen) given 0.



Photograph 2-A

Photograph 2-B



at least 36 months at 4°C was noted (19). S. lactis strain C_2 (both slow and fast) was not totally resistant in contrast to earlier reports (10,23). Nevertheless, according to our test results also reported in a subsequent paper (22), they are among the least agglutinin-susceptible strains.

At least two possible sources exist for the seasonal increased levels of the immunoglobulins in milk, which contribute to milk sedimentation defects. These are contamination of normal milk with colostrum or mastitic milk with their high levels of immunoglobulins. According to our immunofluorescent studies (22), and according to George et al. (14), the Rose bengal stained antigen plate test is capable of detecting the presence of both IgG and IgM immunoglobulins. Colostrum contains about 80 times more immunoglobulins (IgG and IgM) than that found in normal milk (3,4,5,23). Evidently colostrum can be the most possible source of the contamination since mastitic milk (with high leucocytic counts) contains appreciable amounts of the immunoglobulin IgG subclasses IgG_1 and IgG_2 (6). However, the very few mastitic cases that showed positive results with our test might be attributed t o increased levels of IgG or IgM immunoglobulins, while the negative cases were without elevated levels of these immunoglobulins.

Cheese milk which agglutinated (settled) in the vat was sought throughout this study. This spring (1981) happened to be a year in which the northwest cottage cheese plants were free of the sediment defect. Suspect samples no doubt will be available in the future for use to confirm the applicability of this improved agglutination test.

Stained antigens from fast cells were suspended (1 ml each contained approximately 6% pcv) in the different buffer types and concentrations were cross-checked against colostrum whey samples, using the plate antigen testing procedure described by George et al. (14). Results of the study showed that 0.4 and 0.5 molar tris-maleate buffer (pH 7.0) was the best buffer with the appropriate molarity. Figures 1 and 2 show equipment, procedure and results obtained in carrying out the test.

Table 2.1 Determination of Cell and Stain Concentrations Using Fast Growing Lactic Streptococcal Antigens. Numbers Represent Relative Intensity of the Agglutination Reactions from 4 (Strong Positive), 3 (Moderately Positive), 2 (Positive), 1 (Weak Positive) to 0 (Negative).

Strain	Stain Concentration	Whey Sample Dilutions	% Concentration of Cells					
			1%	2%	3%	4%	5%	6%
Fast HP	3%	undiluted	4	4	4	4	4	4
		1/2	3	3	3	3	3	3
		1/4	3	3	3	2	2	2
		1/8	3	2	2	2	1	1
		control	0	0	0	0	0	0
	4%	undiluted	4	4	4	4	4	4
		1/2	4	4	3	3	3	3
		1/4	3	3	2	2	2	2
		1/8	3	2	1	1	1	1
		control	0	0	0	0	0	0
	5%	undiluted	4	4	4	4	4	4
		1/2	4	4	4	3	3	3
		1/4	3	3	2	1	1	1
		1/8	3	0	0	0	0	0
		control	0	0	0	0	0	0
Fast C_2	3%	undiluted	2	2	2	2	2	2
		1/2	±	±	0	0	0	0
		1/4	0	0	0	0	0	0
		1/8	0	0	0	0	0	0
		control	0	0	0	0	0	0
	4%	undiluted	2	2	2	2	2	2
		1/2	1	1	0	0	0	0
		1/4	0	0	0	0	0	0
		1/8	0	0	0	0	0	0
		control	0	0	0	0	0	0
	5%	undiluted	2	2	2	2	2	2
		1/2	0	0	0	0	0	0
		1/4	0	0	0	0	0	0
		1/8	0	0	0	0	0	0
		control	0	0	0	0	0	0
Fast KH	4%	undiluted	4	4	4	4	4	4
		1/2	4	4	3	3	3	3
		1/4	3	3	2	2	2	1
		1/8	2	2	1	1	1	0
		control	0	0	0	0	0	0

Table 2.1 (continued)

Strain	Stain Concentration	Whey Sample Dilutions	% Concentration of Cells					
			1%	2%	3%	4%	5%	6%
	5%	undiluted	4	4	4	4	4	4
		1/2	4	4	4	4	3	3
		1/4	4	3	3	3	2	2
		1/8	3	2	2	2	2	2
		control	0	0	0	0	0	0

Table 2.2 Determination of Stain and Cell Concentrations Using Slow Growing Lactic Streptococcal Antigens. Numbers Represent Relative Intensity of the Agglutination Reactions from 4 (Strong Positive), 3 (Moderately Positive), 2 (Positive), 1 (Weak Positive) to 0 (Negative).

Strain	Stain Concentration	Whey Sample Dilutions	% Concentration of Cells					
			1%	2%	3%	4%	5%	6%
		undiluted	1	2	3	3	3	3
		1/2	2	3	3	3	3	3
	3%	1/4	2	3	3	3	3	3
		1/8	2	3	3	3	3	3
		control	0	0	0	0	0	0
		undiluted	1	2	3	3	3	3
		1/2	1	3	3	3	3	3
Slow R_1	4%	1/4	2	3	3	3	3	3
		1/8	2	3	3	3	3	3
		control	0	0	0	0	0	0
		undiluted	3	3	3	3	3	3
		1/2	3	3	3	3	3	3
	5%	1/4	3	3	3	3	3	3
		1/8	3	3	3	3	3	3
		control	0	0	0	0	0	0
		undiluted	3	3	3	3	3	3
		1/2	1	2	0	0	0	0
	3%	1/4	0	0	0	0	0	0
		1/8	0	0	0	0	0	0
		control	0	0	0	0	0	0
		undiluted	2	3	3	3	3	3
		1/2	1	2	2	1	0	0
Slow C_2	4%	1/4	0	0	0	0	0	0
		1/8	0	0	0	0	0	0
		control	0	0	0	0	0	0
		undiluted	2	3	3	3	3	3
		1/2	2	3	3	2	2	0
	5%	1/4	1	1	0	0	0	0
		1/8	0	0	0	0	0	0
		control						
		undiluted	2	3	3	3	3	3
		1/2	2	3	3	3	3	3
	3%	1/4	2	3	3	3	3	3
		1/8	2	3	3	3	3	3
		control	0	0	0	0	0	0

Table 2.2 (continued)

Strain	Stain Concentration	Whey Sample Dilutions	% Concentration of Cells					
			1%	2%	3%	4%	5%	6%
		undiluted	3	3	3	3	3	3
		1/2	3	3	3	3	3	3
	5%	1/4	3	3	3	3	3	3
		1/8	3	3	3	3	3	3
		control	0	0	0	0	0	0

Table 2.3 Titration of Colostrum Samples to Agglutination End Points Using 2% Cell Concentration (suspended in 0.5 M tris-maleate buffer at pH 7.0). Numbers Represent Relative Intensity of the Agglutination Reactions from 4 (Strong Positive), 3 (Moderately Positive), 2 (Positive), 1 (Weak Positive) to 0 (Negative).

	Stain Concentration	Whey Sample Dilutions							
		1/2	1/4	1/18	1/16	1/32	1/64	1/128	Controls
Fast Strains									
BA-1	5%	4	4	4	3	3	3	2	0
BA-1M	5%	3	3	3	2	1	0	0	0
	3%	4	3	3	3	2	1	0	0
HP	4%	4	3	3	2	1	0	0	0
	5%	4	3	3	2		0	0	0
	3%	2	0	0	0	0	0	0	0
C_2	4%	3	2	0	0	0	0	0	0
	5%	3	0	0	0	0	0	0	0
	3%	4	3	3	2	1	0	0	0
KH	4%	4	3	3	2	1	0	0	0
	5%	4	3	3	2		0	0	0
Slow Strains									
	3%	3	0	0	0	0	0	0	0
C_2	4%	2	0	0	0	0	0	0	0
	5%	3	2	0	0	0	0	0	0
	3%	3	3	3	2	2	0	0	0
C_1	4%	3	3	3	3	3	2	0	0
	5%	3	3	3	3	3	2	0	0
	3%	3	3	3	3	2	1	0	0
R_1	4%	3	3	3	3	2	0	0	0
	5%	3	3	3	3	1	0	0	0

REFERENCES

1. Alton, G. G., J. Maw, B. A. Rogerson and G. G. Mcpherson (1975). Serological diagnosis of bovine brucellosis. Aust. Vet. J. 51(2), 57-63.
2. Anon. U.S. Agriculture Dept. National Animal Disease Laboratory Manual 65C and 65E.
3. Boyed, J. W. (1972). The relationship between serum immunoglobulin deficiency and disease in calves. The Vet. Record 90, 645.
4. Butler, J. E. (1971). Review of the bovine immunoglobulins. J. of Dairy Sci. 54, 1315.
5. Butler, J. E. (1973). Synthesis and distribution of immunoglobulins in cattle. J. of Am. Vet. Med. Assoc. 163, 795-798.
6. Collins, J. C. (1976). The role of different immunoglobulins in the milk ring test, used for detection of brucellosis. Am. de Microbiol. (Inst. Pasteur) 127 B, 177-187.
7. Davis, G. (1971). The Rose bengal test. Vet. Record 88, 447-449.
8. Ellerman, J. A. G. (1975). Testing the lactic streptococci for agglutinin titers in cow's milk. Austral. J. Dairy Technol. 30(3), 111-113.
9. Emmons, D. B., J. A. Elliot and D. C. Beckett (1963). Agglutination of starter bacteria, sludge formation of slow acid development in cottage cheese manufacture. J. Dairy Sci. 46, 600.
10. Emmons, D. B., J. A. Elliot and D. C. Beckett (1965). Sensitive test for lactic streptococcal agglutinins. J. Dairy Sci. 48, 1245.

11. Emmons, D. B., J. A. Elliot and D. C. Beckett (1966). Effect of lactic streptococci agglutinins in milk on curd formation and manufacture of cottage cheese. J. Dairy Sci. 49, 1357.

12. Emmons, D. B., J. A. Elliott and D. C. Beckett (1966). Lactic streptococci agglutinins in milk and blood. Intr. Dairy Congr. XV11(D_2), 499.

13. George, L. W. and L. W. Carmichael (1974). A plate agglutination test for the rapid diagnosis of canine brucellosis. Am. J. of Vet. Res. 34, 905-909.

14. George, L. W. and L. E. Carmichael (1978). Development of a Rose bengal stained plate test antigen for the rapid diagnosis of brucella canis infection. Cornell Veterinarian 68(4), 503-543.

