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SOME OF THE RELATIONSHIPS AMONG THE ORGANISMS IN BUTTER CULTURES

BY

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INTRODUCTION

The results of various investigations show that satisfactory butter cultures are not pure cultures of Streptococcus lactis but that a second type capable of fermenting citric acid with the production of certain volatile acids is also present. This second type includes organisms of at least two species, Streptococcus citrovorus and Streptococcus paracitrovorus, and is often referred to in discussions of the bacteriology of butter cultures as the "associated organisms".

In the fermentation taking place during the ripening of butter cultures each of the two types of organisms is responsible for certain changes and the production of a desirable flavor and aroma is the result of an associative action. It is obvious that under these conditions, factors that affect the growth of either type will also affect the quality of the butter cultures produced.

The studies that have been made on the bacteriology of butter cultures have, for the most part, dealt with factors which influence the quality but they have not been approached from the angle of their effects on the growth of the individual types of organisms present. This is because there has been no satisfactory method available for the study of the

relative numbers of these types. Morphologically, S. lactis and the associated organisms are not distinguishable and butter cultures appear as pure cultures when stained preparations are examined. The other characters of the two types of organisms are also alike to the extent that the organisms cannot be identified without being isolated and observed in pure culture. The difficulties of distinguishing between them was undoubtedly a factor in the failure of early investigators to recognize the presence of the two types in butter cultures.

Problems concerned with the development of new butter cultures have to do with obtaining and maintaining the correct growth balance between the two types of organisms. Defects, such as off flavors, lack of flavor or slow coagulation, often occur in butter cultures that are being transferred regularly and an understanding of these would be greatly helped by information concerning the growth relationships of the types in satisfactory and in unsatisfactory cultures.

In creameries where conditions for transferring butter cultures are not the best, cultures often become unsatisfactory and it is necessary to secure fresh cultures from some laboratory. Such a laboratory may be located at a considerable distance from the creamery and this means the cultures must be in the mail for from one to several days during which time they may be subjected to temperature conditions unfavorable for holding. Packing in ice or in containers designed

to maintain low temperatures during shipment is rather expensive and has not been practiced to any extent. A method which would reduce deterioration in butter cultures during periods of holding as during shipment, would be desirable for several reasons. Such a method would permit the maintainance of a number of butter cultures in laboratories with less work and expense. Butter cultures that are being carried but are not needed for immediate use would not require regular transferring. Such a method would also permit butter cultures to be stored in creameries for a certain time without appreciable deterioration. This would be helpful because the butter cultures would be available when most needed. As it is, in many plants new butter cultures do not arrive when it is most desirable to have them.

STATEMENT OF PROBLEM

The work herein reported is divided into two parts. Part I. is a study of the numbers of the two types of organisms present in butter cultures under various conditions. It involves first, attempts to find a method for determining the numbers of each of the two types of organisms present, and, second, studies on the variations occurring in the growth relationships of the organisms in butter cultures under different conditions, including those existing when new cultures are being developed. Part II. involves studies on the effect of the addition of calcium carbonate to milk on the keeping qualities of butter cultures made from it.

PART I.

STUDIES ON THE NUMBERS OF EACH OF THE TWO TYPES OF ORGANISMS PRESENT IN BUTTER CULTURES UNDER VARIOUS CONDITIONS

HISTORICAL

The lack of knowledge on the part of the early investigators concerning the presence of the associated organisms in butter cultures suggests that S. lactis is normally present in the larger numbers.

Hammer and Bailey (11) observed that by gradually diluting a butter culture a quantity of culture could be secured which would coagulate milk but would give only a low volatile acidity. This indicates that the organism responsible for a high volatile acidity had been diluted out and accordingly that Bacterium lactis acidi was present in the larger numbers.

Hammer (10) in studying the comparative numbers of the two types plated butter cultures on whey agar and picked colonies into litmus milk, later classifying the organisms on the basis of the changes produced. The results showed that S. lacticus constituted from 63 to 94 per cent of the flora while the associated organisms constituted from 6 to 37 per cent. Pickings made from butter cultures carried by different experienced persons gave similar results, the S. lacticus

varying from 87 to 99 per cent and the associated organisms from 1 to 13 per cent.

Orla-Jensen, Orla-Jensen and Spur (19) studied the numbers of the two types by plating on litmus gelatin on the theory that colonies of associated organisms would not produce as much acid as would colonies of S. lactis and could therefore be distinguished. This method failed because the differences were not great enough to be useful. These investigators also used a casein peptone gelatin, with yeast extract, on the assumption that the growth of the associated organisms would be favored to the extent that the colonies could be distinguished but this also was unsatisfactory. They concluded that the best way to study the numbers was to plate the butter cultures and to pick large numbers of colonies and study these in detail.

It was pointed out at an early date by Conn (6), (7), (8), and Weigmann (21) that pure cultures would not produce the flavors wanted in ripened cream. Attempts to develop flavor and aroma in dairy products by combining different species of bacteria were made by Marshall and Ferrand (16) and Evans, Hastings and Hart (9) but the combining of the two types of organisms in a systematic way was not possible until later when the organisms associated with S. lactis in butter cultures were definitely known.

Hammer and Bailey (11) isolated organisms from butter cultures which in combination with Bacterium lactis acidi would

produce volatile acidities approximately those of satisfactory butter cultures. They referred to organisms of this type as "associated organisms". These investigators suggested, however, that all cultures of Bacterium lactis acidi are not equally adapted for use in combinations of this kind. They assumed that this should be expected because other variations existed, for example, variations in the ability of different cultures to produce ropiness as a result of associative action.

Storch (20) also reported that all strains of Streptococci and Betacocci are not equally adapted for living in symbiosis. He observed that pure cultures of Streptococci will apparently become contaminated under natural conditions with the aroma producing organisms and that a high volatile acid will result.

Boekhout and Ott de Vries (5) studied five cultures of aroma forming bacteria and three of lactic acid organisms. Three of the aroma organisms gave good results when combined with any of the lactic acid organisms while the other two gave good results with only one. In regard to methods of combining the two types of organisms these investigators reported that mixing 30 cubic centimeter cultures of each gave good results; also, that inoculating the lactic acid organisms with a needle into a 30 cubic centimeter culture of the aroma forming organisms was satisfactory.

Hammer (10) found that associated organisms could be

combined with S. lacticus from different sources and the mixtures would produce high volatile acidities. This suggested that this ability was not peculiar to certain cultures of S. lacticus. At this time Hammer studied methods of combining the two types also and found that there was an advantage, as measured by the production of a high volatile acidity, in adding the associated organisms first and giving them time to grow before adding the S. lacticus. When S. lacticus was inoculated first only a low volatile acidity was produced. However, if the S. lacticus grew slowly a high volatile acidity was sometimes produced.

Variations in cultures of S. paracitrovorus were reported by Hammer and Baker (12). However, they did not correlate the variations with the ability of the organisms to produce satisfactory butter cultures when combined with S. lactis.

The S. lactis group of organisms was studied by Hammer and Baker (13) who reported distinct variations between cultures. Besides typical S. lactis, four varieties were designated. These were not studied from the angle of their abilities to produce desirable butter cultures when combined with the associated organisms but some are obviously not suited for this. For example, S. lactis var. maltigenes produces an objectionable flavor and S. lactis var. tardus is a slow acid producer.

Knudsen and Sørensen (15) believe that the buffer action of the milk used is important in determining whether a good butter culture is obtained when the two types of organisms are combined. Their theory is that when the buffer action is low S. cremoris (S. lactis) produces a change in the hydrogen ion concentration so rapidly that it hinders the growth of the associated organisms.

METHODS USED

The beef infusion agar was prepared from the infusion obtained by holding lean finely chopped beef in water for about 15 hours at approximately 5°C. The beef was used at the rate of one pound for each liter of medium being made. It was added to one half that volume of water and after the holding the liquid was recovered by straining through cheese cloth. The other one half of the volume of water was used for dissolving the agar and peptone which were added at the rates of 1.5 and 0.5 per cent respectively. After the agar and peptone were dissolved the temperatures of the two solutions were adjusted so that when mixed the temperature of the mixture would be about 50°C. This was to avoid coagulation of the protein due to a high temperature and also to avoid gelation of the agar due to a low temperature. Prevention of the coagulation of the protein at this point permitted its use as

a clearing agent when the medium was heated in the autoclave later. The reaction was adjusted to plus one according to Fuller's scale. In some instances two per cent of lactose was added to stimulate the production of acid.

The whey agar was made using whey recovered from fresh skimmed milk with the aid of rennet. The other ingredients were 1.5 per cent agar and 0.5 per cent peptone. The reaction was for the most part adjusted to plus one according to Fuller's scale although in some instances it was adjusted almost to neutrality in the hope of making the changes in acidity occurring near the colonies more evident. The medium was cleared with eggs.

Two methods for determining the volatile acidity produced were used. The first was that described by Hammer (10). It involves the distillation with steam of 250 grams of milk culture after the addition of 15 cubic centimeters of approximately normal sulfuric acid and enough water to bring the volume to about 500 cubic centimeters. The results are expressed as the cubic centimeters of tenth-normal sodium hydroxide required to neutralize the first liter of distillate, phenolphthalein being the indicator.

A shorter method of determining the volatile acidity was used after comparing it with that described above in order that interpretations of results would be uniform. This consisted of distilling 50 grams of milk culture with steam after the addition of three cubic centimeters of approximately

normal sulfuric acid and 50 cubic centimeters of boiled distilled water. The first and second ten cubic centimeter portions were collected and titrated separately against twentieth-normal sodium hydroxide using phenolphthalein as the indicator. The results are expressed as the sum of the amounts required to neutralize the two ten cubic centimeter portions.

RESULTS OBTAINED

Studies on the Numbers of each of the Two Types of Organisms in Butter Cultures.

The studies on the numbers of each of the two types of organisms in butter cultures involved trials with various methods which it appeared might be useful for giving information along this line. These trials included (1) attempts to distinguish between colonies of the two types on agar to which indicator had been added (2) attempts to detect the presence of associated organisms in combinations with S. lactis by the aroma produced in litmus milk and (3) detection of the presence of associated organisms in combinations with S. lactis by the volatile acidity produced in sterilized milk.

