SEROLOGICAL PROPERTIES OF MILK AND THEIR ROLE IN REGARD TO THE INHIBITION OF CERTAIN STRAINS OF *STREPTOCOCCUS CREMORIS*

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SUMMARY

Serological aspects involved in the inhibition of certain strains of *Streptococcus cremoris* have been examined. The study has included an investigation into the presence of antibodies occurring naturally in milk as well as an examination of the effects of adding homologous antisera to milk containing either susceptible or resistant strains.

Whey agglutination tests showed that there were no antibodies present for the resistant strains, but tests carried out on susceptible strains were unsatisfactory because of the instability of the antigen.

A modified ring test gave positive results for the susceptible strains but was negative for the resistant strains. As the susceptible strains were found to be rough variants and the resistant ones were smooth, it is considered that the positive tests were due to non-specific agglutination.

Microscopic examination revealed that all susceptible strains were agglutinated in both pasteurized wholemilk and skim-milk. Only the long-chain resistant strains, however, showed evidence of agglutination. Long-chain susceptible strains exhibited a tendency to agglutinate in sterilized milk, but short-chain susceptible strains did not. Such agglutination in itself had little effect on acidity production.

Addition of homologous antiserum to pasteurized milk inoculated with a resistant strain resulted in it behaving in all respects like a susceptible strain in pasteurized milk.

By adding homologous antibody to recombined milk (sterile skim-milk and heated cream) inoculated with a susceptible strain, it was shown that the addition of specific antibody caused the susceptible strain to react in the same way as in whole separated milk. However, in this case the resulting inhibition was not quite so marked.

Inoculation of susceptible strains into sterile skim-milk containing cream washings gave results which showed that the inhibitory property was transferable. It was also found that when a short-chain susceptible strain was used there was no evidence of any agglutination of the starter cells.

Conclusions have been drawn from the data obtained and an explanation for the inhibition of the susceptible strains has been advanced. It appears that both skim-milk and cream fractions donate factors which separately cause inhibition of acidity production by the susceptible strains but both these factors are required for maximum inhibition.

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I. INTRODUCTION

Over the past 60 years, numerous observations have been made regarding antibodies in milk and their possible link with the inhibitory properties of milk. Ehrlich (1892) discovered that antibodies of various types may be found in milk. Marrack (1947) reported that the young animal depends on the antibodies in colostrum or milk for its passive immunity. He quoted that a number of investigators, including Little and Orcutt (1922) and Mason, Dalling, and Gordon (1930), agree that colostrum is the main source of antibodies. As lactation advances, the antibody level appears to fall away. Campbell, Humphrey, and Work (1953) were able to demonstrate by using labelled methionine that antibodies are transferred directly from blood to milk.

Heinemann and Glenn (1908) and Rosenau and McCoy (1908) found that *Salmonella typhi* was agglutinated in milk, and they thought that agglutination was responsible for the apparent decrease in the number of bacteria in fresh milk. Heinemann and Glenn were also able to show that species from several genera were agglutinated; they therefore thought this agglutination was probably unspecific, a finding which is verified in the present work. Chambers (1920), however, was unable to find a correlation between agglutination and bacterial inhibition. In support of this later finding, Jones and Little (1927) by various means were able to rule out the action of agglutinin on a non-haemolytic mastitis Streptococcus. They found that all milks inhibited this Streptococcus. More recently, Hobbs (1939) presented evidence that there are two inhibitory factors in milk, the first responsible for the clumping of the growing organism and the second for the bacteriostatic effect which inhibits bacterial multiplication for a variable length of time. Wright and Tramer (1957) noted that cheese starters affected by cream rising in wholemilk were agglutinated. However, as they presumed this agglutination was also present in homogenized or separated milk which was no longer inhibitory, they concluded that agglutination in itself had no effect on acidity production. This finding was confirmed by Keogh (1958).

It is of interest also that Morris and Edwards (1949) found that cultures of *Streptococcus lactis* varied considerably in morphology when grown in raw and in heated milk. They considered this morphological variation to be the cause of the different colony counts obtained with the two types of milk. These workers showed that the clumps formed were due to the proliferation of the growing organism, and were not a result of agglutination. Wright and Tramer (1957) also noted a difference in chain length of the slow starters, the chains being longer in pasteurized milk than in milk heated to higher temperatures. However, the latter pair of workers did not give any information regarding nomenclature of the starters.

