ROLE OF THE FAT GLOBULES IN THE INHIBITION OF CERTAIN STRAINS OF *STREPTOCOCCUS CREMORIS* IN PASTEURIZED MILK

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SUMMARY

Removal of the fat from pasteurized wholemilk either by centrifugation or by gravity resulted in a skim-milk fraction in which susceptible strains produced greater acidity than in pasteurized wholemilk but to a lesser extent than in sterilized milk.

The cream fraction was found to be markedly inhibitory to all strains and it has been possible to transfer this inhibition to sterilized skim-milk by the addition of cream. The amount of cream added, however, greatly modified the inhibitory level. It appears likely that two separate factors were operating in the inhibition brought about by cream. The first factor, occurring with high dilutions of cream, was active only against the susceptible strains, while the other, active at low dilutions of cream, was inhibitory to both groups of organisms. This factor was probably due simply to a shortage of bacterial nutrients.

Resuspension of cream in skim-milk and water followed by subsequent inoculation with susceptible and resistant strains showed that in both cases the susceptible cells rose with the fat globules and remained in the cream layer. The resistant strain on the other hand was uniformly distributed between both layers.

Prevention of milk creaming by constant rotation of the tests resulted in complete loss of inhibition, so that both resistant and susceptible strains produced similar quantities of acid.

The inhibitory factor was washed out of the cream fraction by three washings with sterile water. The inhibitory factors in skim-milk could be removed by adsorption onto the washed cream.

I. INTRODUCTION

Recently attention has been focussed on the correlation between the creaming of milk and the inhibition of certain organisms, including starters, when this phenomenon takes place. This observation was first made by Wright and Tramer (1957). The following year Keogh (1958) proposed the hypothesis that the effect of creaming was a physical one, removing the agglutinated organisms from the bulk of the milk. In view of these results there seemed to be some need to investigate the actual role of the fat globules in the inhibition of starters affected by cream rising. A study of the resulting inhibition whether real or apparent (i.e. lack of nutrients) was a natural outcome of such an examination.

Some of the starters used were "susceptible" to an inhibitory factor in wholemilk, but which displayed good acidity production when creaming was prevented by the addition of rennet or agar, and some which were "resistant" to this factor.

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In every instance, acid production of cultures was measured by the activity test (Anderson and Meanwell 1942). In this test the test medium is inoculated at the 1 per cent. level with starter and incubated for 6 hr at 30°C. Acidity was determined by titrating 9 ml with ·1N NaOH, using phenolphthalein as indicator.

II. FAT CONTENT AND THE INHIBITORY PROPERTY

The correlation between fat content and the inhibitory property has been studied from three angles:—(1) An examination of the acidity production by these starters in skim-milk; (2) an examination of these organisms when grown in milk to which various quantities of cream has been added; and (3) prevention of formation of a cream layer.

(a) Methods

Separation was accomplished in several ways.

For separation by centrifugation, a portion of milk which had previously been found to be inhibitory was centrifuged at 3,000 r.p.m. on a Christ angle-head Junior 0 centrifuge for half an hour. The cream layer obtained was carefully removed and the skim-milk tested for inhibitory properties. Separation was also effected using a small dairy separator. Inhibitory milk was heated to 37°C and then separated. The milk portion was retained.

In separation by gravity creaming, pasteurized milk was dispensed in separating funnels in 160 ml quantities and refrigerated for various lengths of time to allow the cream to separate. At the end of each time interval the volume of the cream fraction was measured and the fat content of the skim-milk fraction determined. The skim-milk was retained for testing.

In each of the above cases, activity tests were set up, using both susceptible and resistant strains, and acidity determinations recorded after incubation for 6 hr at 30°C.

Tests were also set up in sterilized skim-milk containing various concentrations of cream. Activity tests were then carried out, using both susceptible and resistant strains.

(b) Results

It is apparent from the results in Table 1 that centrifugation and machine separation both gave a skim-milk which supported acidity production by the susceptible strains to a much greater extent than whole pasteurized milk. Both means of separation were equally effective in reducing the inhibitory potency. The resistant strains, however, produced approximately equal quantities of acid in either skim-mlik or wholemilk.

It appears also that allowing milk to separate by gravity resulted in a product which was somewhat less inhibitory than the original milk. There would seem to be a correlation between the degree of inhibition and the amount of fat present in the milk. The gravity-formed skim-milk obtained after 24 hours' storage contained the lowest concentration of fat and resulted in the highest degree of acidity formation by the susceptible strains.