Attempts to distinguish between colonies of the two types on agar to which indicator had been added.

Since S. lactis ordinarily produces more acid than do the associated organisms it was thought that this could be used for distinguishing between colonies of S. lactis and colonies of

the associated organisms on agar in the presence of an indicator. The indicators studied were, brom cresol green, methyl red, brom cresol purple and propyl red. Early trials with propyl red indicated that it was not satisfactory for showing changes in acidity produced by the butter culture organisms in agar and it was not used in further trials. Originally, both beef infusion and whey agars were used but after a number of trials it was decided to use beef infusion only because of its clearness and lack of color. Solutions of the three indicators, brom cresol green, methyl red and brom cresol purple, were at first added to the agar by putting them into the petri plates just before pouring the agar. Approximately saturated alcoholic solutions were used and two-tenths of one cubic centimeter were added to each plate. This amount of the solutions produced colors in the agar that were too dark to show small color changes well. The development of colonies was inhibited also as was shown by comparisons with duplicate plates containing no indicator. Accordingly smaller amounts of the solutions were added to plates with the result that the colonies developed satisfactorily and the color produced in the agar was not so dark. Color changes were produced by colonies in the agar containing methyl red and in that containing brom cresol purple. These changes, however, were too indistinct to be useful for distinguishing between the colonies of the two types. The agar containing brom cresol green showed no color change.

Although the colonies developed satisfactorily on agar containing small amounts of saturated alcoholic solutions of the indicators, aqueous solutions were also tried to see if eliminating the alcohol would have any effect. These were added to the plates in the same way the alcoholic solutions had been added. There was no advantage in the use of the aqueous solutions and there was the disadvantage of having to sterilize them before putting them in the petri plates. Moreover, methyl red is comparatively insoluble in water and it was difficult to get enough in solution to produce a color in the agar that would be useful in showing small changes in acidity.

Another method of adding the indicator solutions was to put them into the agar at the time it was filtered and tubed. It was convenient to add the solutions at this time and it also permitted a uniformity of color in the various tubes. Amounts were added which were thought to produce the most desirable shades of color for showing changes. These amounts were with brom cresol green five-hundredths of one cubic centimeter of saturated alcoholic solution per 100 cubic centimeters of medium, with methyl red one-tenth of one cubic centimeter of saturated alcoholic solution per 100 cubic centimeters of medium and with brom cresol purple one cubic centimeter of a 0.32 per cent aqueous solution per 100 cubic centimeters of medium. An aqueous solution of brom cresol purple was used because it was available and was convenient to use rather than because

there was any advantage in its use.

The colors produced in the plates to which the indicators were added in various ways were as follows. The agar with brom cresol green was of a dark bluish green color and no change occurred near the colonies. This indicator is sensitive within the range of pH 3.8 to 5.4 which is probably too acid for showing changes in acidity produced by the butter culture organisms growing in agar. The agar with methyl red was usually quite similar in color to the agar which contained no indicator and only slight changes were produced by the butter culture organisms growing in it. This indicator changes from yellow to red as the reaction changes from a pH of 6.0 to a pH of 4.4. Thus the medium to begin with was yellow and was expected to turn to red as the pH changed to below 6.0. Only a very slight pink developed however. Besides being faint the color change was not confined to the immediate vicinity of the colonies producing it due, presumably, to the rapid diffusion of the acid through the medium and it was impossible, especially when many colonies were present, to tell which were producing the acid. When the numbers of colonies on plates were small they usually all appeared to be acid producers. The agar with brom cresol purple was of a purple-red color. This indicator changes from purple to yellow as the reaction changes from a pH of 6.8 to 5.2. The agar in the plates containing large numbers of colonies turned to a brownish yellow throughout and no distinc-

tion could be made between them. The two types of colonies could not be distinguished even when there were only small numbers per plate because the color changes were only very slight and were not confined to the area immediately surrounding the colonies.

Trials were also made where the agar was flooded with indicator solution after the colonies had developed. This was to avoid any effect the indicator solutions might have on the growth of the colonies. It was also hoped that a comparatively rapid action of the indicators would show more distinct changes of color than the action of the indicators over a longer period of time. These trials, however, gave the same results as did the trials in which the indicators were present during the growth of the colonies; the color changes were not distinct and were not confined to the immediate location of individual colonies.

The addition of lactose to beef infusion agar did not prove to be of an advantage in stimulating the production of acid by the butter culture organisms growing in the agar. Adjusting the reaction of whey agar to almost neutrality likewise did not prove to be of an advantage for making color changes, produced by the butter culture organisms, more distinct.

The failure of the method involving the addition of indicator to agar was probably due to several factors. The butter culture organisms grow poorly on solid media so that only small

amounts of acid were produced and the changes in color were only slight. The acid that was produced diffused out through the medium so rapidly that the color changes were spread over comparatively large areas. Besides tending to make the changes less intense, this diffusion made it difficult to tell which of the colonies were causing the changes. To avoid difficulty due to the rapid diffusion it was necessary to limit the numbers of colonies per plate so they would be well isolated. This was done but where only a few colonies were present invariably all appeared alike and all showed evidence of acid production. This is as would be expected because S. lactis ordinarily outnumbers the associated organisms in butter cultures and in amounts of culture small enough to allow well isolated colonies when plated the associated organisms were likely diluted out. Another factor to be considered in connection with the method is the small differences in the amounts of acid produced by S. lactis and by certain of the associated organisms. There is no definite dividing line between these two types as regards acid production and differentiation on this basis would be certain to cause confusion at times. The results indicate that the production of acid by colonies on beef infusion agar, to which indicator has been added, cannot be used as a basis for determining the numbers of each of the two types of organisms present in butter cultures.

Attempts to detect the presence of the associated organisms by the aroma produced in litmus milk.

Attempts to determine the numbers of the two types of organisms in butter cultures were made by inoculating varying amounts of butter cultures into litmus milk and using the aroma produced as an index to the presence of the associated organisms and coagulation, or, in some cases, reduction as an index to the presence of S. lactis. Sterile water blanks were used and dilutions of 1/1,000,000, 1/10,000,000, 1/100,000,000 and 1/1,000,000,000 prepared; from each of these 1.0, 0.75, 0.50 and 0.25 cubic centimeter portions were measured into test tubes containing litmus milk. In this way, for each butter culture examined, 16 tubes of litmus milk were inoculated with amounts varying from 1/1,000,000 to 1/4,000,000,000 of one cubic centimeter. These were incubated at room temperature and after coagulation each tube was examined for butter culture aroma. Those that did not coagulate were examined after five days. It was thought that by determining the smallest amount of butter culture which produced the changes characteristic of each of the two types of organisms, when inoculated into litmus milk, the approximate numbers of each type could be calculated.

Nineteen butter cultures of varying qualities were studied. Representative results on seven of these are given in Table I.

The data show considerable irregularity particularly as regards the presence of the associated organisms. In some runs

the aroma produced indicated that these organisms were not present in the lower dilutions but were present in some of the higher dilutions. This may have been the result of uneven distribution of the two types of organisms in butter cultures. It would not be expected that the two types are so evenly distributed in butter cultures that all portions would contain equal proportions of each of the two types. The absence of the associated organisms in 1/1,000,000 of one cubic centimeter of butter culture does not mean that they are absent in all the other dilutions of the culture and it is quite possible that smaller portions would contain them. The aroma produced in the litmus milk inoculated with dilutions from butter cultures was not pronounced and in many cases the presence of the butter culture aroma was questionable. The small amount of milk (about eight cubic centimeters) used in each trial was probably a factor responsible for the indefiniteness of the aroma produced. The heated odor due to sterilization of the milk also interfered. The lack of a definite aroma was probably responsible for some of the irregularities in the results secured.

More definite results were obtained concerning the numbers of S. lactis present. The data show that this organism was present in 1/200,000,000 of one cubic centimeter portions of each of the butter cultures examined and in 1/4,000,000,000 of one cubic centimeter portions of some of the butter cultures examined. This indicates that S. lactis is usually present in

Table I.

The approximate numbers of associated organisms and *S. lactis* in butter cultures as indicated by aroma and coagulation in litmus milk inoculated with varying amounts.

Butter cultures used	c.c. dilution water added to milk	dilutions of butter culture 1/1,000,000 aroma indicating assoc. org.	1/1,000,000 coag. indicating *S. lactis*	1/10,000,000 aroma indicating assoc. org.	1/10,000,000 coag. indicating *S. lactis*	1/100,000,000 aroma indicating assoc. org.	1/100,000,000 coag. indicating *S. lactis*	1/1,000,000,000 aroma indicating assoc. org.	1/1,000,000,000 coag. indicating *S. lactis*
A*	1.00	-	+	?	+	-	+	-	+
	.75	-	+	?	+	-	+	+	+
	.50	-	+	?	+	-	+	-	+
	.25	-	+	-	+	-	+	?	+
D-103	1.00	+	+	?	+	-	+	+	+
	.75	+	+	-	+	-	+	-	+
	.50	+	+	-	+	-	+	-	+
	.25	+	+	-	+	+	+	+	+
D-122	1.00	+	+	+	+	?	+	+	+
	.75	+	+	?	+	?	+	-	●
	.50	+	+	?	+	?	+	-	+
	.25	?	+	-	+	-	+	-	+
A16-1	1.00	?	+	-	+	-	+	-?	+
	.75	?	+	-	+	-	+	-	-
	.50	?	+	-	+	-	+	-	+
	.25	?	+	-	+	-	+	-	+
D-122	1.00	+	+	+	+	-	+	-	+
	.75	+	+	+	+	?	+	-	-
	.50	+	+	?	+	-	+	-	+
	.25	?	+	?	+	?	+	-	+
B*	1.00	+	+	-?	+	-	+	-	-
	.75	+	+	?	+	?	+	-	-
	.50	-	+	+	+	-	+	-	-
	.25	+	+	-	+	-	-	-	-
D-122	1.00	+?	+	-	+	?	+	-	-
	.75	?	+	-	+	-	+	-	+

lated with varying amounts.

