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THE MICROBIOLOGY OF CHEESE-LIKE FLAVORS IN UNSALTED BUTTER[1]

E. O. Herreid, H. Macy, and W. B. Combs

INTRODUCTION

A number of creameries in Minnesota and adjacent territory produce considerable quantities of unsalted butter. This butter is made from sweet cream of the best quality and commands a substantial premium on the market.

Some difficulties have been experienced in the manufacture and storage of this butter. Certain off-flavors have developed, especially those characterized as "cheesy." These cheesy flavors have varied from a mild Cheddar to an inferior Limburger. Some are easy to identify, but others suggest the combined odors and flavors of several varieties of cheese, or are actually putrid.

The cheesy flavors develop rapidly, often within one to three weeks after the butter is manufactured. In fact, they frequently develop before shipments are consigned from the central points of distribution. Creamery operators have conducted their plant operations according to the usual routine, only to learn eventually that a previous shipment had been graded "cheesy." Apparently unsalted butter is most susceptible, altho such flavors have been observed in the salted grades.

These defects have been spasmodic, in the individual creameries, occurring most frequently during the spring and early summer. Creameries in certain localities sometimes have been affected simultaneously.

Because of these circumstances, it has been necessary to select carefully the creameries that could reliably produce unsalted butter of superior keeping quality.

Certain creameries have suffered severe financial losses because of these cheesy flavors, since butter graded "cheesy" invariably scores 88 or lower on the market. Occasionally, such defective butter has been returned from local stores, with the result that consumers and retailers have become prejudiced against particular brands.

The causes of these defects have been so firmly established in certain creameries and have appeared so elusive that the operators have been discharged because of their inability to control these outbreaks.

Individual creameries and large marketing organizations have ap-

[1] The data in this publication are taken from a thesis submitted to the faculty of the Graduate School of the University of Minnesota by E. O. Herreid in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June 1933.

pealed to the dairy division of the University of Minnesota for assistance in eliminating the causes of these objectionable flavors in butter. Members of the dairy staff have made observations at creameries and have followed shipments of butter through the channels of trade in an attempt to obtain significant facts concerning these annoying defects.

It is evident that a definite problem exists and the manifestations of these defects indicate clearly that the problem may be complicated by a number of factors, including biological and biochemical considerations.

The studies reported in the subsequent pages were planned in an attempt to obtain some information regarding the fundamental causes of cheesy flavors, especially those of the Cheddar type, which occur so frequently in unsalted butter.

REVIEW OF LITERATURE

The literature concerning cheesy flavors and related defects in butter has been studied exhaustively and has been reported in detail by Herreid (46), but is too voluminous to warrant a comprehensive review in this publication.

The defect has been reported from the leading butter-producing countries of the world. Krueger (60) isolated a number of organisms from a sample of cheesy butter and reported the presence of *Micrococcus acidi, Bacillus fluorescens nonliquefaciens, Bacillus acidi lactici, Oidium lactis,* and other unidentified bacteria and yeasts. From a sample of butter described as cheesy, Fettick (30) isolated *S. lacticus* in small numbers, representatives of the colon-aerogenes group, a *Micrococcus, Bacillus subtilis, Bacillus fluorescens liquefaciens, Penicillium glaucum,* a species of *Mucor* and yeasts.

Orla-Jensen (77) (78), Ibsen (57), Burr (15), Frielinghaus (32) and others (59) (61) (73) (74) (83) (87) described an "ostesurt" or cheese-sour flavor in butter, which was prevalent in the dairy regions of Europe. Orla-Jensen (78) stated that this flavor reached its most intense form when the lactobacilli in symbiosis with yeasts caused fat hydrolysis. He (77) also stated that the symbiotic activity of lactic acid bacteria and gas producers might be involved. Ibsen (57) found that "cheese-sour" butter contained a greater variety of gases than normal butter, but he was not successful in determining if gas formation was essential in the development of this defect. His results indicated that any physical condition which allowed the incorporation of excessive amount of buttermilk would expedite the onset of "cheese-sour" flavors, because a favorable medium would be provided for the lactobacilli and the gas-formers. Burr (15) found that the amount of

protein was not the important factor in the development of these flavors but believed that this defect originated from bacterial causes which were possibly associated with improper ripening, with the bacterial flora of the water supply and with contaminated salt.

Rosengren (91) (92) encountered yeasty flavors in butter which he believed were identical with the "ostesurt" flavors described by Orla-Jensen (77) (78). Rosengren (92) and Reitz (85) attributed yeasty flavors to the symbiotic activity of yeasts and lactic acid bacteria, while Widen (122) found that the fermentation of lactose resulted in yeasty-flavored butter. Macy and Richie (66) found no definite quantitative correlation between mold and yeast counts and the keeping quality of butter.

Orla-Jensen (76) stated that *Penicillium glaucum* imparted a Roquefort-cheese taste and odor to butter. Morgan (71) reported that *Oidium lactis* imparted a flavor to butter typical of Coulommiers and Pont l'Eveque cheese. Macy (64) demonstrated that *Oospora lactis* produced a Cheddar or a Brick cheese odor in butter, and Stokoe (110) found that this organism produced a strong cheesy flavor on a fat medium after seven days' storage at 60° F. Ruehle (93) was of the opinion that cheesy flavors were not produced by *Oospora lactis,* individually, or when grown in association with *Streptococcus lactis.* Combs (17) found that, when *Oidium lactis* and *Penicillium chrysogenum* made considerable growth and when the cream was subsequently pasteurized at 145° F., the butter became cheesy after 90 days' storage at 50° F. Weigmann (118) described a sample of butter with the Roquefort type of cheesiness in which the microflora consisted of *Oidium lactis,* lactobacilli, yeasts, and cocci.

Rahn (123) believed that cheesy flavors in butter were caused by ripening cream to an abnormally high acidity, holding the coagulated clumps of casein between the butter granules during the churning process and not removing them completely during the washing process, resulting in the eventual decomposition of the casein. This coincides with Deger's (20) viewpoint and also with Bouska's (11), who indicted putrefactive organisms and *Oidium lactis* as the causative agents.

Hunziker (54) cited Cheddar, Roquefort, and Limburger as the most common types of cheesiness. He suggested strict sanitation, efficient pasteurization, accurate neutralization, pure wash water and sterile parchment and liners as control measures.

Under Minnesota conditions Macy (63) observed that the most common cheesy flavors in butter were the old, well-ripened Cheddar, the offensive Limburger, and the Roquefort types.

Rogers and Gray (90) stated that butter from unripened and unpasteurized cream always developed cheesy or rancid flavors. Butter stored at −10° F. was judged cheesy, and they attributed this condition to the probability that enzymes might have been secreted in the cream to cause some undetermined change and also to minute quantities of decomposition products from dead bacterial cells. Gray (34) found that, in general, cheesiness was most prevalent in lightly salted butter during the last stages of the storage period. Hesse (48) commented on a cheesy and yeasty sample of unsalted butter after nine days of storage.

Lauterwald (62) stated that cheesy-flavored butter originated when cream and whey cream were churned together, but he did not determine the underlying factors involved. Anthony (2) ripened sweet cream to an acidity of 0.85 per cent and the resulting butter became cheesy. Dempster (25) encountered curdy or yeasty flavors in cream and attributed these defects to inefficient skimming and insufficient cooling.

Spitzer and Parfitt (107) found that *B. ichthyosmius* and *B. proteus vulgaris*, individually, caused rapid proteolysis in butter and the manifestation of cheesy and related flavor defects. Shutt (103) found that certain members of the *Pseudomonas fluorescens* group produced odors in sterile butter comparable to rancid cheese.

Hammer and Patil (38) indicated that butter infected with proteolytic and non-proteolytic strains of *Streptococcus lactis* developed cheesy flavors during a storage period of six weeks at 21° C. Henneberg (45) found that the casein decomposers and, to a degree, the fat-splitting representatives of *Bacillus cloacae* produced putrid, cheesy, and bitter flavors in butter.

Derby and Hammer (26) reported that certain samples of butter with surface-taint flavors developed cheesiness of one type or another at 21.1° C. The degree of cheesiness varied from Limburger to Cheddar. One sample had an odor and flavor suggesting Swiss Cheese.

Demeter and Maier (22), Palmer and Combs (80), and others (12) (65) (84) (117) made incidental reference to cheesy flavors, while dealing with other phases of the keeping quality of butter.

A group of flavors commonly called "fruity" appear to be concerned in the development of flavors and aromas of the Cheddar cheese type. The organisms associated with these pleasant, yet undesirable aromas, have been described as ester- or aroma-producers.

Grimm (36) described an organism, *Bacillus aromaticus lactis*, which produced a fruity odor in butter which became cheesy within two or three weeks. Another organism, listed as *Bacillus No. 41 Conn*, pro-

duced a weak acid reaction in milk, while the milk gradually became cheesy. Stocker (108) (109) reported that organisms belonging to the *Bacterium fluorescens* group produced in butter an ester-like raspberry odor, which passed through a transitional stage of cheesiness to putridness. Weigmann (119) found a lactose-fermenting yeast which imparted to butter a pronounced fruity taste and odor. He found a number of organisms that produced aromatic substances in butter. Virtanen (115) encountered a species resembling *Bacillus punctatum*, which produced, in butter, a strong flavor of a fruit ester. The organism was sensitive to salt and to high acidities. Sewerin (100) isolated an organism from sour cream which produced a pleasant fruity odor in butter within four to six weeks. He named this organism *Bacillus aromaticus butyri* because it appeared unlike the aroma-producers isolated by other investigators. The ability of this organism to produce fruity flavors in butter was not a constant property.

Cunningham (19) reported the development of an aroma in milk which resembled amyl alcohol. It was observed when the cows were stall-fed, but disappeared during the period of pasture feeding. This defect was associated with white and orange micrococci, which were found on the barn floor.

Cheesy and related flavor defects have been traced to infected water supplies. Sadler and Vollum (96), Shutt (103) (104), and Hood and White (51) reported *Pseudomonas fluorescens* in butters with surface-taint flavors and found that pasteurization at high temperatures and ripening the cream prevented these objectionable flavors. Virtanen (116), Rumment (94) (95), Gilruth (33), and Stocker (109) found *Bacterium fluorescens* and *Bacterium punctatum* in creamery water. McKay and Bower (68) found that creamery waters often produced objectionable flavors in sweet cream butter. Jensen (58) described a putrid condition in butter infected with *Bacillus foetidus lactis* which originated from a patron's water supply. Eckles (27) reported a case of putrid butter which lasted for two weeks. This was so offensive that the butter was rejected on the market. He concluded that this condition was due to contaminated milk.

Busch (16), Demeter and Maier (22), Orla-Jensen (77), and others (1) (11) (15) emphasized the importance of bacteriologically pure water in the creamery. Mrozek and Meetz (72) obtained no definite correlation, while Melick (69) reported a direct relationship between the water supply, bacteriologically and chemically, and the quality of the butter. Vieth (114) and others (57) (78) (118) recommended removing the plasma solids exhaustively by washing to produce butter of superior keeping quality.

In this connection it should be mentioned that Rumment (94) (95) reported results from carefully controlled experiments in which he found that the bacterial count of sweet cream butter was four times greater than that of sour cream butter, when both types of butter were infected through the wash water. By increasing the consistency of the butter and by churning larger granules, the finished butter retained relatively fewer bacteria from the wash water, while soft butter retained correspondingly larger numbers of bacteria.

It is evident from this brief resume of the literature that the bacterial flora of cheesy-flavored butter has differed. In a few instances the organisms have been confined to a restricted flora, but in most cases several genera have been reported. While it was impossible to assemble from the literature a complete synonomy of the genera involved, the alleged causes of the various cheesy flavors often appeared to involve directly or indirectly certain combinations of bacteria, yeasts and molds. Moreover, the flora differed from one geographical location to another, and there was no general agreement as to the species implicated in various countries. Some form of associative activity may have induced the development of these cheesy flavors.

EXPERIMENTAL

General Procedure

In order to carry out controlled laboratory experiments, it was necessary to adopt standard procedures. After a few trials, it was decided to use glass churns of the "Dazey" type. Churning in this equipment was satisfactory as judged by comparisons with commercial churns. The size and the physical appearance of the butter granules were normal and the fat content of the buttermilk was reasonably low. Consequently, the experimental churnings, unless otherwise stated, were made in glass churns of four- and eight-quart capacities, equipped with aluminum dashers and driven by an electric motor (see Fig. 1).

The cream for the experiments was obtained principally from milk produced by the university dairy herd, and was standardized to 32 to 33 per cent fat. This cream was sterilized in two-quart lots by preheating in a water bath to 60 to 65° C. and then autoclaving at 15 pounds pressure for a period of 12 minutes. Regular platings demonstrated that the cream was sterilized by this procedure. Following sterilization, it was immediately cooled to the desired temperature. A cooked flavor was evident in the sterile cream, but this did not seriously interfere with the detection of cheesy flavors in the resulting butter. The temperature of the cream at the beginning of the churning process and the temperature of the wash water were controlled so as to maintain a uniform body

and texture in the butter at all seasons. Sterile, distilled, wash water was used in amounts equal to the buttermilk removed.