15. Huggins, A. R. (1979). J. Dairy Sci. 62, 70. Abstract.

16. Lucas, P. S. (1962). What causes spongy formation in cottage cheese? Am. Milk Rev. 24(10), 74.

17. Morgan, W. T., D. T. MacKinnon, J. R. Lawson and G. A. Cullen (1969). The Rose bengal plate agglutination test in diagnosis of brucellosis. Vet. Record 85, 636-641.

18. Nicoletti, P. (1967). Utilization of the card test in brucellosis eradication. J.A.V.M.A. Vol. 515(12), 1778-1783.

19. Personal communication. Communication letter from Dr. Badakhsh, F. F. Head, Bacteriology Dept. Biological Res. Division. Pitman Moore, Inc. Washington Crossing, N. J. 08560.

20. Sacco, T. F. (1976). Use of the Rose bengal antigen in the milk ring test for brucellosis. Alti della Societa Italian della Scienze Veterinarie 30, 669-671.

21. Salih, M. A. and W. Sandine (1980). Lactic streptococcal agglutinins: A review. J. Food Protection Vol. 43(11), 856-858.

22. Salih, M. A. and W. Sandine. Lactic streptococcal agglutinins II.

23. Sawyer, M., B. I. Osburn, H. D. Knight, and J. W. Kendrick (1973). A quantitative serological assay for diagnosing congenital infections of cattle. Am. J. Vet. Res. 34, 1281.

24. Stadhouders, J. and H. A. Veringa (1962). Some experiments related to the inhibitory action of milk peroxidase on lactic acid streptococci. Neth. Milk Dairy J. 16, 96.

25. Terzaghi, B. E. and W. E. Sandine (1975). Improved media for lactic streptococci and their bacteriophages. App. Microbiol. 29, 804.

Chapter 3

LACTIC STREPTOCOCCI AGGLUTININS: II. CORRELATION OF ROSE BENGAL PLATE TEST, INDIRECT IMMUNOASSAYS AND ACTIVITY STUDIES[a]

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[a]Technical Paper No. _____ Oregon Agricultural Experiment Station.

ABSTRACT

Lactic streptococcal Rose bengal-stained antigens tested against enzyme-free neutral colostrum whey samples (pH 7.0) gave the same results as that of untreated whey samples. The indirect immunoassay revealed the specificity of IgG immunoglobulin in contrast to IgM. Streptococcus lactis (strain C_2) was not totally agglutinin-resistant to IgG but did not conjugate well with IgM. Indirect immunoassay study revealed a potential testing procedure for detecting the presence of lactic streptococcal agglutinins. The effect of milk agitation on the settling of starter bacteria was elucidated. An investigation involving the effect of autoclaving nonfat dry milk on the agglutinability of starter bacteria was also carried out. It was found that autoclaving dramatically reduced the agglutination of starter strains.

INTRODUCTION

The effect of milk agitation on the cottage cheese sediment defect was a controversy between workers in past research (8,15,20). Emmons (7) observed that prolonged agitation of inoculated skim milk promoted starter strain agglutination and retarded acid production. Emmons and Keogh (7,12) concluded that agglutination was not inhibitory per se, but caused uneven distribution of lactic acid in milk and thus negated use of pH as a direct indicator of the time to cut the curd. Kanno et al. (11) reported isolation of a purified protein, with high agglutinability to starter bacteria, from normal milk and they characterized the purified protein as the immunoglobulin IgM.

Butler (1,2) identified three classes of immunoglobulins in bovine milk, IgG, IgA and IgM. According to his studies, IgG comprises 85-90% and IgM 10% of the total whey immunoglobulins. It would appear that an increase in either one, especially IgM, could contribute to the seasonal occurrence of the agglutination problem. The present study was intended to investigate the specificity of the colostrum whey agglutinins by two parameters, indirect immunofluorescent assays and by the Rose bengal-stained antigen plate test. Elucidation of the agglutination behavior of lactic starter bacteria in milk was also sought.

MATERIALS AND METHODS

Three kinds of studies were conducted:

(a) Rose bengal-stained antigen plate test

(b) Indirect immunofluorescent assays

(c) Agglutination behavior of lactic streptococci in nonfat dry milk diluted with enzyme-free colostrum whey samples

Whey Sample Preparation and Plate Test

Second day colostrum milk samples were collected from the Oregon State University Dairy Center. The samples were processed on the same day of collection. The following procedure was carried out for the preparation of colostrum whey samples:

1. Centrifugation of 200-ml milk samples in 250-ml polyethylene centrifuge tubes at 10,000 rpm for 5 min to remove fats. Slight coarse filtration with glass wool was performed to remove the fats and other gross particles.

2. Porcine pepsin (75-80 units/ml-Sigma grade) was added and the skim milk allowed to set at room temperature for 1-2 hr.

3. Recentrifugation of the coagulated milk at 10,000 rpm for 35 min to precipitate the casein proteins, leaving a clear colostrum whey.

4. The pH of the whey samples was adjusted to pH 7.0 with a few drops of 0.1 N NaOH and heated at 30°C for 30 min. This treatment was intended to inactivate residual enzyme in the whey (18).

Eight lactic streptococcal Rose bengal-stained antigens (5 fast strains and 3 slow) were tested against the enzyme-free (neutral pH)

clostrum whey samples and the same results were obtained as reported earlier (17).

Indirect Immunofluorescent Assays

Two fluorescin-conjugated bovine anti-IgG and-IgM immunoglobulins (FITC conjugated anti-IgG and anti-IgM) were purchased from Miles Research Products and diluted to their working dilutions. Two lactic streptococcal antigens (HP and C_2) were the whole bacteria suspended in M17 (17) and one loopful from a cell concentration of 10^8/ml was spread over the slide. The procedure described in the Manual for Clinical Laboratory Procedures (6) was used for the acetone fixation of the antigen, addition of colostrum whey samples and addition of fluorescin conjugated anti-immunoglobulins. Lastly, the slides were stained in the dark for 5 min with Evans blue (1/1000) as staining background. With the aid of the fluorescence microscope, conjugation of FITC - anti-immunoglobulin was traced.

Agglutination Behaviour of lactic Streptococci in Milk

(a) Preparation of nonfat dry milk (NFM). Stocks of NFM were prepared in such a manner that 10% solids could be attained after the addition of whey samples. One stock of the NFM was heat-treated at 80°C for 15 min to eliminate all possible agglutinins. The other stock was autoclaved at 121°C for 15 min. Treatments were done before whey dilutions.

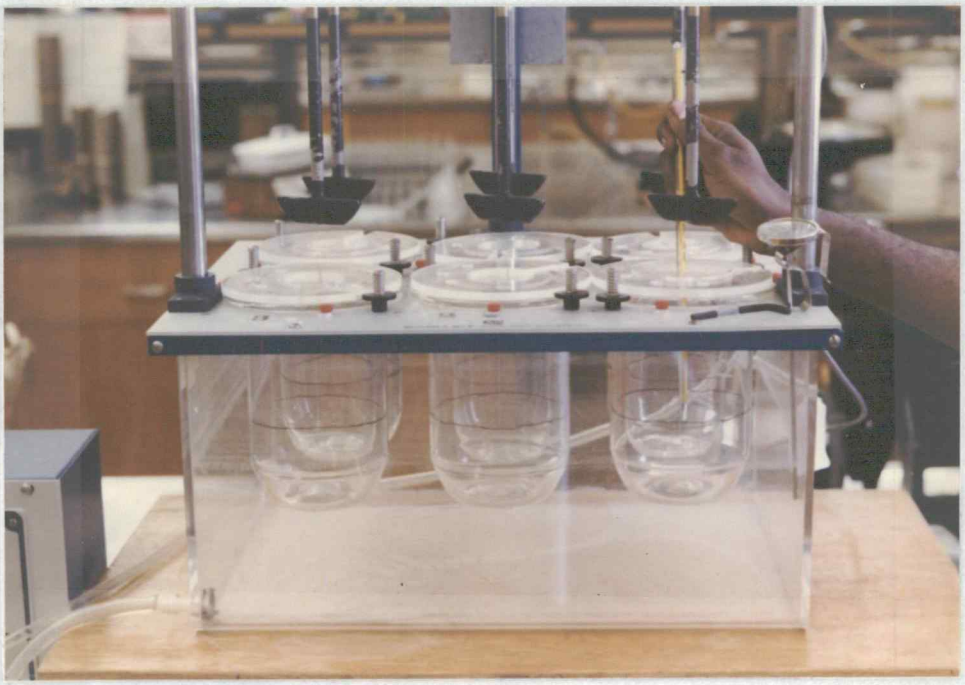
(b) Preparation of whey dilutions. Serial 1:1 dilutions were made of colostrum whey samples with NFM, to achieve 1:2 (50% whey +

50% NFM), 1:4 (25% whey + 75% NFM), 1:8 dilutions, etc. For the controls, colostrum whey samples were steamed for 15 min and checked by the Rose bengal-stained antigen plate test to assure complete elimination of the agglutinins. For the dilutions 1:2 and 1:4, 50% heat inactivated whey samples was used and diluted as above. For 1:8 and 1:16 dilutions, 12.5% whey was added to 87.5% NFM as a control.

(c) Procedure. One-liter capacity dissolution flasks (Van-Kel Industries, Inc., Chathan, NJ) were used after their vertical height had been marked into 3 parts (top, middle and bottom) as pH measurement location points (Figure 3). Agitation of the inoculated milk was carried out for 75 minutes at 150 rpm in a dissolution test apparatus (Model RL-Hanson Research Corporation, Northridge, CA). The pH was followed at the three locations with the aid of a Corning pH-meter-125 over a maximum period of 5 hours.