Butter cultures used	c.c. dilution water added to milk	dilutions of butter culture 1/1,000,000 aroma indicating assoc. org.	1/1,000,000 coag. indicating S. lactis	1/10,000,000 aroma indicating assoc. org.	1/10,000,000 coag. indicating S. lactis	1/100,000,000 aroma indicating assoc. org.	1/100,000,000 coag. indicating S. lactis	1/1,000,000,000 aroma indicating assoc. org.	1/1,000,000,000 coag. indicating S. lactis
A*	1.00	-	+	?	+	-	+	-	+
	.75	-	+	?	+	-	+	+	+
	.50	-	+	?	+	-	+	-	+
	.25	-	+	-	+	-	+	?	+
D-103	1.00	+	+	?	+	-	+	+	+
	.75	+	+	-	+	-	+	-	+
	.50	+	+	-	+	-	+	-	+
	.25	+	+	-	+	+	+	+	+
D-122	1.00	+	+	+	+	?	+	+	+
	.75	+	+	?	+	?	+	-	+
	.50	+	+	?	+	?	+	-	+
	.25	?	+	-	+	-	+	-	+
A16-1	1.00	?	+	-	+	-	+	-?	+
	.75	?	+	-	+	-	+	-	-
	.50	?	+	-	+	-	+	-	+
	.25	?	+	-	+	-	+	-	+
D-122	1.00	+	+	+	+	-	+	-	+
	.75	+	+	+	+	?	+	-	-
	.50	+	+	?	+	-	+	-	+
	.25	?	+	?	+	?	+	-	+
B*	1.00	+	+	-?	+	-	+	-	-
	.75	+	+	?	+	?	+	-	-
	.50	-	+	+	+	-	+	-	-
	.25	+	+	-	+	-	-	-	-
D-122	1.00	+?	+	-	+	?	+	-	-
	.75	?	+	-	+	-	+	-	+
	.50	-	+	-	+	-	+	-	-
	.25	-	+	-	+	-	-	-	-

*A and B were commercial cultures while the others were made up at the laboratory of the Dairy Industry Section of the Iowa Agricultural Experiment Station.

butter cultures in larger numbers than is the associated organism.

The results show that the numbers of each of the two types of organisms present in butter cultures cannot be satisfactorily determined by inoculating varying amounts of butter culture into litmus milk and after incubation at 21°C. examining the litmus milk for aroma and coagulation.

Detection of the presence of the associated organisms in butter cultures by determining the volatile acidity produced in milk.

It was evident after the failure to determine satisfactorily the presence of associated organisms in butter culture by observing the aroma in litmus milk that a method which would measure the products of growth of these organisms more accurately would be necessary. This suggested the determination of the amount of volatile acid produced.

In studies where the production of volatile acidity was used as an index to the presence of the associated organisms, inoculations of varying amounts of each butter culture examined were made into a series of flasks containing 200 cubic centimeters of sterile milk. The amounts inoculated varied from one cubic centimeter to 1/1,000,000,000 of one cubic centimeter; those smaller than one cubic centimeter were measured with the aid of sterile water blanks. Dilutions over this entire range were not made on each butter culture examined because the aroma of some indicated that the higher dilutions would not be needed. Incubation was at room temperature for seven days in order to

permit the production of a maximum amount of volatile acidity. After incubation the cultures were examined for coagulation and then distilled using the method consisting of the distillation of 50 grams of milk culture with steam and the titration of the first and second ten cubic centimeter fractions of the distillate. When more than six-tenths of one cubic centimeter of twentieth-normal sodium hydroxide were required to neutralize the volatile acid in the distillate it was considered that associated organisms were present.

Table II. shows results secured on 32 butter cultures that were of satisfactory quality. The data were collected over a period of three and one-half years, studies being made during each of the seasons to determine whether factors associated with the different seasons affected the growth relationships of the two types of organisms. The approximate numbers of each type, as determined by the smallest amount of butter culture which produced a high volatile acidity and coagulation when inoculated into sterile milk, are shown. The numbers with "at least" before them show that the organisms concerned were present in the highest dilution made and that accordingly the numbers present might have been still higher. The approximate numbers of associated organisms per cubic centimeter varied from 10,000 to at least 100,000,000 with 53 per cent of the butter cultures containing 10,000,000 or more. The numbers of S. lactis per cubic centimeter varied from 1,000,000 to at

Table II.

Approximate numbers of associated organisms and S. lactis in satisfactory butter cultures as indicated by volatile acid production and coagulation in sterile milk inoculated with varying quantities.

Examination number	Date of inoculation	Approx. no. of assoc. org. per c.c.	Approx. no. of S. lactis per c.c.	Approx. ratio of assoc. org. to S. lactis
1	12- 6-27	10,000	at least 10,000,000	1:1000
2	12- 7-27	100,000	" " 10,000,000	1:100
3	12- 8-27	100,000	" " 100,000,000	1:1000
4	12-13-27	1,000,000	" " 10,000,000	1:10
5	3-27-28	10,000,000	" " 10,000,000	1:1
6	3-27-28	10,000,000	" " 10,000,000	1:1
7	4- 4-28	10,000,000	" " 10,000,000	1:1
8	4-11-28	10,000,000	" " 100,000,000	1:10
9	4-13-28	50,000,000	" " 100,000,000	1:2
10	4-18-28	50,000,000	" " 100,000,000	1:2
11	5-25-28	100,000	" " 100,000,000	1:1000
12	5-25-28	100,000	" " 100,000,000	1:1000
13	10-16-28	100,000	" " 100,000,000	1:1000
14	10-16-28	100,000	" " 100,000,000	1:1000
15	12- 4-28	1,000,000	1,000,000	1:1
16	3-16-29	1,000,000	at least 100,000,000	1:100
17	3-16-29	1,000,000	" " 100,000,000	1:100
18	3-21-29	1,000,000	" " 100,000,000	1:100
19	3-25-29	10,000,000	" " 100,000,000	1:10
20	5-21-30	at least 100,000,000	" " 100,000,000	1:1
21	5-21-30	1,000,000	" " 100,000,000	1:100
22	6-13-~~26~~ 30	10,000,000	" " 100,000,000	1:10
23	6-30-30	1,000,000	" " 100,000,000	1:100
24	7-14-30	10,000	" " 100,000,000	1:10,000
25	9- 2-30	10,000,000	" " 100,000,000	1:10
26	9-16-30	10,000,000	" " 100,000,000	1:10
27	10- 7-30	100,000,000	" " 1,000,000,000	1:10
28	11-13-30	at least 100,000,000	" " 100,000,000	1:1
29	12-16-30	10,000,000	100,000,000	1:10
30	1- 8-31	10,000,000	at least 100,000,000	1:10
31	1-14-31	10,000,000	" " 1,000,000,000	1:100
32	1-14-31	10,000,000	" " 1,000,000,000	1:100

least 1,000,000,000 with 78 per cent of the butter cultures containing 100,000,000 or more. The approximate ratios of the associated organisms to S. lactis varied from 1:1 to 1:10,000.

The data on satisfactory butter cultures show many variations in the numbers of each of the two types of organisms as well as in their ratios. It is possible that uneven distribution of the two types of organisms in the butter cultures caused some of the variations although one cubic centimeter samples were always used for preparing the dilutions and it would be expected that samples of this size would be representative. There was no relationship between the time of the year and the variations in the numbers of the two types of organisms or in their ratios.

Data, comparable to those secured on satisfactory butter cultures and reported in Table II, were secured on 17 butter cultures which were not satisfactory and are presented in Table III. The unsatisfactory butter cultures examined were for the most part new cultures which were lacking in flavor although cultures that were not new but were unsatisfactory were also examined. The data were collected over a period of two years and represent results secured at different seasons of the year. The numbers of associated organisms per cubic centimeter varied from 100 to at least 100,000,000 with 23 per cent of the butter cultures containing 10,000,000 or more while the numbers of S. lactis per cubic centimeter varied from 1,000,000 to at

Table III.

Approximate numbers of organ
types present in butter cult
not satisfactory

examination no.	date of inoc.	approx. no. of assoc. org. per c.c.	approx. no. of *S. lactis* per c.c.	appro: of as: to S.
1	3-22-28	100,000	at least 50,000,000	1:500
2	4- 6-28	10,000,000	" " 10,000,000	1:1
3	10-12-28	100	" " 100,000,000	1:1,0(
4	10-12-28	100	" " 100,000,000	1:1,0(
5	12- 7-28	100,000	" " 1,000,000	1:10
6	12-13-28	10,000	" " 100,000,000	1:10,(
7	12-13-28	10,000	" " 100,000,000	1:10,(
8	12-15-28	10,000	" " 1,000,000	1:100
9	12-22-28	10,000	" " 100,000,000	1:10,(
10	12-22-28	10,000	" " 100,000,000	1:10,(
11	12-29-28	10,000	" " 100,000,000	1:10,(
12	3-28-29	1,000,000	" " 100,000,000	1:100
13	3-28-29	1,000,000	" " 100,000,000	1:100
14	6-12-29	100,000,000	" " 100,000,000	1:1
15	6-12-29	100,000,000	" " 100,000,000	1:1
16	9- 2-29	10,000	" " 100,000,000	1:10,(
17	3-25-30	10,000,000	" " 100,000,000	1:10

Table III.

pproximate numbers of organisms of the two ypes present in butter cultures that were not satisfactory.

approx. no. of *S. lactis* per c.c.	approx. ratio of assoc. org. to S. lactis	remarks
t least 50,000,000	1:500	lacking in flavor
" 10,000,000	1:1	very slow to coagulate
" 100,000,000	1:1,000,000	lacking in flavor
" 100,000,000	1:1,000,000	" " "
" 1,000,000	1:10	" " "
" 100,000,000	1:10,000	a new culture, lacking in flavor
" 100,000,000	1:10,000	" " " " " "
" 1,000,000	1:100	" " " " " "
" 100,000,000	1:10,000	" " " " " "
" 100,000,000	1:10,000	" " " " " "
" 100,000,000	1:10,000	" " " " " "
" 100,000,000	1:100	slow to coagulate and lacking in flavor
" 100,000,000	1:100	" " " " " " "
" 100,000,000	1:1	a new culture, slow to coagulate
" 100,000,000	1:1	" " " " " "
" 100,000,000	1:10,000	a new culture, lacking in flavor
" 100,000,000	1:10	lacking in flavor

least 100,000,000 with 77 per cent of the butter cultures containing 100,000,000 or more. The approximate ratios of the associated organisms to the S. lactis varied from 1:1 to 1: 1,000,000 being 1:10,000 or greater in 47 per cent of the runs.

