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A report in the literature suggesting that the use of cottage cheese dressing cultured with S. diacetilactis would afford enhanced flavor and shelf life in the finished product prompted this study to examine reasons for its effectiveness. The method used involved addition of a culture of S. diacetilactis 18-16 to 12 percent cream at the rate of one percent with incubation at 21° C. for six hours; this dressing was then used to cream dry Cottage cheese curd.

It was found that the dressing was effective in suppressing the growth of organisms in the Bacillus, Pseudomonas, Proteus, Alcaligenes, Aerobacter, and Staphylococcus genera when the dressing was used on Cottage cheese curd that had been heavily contaminated with these organisms.

The disc assay method was used to determine what effect various culture fractions would have against the growth of these

spoilage organisms. The culture fractions tested were: cell suspension of S. diacetilactis (2×10^{10} cells/ml.); sonicated cell suspension (2×10^{8} viable cells/ml.); broth supernatant of 24 hour culture; broth supernatant of 24 hour culture concentrated ten times by lyophilization; this same concentrate, dialyzed to remove salts and sugars; uninoculated broth, single strength, concentrated, and concentrated-dialyzed; 24 hour milk culture; 24 hour broth culture. Buffered lactic acid at pH 4.5, 5.0, 5.5 or 7.0 was also tested. In all cases, there was no inhibition from these culture fractions except where there were viable cells of the lactic organisms present. Cross streaking with S. diacetilactis 18-16 against the various spoilage organisms also showed inhibition of the bacteria at the point of intersection; not all organisms were inhibited to the same degree.

Competitive growth studies were also conducted between S. diacetilactis and S. aureus and between S. diacetilactis and P. fragi in milk and broth. Growth repression of P. fragi ranged from one to ten percent of the control growth in the presence of viable cells of S. diacetilactis; there was no inhibition in the presence of heat-killed cells. Similar results were found with S. aureus grown in the presence of S. diacetilactis. Maintenance of the pH at 7.0 did not alter the inhibitory effect, suggesting that the lowered pH caused by acid produced by the lactic organisms was not a contributing factor.

The minimum growth temperature of S. diacetilactis 18-16

was found to be approximately 7.5° C.; the cells were still metabolicly active at this temperature. Inhibition of P. fragi did occur when grown with S. diacetilactis at 7.5° C., suggesting that active metabolism and not cell growth per se was responsible for the inhibitory effect.

Experiments were conducted with Cottage cheese curd that had been heavily contaminated with spoilage organisms and creamed with cultured S. diacetilactis dressing or plain cream. Results showed that the cultured dressing was effective in retarding loss of aroma and slimy curd defect when the cheese was held at 7.5° C. for 30 days, or for two weeks at 10° C. and even when held at 21° C. for as long as six days.

Studies on the Inhibition of Spoilage Organisms in Cottage Cheese by Lactic Streptococcus Organisms

by

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INTRODUCTION

Due to the high moisture, protein and carbohydrate content of Cottage cheese, the product presents an ideal medium for the growth of almost all types of bacteria, yeasts and molds that might gain entrance to the product during manufacture and processing. Consequently, one of the most important problems facing manufacturers of Cottage cheese is their inability to produce a uniform product with sufficient keeping quality for retail distribution and home consumption.

The most apparent but possibly the most difficult solution to this problem is to prevent contaminating organisms from entering the product. Maintenance of high standards of sanitation in the plant will help markedly in reducing the level of contamination in the product. This will increase the shelf-life since it will take longer for the smaller numbers of organisms to reach levels where they can cause noticeable off-odors or changes in appearance. However, this approach will be of little avail if the product is improperly handled at some later stage which would allow for the rapid growth of the contaminating organisms and subsequent spoilage.

Another approach to the problem is to prevent the contaminating

organisms from growing after they have gained entrance to the product. One method that has been used is to maintain a low pH in the product (4.8 or lower), which will prevent the growth of all but the most acid-tolerant organisms. Another method that has been used is to exclude all air from the product by packaging under nitrogen; since many of the spoilage bacteria are primarily aerobic, this method will retard or halt the growth of many spoilage bacteria, subsequently increasing the shelf-life.

In 1963 a report from Mr. W. K. Moseley of Indianapolis, Indiana suggested that the use of cottage cheese dressing cultured with S. diacetilactis might afford enhanced flavor and shelf life in the finished product. Preliminary studies substantiated this hypothesis and suggested further investigation.

The present investigation involved an examination of this possibility and the probable reasons for its effectiveness. It was suggested that the increased keeping quality was due to the production of an antibiotic agent by the streptococci which would inhibit the growth of the spoilage bacteria. The objectives of this study were to determine whether or not an antibiotic agent was being produced by S. diacetilactis; if so, possibly to determine the nature of the agent; if not, then to determine what other modes of action would account for the apparent inhibition of the spoilage bacteria by the presence of S. diacetilactis in the creaming mixture.

HISTORICAL REVIEW

Cottage Cheese

Cottage or Pot cheese is a soft, unripened, fermented food made from skimmilk. It is edible as soon as made and contains a large amount of water, a fact which contributes to its softness and short keeping time. The finished product may be marked as "dry curd," to it may be mixed with cream, pineapple, chives, or other fruit. Production of Cottage cheese and Baker's cheese, a low-fat product used in the baking industry, exceeds 300,000,000 pounds per year. The maximum amount of moisture allowed in Cottage cheese is 80 percent.

Manufacture of Cottage Cheese Curd

Manufacture of Cottage cheese begins when high quality pasteurized milk is inoculated with a mixed strain lactic starter culture containing lactic streptococci and the associative Leuconostoc species. A small amount of rennet extract may be added if desired. Time is allowed for curdling of the milk due to the acid produced by the lactic streptococci. A short-setting period may be used in which the milk is warmed to about 32° C. and inoculated with four to five percent of active starter. The curd is ready to cut within four to

six hours after the starter is added. Sometimes it is more convenient to use the long-set method in which the milk is held near 21° C. and inoculated with 0.3 to 1.0 percent of an active culture; curdling takes place in about 16 hours. The long-set method is more likely to result in more pleasing aroma in the final cheese because it provides better conditions for growth of Leuconostoc, which convert milk citrate to diacetyl.

When the titratable acidity of the whey within the curd mass reaches 0.5 to 0.6 percent, the curd is cut into cubes and slowly heated to a temperature of 43° C. to 54° C. where it is cooked over a period of about an hour. The whey is drained and the curd is washed two or three times with cold water in order to increase its firmness, remove the sour whey, and inhibit further acid formation. The wash water is drained and salt may be added at a rate of 0.5 to 1.0 percent. Sometimes salt is not added, or it may be added later during creaming. The curd is then creamed, packaged, and marketed. Under these conditions it can be kept for one to two weeks at most. Experiments have shown that uncreamed cottage cheese curd can be preserved under a two to four percent salt brine at -1.1° C. to 4.4° C. for periods up to three months. Freezing of the curd can also be used to preserve the curd for several weeks, but

the thawed curd has an undesirable tendency to break down (18, p. 355-357).

Aroma and Flavor in Cottage Cheese

Mixed strain starter cultures frequently contain species of Leuconstoc (L. citrovorum or L. dextranicum) which are hetero-fermentative organisms. These organisms are capable of fermenting glucose to acetic acid, ethanol, carbon dioxide, and lactic acid. Milk citrate or added citric acid is fermented to diacetyl, acetoin, 2,3-butylene glycol, acetic acid and carbon dioxide. These bacteria are unable to coagulate milk because they do not ferment lactose to any great degree. S. lactis and S. cremoris are unable to ferment citrate but S. diacetilactis does have this ability. Moreover, S. diacetilactis is able to produce diacetyl in pure milk culture, while the Leuconostoc can ferment citrate only under acid conditions (when the pH is lower than 4.7). This can be achieved by growing Leuconostoc in association with S. lactis or S. cremoris or by artificially lowering the pH by the addition of lactic or citric acid. The production of diacetyl by these bacteria is what gives the culture a delectable buttery odor and flavor. Leuconostoc organisms can be used successfully in starter cultures for setting the curd for cottage cheese, however, S. diacetilactis should not be used because of the large amounts of carbon dioxide that are produced by this bacterium

during the fermentation of milk citrate. This usually will result in a floating curd defect (49, p. 2-4).

In order to achieve the desirable odor and flavor that these bacteria produce in cottage cheese without having too much gas in the curd, several procedures have been developed. In 1959, Mather and Babel (32) described a process to increase and standardize the amount of flavor in cottage cheese. This process involves growing Leuconostoc citrovorum (Streptococcus citrovorus) in skim milk for 24 hours at 21° C., then acidifying the milk to pH 4.3 with sterile citric acid and reincubating the culture for an additional 24 hours. This culture is then used to standardize the fat content of 20 percent cream to about 12 percent which is used to cream the dry Cottage cheese curd. By varying the amount of cultured milk and cream, the diacetyl can be standardized in the final creaming mixture, giving a product of consistant aroma and flavor.

In 1961, Erik Lundstedt filed for a patent on a similar process (29). The purpose in this case was mainly to enhance the flavor of such dairy products as Cream cheese, Neufchatel cheese, Cottage cheese, butter and margarine. His process involves the use of cottage cheese whey as the medium for growing a culture of S. diacetilactis. Sodium citrate is added to the cheese whey, the pH is adjusted to approximately 6.0, then it is sterilized. The citrated medium is then inoculated with a pure culture of S. diacetilactis and

is ripened for 18 hours at 22° C. (72° F.). This product, which is high in diacetyl, is added in small amounts (one-half of one percent by weight) to Cottage, Cream and Neufchatel cheeses, and one to five percent by weight to butter and margarine.

In 1963, William K. Moseley described a process which was directed towards the improvement of the aroma of Cottage cheese (35). This process is used for Cottage cheese and is much simpler than either of the above mentioned processes. This process involves pasteurizing 12 percent cream, cooling to 21° C., inoculating with a pure culture of S. diacetilactis and ripening for six hours at 21° C. The Cottale cheese curd is then creamed directly with this cultured cream. The resulting Cottage cheese has a very uniform aroma and flavor and a much enhanced keeping quality. An added advantage of this process is the simplicity and convenience to the manufacturer; the only difference from the normal processing procedure for Cottage cheese is the addition of the culture to the pasteurized cream and holding for about six hours. The inoculation of the cream can be done by the direct seed method with a lyophilized culture (49, p. 7). Thus the manufacturer does not have to maintain the mother culture in the dairy plant. Frozen cultures also can be used very effectively with this process.

Defects of Cottage Cheese

There are a number of defects that can occur in Cottage cheese as a result of faulty manufacturing methods, most of which cause changes in the physical characteristics of the curd. Some of these defects also can affect the flavor or keeping quality of the finished product. If the curd is cut before sufficient acid is developed or if the curd is washed with alkaline water, the finished curd may have a "flat" flavor and a high pH that will encourage growth of spoilage organisms. Insufficient washing of the curd can result in a sour cheese to which the consumer might object. The growth of spoilage bacteria can result in two serious defects that will be the main consideration here: a) loss of aroma due to reduction of diacetyl to flavorless compounds; b) gelatinous curd produced by the growth of psychrophilic bacteria.