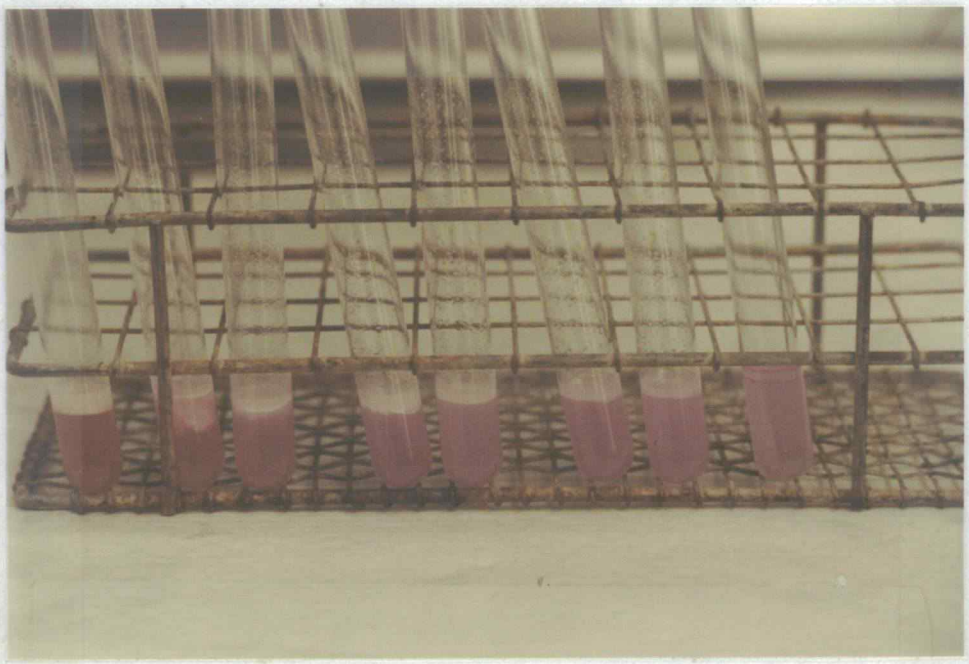
Figure 3. Part of the dissolution station which was used for the pH measurements as a parameter for following the pattern of starter bacteria settling. The dissolution flasks were vertically divided into three portions; top, middle and bottom.

Figure 4. The Ring test. 50 µl of S. cremoris (HP) Rose bengal-stained antigens were mixed with 1/2 serially diluted whey samples and a fatty acid layer added and the whole was well mixed, incubated at 37°C for 30 minutes. A white band is clear on top of the aqueous fluid in the fatty layer indicating a positive agglutination. The right end tube is the control (tris-maleate buffer, antigen and a fatty acid layer) shows no white band but a clear solution monitoring the absence of agglutinin.



Photograph 3

Photograph 4



RESULTS AND DISCUSSION

When antibodies bind particulate antigens (at equivalencies), large lattice-like complexes will be formed resulting in decreased affinity for water due to interaction of solubilizing polar groups and loss of solubility leading to agglutination or precipitation (16). Since lactic acid production by starter bacteria is not affected by agglutination per se (7,12), pH measurement at different levels will reflect agglutination behaviour in milk. According to Figures 1A and B, distinct differences in acid production between top and bottom layers of the milk compared with that of the control (Figure 1C) occur and confirm this idea. The behaviour of strain C_2 (S. lactis) was especially interesting (Figures 2A and 2B). Strain C_2 was not resistant to agglutination, especially with high levels of immunoglobulins, but as the immunoglobulins were diluted out (1:4 dilution, Figure 2B) an alteration occurred in the pattern of agglutination. We considered this disburbance as an indication of agglutination resistance. With lower dilutions 1:8 and 1:16 (Figure 3A,B and 4A,B), the effect of agitation started to show up and no setting of bacteria was detectable. This might reveal that under high agglutinin levels the large lattice-like complex is more resistant to agitation but, with low agglutinin levels the small size of the lattice-like complex does not allow settling.

Surprisingly, with autoclaved (NFM) milk (agitated for 5 min) there was a dramatic reduction in agglutination (Figures 5 A,B) and strain C_2 (Figure 6A,B) was absolutely resistant (9,10). This reduction might be

attributed to a change in the microenvironment of the agglutinins resulting either from increased density of the milk or increased solubility of antigen-antibody complexes.

Results of the rose bengal-stained antigen tests were in agreement with the agglutination behaviour of the starter bacteria in the unautoclaved milk. The indirect immunofluorescent assays revealed the most astonishing results in relation to the specificity of the agglutinins. Two lactic streptococcal bacteria were very susceptible and specific to IgG immunoglobulins (Figures 5 and 6). Their conjugation with FITC anti-IgM was very faint in comparison to FITC anti-IgG fixation and strain C_2 did not bind at all with IgM. These immunofluorescent findings confirmed the previous Rose-bengal stained antigen plate test results if we consider that Rose bengal-stained plate antigens have a higher affinity for IgG than IgM (3,4,5,13,14). The indirect immunofluorescent assays represent another technique for detecting the presence of lactic streptococcal agglutinins in milk but further studies on its usefulness need to be carried out.

The pattern of agglutination behaviour of starter bacteria followed by pH measurement in the dissolution station using unautoclaved milk.

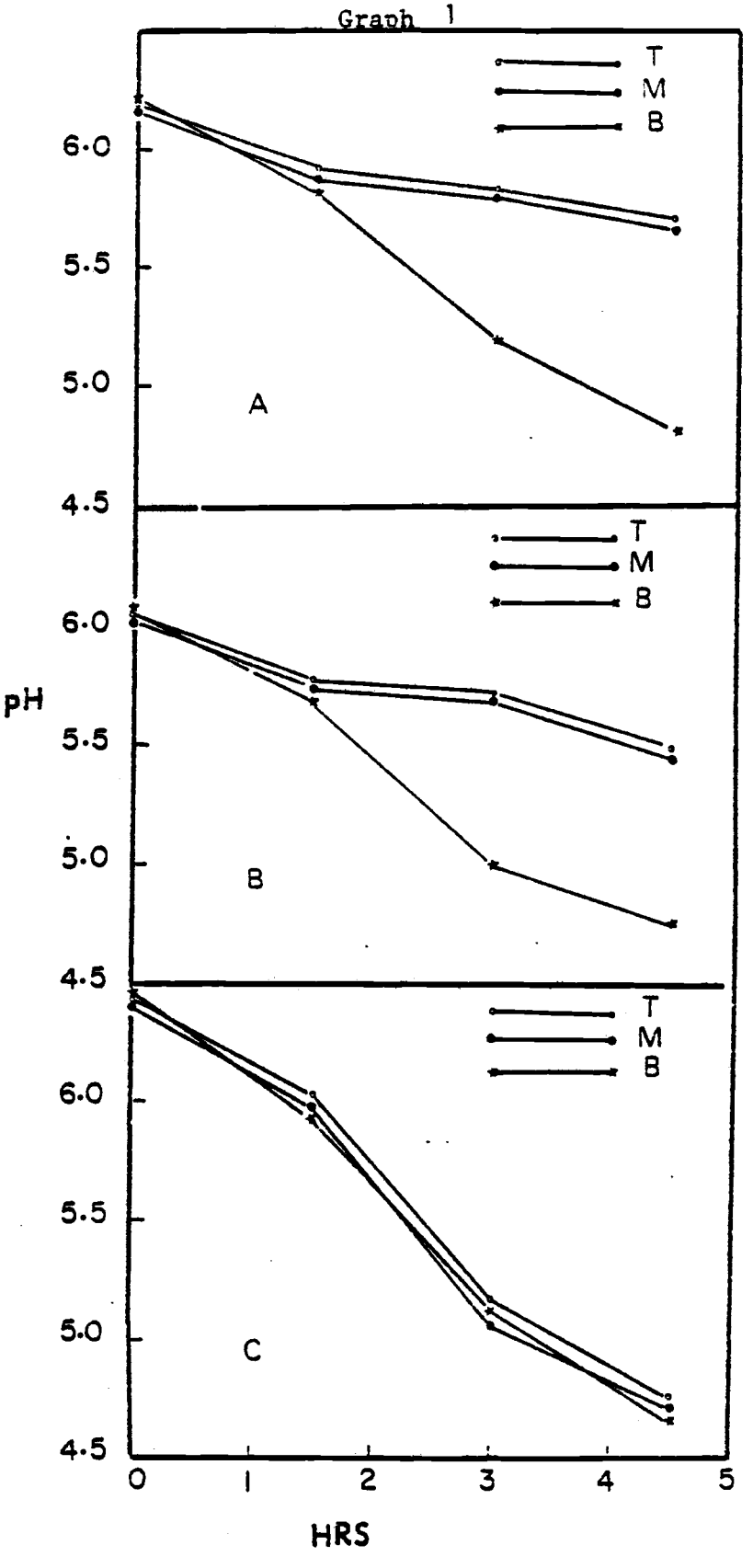
Graph 1. Streptococcus cremoris:

A. 1/2 whey dilution. It reveals the sedimentation of the agglutinated starter bacteria indicated by the sharp drop in pH at the bottom layer.

B. 1/4 whey dilution. It shows sedimentation of the starter bacteria with no sharp differences from 1/2 whey dilutions.

C. Control. Almost the lines coincide with each other indicating even distribution of starter bacteria due to the even production of lactic acid. The controls contain steamed whey samples and virtually no antibodies.

Graph 1



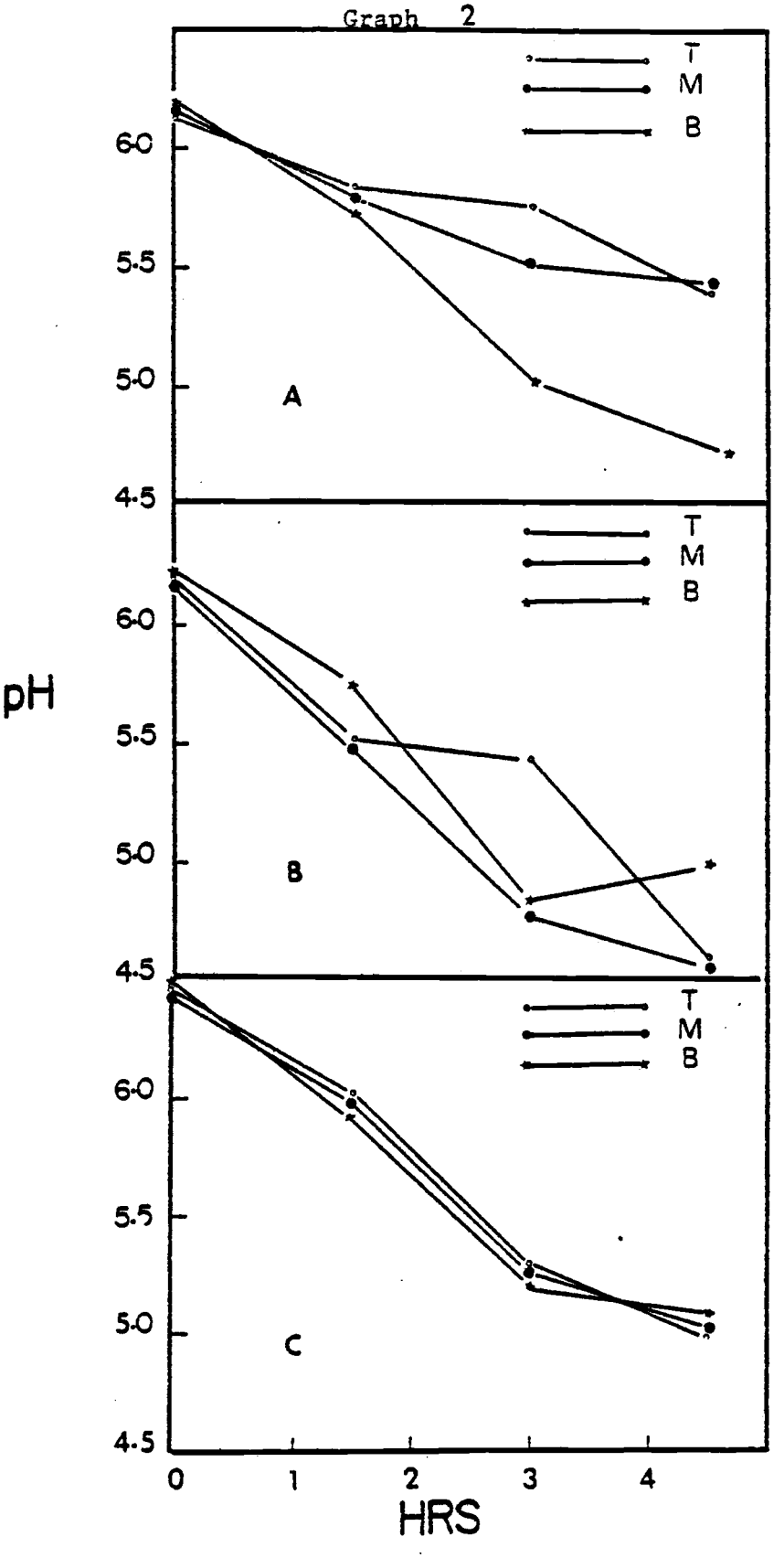
Graph 2. *S. lactis* (C_2):