From the foregoing evidence it is clear that further examination of the serological role in the inhibitory phenomenon was necessary. Such an examination has been made in the present work. There were two main aspects of the investigation, viz:—

(1) The presence of antibodies in inhibitory milk.

(2) The effects produced on starter streptococci by the addition of homologous antisera.

Throughout the work the starters used were strains of *Streptococcus cremoris*. These were labelled (*a*) "susceptible" (e.g. HP and R1, which were inhibited in whole, raw or pasteurized milk allowed to stand but produced acidity normally in homogenized or sterile milk) and (*b*) "resistant" (C13 and C3, strains which produced acid normally in all milks).

II. PRESENCE OF ANTIBODIES IN INHIBITORY MILK

(a) Methods

Several methods were used for testing for the presence of antibodies in milk. These were agglutination, precipitation and ring tests.

(i) Agglutination Tests

Agglutination tests were carried out by the following two methods:

(1) Whey was prepared according to the method of Hall and Learmonth (1933). The clear whey so obtained was serially diluted with 0·85 per cent. saline. An equal volume of a standardized suspension of formalized cells (1,000 x 10^6 organisms per ml) was added to each dilution. As some of the organisms did not form stable suspensions in 0·85 per cent. saline, they were resuspended in decreasing concentrations of saline down to and including 0·15 per cent. All strains were shaken with glass beads in an endeavour to obtain stable suspensions. Tests were incubated (*a*) by overnight incubation at 55°C as used by Hobbs (1939), and (*b*) according to the method of Hucker (1932), who allowed antigen-serum mixtures to be incubated at 37°C for 4 hr. The tests were then removed from the water-bath, shaken, and held in a refrigerator overnight. Tests were then read for the degree of agglutination.

(2) As several workers have used the microscopic test as a means of judging whether agglutinins are present in the milk, a similar study of the growth of both susceptible and resistant strains in pasteurized wholemilk, pasteurized skim-milk and sterilized wholemilk was carried out. Smears were made from activity tests of susceptible and resistant strains which had been set up to include all three types of milk. Smears were made after 3 hr and 6 hr. These were stained with Newman's stain and examined.

(ii) Precipitation Tests

The Lancefeld precipitation test was used with Fuller's (1938) formamide modification. The antigen after extraction was carefully layered onto the surface of the whey, so that the line of demarcation between the two liquids was easily visible. A ring of precipitate at the junction of the two liquids was regarded as a positive test.

(iii) Ring Tests

The ring test, as used for the detection of Brucella agglutinins, was employed, and the method described by Wood (1948) was adapted for the purpose. This test utilizes a stained antigen. If the test is positive this antigen forms a highly coloured ring in the cream layer. A negative test is denoted by a uniform mauve colour throughout the milk.

(b) Results

Results were obtained over a range of milks, using a number of resistant and susceptible strains. All milks gave fair uniformity of results.

Agglutination tests were partially unsatisfactory because it was impossible to obtain stable suspensions of the susceptible strains. However, results with these strains after four hours indicated that no specific antibodies were present. Agglutination tests when the resistant strains were used gave completely negative results.

Microscopic examinations revealed several interesting features. Starters of both types which formed long chains in pasteurized milk (whole or skim) did so in sterilized milk. Both long-chain and short-chain susceptible strains were agglutinated in wholemilk and skim-milk, but the agglutination of short-chain susceptible strains was completely absent in sterilized milk. The long-chain susceptible organisms displayed a tendency to clump in both pasteurized and sterilized milk, this tendency being more pronounced in pasteurised milk.

In the case of resistant strains, however, short-chain organisms did not agglutinate in any of the milks, but strains forming long chains clumped in all milks, this clumping being more pronounced in the pasteurized than in the sterilized milk. It is also noteworthy that it was not possible to discern any difference between the microscopic results after three and six hours.

The results are summarized in Table 1.