Table 1

PERCENTAGE ACIDITY PRODUCED BY STRAINS OF STREPTOCOCCUS CREMORIS IN PASTEURIZED WHOLE-MILK AND THE SAME MILK PARTIALLY SKIMMED

(*a*) *Skim-milk obtained by centrifugation*

(1) By centrifuging in a Christ Laboratory centrifuge—3000 r.p.m.

Starter	Percentage Acidity in Wholemilk	Percentage Acidity in Skim-milk
HP (susceptible strain) ..	·30	·44
R6 „ „	·31	·47
C1 (resistant strain) ..	·56	·59
C13 „ „	·48	·47
Uninoculated control ..	·20	·19

(2) By passing through a small dairy separator

Starter	Percentage Acidity in Wholemilk	Percentage Acidity in Skim-milk
R6 (susceptible strain) ..	·28	·44
C13 (resistant strain) ..	·55	·56
Uninoculated control ..	·18	·18

(*b*) *Skim milk obtained by gravity separation of cream by storage at refrigeration temperatures for periods up to* 24 *hours*

Duration of Storage (hr)	Fat Content of Skim-milk Fraction (%)	Volume of Cream Layer (%)	Percentage Acidity in Skim-milk	
			Susceptible Strain R6	Resistant Strain C13
0	3·9	·6	·21	·44
2	3·9	5·0	·22	·44
3	3·4	5·0	·22	·44
4	3·2	5·0	·22	·44
5	3·1	5·0	·22	·44
6	3·1	5·75	·23	·44
24	2·4	6·25	·29	·44

This correlation between fat content and acidity production takes more point when it is considered that centrifugation resulted in milks containing approximately 0·4 per cent. fat.

An examination of Figure 1 shows a marked difference in the behaviour of resistant and susceptible strains in response to increasing cream concentration. When a small proportion of cream was added to sterilized skim-milk the susceptible strains were inhibited, but the resistant strains showed no effect. As the proportion of cream to skim-milk was increased, the susceptible strains were further inhibited, reaching a maximum degree of inhibition (minimum acidity production) at 10 per cent. The maximum was maintained until 25 per cent. concentration of cream had been attained. With further increases in the proportion of cream the degree of inhibition with the susceptible strain was reduced and was at its lowest at 75 per cent. cream. In the case of the resistant strain, increases in cream concentration produced first a slight stimulation, then inhibition, and finally marked inhibition at concentrations exceeding 75 per cent. When the concentration of cream reached 100 per cent., both susceptible and resistant strains were equally and strongly inhibited. These results therefore show that, under the conditions of the experiment, the degree of inhibition changed with the percentage of cream in the mixture.