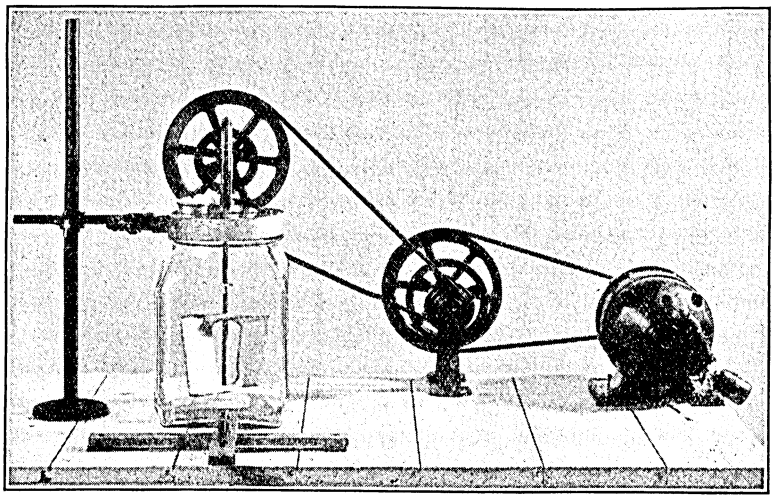

Fig. 1. Apparatus Used for Regular Churning Procedure

Considerable difficulty was experienced in obtaining suitable butter working equipment which could be easily sterilized. Glass, porcelain, and roughened aluminum were submitted to exhaustive trials, but the butter showed a tendency to stick to such surfaces. Finally, a suitable apparatus was made from wood. Birch boards (13″ x 18″ x 1″) were obtained and the butter was placed on them during the working process. An appliance to work the butter was made by cutting in half the horizontal bars of an old household butter working apparatus. The working process consisted of gathering the butter into a roll, leveling this roll out on the board and repeating this process until the desired body and texture were obtained. In general, this procedure was uniform for each experimental series. However, it was necessary to vary the extent of the working somewhat from one experiment to another, because of seasonal factors, which were accompanied by changes in the composition of the fat and in the temperature of the room. Butter was worked in a chamber covered with glass to eliminate, as far as possible, contamination from the air. The finished butter was placed in g'ass jars by means of a wooden spatula, and packed with a wooden stamper (see Fig. 2).

Sterile equipment was used throughout. The glass churns were covered with paper and sterilized by dry heat for at least five hours at 170 to 180° C. The wooden equipment and aluminum dashers wrapped in paper, the glass sample jars, and the wash water, were all autoclaved for 45 minutes at 15 pounds pressure. After autoclaving, the paper was

removed from the wooden equipment and the individual pieces were immersed in an earthenware jar containing a cold hypochlorite or chloramine-T solution and allowed to cool to 9-11° C. The chlorine solution was maintained at a strength of 150 to 175 parts per million of available chlorine. Immediately after removal from this solution, the wooden pieces were always rinsed. A complete set of sterile wooden equipment was available for each churning. At all times precautions were taken to reduce possibilities of contamination from any source.

Employing these procedures and technic, it was observed that the butter obtained from sterile cream did not show any significant contamination even after 14 and 28 days of storage.

The experimental butters were judged by certain members of the staff of the dairy division, assisted on different occasions by federal butter graders. The butter was judged and plated at intervals of two and four weeks and in some instances more frequently, as it was deemed necessary. Descriptive adjectives are used in recording the defects of the experimental butters. Some of the terms used are very general in their meaning, but this system is followed because a better one is lacking. Butters scoring 90 or above, according to commercial practice, are termed "satisfactory." The usual storage temperatures were 5° C. and 10° C., but occasionally samples of butter were kept at 21° C. The fluctuations in temperature from the desired point rarely exceeded ± 1.5° C.

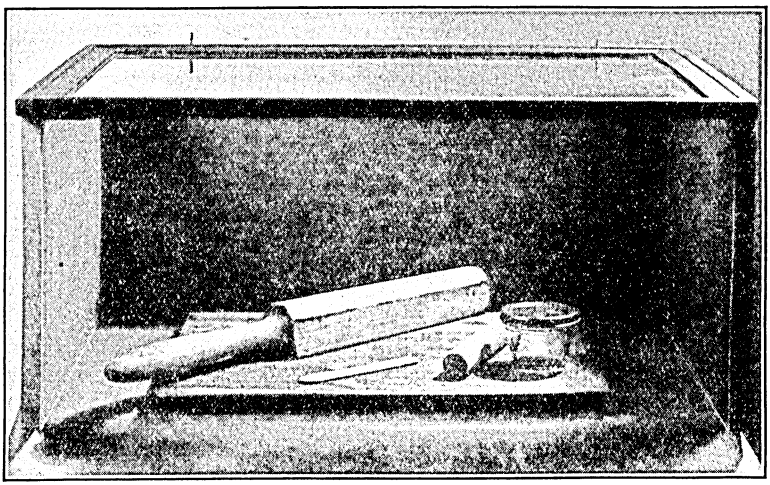

Fig. 2. Apparatus Used for Working and Packing Butter, with Glass Jar in Which Butter Was Stored

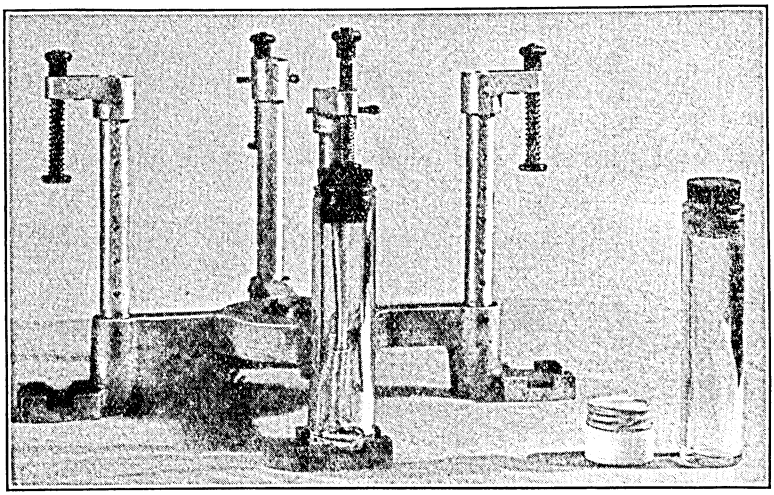

Fig. 3. Apparatus Used for Churning Small Quantities of Cream, and Small Porcelain Jar in Which Butter Was Stored

It was necessary in some of the experiments to churn cream inoculated with pure cultures. The use of glass churns required large amounts of cream. This was neither practical nor expedient. Therefore, it was decided to use another apparatus (see Fig. 3) whereby the amount of cream could be reduced to a minimum. The individual cultures (24-48 hours old) were inoculated into 25 milliliters of sterile cream contained in thick-walled, cotton-stoppered glass tubes. These were incubated at 10° C. and 21° C. for 24 to 48 hours. The inoculated creams were adjusted to suitable churning temperatures. The cotton plugs were replaced with sterile, rubber stoppers and the contents churned by means of a shaker attached to a No. 2 International centrifuge. This shaking device produced rapid, vertical oscillations, and churning was usually completed within five minutes. The butter granules, however, were packed together in a more or less compact mass. The butter was washed with a suitable amount of sterile water and worked on sterile parchment paper by means of a moist, sterile, wooden spatula and packed in small porcelain ointment jars.

PRELIMINARY OBSERVATIONS ON THE OCCURRENCE OF CHEESY FLAVORS IN BUTTER IN MINNESOTA AND ADJACENT TERRITORY

No attempt was made to survey all the creameries experiencing spasmodic outbreaks of cheesy flavors in butter. However, owing to the prevalence of these defects, certain members of the dairy division have visited many of the affected plants. The creameries in question were all confronted with the same general problem, but the underlying causes in

each may have been different. A few cases will be cited to indicate the seriousness of the problem.

A certain creamery experienced intermittent trouble with cheesy flavors in unsalted butter. A careful survey was made of the entire plant, but the source of trouble was not located. Another creamery reported similar difficulties and a survey at this plant indicated quite clearly that the equipment was contaminated from some unknown source. Both of these creameries abandoned the manufacture of unsalted butter.

One creamery suffered a series of annoying outbreaks of cheesy and putrid flavors in butter extending over a period of three years. Rather extensive experiments were completed at the plant and various specimens were taken to the laboratory for further study. The results showed quite definitely that infected water was the source of the trouble. When a new and satisfactory water supply was provided no further difficulties were reported.

Complaints were also received from creamery organizations in other states. Inoculation of sterile cream with water received from these creameries has invariably led to the development of cheesy flavors of one type or another in the resulting butter. Some of these specimens were submitted to detailed study to obtain some information concerning the fundamental causes of the defects. These experiences and observations have aided in planning the present study.

PRELIMINARY EXPERIMENTS WITH RAW CREAM AND BACTERIAL CULTURES

A source of raw cream that would consistently produce butter with cheesy flavors and aromas was one of the first considerations. It was needed as a basis for studying some of the biological factors responsible for these defects.

Through the co-operation of a large creamery organization in Minnesota, eleven samples of raw cream were obtained from different points in the state. The butter churned from these creams was judged for flavor defects. More than one-half of the samples developed cheesy flavors of one type or another.

A number of churnings were made with raw cream from the university dairy. The results are recorded in Table 1. It will be noted that all of the butters were graded "cheesy" at some time during the storage period. These flavors apparently were not stable, but gradually passed through various stages of development.

Table 1

Comments on Unsalted Butter Prepared from Raw Cream

Churning No.	Flavor and aroma of butter after storage for:			
	14 days at		28 days at	
	5° C.	10° C.	5° C.	10° C.
1	Slight Cheddar	Slight Cheddar	Mild Cheddar	Mild Cheddar
2	Slight Cheddar	Slight Cheddar	Slightly off	Cheddar, high acid
3	Slight Cheddar	Slight Cheddar	Cheddar	Doughy and Roquefort
4	Satisfactory	Slightly off	Slight Limburger	Limburger
5	Slightly fishy	Cheesy	Slight cheesy	Fishy and tallowy
23	*	*	Cheddar	Cheddar
24	*	*	Slight Cheddar	Unclean
25	*	*	Cheddar	Cheddar and putrid
29	Overripe cheese	Urinary	Cheddar and high acid	Cheddar and high acid
30	Bitter	Unclean	Satisfactory	Slight Cheddar
36	Slightly off	Slight cheesy	Satisfactory	Satisfactory
37	Mild Cheddar	Cheesy	Mild Cheddar	Cheesy
38	Satisfactory	Satisfactory	Slight Cheddar	Cheddar and surface taint
39	Satisfactory	Satisfactory	*	Cheesy

* Not examined.

The question obviously arose as to the possible causes of these cheesy flavors. Owing to the fact that a large number of defects in milk products are caused by microorganisms, it was natural to suspect that bacteria might be the causative agents. Consequently, during the course of this preliminary work, 450 cultures were picked from platings of cheesy-flavored butter and from suspected specimens of water sent to this laboratory. These cultures were carried on whey agar slants and their ability to produce cheesy flavors was determined according to the procedure described on page 11.

Sixty-eight of these cultures produced flavors suggesting some type of cheese. The butters from 14 churnings of raw cream were graded cheesy, and 40 cultures picked from platings of these butters reproduced similar flavors. In general, a majority of the cultures obtained from cheesy butter reproduced such flavors. However, certain discrepancies appeared and they are difficult to explain. For example, four churnings of raw cream butter were not cheesy, but the cultures isolated from such butter produced cheesy flavors. These inconsistencies might be attributed to the probability that the original raw cream butter may have passed through the cheesy stage prior to, or even after, the butter was judged. It is also possible that other biochemical changes may have predominated and masked or even inhibited the development of cheesy flavors and aromas in the butter.

The evidence indicated that pure cultures might cause cheesy flavors in butter. Sixty-eight cultures were retained for further study, because they had produced such flavors. However, the possibility existed that

some of these cultures might be duplicates, so it was considered advisable to reduce the number still further. The action of these cultures was studied on different media, and on the basis of their physiological reactions and morphology they were segregated into eight general groups which are listed in Table 2.

Table 2

Characteristics of Cultures Isolated from Cheesy Butter

Group	Culture	Morphology	Gram stain	Action on:				
				Gelatin	Litmus milk	Glucose broth	Lactose broth	Sucrose broth
I	72, 331 435, 438	Rods	—	+	P	—	—	I
II	186, 213	Rods	—	—	I or Al	—	—	—
III	269	Rods	±	—	P	—	—	—
IV	190, 233	Rods	—	—	A	AG	AG	AG
V	35, 88 192, 221	Rods	—	+	Al	AG	—	AG
VI	241, 246	Rods	—	+	Al	AG	—	—
VII	116	Cocci	+	—	I	—	—	—
VIII	21, 28 411	Yeasts	+	+	P	A	A	A

Legend: (—) negative; (+) liquefying; (±) variable; (A) acid; (G) gas; (P) proteolysis; (I) inert; (Al) alkaline.

It was thought advisable to repeat the previous work with pure cultures, using larger amounts of cream and churning with the larger glass churns. This repetition was necessary in order to ascertain if the pure cultures would consistently produce cheesy flavors in unsalted butter. This information would be necessary before planning subsequent experiments concerning the technical and the practical phases of the problem.

The cultures listed in Table 2 were transferred twice in cream and thereafter two liters of cream were infected with 200 milliliters of a one-day cream culture of each organism. The inoculated creams were incubated at 10 to 11° C. for 24 to 48 hours before churning. Colonies were picked from petri plates prepared at the end of two and four weeks' storage and smears were stained by Gram's method to ascertain if any noticeable contamination had occurred. The results indicated that, with the exception of the organisms in Group VIII, pure cultures did not consistently produce cheesy flavors and aromas. The cultures in Group VIII produced flavors suggestive of Cheddar cheese and this was particularly true of Culture 411, which produced a fine Cheddar cheese

flavor. In the other groups, except V and VII, cheesy flavors of various descriptions were detected at some time during the storage period. The bacterial counts of the butter were sufficiently high so that lack of numbers was not a factor in the production of cheesy flavors and aromas.

It has been mentioned previously that cheesy flavor defects have been attributed to organisms in the water supplies of certain creameries. Because these defects were not consistently produced by inoculating the suspected organisms into sterile cream, butter made from sterile cream was washed with infected water. Cultures representing most of the groups in Table 2 were selected for this experiment. (Cultures 28, 72, 411, 186, 21, 246, 269.) One loopful of a one- or two-day-old culture was added to five milliliters of cold, sterile cream, and the suspension added to the wash water just before the butter granules were washed. The results confirmed the findings previously reported that pure cultures did not consistently produce cheesy flavors during a storage period of four weeks, when sterile butter was infected through the wash water. Duplicates of these samples were held in storage and examined later. The butters containing Cultures 28, 186, and 411, stored at 10° C., were cheesy after eight weeks' storage. Bacterial growth had occurred to such an extent that lack of numbers should not have been the limiting factor.