Table 1

MICROSCOPIC APPEARANCE OF "SUSCEPTIBLE" AND "RESISTANT" STRAINS IN PASTEURIZED WHOLEMILK AND SKIM-MILK AND STERILIZED WHOLEMILK

Milk	Resistant Strain		Susceptible Strain	
	Long-chain Type C13	Short-chain Type C3	Long-chain Type HP	Short-chain Type R1
Pasteurized wholemilk ..	++	−	++	++
Skim-milk	++	−	++	++
Sterilized wholemilk ..	+	−	+	−

++ Pronounced clumping.
+ Slight clumping.
− No clumping.

As was to be expected in view of the results obtained with the agglutination tests, the precipitation tests using Lancefeld's extracts were found to be negative for all strains of organisms examined.

The ring test, on the other hand, was shown to be positive for all susceptible strains, but negative for the resistant ones. All tests were negative when carried out with sterilized milk.

The ring test, which was designed for the detection of brucellosis, is comparatively new and much work has been done with it over the past few years. Some of the data regarding it are conflicting. Diernhofer and Simetsberger (1953) found that although agreement between all the tests for detecting Brucella antibodies were good, the ring test was the least sensitive. On the other hand, Krejci (1956) found the ring test to be the most sensitive of all tests. Tallman and Herman (1954) in a comprehensive study of this test showed that production of a specific coloured antigen is the most critical step involved in the ring test procedure, and that the selection of a smooth *Brucella abortus* culture is of paramount importance. Antigen specificity was decreased when antigens were prepared from rough strains. It is of interest that Kastli (1955) found that Brucella cultures tested by him could be divided into two main groups differing in sensitivity.

Apparently Fleischhauer (1954) has been the only worker to adapt the ring test for the detection of serological infections caused by genera other than *Brucella*. He showed that this test did not yield consistent results in the examination of the sera of cows and horses for salmonellae infections. Keogh (1958) and McPhillips (1958) adapted the ring test in the same way as described herein, but gave no information as to the specificity of the antigens used by them.

In view of these results it seemed necessary that the starter cultures should be examined in order to determine whether they were "rough" or "smooth" in type. Acriflavine tests were therefore carried out on all organisms tested according to the method described by Stokes (1955). It is noteworthy that all susceptible strains tested were rough and all resistant strains were smooth.

Attempts were made to obtain smooth resistant variants from the rough susceptible strains by the use of (*a*) homologous antisera and (*b*) bacteriophage, but these were unsuccessful. In cases where smooth cultures were produced, the activity of these organisms in sterilized milk was low and therefore such cultures were useless for further testing for acidity production. Consequently, it was impossible to determine whether smooth-type cells obtained from the susceptible strains would behave like the resistant strains in pasteurized milk.

During the course of the work opportunity was taken to examine a number of the experimental bulk milks for the presence of agglutinins active against *Brucella abortus*. All such tests were positive, some presumably as a result of

Strain 19 vaccination. In view of this result it has been considered whether the positive ring test obtained with lactic streptococci was the result of cross-agglutination with *Brucella abortus* antibodies. In order to prove this point some preliminary testing was carried out with milk from experimental cows not giving positive agglutination tests. Milk from these cows gave a positive ring test (using a Streptococcus coloured antigen) and were found to be inhibitory to susceptible streptococcal strains. In the face of these results it can no longer be supposed that the positive ring tests were due to reaction with Brucella agglutinins.

III. EFFECT OF ADDING HOMOLOGOUS ANTISERA

As it is evident that the susceptible strains are carried up with the fat into the cream layer, presumably as a result of non-specific agglutination, it seemed desirable to determine the behaviour of a resistant culture in the presence of its specific antibody. Such an investigation was therefore carried out.

(a) Methods

Antisera were prepared in rabbits according to the method of Hucker (1932). The animals were inoculated on the 1st, 5th, 7th, 11th and 13th days. Increasing inocula of a standardized suspension of cells were used. At the first inoculation only 0·5 ml was given; the dose was increased by 0·5 ml at each inoculation. Eight days following the final inoculation, a test bleeding was made and the serum titre determined. Titre obtained was 1 in 1,250. Antisera were prepared against both susceptible and resistant strains. In the preparation of these antisera it was found that agglutination tests for the susceptible strain had to be read after four hours because these organisms did not form stable suspensions and auto-agglutinated on prolonged standing.