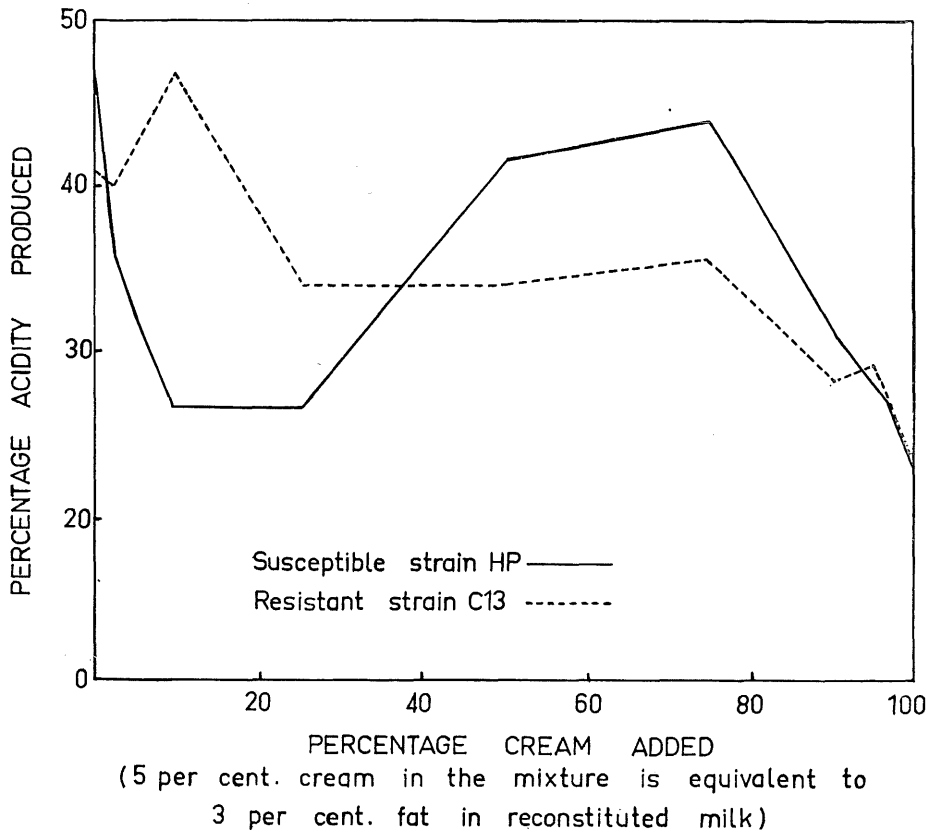

Fig. 1.—Effect of increasing cream concentration on resistant and susceptible strains.

It is pointed out that additions of cream at a level of 5–8 per cent. to sterilized skim-milk yields milk with a fat content approximating that of normal milks.

III. PREVENTION OF MILK CREAMING BY CONSTANT ROTATION OF TESTS

Wright and Tramer (1957) were able to show that homogenization and inversion of the tubes every 30 min resulted in increased acidity production. An experiment was therefore devised to completely prevent the formation of a cream layer.

(a) Methods

Two 100 ml portions of pasteurized milk were inoculated at the 1 per cent. level, one with a susceptible strain and the other with a resistant strain. After thorough mixing each milk was pipetted into a wide-necked 1 oz McCartney bottle so that each bottle was completely filled. In order to prevent inclusion of air at the top of each container, a rubber stopper fitted with a piece of glass tubing and stopcock was placed in the neck of each bottle. The excess milk then flowed into the glass tubing, carrying away any residual air. After all the tubing was filled with milk the stopcock was closed. The stoppers were then securely tied down to the bottles. Both bottles were clamped down to a revolving spindle travelling at 10 r.p.m. Because of the absence of an air bubble, no agitation of the milk occurred during inversion. Both samples were allowed to rotate for 6 hr at room temperature (26-28°C). Another two bottles were set up as controls without constant inversion. At the end of 6 hr, smears were made and the acidity percentage of each milk determined.

(b) Results

The results of two separate tests are set out in Table 2.

It is obvious from these results that inhibition of the susceptible strains occurred only when a cream layer was allowed to form on the top of the milk and not when the test bottles were constantly inverted to prevent creaming.

Table 2

ACIDITY PRODUCTION OF "SUSCEPTIBLE" AND "RESISTANT" STRAINS IN MILKS ALLOWED TO CONSTANTLY ROTATE DURING A 6-HOUR INCUBATION PERIOD AT ROOM TEMPERATURE (26–28°C)

	Susceptible Strains		Resistant Strains	
	HP	R6	C13	C3
Bottles constantly revolving to prevent formation of cream layer	·54	·40	·44	·45
Bottles allowed to stand to facilitate the formation of cream layer	·22	·22	·40	·47

Smears made from one set of tests (using HP and C3) which had been allowed to rotate revealed that the susceptible strain (HP) was agglutinated and that large numbers of the organisms were congregated around the fat globules. It was also noted that individual chains were situated around fat globules. However, the resistant strain (C3) showed no evidence of agglutination. These results show that agglutination in itself is without effect on the susceptible strain. Inhibition is manifest only with the formation of a cream layer when the cells are taken up with the rising fat globules.

It is to be noted, moreover, that in the bottles constantly inverted, agitation and aeration of the milk did not occur because all air had been removed. In these tests the pasteurized milk had been stored in a refrigerator for 48 and 72 hr respectively prior to testing, so that clumping of the fat could be expected to have taken place.

The results obtained here agree with those of Wright and Tramer (1957) but go further in that external influences such as aeration and agitation are completely eliminated.

IV. ASSOCIATION OF "INHIBITORY" FACTOR WITH THE CREAM LAYER

There are indications that the factor causing the concentration of the susceptible strain in the cream layer must in part, at least, be associated with the fat globule; this assumption is supported by the fact that additions of cream to sterilized milk reduced the acidity production of the susceptible strains. Tests were therefore set up to determine whether the susceptible organism rose with the cream when resuspended in water.