Culture 411 previously had produced a fine Cheddar cheese flavor in butter, but when this culture was inoculated into sterile butter through the wash water no pronounced changes in flavor were observed. This inconsistency was difficult to explain. The possibility existed that the numbers of organisms might have influenced the results, and also that the cheesy flavors might have developed and disappeared suddenly and were missed during the regular observations at the end of two and four weeks' storage. Consequently, an experiment was planned in which the amount of inoculum was varied in an attempt to control the number of organisms in the butter at the beginning of the storage period. A cream inoculum of Culture 411 was prepared and allowed to incubate at 5 and 10° C. for about 24 hours. Decreasing amounts of this culture were added to each of four lots of sterile cream, which were churned immediately. The butters were observed for flavor defects at seven-day intervals and the results showed that the ability of Culture 411 to produce flavors of the Cheddar cheese type was not a constant property. Flavors suggesting cheesiness were observed in only two of the samples. The desired variations in the bacterial content of the fresh butter were not obtained. However, it is questionable if the number of bacteria was the important factor in the production of cheesy flavors in the experimental butters. This culture (411) produced a fine Cheddar cheese

flavor at one time and the bacterial counts were not significantly different from those obtained when no such flavor was produced.

STUDIES WITH ASEPTICALLY-DRAWN CREAM AND ENZYME PREPARATIONS

Babcock and coworkers (3) (7) (8) reported that the enzyme galactase was associated with the ripening of cheese. Von Freudenreich (31) confirmed Babcock's work. On the contrary, Boekhout (10) found that no ripening occurred in Edam cheese, which was prepared from aseptically-drawn milk. Babcock and associates suggested that galactase might cause decomposition in butter because it was adsorbed on the surface of the fat globules. This was in general accord with the statement made by Rogers, Berg and Davis (88) that the normally occurring enzymes of milk are incorporated in the butter during churning. Babcock, Russell and Vivian (6) found that galactase was inactivated by heating the milk at 71° C. for ten minutes, while Hippius (49) reported that this enzyme withstood an exposure of 65° C. for thirty minutes. Thatcher and Dahlberg (112) concluded that salt and low storage temperatures inhibited the activity of galactase. On the basis of these facts and suggestions it was decided to plan experiments to ascertain if the biological factors in normal milk were responsible for the development of cheesy flavors in butter.

Two cows were selected from the University herd on the basis of the low bacterial content of their milk. The cows were milked under aseptic conditions with a sterile milking machine and the milk was skimmed in a sterile separator. After separation the cream was divided into lots treated as follows:

Series I Churned immediately, butter not washed.
Series II Held 13 hours, churned, butter washed once.
Series III Held 24 hours, churned, butter washed once.
Series IV Held 24 hours, churned, butter washed twice.
Series V Held 24 hours, churned, butter washed once and salted.
Series VI Pasteurized immediately at 60° C. for 20 minutes, cooled and churned.
Series VII Held 24 hours, pasteurized at 60° C. for 20 minutes, churned.
Series VIII Pasteurized at 60° C. for 20 minutes, cooled and held for 13 hours before churning.

At least two churnings were made in each series. The cream was held at 6 to 7° C.

It was found that the biological factors in butter from aseptically-drawn cream were not important agents in the development of cheesy

flavors. There were no evidences of these flavors during a storage period of four weeks. This experiment included only two cows, a Jersey and a Holstein. However, this should be representative, because Babcock (5) concluded that galactase is a normal constituent of cow's milk. Samples of butter from Series I and II were observed for flavor defects after eight weeks' storage, but no sign of cheesiness was evident; in fact, these butters were considered satisfactory. Seventeen churnings were made in this experiment and only two samples of butter were judged rancid, which would tend to indicate that the enzyme lipase was not present in sufficient quantities to cause rancidity in the butter made from aseptically-drawn milk. These results may also be interpreted to support Palmer's (79) contention that lipase is not a normal constituent of cow's milk, providing it can be assumed that most of the alleged enzymes of milk pass into the cream and are finally incorporated in the butter. On the contrary, Rice and Markley (86) contended that milk normally contains a fat-splitting lipase.

It is evident that the udder flora did not thrive at the low temperatures as shown by relatively low counts of the butters. It was difficult to determine if the flora included only the udder types; however, stained preparations of the plasma of the fresh and the storage butters indicated that no serious contamination had occurred. Petri plates of the fresh butter were saved and compared with the plantings made after two and four weeks' storage. The plates were stored at 5 and 6° C. and held their original appearance for as long as 90 days. In most of the churnings, the flora was fairly constant throughout the storage period of 28 days. It was necessary to repeat some of the experimental procedures with aseptically-handled cream, because accidental contamination sometimes occurred during the manufacturing process.

A study was made to determine the effect of certain animal proteases, such as pepsin, trypsin and the enzyme rennin, as possible contributing factors in the development of cheesy flavors in butter. Cream was produced under aseptic conditions and the churnings were reduced to a minimum number by dividing the butter from each churning into three parts of about 150 grams each. The enzymes were prepared in 0.1 per cent suspensions immediately before using, except the rennet extract, which was diluted to one-half strength with sterile distilled water. The enzyme preparations were plated and found sterile, except the rennet extract, which showed five colonies per milliliter. A rennin-coagulated curd was obtained by adding rennet extract to aseptically-drawn milk, and a portion of this curd was incorporated into one sample of butter. The enzyme suspensions of pepsin and trypsin were added to the butter

granules in 15 milliliter quantities in each case and incorporated in the butter during the working process. The results obtained were negative. Cheesy flavors did not develop in the butter containing the animal proteases, individually, or in different combinations. The same was true of rennin.

The studies with galactase were continued in a series of experiments in which specially prepared enzyme concentrates were used. The methods for isolating the crude enzyme preparation were as follows:

Method I:—Thirty milliliters of chloroform were added to 30 grams of fresh separator slime. The contents were equally divided in two thick-walled glass tubes and agitated vigorously for one hour with the shaking device in a No. 2 International centrifuge. The centrifuge was stopped at the end of 30 minutes to release the vapor pressure. The excess chloroform was removed by centrifuging in a separatory funnel.

The separator slime was treated with 50 milliliters of 60 per cent alcohol, and each alcohol washing was filtered on a Buchner filter through a No. 589 filter paper. This process was repeated four times. Finally the separator slime was placed on the Buchner funnel and washed with an additional 100 milliliters of 60 per cent alcohol.

The alcohol washings were evaporated in a Claisen flask under reduced pressure at 38 to 39° C.

In preparing galactase, Babcock and coworkers (6) allowed the slime to remain in contact with chloroform for 40 hours, which they claimed made the extraction of galactase easier. This procedure is open to criticism in that bacterial proteases may have been liberated from the dead cells and thus have contaminated the enzyme preparation. The magnitude of this factor would be reduced considerably by the procedure described above, because less than five hours elapsed from the time the sample of fresh slime was taken until the crude enzyme preparation was obtained in final form. A small portion of this enzyme preparation was added to sterile milk and very slight proteolysis was detected in three weeks at room temperature. Check platings showed that the milk was sterile.

Method II:—A portion of fresh separator slime (30 grams of slime and 100 milliliters of sterile water) was shaken with an excess of chloroform (5 per cent) according to the procedure used in Method I, and was allowed to remain in contact with chloroform for two hours. The excess chloroform was drained off and the mass was agitated three times with acetone, which was removed by centrifuging. The final preparation was dried under reduced pressure at 38 to 39° C.

Method III:—Thirty grams of fresh separator slime were agitated with 100 milliliters of 60 per cent alcohol. The contents were centrifuged and the alcohol solution was decanted off and filtered through an N-type Berkefeld. The filtrate was concentrated under reduced pressure at 38 to 39° C. and kept in a dry state until used.

The crude enzyme preparations were made under aseptic conditions and sterile equipment was used throughout. The slime was collected on three consecutive mornings from the separator used in the university dairy, and was prepared immediately in the desired form. Fifty milliliters of sterile water were added to each fraction of the dry enzyme preparation and the contents added, in 25 milliliter quantities, directly to the granules of butter after they were washed. The butter was worked exhaustively, to insure more complete incorporation of the enzyme suspension. Duplicate churnings were made with each enzyme preparation. Twenty milliliters of galactase prepared by Method III were also added directly to two liters of cream and immediately churned. The results obtained with galactase were negative with respect to the development of cheesy flavors in the experimental butters. The aqueous enzyme preparations obtained by Methods I, II, and III were not sterile, but contained 2, 18, and 12 bacteria per milliliter, respectively. The bacterial counts of the butters containing galactase were low, showing an average of less than 100 colonies per milliliter. Consequently, it is improbable that the number of organisms present in the original enzyme preparation were of any significance in this experiment.

Cheesy flavors did not develop in the experimental butters containing the different preparations of galactase. Furthermore, it is quite apparent from this experimental evidence that the biological factors responsible for cheesy flavors and aromas were not present in butter made from cream produced under aseptic conditions. An experiment was planned to confirm these findings by attacking the problem from another angle. Milk was obtained under aseptic conditions from the same animals previously used, but it was separated in the university dairy after the milk from the regular herd was skimmed. Portions of cream were held at 10° C. for 24 and 26 hours, respectively, and duplicate churnings were made in each instance. The butter from every churning developed, at some time during storage for 28 days, cheesy flavors of the Cheddar type. These results and those recorded in Table 1 suggest very strongly that a mixed flora is involved in the consistent production of flavors of the Cheddar cheese type, and that such microorganisms might find a favorable habitat in equipment, which could serve as one focus of infection.

EXPERIMENTS WITH PURE AND NATURALLY-MIXED CULTURES OF BACTERIA

The evidence presented in the foregoing pages suggested that several types of bacteria might be involved in bringing about the proper biological circumstances necessary to induce the development of cheesiness in butter. Judging from the consistency of the results in Table 1, the cream from the university dairy fulfilled the requirements in this respect. Consequently, a more intensive study was made of the mixed microflora from this and other sources. Raw cream from the university dairy was churned (Churning 124) and the resulting butter became cheesy. A mixed culture was assembled from colonies picked from a plating of this butter. This mixture was inoculated into 10 milliliters of sterile cream and incubated at 8 to 10° C. for approximately 12 hours. Five milliliters of this cream culture were then added to two liters of sterile cream which was then incubated at 8 to 10° C. for 12 hours and afterwards churned (Churning 150). The remainder of the culture was inoculated into sterile butter (Churning 151) through the wash water.

Table 3

Comments on Butter Prepared from Sterile Cream Inoculated with Mixed Cultures from Different Sources

Churning No.	Fresh	Flavor and aroma of the butter after storage for:			
		14 days at		28 days at	
		5° C.	10° C.	5° C.	10° C.
		Raw cream butter			
124	Satisfactory	Good Cheddar	Fruity, cheesy	Unclean	Fruity, cheesy
		Sterile cream inoculated with mixed culture from plating of butter, Churning 124			
150	Cheesy aroma	Cheesy	Cheesy	Cheesy, fruity	Acetic, Cheddar
		Sterile butter washed with water inoculated with mixed culture from plating of butter, Churning 124			
151	Acid	Fruity, unclean	Putrid	Acetic, cheesy	Cheesy, unclean
		Sterile butter washed with infected water			
126*	Satisfactory	Good Cheddar	Good Cheddar	Unclean	Unclean
		Sterile cream inoculated with mixed culture from plating of butter, Churning 126			
152	Satisfactory	Satisfactory	Cheesy	Unclean†	Cheesy, unclean
		Sterile butter washed with water containing mixed culture from plating of butter, Churning 126			
153	Satisfactory	Unclean	Fruity, cheesy	Cheesy, unclean	Cheesy, acetic

* After three months' storage the butter was graded "Limburger."

† After three months' storage a duplicate sample had a green cheese flavor.

Churning 126 was prepared from sterile cream, but the butter was washed with infected water from a Minnesota creamery which had suffered losses due to the development of cheesy flavors in several shipments of unsalted butter. A mixed culture was prepared from platings of this butter according to the technic described in the preceding paragraph and used for Churnings 152 and 153.

The results in Table 3 indicate clearly that the mixed flora from samples of cheesy butter reproduced this defect when inoculated into sterile cream and into sterile butter through the wash water. The plate counts recorded in Table 4 revealed the interesting fact that the bacterial content of the fresh butters was very low, indicating that the numbers of bacteria may be unimportant in the production of the cheesy flavors and aromas.

Table 4

Changes in the Bacterial Counts of Butter Prepared from Sterile Cream Inoculated with Mixed Cultures from Different Sources

(Numbers per milliliter)

Churning No.	Fresh	Bacterial counts of the butter after storage for:			
		14 days at		28 days at	
		5° C.	10° C.	5° C.	10° C.
		Raw cream butter			
124	17,000,000	10,500,000	3,900,000	12,800,000	9,000,000
		Sterile cream inoculated with mixed culture from plating of butter, Churning 124			
150	8,800	5,800,000	212,000	4,900,000	2,710,000
		Sterile butter washed with water inoculated with mixed culture from plating of butter, Churning 124			
151	10,800	240,000	900,000	3,500,000	20,000,000
		Sterile butter washed with infected water			
126	2,400	20,600,000	17,600,000	57,200,000	17,000,000
		Sterile cream inoculated with mixed culture from plating of butter, Churning 126			
152	1,800	37,700,000	40,600,000	11,800,000	1 290,000
		Sterile butter washed with water containing mixed culture from plating of butter, Churning 126			
153	1,400	*	*	16,280,000	20,700,000

* Unsatisfactory plates.

Churning 150 (Table 3) was graded "cheesy" at the termination of 14 and 28 days' storage. The microflora from this butter was studied more intensively. By means of sterile trier, 10 to 15 grams of butter were taken from the samples stored at 5 and 10° C. After melting at 20° C., these specimens were emulsified, individually, with about 30

milliliters of sterile skimmilk. Each emulsion was added to a batch of sterile cream which was incubated at 14 to 15° C. for 12 hours and then at 10° C. for the same period of time. The butter from Churning 150 was also plated. Pure and mixed cultures, picked from the plates, were inoculated into sterile cream, which was held at room temperature for eight hours. Two liters of sterile cream were also inoculated with these cream cultures and incubated at 8 to 10° C. for 12 hours.