The data on the unsatisfactory butter cultures show that there were wide variations in the numbers and also in the ratios of the two types of organisms. There was a general correlation between large numbers of associated organisms in butter cultures and slow coagulation. There was also a general correlation between a wide ratio of the numbers of associated organisms to the numbers of S. lactis present in butter cultures and lack of flavor.

A comparison of the ratios of the two types of organisms in the satisfactory butter cultures and in the unsatisfactory butter cultures is shown in Table IV. The ratio of the numbers of associated organisms to the numbers of S. lactis was less than 1:10,000 in 97 per cent of the 32 satisfactory butter cultures examined while it was less than 1:10,000 in only 53 per cent of the 17 unsatisfactory butter cultures examined. Data given in Tables II. and III., however, show that there is no constant relationship between the quality of butter cultures and the ratio of the associated organisms to S. lactis because both low and high ratios are found in satisfactory as well as unsatisfactory butter cultures.

Table IV.

Summary of the data shown in Tables II and III.

quality of butter cultures	no. of trials	per cent of butter cultures with ratios of assoc. org. to S. lactis less than 1:10,000	per cent of butter cultures with ratios of assoc. org. to S. lactis 1:10,000 or more
satisfactory	32	97	3
unsatisfactory	17	53	47

Studies on Combining Different Proportions of the Two Types of Organisms in the Development of Butter Cultures.

The relationship between the proportions of the two types of organisms present when the combinations were prepared and the volatile acidity produced.

Since the associated organisms are largely responsible for the production of volatile acidity in butter cultures, it is reasonable to suppose that the amounts of volatile acid produced would be in direct proportion to the numbers of the associated organisms present. The development of butter cultures by combining the two types of organisms often fails because of lack of butter culture flavor. This suggests that in these cases the associated organisms are not growing. With this in mind studies were made to determine whether or not there was a relationship between the proportions of the two types of organisms present at the time of combining and the volatile acidity produced in mixtures.

In studying the effect of variations in the proportions of the two types at the time of preparing the combinations on the amount of volatile acidity produced, inoculations of both types were made into series of flasks, each flask containing 200 cubic centimeters of sterile milk. Fourteen series of combinations were made in 13 of which the inoculations of the associated organisms were constant and the inoculations of S. lactis were varied. In one series (2) the inoculations of S. lactis were constant while the inoculations of the associated

organisms were varied. The cultures from which inocula were taken were in milk and the S. lactis cultures contained more organisms per cubic centimeter than did the associated organisms. This did not allow the calculation on the basis of the amounts of inoculum added, of the actual proportions of the two types present at the time of combining but did permit comparisons to be made as described. In each series pure culture checks of each the associated organisms and S. lactis were prepared. Incubation was at room temperature for seven days, after which the cultures were examined for coagulation and then distilled using the method consisting of the distillation of 50 grams of milk culture with steam and the titration of the first and second ten cubic centimeter fractions of the distillate. The results secured are shown in Table V.

In eight of the 14 series the amounts of volatile acidity produced in the different mixtures definitely increased as the proportion of associated organisms added increased. This relationship was not constant, however, for in some series the mixture containing the highest proportion of associated organisms when prepared did not produce the highest volatile acidity. In six of the series the amounts of volatile acidity produced in the different mixtures did not definitely increase as the proportion of associated organisms added increased.

These variations cannot be correlated with the cultures used in preparing the mixtures because the results from similar

Table V.

The effect of varying the proportions of the two types of organisms at the time of combining them on the volatile acidity. Incubation seven days at room temperature.

series	c.c. of milk culture added		coagulation	volatile acidities after 7 days
	S. lactis	assoc. org.		
series 1	1/10,000	1/10,000	+	.75
	1/100,000	"	+	.95
	1/500,000	"	+	1.40
S. lactis 1	1/1,000,000	"	+	1.70
	1/10,000,000	"	+	1.60
assoc. org. 1	1/100,000,000	"	+	1.80
	none	"	-	1.80
	1/10,000	none	+	.35
	1/10,000	1/10,000	+	.30
	"	1/100,000	+	.30
series 2	"	1/500,000	+	.30
	"	1/1,000,000	+	.30
S. lactis 1	"	1/10,000,000	+	.25
	"	1/100,000,000	+	.20
assoc. org. 1	"	none	+	.20
	none	1/10,000	-	1.55
	1/10,000	1/10,000	+	.45
series 3	1/100,000	"	+	.95
	1/500,000	"	+	.40
S. lactis 1	1/1,000,000	"	+	1.55
	1/10,000,000	"	+	1.60
assoc. org. 1	1/100,000,000	"	+	1.45
	none	"	-	1.35
	1/10,000	none	+	.35
	1/10,000	1/10,000	+	1.45
series 4	1/100,000	"	+	1.60
	1/500,000	"	+	1.40
S. lactis 1	1/1,000,000	"	+	1.60
	1/10,000,000	"	+	1.70
assoc. org. 2	1/100,000,000	"	+	1.25

	none	1/10,000	−	1.55
	1/10,000	1/10,000	+	.45
series 3	1/100,000	"	+	.95
	1/500,000	"	+	.40
S. lactis 1	1/1,000,000	"	+	1.55
	1/10,000,000	"	+	1.60
assoc. org. 1	1/100,000,000	"	+	1.45
	none	"	−	1.35
	1/10,000	none	+	.35
	1/10,000	1/10,000	+	1.45
series 4	1/100,000	"	+	1.60
	1/500,000	"	+	1.40
S. lactis 1	1/1,000,000	"	+	1.60
	1/10,000,000	"	+	1.70
assoc. org. 2	1/100,000,000	"	+	1.25
	none	"	−	.50
	1/10,000	none	+	.30
	1/100	1/10,000	+	.25
series 5	1/10,000	"	+	.30
	1/100,000	"	+	.30
S. lactis 1	1/1,000,000	"	+	.30
	1/10,000,000	"	+	.25
assoc. org. 2	1/100,000,000	"	+	.30
	none	"	−	.75
	1/10,000	none	+	.35
	1/100	1/10,000	+	.30
series 6	1/10,000	"	+	.45
	1/100,000	"	+	.30
S. lactis 1	1/1,000,000	"	+	.40
	1/10,000,000	"	+	.40
assoc. org. 3	1/100,000,000	"	+	.40
	none	"	−	1.20
	1/10,000	none	+	.40
	1/100	1/10,000	+	.25
series 7	1/10,000	"	+	.30
	1/100,000	"	+	.35
S. lactis 1	1/1,000,000	"	+	.45
	1/10,000,000	"	+	.45
assoc. org. 4	1/100,000,000	"	+	.65
	none	"	+	1.25
	1/10,000	none	+	.35

Table V. (Continued)

series	c.c. of milk culture added		coagulation	volatile acidities after 7 days
	S. lactis	assoc. org.		
	1/100	1/10,000	+	.25
series 8	1/10,000	"	+	.25
	1/100,000	"	+	.30
S. lactis 1	1/1,000,000	"	+	.25
	1/10,000,000	"	+	.25
assoc. org. 3	1/100,000,000	"	+	.25
	none	"	-	1.55
	1/10,000	none	+	.25
	1/100	1/10,000	+	.25
series 9	1/10,000	"	+	.40
	1/100,000	"	+	1.80
S. lactis 2	1/1,000,000	"	+	1.65
	1/10,000,000	"	+	1.55
assoc. org. 5	1/100,000,000	"	+	1.50
	none	"	+	1.45
	1/10,000	none	+	.35
	1/100	1/10,000	+	.35
series 10	1/10,000	"	+	.30
	1/100,000	"	+	.35
S. lactis 2	1/1,000,000	"	+	.45
	1/10,000,000	"	+	.40
assoc. org. 1	1/100,000,000	"	+	.45
	none	"	+	1.65
	1/10,000	none	+	.45
	1/100	1/10,000	+	.30
series 11	1/10,000	"	+	.20
	1/100,000	"	+	.40
S. lactis 2	1/1,000,000	"	+	.75
	1/10,000,000	"	+	1.20
assoc. org. 4	1/100,000,000	"	+	1.35
	none	"		
	1/10,000	none	+	.55
	1/100	1/10,000	+	.30 (8)*
series 12	1/10,000	"	+	.40
	1/100,000	"	+	.40
S. lactis 2	1/1,000,000	"	+	.35

	none	"	-	.45	
	none	"	+	1.65	
	1/10,000	none	+	.45	
	1/100	1/10,000	+	.30	
series 11	1/10,000	"	+	.20	
	1/100,000	"	+	.40	
S. lactis 2	1/1,000,000	"	+	.75	
	1/10,000,000	"	+	1.20	
assoc. org. 4	1/100,000,000	"	+	1.35	
	none	"			
	1/10,000	none	+	.55	
	1/100	1/10,000	+	.30	(8)*
series 12	1/10,000	"	+	.40	
	1/100,000	"	+	.40	
S. lactis 2	1/1,000,000	"	+	.35	
	1/10,000,000	"	+	.45	
assoc. org. 1	1/100,000,000	"	+	.85	(9)
	none	"	-	.70	
	1/10,000	none	+	.40	
	1/100	1/10,000	+	.20	
series 13	1/10,000	"	+	.40	
	1/100,000	"	+	.80	(10)
S. lactis 3	1/1,000,000	"	+	1.65	
	1/10,000,000	"	+	1.65	
assoc. org. 6	1/100,000,000	"	+	1.95	(11)
	none	"	-	1.45	
	1/10,000	none	+	.50	
	1/100	1/10,000	+	.30	
series 14	1/10,000	"	+	.40	
	1/100,000	"	+	.40	
S. lactis 4	1/1,000,000	"	+	.40	
	1/10,000,000	"	+	.60	
assoc. org. 6	1/100,000,000	"	+	1.40	(18)
	none	"	-	1.10	
	1/10,000	none	+	.55	

*Numbers in parentheses refer to butter cultures as shown in Table VIII.

combinations varied; for example, in series 2 "S. lactis 1"and "associated organism 1" were used and the volatile acidities were all low while in series 1 and 3 high volatile acidities were produced with the same cultures. In series 1 and 4 high volatile acidities were obtained in the mixtures containing equal amounts of inoculum of each of the two types of organisms. This amounted to an excess of S. lactis and indicates that under some conditions the associated organisms grow well even when added in the minority at the time of preparing the mixtures.