(a) Methods

Pasteurized milk was collected and stored in the refrigerator for 24 hr prior to use. The milk was skimmed in a dairy separator and the cream and skim portions retained. Four sterile separating funnels were filled with 140 ml of sterile tap water and four with 140 ml of skim-milk; 14 ml of cream were added to each of the separating funnels. All funnels were inoculated with starter at the rate of 1 per cent., the contents thoroughly mixed and the plate counts done on each one immediately. The funnels were incubated for 6 hr at 32°C and the plate counts and acidity titrations were then performed. Two funnels from each pair were used as controls.

(b) Results

The results are set out in Tables 3 and 4.

Table 3

DISTRIBUTION OF STARTER ORGANISMS FOUND IN GRAVITY CREAM AND SKIM-MILK WHEN AN INOCULATED RECOMBINED MILK IS STORED FOR 6 HOURS AT 30°C

Strain	Pre-Storage Initial Count (x 10^6)	After 6 Hours Storage at 30°C				Percentage Volume Cream
		Percentage Acidity		Final Count (x 10^6)		
		Skim	Cream	Skim	Cream	
Susceptible Strain—R6 ..	1·4	·25	·36	20	162	2·8
Susceptible Strain—R6 ..	1·5	Milk after remixing—·25		Milk after remixing—6		
Resistant Strain—C3 ..	9·0	·68	·41	640	292	2·8
Resistant Strain—C3 ..	6·0	Milk after remixing—·55		Milk after remixing—492		

Table 4

DISTRIBUTION OF STARTER ORGANISMS FOUND IN GRAVITY CREAM AND THE AQUEOUS PHASE WHEN AN INOCULATED SUSPENSION OF CREAM AND WATER IS STORED FOR 6 HOURS AT 30°C

Strain	Pre-Storage Initial Count (x 10^6)	After 6 Hours Storage at 30°C				Percentage Volume Cream
		Percentage Acidity		Final Count (x 10^6)		
		Aqueous Phase	Cream	Aqueous Phase	Cream	
Susceptible Strain—R6 ..	·7	·04	·11	1·2	18	15
Susceptible Strain—R6 ..	1·1			After remixing 7		
Resistant Strain—C3 ..	7	·04	·11	18	21	15
Resistant Strain—C3 ..	8			After remixing 22		

It is evident that the susceptible strain was carried up with the rising fat whether it was suspended in skim-milk or in tap water. The resistant strain was nearly equally divided between the aqueous phase and the cream. However, the cream volume in water was approximately five times as great as in skim-milk. Another interesting feature of the results is that the susceptible strain behaved in exactly the same manner in cream and skim-milk recombined as in wholemilk.

Table 4 shows that the development of acidity in the cream layer was very slow for both susceptible and resistant strains. This result confirms that already obtained when both strains were grown in cream, and it seems probable that the growth of organisms in a complete medium of cream involves a separate investigation.

The explanation of the cream rising in tests where it was recombined with water seems to be due to the fact that the milk prior to skimming had been standing for 24 hr, which was sufficiently long for the fat globules to have clumped and risen to the surface of the milk. Once this has happened, it is probable that

even after stirring and skimming at 37°C the fat globules were aggregated together and therefore rose to the surface of the liquid whether the cream had been resuspended in milk or water.

It was found that if the milk was heated to 55°C prior to separation, the cream did not rise to form a layer on the top of the aqueous phase. This observation, of course, further confirms the conclusions of Sharp and Krukovosky (1939).

V. ELIMINATION OF THE INHIBITORY SUBSTANCE FROM CREAM BY WASHING

As the inhibitory factor of milk is associated with the fat globule, it seemed possible that a substance causing inhibition of the susceptible strains might be adsorbed on the fat globule membrane. Isolation of the fat globule membrane involves its separation from the aqueous phase of the milk (plasma proteins, lactose and salts) and from the material contained inside the fat globules. The first aim is achieved by washing the fat globules with water and the second by extraction with fat solvents. Preliminary investigations showed that the inhibitory factor could be separated from the cream merely by washing with water. This section contains a description of this process and its implications.

Several workers (Rimpila and Palmer 1935; Jack and Dahle 1937; and Brunner, Duncan, and Trout 1953) have shown that most of the milk plasma protein is washed out of the cream after the first washing. After the third washing practically all the plasma protein has been diluted out of the cream and any further lowering of the protein: fat ratio is insignificant. Rimpila and Palmer (1935) and Heinemann (1939) found that the decrease in the phospholipid content after the first washing is not so pronounced, being about 80 per cent. of the original content. After the third washing, however, both protein and phospholipid contents remain almost constant.