A. 1/2 whey dilutions. Sharp drop in pH in the bottom layer indicates the large concentration of starter bacteria resulting from sedimentation of agglutinated organisms.

B. 1/4 whey dilutions. Drop in pH at the beginning then followed by a sudden drop in upper layer's pH. It shows an upset in the pattern of agglutination indicating the start of the resistant response.

C. Control. The three lines almost coincide to reflect even distribution of the starter bacteria.

Graph 2



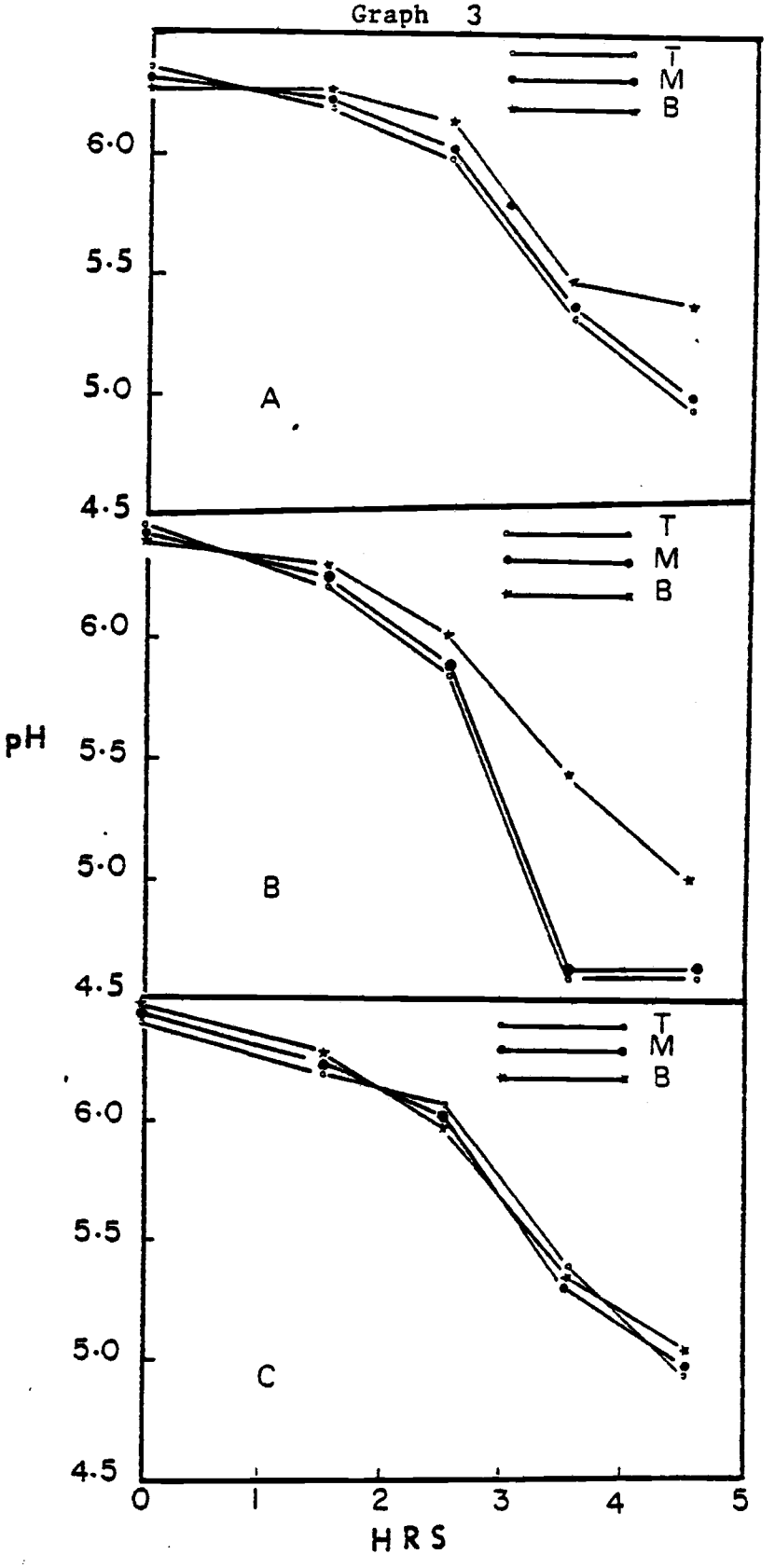
Graph 3. S. cremoris:

A. 1/8 dilution. The lines are somewhat closer to each other but no sedimentation is taking place.

B. 1/16 dilution. Higher pH started to show up in the upper layers of the milk and no sedimentation occurring.

C. Control. Almost even distribution of the starter bacteria.

Graph 3



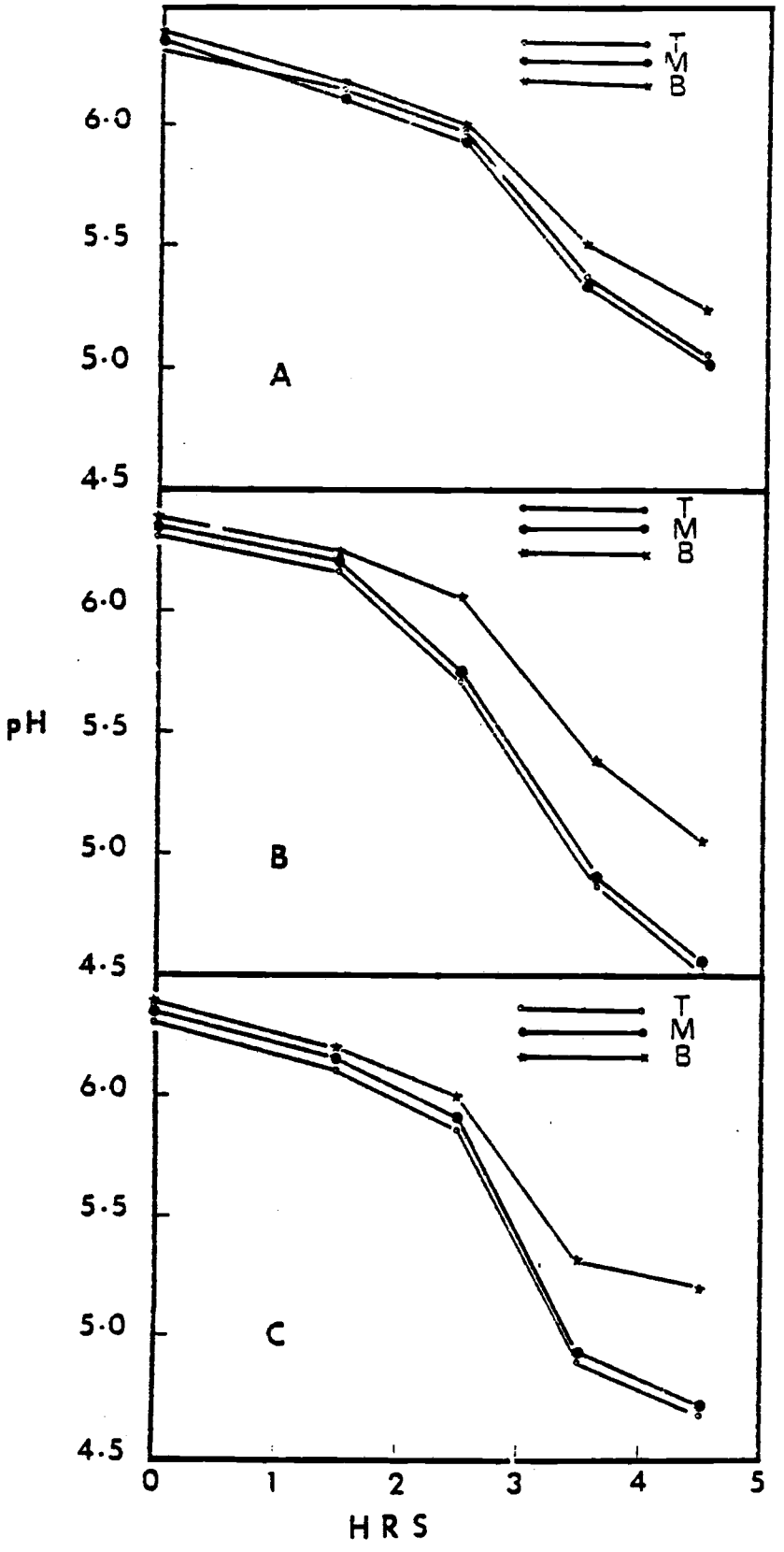
Graph 4. S. lactis (C_2):

A. 1/8 dilution. No sedimentation is occurring and no sharp differences between bottom layers and top layers of the milk.