The aroma of each mixture was observed just before distillation and while in most instances when a high volatile acidity was produced it could be detected, the aroma was in no case similar to that produced by a satisfactory butter culture. This may have been due in part to the long time that the mixtures were ripened and also to the sterilized milk which was used.

The data show that adding the associated organisms in large numbers at the time of combining the two types tends to increase the amount of volatile acid that will be produced as determined after seven days. This relationship was not constant, however, and the mixture containing the highest proportions of associated organisms in each series at the time of preparation did not always produce the highest volatile acidity.

The relationship between the proportions of the two types of organisms present when the combinations were prepared and the time required for the production of a high volatile acidity.

In developing new butter cultures a lack of flavor and aroma is common due presumably in part to the failure of the growth of the associated organisms. The usual procedure in starting new combinations of the two types of organisms is to inoculate the associated organism into a tube of sterile milk and after about 24 hours incubation at 21°C. to inoculate S. lactis. The delay in inoculating S. lactis is to give the associated organism a chance to grow so it will not be inhibited by the development of acid to the extent of losing its influence in the production of flavor and aroma. The data given in Table V. show the amount of volatile acid produced after seven days. It is desirable to know how long a time is required for the production of a high volatile acidity because when developing new butter cultures this information would be useful in arranging the time between the initial combining of the organisms and the beginning of the regular transfers. This information would be useful also in determining when regular transfers of butter cultures are satisfactorily ripened. Accordingly combinations of the two types of organisms similar to those described in Table V. were prepared in quadruplicate so determinations could be run after one, two, four and seven days respectively. The results secured are shown in Table VI.

Table VI.

The effect of varying the proportions of the two types of organisms at the time of combining them on the rate of volatile acid production. Incubation at room temperature.

series	c.c. of milk culture added		volatile acidities			
	S. lactis	assoc. org.	after 1 day	after 2 days	after 4 days	after 7 days
series 1	1/100	1/10,000		.30	.25	.50
	1/10,000	"	.35	.40	.40	.55
	1/100,000	"	.30	.40	.65	1.80
S. lactis 6	1/1,000,000	"	.30	.55	1.60	2.10
	1/10,000,000	"	.40	1.00	1.85	1.95
assoc. org. 6	1/100,000,000	"	.30	1.30	1.90	2.00
	none	"	.30	1.50	1.85	2.15
	1/10,000	none	.30		.55	.55
series 2	1/100	1/10,000	.30	1.25	1.80	1.90
	1/10,000	"	.20	1.65	2.10	2.25
	1/100,000	"	.15	1.60	2.10	2.15
S. lactis 7	1/1,000,000	"	.20	1.30	2.20	2.05
	1/10,000,000	"	.30	.95	2.15	2.15(21)*
assoc. org. 6	1/100,000,000	"	.25	.85	2.25	2.05
	none	"		.80	2.30	2.10
	1/10,000	none	.15	.40	.45	.50
series 3	1/100	1/10,000	.30	.20	.60	1.20(28)
	1/10,000	"	.25	.30	1.15	1.55
	1/100,000	"	.20	.75	1.55	1.70
S. lactis 9	1/1,000,000	"	.20	1.00	1.50	1.65
	1/10,000,000	"		.75	1.60	1.65
assoc. org. 6	1/100,000,000	"		1.10	1.60(23)	1.65(29)
	none	"	.30	1.15	1.85	1.70
	1/10,000	none		.35		.35
series 4	1/100	1/10,000	.30	.30	.30	.30
	1/10,000	"	.30	.20	.45	.60
	1/100,000	"	.30	.40	.65	1.10
S. lactis 4	1/1,000,000	"	.30	.45	1.30	1.90
	1/10,000,000	"		.80	1.85	2.15
assoc. org. 6	1/100,000,000	"		1.05	1.90(27)	1.95(31)
	none	"		.60	1.10	1.65

	1/10,000	none	.15	.40	.45	.50
	1/100	1/10,000	.30	.20	.60	1.20(28)
series 3	1/10,000	"	.25	.30	1.15	1.55
	1/100,000	"	.20	.75	1.55	1.70
S. lactis 9	1/1,000,000	"	.20	1.00	1.50	1.65
	1/10,000,000	"		.75	1.60	1.65
assoc. org. 6	1/100,000,000	"		1.10	1.60(23)	1.65(29)
	none	"	.30	1.15	1.85	1.70
	1/10,000	none		.35		.35
	1/100	1/10,000	.30	.30	.30	.30
series 4	1/10,000	"	.30	.20	.45	.60
	1/100,000	"	.30	.40	.65	1.10
S. lactis 4	1/1,000,000	"	.30	.45	1.30	1.90
	1/10,000,000	"		.80	1.85	2.15
assoc. org. 6	1/100,000,000	"		1.05	1.90(27)	1.95(31)
	none	"		.60	1.10	1.65
	1/10,000	none		.40	.45	.50
	1/10,000	1/100	.15	.95(30)	1.85	1.80
series 5	"	1/10,000	.15	.75	2.00	2.05
	"	1/100,000	.20	.55	2.05(32)	2.05
S. lactis 7	"	1/1,000,000	.15	.25	1.70	1.75
	"	1/10,000,000	.20	.25	1.85	1.75
assoc. org. 6	"	1/100,000,000	.15	.20	.40	1.50
	"	none	.15	.25	.35	.40
	none	1/10,000	.15	.45	1.15	1.55
	1/10,000	1/100	.30		1.80	1.50
series 6	"	1/10,000	.25		1.90	1.80
	"	1/100,000	.25		1.40	1.70
S. lactis 4	"	1/1,000,000	.25		.60	.70
	"	1/10,000,000	.25		.45	.45
assoc. org. 6	"	1/100,000,000	.30		.45	.45
	"	none	.20		.50	.55
	none	1/10,000	.20		1.10	1.80
	1/100	1/10,000	.30		1.30	1.80
series 7	1/10,000	"	.20		1.65	2.05
	1/100,000	"	.20		1.75	1.75
S. lactis 8	1/1,000,000	"	.20		1.80	1.95
	1/10,000,000	"	.20		1.80	2.00
assoc. org. 6	1/100,000,000	"	.20		1.95	2.15
	none	"	.20		1.10	2.05
	1/10,000	none	.20		.45	.50

*Numbers in parentheses refer to butter cultures as shown in Table VIII.

After the first day no high volatile acidities were secured. After the second day there were increases in the volatile acidity with most of the mixtures, however, in only two of the 30 mixtures were the amounts as high as those normally produced in satisfactory butter cultures.*

After four days the volatile acidities secured were generally comparatively high and in many of the mixtures amounts comparable to those present in satisfactory butter cultures were obtained. After seven days there was a further increase in the volatile acidities in many of the mixtures although in some the maximum was reached after four days.

The data show that, although there was considerable variation in the different series, in general the time required for the production of a high volatile acidity decreased as the proportions of the associated organisms present in the combinations when prepared increased.

Table VII. summarizes the data presented in Table VI. and shows that on the average the volatile acidities increased more rapidly and reached a higher maximum in the combinations containing the higher proportions of associated organisms.

The amounts of volatile acid produced by the pure cultures of associated organisms were, on the average, higher than the amounts produced by all of the mixtures but were not

*The amounts of volatile acid normally produced in satisfactory butter cultures varies from 1.6 to 2.2 as determined by the method used after seven days incubation.

Table VII.

Summary of data presented in table VI. A comparison of the average volatile acidities produced by mixtures and by pure cultures of associated organisms after different incubation periods.

times of holding	average volatile acidities			
	all mixtures	mixtures of each series containing the lowest percentage of assoc. org.	mixtures of each series containing the highest percentage of assoc. org.	assoc. org. alone
1 day	.25	.26	.23	.22
2 days	.67	.45	.94	.80
4 days	1.31	.68	1.71	1.33
7 days	1.45	.96	1.65	1.65

as high as the amounts produced by the mixtures containing the highest proportions of associated organisms. Presumably S. lactis has a restraining action on the associated organisms especially in combinations when the associated organisms are not in the majority.

The selection of combinations of the two types of organisms on the basis of their ability to produce a high volatile acidity, for use in the development of butter cultures.

In order to compare the ability of mixtures of the two types of organisms to produce high volatile acidities with their ability to produce satisfactory butter cultures, thirteen of the mixtures* used in securing the data reported in Tables V. and VI. were selected and transfers from each made to milk pasteurized at a temperature of 85°C. or higher for 30 minutes. The volatile acidities of the mixtures at the time the transfers were made to pasteurized milk varied from 0.30 to 2.15 which are comparatively low and high values respectively. The aroma of the mixtures which was noted at the time the transfers were made to the pasteurized milk was in no instance similar to to that of a satisfactory butter culture. Each of the mixtures selected was carried through a series of transfers in pasteurized milk and the quality of each transfer studied. The results are shown in Table VIII.

Although nine of the thirteen mixtures used had produced volatile acidities in amounts comparable to the amounts normally

*The mixtures used are designated in Tables V. and VI.

Table VIII.