The term "psychrophilic" as commonly used in the dairy industry, refers to those bacterial species which are capable of relatively rapid growth at low temperatures, generally within the range of 1.7 ^{o} C. (35^{o} F) to 7.2^{o} C. (45^{o} F.). These are the organisms which are of major importance in affecting the keeping quality of dairy products stored at low temperatures.

Loss of Aroma

It has been shown (50) that diacetyl produced in mixed strain starter cultures, incubated at 21° C., increases to a maximum within about 12 hours and then rapidly decreases. Investigation of the cause for the rapid decrease in diacetyl led to the discovery that starter bacteria, and especially S. diacetilactis, contain an enzyme (diacetyl reductase) that converts diacetyl to acetoin, a flavorless and odorless compound. Psychrophilic bacteria also contain the enzyme and the first indication of psychrophilic spoilage of Cottage cheese therefore usually is loss of diacetyl aroma. It is paradoxical that the use of S. diacetilactis to produce aroma in cottage cheese may actually result in a flat product due to diacetyl destruction. Two approaches have been suggested to overcome this difficulty. The first would be the use of an inhibitor to inhibit diacetyl reductase but so far this has not been successful. A second approach, cooling the cultures when diacetyl is at its maximum level and thereby inhibiting activity of the diacetyl reductase, was used successfully by Pack (39).

Gelatinous Curd

This defect sometimes developes in cottage cheese during storage. It is characterized by a soft slimy film that coats the

curd particles. The slime may present a varied appearance--it may be white, yellow, brown or greenish-brown; it may be watery; sometimes it is ropy. Often, but not always, it is accompanied by a rancid, putrid, or fruity odor (10, 11, 43). As might be assumed from the diverse appearance of the defect, it can be caused by any of several organisms. Species of Alcaligenes, Pseudomonas, Aerobacter, Proteus, and Achromobacter have been implicated in outbreaks. The defect may develop in 24 hours during refrigerated storage or may be delayed for several days. Creamed curd usually has shown more frequent and rapid spoilage than uncreamed. When this happens there is a natural tendency to associate the defect with the cream or the creaming equipment and to attempt to prevent the trouble by more careful handling of the cream and more thorough sanitization of the creaming equipment. Although the creaming operation may indeed be at fault, the organisms responsible for the defect often are present in the plain curd but are unable to grow appreciably until some of the acid is neutralized by adding sweet cream. This addition usually raises the pH of the cheese by 0.25 to 0.35 of a pH unit (18, p. 359).

Collins (7) confirmed that a low temperature was very effective in controlling gelatinous curd defects. Prolonged incubation permitted cultures of P. fragi, P. viscosa and A. metalcaligenes to cause surface spoilage of Cottage cheese at initial pH values as low

as 4.6 and at temperatures as low as 3.5° C., but the defect developed slowly at low pH values and very slowly at 3.5° C. The effect was accumulative; the low pH was more effective at the lower temperatures than the higher pH at the lower temperatures or the lower pH at the higher temperatures. Davis and Babel (10) have shown that the temperature at which the cheese was held had a greater effect on the rate of slime formation than did the number of bacteria in the wash water. Thus, the slimy curd defect developed rapidly at 21° C., but did not develop at 4.4° C. Cultures of Proteus, Pseudomonas and Klebsiella isolated from cheese showing slimy curd, produced the spoilage in experimental Cottage cheese in one to four days when held at 21° C., and in seven to eleven days at 4.4° C.

Parker et al. (43) have shown the effect of pH of uncreamed, unsalted cottage cheese on the development of gelatinous curd defect at 15° C. in 72 hours. A. metalcaligenes, P. viscosa and P. fragi were used in the trials with several strains of each organism used. P. viscosa showed growth in some trials at pH 5.0 but not at pH 4.8. None of the strains of A. metalcaligenes or P. fragi grew at pH 5.0 or below. All of the strains of all three organisms grew at least slightly at pH 5.2. They also showed the effect of salt on the growth of the same organisms. Results indicated that P. viscosa was the most salt-tolerant and P. fragi the least salt-tolerant of the three

species. All three species grew in two percent salt at pH 5.2. Increasing the salt concentration to 2.5 to 3.0 percent at pH 5.2 produced marked inhibition of all three species. This method would not be a practical control method on a commercial basis since concentrations much above one percent salt are found to be objectionable by the consumer.

The trend toward a more bland-flavored cottage cheese with lower acid and higher final pH may explain part of the increased difficulty with this defect in recent years. Another factor may be centralization of Cottage cheese plants with longer marketing time required (43).

Source of Spoilage Bacteria

Soil and water appear to be the most common sources of the spoilage bacteria. They have been isolated from plant and city water supplies and dust that enters the plant and settles on equipment. They also have been isolated from all of the equipment that is used in the manufacture of the Cottage cheese. In this case the organisms most likely have grown in milkstone deposits remaining after improper cleaning and sanitizing operations. Studies have shown that they are destroyed by proper pasteurization (12).

Dust that contaminates the raw milk and also enters the dairy plant can be a source of yeasts and molds as well as psychrophilic

spoilage bacteria. The destruction of yeasts and molds by pasteurization and equipment sanitization is very important, since their utilization of lactic acid for growth may raise the pH to a point (above 5.0) where spoilage bacteria are able to develop. Molds and yeasts are readily destroyed by approved pasteurization treatment of dairy products. Both molds and yeasts form spores, but in contrast to the bacteria, their spores do not survive approved pasteurization procedures. The fact that they are able to grow at temperatures below 5° C. is also reason to insure that they are destroyed by pasteurization and proper sanitation and are not allowed to re-enter the product before packing (13).

Staphylococcal Food Poisoning

Sources of Staphylococci

Staphylococci are extremely widespread in air, water, milk and sewage, but their main source is the animal body, normally on the skin and in the intestinal and respiratory tracts. It is generally believed that those strains of S. aureus that are found in boils, infected cuts, sores, post-nasal drip, expelled sprays from coughing and sneezing are most likely to produce enterotoxin in contaminated foods. The bacteria producing the toxin enter milk and milk products through human handlers or directly from cows with mastitis caused by these organisms.

Growth and Toxin Production

The staphylococci are fairly heat-resistant and are not always killed by pasteurization if present in high concentration. Even though staphylococci may survive the pasteurization of the milk used to make cottage cheese, it is doubtful that heat damaged cells would be able to grow enough in competition with the lactic starter bacteria to reach populations that are considered high enough to produce toxin. Appreciable levels of enterotoxin are produced only after the staphylococci attain a population of at least several millions per milliliter or gram. Enterotoxin has been demonstrated in an adequate growth medium within three days at 18° C. and 12 hours at 37° C., but not within three days at 15° C., seven days at 9° C., or four weeks at 4.0 to 6.7° C. (19, p. 297).

The enterotoxin produced by some strains of S. aureus grown under favorable conditions is a heat stable exotoxin. It is able to withstand heating at 100° C. or higher for 30 minutes or more; therefore, it would survive pasteurization if it were produced in the raw milk.

Competitive Inhibition of Staphylococci

It has been observed that production of enterotoxin by the staphylococci is more likely when competing organisms are absent

(19, p. 397). Troller and Frazier (56) reported that the maximum inhibition of S. aureus by food bacteria was at about 20° C. to 25° C. in a good medium. Frazier (19, p. 397) has reported that enterotoxin production by S. aureus is greatest in the range from 21° C. to 36° C. It is of great practical significance that the maximum inhibition should also occur in the same range. It is of particular significance since this is the range that milk is held during the incubation period for the manufacture of cottage cheese (29° C. to 32° C. for the short-set; 21° C. for the long-set). Troller and Frazier (56) also have shown that the greater the staphylococci are outnumbered by the food bacteria, the greater is the inhibition which occurs. When the numbers of effector organisms and staphylococci were equal, the staphylococci were able to multiply about 1,000 to 3,000 times in 24 hours at 30° C.; the control staphylococcus culture was able to increase 30,000 to 40,000 times. When the effector organisms were initially 100 times greater, the staphylococci were able to multiply about 100 fold during the first six hours, with some of the cultures declining in numbers or holding steady after the first 10 hours.

Troller and Frazier (57) have shown that the repression of growth of S. aureus by food bacteria (Serratia marcescens, Pseudomonas sp., Bacillus cereus, Proteus vulgaris, Escherichis coli H-52, Aerobacter aerogenes and Achromobacter sp.) was not

caused by changes in pH, oxidation-reduction potential, or production of peroxide or fatty acids. The first two, S. marcescens and Pseudomonas sp., apparently inhibited the staphylococci by out-competing it for nutrients. The other five inhibited by means of antibiotic substances which were Seitz-filtrable, dializable, and stable at 90° C. for 10 minutes. The concentrated antibiotic material from E. coli H-52 contained amino acids but not peptides and was especially effective against staphylococci and micrococci.

Since S. aureus requires cystine, valine, glycine, proline, arginine, and aspartic acid, any of these amino acids might be used by the competing food bacteria, with resulting inhibition of the staphylococci. This would support the contention from 1931 of Barnes (2) who concluded that the inhibition of one organism by another could be due to alteration of necessary nutrient materials rather than by production of inhibitory agents. Lockhart (28) also supported this and concluded that staling of culture media was a manifestation of the stationary growth phase and not caused by specific inhibitory agents but rather probably by the exhaustion of some factor or factors essential to the anabolic processes of the cell. Recently, Iandolo, Clark, Bluhm, and Ordal (26) showed competitive inhibition of S. aureus by lactic acid bacteria by exhaustion of nicotinamide.

Effect of Environmental Conditions

If pasteurized milk or low-heat, skim milk powder containing staphylococci were subsequently made into Cottage cheese, incubation temperatures and time could be conducive to considerable bacterial growth with accompanying enterotoxin production during manufacture. However, the high acidity encountered during manufacture of the Cottage cheese might be expected to inhibit staphylococcal growth. Foltz et al. (17) isolated staphylococci from cultured buttermilk with a pH of 4.3 to 4.5. The isolation of staphylococci from cultured buttermilk points out that some strains of the organism apparently tolerate acidity levels equal to or greater than those encountered in the manufacture of Cottage cheese (34). This does not indicate that the staphylococci are able to grow at this low pH, but merely survive. Hall and Fraser (23) reported that while the general pH tolerance range of the staphylococci group extends from 2.6 to 10, it is well known that primarily low acid food products will support the growth of food-poisoning bacteria. Even though the staphylococci may survive in high acid foods, they probably will not grow, or will grow at a limited rate. It would be expected, therefore, that the repression by competition would be greater under these circumstances where the pH would put the staphylococci at a competative disadvantage.