Palmer (1944) pointed out that the agitation and dilution effect involved in the washing of cream probably washes out all but the most tenaciously adsorbed materials from the fat.

It is noteworthy, also, that the standard methods for the isolation of membrane material, as reported by King (1955), stipulate six washings of the cream prior to further treatment for the isolation of the membrane material.

(a) Methods

Pasteurized milk was collected and separated by passing it through a small dairy separator at 37°C. The cream fraction was retained. This was washed by two methods as follows:—

(1) A portion of cream was diluted with an equal quantity of sterile tap water and both fractions were mixed by gently rotating the flask, which was then

heated to 37°C. The contents of the flask were then separated and both cream and the washings retained. A sub-sample of approximately 20 ml was taken from the cream and the remainder was again washed twice more in the manner described above. Sub-samples of the cream were again withdrawn after each of these washings. Each wash water was retained.

(2) A portion of the cream was diluted with an equal quantity of sterile tap water and placed in a separating funnel in the refrigerator. After a few hours a sharply defined cream layer had formed and the aqueous phase was withdrawn and retained. A sub-sample of cream was taken (to do this the cream had to be warmed to 37°C). The cream was washed twice more and sub-samples of the cream after each washing retained. Each wash water was also kept. It had previously been found that additions of cream from 10 to 25 per cent. to sterilized milk resulted in a maximum inhibition of a susceptible culture. Therefore both 10 and 25 per cent. additions of each cream were made to sterilized milk. At the same time, tests were set up wherein similar amounts of wash water were added to the sterile milk. All tests were inoculated with a susceptible strain (R6) at the 1 per cent. level and the usual activity tests performed.

Microscopic examinations were also made of the skim and cream layers of the tests containing the creams after successive washings, using long-chain strain R6 and short-chain strain R1. Smears were also made of the tests containing the washings, which were stained and examined. These smears were simply stained with Newman's stain.

(b) Results

The results are set out in Table 5. In this table, there are indications that the inhibitory factor can be washed out of the cream. One washing was insufficient to remove any of the inhibitory properties. However, after a second washing the cream appeared to be less inhibitory. The third washing resulted in a cream which when added to skim-milk caused stimulation of the susceptible strain at concentrations of cream at the 10 and 25 per cent. levels, although stimulation was not quite as marked with the test containing 25 per cent. of cream as with that containing 10 per cent.

The table also shows that the washings may contain the inhibitory properties when added to sterile skim-milk. The first washing was actually stimulatory, whereas the second contained inhibitory factors to a marked extent. The third washing, on the other hand, had no effect on the acidity production by the susceptible strain. Washings obtained by both methods gave similar results.

Microscopic examinations of washed creams following resuspension with sterile skim-milk and inoculated with susceptible strains confirmed the above results. The cream layer which formed when the cream after one washing only

Table 5

PERCENTAGE ACIDITY FORMATION OF A "SUSCEPTIBLE" STRAIN IN STERILE MILK CONTAINING (*a*) CREAM BEFORE AND AFTER EACH OF THREE SUCCESSIVE WASHINGS WITH STERILE TAP WATER AND (*b*) EACH CREAM WASHING

—	Percentage Acidity Susceptible Strain R6	—	Percentage Acidity Susceptible Strain R6
Sterile skim containing 25% cream ..	·28	Sterile skim containing 10% cream	·28
Sterile skim containing 25% cream (after 1st washing)	·28	Sterile skim containing 10% cream (after 1st washing)	·28
Sterile skim containing 25% cream (after 2nd washing)	·35	Sterile skim containing 10% cream (after 2nd washing)	·44
Sterile skim containing 25% cream (after 3rd washing)	·45	Sterile skim containing 10% cream (after 3rd washing)	·50
Sterile skim—	·40		
Sterile skim containing 25% .. 1st wash-water	·42	Sterile skim containing 10% .. 1st wash-water	·52
Sterile skim containing 25% .. 2nd wash-water	·26	Sterile skim containing 10% .. 2nd wash-water	·37
Sterile skim containing 25% .. 3rd wash-water	·40	Sterile skim containing 10% .. 3rd wash-water	·41
Sterile skim containing 25% sterile tap water—control	·40	Sterile skim containing 10% sterile tap water—control	·40

Fat Content First wash-water ·9% ; second wash-water ·25% ; third wash-water ·25%.

was used contained most of the organisms, but the skim-milk contained relatively few. However, when creams were used following the second and third washings, only a few organisms were found in the cream layer and the majority remained in the skim-milk. There was no evidence of agglutination of the short-chain strain in any of the tests but interpretation of agglutination with the long-chain strain was obscure.