Activity tests were set up, using a resistant strain in pasteurized milk with homologous antiserum of determined titre, and sufficient to cause agglutination of this strain was employed.

Unfortunately, a limited supply of rabbits allowed the preparation of anti-serum against only one resistant and one susceptible strain.

Further information was needed to ascertain whether the resistant strain in the presence of its antibody was being taken up with the fat. Tests to determine this point were therefore set up in pasteurized milk, using separating funnels as culture bottles. Parallel tests were also set up with the susceptible strain and its homologous antibody. Smears were made of all tests at hourly intervals from three hours onwards. The smears were stained with Sudan IV and counterstained with Loeffler's methylene blue, then mounted in glycerine jelly. After six hours' incubation, the milks were taken out of the incubator and the skim-milk and cream fractions separated and retained. Titrations were then performed on all samples.

Photomicrographs were taken of the smears, using an "Ortholux" microscope and a "Praktica" camera.

(b) Results

Results of the preliminary tests with the resistant strain are set out in Table 2.

Table 2

PERCENTAGE ACIDITY PRODUCED BY A RESISTANT STRAIN (C3) IN PASTEURIZED MILK CONTAINING HOMOLOGOUS ANTISERUM

—	Percentage Acidity Produced by Resistant Strain C3
Pasteurized milk	·70
Pasteurized milk containing homologous antibody ..	·28

The results show that the presence of its antibody caused a great drop in acidity production by the resistant strain. Further tests, involving separation of cream and skim-milk fractions, revealed quite definitely that agglutination of the resistant strain by its homologous antibody had taken place and had caused most of the cells to be taken up into the cream layer by the rising fat globules. Thus it caused the resistant strain to behave in all respects like a susceptible strain. These results are set out in Table 3.

Table 3

ACIDITY PRODUCTION OF SUSCEPTIBLE AND RESISTANT STRAINS IN MILK CONTAINING HOMOLOGOUS ANTIBODY

Milk	Percentage Acidity			
	Susceptible Strain		Resistant Strain	
	Skim-milk	Cream	Skim-milk	Cream
Control milk	·22	·40	·39	·32
Test milk (containing homologous antibody) ..	·22	·43	·26	·47
	Wholemilk		Wholemilk	
Control milk (mixed at end of test)	·22		·39	
Test milk (mixed at end of test)	·22		·26	

Susceptible Strain—HP
Resistant Strain—C3

It is quite evident also that the presence of homologous antibody had no effect on the acidity production of the susceptible strain in pasteurized milk. Results were the same, indicating that the concentration of the inhibitory properties native to milk were of such an order that additions of homologous antibody to the susceptible strain had no further effect on acidity production.

Microscopic examination of smears showed that both resistant and susceptible strains in the presence of their homologous antibodies were agglutinated in the skim-milk fractions following three hours' incubation. After six hours, the majority of organisms were in the cream layer and only a few remained in the

skim fraction. This result is identical with that already obtained when the susceptible strain was grown in pasteurized milk without antiserum. The resistant strain when grown in pasteurized milk without antibody followed the same pattern as has been described previously, i.e. the bulk of the organisms remain in the skim-milk fraction.

Photomicrographs of the resistant strain in the skim-milk fraction both with and without antibody serve as an interesting comparison with the tabulated results (Figures 1 and 2).