Mickelson et al. (34) have shown the results of adding staphylococci to milk that is being made into Cottage cheese. In five trials with the short-set method, where the staphylococci were added in numbers ranging from 15,000 to 2,000,000, the numbers decreased by cutting time and were virtually eliminated by the cooking process. There was no increase in numbers during the holding period of 144 hours at 0° C., 4.4° C. to 10° C. or 21° C. The same was true for the four trials using the long-set method except when the initial count was over 200,000 per gram. In this case the numbers did increase by about 10 times, at the most, by cutting time. The numbers were also reduced to almost zero by the cooking process. Few survived to show up during the holding period and there was no noticeable increase in the few that did survive.

Coagulase positive staphylococci were isolated from 14 percent of the 66 commercial Cottage cheese samples by Mickelsen et al. (33). No mention was made of the numbers of staphylococci found in these samples. In a survey of Cottage cheese quality, Martin et al. (30) found no staphylococci in 142 samples of retail cheese tested.

Foltz et al. (17) examined various fluid milk and milk products such as chocolate milk, buttermilk, half and half, coffee cream and whipping cream. Of these, 3.4 percent contained coagulase positive

staphylococci; all classes contained staphylococci but not all samples in a class. Presence of coliform and staphylococci did not correlate positively, leaving some question as to the source of contamination.

All of the evidence suggests that the conditions under which Cottage cheese is manufactured are not conducive for the growth of staphylococci or the production of enterotoxin. The main concern over food-poisoning in Cottage cheese is if the raw milk has been held under conditions favorable for the growth of the staphylococci and the subsequent production of the heat-stable toxin, which would withstand pasteurization and would probably remain toxic in the finished product.

Reflection on the incidence of staphylococcal food poisoning indicates that a majority of the cases occur in foods which have been treated to drastically reduce the bacterial population; staphylococci subsequently inoculated are without competition. Staphylococcal food poisoning also occurs in foods which favor selectively the growth of staphylococci by sharply inhibiting growth of other genera, or in foods such as eggs, starches and lipids which have a protective action on staphylococci.

The only means of preventing occurrence of the intoxication is to prevent the entrance of the staphylococci into dairy products (which is extremely difficult) and/or to prevent their growth prior

to pasteurization by means of adequate refrigeration of the product. Entrance and growth of toxin-producing bacteria after pasteurization have resulted in serious food-poisoning outbreaks in dairy products and emphasize the need for rigid sanitary care after pasteurization.

PART I

DETECTION OF SUSPECTED ANTIBIOTIC OR INHIBITORY MATERIAL PRODUCED BY *STREPTOCOCCUS DIACETILACTIS* 18-16

Experimental Methods

Source of Cultures

The following cultures were used in the experiments relating to the problems under consideration in this thesis: *Streptococcus diacetilactis* 18-16, *S. diacetilactis* 4R5, *S. diacetilactis* 31_I, *S. lactis* C_2S, *S. lactis* C_2F, *S. cremoris* M1-3, *Leuconostoc citrovorum* 91404, *Pseudomonas fragi*, *P. viscosa*, *P. putrifaciens*, *P. fluorescens*, *Alcaligenes metalcaligenes*, *Chromobacterium lividum* (51), *Staphylococcus aureus* (coagulase positive, and beta-hemolytic), *Salmonella typhosa*, *Shigella dysentariae*, *Aerobacter aerogenes*, *Escherichia coli* 198, and *Proteus vulgaris*.

All of the above listed bacteria except *S. aureus*, *S. typhosa* and *S. dysentariae* were obtained from the culture collection maintained in the Department of Microbiology, Oregon State University. The cultures of *S. aureus*, *S. typhosa* and *S. dysentariae* were obtained from Dr. K. S. Pilcher, Department of Microbiology, Oregon State University. These three cultures are used in the laboratory exercises in a Pathogenic Microbiology course and have

been shown to give typical reactions to the test media used.

Culture Propagation

The cultures of S. diacetilactis, S. lactis, S. cremoris, and L. citrovorum were maintained by weekly transfers at the rate of one percent to sterile nonfat milk, with incubation at 30° C. for 24 hours. The organisms were stored at 4° C. between transfers. The milk used was dry milk powder reconstituted to 11 percent solids with distilled water. In small quantities, 20 milliliters or less, the milk was autoclaved for eight to ten minutes at 121° C. at 15 pounds per square inch of pressure. In volumes of 20 to 200 milliliters, it was autoclaved for 10 to 12 minutes at 121° C., and in quantities of more than 300 milliliters, it was autoclaved for 15 minutes at 121° C. These times will render the milk sterile without causing excessive caramelization if the milk powder is not contaminated with large numbers of spore-forming bacilli. In cases where it was necessary to use a clear medium, the cultures were grown in lactic broth (14) at 30° C. for 24 hours unless otherwise stated. The composition of lactic broth is shown in Table 1.

Growth curve studies of S. diacetilactis 18-16 indicated that the organisms were in the log phase of growth from about one hour to six hours after inoculation. The maximum numbers were reached shortly after six hours and were usually about 1×10^9 cells per

milliliter in milk, lactic broth or trypticase soy broth. This organism also grows quite well in tryptone-glucose-yeast extract (TGY) broth or on TGY agar. The composition of trypticase soy broth and TGY broth and agar are shown in Table 2 and Table 3, respectively.

Table 1. Composition of Lactic Broth Culture Medium (14)*

Ingredient	Grams per Liter
Tryptone	20.0
Yeast Extract	5.0
Glucose	5.0
Lactose	5.0
Sucrose	5.0
Gelatin	2.5
Sodium Chloride	4.0
Sodium Acetate	1.5
Ascorbic Acid	0.5
Autoclave at 121° C. for 15 minutes. pH 6.8 to 7.0	

*Solid lactic agar was prepared by adding 15.0 grams of agar per liter.

The cultures of P. fragi, P. viscosa, P. putrifaciens, P. fluorescens, A. metalcaligenes, and C. lividum were maintained by monthly transfers on TGY agar slants with incubation at 21° C. for

Table 2. Composition of Tryptone-Glucose-Yeast Extract (TGY) Broth*

Ingredient	Grams per Liter
Tryptone	5.0
Glucose	1.0
Yeast Extract	2.5
Adjust pH to 7.0. Autoclave at 121° C. for 15 minutes	

*Solid TGY agar was prepared by adding 15.0 grams of agar per liter.

Table 3. Composition of BBL Trypticase Soy Broth*

Ingredient	Grams per Liter
Casien Peptone	15.0
Soy Peptone	5.0
Sodium Chloride	5.0
Autoclave at 121° C. for 15 minutes. pH 7.0 ±	

*Solid T. S. agar was prepared by adding 15.0 grams of agar per liter.

24 hours and storage at 4° C. between transfers. For some experiments these organisms were transfered to sterile milk or TGY broth with incubation at 21° C. for 24 hours and storage at 4° C.

The cultures of S. aureus, S. typhosa, S. dysentariae, A. aerogenes, E. coli and P. vulgaris were maintained by monthly transfers on TGY agar slants with incubation at 37° C. for 24 hours and storage at 4° C. between transfers. For some experiments these organisms were transferred to sterile milk or trypticase soy broth with incubation at 37° C. for 24 hours and storage at 4° C.

When these cultures were used in experiments, fresh transfers were made to the proper medium and the cultures were incubated at their optimum temperature for 24 hours at which time a fresh transfer was made to the same medium and the culture incubated for an additional 24 hours. A third transfer was made with appropriate incubation to insure that the organisms were acclimated to the medium. If a temperature other than the optimum for the organism was used, the last two transfers were incubated at the test temperature.

Disc Assay Method

This is a standard method of testing the possible inhibitory property of certain materials against a test organism. The material

to be tested is put into solution or suspension (usually in water); a small, sterile filter paper disk (about 0.5 centimeters in diameter) is dipped into the solution and then touched to a dry sterile piece of filter paper to drain off the excess liquid. The disk is then placed on the surface of a solidified agar plate that has been seeded with the test organism, and the plate incubated in an inverted position (1, p. 183-186). If the material is inhibitory to the test organism, the bacterium will not grow in the vicinity of the disk. The larger the zone of inhibition, the more inhibitory the substance; the amount of inhibition is directly proportional (within limits) to the area of the zone of inhibition. Different substances can not be compared by this method since the size of the zone of inhibition is also dependent on the rate of diffusion of the material through the agar, which depends on the size of the molecules, temperature, concentration, etc.

Preparation of Assay Materials

a. Cell suspension. An active culture of S. diacetilactis 18-16 was inoculated into lactic or TGY broth at the rate of one percent and incubated for 24 hours at 30° C. Usually one liter of broth was used since this would yield sufficient material to wet 100 disks. The broth culture was then centrifuged at 4,080 x g for 15 minutes in a refrigerated centrifuge. The supernatant was poured off and the cells were resuspended in distilled water and centrifuged again

at 4,080 x g for 15 minutes. The wash water was poured off and the cells resuspended in about five milliliters of sterile distilled water. This suspension contained about 2×10^{10} cells per milliliter.

b. Sonicated cell suspension. About 15 milliliters of the above cell suspension were placed in the water-cooled chamber of the sonic oscillator (Ratheon Sonic Oscillator, Model DF 101) and the power was adjusted to maximum according to directions and allowed to run for 30 minutes. The resulting suspension was stored at -20° C. between experiments. The sonication process destroyed approximately 99 percent of the viable cells; standard plate count showed approximately 2×10^{8} cells per milliliter were able to form colonies after this treatment.

c. Lactic broth supernatant. The supernatant from a broth culture that was centrifuged to obtain the above cell suspension was filtered through a Millipore filter, with a pore diameter of 0.45 micron, to remove any cells remaining in the broth. This sterile broth was transferred to a sterile screwcap bottle and stored at -20° C. until used.

d. 10X concentrated lactic broth supernatant. In order to increase any possible inhibitory effect that might be produced by some agent in the broth, the lactic broth was concentrated to ten times the usual amount of dry weight solids. This was accomplished

lyophilizing (freeze-drying) one liter of the lactic broth supernatant mentioned above to a dry powder and then reconstituting to 100 milliliters with distilled water. The resulting concentrate was then sterilized by filtration through a Millipore filter. This concentrate was stored at -20° C. until used.

e. Ten-X concentrated lactic broth. One liter of uninoculated sterile lactic broth was concentrated to one-tenth of its original volume by lyophilization and then reconstituted to 100 milliliters with sterile distilled water. This concentrate was used as a control for the concentrated lactic broth supernatant to see if the concentrated sugars or salts in the medium might have an inhibiting effect on some organisms. The concentrate was stored at -20° C. until used.

f. Dialyzed concentrated supernatant. The concentrated supernatant ("d" above) was placed in a length of dialisis tubing and dialyzed against three 20-liter changes of distilled water at 2° to 5° C. The water was changed every eight hours. The resulting concentrate was again lyophilized and reconstituted to 100 milliliters. The purpose of this step was to remove all of the sugars and salts from the concentrated broth. This would also give some idea of the molecular size of any inhibitory substance.

g. Concentrated TGY supernatant. A culture of S. diacetilactis 18-16 was grown in TGY broth for three days at 30° C. through three

successive transfers. The culture was then transferred to one liter of TGY broth at the rate of one percent and incubated for 24 hours at 30° C. The broth culture was centrifuged at 4,080 x g in a refrigerated centrifuge. The resulting supernatant was decanted and sterilized by filtration through a Millipore filter (0.45 micron). The sterile supernatant was then lyophilized and reconstituted to 100 milliliters with distilled water and filter-sterilized. The concentrate was stored at -20° C. until used.

h. Dialyzed concentrated TGY supernatant. One sample of the above mentioned concentrated TGY supernatant was dialyzed in the same manner as the concentrated lactic supernatant in "f" above. The dialyzed material was lyophilized and reconstituted to 100 milliliters and filter-sterilized. The concentrate was stored at -20° C.

i. Milk culture. Sterile milk (11 percent solids) was inoculated with an active milk culture of S. diacetilactis 18-16 or L. citrovorum 91404 at the rate of one percent; the culture was incubated at 30° C. for 24 hours and used immediately without storage.

j. Lactic broth culture. Sterile lactic broth was inoculated with an active broth culture of S. diacetilactis 18-16 or one of the other lactic cultures at the rate of one percent and incubated at 30°C. for 24 hours and used immediately without storage.

k. TGY broth culture. Sterile TGY broth was inoculated with

an active TGY broth culture of the desired organism at the rate of one percent and incubated at the optimum temperature for the organism for 24 hours. The culture was used without storage.