Relationship between the ability of mixtures of the two types of organisms to produce high volatile acidities and their ability to produce satisfactory butter cultures.

butter culture numbers	c.c. of milk culture added to mixtures: assoc. org.	c.c. of milk culture added to mixtures: S. lactis	volatile acidity at time of transferring to pasteurized milk	quality of butter cultures
8	1/10,000	1/100	.30	not good
9	1/10,000	1/100,000,000	.85	not good
10	1/10,000	1/100,000	.80	not good
11	1/10,000	1/100,000,000	1.95	not good
18	1/10,000	1/100,000,000	1.40	fair in first transfer but not in later transfers
21	1/10,000	1/10,000,000	2.15	not good
23	1/10,000	1/100,000,000	1.60	not good
27	1/10,000	1/100,000,000	1.90	not good
28	1/10,000	1/100	1.20	not good
29	1/10,000	1/100,000,000	1.65	not good
30	1/100	1/10,000	.95	not good
31	1/10,000	1/100,000,000	1.95	not good
32	1/100,000	1/10,000	2.05	fair in first transfer but not in later transfers

produced by satisfactory butter cultures, no satisfactory butter cultures were obtained. Two of the mixtures (8 and 32) produced some flavor and aroma in the first transfer but not in subsequent transfers.

The results indicate that the ability of combinations of associated organisms and S. lactis to produce high volatile acidities is not a satisfactory basis on which to select them for use in butter cultures.

The use of freshly isolated cultures of S. lactis for combining with associated organisms in the development of new butter cultures.

The failure of many mixtures of an associated organism and S. lactis to produce desirable butter cultures may be due in part to differences which exist between individual cultures. Different varieties of S. lactis have been described and undoubtedly there are variations that are not understood that are important from the standpoint of the flavor and aroma produced when a culture of S. lactis is combined with an associated organism. Variations are also known to exist between cultures of the associated organisms but have not been studied from the angle of their influence on the development of flavor and aroma.

When new combinations of the associated organisms and S. lactis are prepared the cultures of each type used are usually those that have been maintained as pure cultures for some time. The frequency of butter culture flavor and aroma in milk and cream souring under natural conditions indicates that organisms

of the desired types are commonly present in milk and cream in considerable numbers and that isolation of satisfactory cultures of each type should be comparatively easy. The failure of many mixtures of the two types to produce satisfactory butter cultures suggests the possibility that certain cultures of the associated organisms or S. lactis may undergo changes, when carried in the laboratory as pure cultures, that influence their usefulness in butter cultures.

Experiments were carried out in which freshly isolated cultures of S. lactis were used in combination with associated organisms in an attempt to develop new butter cultures. Twenty cultures of S. lactis and eight cultures of associated organisms were used. Freshly isolated cultures of associated organisms were not used because the time required for their identification would make it necessary to carry them in pure cultures for several days before using them in mixtures. The cultures of S. lactis used were obtained for the most part by plating sour cream that had a butter culture aroma while in some instances they were obtained by plating butter cultures. Whey agar was the plating medium and, after incubation of the plates, colonies were picked into litmus milk. The cultures which rapidly reduced the litmus were examined by staining; if the morphology was characteristic of S. lactis, transfers were immediately made to young litmus milk cultures of associated organisms. Forty-two mixtures were prepared and, as soon as coagulation

occurred, were transferred to milk that had been heated to at least 85°C. for 30 minutes and cooled to 21°C. Incubation was at 21°C. Subsequent transfers were then made as regularly as coagulation occurred each transfer being examined for flavor and aroma until it was known whether or not satisfactory butter cultures could be produced. Eight of the S. lactis cultures were carried as pure cultures for two months and were then again combined with the associated organisms that they had been combined with immediately after isolation. These mixtures were also run through a series of transfers to determine whether or not satisfactory butter cultures could be produced. The results obtained are shown in Table IX.

Twelve of the 42 combinations prepared using freshly isolated S. lactis produced some butter culture flavor and aroma, however, only one of these produced a satisfactory butter culture. Nine of the 16 combinations prepared after two months produced some butter culture flavor and aroma. In six of the combinations better flavor and aroma were produced in the mixtures containing freshly isolated S. lactis while in two of the combinations better flavor and aroma were produced in the mixtures prepared after two months.

The results secured indicate that freshly isolated cultures of S. lactis had no advantage, over the same cultures of S. lactis that had been maintained for two months in pure culture, for use in developing new butter cultures.

Table IX.

The use of freshly isolated cultures of *S. lactis* as compared to the use of old cultures of *S. lactis* for combining with associated organisms.

culture of *S. lactis* used	culture of assoc. org. used	quality of butter cultures obtained with freshly isolated *S. lactis*	quality of butter cultures obtained with the same cultures of *S. lactis* after 2 months in pure culture
4	1	poor	
4	2	"	
4	3	"	
5	4	"	
5	5	"	
5	6	"	
7	6	"	
7	6	"	
8	7	"	
8	8	"	
9	7	fair	poor
9	8	"	"
10	7	poor	
10	8	"	
11	7	some flavor	poor
11	8	" "	"
12	7	fair	fair
12	8	some flavor	poor
13	7	poor	
13	8	"	
14	7	fair	some flavor
14	8	"	" "
15	7	poor	
15	8	"	
16	7	fair	some flavor
16	8	poor	poor
17	7	fair	some flavor
17	8	poor	" "
18	7	"	
18	8	"	
19	7	fair	satisfactory

8	7	"	
8	8	"	
9	7	fair	poor
9	8	"	"
10	7	poor	
10	8	"	
11	7	some flavor	poor
11	8	" "	"
12	7	fair	fair
12	8	some flavor	poor
13	7	poor	
13	8	"	
14	7	fair	some flavor
14	8	"	" "
15	7	poor	
15	8	"	
16	7	fair	some flavor
16	8	poor	poor
17	7	fair	some flavor
17	8	poor	" "
18	7	"	
18	8	"	
19	7	fair	satisfactory
19	8	satisfactory	some flavor
20	7	poor	
20	8	"	
21	7	"	
21	8	"	
22	7	"	poor
22	8	"	fair
23	7	"	
23	8	"	
24	7	"	
24	8	"	

DISCUSSION OF RESULTS FOR PART I.

The failure to distinguish between the two types of organisms in butter culture by plating on agar to which indicator had been added was due to several factors. S. lactis does not grow well on agar and the rate of acid production by the colonies was so slow that diffusion of the acid through the agar took place almost as rapidly as the acid was produced so that the color changes were not confined to the immediate location of the colonies and were not distinct. When the numbers of colonies per plate were small so that the color changes produced by the individual colonies could be distinguished, the colonies all appeared to have produced some acid. Work done by Hammer and Bailey (11) and by Hammer (10) show that S. lactis is usually present in greater numbers in butter cultures than are the associated organsims and data presented in this report (Table III.) indicate that the ratio of the numbers of the associated organisms to the numbers of S. lactis may, in some instances, be as high as 1:1,000,000. It is probable, therefore, that the associated organism would be diluted out in plating amounts small enough to give only a few colonies on agar; and that even though the colonies of the two types could be differentiated by plating on agar to which indicator had been added there would be many platings with only S. lactis

colonies present.

There is no definite dividing line on the basis of acid production between the two types of organisms present in butter cultures. Certain of the associated organisms produce approximately as much acid as do some cultures of S. lactis and, altho the rate is ordinarily slower, this further complicates differentiation of the two types on this basis.

The indistinctness of the aroma produced when varying amounts of butter culture were inoculated into litmus milk was undoubtedly due in part to the small amount of litmus milk used for each trial and also to the interference of the odor caused by sterilization of the milk.

The amounts of volatile acid produced in portions of milk which had been inoculated with small amounts of butter culture were satisfactory as an index to the presence of an associated organism in the amounts of butter culture used. The coagulation was also definite and the production of a high volatile acidity and coagulation, in series of milk samples which had been inoculated with varying amounts of butter culture, were used for determining the approximate numbers of each the associated organisms and S. lactis present. The general correlation which was found between high numbers of the associated organism in butter cultures and slow coagulation is in agreement with the work of Hammer (10) who showed that associated organisms had a restraining action on the growth of S. lactis especially in

combinations where the numbers of the associated organism were high. The general correlation between a wide ratio of the numbers of the associated organism to the numbers of S. lactis in butter cultures and a lack of flavor is also in agreement with the work of Hammer (10) who found that the presence of excessive amounts of lactic acid in mixtures prevented the production of large amounts of volatile acid by the associated organisms. Slow coagulation and a lack of flavor are both defects commonly found in butter cultures and they are undoubtedly caused by unusual ratios between the two types of organisms present.

The variations in the ratios of the numbers of the associated organism to the numbers of S. lactis in satisfactory as well as in unsatisfactory butter cultures indicate that factors other than the relationships of the numbers of the two types of organisms are important in the production of a desirable flavor and aroma. It is possible, of course, that some of the variations found were due to unrepresentative samples of butter cultures being used in the preparation of the dilutions employed for inoculation. While it is probable that the two types of organisms are not evenly distributed in butter cultures it is not likely that uneven distribution was an important factor causing the variations obtained because one cubic centimeter samples of butter culture were always used and it is reasonable to suppose that samples of this size would be representative.

The increase in the rate of volatile acid production as well as in the amount that was produced in from four to seven days in mixtures containing a high proportion of the associated organism as compared to mixtures containing a low proportion of the associated organism shows the necessity of having large numbers of an associated organism in butter cultures. Butter cultures that lose flavor and aroma can be brought back to normal by over-ripening, which presumably increases the proportion of associated organism present.

The lack of correlation between the ability of mixtures of an associated organism and S. lactis to produce high volatile acidities in sterilized milk and their ability to produce satisfactory butter cultures suggests that the production of a high volatile acidity is only part of the requirements for the development of satisfactory butter cultures. This suggests further that the failures of certain mixtures of an associated organism and S. lactis to produce satisfactory butter cultures are due to characters, of the organisms, which are not directly concerned with the production of volatile acidity. It should be pointed out, however, that the conditions existing in sterilized milk held four to seven days at room temperature and the conditions existing in butter cultures are quite different and it is probable that mixtures could produce high volatile acidities in the sterilized milk and yet could not produce high volatile acidities under the conditions existing

in regularly transferred butter cultures.