B. 1/16 dilution. No sedimentation.

C. Control. The lines are not very much coinciding as before.

Graph 4



The pattern of agglutination behaviour of starter bacteria followed by pH measurement using autoclaved nonfat dry milk.

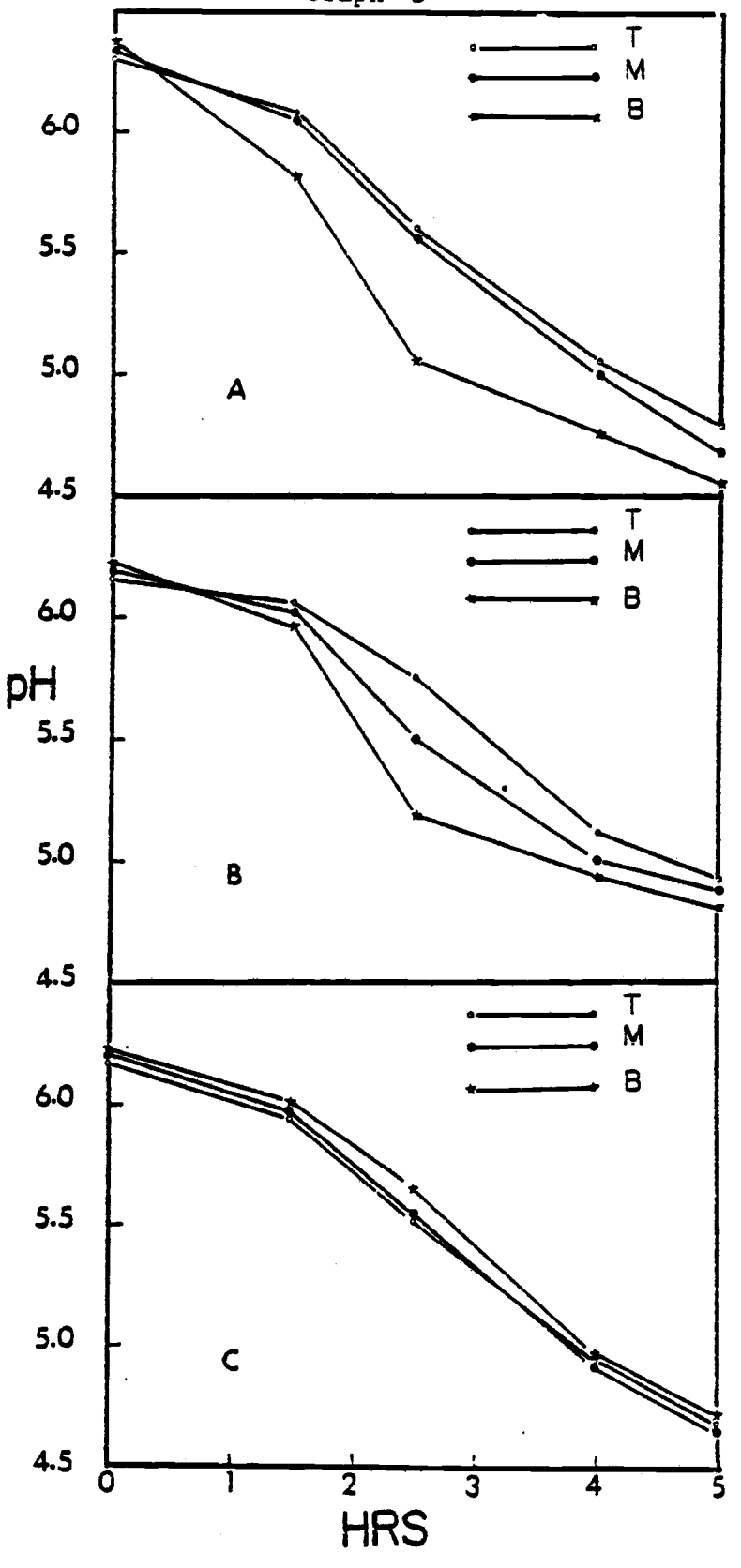
Graph 5. S. cremoris (HP):

A. 1/2 whey dilution. Settling of the starter bacteria occurred but with a noticeable reduction in size compared to the 1/2 dilution of the unautoclaved milk.

B. 1/4 whey dilution. Even more reduction in the size of agglutination but there is sedimentation.

C. Control. Even distribution of starter bacteria with even lactic acid distribution.

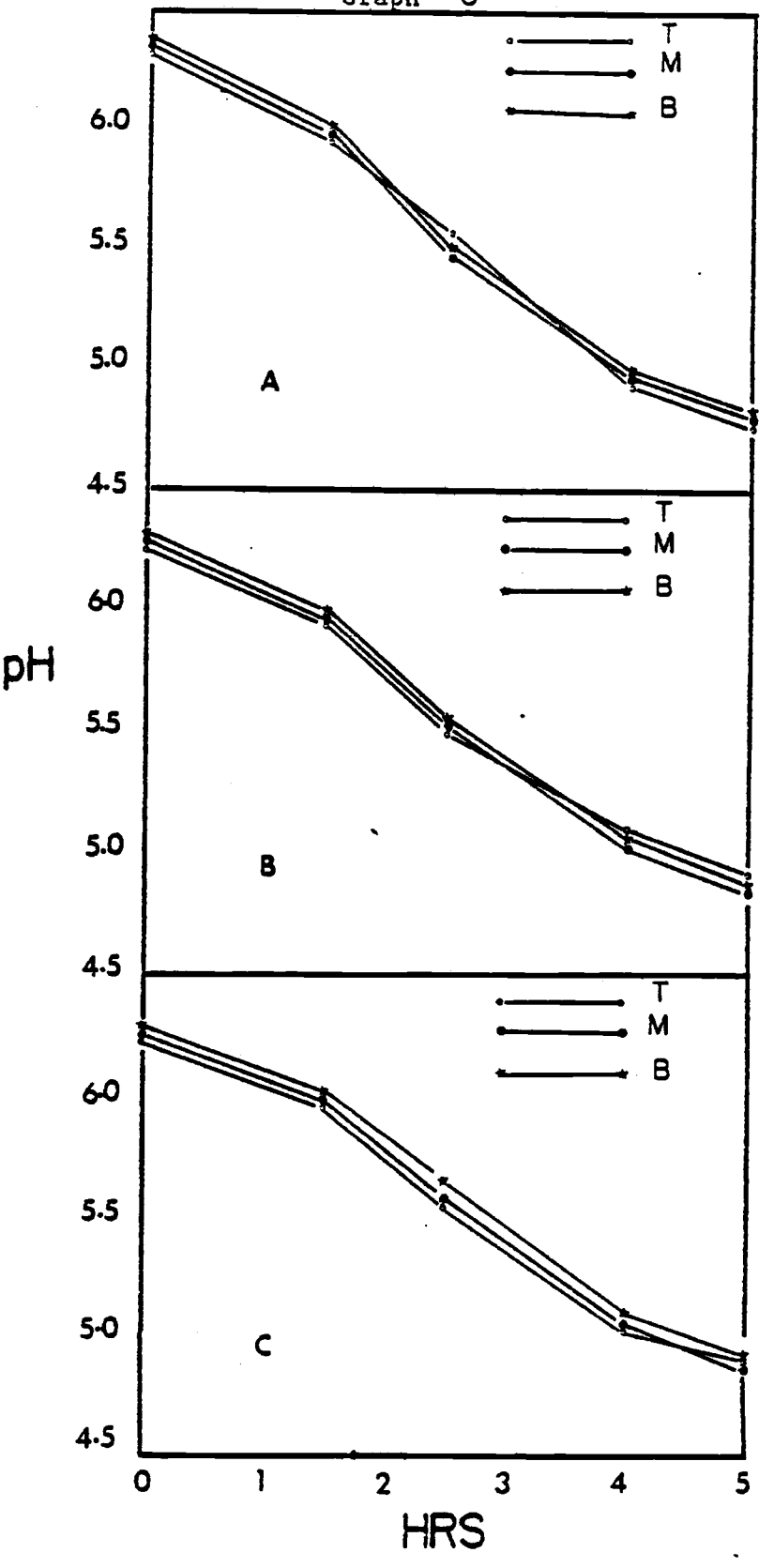
Graph 5



Graph 6. *S. lactis* (C_2):

A, B, and C. All the graphs appear the same and no agglutination was eminent in either A or B, indicating that autoclaving the milk (without antibodies in it) alone can lead to the reduction and elimination of agglutination.

Graph 6



Immunofluorescent Assays

Figure 5.

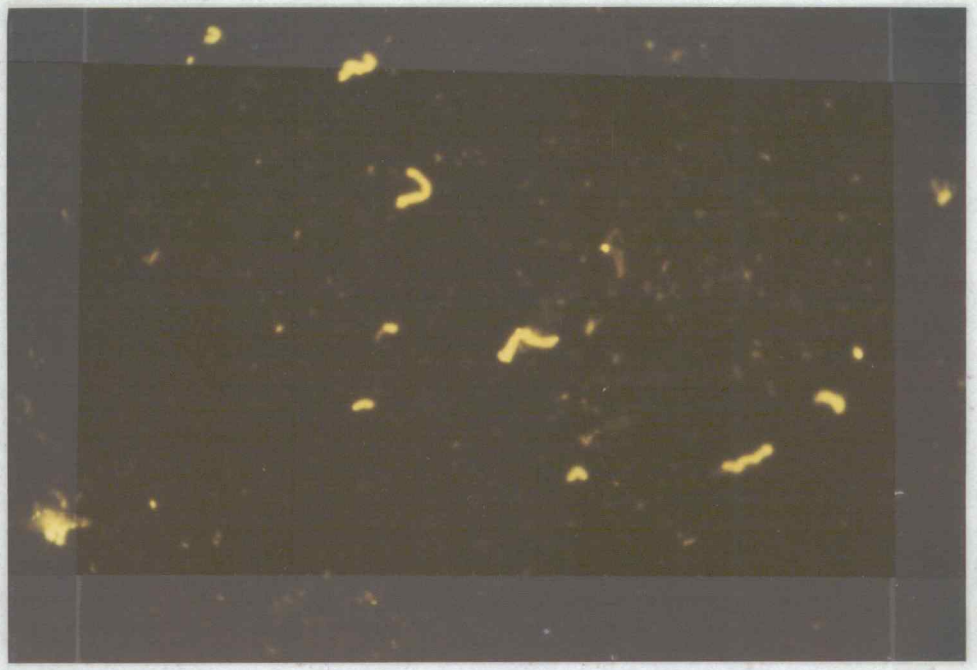
A. S. lactis (strain C_2). Binding the fluorescin conjugated anti-antibody (immunoglobulin IgG). Only some of the organisms bound the immunoglobulin. Organisms that bound the fluorescin conjugated immunoglobulin IgG are shining yellow and increasing in size.