VI. ELIMINATION OF INHIBITORY FACTORS FROM SKIM-MILK BY ADSORPTION ONTO WASHED CREAM

As the inhibitory factor may be washed out of the cream, it seemed likely that addition of such washed cream to skim-milk would result in some of the inhibitory factors present in the skim-milk being adsorbed onto the washed cream. This possibility is explored in this section.

(a) Methods

Milk was skimmed and both skim and cream fractions were retained. The cream was washed three times and separated after each washing. This washed cream was then added to the skim-milk and left in the refrigerator overnight. The following morning the milk was warmed to 37°C and separated. Both fractions were retained.

Activity tests were set up, using two susceptible strains. Acidity developed by each was estimated in the original skim-milk and skim-milk obtained after recombining with washed cream. Reconstituted milk was formed from the different combinations of skim-milk and cream.

(b) Results

The results are set out in Table 6. They indicate that the inhibitory factor in milk is adsorbed onto the fat globules. Such adsorption is reflected in the increased acidity production by both strains in skim-milk obtained after such adsorption had been allowed to take place. It appears also that inhibitory properties in the skim-milk primarily determine the acidity production of the recombined milk. However, as is seen by the recombination with the adsorbed cream, both fractions exert a cumulative action.

Table 6

DISTRIBUTION OF INHIBITORY FACTORS IN SKIM AND CREAM FRACTIONS AFTER RECOMBINING SKIM AND WASHED CREAM

Milk	Percentage Acidity Produced in Susceptible Strains	
	HP	R6
Skim-milk	·35	·31
Skim-milk A	·50	·40
Skim-milk containing 10% washed cream	·30	·35
Skim-milk containing 10% cream A	·28	·30
Skim-milk A containing 10% washed cream ..	·38	·37
Skim-milk A containing 10% cream A	·35	·31

Skim-milk A—obtained after recombining skim-milk with washed cream.
Cream A—obtained after recombining skim-milk with washed cream.

VII. DISCUSSION

It is of interest that skim-milk obtained by gravity and centrifugal separation was less inhibitory than the original product. This indicates that the inhibition has been partially removed with the fat.

On the other hand, as demonstrated by Wright and Tramer (1957), when milk was recombined following heat treatment of either or both fractions, it was the skim-milk fraction which determined the final degree of inhibition of the susceptible strains.

Jago (1954) was the first to note that the inhibitory substance was closely associated with the fat globule. However, he added cream at the rate of 1–5 per cent. to sterilized milk. He therefore did not discover the presence, shown herein, of a stimulatory zone followed by a second inhibitory level when increasing concentrations of cream were added. Both resistant and susceptible strains are affected by the second inhibitory zone, which is probably the result of a nutritional deficiency in the cream.

The presence of stimulatory factors in milk has been noted on a number of occasions. Jago (1954) found that separator slime was stimulatory to "susceptible" starter strains studied by him. Czulak and Meanwell (1951) merely presented an hypothesis that presence or absence of stimulatory substances in the milk determined "winter slowness" of cheese starters. Although in this present work it has been possible to stimulate susceptible strains in milk by addition of separator slime, it seems unlikely that sufficient of these substances would still be in the cream to account for the stimulatory level.

It is clear from the results that any change in the potency of the inhibitory factor in the milk parallels, at least in part, changes in the fat content of the milk. However, it is likely that some other factor is involved also. This is shown by the fact that violent agitation of inhibitory milk resulted in complete destruction of the inhibitory factor only after maximum degree of churning had been reached (Gillies 1959).

There appears to be little doubt that the physical state of the milk plays an important role in determining the inhibitory nature of pasteurized milk. Although Wright and Tramer (1957) would appear to be correct in this regard, their theory regarding the cause of inhibition seems to have little justification.

It is also noteworthy that constant rotation of wholemilk resulted in the removal of the inhibitory properties even though there was considerable evidence of gross agglutination of the susceptible strains. Therefore, if agglutination is concerned with the inhibitory phenomenon, it must only be when a cream layer is allowed to form.