The failure of freshly isolated cultures of S. lactis to be more useful than old cultures of S. lactis for combining with associated organisms in developing new butter cultures indicates that it is not the loss of some character or characters incident to carrying S. lactis in pure cultures that is responsible for the frequent failure of combinations of the two types of organisms to produce satisfactory butter cultures. This suggests also that the inherent characters of individual cultures of S. lactis or of the associated organisms may be important in determining whether or not combinations of the two types will produce satisfactory butter cultures.

CONCLUSIONS FOR PART I.

1. The production of acid by colonies on agar to which indicator had been added could not be used satisfactorily as a basis for determining the numbers of each of the two types of organisms present in butter cultures.

2. The production of aroma and coagulation in litmus milk inoculated with varying amounts of butter culture could not be used satisfactorily for determining the numbers of each of the two types of organisms present in butter cultures.

3. The approximate numbers of the associated organisms and of S. lactis in butter cultures were satisfactorily deter-

mined by inoculating series of flasks containing sterile milk with varying amounts of butter culture and determining the smallest amount of butter culture which produced a high volatile acidity and coagulation respectively.

4. There were wide variations in the numbers as well as in the ratios of the two types of organisms in satisfactory and also in unsatisfactory butter cultures.

5. Adding the associated organisms in large numbers at the time of combining the two types tended to increase the amount of volatile acid produced as well as the rate of volatile acid production.

6. The ability of combinations of the associated organisms and S. lactis to produce high volatile acidities in sterilized milk in four to seven days was not a satisfactory basis on which to select combinations for use in butter cultures.

7. Freshly isolated cultures of S. lactis had no advantage, over the same cultures of S. lactis that had been maintained for two months in pure culture, for use in developing new butter cultures.

PART II.

THE EFFECT OF THE ADDITION OF CALCIUM CARBONATE TO MILK ON THE KEEPING QUALITIES OF BUTTER CULTURES MADE FROM IT.

HISTORICAL

The period that acid producing bacteria can be held in milk cultures can be increased by the addition of calcium carbonate to the milk before inoculation. This procedure is quite satisfactory because the calcium carbonate can be added in sufficient quantities to neutralize all the acid that will be formed and yet, because of its insolubility in milk, it will not alter the reaction of the milk to the extent of interfering with the growth of the bacteria.

A number of investigators, Orla-Jensen (17), Barthel (2) Anderegg and Hammer (1) and Hammer and Patil (14), have added calcium carbonate to milk in studying the action of lactic acid organisms on the proteins. The calcium carbonate was added for the purpose of neutralizing the acid as it was formed and thus allowing the organisms to grow longer than they otherwise would.

Belonovsky (4) reported that the addition of calcium carbonate to milk in which Lactobacillus bulgaricus was grown prolonged the period the organism would remain alive at 35°C.

This investigator used amounts of calcium carbonate varying from eight-tenths to ten per cent and found that when four per cent or more was added to the milk the organisms remained alive for four months.

It was pointed out by Orla-Jensen (18) that lactic acid bacteria could be preserved for several months in milk to which calcium carbonate had been added. He did not use this method extensively, however, because it required the use of relatively large flasks so the contents could be shaken frequently to distribute the calcium carbonate. He thought these conditions favored contamination.

Barthel (3) reported that lactic acid bacteria could be kept alive for nine years, without transfer, in milk to which chalk had been added.

METHODS USED

Some of the butter cultures used in the study of the effect of adding calcium carbonate were prepared in pasteurized milk while others were prepared in sterile milk. When prepared in pasteurized milk the procedure was as follows. Approximately 150 cubic centimeters of milk were put into each of a number of six ounce small-mouth glass-stoppered bottles and to each of one-half of the bottles approximately two grams of precipitated calcium carbonate were added. The bottles of milk were then heated in a water bath to above 85°C. for 30 minutes after which

they were cooled to 21°C. by running cold water into the hot water surrounding the bottles. When the butter cultures were prepared in sterilized milk, erlenmeyer flasks were used instead of glass-stoppered bottles and 3.2 per cent of precipitated calcium carbonate was added before sterilization. Each of the bottles of pasteurized milk and flasks of sterilized milk was inoculated with a butter culture and then incubated at 21°C. After incubation the butter cultures containing calcium carbonate were shaken thoroughly to insure its even distribution. The butter cultures, both with and without added calcium carbonate, were put into small bottles (capacity 25 cubic centimeters) of the type used for sending butter cultures through the mails, the bottles stoppered with sterile corks and then sealed with sealing wax.

The temperatures of holding the bottled cultures varied in different comparisons, room temperature, 21°C. and 37°C. all being employed. Room temperature and 21°C. are mentioned separately because the former varied considerably during some of the holding periods. After the holding periods, each butter culture was run through a series of transfers in pasteurized milk and each transfer was examined for flavor and aroma until it was determined whether or not satisfactory butter cultures could be produced.

The designations of quality used are for the most part self explanatory. Cultures classified as "good" were not all

equal in quality but were considered to be very satisfactory. Those classified as "fair" either lacked sufficient flavor and aroma or had a slight off flavor. These probably would have developed into satisfactory butter cultures if they had been carried through more transfers. Those classified as "poor" had a small amount of flavor but not enough to be considered satisfactory. While these might have improved on later transfers it is uncertain that they would and butter cultures of this type could not be carried to advantage under practical conditions. Those classified as "bad" had no butter culture flavor whatever and some failed to coagulate even after several days. Many that coagulated very slowly showed, when stained mounts were made, organisms other than the butter culture organisms. These were usually gram positive rods and were presumably resistant types that survived the pasteurization.

RESULTS OBTAINED

The Effect of the Addition of Calcium Carbonate on the Keeping Qualities of Butter Cultures Held at Room Temperature.

In the studies on the effect of the addition of calcium carbonate on the keeping qualities of butter cultures, comparisons were made at room temperature between butter cultures prepared in milk with added calcium carbonate and butter cultures prepared in milk without added calcium carbonate. Room tempera-

ture was used for holding because under practical conditions when cultures are held, as in shipping, this would be the approximate temperature encountered. However, during some of the holding periods the room temperature was quite high, being above 31°C. on a number of days. Data on the exact number of days in the holding periods were recorded but for convenience in comparison only three groups of holding periods are reported. The first, "five days or less", represents a length of time comparable to that which would elapse when cultures are sent to plants by mail. The other two are longer and the third group, "more than eleven days", includes some periods as long as 93 days. Results obtained with six different butter cultures are presented in Table X.

In general the number of satisfactory butter cultures which were obtained from the material held decreased as the holding periods increased. With calcium carbonate added the percentages of the trials resulting in "good" butter cultures were 49, 42 and 33 when the holding periods were "five days or less", "six to eleven days" and "more than eleven days" respectively. If the trials that resulted in satisfactory ("good" or "fair") butter cultures are compared with the trials that resulted in unsatisfactory ("poor" or "bad") butter cultures the differences in favor of the shorter holding periods are still more pronounced.

Without calcium carbonate the percentages of the trials

Table X.

The effect of the addition of calcium carbonate on the keeping quality of butter cultures held at room temperature.

holding periods	no. of comparisons	butter cultures held with added $CaCO_3$				butter c
		satisfactory		unsatisfactory		satisfa
		% good	% fair	% poor	% bad	% good
5 days or less	33	49	36	9	6	46
6 to 11 days	38	42	16	32	10	24
more than 11 days	33	33	9	9	49	0
totals	104	41	20	17	22	23

Table X.

effect of the addition of calcium carbonate
ne keeping quality of butter cultures held
at room temperature.

tter cultures held with added $CaCO_3$				butter cultures held without added $CaCO_3$			
atisfactory		unsatisfactory		satisfactory		unsatisfactory	
% od	% fair	% poor	% bad	% good	% fair	% poor	% bad
49	36	9	6	46	30	9	15
42	16	32	10	24	18	24	34
53	9	9	49	0	6	3	91
41	20	17	22	23	18	13	46

resulting in "good" butter cultures were, 46, 24, and 0 when the holding periods were "five days or less", "six to eleven days" and "more than eleven days" respectively. This shows a greater deterioration, on continued holding, in the cultures without added calcium carbonate than in the cultures with added calcium carbonate. In some series of trials the keeping qualities of all the butter cultures used were definitely better than in other series of trials. This indicates that factors which vary with different trials are important in this respect.

The results secured show that the addition of calcium carbonate to milk increased the keeping qualities of butter cultures made from it. The advantage due to the presence of calcium carbonate was greater in the butter cultures held for the longer periods than in the butter cultures held for the shorter periods. In the short holding periods (5 days or less) the advantage due to the presence of calcium carbonate was only slight.

To determine whether or not the addition of calcium carbonate affects the keeping qualities of various butter cultures differently the data secured with the four cultures used most frequently were arranged so the cultures could be compared. The results are presented in Table XI.

On the basis of the total trials made the keeping qualities of the four butter cultures were increased to approximately the same extent by the addition of calcium carbonate. On the basis

Table XI.