B. S. lactis (strain C_2). Control. No binding of the fluorescin conjugated immunoglobulin IgG is eminent (only clusters of unbound fluorescin conjugated immunoglobulin). Some of the organisms are green in color taking the counter-stain Evan's blue.

Figure 6. S. Cremoris (HP).

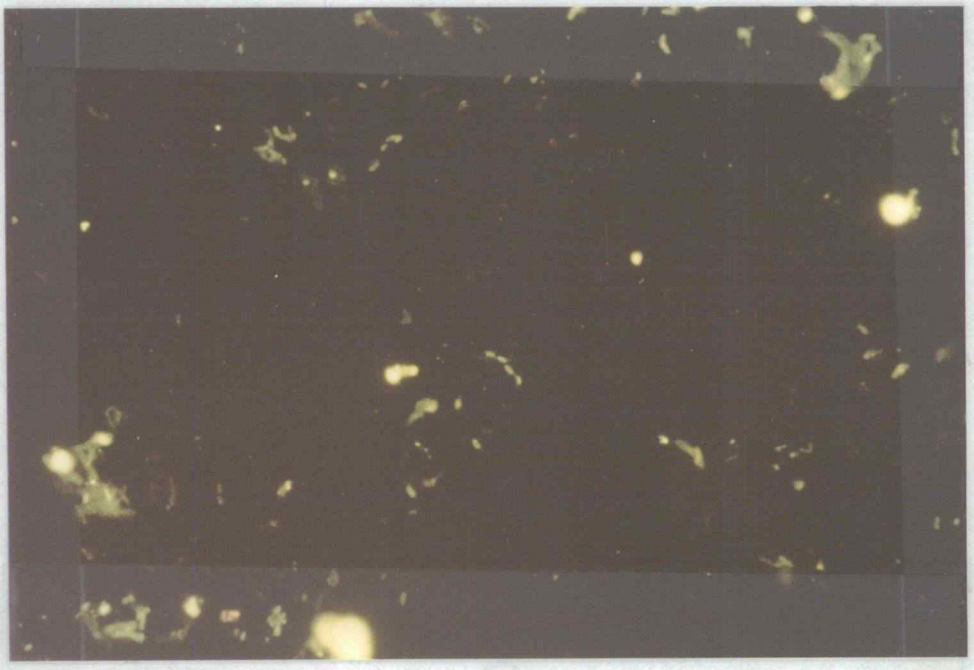
A. Organisms that bind the immunofluorescent IgG are shining yellow and thickened. A chain of streptococci is binding the fluorescin conjugated anti-antibody.

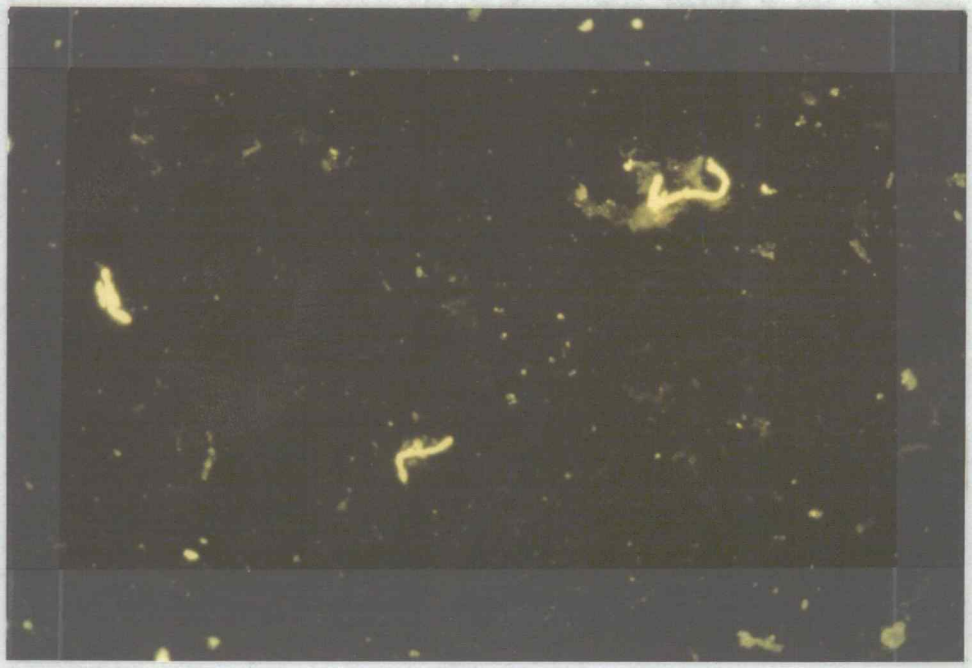
B. S. cremoris (HP). Control. No antibodies added only tris-maleate buffer was used. No binding of the immunoglobulin could be seen. Organisms appear green in color.



Photograph 5-A

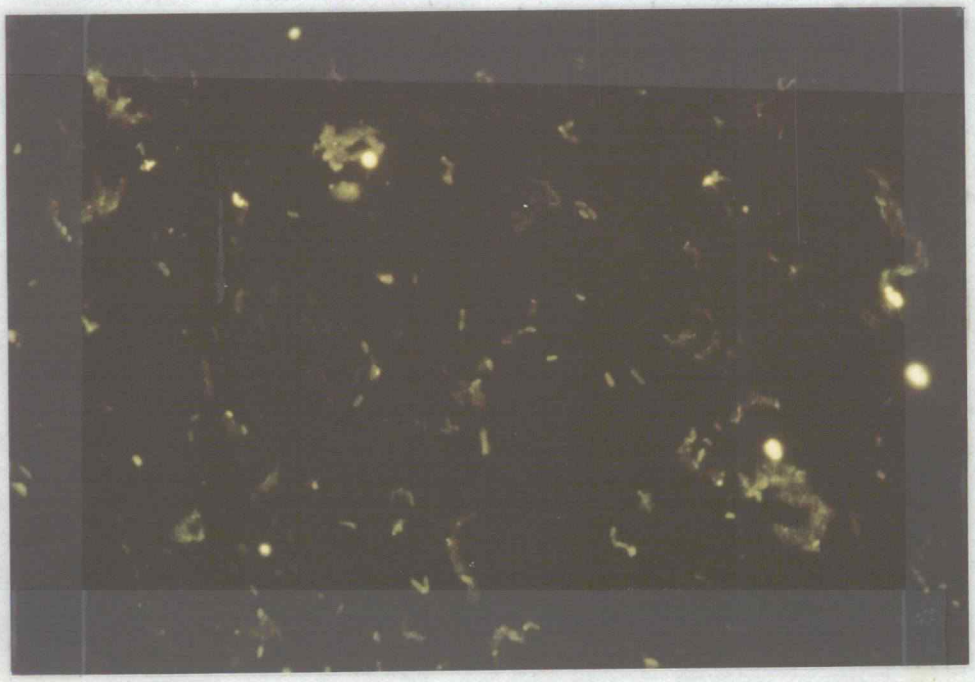
Photograph 5-B





Photograph 6-A

Photograph 6-B



REFERENCES

1. Butler, J. E. (1969). Bovine immunoglobulins: A review. J. Dairy Sci. 52:1895.
2. Butler, J. E. and C. F. Macewell (1972). Preparation of bovine immunoglobulins and free secretory components and their specific antisera. J. Dairy Sci. 55:151.
3. Corbel, M. J. (1972). Characterization of antibodies active in the rose bengal test. Veterinary record 90(17):484.
4. Corbel, M. J. (1972). Identification of the immunoglobulins class active in the rose bengal plate test for bovine brucellosis. J. of Hygiene 70(4)779.
5. Corbel, M. J. (1973). Studies on the mechanism of the rose bengal plate test for bovine brucellosis. Brit. Vet. J. 129:517.
6. CRC - Manual of Clinical Laboratory Proceedures: 2nd edition. 320.
7. Emmons, D. B., J. A. Elliott and D. C. Beckett (1966). Effect of lactic streptococci agglutinins in milk on curd formation and manufacture of cottage cheese. J. Dairy Sci. 49:1357.
8. Gillies, A. J. (1959). Inhibitory factors in cheese milk. XVth Inter. Dairy Congr. 2:523.
9. Hobbs, B. C. (1939). The part played by the bacteria in the reduction of methylene blue in milk. J. Dairy Res. 10:35.
10. Hucker, G. J. (1932). Agglutination as a means of differentiating the species of streptococcus and leuconostoc. New York Agr. Sta. Technol. Bull. 190.

11. Kanno, C., D. B. Emmons, N. R. Harwalker and J. A. Elliott (1976). Purification and characterization of the agglutinating factor for lactic streptococci from bovine milk: IgM immunoglobulin. J. Dairy Sci. 59:2036.

12. Keogh, B. P. (1958). Variations in lactic acid production in milk. Austral. J. Dairy Technol. 13:132.

13. Morgan, W. J. B., D. J. MacKinnon, J. R. Lawson and G. A. Cullen (1969). The rose bengal plate agglutination test in the diagnosis of brucellosis. Vet. Rec. 85:636.

14. Pietz, D. E. and E. A. Schiff (1968). A rapid brucellosis card test using buffered brucella antigen (BBA). J. A. V. M. A. Vol. 515(12):1783.

15. Stadhouders, J. (1963). Inhibitory effect of lactenin 3 on acid production in milk by S. cremoris 803. Meth. Milk Dairy J. 17:96.

16. Stewart, Sell (1980). Immunology, immunopathology and immunity. 3rd edition by Harber and Row.

17. Salih, M. A. and W. E. Sandine (1981). Lactic streptococcal agglutinins I.

18. Terzaghi, B. E. and W. E. Sandine (1975). Improved media for lactic streptococci and their bacteriophages. Appl. Microbiol. 29:807.

19. Thunell, R. K., J. W. Duersch and C. A. Ernstrom (1979). Thermal inactivation of residual milk clotting enzymes in whey. J. Dairy Sci. Vol. 62(3):373.

20. Wright, R. C. and T. Trammer (1957). The effect of cream rising upon the activity of bacteria in heat treated milk. J. Dairy Res. 24:124.

Chapter 4

TECHNICAL NOTE

A SIMPLE RING TEST FOR LACTIC STREPTOCOCCAL AGGLUTININS[a]

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[a]Technical Paper No. _____, Oregon Agricultural Experiment Station.