Preparation of Lawn of Test Organisms

An active TGY broth culture was diluted one to ten in TGY broth; one milliliter of this suspension was added to 200 milliliters of previously melted TGY agar that had been cooled to 45° C. The seeded agar was then poured into flat-bottom petri dishes at the rate of approximately 12 milliliters per dish. The seeded agar was poured immediately to prevent excessive kill of some of the more heat sensitive organisms. The plates were placed on a flat surface to insure that the agar would solidify in a uniformly thick layer. The paper disks that had been charged with the appropriate material were then placed on the plate. The disks were usually placed on the plate in groups of six, about two centimeters apart and two centimeters from the center. A sterile uninoculated disk was usually placed in the center of the petri dish to act as a control and to assist in placement of the other six disks. When fewer disks were used, they were placed two centimeters from the center and equidistant from each other. Incubation was at 10° C., 21° C. or 30° C. depending on the experiment.

Effect of pH

One experiment was carried out to measure the possible effect of pH on growth of spoilage bacteria and to see whether or not other lactic organisms had the same effect on the test organisms as S. diacetilactis 18-16. The following organisms: S. diacetilactis 18-16, S. diacetilactis 31_I, S. lactis C_2S, L. citrovorum 91404, and S. cremoris M1-3, were grown in TGY broth (one liter each) at 30° C. for 24 hours; the cells were spun out and the supernatant filter-sterilized, lyophilized, dialyzed, lyophilized again and reconstituted to 100 milliliter. The supernatant was divided into two parts; one part was kept at its natural pH and the other part was adjusted to pH 5.5. A 24 hour milk culture of each organism was also prepared. One-tenth molar phosphate buffer was prepared and divided into four parts; each was adjusted to pH 4.5, 5.0, 5.5, or 7.0 with lactic acid and sterilized by filtration. Table 4 shows the pH of each of the cultures at the end of the 24 hour period and again after lyophilization and dialysis.

The following assay materials for each of the above listed organisms (Table 4) were placed on lawns of P. fragi, P. viscosa, A. metalcaligenes, S. aureus, S. typhosa, and S. dysentariae: 1. fresh cell suspension; 2. milk culture; 3. concentrated TGY broth supernatant at natural pH (Table 4); 4. concentrated TGY

broth supernatant adjusted to pH 5.5; 5., 6., 7., and 8. buffered lactic acid at pH 4.5, 5.0, 5.5, and 7.0 respectively.

Table 4. pH of Several Cultures of Lactic Acid Bacteria Grown in TGY Broth Before and After Lyophilization and Dialysis

Organism	pH after growth for 24 hours	pH after lyophilization and dialysis
S. diacetilactis 18-16	4.80	4.90
S. diacetilactis 31_I	4.60	4.50
S. lactis C_2S	4.65	4.40
S. cremoris M1-3	4.55	4.20
L. citrovorum 91404	4.70	4.30
TGY broth (uninoculated)	6.75	6.35

Cross-Streaking to Show Inhibition

Thirty petri dishes were poured with about 15 milliliters of agar each; ten with lactic agar, ten with TGY agar and ten with milk agar. The milk agar was prepared by mixing sterile milk (11 percent solids) with an equal volume of melted three percent agar solution. When the agar had solidified, and active culture of the test organism was streaked across the diameter of the plate with a wire loop. An active culture of S. diacetilactis 18-16 was streaked

across the diameter of the plate at a 90 degree angle to the first streak. The plates were inverted and incubated at room temperature (25° C.) for 48 hours.

The cultures that were used as test organisms in this experiment were: P. fragi, P. viscosa, A. metalcaligenes, P. putrifaciens, C. lividum, E. coli 198, A. aerogenes, S. aureus, S. typhosa, and S. dysentariae.

Results and Discussion

Disc Assay Experiments

The results shown in Table 5 are the compiled data from six different experiments. Not all of the assay materials were run against all of the test organisms at one time. The plates that used P. fragi, A. metalcaligenes and P. viscosa as test organisms were incubated at 21° C.; the other plates were incubated at 30° C. One experiment was done where plates with the three above mentioned organisms were held at 10° C. for six days. The results of this experiment were very similar to those where the plates were incubated at 21° C.

As can be seen in Table 5, the only cases where inhibition was evident (with the exception of S. dysentariae) occurred when viable cells of S. diacetilactis 18-16 were present. Where the cell

Table 5. Results of Disk Assay for Inhibitory Substance Produced by S. diacetilactis 18-16

Assay Material	I[a]	II	III	IV	V	VI	VII	VIII
a. cell suspension[c]	++[b]	++	+++	+++	++	+++	+++	++++
b. sonicated cell suspen.	+	+	++	++	++	++	++	+++
c. lactic supernatant	-	-	-	-	-	-	-	-
d. conc. lactic supernatant	-	-	-	-	-	-	-	±
e. conc. lactic broth	-	-	-	-	-	-	-	±
f. dialyzed conc "d"	-	-	-	-	-	-	-	±
g. conc. TGY supernatant	-	-	-	-	-	-	-	-
h. dialyzed conc. "g"	-	-	-	-	-	-	-	-
i. milk culture (S. d.[d] 18-16)	++	++	++	++	++	++	+++	++++
j. TGY culture (S. d. 18-16)	++	++	++	++	++	++	+++	+++
k. lactic culture (S. d. 18-16)	+++	++	++	+++	++	++	+++	++++
l. "b" filter-sterilized	-	-	-	-	-	-	-	-

[a] I = P. fragi; II = A. metalcaligenes; III = P. viscosa; IV = E. coli 198; V = A. aerogenes; VI = S. aureus; VII = S. typhosa; VIII = S. dysentariae

[b] + = relative inhibition; - = no inhibition; ± = slight inhibition

[c] pp. 26-30

[d] S. d. = S. diacetilactis

suspension was sonicated to destroy most of the cells, there was only slight inhibition, presumably due to the number of cells that survived. When this sonicated suspension was filter-sterilized to remove these viable cells, there was no inhibition. The slight inhibition of S. dysentariae perhaps was due to the high concentration of salts, sugars, and protienatious material. The TGY concentrated supernatant did not have any inhibitory effect on the Shigella. This very well could be due to the much lower amount of tryptone in TGY broth concentrate as compared to the lactic broth concentrate (5 percent in TGY; 20 percent in lactic broth).

Figure 1, page 36 shows typical results of a plate that was seeded with P. fragi and incubated at 21° C. The disks held the following assay materials counterclock-wise from the top: a) cell suspension of S. diacetilactis 18-16, b) supernatant from a lactic broth culture of S. diacetilactis 18-16, 24 hours old. c) sonicated cell suspension of S. diacetilactis 18-16 (contains about 2×10^8 viable cells per milliliter). d) 24 hour milk culture of S. diacetilactis 18-16. e) 24 hour milk culture of L. citrovorum 91404. f) sterile, uninoculated lactic broth. center disk: sterile, uncharged disk control.

Figure 2 shows the results of the assay disks placed on a lawn of P. fragi held at 10° C. The disks were charged with the following materials counterclock-wise from the top: a) cell suspension of

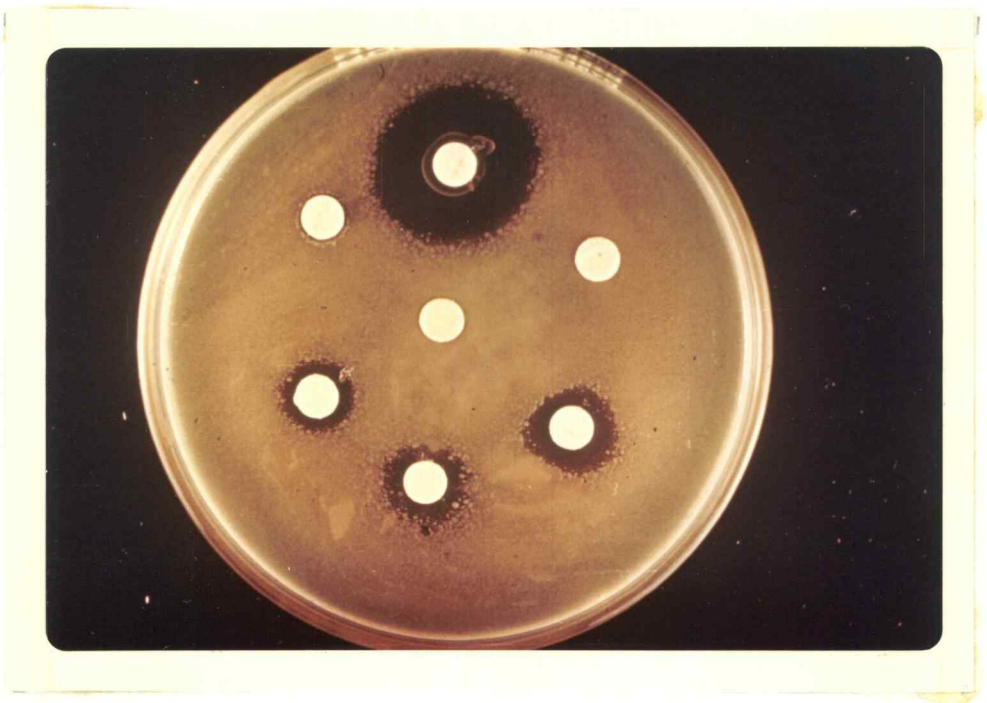

Figure 1. Disk assay method showing inhibition of P. fragi (lawn) by S. diacetilactis 18-16 and L. citrovorum 91404, at 21° C.