The effect of the addition of calcium carbonate the keeping qualities of different butter cultur when held at room temperature.

butter culture number	holding periods	no. of comparisons	butter cultures held with $CaCO_3$		butte
			% satisfactory	% unsatisfactory	% satisf
D-103	5 days or less	6	84	16	6
	6 to 11 days	6	17	83	
	more than 11 days	7	43	57	1
	total	19	47	53	2
D-122	5 days or less	16	94	6	7
	6 to 11 days	16	81	19	5
	more than 11 days	10	50	50	
	total	42	79	21	5
D-144	5 days or less	3	33	67	3
	6 to 11 days	5	20	80	2
	more than 11 days	6	50	50	
	total	14	36	64	1
D-146	5 days or less	8	87	13	10
	6 to 11 days	9	78	22	5
	more than 11 days	6	50	50	1
	total	23	74	26	6

Table XI.

he effect of the addition of calcium carbonate on
he keeping qualities of different butter cultures
when held at room temperature.

	no. of comparisons	butter cultures held with $CaCO_3$		butter cultures held without $CaCO_3$	
		% satisfactory	% unsatisfactory	% satisfactory	% unsatisfactory
	6	84	16	67	33
	6	17	83	0	100
ays	7	43	57	14	86
	19	47	53	26	74
	16	94	6	75	25
	16	81	19	56	44
ays	10	50	50	0	100
	42	79	21	50	50
	3	33	67	33	67
	5	20	80	20	80
ays	6	50	50	0	100
	14	36	64	14	86
	8	87	13	100	0
	9	78	22	55	45
ays	6	50	50	16	84
	23	74	26	61	39

of the different holding periods the keeping qualities showed irregularities; for example, a larger percentage of the trials in which butter culture D-103 was held for "more than eleven days" yielded satisfactory butter cultures than did the trials in which this butter culture was held for "six to eleven days". With butter culture D-144 the trials in which the holding periods were longer than eleven days yielded a larger percentage of satisfactory butter cultures than did the trials in which the holding periods were "six to eleven days" or even "five days or less". These differences are not significant, however, because of the small number of comparisons made with butter cultures D-103 and D-144.

The results secured show that there were no significant differences in the effect of calcium carbonate on the keeping qualities of the different butter cultures used. The differences between the keeping qualities in different trials with the same butter cultures were as great as the differences between the keeping qualities of the different butter cultures.

Relationship of Temperature to the Effect of the Addition of Calcium Carbonate on the Keeping Qualities of Butter Cultures.

The relationship of temperature to the effect of the addition of calcium carbonate on the keeping qualities of butter cultures was studied by preparing four different butter cultures both with and without added calcium carbonate, transferring each

preparation to a number of small bottles and holding one portion of each set of bottles at 21°C. and the other at 37°C. After various holding periods the contents of the bottles were inoculated into pasteurized milk and each culture secured was run through a series of transfers to determine whether or not satisfactory butter cultures could be produced. Twenty trials at each temperature were made. The results obtained are presented in Table XII.

Ninety per cent of the butter cultures held at 21°C. in milk with calcium carbonate and ten per cent of the butter cultures held at 37°C. in milk with added calcium carbonate were satisfactory when transferred into pasteurized milk. Thirty-five per cent of the butter cultures held at 21°C. in milk without calcium carbonate and 20 per cent of the butter cultures at 37°C. in milk without calcium carbonate were satisfactory when transferred to pasteurized milk.

The results obtained show that the addition of calcium carbonate increased the keeping qualities of the butter cultures held at 21°C. but did not increase the keeping qualities of the butter cultures held at 37°C. The temperature of holding had a greater influence on the keeping qualities than did the presence of calcium carbonate.

Table XII.

The relationship of temperature to the effect of the addition of calcium carbonate on the keeping qualities of butter cultures.

quality of the butter cultures	butter cultures held at 21°C.		butter cultures held at 37°C.	
	with $CaCO_3$	without $CaCO_3$	with $CaCO_3$	without $CaCO_3$
% satisfactory	90	35	10	20
% unsatisfactory	10	65	90	80

Studies on the Keeping Qualities of Butter Cultures Held for Long Periods of Time in Milk with Added Calcium Carbonate.

Studies on the keeping qualities of butter cultures held for long periods in milk with added calcium carbonate were made by preparing butter cultures in sterile milk to which 3.2 per cent calcium carbonate had been added, transferring each preparation to small bottles and after varying holding periods (154 to 272 days) at room temperature inoculating the contents of each of the bottles into pasteurized milk. Each butter culture thus secured was then run through a series of transfers to determine whether or not satisfactory flavor and aroma could be produced. The results obtained from 28 trials are presented in Table XIII.

In general the butter cultures obtained after the holding periods were unsatisfactory. In trial 24 a satisfactory butter culture was secured and in trials 5, 14, 17, and 23 some butter culture flavor and aroma were produced, however, in the other trials, no butter culture flavor and aroma were produced and in some coagulation did not even occur. Microscopic examination of the material in the trials that failed to produce butter culture flavor and aroma usually showed gram positive rods which presumably had survived the pasteurization incident to the preparation of the milk for inoculation.

Examinations for the numbers of each the associated organisms and S. lactis were made on the material held in trials

Table XIII.

Keeping qualities of butter cultures held for long periods at room temperature in milk to which calcium carbonate had been added.

trial number	days held at room temperature	quality of butter cultures secured
1	154	lacking in flavor
2	154	" " "
3	154	" " "
4	154	" " "
5	161	some flavor but not satisfactory
6	161	lacking in flavor
7	161	" " "
8	161	" " "
9	166	very slow to coagulate
10	166	failed to coagulate
11	166	" " "
12	166	lacking in flavor
13	174	bitter
14	174	some flavor, but not satisfactory
15	174	lacking in flavor
16	174	off flavor
17	174	some flavor, but not satisfactory
18	174	bitter
19	174	off flavor
20	174	bitter
21	192	failed to coagulate
22	192	lacking in flavor
23	204	some flavor, but not satisfactory
24	204	fairly satisfactory flavor and aroma
25	272	lacking in flavor
26	272	" " "
27	272	bitter, slow to coagulate
28	272	off flavor

5, 6, 23 and 24. In trial 5 there were approximately 10,000 associated organisms and 100 S. lactis per cubic centimeter; in trial 6, approximately one associated organism and 1,000,000 S. lactis per cubic centimeter; in trial 23, approximately 10,000,000 associated organisms and 100,000 S. lactis per cubic centimeter and in trial 24, which produced a satisfactory butter culture, no associated organisms and approximately 10,000 S. lactis per cubic centimeter. These results again show that more than the mere presence of the associated organisms in mixtures is necessary for the production of a butter culture flavor.

The data secured show that butter cultures could not successfully be held for long periods at room temperature in milk to which calcium carbonate had been added; only one of the 28 trials resulted in the production of a satisfactory butter culture.

Difficulty of Holding Butter Cultures, Prepared in Milk with Added Calcium Carbonate, in Stoppered Bottles.

Butter cultures prepared in milk containing calcium carbonate produce considerable carbon dioxide and when held in tightly stoppered bottles the stoppers are frequently blown out. This is especially apt to happen when the butter cultures are allowed to warm up in the bottles after the bottles have been sealed. Agitation of the sealed bottles favors the release of carbon dioxide and is often the immediate cause of blowing the

stoppers. Both warming up and agitation often occur during shipment of butter cultures and when calcium carbonate is used in the milk the bottles must be carefully sealed to avoid loss from this source.

DISCUSSION OF RESULTS FOR PART II.

The small increases obtained in the keeping qualities of butter cultures held at room temperature (25° - 32°C.) in milk with added calcium carbonate indicate that this procedure would not be of value when butter cultures are held for only short periods and consequently it is doubtful whether or not it would be of value in mailing butter cultures. The blowing of stoppers is very objectionable and lessens the value of this procedure for preparing butter cultures for mailing. For longer holding periods the increases in the keeping qualities obtained in milk with added calcium carbonate were quite definite, however, the percentages of the trials in which good butter cultures were obtained, even when calcium carbonate was added, were too small to justify the use of this procedure for holding butter cultures at temperatures between 25° and 32°C.

A comparison of the results obtained when butter cultures were held at 21°C. with those obtained when butter cultures were held at 37°C. shows that the temperature of holding is very important. At 21°C. with added calcium carbonate it was possible

to hold butter cultures for periods up to at least 20 days without serious deterioration but at 37°C. deterioration was very rapid and only a small per cent of the trials resulted in satisfactory butter cultures even when the holding periods were short. This suggests that the deterioration of butter cultures during the time held in the mails could be reduced by maintaining lower temperatures.

The differences in the keeping qualities of the butter cultures in different series of trials show that factors which varied with the different series of trials were important. This suggests that variations could be expected in the keeping qualities of butter cultures prepared regularly and that a study of factors which vary in butter cultures from day to day such as rate of acid production or amount of acid produced might give information which would be useful along this line.

The deterioration of the butter cultures held for long (154 to 272 days) periods at room temperature in milk to which calcium carbonate had been added shows that this procedure was not satisfactory. However, the fact that a satisfactory butter culture was obtained from the material held in one trial and some butter culture flavor and aroma were obtained from the material held in four other trials suggests that it would be possible under different conditions to hold butter cultures satisfactorily for long periods of time. The room temperature, during the holding periods of the trials made, varied from

approximately 25°C. to approximately 33°C. and it is quite probable that had lower temperatures been employed the deterioration would have been considerably less.

The presence of large numbers of associated organisms in some of the butter cultures held for long periods shows that the mere presence of the associated organisms in combinations is not all that is necessary for the production of a desirable flavor and aroma. The associated organisms and S. lactis present after the holding periods were of the same strains as those present before the holding periods when the flavor and aroma were satisfactory. This suggests that the failures to obtain satisfactory butter cultures after the holding periods were due to changes that had taken place in the organisms themselves or in their growth relationships.

The calcium carbonate added to the milk did not neutralize all of the acid formed although there was calcium carbonate left in the butter cultures after the holding periods. The insolubility and settling out of the calcium carbonate in the milk interfered with the neutralization of the acid. It is, therefore, not surprising that deterioration occurred even in the presence of calcium carbonate, due to the accumulation of acid. It is quite probable that frequent shaking of the butter cultures during the holding periods would have increased the amounts of acid neutralized by the calcium carbonate and thus would have decreased the extent of deterioration.

CONCLUSIONS TO PART II.

1. The addition of calcium carbonate to milk increased the keeping qualities of butter cultures made from it when held at room temperature. However, the percentage of the cultures held at this temperature from which satisfactory butter cultures were obtained was small even when calcium carbonate was added.

2. There were no significant variations in the effect of the addition of calcium carbonate on the keeping qualities of different butter cultures.

3. The temperatures of holding influenced the effect of calcium carbonate on the keeping qualities of butter cultures. At 21°C. the keeping qualities were increased to a considerable extent but at 37°C. the keeping qualities were not increased.

4. Butter cultures could not successfully be held for long periods at room temperature in milk to which calcium carbonate had been added.

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