Because the susceptible strains are congregated in the cream layer of unagitated wholemilk even after one hour (Wright and Tramer 1957), it is likely that the organisms are attracted to the surface of the fat globules as they rise to form the cream layer. Further proof of such an attraction is available, as the susceptible strain rose with the cream when suspended in water.

As it has been found possible to wash an inhibitory substance from the cream after the second washing with tap water or saline, it is impossible to consider such a substance as an integral part of the fat globule membrane. In the classical methods of examining the fat globule membrane substances, as many as six preliminary washings have been employed. It is likely that there is a loose linkage between the inhibitory substance and some component or components of the fat globule membrane, which linkage is destroyed by washing with tap water or isotonic saline. It seems probable that a milk serum protein is involved as an essential component leading to inhibition, this component becoming enmeshed with the fat globules as they rise to form a cream layer. It is clear, however, that some bonding stronger in nature than a mere enmeshment is involved, because marked inhibition was found to occur when cream was added

to sterilized skim-milk. In addition, simple enmeshment suggests that this material would be at least partially removed from the fat globule membrane by the first washing, whereas in fact it was not removed until the second washing.

It has been possible to transfer the inhibitory property to sterile skim-milk by the addition of cream washings. From these results it is possible to propose that inhibition is due to the presence of at least one chemical substance and is not a result of overcrowding or lack of nutrients when a cream layer is formed. As the fat content of the washings was as low as 0·2 per cent., it is impossible to correlate inhibition with the presence of a cream layer when such washings are added to sterile skim-milk. It is also of interest that there was no evidence of agglutination when a short-chained susceptible strain was grown in such mixtures. The latter observation indicated that agglutination is quite independent of this type of inhibition.

The inhibitory property of milk for the susceptible strains appears to be a complex one and is most evident when the organisms are grown in unagitated wholemilk. There are numerous indications that the inhibitory properties of milk are associated with both cream and skim fractions.

Skim-milk itself is much less inhibitory than wholemilk. Such a modification can be thought to be correlated with the great reduction in fat content. Nevertheless, there are indications that the skim fraction contains a potential inhibitor which determines the final degree of inhibition in wholemilk. There is much evidence for such an hypothesis. For example, the degree of heat treatment of the skim-milk fraction, not that of the cream, determined the degree of inhibition of the susceptible strains in whole recombined milk. These results were paralleled when washed cream was added to skim-milk.

On the other hand, there is ample evidence available demonstrating the inhibitory properties of the cream fraction. This is shown most dramatically when cream is added to sterilized milk and also by the fact that the inhibitory property can be removed from cream by mere washing with water. Further indications that the bacterial cells are attracted to the fat globules are provided when cream is resuspended in water. Such a mixture gives results which show quite definitely that the susceptible strains are attracted to the fat globules and that they will rise with the cream in the absence of skim-milk.

Although the presence of a cream layer is necessary for inhibition of a susceptible strain, the mechanism of inhibition is not clear. The cells of the susceptible strain rose continually as the cream layer formed, but the final bacterial numbers in the cream were comparable with those of the resistant strain. It is probable that agglutination was responsible for such plate counts in the cream layer. Other possible causes of lowered bacterial numbers are that the numbers of the susceptible bacteria in the cream layer exceeded the M-concentration, and

that the inhibition was associated with the unavailability of nutrients. Nevertheless, it is difficult to reconcile these theories with the fact that an active inhibitory factor was washed out of the cream and that this when added to sterile skim-milk was found to cause inhibition of the susceptible strains. It is also noteworthy that there was no evidence of agglutination when a short-chained susceptible strain was grown in such mixtures. Therefore, it is concluded that agglutination is not necessarily an integral part of inhibition but rather tends to accompany it.

To sum up: skim-milk would appear to donate a factor which combined with another on the fat globule membrane can result in inhibition if creaming is permitted. Both are necessary for maximum inhibition. Creaming if permitted allows maximum interaction between the two. The addition of cream washings to sterile skim-milk merely permits the interaction without the necessity of creaming.

VIII. ACKNOWLEDGEMENTS

The author is indebted to Mr. V. R. Smythe (Senior Bacteriologist, Dairy Research Branch, Department of Agriculture and Stock) for his advice and interest in this work.

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(Received for publication September 27, 1960)