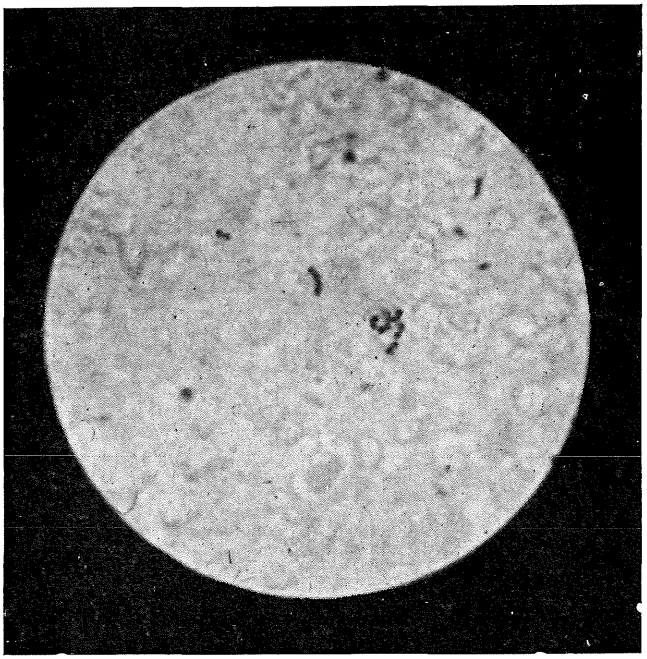

Fig. 1.—Resistant strain (C3) grown in pasteurized milk in presence of antibody for six hours at 30°C. Smear made of skim layer. Agglutination of the organisms has resulted in their rising with the fat. Sudan IV and methylene blue. X1140.

Some difficulty was experienced in obtaining smears uniformly stained by means of the Sudan IV method. This difficulty was aggravated by decolourization in preparing permanent mounts in glycerine jelly.

IV. EFFECT OF SPECIFIC AGGLUTININS

As no clear-cut evidence as to whether the presence of specific agglutinins in pasteurized milk was a possible cause of the susceptible strains rising with the fat was forthcoming from the trials, a series of tests was set up to investigate this problem.

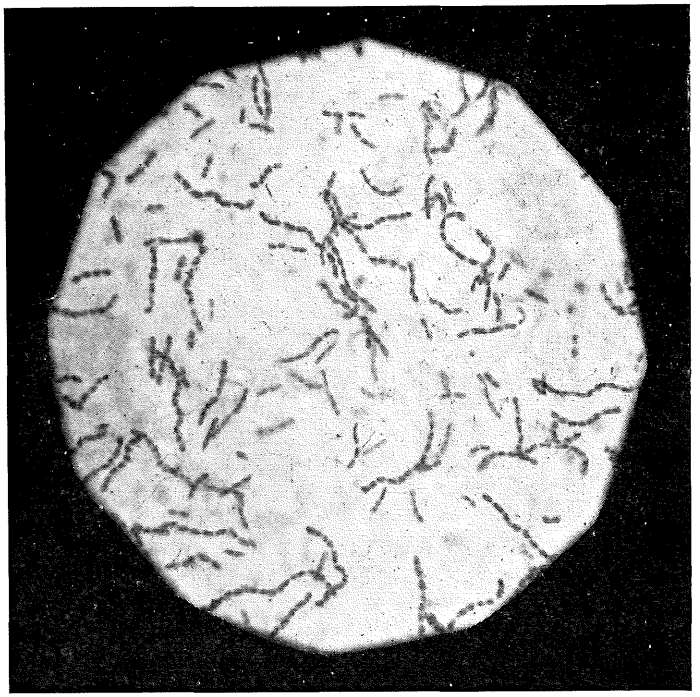

Fig. 2.—Resistant strain (C3) grown in pasteurized milk without antibody. Smear made of skim layer. Without agglutination the organisms remain evenly distributed. Sudan IV and methylene blue. X1140.

(a) Methods

Milk was recombined, using 3 parts of sterilized skim-milk to 1 part of cream from pasteurized milk. It was found that this cream had to be heated at 76°C for 30 min in order to destroy any inhibitory properties attached to the fat globules, which were found to interfere with the tests. Activity tests were set up, using a susceptible strain, so that one test acted as a control while the other was inoculated with homologous antibody. A parallel series of tests was set up, using sterile skim-milk only. Microscopic checks were made for evidence of agglutination in the tests containing antibody.

(b) Results

Results of the tests are given in Table 4.

Acidity production by the susceptible strain in sterile skim-milk was unaffected by the presence of antibody, even though there was visible evidence of agglutination (shown by microscopic examination). However, the presence of antibody in recombined milk caused a decrease in acidity production; this was found to be due to the aggregation of the susceptible strains in the cream. This

Table 4

PERCENTAGE ACIDITY PRODUCED BY A SUSCEPTIBLE STRAIN (HP) IN RECONSTITUTED MILK CONTAINING HOMOLOGOUS ANTISERUM

Milk	Percentage Acidity Produced by Susceptible Strain
Sterile skim-milk	·45
Sterile skim-milk + antibody ..	·44
Recombined milk	·45
Recombined milk + antibody ..	·37

aggregation and the degree of inhibition, however, did not appear to be as marked as would be expected in pasteurized wholemilk. It is possible that this was due to the dilution of the antibody or alternatively the denaturation of the fat globule membrane by heat treatment, which may have interfered with the carrying-up of the organisms. This latter possibility is unlikely in view of the results previously obtained with heated cream and unheated skim-milk (Wright and Tramer 1957). It must be pointed out that the presence of skim-milk is a possible complicating factor.