The results which are tabulated in Table 5 show definitely that the mixed flora from Churning 150 reproduced cheesy flavors when inoculated into sterile cream. The organisms were active even after the butter had remained in storage for five weeks. Pure cultures from platings of this butter, with one exception, also produced cheesiness, but when they were all combined, the flavors and odors observed were not the same as those obtained with the original pure cultures. However, there was a considerable degree of similarity in the flavors produced by several of the pure cultures and those produced by the mixed flora.

Table 5

Comments on Butter Prepared from Sterile Cream Inoculated with Butter and with Cultures from a Cheesy Sample

Churning No.	Fresh	Flavor and aroma of the butter after storage for:			
		14 days at		28 days at	
		5° C.	10° C.	5° C.	10° C.
		Inoculated with butter from Churning 150, held at 5° C. for 3 weeks			
172	Cheesy aroma	Fruity	Cheesy	Acetic, Cheddar	Green Cheddar
		10° C. for 3 weeks			
173	Cheesy aroma	Yeasty, cheesy	Cheesy aroma	Unclean, cheesy	Unclean, cheesy
		10° C. for 4 weeks			
182	Cheesy, fruity	Cheesy	Cheddar	Fruity, cheesy	Fruity, Cheddar
		10° C. for 5 weeks			
183	Cheesy	Ripe Cheddar	Green curd	Fruity, cheesy	Acetic
		Inoculated with cultures from Churning 150, butter held at 10° C. for 3 weeks			
		Pure Culture 469			
176	Cheesy aroma	Good Cheddar	Swiss cheese	Fruity, cheesy	Cheddar
		Pure Culture 470			
177	Cheesy aroma	Acetic	Aromatic	Sour dough	Musty
		Pure Culture 471			
178	Cheesy aroma	Cheddar	Unclean	Cheddar	Green Cheddar
		Pure Cultures 469, 470, 471, mixed			
175	Sour aroma	Unclean	Acetic, Cheddar	Acid, cheesy	Acetic

Inasmuch as inconsistent results had been obtained with pure cultures in the past, the churnings with these pure cultures were repeated. Cultures 469, 470, and 471 (24 to 48 hours old) were inoculated from whey agar slants into sterile cream which was then churned, duplicating the previous experimental conditions as nearly as possible. These cultures were about 60 days old at the time of this experiment. The results showed that none of the cultures produced cheesy flavors and aromas in unsalted butter during a storage period of 28 days. These results are in line with those previously reported with pure cultures. The possible explanations for these results will be considered in a later discussion.

Table 6

Comments on Butter Prepared from Sterile Cream Inoculated with Butter and with Cultures from Cheesy Samples

Churning No.	Fresh	Flavor and aroma of the butter after storage for:			
		14 days at		28 days at	
		5° C.	10° C.	5° C.	10° C.
Sterile butter inoculated with infected water					
126	Satisfactory	Good Cheddar	Good Cheddar	Unclean,	Unclean
Sterile cream inoculated with butter from Churning 126					
126a	Satisfactory	Cheddar	Good Cheddar	Cheddar	Unclean
Sterile cream inoculated with a mixed culture from Churning 126					
152	Satisfactory	Satisfactory	Cheesy	Unclean	Cheesy, unclean
Sterile cream inoculated with butter from Churning 152					
174	Satisfactory	Acetic	Acetic	Acetic	Acetic
Cultures from Churning 152 Pure Culture 473					
180	Slight cheese	Acetic	Acetic	Acetic	Acetic
Pure Culture 475					
181	Satisfactory	Acetic	Unclean, acetic	Unclean, acetic	Acetic
Pure Cultures 473 and 475					
179	Satisfactory	Acetic	Acetic	Acetic, cheesy	Acetic

The butters from Churnings 126 and 152 (see Table 3) were graded "cheesy." Specimens of these butters and individual and mixed cultures from them were submitted to a detailed study, according to the technic previously described. The results recorded in Table 6 are not so consistent since the microorganisms in the infected butter and the individual or mixed cultures taken from platings of Churning 152 did not reproduce flavors suggesting any type of cheesiness. The marked

similarity in the comments on the butter from Churnings 174, 179, 180, and 181 would tend to indicate that the causative flora became inactivated in some manner before the inoculations were made. It was also evident that the organisms did not thrive during the storage period because of the low bacterial counts of the infected butters.

A number of samples of cheesy butter were plated on Bacto nutritive caseinate agar, which was useful for detecting the proteolytic properties of certain organisms. The presence of clarified areas surrounding the colonies indicated that proteolysis had occurred. It was revealed by this technic that the mixed flora from the cheesy-flavored butters included such proteoclastic types.

A can of cream that had a very distinct cheesy flavor and aroma was obtained from a Minnesota creamery. This was typical centralizer cream, and was submitted to a detailed study. Twenty-five milliliters of this cream were inoculated into sterile cream, which was incubated at 14 to 15° C. and at 10 to 11° C. for 12 and for 24 hours, respectively, and before churning, 25 milliliters of this cream were inoculated into another portion of sterile cream and incubated in the same manner. The titrable acidities of the original samples of the raw and the inoculated creams before churning were 0.44, 0.65, and 0.48 per cent, respectively. It will be recalled that the cheesy flavors encountered in the past were most often associated with sweet cream butter. It was evident from the comments on the butters during the storage period that the heterogeneous microflora in the original specimen of cheesy cream was able to produce these flavors and aromas in unsalted butter, and that this flora was also carried through into sterile cream butter by subsequent inoculations. No attempt was made to identify this microflora completely, but 24 colonies were picked from whey agar platings of the original raw cream butter, including surface and subsurface colonies. These were stained according to Gram's method and the distribution of the types was as follows: Six were Gram positive cocci; five were Gram positive rods; seven were Gram negative rods; five were yeasts; and one was a variety of *Oospora lactis*.

STUDIES OF THE EFFECT OF CERTAIN ENVIRONMENTAL CONDITIONS ON THE DEVELOPMENT OF CHEESINESS IN BUTTER

A resumé of the experimental results up to this point suggested certain methods of approach which aided in planning subsequent experiments to study the fundamental causes of the cheesy flavors. For example, the work with pure cultures had shown that individual organisms were unable to induce, consistently, the development of cheesiness in

unsalted butter, and their inconsistency in this respect suggested that other biological factors might be involved. On the other hand, the work with a mixed or a heterogeneous flora had uniformly caused the appearance of these flavors within the usual time associated with their development under practical conditions. This indicated that some degree of symbiosis might be necessary to fulfill the biological requirements for the production of these characteristic flavors and aromas.

On the basis of these results, it seemed logical to continue the work with a mixed flora, because in order to plan more elaborate experiments involving a study of some of the technical and practical phases of the problem, it would be necessary to have a fair degree of assurance that these defects would manifest themselves at all times if the proper conditions were provided. Furthermore, this procedure was in accord with the results reported by Evans (28) and Evans, Hastings, and Hart (29), Hucker (53) and others (41) (43) who found that several genera were present in sufficient numbers in Cheddar cheese to warrant consideration in the ripening process.

A mixed flora was obtained by emulsifying into sterile cream about ten grams of cheesy butter from Churning 173. This mixture was kept at a temperature of 9 to 10° C. and transfers were made weekly. This culture was carried in sterile cream and will be referred to as Mixed Culture 173. It was thought advisable to propagate this flora in cream that was as near a natural habitat as it was practical to obtain, and particularly so in view of the attention which has been given to the phenomenon of bacterial dissociation (14) (37) (56) (101) (102) in which a group of investigators (37) (56) have presented excellent evidence that certain genera may actually change their characteristics when submitted to variable environments. Mixed Culture 173 was plated on whey, beef extract, beef infusion, milk digest, and Bacto nutritive caseinate agar. The last three media gave equally good results and the growth was more profuse than that observed on whey or beef extract agar. Beef infusion medium was adopted in place of whey agar for plating the butters during the remainder of the experimental work.

Having obtained a heterogeneous microflora that showed promise of producing cheesy flavors of the Cheddar type consistently, it was deemed advisable to ascertain, if possible, the environmental conditions under which these flavors would develop to the best advantage.

Since Sammis and Bruhn (97) reported that Cheddar cheese prepared from pasteurized milk did not have the characteristic flavor and aroma, an attempt was made to determine if previous heat treatments of the cream would in any way modify the development of these flavors

in unsalted butter. This was accomplished by submitting fresh, raw cream to the following heat treatments:

1. Cream pasteurized at 63 to 65° C. for 20 minutes.
2. Cream pasteurized at 71 to 73° C. for 20 minutes.
3. Cream pasteurized at 80° C. for two minutes.
4. Cream preheated to 62.5° C. and then autoclaved at 15 lbs. pressure for 12 minutes.

The treated cream was immediately cooled to 10 to 11° C. and each portion was inoculated with 10 milliliters of a 72-hour-old Mixed Culture 173 which was carried in cream. The infected cream was incubated for 48 hours at 10 to 11° C.

It was found that the previous heat treatments of the cream, prior to inoculation, had no noticeable effect on the subsequent development of the cheesy flavors and aromas. All of the creams subjected to the different heat treatments yielded butters with flavors and aromas of varying gradations of cheesiness, which bordered on the Cheddar type.

Table 7

Comments on Butter Prepared from Sterile Cream Inoculated with Mixed Culture 173

Churning No.	Storage temperature	Flavor and aroma of the butter				
		Fresh	7 days	14 days	28 days	35 days
		Cream incubated at 5° C. for 48 hours				
193	5° C.	Satisfactory	Fruity, cheesy	Fruity, cheesy	Fruity, cheesy	Cheddar
	10° C.	Satisfactory	Fruity, cheesy	Cheddar	Brick, Swiss	Cheddar
		Cream incubated at 10° C. for 48 hours				
194	5° C.	Cheesy	Fruity	Cheddar, Brick	Bitter, Cheddar	Fruity, cheesy
	10° C.	Cheesy	Brick	Cheddar, unclean	Unclean	Cheddar
		Cream incubated at 21° C. for 48 hours				
195	5° C.	Cheesy	Sour dough	Fatty acid	Slight soap	Sour dough
	10° C.	Cheesy, Swiss	Yeasty	Acetic, cheesy	Musty	High acid
		Check, sterile cream				
195a	5° C.	Cooked	*	Satisfactory†	Satisfactory†	*
	10° C.	Cooked	*	Satisfactory†	Satisfactory†	*

* Butter was not judged.
† Butter also had a cooked flavor.

Cheesy flavors have been observed in unsalted butter stored at low temperatures and the results indicate that the causative flora also thrives in cream at these low temperatures. The care that cream receives on the farm varies considerably as some producers cool their cream efficiently while others are lax in this respect. An experiment was planned to note the effect of temperatures on the development of cheesy flavors in cream and to follow such changes in the butter. In this experiment three lots of sterile cream were cooled to 5, 10, and 21° C., respectively, and inoculated with 15 milliliters of Mixed Culture 173. The inoculated creams were held at these temperatures for 48 to 50 hours. The results in Table 7 show clearly that the typical cheesy flavors developed in the butter prepared from cream incubated at the lower temperatures of 5° and 10° C., while at 21° C. the flavors were not definitely cheesy.

Table 8

Comments on Butter from Sterile Cream Inoculated with Mixed Culture 173 Incubated at Various Temperatures

Churning No.	Storage temperature	Flavor and aroma of the butter			
		Fresh	14 days	28 days	60 days
		Inoculum held at 10° C. for 24 hours			
238	5° C.	*	Cheddar	Cheddar	Unclean, cheesy
	10° C.	*	Good Cheddar	Unclean Cheddar	Unclean, cheesy
		Inoculum held at 21° C. for 24 hours			
238a	5° C.	*	Nut-like	Fruity	Cheesy, fruity
	10° C.	*	Fruity, nut-like	Fruity	Cheesy, fruity
		Inoculum held at 37° C. for 24 hours			
239	5° C.	*	Fruity, cheesy	Fruity	Fruity
	10° C.	*	Quince-like	Musty†	Musty†

* Butter was satisfactory, but had a cooked flavor.
† Mold growth was evident on the surface.

The possibility existed that Mixed Culture 173 might contain organisms whose optimum temperatures for growth were considerably higher than those already noted. For this reason an experiment was initiated to observe the effect of different temperatures on this mixed culture by inoculating separate portions of sterile cream and holding them at 10°, 21°, and 37° C., respectively, for 24 hours. Approximately 100 milliliters of each inoculum were added to separate lots of sterile cream which were immediately churned. The results in Table 8 again disclose the fact that the cheesy flavors were evident in the butter from the cream that was infected with the inoculum held at 10° C., while the inoculum incubated at the higher temperatures yielded butter with flavors suggestive of cheesiness, but certainly not typical. This in-

dicated that the flora became unbalanced and organisms other than the cheesy-flavor-producing types predominated. This statement was substantiated by the observation that platings of Mixed Culture 173 on beef infusion agar revealed a number of large, glistening colonies which grew abundantly on the surface. This same macroscopic picture was evident on platings of the butters stored at 5 and 10° C. A few of these large, glistening colonies were found on the platings of the inoculum held at 21° C., but they were entirely missing in the one incubated at 37° C. The titrable acidities (calculated as lactic acid) of the mixed cultures held at 10°, 21°, and 37° C. were 0.27, 0.60, and 0.57 per cent, respectively.

It is apparent that temperature was an important factor in the development of cheesy flavors in the butters containing Mixed Culture 173. The effect of temperature may be due to the possibility that the lower ranges may be optimum for the causative organisms to develop in their associative relationship. On the other hand, certain types may have predominated at the higher temperatures and induced the formation of compounds that checked the development of, or masked, the cheesy flavors so that they were not detectable, or they may have been produced for the moment and the substances responsible for them quickly transformed. It appeared certain that Mixed Culture 173 produced the most characteristic cheesy flavors in butter when it was incubated at 5 or 10° C.