Counterclockwise from the top:

1. cell suspension of S. diacetilactis 18-16
2. supernatant of lactic broth culture of S. diacetilactis 18-16
3. sonicated cell suspension of S. diacetilactis 18-16 (2×10^8 viable cells)
4. milk culture of S. diacetilactis 18-16
5. milk culture of L. citrovorum 91404
6. sterile, uninoculated lactic broth

center disk: sterile, uncharged disk

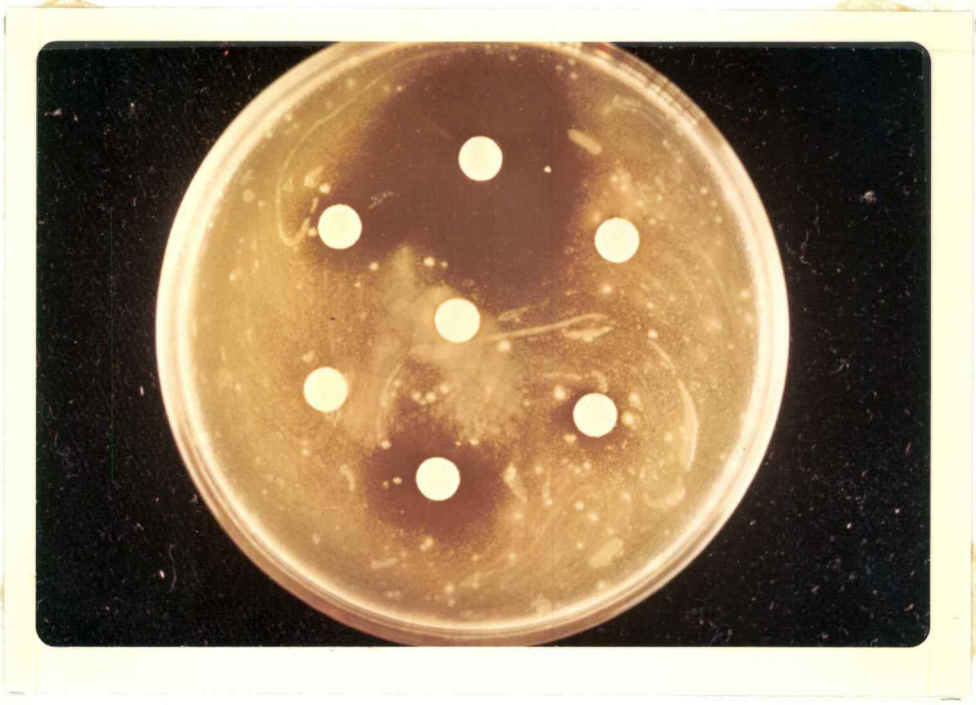

Figure 2. Disk assay method showing inhibition of P. fragi (lawn) by S. diacetilactis 18-16 and L. citrovorum 91404 at 10° C.

Counterclockwise from the top:

1. cell suspension of S. diacetilactis 18-16
2. sonicated cell suspension of S. diacetilactis 18-16
3. supernatant of lactic broth culture of S. diacetilactis 18-16
4. milk culture of S. diacetilactis 18-16
5. milk culture of L. citrovorum 91404
6. sterile, uninoculated lactis broth

centerdisk: sterile, uncharged disk

S. diacetilactis 18-16. b) sonicated cell suspension of S. diacetilactis 18-16 (containing 2×10^8 viable cells). c) supernatant from a 24 hour lactic broth culture of S. diacetilactis 18-16. d) milk culture (24 hours) of S. diacetilactis 18-16. e) 24 hour milk culture of L. citrovorum 91404. f) sterile uninoculated lactic broth. center disk: sterile, uncharged disk control.

These figures clearly show that the greater number of cells present in disk "a" in Figure 1 produced a much larger zone of inhibition than the other disks where there were less viable cells present. Disk "c" in Figure 1 does not have as large a zone of inhibition as disk "a" even though it held the same amount of cellular material. This indicated that the active metabolism of the cell was necessary to produce inhibition. This was substantiated by the results shown in Table 5 where the filter-sterilized, sonicated cell suspension revealed no inhibition, indicating that the 2×10^8 viable cells that remained in the "b" sample were responsible for the inhibition that resulted.

Table 6 shows results of the experiment where the supernatant from several lactic acid bacteria was concentrated and tested along with cell suspensions and milk cultures of these same organisms. Also included were disks charged with phosphate buffer that had been acidified with lactic acid to four different pH levels. These results show that all of the cultures acted much the same against the test

Table 6. Effect of Cells and Cell-free Extracts of Various Lactic Acid Bacteria and Buffered Lactic Acid on Various Spoilage and Pathogenic Organisms.

Organism and assay material		I[a]	II	III	IV	V	VI
S. diacetilactis 18-16							
	1[b]	+[c]	+	+	++	++	+++
	2	+	+	+	+	++	+++
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
S. lactis C_2S							
	1	+	+	+	++	++	+++
	2	+	+	+	++	++	+++
	3	-	-	-	-	-	-
	4	-	-	-	-	-	-
L. citrovorum 91404							
	1	+	+	+	++	+++	+++
	2	+	+	+	++	++	+++
	3	-	-	-	±	±	-
	4	-	-	-	-	-	-
Buffered lactic acid							
	pH 4.5	±	±	±	-	±	-
	pH 5.0	-	-	-	-	-	-
	pH 5.5	-	-	-	-	-	-
	pH 7.0	-	-	-	-	-	-

[a] I = P. fragi
II = P. viscosa
III = A. metalcaligenes
IV = S. aureus
V = S. typhosa
VI = S. dysentariae

[b] 1 = cell suspension
2 = milk culture
3 = concentrated, dialyzed lactic broth supernatant @ normal pH
4 = concentrated, dialyzed lactic broth supernatant @ pH = 5.5

[c] + = relative inhibition
- = no inhibition
± = slight inhibition

organisms. The cell suspensions and the milk cultures showed a definite inhibition against all of the test organisms; the amount of inhibition varied with the organism, with the pathogenic organisms being inhibited to the greatest degree. The disks with the buffered lactic acid revealed that pH can affect the growth of the organisms since the disks at pH 4.5 did show a slight amount of inhibition. However, this was much less inhibition than that shown by the cell suspensions or the milk cultures. The milk cultures of these organisms do not reach a pH of 4.5 for approximately 48 hours and organisms such as L. citrovorum never produce enough acid to reach a low pH.

Figure 3 shows the inhibition of S. typhosa by the cell suspension and the milk culture of S. diacetilactis but not by the concentrated supernatants using the disk assay technique. Adjusting the pH of the supernatant to pH 5.5 did not make any difference. (The dark area around the disk on the left side is growth of a contaminate; this picture was reproduced as a negative, consequently the normally white disks appear black, etc.)

Cross Streaking Experiments

Figures 4 through 8 show the results of five of the plates that were cross-streaked with S. diacetilactis 18-16 and the test organisms. Figures 4, 5, and 6 all show significant inhibition by

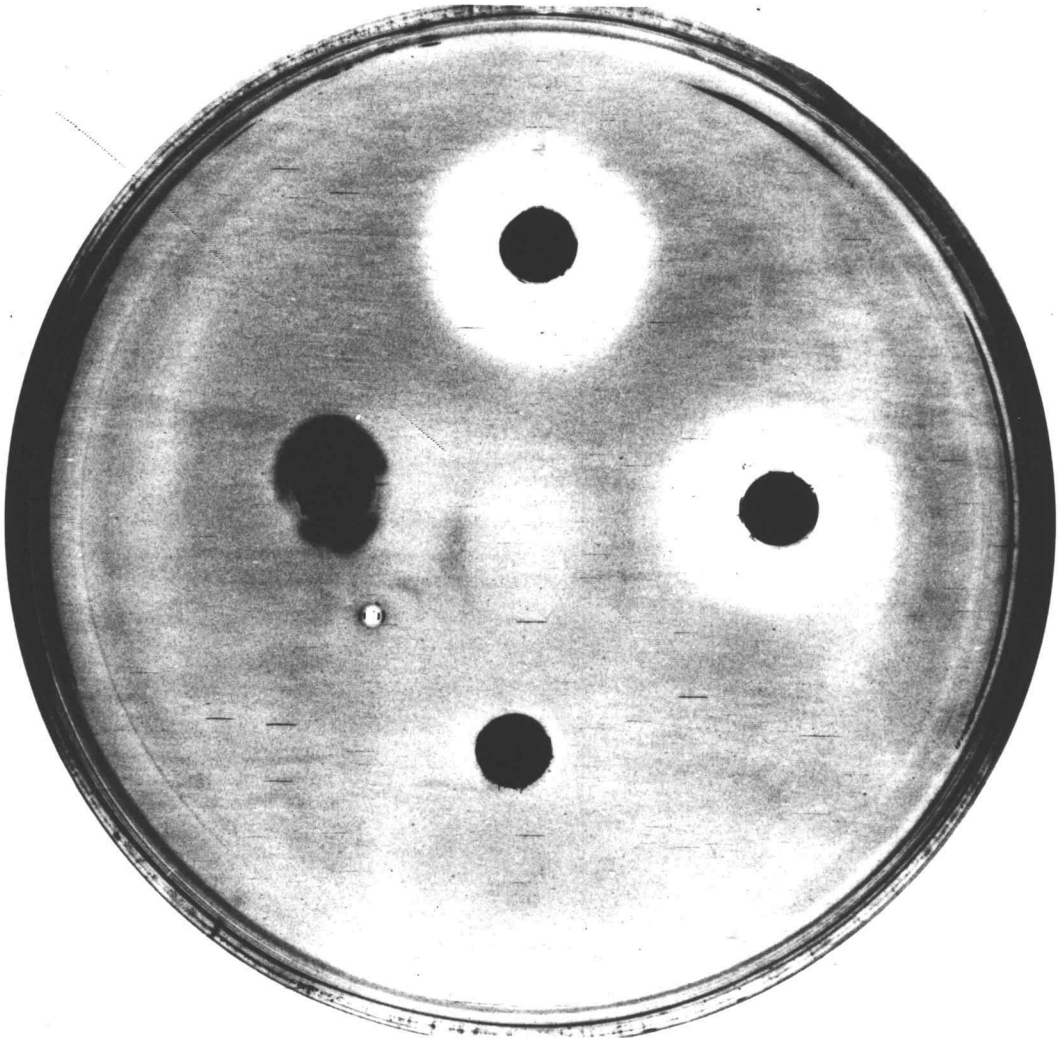

Figure 3. Disk assay method showing inhibition of S. typhosa (lawn) by viable cells of S. diacetilactis 18-16 but not by concentrated supernatant of culture of S. diacetilactis.

Clockwise from the top:

1. cell suspension of S. diacetilactis 18-16
2. milk culture of S. diacetilactis 18-16
3. concentrated supernatant of culture of S. diacetilactis 18-16 at pH 4.65
4. concentrated supernatant of culture of S. diacetilactis 18-16 at pH 5.50

This picture was reproduced as a negative, therefore the normally white disks are black; the zones of inhibition are white; the dark area around #4 disk is growth of a contaminate.