ABSTRACT

A simple tube testing procedure for detecting the presence of lactic streptococcal agglutinins in milk was developed. Bacterial cells were stained with Rose bengal, Ravinol and hematoxylin stains. Ravinol and hematoxylin stained cells gave faint blue colorations to the milk layers. After incubation at 37°C for 30 minutes most of the cells precipitate and agglutinated bacterial cells adhering to the fatty layer were reduced in number. The ring formation is due to adherence of agglutinated bacterial cells at the fatty acid layer. Rose bengal-stained cells yielded better results than either Ravinol or hematoxylin-stained antigens. A fatty acid layer composed of palmitic, stearic and oleic acids in the ratio of 2:1:2 was used instead of the bovine cream fat layer for the adherence of the agglutinins.

INTRODUCTION

In 1937, Fleischhauer introduced a simple agglutination test (the milk ring test) for the detection of Brucella abortus agglutinins in milk to identify cattle with brucellosis. Since that time, the ring test has been one of the most-used, simple agglutination tests and widely-used in erradication of Brucellosis (2,3,5,6,8,9). Furthermore, the modified ring test that utilizes the negative milk samples to dilute out and test serum and whey samples from infected animals was recognized and being used world-wide (7). Taking advantage of the mechanism involved in the ring test, a simple modified procedure for detecting the presence of lactic streptococcal agglutinins was sought.

MATERIALS AND METHODS

Antigen preparations

Lactic streptococci bacterial cells were pelleted, washed and prepared for staining according to Salih and Sandine (10). Three different staining procedures were used to stain lactic streptococcal cells.

(a) Haematoxylin staining: this was done according to the Woods (11) procedure in staining Brucella antigens for the milk ring test. The stained cells were suspended in phenolized normal saline.

(b) Ravinol staining: with a 6-month old crystal violet solution (1), lactic streptococci were stained and suspended in 0.1 M phosphate buffer pH 7.0 (1,4).

(c) Rose bengal staining: the procedure of Salih and Sandine (10) was used for staining the lactic streptococcal antigens and suspending them in 0.5 M Tris-maleate buffer (pH 7.0).

(d) Whey sample preparations: whey samples were separated for normal milk samples according to Emmons' method (3).

(e) Fatty acids combinations: three different fatty acids (purchased from Sigma) were prepared and combined as follows: 10% palmitic acid dissolved in propyl alcohol (w/v) and 10% stearic acid dissolved in toluene (w/v). Oleic acid butyl ester solution, stearic acid solution and palmitic acid solution were combined in the ratio of 2:1:2 and used as a fatty acid layer in the diluted

whey samples. Bovine creams were prepared and steamed for 15 min to inactivate possible agglutinins and used as a fatty acid layer in whey samples.

Testing procedure

The same buffers in which the antigens were previously suspended were used to dilute out the whey samples in 1/2 serial dilutions. The controls were made of the buffer solution without any added whey. To one ml of each dilution and control, 25-50 µl of the stained antigens were added. Then, 0.5 ml of the fatty acids were added to each test tube and the contents well agitated on a vortex mixer. Incubation of the test tubes was carried out for 30 min at 37°C. Strong whitish bands with some blue (in case of Ravinol stain and hematoxylin stain) or pinkish color (in case of Rose bengal) were scored as 4, moderate bands with blue or pinkish coloration as 3, and whitish bands as 2 or 1, depending on intensity; the negatives were given 0 when there were no whitish bands at all.

RESULTS AND DISCUSSION

The physical mechanism involved in the milk ring tests depends essentially on the presence of agglutinins in milk. The agglutinated bacteria will adhere to the fat droplets and will be carried to the surface of the milk. The use of stained bacterial cells is therefore a differential color indicator revealing the presence or absence of these agglutinins (6,8,9,11). Addition of bovine cream to diluted whey samples was found to be of no value in the attraction of lactic streptococcal agglutinins to the surface. Most probably the steaming of the cream altered the attractive sites or affected the size and dispersity of the fat globules (9). The combined fatty acids (palmitic, stearic and oleic acids) are the major fatty acids existing in milk fats with the ratios of 2:1:2. The acidic nature of the combined fatty acids poses a decolorization problem of some stained antigens on the top layers. Attempts were made to raise the pH of the whey to 7.0 or to add some basic amino groups to the fatty acid combination. The Rose bengal stained cells were found to yield better results with high agglutination titers than either Ravinol or hematoxylin stained cells (Tables 1 and 2).

No agglutination differences were detected with all tested antigens, between fast growing strains and slow growing strains, confirming our previous findings (10). However, additional studies are needed to find out if any correlation exists between the titers of this test and the settling of starter bacteria in cheese vats. Furthermore, studies to

find a chemical combination which will allow the stained bacterial cells to float in the cream layer according to their densities is needed.

Table 4.1 Hematoxylin stained antigens tested against normal milk samples (strong white band given 4 and no band given 0).

Lactic Streptococcal Strains	Dilutions 1/2	1/4	1/8	1/16	Controls
Fast Strains					
HP	4	3	2	1	0
C_2	3	1	0	0	0
KH	4	3	2	1	0
US_3	2	0	0	0	0
C_{13}	0	0	0	0	0
R_6	4	3	2	1	0
Slow Strains					
R_6	4	3	2	1	0
TR	4	3	2	1	0
Z8	3	2	1	0	0
R_1	3	2	1	0	0
ML_1	2	1	0	0	0
C_1	2	1	0	0	0
US_3	2	1	0	0	0
E8	4	3	2	1	0
C_2	3	2	0	0	0
HP	4	3	2	1	0
CH	2	1	0	0	0

Table 4.2 Rose bengal and Ravinol stained antigens tested against normal milk samples (4 = strong white band; 0 = no band).

Lactic Streptococcal Strains	Dilutions						
	1/2	1/4	1/8	1/16	1/32	1/64	Controls
Ravinol Staining							
Fast Strains							
HP	2	2	2	0	0	0	0
C_2	3	2	0	0	0	0	0
KH	2	2	2	0	0	0	0
C_{13}	2	0	0	0	0	0	0
Slow Strains							
HP	2	2	2	0	0	0	0
C_2	3	2	0	0	0	0	0
KH	2	2	2	0	0	0	0
C_{13}	1	0	0	0	0	0	0
US_3	3	2	2	0	0	0	0
R_1	2	1	1	0	0	0	0
Rose Bengal Stainins							
Fast Strains							
HP	4	3	3	2	1	1	0
C_2	3	2	0	0	0	0	0
KH	4	3	3	2	1	1	0
C_{13}	2	1	0	0	0	0	0
US_3	3	3	2	2	1	1	0
Slow Strains							
HP	4	3	3	2	1	1	0
C_2	3	2	0	0	0	0	0
C_{13}	2	1	0	0	0	0	0
US_3	3	3	2	2	1	1	0

References

1. Anon. U.S. Dept. of Agriculture. National Veterinary Services Laboratories. Diagnostic Reagents Manual 65E. Ames, Iowa.
2. Bruhny, P. A. (1948). The Brucella abortus ring test. Am. J. Vet. Res. Vol. IX(33):360.
3. Emmons, D. B., J. A. Elliott and D. C. Beckett (1965). Sensitive test for lactic streptococcal agglutinins. J. Dairy Sci. 48:1245.
4. Fleischhauer, G. (1937). Die abortus-bang-ringprobe (ABR). Zur Feststellung von Bangverdachtigen Vollmilchproben. Ber. Tierazel. Wehnschr. 53:527-528.
5. George, L. W. and L. W. Carmichel (1974). A plate agglutination test for the rapid diagnosis of canine Brucellosis. Am. J. of Vet. Res. 34:905-909.
6. Hamilton, A. V. and A. V. Hardy (1950). The Brucella ring test. Am. J. Publ. Health. Vol. (40):321.
7. Hill, W. K. W. (1966). Een oderzock over de waarde van de abortus bang ring test by melkmonsters uit melktanks tijdschr. Diergneesk 91:885.
8. Manatove, S. M. (1975). Veterinariya, Moscow. Vol. 7:96-
9. Moore, T. (1951). The Brucella abortus ring test. Canadian J. of Comp. Medicine. Vol. XV(2):39.

10. Ogonwski, K. (1955). Brucellosis: A note on the relationship between the milk ring test results and the size and dispersity in size of milk fat globules. The Vet. Rec. p. 1127-

11. Salih, M. A. and W. E. Sandine (1981). Development of a Rose bengal test for detection of lactic streptococcal agglutinins.

12. Wood, R. W. (1948). A new and rapid method for the preparation and standardization of Brucella ring test antigen. Am. J. of Publ. Health. Vol.(38):1225.

Overall Bibliography

1. Alton, G. G., J. Maw, B. A. Rogerson, and A. A. McPherson. 1975. Serological diagnosis of bovine brucellosis. Aust. Vet. J. 51(2), 57-63.
2. Anon. U.S. Agriculture Dept. National Animal Disease Laboratory Manual. 65C and 65E.
3. Auclair, J. E. and A. Hirsch. 1953. The inhibition of micro-organisms by raw milk. The occurrence of inhibitory and stimulatory phenomenae. J. Dairy Res. 20, 45.
4. Auclair, J. E. and Y. Vassal. 1963. Occurrence of variants sensitive to agglutinins and to lactoperoxidase in alactenin resistant strain of S. lactis. Dairy Res. 30, 345.
5. Balakrishnan, R., and A. R. Vedanayakam. 1973. The agglutinins of cow and buffalo milk. The nature and properties of the agglutinins. Indian Vet. J. 50, 111-124.
6. Boyed, J. W. 1972. The relationship between serum immunoglobulin deficiency and disease in calves. The Vet. Record 90, 645.
7. Briggs, C. A. E. and L. G. M. Newland. 1953. The serological classification of S. cremoris. J. Dairy Res. 19, 160.
8. Bruhny, P. A. 1948. The Brucella abortus ring test. Am. J. Vet. Res. Vol. 1X(33), 360.
9. Butler, J. E. 1969. Bovine immunoglobulins: A review. J. Dairy Sci. 52, 1895.

10. Butler, J. E. and C. F. Mascwell. 1972. Preparation of bovine immunoglobulins and free secretory components and their specific antisera. J. Dairy Sci. 55, 151.

11. Butler, J. E. 1973. Synthesis and distribution of immunoglobulins in cattle. J. of Am. Vet. Assos. 163, 795-798.

12. Collins, J. C. 1976. The role of different immunoglobulins in the milk ring test, used for the detection of brucellosis. Am. de Microbiol. (Inst. Pasteur) 127 B, 177-187.