Table 9

Comments on Butter from Sterile Cream Inoculated with Mixed Culture 173 and Incubated for Different Periods

Churning No.	Storage temperature	Flavor and aroma of the butter				
		Fresh	7 days	14 days	28 days	42 days
		Cream incubated at 10° C. for 24 hours				
189	5° C.	Cooked	Cheesy	Unclean	Fruity, Cheddar	Slight unclean
	10° C.	Cooked	Fruity	Acetic	Fruity. Cheddar	Unclean, Brick cheese
		48 hours				
190	5° C.	Unclean	Satisfactory	Unclean, fruity	Old Cheddar	Doughy, unclean
	10° C.	Unclean	Satisfactory	Unclean, Cheddar	Old Cheddar	Acetic
		72 hours				
191	5° C.	Cheddar	Satisfactory	Slight cheesy, slight fruity	Doughy, unclean	Unclean, acid
	10° C.	Cheddar	Sour cream	Slight cheesy, slight acetic	Unclean, cheesy	Cheddar

The question next arose as to the effect of the duration of incubation at these low temperatures on the ultimate development of cheesy flavors in unsalted butter. This time factor was noted by inoculating three portions of sterile cream with 15 milliliters each of Mixed Culture 173 and allowing each portion to incubate at 10° C. for 24, 48, and 72 hours, respectively. The results in Table 9 indicated that there were no specific differences in the manifestation of cheesy flavors in unsalted butter when the cream was incubated for periods of 24 to 72 hours. The titrable acidity of the creams in Churnings 189, 190, and 191 prior to churning were 0.24, 0.31, and 0.40 per cent, respectively.

During the course of this work it was observed that, in general, the cheesy flavors and aromas and particularly those of the Cheddar type were not stable, but gradually passed through various stages of development and finally lost their original identity. The transient character of these flavors is shown in Tables 1, 3, and 5.

FURTHER STUDIES WITH PURE CULTURES AND COMBINATIONS OF PURE CULTURES

An intensive study was made of Mixed Culture 173 for the purpose of obtaining more specific information concerning the individual organisms which it contained.

A cream inoculum of Mixed Culture 173, 24 hours old, was plated on beef infusion agar and after incubating the plates at 10° C. for 48 to 50 hours and at room temperature for 12 hours, 20 surface and subsurface colonies were picked. Anaerobic plates were also prepared and colonies picked from them. The activity of the cultures was studied on various media. On the basis of their biochemical and physiological reactions in these media and their differences in morphology, they were identified as distinct species or varieties of the following genera: *Achromobacter* (Cultures O-173, E-173, A-173r, T-173 and B-173s), *Proteus* (Culture K-173), *Streptococcus* (Culture Y-173), *Escherichia* (Culture Q-173), and Cultures L-173 and U-173 had characteristics most closely resembling those of *Salmonella*.

These cultures, 10 in number, were carried on beef infusion slants and transferred monthly. Duplicates were also kept in cream and transferred weekly.

Cream cultures of these organisms were inoculated into sterile cream, individually, according to the technic previously described, in order to determine if any one of them would produce, in unsalted butter, cheesy flavors comparable to those produced by Mixed Culture 173. It was found that more than half of the cultures produced cheesy

flavors of one type or another and some of the flavors simulated those of typical Cheddar cheese.

These cultures had been carried less than a week on beef infusion agar prior to their inoculation into the sterile cream. The experimental work previously reported with pure cultures was initiated after the organisms had been carried on whey agar for a considerable period, and during this time the cultures might have lost their ability to induce the development of the cheesy flavors in butter. To determine the influence of this age factor and to observe whether they would consistently produce cheesiness in butter, the work with some of the cultures that had produced Cheddar cheese flavors in the preceding experiment was repeated. For this purpose Cultures A-173r, B-173s, and O-173 were inoculated into separate lots of sterile cream according to the technic already described. The results obtained corroborated the earlier findings in that the pure cultures obtained thus far did not consistently induce the development of cheesy flavors. It is improbable that these flavors developed and were missed, because frequent observations were made on the experimental butters. Undoubtedly, some other factor is involved.

It appeared certain that Mixed Culture 173 contained a flora that collectively was able to cause biochemical changes resulting in the manifestation of flavors of the Cheddar cheese type. It seemed logical, thereafter, to approach the problem from another angle, by inoculating different combinations of the pure cultures into sterile cream and observing their combined effect on the resulting butter. However, this method of attack has very definite limitations. In the first place, 10 pure cultures were isolated from Mixed Culture 173. The number of possible combinations with 10 cultures, according to the formula, $N=2^n-1$, would be 1,023. According to this scheme it would be necessary to make 1,023 churnings in order to test the cultures in all their possible combinations. This obviously would not be a practical procedure.

Fortunately, the investigations dealing with the bacteriology and the biochemistry of cheese-ripening have shown that microorganisms are associated with certain changes. For example, the flora of Cheddar cheese has been differentiated into proteolytic and aroma- and acid-producing types. These suggestions were applied in the studies described below.

Preparatory to conducting the experiments with these different combinations, the cream cultures of the individual organisms were carefully observed for their characteristic flavors and aromas. The titrable acidities were also determined. The results were as follows:

Culture	Comments	Titrable acidity calculated as per cent lactic acid
A-173r*	Nothing definite	0.22
B-173s	Stale	0.23
E-173*	Cheesy, putrid	0.35
K-173†	Acid aroma	0.39
L-173	Foetid	0.21
O-173	Stale	0.19
Q-173	Very fruity	0.25
T-173*	Putrid, cheesy	0.32
U-173	Acid aroma	0.21
Y-173†	Acid	0.39

* Produced rapid proteolysis of milk.
† Produced acid in milk.

These individual cultures were carried in cream from the time they were first isolated and were held at a temperature of 10° C., so that their environment, within certain limits, was comparable to that of Mixed Culture 173. Attempts were made to "synthesize" a flora that would produce cheesy flavors comparable to those produced by Mixed Culture 173. This was tried by preparing different combinations of these cultures and noting the flavors and aromas produced in cream. A number of combinations were made, but only those showing some suggestion of cheesiness in cream were used for experimental purposes. As a matter of convenience, these cultures are listed with their prefix letters because they were all isolated from Mixed Culture 173. The small suffix letters of Cultures A and B represent cultures from rough and smooth colonies, respectively. The different combinations and the characteristic flavors and aromas that they produced in cream are listed as follows:

Mixed cultures used	Comments on flavors and aromas produced in cream
QY	Fruity; Cheddar
AQ	Bitter; cheesy
AQY	Fruity; Cheddar
AKQY	Bitter; cheesy; fruity
Check*	Cheddar cheese
All cultures in 173†	Putrid
All cultures in 173‡	Putrid
AKQYT	Typical Cheddar
T	Putrid; cheesy
Rough and a smooth culture	Fruity; slightly cheesy
Rough and a smooth culture	Cheesy

* Naturally-mixed culture.
† Inoculated from beef infusion slants.
‡ Inoculated from cultures carried in cream.

Cream cultures of these different combinations were inoculated (15 to 20 milliliters) into sterile cream and incubated at 10° C. for 48 hours. These "synthetic," mixed cultures are further identified in the captions of Table 10, which presents a resumé of the comments on butters containing these different combinations. The butters from Churnings 305 and 351 are of interest because they contained four organisms that produced different biochemical reactions, namely, a proteolytic type (A), an ester- or aroma-producer (Q), and acid producers (K), (Y). The flavors and the aromas produced, while not typically cheesy, were sufficiently outstanding in this respect so that they could be placed in this category. The comments relative to the butters from Churnings 306 and 307a suggest that a proper balance was not obtained for the flora that constituted the cheesy-flavor-producing types. However, the butters from both churnings possessed similar flavors and aromas. This would indicate that the medium on which the cultures were carried prior to preparing these "synthetic" mixtures was not the important factor which caused the deviations from the flavors and aromas of the Cheddar type, so characteristic of the natural bacterial population of Mixed Culture 173. In addition to the flora used in Churnings 305 and 351, Churning 307 contained Culture T. Its presence was accompanied by a marked change in the character of the flavors and aromas, which were outstanding because of the predominating odors of certain fatty acids. In general, the comments on the butters from Churnings 306, 307, 307a, and 308 are similar. These churnings were repeated and it was found that Culture T was responsible for the divergences from the mild, fruit-like and cheesy flavors observed in butter not containing it. The comments on the butters from the remaining churnings require no further discussion, with the exception of Churning 296. The flora of this butter consisted of a rough and a smooth colony which had grown together on the surface of a beef infusion agar plate. A loopful was taken from this rough-smooth colony and inoculated into sterile cream. The comments on Churning 296 indicate cheesiness. The butter from the check, Churning 350, containing Mixed Culture 173, did not reveal cheesy flavors of the Cheddar type; this is the first exception.

The action of Culture T on butter, resulting in the manifestation of these offensive flavors and aromas, involving lipolysis and proteolysis, are of interest because of the fact that this organism was placed in the genus *Achromobacter* with Cultures A, B, E, O. Cultures E and T produced comparable changes while Cultures A, B, and O were similar in their action on butter.

Table 10

Comments on Butter from Sterile Cream Inoculated with Combinations of Organisms Isolated from Mixed Culture 173

Churning No.	Cultures	Flavor and aroma of the butter after storage for:			
		14 days at		28 days at	
		5° C.	10° C.	5° C.	10° C.
308a	Check*	Good Cheddar	Unclean, Cheddar	Cheesy, fruity	Cheesy, fruity
351	AKQY	Unclean	Unclean, grass-like	Fruity, cheesy	Fruity, cheesy
305	AKQY	Unclean, oxidized	Stale	Cheesy, doughy	Cheesy, unclean
306	173†	Slight Limburger	Limburger, Brick	Fatty acids	Bitter
307a	173‡	Cheesy, putrid	Limburger, Cheddar	Fatty acids, rotten	Very rotten
307	AKQTY	Fatty acids, unclean, bitter, rancid	Cheesy, bitter, fatty acids	Fatty acids, caprylic	Intense fatty acids, bitter
308	T	Strong acetic	Strong acetic	Putrid, fatty acids	Putrid, fatty acids
350	Check*	Unclean	Unclean, grass-like	Fruity, cheesy	Doughy, grass-like
352	AQY	Yeasty, cheesy	Stale meat	Unclean	Unclean
353	AQ	Cheesy, fruity	Fruity, doughy	Doughy	Unclean, grass-like
354	QY	Fruity, doughy	Fruity, doughy	Doughy	Fruity, nut-like
296	RS§	Fruity, Cheddar	Fruity, Cheddar	Fruity	Cooked, dried-milk
311	RS§	Unclean, oxidized	Flat, cooked	Unclean	Satisfactory

* Natural cream culture of 173.
† Includes the 10 cultures from Mixed Culture 173 and carried on beef infusion slants.
‡ Includes the 10 cream cultures from 173.
§ Rough and smooth culture.

A classification of the different genera which constituted the various combinations of "synthetic" mixed cultures is outlined in Table 11. The evidence which has been presented to show that several genera are necessary to cause consistent cheesiness in unsalted butter is corroborated in this table. It is interesting to note that the genus *Escherichia* is represented in every combination. It will be recalled that the basis for selecting these different combinations was suggested by statements in the literature on cheese ripening, in which it was inferred that several genera were concerned in the ripening process and that these included proteolytic types, ester-, or aroma-, and acid-producers. It was possible to select cultures that would fulfill these different requirements. A number of combinations were prepared, but only those producing flavors in cream, suggestive of cheesiness, were used for experimental purposes.

The limitations of using all the possible combinations from Mixed Culture 173 were discussed previously.

Table 11

Distribution of the Genera in the Combinations that Induced the Development of Cheesy Flavors in Unsalted Butter

Churning No.	Culture	Genus
305 and 351	A	Achromobacter
	Q	Escherichia
	K	Proteus
	Y	Streptococcus
306	Mixed culture 173*	
307a	Mixed Culture 173†	
307	A	Achromobacter
	T	Achromobacter
	Q	Escherichia
	K	Proteus
	Y	Streptococcus
352	A	Achromobacter
	Q	Escherichia
	Y	Streptococcus
353	A	Achromobacter
	Q	Escherichia
354	Q	Escherichia
	Y	Streptococcus
296	Rough and smooth culture	Not identified

* Individual cultures picked from beef infusion slants.
† Individual cream cultures.

In spite of the fact that these various combinations of "synthetic" mixed cultures contained different organisms, it was impossible to differentiate one flora from another on the basis of the appearance of the colonies on beef infusion medium.

AN OBSERVATION ON THE HEAT STABILITY OF CHEESY FLAVORS AND AROMAS

In the experimental work conducted thus far, it has been observed that the flavors and aromas of the Cheddar type have been more or less volatile, and by means of these volatile substances it has been possible to identify the different cheesy flavors. It usually has been assumed that because these flavors are volatile they are also unstable. To obtain some information on this point, a few of the combinations of mixed cultures which were prepared for experimental purposes were autoclaved at 15 pounds pressure for 15 minutes in cotton-stoppered flasks. The cultures were cooled to 21° C. and observed for flavors and aromas. The results were as follows:

Combination of cultures	Comments
AKQY	Cheddar
AKQYT	Putrid Cheddar
T	Bread crust
Naturally Mixed Culture 173	Cheddar
Synthetic culture of all the organisms in Mixed Culture 173	Unclean, putrid

The cream containing Culture T, before heating, had a very characteristic, putrid odor, indicating proteolysis and lipolysis. On autoclaving, however, these odors had disappeared, leaving a residual aroma, which was called "bread crust."

It is evident that cream cultures of combinations that produced flavors and aromas of the Cheddar type are sufficiently stable to withstand autoclaving temperatures, which indicates that the substances responsible for these flavors may be bound in some type of stable physical and chemical combination. On first thought these results may seem inconsistent, yet a reflection on the practices used in making processed cheese, where high temperatures are employed, tends to place these results in line with the expected.

STUDIES CONCERNING THE CONSTITUENTS OF CREAM INVOLVED IN THE PRODUCTION OF CHEESY FLAVORS

There are two important factors involved in ascertaining the fundamental causes of cheesy flavors in unsalted butter, namely, the active biological agents and the substrates which they utilize in producing these characteristic flavors and aromas.