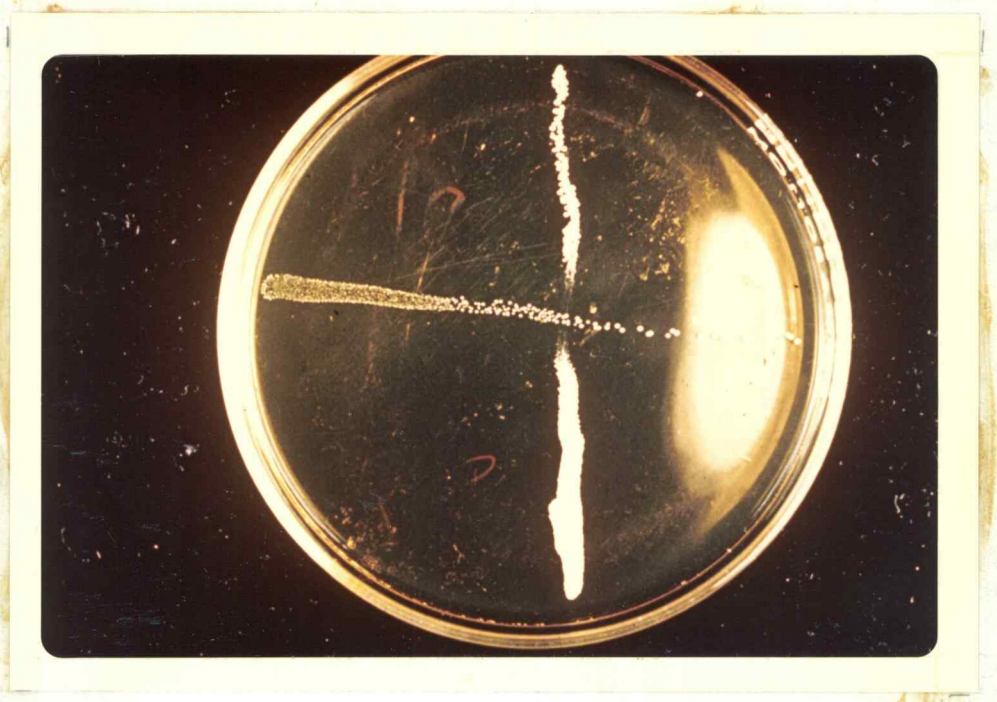

Figure 4. Evidence of inhibition of S. aureus (from bottom to top) as a result of cross streaking against S. diacetilactis 18-16 (from left to right) on TGY agar.

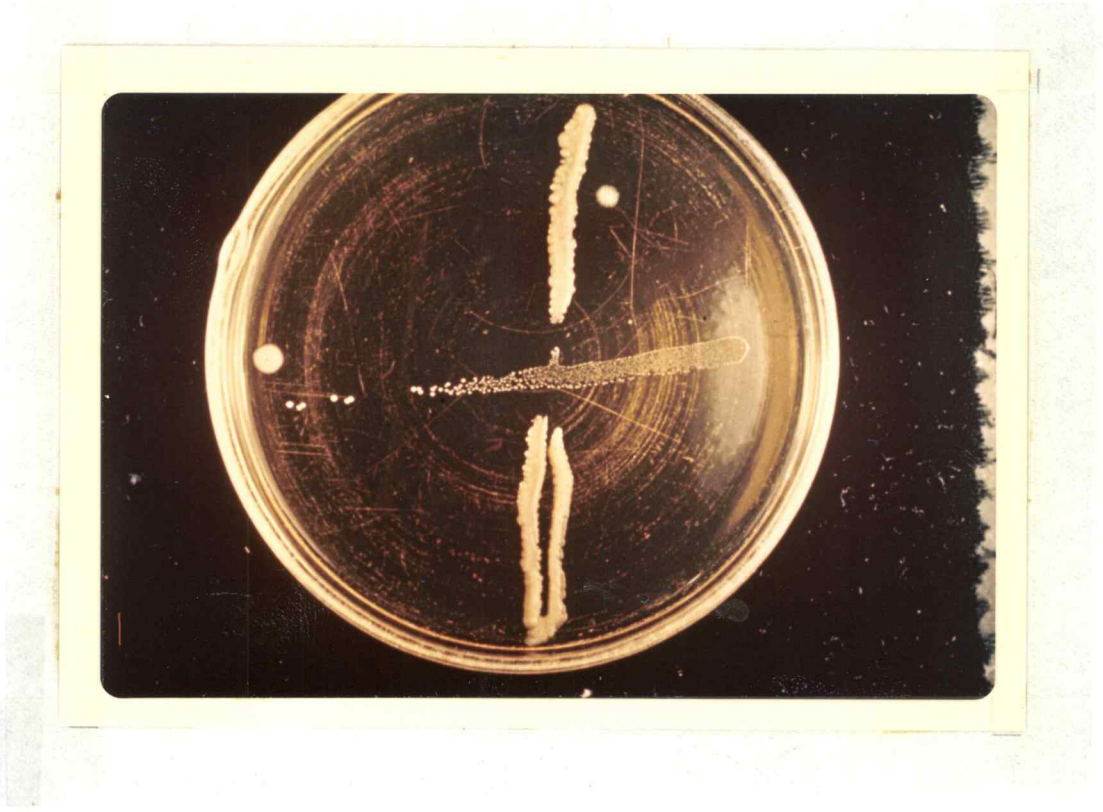

Figure 5. Evidence of inhibition of A. metalcaligenes (from bottom to top) as a result of cross streaking against S. diacetilactis 18-16 (from left to right) on TGY agar.

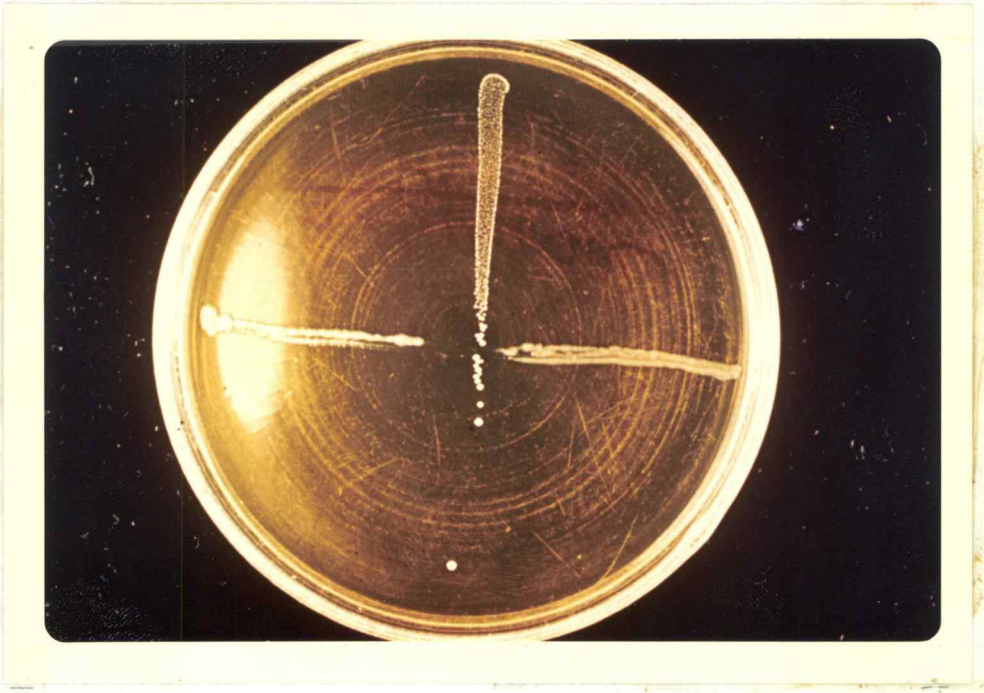

Figure 6. Evidence of inhibition of S. typhosa (from left to right) as a result of cross streaking against S. diacetilactis 18-16 (from top to bottom) on TGY agar.

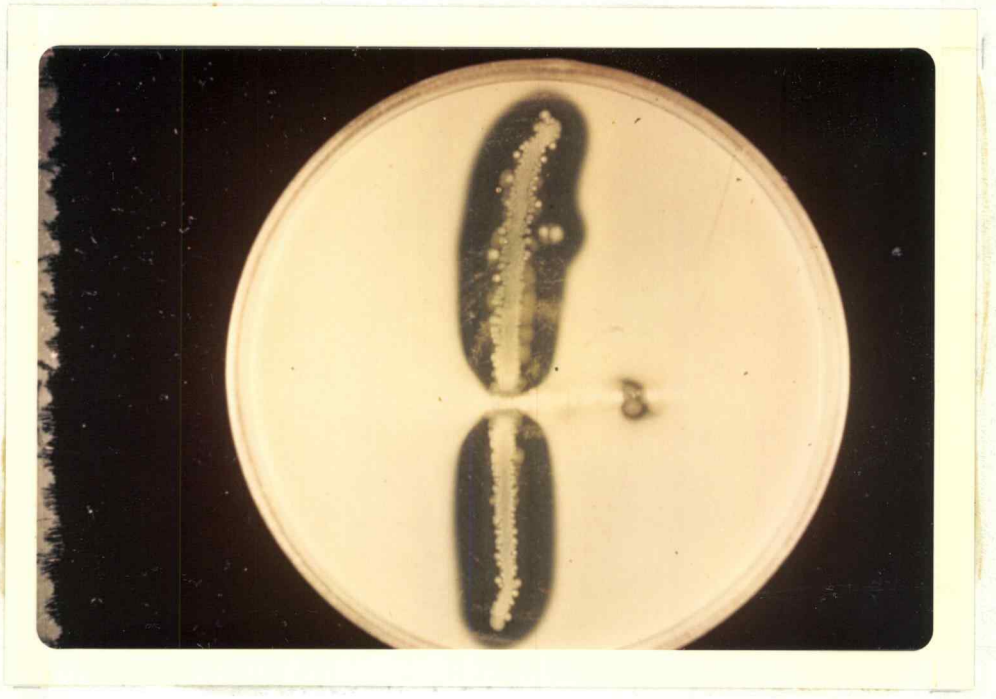

Figure 7. Evidence of inhibition of growth and proteolysis of casien by P. viscosa (from bottom to top) as a result of cross streaking against S. diacetilactis 18-16 (from left to right) on milk agar.