V. DISCUSSION

As the susceptible strains were found to be rough in type while the resistant strains were smooth, any agglutination of the susceptible organisms would probably be non-specific.

In spite of the difficulties encountered in testing for the presence of agglutinins to the susceptible strains in the milk supplies, there is some evidence from both the direct microscopic test and the ring test which points to their presence. On the other hand, all tests used (agglutination, precipitation and ring tests) have consistently failed to show the presence of antibodies to any of the resistant strains in the milk supplies.

The agglutination test, although of limited use owing to the auto-agglutination of the susceptible strains, was still of value in estimating the antibody content of antiserum prepared against a susceptible strain. The results of agglutination tests (using various dilutions) differ from those obtained by Portmann and Auclair (1959), who obtained positive slide agglutination tests when several starter strains were used.

Because the agglutination tests were negative it would be expected that the precipitation tests would give similar results. This was found to be the case.

The use of the direct microscopic test for the study of the presence of agglutinins has, however, a very limited value, due to the fact that all long-chain organisms have a tendency to clump irrespective of the medium of growth. In

the case of the long-chain resistant strains, it has been proved that the increased clumping in pasteurized milks bears no relation to the presence of agglutinins. With the short-chain susceptible strains, it is likely that clumping in pasteurized milk is indicative of agglutination.

Although the specificity of the ring test is open to question in view of the fact that "rough" antigens were used, it is significant that negative results were obtained in sterilized milk even though the same antigens were employed. It is also of interest that McPhillips (1958) presented evidence demonstrating the specificity of the agglutination of "slow" starter cultures.

In this present work it was possible to bring about inhibition of the resistant strain by the addition of homologous antibody to raw or pasteurized wholemilk and this is proof in itself that specific antibodies are capable of bringing about an inhibition which is similar in every way to that normally occurring with the susceptible strains in pasteurized milk. These results were confirmed by microscopic evidence because the resistant strain plus homologous antiserum was agglutinated and carried up with the cream layer in the same way as a susceptible strain grown in pasteurized milk (without added antiserum). The similarity is even more marked when it is considered that the behaviour of a susceptible strain in the presence of homologous antibody shows the return of slight inhibition in milk reconstituted from fractions heat-treated to destroy inhibition.

It is also of interest that the addition of homologous antiserum to sterile skim-milk containing a susceptible strain caused marked visible agglutination but at the same time did not bring about any inhibition in acid production by the susceptible strain. Inhibition was only apparent when a cream layer was present. These results indicate that agglutination in itself is not responsible for inhibition and confirm conclusions reached by Wright and Tramer (1957) and Keogh (1958).

The agglutination in milk and whey of such a wide spectrum of bacteria as has been mentioned in the relevant literature has caused some speculation and has not been satisfactorily explained. Recently Portmann and Auclair (1959) found a constant relationship between the inhibition and agglutination of certain strains of *Streptococcus cremoris* and they suggested that both may be due to the same cause. Most workers, including Hobbs (1939), Rosenau and McCoy (1908) and Chambers (1920), have assumed that agglutination is non-specific. Indeed, it seems quite likely that many of the recorded instances of agglutination are merely the result of non-specific immunity occurring in the cow. Nevertheless, there is no doubt that the presence of agglutinins to such organisms as *Brucella abortus* and the pyogenic streptococci are the result of active infection. Although the results herein show that the positive ring test obtained with the lactic streptococci was not the result of cross-agglutination with *Brucella abortus* antibodies, the possibility exists that there may well be shared antigens between these strains of *Streptococcus cremoris* and with other Streptococci.

Although it is evident that the presence in milk of specific antbiodies to resistant and susceptible strains can result in their inhibition, there is no conclusive proof available at present that the inhibition of the susceptible strains as studied is due solely to antibodies (specific or non-specific).

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