The first phase of this problem was solved to the extent that certain organisms, combinations of pure cultures and naturally-occurring mixtures of different species induced the condition known as cheesiness in unsalted butter. However, no definite information is available regarding the substrates acted upon by these organisms in the development of these cheesy flavors. This phase of the problem certainly merits attention, because such knowledge may eventually lead to further information regarding some of the fundamental biochemical changes which occur in butter.

It was thought that some clues might be obtained with rather concentrated mixtures of the major components of butter. Therefore, calcium caseinate was prepared by adding calcium hydroxide to moist, grain-curd casein and triturating to a thick, pasty mass. Water was added to make the mixture reasonably fluid. It was then acidified with three per cent phosphoric acid until the reaction was acid to litmus. Whey, from which the casein was removed, was adjusted to about

pH 6.8 with a calculated amount of N/1 sodium hydroxide, using brom thymol blue as the indicator; then the whey was autoclaved at 15 pounds pressure for 15 minutes. The precipitated proteins, consisting chiefly of lactalbumin and globulin, were placed in sterile containers. Refined preparations of lecithin and cephalin were available. Butterfat was prepared by rendering fresh unsalted butter and removing the moisture by centrifuging. Bacto-lactose prepared by the Digestive Ferments Company was the source of the milk sugar.

The phospholipides, prepared in one per cent sols, the lactose in five per cent solution, and the pure butterfat were all sterilized by autoclaving for 15 minutes at 15 pounds pressure, while the protein suspensions were sterilized by heating to 62 to 65° C. for 30 minutes on three consecutive days.

The proteins and the fats were used in five-gram quantities, individually, except when different combinations were prepared with fat as the base, in which case they were mixed in the approximate proportion in which they occurred in butter. Lactose and the phospholipides were added in 0.5 and 0.1 milliliter quantities, respectively.

Concentrated substrates and combinations were prepared in sterile petri plates and were inoculated with 0.1 milliliter of cream containing Mixed Culture 173. The plates were stored at 10° C. and observed for flavors and aromas. The comments on these synthetic mixtures are tabulated as follows:

Combination of substrates	Comments on aroma*
Calcium caseinate	Musty, yeasty
Calcium caseinate plus lactose	Musty, aromatic
Calcium caseinate, lecithin and cephalin	Flat, tallowy
Calcium caseinate, lactalbumin-globulin, lecithin and cephalin	Unclean, putrid
Calcium caseinate, lactalbumin-globulin, cephalin and lactose	Unclean
Calcium caseinate, lactalbumin-globulin, cephalin, lactose and butterfat	Yeasty, cheesy
Lactalbumin-globulin and lactose	Fruity, musty
Lactalbumin-globulin	Yeasty, cheesy
Lactalbumin-globulin, cephalin, lactose and butterfat	Flat, oxidized
Butterfat and water	Nothing detectable

* After three weeks.

According to these results, no single substrate could be incriminated as being the source of cheesy flavors and aromas. Where these flavors were detected, more than one substance was present in the substrate. Calcium caseinate yielded a yeasty odor which is often

classified in the category of cheesiness because of its close similarity to the first or incipient stages of cheesiness.

Obviously, these qualitative mixtures of the substances inherent in butter did not simulate a normal condition. However, another method of approach was suggested by the work of Weise and Palmer (120) which held promise of serving as a basis for obtaining information regarding the substrates utilized by the causative flora in the production of these cheesy flavors and aromas. They removed the plasma colloids from cream by dilution with four volumes of distilled water to one of cream and separated the fat phase with a centrifugal separator. As a matter of fact, they repeated this process eight times without destroying the cream emulsion.

On the supposition that washed cream inoculated with the causative flora did not supply the substances necessary for the production of cheesy flavors and aromas, it would serve as a base for adding the remainder of the constituents in more or less purified form. The particular advantage of this technic is that the physical condition of the synthetic cream prepared in this manner would more nearly approach that of the normal product. An experiment was planned to test the validity of this hypothesis.

Fifty pounds of cream were pasteurized by heating to 70° C. for 20 minutes on two consecutive days. The cream was mechanically agitated during the heating process. This cream was warmed to 32° C. and washed with sterile tap water which was held at a constant temperature of 40 to 41° C., using four volumes of water to one of cream, and re-separating it in a sterile separator. This process was repeated five times. Four liters of cream were retained from each washing and divided into two equal portions; one was inoculated and the other served as a control. All utensils used in handling the cream, including the separator parts, were sterilized by appropriate methods.

It was obvious that cream could not be used as an inoculum because it might be added in sufficient amounts to the washed cream to nullify the effect of removing the plasma solids and thereby contribute to the production of cheesy flavors and aromas. Consequently, it was necessary to procure another carrier which would provide a medium similar to that of cream for the cheesy-flavor-producing flora. This consisted of preparing a beef infusion medium containing 2.5 per cent agar. One hundred and fifty milliliter lots of this were placed in half-liter Erlemeyer flasks and autoclaved for 15 minutes. When the medium was cooled near the point of solidification, 30 milliliters of sterile 32 per cent cream were added and the mixture shaken in cold water until the agar was solidified. Approximately 0.2 milliliter of Mixed Culture 173 from cream was dropped on the roughened surface of this cream-

beef infusion medium, followed by the addition of one milliliter of sterile distilled water to facilitate spreading the inoculum uniformly over the surface. This inoculum was allowed to incubate at 10° C. for 48 hours. When the washed creams were ready for inoculation, 100 milliliters of sterile distilled water were added to the flask containing the inoculum and the contents shaken, carefully. Five milliliters of this suspension were added to each of the experimental portions of washed cream and incubated at 10° C. for 48 hours. This mixed flora was in this aqueous suspension not to exceed five minutes before all the inoculations were made; therefore, it is improbable that any serious effects such as plasmoptysis of the bacterial cells had occurred. Sufficient cream remained from the fifth washing and was autoclaved and inoculated in the same manner, except that the bacterial suspension was five to six hours old at the time.

Table 12

Comments on Butter from Washed Cream Inoculated with Mixed Culture 173

Churning No.	Treatment of the cream	Flavor and aroma of the butter after storage at 10° C. for	
		14 days	28 days
		Unwashed	
341	Uninoculated	Satisfactory	Satisfactory
341a	Inoculated	Fruity, cheesy	Fruity, old Cheddar
		Washed once	
331	Uninoculated	Oxidized, oil-like	Washed out, flat
332	Inoculated	Unclean, slight fruit	Slight fruit, slight cheese
		Washed twice	
333	Uninoculated	Oxidized	Oxidized, cardboard
334	Inoculated	Oil-like	Slight fruit
		Washed three times	
335	Uninoculated	Oil-like	Oil-like
336	Inoculated	Oil-like	Cooked, flat
		Washed four times	
337	Uninoculated	Oil-like, dried milk	Dried milk, oxidized
338	Inoculated	Slight oil-like	Dried milk, oxidized
		Washed five times	
339	Uninoculated	Strong oil	Oxidized, unclean
340	Inoculated	Slight oil-like	Old, flat, stale
342	Inoculated*	Oxidized, tallow	Unclean, oxidized

* Cream was autoclaved.

The results are summarized in Table 12 and clearly incriminate the plasma solids removed during the washing process as necessary for the manifestation of cheesy flavors and aromas. There was no sug-

gestion of cheesiness in the butters prepared from cream washed more than twice, but the predominating defects were indicative of certain changes in the butterfat. Presumably, the substances attacked by this mixed culture, resulting in the production of acids, were removed during the washing process, as indicated by the results tabulated below:

Churning No.	Treatment of the cream	Per cent of acidity calculated as lactic acid
	Unwashed	
341	Uninoculated	0.17
341a	Inoculated	0.35
	Washed once	
331	Uninoculated	0.03
332	Inoculated	0.09
	Washed twice	
333	Uninoculated	0.01
334	Inoculated	0.03
	Washed three times	
335	Uninoculated	0.01
336	Inoculated	0.02
	Washed four times	
337	Uninoculated	0.01
338	Inoculated	0.02
	Washed five times	
339	Uninoculated	0.01
340	Inoculated	0.01
342	Inoculated*	†

* Cream was autoclaved.
† Acidity was not determined.

It is evident that after the third washing there were no significant differences between the titrable acidities of the check and the inoculated creams.

A portion of the cream from the fifth washing was autoclaved at 15 pounds pressure for 15 minutes. The interesting observation was made that the emulsion was stable even after exposure to such high temperatures and the degree of "oiling off" was not in excess of that ordinarily observed in normal cream. This cream was inoculated (Churning 342) and the comments on the butter were similar to those of Churning 340. Another specimen of autoclaved cream from this washing was stored at 10° C. and at the end of three weeks it had a very bitter, tallowy taste and odor.

The creams from the different washings churned normally, and the physical appearance of the butter granules was natural. However, the churning time was decreased by about one-third to one-fourth in comparison with unwashed cream.

The bacterial counts of the butter from the different fractions of washed cream are summarized in Table 13. A comparison of the trend of the counts of the fresh and the storage butter indicate that bacterial growth was less prolific in the butters where the plasma solids were removed more exhaustively. Fehling's test for lactose on a small portion of the plasma from the fifth washing was faintly positive. This small quantity of lactose might account for the low titrable acidities in the creams washed more than three times, because the bacterial flora probably lacked a suitable substrate from which to form acid-reacting substances. A portion of the fifth washing was tested for proteins, and the biuret test was negative. As the creams were washed more exhaustively, there was a gradual decrease in the bacterial counts of the corresponding butters, suggesting that certain substances were removed from the active substrate to retard the rate of bacterial development. The macroscopic picture of the flora from the platings of all the inoculated washed cream butters was identical and typical of Mixed Culture 173.

A Kohman analysis was made of the butters in this experimental series with the results reported as follows:

Churning No.	Treatment of the cream	Kohman Analysis		
		Fat	Moisture	Curd
		per cent	per cent	per cent
341	Unwashed	83.90	15.20	0.90
331	Washed once	84.95	14.50	0.55
332	.....	....	....	....
333	Washed twice	86.25	13.30	0.45
334	.....	....	....	....
335	Washed three times.....	85.00	14.70	0.30
336	.....	....	....	....
337	Washed four times.....	86.25	13.50	0.25
338	.....	....	....	....
339	Washed five times.....	86.95	12.80	0.25
340	.....	....	....	....

These results are of interest because the fraction designated as "curd" was different from the similar fraction of normal butter, in that this residue possessed a fluffy, brush-heap structure and did not change its physical appearance on heating during the evaporation of ether in the Kohman analysis. The creams which were washed more than twice yielded butters containing approximately the same amount of this ether-insoluble substance, indicating that most of the plasma solids of which there is any specific knowledge were removed in the first two washings.

Table 13

Changes in the Bacterial Counts of Butter from Washed Cream Inoculated with Mixed Culture 173

(Numbers per milliliter)

Churning No.	Treatment of the cream	Bacterial counts of the butter		
		Fresh	After storage at 10° C. for	
			14 days	28 days
		Unwashed		
341	Uninoculated	†	22	9
341a	Inoculated	60,000,000	94,000,000	43,000,000
		Washed once		
331	Uninoculated	†	800	37,000
332	Inoculated	7,900,000	23,300,000	8,800,000
		Washed twice		
333	Uninoculated	†	‡	§
334	Inoculated	5,800,000	12,400,000	14,500,000
		Washed three times		
335	Uninoculated	†	1,200	11,500
336	Inoculated	3,800,000	9,000,000	8,000,000
		Washed four times		
337	Uninoculated	†	330	37,500
338	Inoculated	9,500,00	6,100,000	7,700,000
		Washed five times		
339	Uninoculated	†	2	14,000
340	Inoculated	4,000,000	5,700,000	6,200,000
342	Inoculated*	‡	6,600,000	1,700,000

* Cream was autoclaved before it was inoculated.
† No visible colonies.
‡ Dilutions were unsatisfactory.
§ Plates were contaminated.

It was evident that cream washed more than twice did not contain the substrates which would support the development and the ultimate manifestation of cheesy flavors and aromas in unsalted butter containing Mixed Culture 173. For this reason it was deemed advisable to check these findings by inoculating the cream prior to washing and to observe if these flavors could be detected in the resulting butter.

Forty-five pounds of cream were pasteurized by heating to 70° C. for 20 minutes on two consecutive days. After a check sample was taken this cream was inoculated with 180 to 200 milliliters of Mixed Culture 173 and allowed to incubate at 10 to 11° C. for 48 hours. A portion was taken for a check churning and the remainder was washed according to the technic described on page 37. A sample of cream was taken from each washing and cooled immediately. The entire washing

procedure did not exceed one hour and the creams were churned in the order in which they were washed.

The comments are summarized in Table 14 and are similar to those recorded in Table 12 except that flavors suggestive of cheesiness were not detected in the butters from the washed creams. These creams had a flat, watery taste, signifying that substances essential to the development of the flavors in normal cream were removed during the washing process. This is in accord with Thurston's work (113).

Table 14

Comments on Butter from Cream Inoculated with Mixed Culture 173 and Washed

Churning No.	Treatment of the cream	Flavor and aroma of the butter after storage at 10° C. for:	
		14 days	28 days
324	Check, sterile	Satisfactory	Satisfactory
325	Check, inoculated	Root-like	Cheddar, slight bitter
325a	Check,* inoculated	Cheesy, fruity	Cheesy, doughy
326	Washed once	Unclean	Unclean
327	Washed two times	Satisfactory	Bitter
328	Washed three times	Satisfactory	Oxidized, cardboard
329	Washed four times	Satisfactory	Very oxidized
330	Washed five times	Very oxidized	Washed out
330a	Washed six times	Satisfactory	Very oxidized

* Taken when cream reached 32° C. prior to washing.

Table 15

Changes in the Bacterial Counts of Butter from Cream Inoculated with Mixed Culture 173 and Washed

(Numbers per milliliter)

Churning No.	Treatment of the cream	Bacterial counts of the butter		
		Fresh	After storage at 10° C. for:	
			14 days	28 days
324	Check, sterile	†	6	†
325	Check, inoculated	4,200,000	61,000,000	46,500,000
325a	Check,* inoculated	3,600,000	61,000,000	43,000,000
326	Washed once	11,700,000	29,600,000	19,800,000
327	Washed two times	6,600,000	9,100,000	8,500,000
328	Washed three times	5,420,000	6,700,000	4,450,000
329	Washed four times	4,440,000	4,400,000	1,600,000
330	Washed five times	1,470,000	2,150,000	990,000
330a	Washed six times	1,000,000	2,190,000	1,500,000

* Taken when cream reached 32° C. prior to washing.