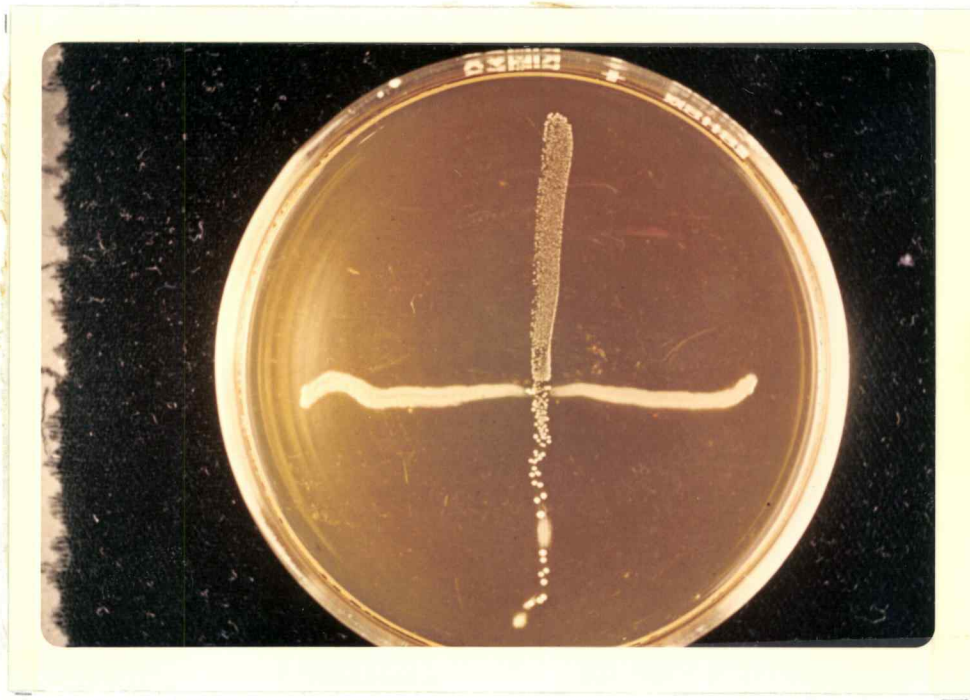

Figure 8. Evidence of slight inhibition of E. coli 198 (from left to right) as a result of cross streaking against S. diacetilactis 18-16 (from top to bottom) on TGY agar.

S. diacetilactis of S. aureus, A. metalcaligenes and S. typhosa. Figure 6 shows inhibition of proteolysis of casien by P. viscosa in the area where S. diacetilactis is growing. Figure 8 shows the cross-streak against E. coli; the amount of inhibition is not great in this case, however, the streak of E. coli is not continuous in the area where the S. diacetilactis is growing.

General Conclusions

The results of these experiments indicate that it is not an antibiotic or other inhibitory substance produced by S. diacetilactis that causes the inhibition of the spoilage organisms or the pathogens used in these studies. Neither is the production of lactic acid or the low pH produced by the lactic acid a contributing factor to the inhibition of these organisms. Since the surface of the agar plates are exposed to free oxygen, it also is unlikely that the test organisms could be inhibited at such a great distance from the disk (up to one centimeter) by a lowered oxidation-reduction potential of the medium.

It seems therefore that a reasonable explanation for the inhibitory effect observed is that the lactic organisms were capable of faster growth and therefore were able to out-compete the other organisms for essential nutrients. The exact nature of this competitive effect was not determined though it may be the same or similar to that reported by Iandolo et al. (26). In this case, it was shown

that a culture of S. aureus was inhibited by S. diacetilactis when these organisms were grown in association. Inhibition was primarily due to nutrient depletion. It was reported that the addition of nicotinamide would reverse the inhibition. Another contributing factor mentioned was that the degree of inhibition was dependent on the initial ratio of the two organisms (cells per milliliter); the higher the proportion of S. diacetilactis to S. aureus, the greater the inhibitory effect, although repression of the maximal possible numbers was noted regardless of this ratio. A third contributing factor was the pH of the medium; the biological availability of nicotinamide was found to be a function of the pH, being less available at pH 7.3 than at 5.5. It is presumed that S. diacetilactis was able to metabolize this compound at a rapid rate, leaving insufficient amounts for the growth of this particular strain of S. aureus, even if the pH is lowered by the acid that is produced by the S. diacetilactis. It is also suggested that amino acids or other vitamins, etc., might be the limiting factor with other organisms or strains that might be encountered under natural conditions.

PART II

COMPETITIVE GROWTH STUDIES

Experimental Methods

Introduction

After the disk assay experiments indicated that there apparently was no antibiotic substance produced by S. diacetilactis, it was decided to conduct experiments in which various spoilage bacteria (test organisms) would be grown in liquid media in association with the lactic streptococci to determine what effect competitive growth would have on the test organisms. The proportion of test organisms to S. diacetilactis was also an important factor to be considered.

Studies on Growth of S. diacetilactis 18-16

S. diacetilactis 18-16 was grown in active culture at 30° C. and then inoculated into sterile milk which had been cooled to the test temperature and held for at least six hours. The inoculation was at the rate of one percent into bottles containing 100 milliliters of milk. Methylene blue was also added to the milk at the rate of four parts per million (1, p. 107). A control bottle of milk containing

methylene blue but not the S. diacetilactis also was observed. Biological Oxygen Demand (BOD) incubators were used to maintain the low temperatures desired. These incubators will hold a constant temperature ± 0.5° C. down to about 3° C. and as high as 50° C. The test temperatures used were 6.5° C., 7.5° C., 8.5° C. and 10.0° C. Standard plate counts (1, p. 72-79) were made at the time of inoculation and again when the methylene blue showed signs of reduction. This was usually from four to six days at the lower temperatures and about two days at 10° C. The plates were incubated at 30° C.

Selective Plating Media

Since in these experiments two organisms were grown together in the same medium in different numbers, it was necessary to use selective plating media to determine the exact numbers of each organism. The first problem was to select a medium that would allow Pseudomonas species to grow but inhibit the streptococci; conversely a medium that would inhibit the pseudomonads but not the streptococci was sought. Since sodium azide is used in the isolation of fecal streptococci from milk (1, p. 169, 403), this was tried to determine if the streptococci would grow without inhibition while the pseudomonads would be completely inhibited. The recommended level of sodium azine is 200 parts per million. A standard plate

count was done on an active culture of P. fragi and S. diacetilactis 18-16 using TGY agar with sodium azide added at concentrations of 0, 5, 10, 25, 50, 100, and 200 parts per million. A spreading technique was used in order to get all surface colonies to facilitate identification of typical colonies. Plates were incubated at 21° C. for 48 hours. The agar plates were poured 24 hours before use and porous ceramic lids were used until the agar had cooled and hardened to assure a dry surface. The ceramic lids were replaced with regular sterile glass lids about one-half hour after the agar was poured.

Crystal violet has been used in selective media to inhibit Gram positive organism; it has been used at two parts per million (1, p. 420). A standard plate count was made of P. fragi and S. diacetilactis using TGY agar with and without two parts per million added crystal violet. A spreading technique was used; the plates were prepared as mentioned above. The plates were incubated at 21° C. for 48 hours and then counted to determine whether or not the crystal violet had an inhibitory effect on the P. fragi.

A third selective medium was needed to differentiate between S. aureus and S. diacetilactis. Since the staphylococci are resistant to sodium chloride in concentrations up to 10 percent, TGY agar was made up containing sodium chloride at concentrations of 0, 1, 3, 5, 7, and 10 percent. A standard plate count was made of active

cultures of S. aureus and S. diacetilactis using a spreading technique with each of the six different concentrations of salt. The plates were prepared as above. Incubation was at 25° C. for 48 hours. The size of the colonies and pigmentation also was used to differentiate between the two organisms.

Growth of S. diacetilactis 18-16 and P. fragi in milk at 7.5° C.

These experiments measured the effect of growing S. diacetilactis 18-16 in milk upon the subsequent growth of P. fragi. Powdered milk was made up to 11 percent solids in sterile water in a sterile container using aseptic techniques to prevent contamination. The milk (100 mls.) was put into sterile milk dilution bottles and pasteurized at 63° C. for 30 minutes. Six of the bottles of pasteurized milk were inoculated with an active culture of S. diacetilactis 18-16 at the rate of one percent and incubated at 21° C. for six hours. At the end of the six hours, three of these bottles were pasteurized again to kill the viable cells of S. diacetilactis. The three bottles with the heat-killed cells, the three with the viable cells and three uninoculated bottles were then cooled in ice water to about 5° C. One bottle of each of the three groups was inoculated with approximately 20,000 P. fragi cells per milliliter. A second bottle of each of the three groups was inoculated with approximately 2,000 cells per milliliter, while a third bottle from each group was

left uninoculated for a control. The bottles were then placed in a BOD incubator at 7.5° C. Standard plate counts were made at 0 hours, 24 hours, 48 hours, 96 hours and 7 days. A spreading technique was used; duplicate plates were made on TGY agar with two parts per million crystal violet, TGY agar plus 15 parts per million sodium azide, and plain TGY agar. All of the plates were incubated at 25° C. for 48 hours before counting.

Growth of S. diacetilactis 18-16 and S. aureus in T. S. broth at 30° C.

S. diacetilactis 18-16 and S. aureus were grown in pure culture in Trypticase-Soy (T. S.) broth at 30° C. for three successive transfers, 24 hours apart. The S. diacetilactis was inoculated into one liter of T. S. broth at 30° C. to give a concentration of approximately 2×10^6 cells per milliliter. S. aureus was inoculated into T. S. broth at 30° C. to give a concentration of approximately 2×10^5 cells per milliliter. Standard plate counts were made on both of these cultures at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, and 24 hours. A spreading technique was used. The S. diacetilactis was plated out on TGY agar while the S. aureus was plated out on both TGY agar and on TGY agar with seven percent sodium chloride added.

A third flask of T. S. broth was inoculated with S. diacetilactis at approximately 2×10^6 cells per milliliter and S. aureus at about

2×10^5 cells per milliliter. The flask was incubated at 30° C. and plate counts were made at 0, 2, 4, 6, 9, 12, 24, and 48 hours. A spreading technique was used on plates of TGY agar and TGY agar with seven percent added sodium chloride. All of the plates were incubated at 30° C. for 24 hours, 10° C. for 12 hours, and 25° C. for 24 hours. The incubation at 30° C. was to enhance initial colony growth while incubation at 10° C. and 25° C. was intended to enhance pigmentation of the S. aureus.

Effect of pH on S. diacetilactis and S. aureus in Milk at 30° C.

S. diacetilactis 18-16, which is a slow acid-producer and takes about 48 hours to coagulate milk at 30° C., and S. diacetilactis 4R5, which is a fast acid-producing strain, were grown in milk with S. aureus at 30° C. according to the technique of Reiter et al. (48). Four different lots (A, B, C, and D) of milk of one liter each were Tyndallized; after they had been cooled to 30° C., two of the batches (B and C) were inoculated with active cultures of S. aureus at the rate of about 3×10^4 cells per milliliter. The other two were inoculated with both S. diacetilactis and S. aureus; the former at one percent of an active culture and the later at about 3×10^4 cells per milliliter.

Cultures "A" and "D" were inoculated with both organisms, while cultures "B" and "C" were inoculated with only S. aureus. Cultures "A" and "C" were allowed to develop the normal amount of

acid; the pH was not adjusted in any way. Culture "B" which contained only the S. aureus, was adjusted with sterile 10 percent lactic acid to the same pH as "A." Culture "D" was adjusted with sterile one normal sodium hydroxide to the same pH as "C." This was designed to show whether or not the acid produced by the culture of viable S. diacetilactis cells was a factor in the inhibition of S. aureus by S. diacetilactis.