13. Corbel, M. J. 1972. Characterization of antibodies active in the Rose bengal test. Veterinary Record 90(17), 484.

14. Corbel, M. J. 1972. Identification of the immunoglobulins class active in the Rose bengal plate test for bovine brucellosis. J. of Hygiene 70(4), 779.

15. Corbel, M. J. 1973. Studies on the mechanism of the Rose bengal plate test for bovine brucellosis. Brit. Vet. J. 129, 517.

16. CRC - Manual of Clinical Laboratory Procedures. 2nd edition. 320.

17. Czulak, J. and L. J. Meanwell. 1951. Seasonal variation in cheese starter activity. Proc. Soc. Appl. Bacteriol. 14, 1.

18. Davis, G. 1971. The Rose bengal test. Vet. Record 88, 447-449.

19. Ellerman, J. A. G. 1975. Testing the lactic streptococci for agglutinin titers in cows milk. Austral. J. Dairy Technol. 30(3), 111-113.

20. Emmons, D. B., J. A. Elliott, and D. C. Beckett. 1963. Agglutination of starter bacteria, sludge formation and slow acid development in cottage cheese manufacture. J. Dairy Sci. 46, 600.

21. Emmons, D. B., J. A. Elliott and D. C. Beckett. 1965. Sensitive test for lactic streptococcal agglutinins. J. Dairy Sci. 48, 1245.

22. Emmons, D. B., J. A. Elliott and D. C. Beckett. 1966. Effect of lactic streptococcal agglutinins in milk on curd formation and manufacture of cottage cheese. J. Dairy Sci. 49, 1357.

23. Emmons, D. B., J. A. Elliott and D. C. Beckett. 1966. Lactic streptococcal agglutinins in milk and blood. Inter. Dairy Congr. XVII D2, 499.

24. Fleischhaur, G. 1937. Die abortus-bang-ring probe (ABR). Zur Feststellung von Bangverdachtigen Vollmilchproben. Ber. Tierazel. Wehnschr 53, 527, 528.

25. George, L. W. and L. W. Carmichael. 1974. A plate agglutination test for the rapid diagnosis of canine brucellosis. Am. J. of Vet. Res. 34, 905-909.

26. George, L. W. and L. E. Carmichael. 1978. Development of a Rose bengal stained plate test antigen for the rapid diagnosis of brucella canis infection. Cornell Veterinarian 68(4), 503-543.

27. Gillies, A. J. 1959. Inhibitory factors in cheese milk. XVth Inter. Dairy Congr. 2, 523.

28. Gough, P. M., R. Jenness, and R. K. Anderson. 1966. Characterization of immunoglobulins. J. Dairy Sci. 49, 713 (Abst.).

29. Hamilton, A. V. and A. V. Hardy. 1950. The brucella ring test. Am. J. Publ. Health Vol. 40, 321.

30. Hesse, W. 1894. Z. Hyg. Infekrkr. 17, 238.

31. Hill, W. K. W. 1966. Een oderzock over de waarde van de abortus bang ring-test by melkmonsters vit melktans tijdschr. Diergnessk 91, 885.

32. Hobbs, B. C. 1939. The part played by the bacteria in the reduction of methylene blue in milk. J. Dairy Res. 10, 35.

33. Hucker, G. J. 1932. Agglutination as a means of differentiating the species of Streptococcus and Leuconostoc. New York Agr. Expt. Sta. Tech. Bull. 190.

34. Huggins, A. R. 1979. J. Dairy Sci. 62, 70. Abstract.

35. Jago, G. R. 1954. Factors influencing the lactic acid producing properties of Streptococci used in the manufacture of Cheddar cheese. J. Dairy Res. 21, 111.

36. Jenness, R., R. K. Anderson, and P. M. Cough. 1965. Fractionation of bovine milk and blood agglutinins for Brucella by gel-filtration and specific absorption techniques. Fred. Proc. 24, 503.

37. Jones, F. S. and R. B. Little. 1927. The bacteriocidal property of cow's milk. Exptl. Med. 45, 319.

38. Jones, F. S. and H. S. Simms. 1930. Bacterial growth inhibitory (Lactenin) of milk. J. Exptl. Med. 51, 327.

39. Kanno, C., D. B. Emmons, V. R. Harwalker, and J. A. Elliott. 1976. Purification and characterization of the agglutinating factor for lactic streptococci from bovine milk IgM immunoglobulin. J. Dairy Sci. 50, 2036-2045.

40. Keogh, B. P. 1958. Variations in lactic acid production in milk. Austral. J. Dairy Technol. 13, 132.

41. Klaus, G. G. B., A. Bennett, and E. W. Jones. 1969. A quantitative study of the transfer of colostral immunoglobulins to the newborn calf. Immunology 16, 293.

42. Kosikowski, F. V. 1963. Some distribution patterns of cottage cheese particles and conditions contributing to curd shattering. J. Dairy Sci. 46, 391.

43. Lucas, P. S. 1962. What causes spongy formation in cottage cheese? Am. Milk Rev. 24(10), 74.

44. Manatove, S. M. 1975. Veterinaryia, Moscow, Vol. 7, 96.

45. McPhilips, J. 1958. Specificity of agglutinins in milk. Nature 182, 869.

46. Moore, T. 1951. The Brucella abortus ring test. Canadian J. of Comp. Medicine. Vol. XV(2), 39.

47. Morgan, W. J. B., D. J. Mackinon, J. R. Lawson, and G. A. Cullen. 1969. The Rose bengal plate agglutination test in the diagnosis of brucellosis. Vet. Rec. 85, 636.

48. Nicoletti, P. 1967. Utilization of the curd test in brucellosis eradication. J. A. V. M. A. Vol. 515(12), 1778-1783.

49. Ogonwski, K. 1955. Brucellosis. A note on the relationship between the milk ring test results and the size and dispersity in size of milk fat globules. The Vet. Rec. p. 1127.

50. Personal communication. Communication letter from Dr. Badakhsh, F. F. Head, Bacteriology Dept., Biological Res. Division, Pitman Moore, Inc., Washington Crossing, N. J. 08560.

51. Pietz, D. E. and D. A. Schift. 1968. A rapid brucellosis curd test using buffered brucella antigen (BBA). J. A. V. M. A. Vol. 515(12), 1783.

52. Portman, A., Y. Gate, and J. Auclair. 1962. Influence of lactoperoxidase and agglutinins of milk on the activity of thermophile starter bacteria. XVI Int. Dairy Congr. (B), 729.

53. Randolph, H. E. and I. A. Gould. 1968. Characterization of the natural inhibitors in skim milk affecting lactic acid bacteria. J. Dairy Sci. 51, 8.

54. Reiter, B. and A. M. Madsen. 1963. Reviews of the Progress of Dairy Sci. Section B. Cheese and butter starters. J. Dairy Res. 30, 419.

55. Sacco, T. F. 1976. Use of the Rose bengal antigen in the milk ring test for brucellosis. Alti della Societa Italian delta Scienze Veterinarie 30, 669-671.

56. Salih, M. A. and W. E. Sandine. 1980. Lactic streptococcal agglutinins: A review. J. Food protection. Vol. 43(11), 856-858.

57. Salih, M. A. and W. E. Sandine. 1981. Unpublished data. Lactic streptococcal agglutinins II.

58. Salih, M. A. and W. E. Sandine. 1981. Development of a Rose bengal test for detection of lactic streptococcal agglutinins.

59. Sandine, W. E. 1977. New techniques in handling cultures to enhance their performance. J. Dairy Sci. 60, 822-828.

60. Sandine, W. E., A. W. Anderson, P. R. Elliker, and R. W. Stein. 1963. Observations on citrate utilization by mixed strain starter cultures and protein stability of cheese milk. J. Dairy Sci. 46, 610.

61. Sawyer, M., B. I. Osburn, H. D. Knight, and J. W. Kendrick. 1973. A quantitative serological assay for diagnosing congenital infections of cattle. Am. J. Vet. Res. 34, 1281.

62. Smith, E. C. 1946. Isolation and properties of immunolactoglobulins from bovine whey. J. Biol. Chem. 165, 665.

63. Stadhouders, J. 1963. Inhibitory effect of Lactenin L_3 on acid production in milk by S. cremoris 803. Neth. Milk Dairy J. 17, 96.

64. Stadhouders, J. and G. Hup. 1970. Complexity and specificity of englobulins in relation to inhibition of bacteria and to cream rising. Neth. Milk Dairy J. 24, 79.

65. Stadhouders, J. and H. A. Veringa. 1962. Some experiments related to the inhibitory action of milk peroxidase on lactic acid streptococci. Neth. Milk Dairy J. 16, 96.

66. Steward, Sellu. 1980. Immunology, immunopathology and immunity. 3rd edition by Harber and Row.

67. Stocking, W. A., Jr. 1904. The so called germicidal property of milk. 16th Annual Report of Storrs Conn. Agr. Expt. Sta. 16, 89.

68. Terzaghi, B. E. and W. E. Sandine. 1975. Improved media for lactic streptococci and their bacteriophages. Appl. Microbiol. 29, 807.

69. Thunnell, R. K., J. W. Duersch, and C. A. Ernstrom. 1979. Thermal inactivation of residual milk clotting enzymes in whey. J. Dairy Sci. Vol. 62(3), 373.

70. Vedamuthu, E. R., G. W. Reinbold, and E. G. Hammond. 1967. Inhibitory activity of acid and rennet whey on propionibacteria. J. Dairy Sci. 51(4), 503-510.

71. White, C. H. and B. W. Ray. 1977. Influence of heat treatment and methods of acidification on manufacture and properties of cottage cheese. J. Dairy Sci. 60, 1236.

72. Wilson, A. T. and H. Rosenblum. 1952. The antistreptococcal property of milk. J. Expt. Med. 95, 25.

73. Wilson, C. S. and A. A. Miles. 1975. Toply and Wilson principals of bacteriology and immunity, 6th ed., Vol. I, Williams and Wilkins Co., Baltimore.

74. Wood, R. W. 1948. A new and rapid method for the preparation and standardization of Brucella ring test antigen. Am. J. Publ. Health. Vol. 38, 1225.

75. Wright, R. C. and J. Trammer. 1957. The influence of cream rising upon the activity of bacteria in heat-treated milk. J. Dairy Res. 24, 124.

76. Wright, R. C. and J. Trammer. 1959. Factors influencing the activity of cheese starters. The role of milk peroxidase. J. Dairy Res. 25, 104.