† No visible colonies.

There is a resumé of the bacterial counts in Table 15 which portrays an interesting picture of the trends in the fresh and storage butters. The numbers decreased during the storage period in each instance, and

there was also a gradual decrease in the counts of the butters as the number of washings was increased. However, the bacterial counts of the fresh butter from each series of washed cream are of particular significance. In spite of the excessive dilution with water, the bacterial counts of the butter from the cream washed six times were actually decreased only one-tenth as compared with the butter from the first washing of cream. The washing procedure took approximately one hour; therefore, it is improbable that increases in numbers of bacteria could account for the relatively high count in the butter from the final washing. The logical explanation, and the one involving fundamental considerations, would tend to indict the fat and the materials associated with the stable emulsion of fat as the factors responsible for the retention of such large numbers of bacteria in butter prepared from cream which was washed six times. The higher count of the butter from the first washings, as compared to the check sample, can be attributed to the fact that it took considerable time to warm the cream from the incubation temperature (10° C.) to the temperature (32° C.) at which the cream was washed, due to a lack of warm water. There also may have been apparent increases due to the breaking of bacterial clusters.

The cream was analyzed for fat and for total solids by the Mojonnier method and a Kohman analysis was made on the butter. The results for milk-solids-not-fat in both instances, together with the acidity of the cream, are summarized in the following tabulations:

Churning No.	Treatment of cream	Acidity	Milk-solids-not-fat of	
			Cream	Butter
		per cent	per cent	per cent
325	Check, inoculated.....	0.44	5.33	0.90
326	Washed once	0.09	0.95	0.60
327	Washed two times.....	0.02	0.73	0.50
328	Washed three times.....	0.02	0.51	0.35
329	Washed four times.....	0.01	0.55	0.25
330	Washed five times.....	0.01	0.55	0.20
330a	Washed six times.....	0.01	....	....

The results indicate that the greater proportion of the plasma solids was removed in the first washing, including the acid-reacting substances and the substrates acted upon by the causative flora in the manifestation of the cheesy flavors and aromas. It is of interest to note that the plasma solids were decreased by about one-tenth in the cream from the fifth washing, as compared with the normal, while the same fraction in the butter from this cream was decreased by approximately one-fourth to one-fifth.

These experiments have shown that cream washed more than twice did not contain the substrates necessary for the development of cheesiness in unsalted butter. Therefore, it seemed logical to use washed cream as a base and to add to it, in more or less purified form, some of the important substances which are found in normal cream in order to ascertain if these materials, singly or in different combinations, might constitute the active substrates necessary for the formation of these characteristic flavors and aromas. Consequently, experiments were planned in this direction.

Casein was prepared by the grain-curd method and was reprecipitated twice from ammonium hydroxide solution. It was washed with five volumes of distilled water that was adjusted to a pH of 4.6 and soaked in similar water at 5° C. for 72 hours. This casein was preserved in a moist, frozen state so that it could be more easily dispersed. The casein was triturated with excess calcium hydroxide and the surplus calcium was removed by adding oxalic acid until the mixture was just acid to phenolphthalein (pink color disappeared). Sufficient water was added to obtain an approximate concentration of three per cent casein and the sol was centrifuged. Three per cent phosphoric acid was added slowly to the sol, which was stirred rapidly until the contents reacted just acid to litmus. The sol, which contained 2.02 per cent total solids, had a few visible particles of coagulated casein; otherwise it was satisfactory. Sufficient calcium caseinate was prepared for the experimental series to follow.

Lactalbumin powder was obtained from Dr. L. S. Palmer. It contained 35.37 per cent protein (N x 6.38), 0.195 per cent ash, and 64.44 per cent lactose (by difference). It was then necessary to prepare a lactose-free lactalbumin. This was accomplished by preparing a 20 per cent solution of the lactalbumin powder and dialyzing it in a hardened collodion bag against cold tap water for 72 hours. Some sediment was present in the bottom of the bag, but this was removed by filtration. The sol reacted positive to the Fehling's test. The lactalbumin preparation was pasteurized at 62 to 63° C. for 20 minutes and when plated showed a count of 400 colonies per milliliter. Dialysis was continued against tap water for 48 hours and against distilled water at 5 to 6° C. for 48 hours, and the sol was again pasteurized at 62 to 63° C. for 30 minutes on two consecutive days. The counts were 26 and 2 colonies per milliliter, respectively. The total solids of this lactalbumin sol were 8.76 per cent as compared wtih the initial concentration of 20 per cent. It showed a slightly positive Fehling's test.

Bacto-lactose was used as the source of milk sugar. Highly purified preparations of lecithin and cephalin were available in the laboratory.

Raw cream containing 42 per cent fat was washed five times, employing the technic previously described. This washed cream contained 59 per cent fat.

The inoculum was prepared according to the method described on page 37. It was impossible to make all the churnings on the same day. Consequently, they were made on three different days, with two days intervening, to make it possible to prepare sterile equipment and other materials. A fresh inoculum was available for each series of churnings and inoculations were made according to the technic previously described.

The different constituents were added to the washed cream (59 per cent fat) in amounts to approximate their proportions in normal cream, and the calcium caseinate sol was used to standardize the final volume to 30 per cent fat. The synthetic creams were calculated to contain about one per cent of casein. When casein was not added, distilled water was used to adjust the fat content to 30 per cent. The lecithin and the cephalin solutions were added in five milliliter quantities, each, to the synthetic creams. The synthetic creams were not stable at autoclaving temperatures; consequently it was necessary to pasteurize them by heating for 20 minutes at 65° C. on two consecutive days. The remainder of the procedure is described in the sub-headings of Table 16.

The results which are summarized in Table 16 tend to show that no single constituent of cream can be indicted as being the source of cheesy flavors in butter. The simplest combination (Churning 359) showing any degree of cheesiness was that containing casein and lactose. Otherwise, the butters exhibiting any manifestation of these flavors contained a number of substrates of which casein was one of the constituents. Considerable acid development was evident in the cream of Churning 377. It will be recalled, however, that the crude lactalbumin originally used in preparing this cream contained 64.44 per cent lactose. The calculated amount of lactose present in the final synthetic cream was about one per cent, which was probably sufficient sugar for the acid-producing types.

There was no consistency in the bacterial counts of the fresh butters prepared from these synthetic creams (see Table 17). This is to be expected, because the physical condition of the creams was undoubtedly affected by the addition or the absence of certain constituents, which might have influenced the number of organisms retained by the finished butter. It is also possible that certain combinations of substrates were more suitable than others for bacterial growth.

Table 16

Comments on Butter Prepared from Synthetic Cream

Churning No.	Acidity of the cream*	Treatment of the cream	Flavor and aroma of the butter after storage at 10° C. for:	
			14 days	28 days
	Per cent	Unwashed sterile cream		
355a	0.28	Inoculated	Very fruity, slight cheesy	Unclean, cheesy
		Washed cream		
355	0.01	Uninoculated	Washed out	Tallowy
		Plus calcium caseinate		
356	0.04	Uninoculated	Washed out	Slight tallow
357	0.05	Inoculated	Unclean	Doughy, unclean
		Plus calcium caseinate and lactose		
358	0.05	Uninoculated	Oxidized, tallowy	Very oxidized
359	0.11	Inoculated	Unclean	Slightly cheesy, and fruity
		Plus calcium caseinate, lactalbumin† and lactose		
364	0.07	Uninoculated	Satisfactory	Satisfactory
365	0.12	Inoculated	Yeasty, doughy	Yeasty, doughy
		Plus calcium caseinate and lecithin		
366	0.06	Uninoculated	Oily	Peculiar aromatic
367	0.04	Inoculated	Unclean, oily	Peculiar oily
		Plus calcium caseinate, lactose and lecithin		
368	0.04	Uninoculated	Tallowy, oxidized	Very oily, unclean
369	0.12	Inoculated	Cardboard	Yeasty, cheesy
		Plus calcium caseinate, lactose, lactalbumin‡ and lecithin		
370	0.08	Uninoculated	Satisfactory	Slight unclean
371	0.18	Inoculated	Unclean, yeasty	Cheesy, fruity
		Plus calcium caseinate and lactalbumin‡		
374	0.06	Uninoculated	Milk powder	Very unclean
375	0.12	Inoculated	Unclean, slight oxidized	Doughy
		Plus calcium caseinate, lactalbumin,‡ lecithin, and cephalin		
372	0.07	Uninoculated	Oily, doughy	Oxidized
373	§	Inoculated	Yeasty, doughy	Slight fruity and cheesy
		Plus lactalbumin†		
360	0.05	Uninoculated	Milk powder	Slight unclean
361	0.03	Inoculated	Unclean	Putrid

Table 16—Continued

Churning No.	Acidity of the cream*	Treatment of the cream	Flavor and aroma of the butter after storage at 10° C. for:	
			14 days	28 days
		Plus lactalbumin† and lactose		
362	0.02	Uninoculated	Rotten	Putrid
363	0.12	Inoculated	Putrid	Limburger, very putrid
		Plus lactalbumin‡		
376	0.09	Uninoculated	Oxidized, milk powder	Oxidized
377	0.18	Inoculated	Unclean	Limburger

* Calculated as lactic acid, using $N/_{10}$ NaOH.
† Specially prepared by dialysis.
‡ Obtained from Dr. L. S. Palmer.
§ Sample accidentally destroyed.

Table 17

Changes in Bacterial Counts of Butter Prepared from Synthetic Cream

(Numbers per milliliter)

Churning No.	Treatment of the cream	Bacterial counts of the butter		
		Fresh	After storage at 10° C. for:	
			14 days	28 days
		Unwashed sterile cream		
355a	Inoculated	990,000	72,000,000	19,500,00
		Washed cream		
355	Uninoculated	13	*	*
		Plus calcium caseinate		
356	Uninoculated	*	2	*
357	Inoculated	600,000	8,000,000	4,200,000
		Plus calcium caseinate and lactose		
358	Uninoculated	11	110	17
359	Inoculated	490,000	11,000,000	1,500,000
		Plus calcium caseinate, lactose and lactalbumin†		
364	Uninoculated	7	*	*
365	Inoculated	298,000	26,000,000	4,400,000
		Plus calcium caseinate and lecithin		
366	Uninoculated	7	110	23
367	Inoculated	9,900,000	84,000,000	7,600,000
		Plus calcium caseinate, lactose and lecithin		
368	Uninoculated	*	*	11
369	Inoculated	11,000,000	140,000,000	70,000,000

Table 17—Continued

Churning No.	Treatment of the cream	Bacterial counts of the butter		
		Fresh	After storage at 10° C. for:	
			14 days	28 days
	Plus calcium caseinate, lactose, lactalbumin and lecithin			
370	Uninoculated	*	*	*
371	Inoculated	7,500,000	500,000,000	89,000,000
	Plus calcium caseinate and lactalbumin			
374	Uninoculated	2	*	*
375	Inoculated	750,000	19,000,000	3,500,000
	Plus calcium caseinate, lactalbumin, lecithin, and cephalin			
372	Uninoculated	11	*	*
373	Inoculated	6,000,000	110,000,00	1,800,000
	Plus lactalbumin			
360	Uninoculated	*	6	*
361	Inoculated	250,000	7,200,000	3,600,000
	Plus lactalbumin and lactose			
362‡	Uninoculated	20,000	12,000,000	3,200,000
363	Inoculated	8,500,000	80,000,000	4,200,000
	Plus lactalbumin			
376	Uninoculated	65	1,100	37
377	Inoculated	600,000	8,800,000	27,600,000

* No visible colonies.

† For differences in lactalbumins used, see footnotes in Table 16.

‡ Obviously some contamination occurred, so it is questionable if these results are of any significance.

DISCUSSION

The fundamental causes of the Cheddar cheese type of flavors and aromas were studied experimentally and the results disclose important facts concerning some of the active biological agencies concerned in the production of these undesirable defects in unsalted butter. For convenience, the discussion will follow the material as it is presented in the context.

Pure cultures were obtained from specimens of cheesy butter for inoculation into sterile cream in attempts to reproduce the defect. Such pure cultures did not consistently reproduce cheesiness in the unsalted butter. These results cannot be attributed to differences in environment, because the inconsistencies were encountered even when the cultures were carried in sterile cream, which was as nearly a natural habitat as it was practical or possible to obtain. It was found that the

development of acid was not an important factor, because the ability of individual cultures to produce acid was often a constant property, irrespective of the flavors which developed in the resulting butter. The flora of the infected butters was not observed to undergo any morphological or dissociative change. Therefore, it is inevitable that explanations for the inconsistencies must be made on more or less theoretical grounds.

If it can be assumed that the manifestation of cheesy flavors and aromas is an expression of the physiological functions and properties of bacteria, then these inconsistencies might be explained on the basis of physiological variations of one sort or another. Possibly, the protoplasm was of such a nature that the metabolic processes of the cells were changed to the extent that by-products of a non-cheesy nature were formed.

On the other hand, the ability of pure cultures to produce cheesy flavors in butter has little practical significance, because a pure culture rarely, if ever, occurs naturally in raw milk or cream. Mixed cultures predominate. However, if single species are able to induce these defects, they must be considered as possibilities. There are conditions under which a pure culture might be localized in creamery equipment, water or other foci, and become a distinct menace. The organisms might resist pasteurization temperatures or be introduced subsequent to pasteurization. Such circumstances were encountered in several creameries.

It was difficult, if not almost impossible, to segregate the causative organisms at individual creameries experiencing trouble with cheesy flavors in butter. In some cases, a moderate degree of success was achieved in reproducing cheesy flavors in butter in the laboratory by using infected material taken from different points in afflicted creameries, or with organisms isolated from such sources, but more often negative results were obtained. Therefore, it is evident that the inconsistencies with pure cultures in the laboratory can be reconciled with what has been observed and experienced in the field.

The work with pure cultures naturally led to other methods of attack in attempts to obtain information regarding the causes of cheesy flavors, because it was very evident that some other unknown factor was playing a role in the development of these defects. Since the original hypothesis assumed that if the causative factors could be isolated and segregated, then by employing the proper technic and procedures one should be able to reproduce these defects consistently in unsalted butter.

Experiments were initiated to study the effect of the enzyme galactase and incidentally to note the effects of other enzymes and the udder flora on the keeping quality of butter. The results failed to re-

veal, in the butter, any suggestion of cheesiness which could be attributed to the enzyme galactase, or to the udder flora, or to a combination of both. Mention should be made of the work of Heiduschka and Komm (44) in which they showed experimentally, with a new technic, that aseptic milk did not contain an inherent protease.

The addition of certain animal proteases to sterile butter did not cause cheesy flavors. It has been generally assumed that the biochemical changes in the ripening of cheese are induced by enzymes of the peptic, tryptic and ereptic types. Such changes might take place in cheesy butter. However, it is doubtful if one could extract bacterial enzymes and simulate a natural condition in cream or in butter, because there is no definite information about the preparation of bacterial enzymes from the different species.

The observation was made that butter from aseptic cream, with one exception, did not become rancid, which would tend to indicate that lipase was not present in sufficient amounts in the butter to cause lipolysis and that this enzyme may not be a normal constituent of cow's milk.

It was obvious that the biological factors actually responsible for the consistent development of flavors and aromas of the Cheddar cheese type in unsalted butter were still undiscovered, since numerous attempts to reproduce them had failed. Consequently, other methods of approach were devised and these involved a study of the natural, mixed flora from samples of butter which were definitely designated as cheesy. It was revealed that such infected butter from a number of different sources, inoculated into sterile cream, consistently caused the development of cheesy flavors. Mixed cultures taken from platings of this butter also produced these flavors with a fair degree of consistency. These facts served to indicate that possibly more than one species was necessary to produce the proper biological circumstances for the development of these characteristic flavors and aromas. This led to experimental studies involving the use of a mixed culture (Mixed Culture 173) which uniformly caused the production of cheesy flavors in unsalted butter.

A study of the influence of certain environmental factors revealed that the typical flavors of the Cheddar type developed most consistently at 5 to 10° C. These results coincided with practical observations, because these defects have been noted in butter prepared from sweet cream which had been cooled and held for 48 hours or less. When incubation and storage temperatures of 21° C. were employed the flavors which developed in butter were somewhat suggestive of cheesiness, altho not typical. These results were to be expected in view of the well-known fact that the temperature to which bacteria are subjected

may influence them in a number of ways, and these influences might be manifested in the extent of growth, the rate of metabolic processes, and the chemical changes ultimately produced. These effects of temperature on Mixed Culture 173 seemed all the more obvious when it is considered that it was a natural, heterogeneous mixture of organisms.

The duration of incubation (from 24 to 72 hours) had no detectable effect on the development of the flavors and aromas of the Cheddar cheese type. The titrable acidity of the creams incubated for 24, 48, and 72 hours was 0.24, 0.31, and 0.40 per cent, respectively, indicating that such acid development, within these limits, was not an important factor.

The ability of Mixed Culture 173 to produce cheesiness in unsalted butter is an example of associative action or symbiosis. The activity of this flora in causing the development of these undesirable flavors and aromas is a complicated biological phenomenon, involving interrelationships of the different species. The consistency of the results obtained in this study indicated that this associative activity was a constant property of this mixed flora.

Attempts were made to resynthesize this mixed culture by recombining all of the individual cultures originally isolated from it. Such a mixture was inoculated into sterile cream from pure cultures carried on agar slants or in cream. The results indicated quite clearly that there was a natural relationship existing between the species which could not be duplicated in the laboratory. Possibly, there was a certain definite order in which these cultures should have been added to sterile cream to simulate the naturally-mixed culture. However, the arrival at the correct combination would be by chance or after a tremendous number of trials. This is clear when it is recalled that the number of possible combinations with ten cultures would be 1,023. If the cultures were inoculated in all the possible orders in the different combinations, with incubation periods intervening, the total number of combinations would be increased to such an enormous figure that the task would be practically impossible within a reasonable period of time.

Another peculiarity of Mixed Culture 173 was the fact that the species which it contained possessed optimum growth temperatures ranging from 25 to 37° C., while the most characteristic Cheddar cheese flavors were produced at 5 and 10° C. It appeared that the individual organisms became acclimated and thrived at these relatively low temperatures in association with each other. It was observed that certain of the component cultures in this mixed flora produced rather pronounced changes in milk. For example, certain types were proteolytic and others were aroma- and acid-producers. These characteristics appeared to coincide with the types isolated from Cheddar cheese. With

these facts at hand, it was possible to synthesize a number of mixed cultures which produced flavors suggestive, though not typical, of those obtained with the naturally-occurring mixture. This further substantiates the contention that there may be some type of associative action which is very difficult to duplicate artificially. From a practical standpoint, it appears probable that a mixed flora may be the cause of cheesiness in butter more often than a pure culture, because the latter is a rare occurrence in natural food products such as milk or cream.

The results with mixed cultures, incriminating them as agencies in the production of flavors of the Cheddar cheese type, are somewhat in accord with the results reported in cheese investigations. Authorities have disagreed as to the exact species of bacteria which have a role in the ripening of Cheddar cheese. This lack of agreement may be due to an inadequate system of classification, whereby it is impossible to identify the organisms on a strictly comparable basis. However, a more likely explanation is that a number of combinations of species are able to contribute to the sum total of the biochemical changes in the ripened cheese. This was adequately demonstrated with unsalted butter when it was shown that a number of different species in various combinations were able to induce the development of cheesy flavors, altho the results obtained with artificially-mixed cultures did not exactly duplicate those encountered with the naturally-mixed flora. It would be an exceptional occurrence if the same causative flora were isolated from cheesy butter in different regions. As a matter of fact, it was shown that the microflora of several samples of cheesy butter produced in Minnesota were notably different, but the end results were the same, in that flavors and aromas of the Cheddar type predominated. Only a few of the natural, bacterial mixtures were studied intensively in the laboratory. However, the number was sufficient to indicate the complexity of the problem.

Anyone familiar with the operation of a creamery realizes the importance of pure water, because it enters into every phase of creamery sanitation and is intimately involved in the manufacture of butter. The source of the trouble, in some cheesy butter outbreaks, was traced directly to infected water supplies. There are conditions under which a contaminated water supply at the creamery might infect the cream cans of individual patrons to such an extent that considerable bacterial growth could occur while the cream is held on the farm. This would be more likely if skimmilk or buttermilk were transported back to the farm in cream cans. As a matter of fact, this condition prevailed in one particular creamery which had experienced an extended series of outbreaks of cheesy and putrid flavors. It is conceivable that infections from contaminated water supplies at individual farms might be carried in the cream and become established in creamery equipment, resulting

in difficulties of considerable magnitude, particularly if the operator did not adhere at all times to the principles of strict sanitation. Experimental work has shown that contaminated water from the creamery well may infect the creamery equipment or the butter, directly, during the washing process. This phase of the problem is being studied more extensively in the University of Minnesota laboratories.

The data revealed the fact that the bacterial counts of the fresh butter prepared from sterile cream and washed with naturally-infected water were relatively low, but during storage the numbers reached proportions which were comparable with the counts of butter from artificially-infected cream. This leads to the conclusion that it is not a question of numbers of bacteria but essentially a matter of specific types so that the qualitative rather than the quantitative aspects of the problem are the most important considerations.

During the progress of this work, it was observed that flavors of the Cheddar cheese type, and others as well, were not stable, but often passed through successive stages of development and in some instances entirely lost their original identity. This was true of butters containing pure and mixed cultures which were obtained from a number of different sources. The transient character of these flavors might account for certain irregularities observed in the field where consignments of butter from individual creameries have been graded "cheesy," and later placed in other categories of flavor defects. To account for the instability of these flavors it would be necessary to compare the distribution of the various substrates in Cheddar cheese and their relationship to each other with a consideration of the same factors in butter.

Investigations (111) on the biochemistry of Cheddar cheese ripening have demonstrated that certain changes occur consistently during the curing process. For example, lactose is transformed rapidly. Certain slight changes appear to occur in the fat, while various alterations take place in the nitrogen-bearing compounds, most of which are alleged to contribute to the characteristic flavor of Cheddar cheese. The water is firmly bound in cheese and is not readily available. The supply of air is also limited. This structure and the proportion of the different substrates may be contrasted with the conditions in butter. The latter has a fat content of about 80 per cent, lactose, salt and protein usually constituting a total of less than one per cent, while the water (approximately 15 to 16 per cent) exists in a more or less free state. The studies with washed cream definitely indicated that the plasma solids were necessary for the production of cheesy flavors and aromas in butter. Therefore, it is conceivable that the causative flora might attack the proteins and lactose to the extent that cheesy flavors become de-

tectable, but that the biochemical changes might proceed to the point where other compounds are formed to nullify or mask completely the cheesy-flavor-bearing substances, which in turn may be transformed into other compounds. With most of the water in a free state, the biochemical reactions might proceed further than it would be possible in cheese which is "buffered" by bound water. Butters containing cheesy-flavor-producing types have developed flavors suggesting fat hydrolysis, indicating that fats may also be attacked by these organisms. Therefore, the instability of cheesy flavors in butter might be explained on the basis of an inequality of substrate and free moisture which allows the chemical reactions to continue past the point where cheesy flavors are detectable.

The data from the experiments with washed cream showed that the cheesy-flavor-reacting substances were neither soluble in fat nor absorbed by it, indicating that they were carried or dispersed in the plasma phase of the cream. The experimental evidence clearly indicted the plasma solids as the substrates necessary for the development of these cheesy flavors and aromas. However, the removal of the incriminated substrates under commercial conditions to prevent the onset of these flavors would not be practical or expedient. Certain constituents of the plasma solids are essential to the normal and pleasant aroma of butter.

The addition of the major constituents of the plasma solids to washed cream revealed the fact that no single component was the source of compounds responsible for cheesy flavors. The inoculated, synthetic cream containing lactose and casein developed in the resulting butter flavors suggesting the first stages of cheesiness of the Cheddar type. If it can be assumed that acid development and proteolysis are necessary in the production of these flavors and aromas, then these results are in line with what might be expected. The other combinations with casein showing any degree of cheesiness contained, in addition, most of the other constituents of cream. The synthetic cream containing lactalbumin and lactose, or lactalbumin which contained lactose (64.44 per cent), yielded flavors resembling those of Limburger cheese.

One difficulty is the fact that a suitable quantitative standard is lacking for evaluating the quality of butter, and the conventional method of judging butter—employing the sense of taste and smell—is open to some criticism because of the personal factor involved. It is recognized that only four tastes are fundamental, namely, acid, bitter, salty, and sweet. All others are combinations, or, what is more common, sensations of taste modified by the sense of smell. In order to affect the taste, the substances must be soluble or highly dispersed. Therefore, it is obvious that these qualitative functions are complicated processes and that they

undoubtedly vary with different individuals. However, in judging butter with such outstanding defects as cheesy flavors of one type or another, the error attributed to the personal factor becomes of somewhat less importance, because flavors typical of certain cheeses stand out so prominently that usually they are not mistaken. Nevertheless, occasions do arise when it is difficult to classify a cheesy flavor defect, because it may partially simulate two or more different varieties of cheese. Furthermore, butters have been examined in which the defects were in the first stages of cheesiness, and difficulty was encountered in classifying them. Such criticisms as "doughy," "yeasty," "fruity," and "unclean" were often associated with the occurrence of flavors of the Cheddar cheese type, particularly in the incipient stages of development.

Much has been accomplished at the sources of production to improve the quality of raw cream, but much remains to be done. On the other hand, in spite of improved sanitation and the application of precautionary measures, it is inconceivable that cream could be delivered to the factory entirely free from detrimental microorganisms. In view of the fact that the experimental work at hand has indicated that a number of different species might be involved in the production of these undesirable flavors and aromas, it appears futile to attempt a study of all the possible natural combinations of bacteria which might be encountered under practical conditions. Such a study would be interesting and instructive, but would lead one into innumerable byways and take one from the immediate and more practical aspects of the problem.

Because of the complications which are involved in a study of these defects from the standpoint of determining all the causative organisms or combinations of them, it is obvious that the solution must be sought in attempts to determine the practices and circumstances that will control or prevent the development of these defects.

CONCLUSIONS

On the basis of the experiments conducted in the laboratories of the University of Minnesota, it is possible to draw the following conclusions:

1. Raw cream, in the majority of cases, contained microorganisms capable of producing cheesy flavors and aromas in unsalted butter.

2. The types of bacteria present in aseptically-drawn milk were not important factors in the production of cheesiness in unsalted butter.

3. The enzyme galactase in cow's milk was not responsible for flavors of the Cheddar cheese type in unsalted butter.

4. Pure cultures of various species of bacteria were able to induce cheesiness in unsalted butter, altho not consistently.

5. Naturally-mixed cultures of bacteria were most consistent in the production of cheesy flavors in unsalted butter.

6. Artificially-mixed cultures were able to produce cheesy flavors suggestive of, but not identical with, those produced by the naturally-occurring mixtures.

7. It appears probable that such flavors are most commonly produced as a result of the associative activity of several species.

8. Gram negative, rod types of bacteria predominated in the naturally-mixed cultures studied as they did among the pure cultures capable of producing flavors and aromas of the Cheddar cheese type in unsalted butter.

9. The development of Cheddar cheese flavors in cream or unsalted butter occurred most typically at temperatures of 10° C. or lower.

10. Creamery water supplies are sometimes contaminated with bacteria which are able to produce the Cheddar cheese and other cheesy flavors in unsalted butter.

11. Naturally-mixed cultures are capable of producing Cheddar cheese flavors in sterile cream butter when such butter is infected through the wash water.

12. The plasma of cream contained the substrates necessary for the development of cheesy flavors and aromas by the causative flora.

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