A plate count was done with a spreading technique using TGY agar and TGY agar plus seven percent sodium chloride. The counts were made at 0, 3, 6, 9, 12, and 24 hours. Plates were incubated at 30° C. for 24 hours, then at 10° C. for 12 hours and then 24 hours at 25° C.

The experiment was repeated using S. diacetilactis 4R5 in place of S. diacetilactis 18-16; this strain is a fast acid producer and is able to coagulate milk in less than 24 hours at 30° C. Use of this strain allowed a better evaluation of the effect of a low pH as produced by S. diacetilactis on the growth of S. aureus.

Effect of pH on S. diacetilactis and P. fragi in Milk at 21° C.

The technique of Reiter et al. (48) was used in this experiment also, except that P. fragi was used as the test organism and the cultures were incubated at 21° C. Standard plate counts were made using TGY agar and TGY agar plus two parts per million crystal

violet. The plates were incubated at 25° C. for 48 hours. The P. fragi was added to cultures "A" and "D" at the rate of about 2×10^3 cells per milliliter final concentration.

Results and Discussion

Studies on Growth of S. diacetilactis 18-16

Table 7 shows the results of the experiments that were designed to determine the lowest temperature at which S. diacetilactis 18-16 was capable of cell multiplication and metabolism. The metabolism of the cell was determined by the reduction of methylene blue in the milk. These tests indicated that S. diacetilactis was capable of cell multiplication at 7.5° C. in four to six days. One test showed that there was some reduction in numbers in four days at 7.5° C., however, the other two tests showed some multiplication (approximately three times in one case and about 12 times in the other case). The plate counts showed that there was some multiplication at 6.5° C. in six days but not in four days. Furthermore, these cells were metabolicly active at 6.5° C. since they reduced the methylene blue at this temperature. This experiment also indicated that the cells were not capable of multiplication or metabolism at 5.0° C. in six days. It is possible, however, that they might be able to multiply or reduce the methylene blue at this

Table 7. Temperature Studies on the Growth of S. diacetilactis 18-16

Temperature	plate count at 0 days	plate count at 4 days	plate count at 6 days	reduction of[(a)] methylene blue
6.5° C.	58×10^5	50×10^5	---	10% in 4 days
7.5° C.	49×10^5	177×10^5	---	50% in 4 days
5.0° C.	47×10^5	19×10^5	---	0% in 6 days
7.5° C.	48×10^5	25×10^5	---	90% in 6 days
10.0° C.	52×10^5	8×10^8	---	100% in 2 days
6.5° C.	210×10^5	---	800×10^5	50% in 6 days
7.5° C.	100×10^5	---	1200×10^5	90% in 6 days

[a]The reduction of methylene blue is used as an indication of active metabolism; the reduction usually proceeded from the bottom irregularly thru the rest of the milk. The extent (percentages) of the reduction is a relative indication of the rate of metabolism.

temperature if given sufficient time. At 10° C. the cells demonstrated active cell growth and metabolism since they were able to reduce the methylene blue completely in two days and reach almost the maximum numbers in four days. This indicated that 6.5° C. was the lowest temperature at which S. diacetilactis 18-16 was able to metabolize or multiply in six days.

Selective Plating Media

The results shown in Table 8 are the actual plate counts of different cultures of P. fragi, S. aureus, and S. diacetilactis that were plated and grown out on the different selective media. In the first set of data, P. fragi gave very similar counts whether plated on the TGY agar with two parts per million crystal violet or on the plain TGY agar. On the TGY agar with 15 parts per million sodium azide, none of the cells were able to grow and form colonies, even though 7×10^{3} cells were spread on the plate. The S. diacetilactis was not inhibited by the presence of the 15 parts per million of sodium azide, but was completely inhibited by two parts per million of crystal violet, even though 3×10^{6} cells were spread on the plate originally.

No satisfactory method was found to inhibit the staphylococci while letting the streptococci grow. However, such a method was not needed since the streptococci were present in such high numbers

Table 8. Plate Counts Showing Effectiveness of Selective Media

Culture	TGY agar + 2ppm crystal violet	plain TGY agar	TGY agar + 15ppm sodium azide
A	75×10^3	74×10^3	0×10^1
P. fragi	68×10^3	86×10^3	0×10^1
B	92×10^2	94×10^2	0×10^1
P. fragi	81×10^2	96×10^2	0×10^1
C	0×10^1	28×10^7	46×10^7
S. diacetilactis	0×10^1	46×10^7	36×10^7
D	0×10^1	37×10^7	51×10^7
S. diacetilactis	0×10^1	50×10^7	39×10^7
E	64×10^3	41×10^3	0×10^1
P. fragi	50×10^3	51×10^3	0×10^1
F	55×10^2	37×10^2	0×10^1
P. fragi	46×10^2	50×10^2	0×10^1

Culture	TGY agar + 7% sodium chloride	plain TGY agar	TGY agar + 5% blood
S. aureus	51×10^4	41×10^4	56×10^4
	57×10^4	57×10^4	73×10^4
S. aureus	41×10^7	49×10^7	53×10^7
	68×10^7	57×10^7	55×10^7
S. diacetilactis 18-16	0×10^1	48×10^5	46×10^5
	0×10^1	55×10^5	48×10^5
S. diacetilactis 18-16	0×10^1	46×10^8	33×10^8
	0×10^1	30×10^8	35×10^8

that when the culture was diluted out for an accurate count, the staphylococci were not present in high enough numbers to be a problem. The S. diacetilactis was completely inhibited by the seven percent sodium chloride in the TGY agar even when 3×10^7 cells were spread on the plate. This high concentration of salt was not inhibitory to the staphylococci however. The TGY agar with five percent added blood was used as a further check on the growth-supporting adequacy of plain TGY agar; similar counts were obtained, showing that the addition of such materials was not necessary to achieve the maximum plate count.

The streptococci were able to grow normally on TGY agar with three percent added sodium chloride but were partially inhibited by five percent salt. The addition of seven percent sodium chloride was used since it gave complete inhibition of the streptococci without inhibiting the staphylococci.

Growth of S. diacetilactis 18-16 and P. fragi in Milk at 7.5° C.

Table 9 shows the results of a typical experiment where P. fragi was grown in milk in which S. diacetilactis 18-16 had been grown for six hours at 21° C. In cultures A-1 and A-2, the six hour culture of S. diacetilactis was pasteurized at 63° C. for 30 minutes to kill all of the streptococci. Since the heat-killed cells were still present but not able to compete with P. fragi for further

Table 9. Growth of P. fragi in Presence of Viable or Heat-Killed Cells of S. diacetilactis in Milk at 7.5°C.

No.	Culture Medium	Plate Count of P. fragi at: 0 day	1 day	2 days	4 days	7 days
A-1	Pasteurized milk culture of S. diacetilactis	2.5×10^4	8.0×10^4	3.0×10^5	3.0×10^7	1.2×10^8
A-2	Pasteurized milk culture of S. diacetilactis	2.5×10^3	9.5×10^3	2.5×10^5	3.0×10^7	2.0×10^8
B-1	Viable milk culture of S. diacetilactis	2.5×10^4	4.5×10^4	1.5×10^5	7.0×10^5	2.0×10^7
B-2	Viable milk culture of S. diacetilactis	2.5×10^3	6.0×10^3	1.2×10^4	1.0×10^5	3.0×10^6
C-1	Pasteurized milk	2.5×10^4	5.5×10^4	---	5.0×10^7	2.0×10^8
C-2	Pasteurized milk	2.5×10^3	5.0×10^3	---	2.0×10^7	1.5×10^8

nutrients in the milk, normal growth of the P. fragi would indicate that there was no heat stable inhibitory substance produced by the S. diacetilactis and there was no depletion of essential nutrients. Comparison of the counts of B-1 and B-2 show that there was no significant difference in the growth rates when a 10-fold difference in numbers existed at zero time. It was found that use of equal numbers of P. fragi and S. diacetilactis resulted in little or no inhibition of the pseudomonads by S. diacetilactis since the former grew rapidly at 21° C. Data from cultures A-1, A-2 and C-1, C-2 show that there was no difference in the growth of the P. fragi when the milk had been pasteurized to kill the streptococci.

When the percent inhibition is defined as equal to 100 - (b/a x 100) where "a" equals the normal plate count and "b" equals the inhibited count, the percent inhibition in this experiment was between 84 and 99.7 percent. When figures after four days, the percent inhibition calculated between A-1 and B-1 was 97.7 percent; between A-2 and B-2 it was 99.7 percent. When figured after seven days, between A-1 and B-1 it was 84 percent; between A-2 and B-2, 98.5 percent. The reason for the decrease is that cultures A-1 and A-2 had reached their maximum numbers while cultures B-1 and B-2 were still growing. Since there was no leveling off, it was assumed that the P. fragi would reach the same maximum numbers if given sufficient time. This experiment does show that the

presence of the high numbers of S. diacetilactis cells retards the rate of growth of the P. fragi even though the spoilage organism has a considerable temperature advantage. This is even more remarkable when previous experiments revealed that S. diacetilactis was able to metabolize and grow only very slowly at 7.5° C. The counts of the streptococci on the azide agar indicated that the S. diacetilactis 18-16 had reached a population of 5×10^8 cells per milliliter after the six hours of incubation at 21° C. and they did not increase during the seven day holding period. In fact, there was a slight decrease to about 3×10^8 cells per milliliter. This showed that the inhibition was not the result of a product of cellular growth but apparently was due to active metabolism.

Growth of S. diacetilactis 18-16 and S. aureus in T. S. Broth at 30° C.

Table 10 shows the results of the experiment in which S. diacetilactis 18-16 and S. aureus were grown in Trypticase-Soy broth both alone and together to determine if there would be inhibition of the staphylococci by the streptococci. The two organism initially were grown in pure culture to establish their rate of growth in the Trypticase-Soy broth. Then they were grown together at approximately the same starting numbers to check their growth rates and establish whether or not inhibition of the staphylococci

Table 10. Growth of S. diacetilactis 18-16 and S. aureus in Trypticase-Soy broth at 30° C.

Time (hours)	S. diacetilactis		S. aureus	
	alone	with S. aureus	alone	with S. diacetilactis
0	1.95×10^6	1.57×10^6	5.6×10^5	1.8×10^5
2	1.33×10^7	3.8×10^6	2.3×10^6	4.0×10^5
4	1.88×10^8	8.2×10^7	1.1×10^7	1.2×10^6
6	1.25×10^9	4.0×10^8	4.1×10^7	9.3×10^6
10	2.4×10^9	1.2×10^9	1.9×10^8	2.3×10^7
24	2.6×10^9	1.5×10^9	5.4×10^8	6.6×10^7

would take place.

The plate counts revealed that there was inhibition of the S. aureus even at the high starting numbers used. The percent inhibition was 87.8 percent when figured between S. aureus grown alone and in conjunction with S. diacetilactis. There was also a slight decrease in the maximum numbers that the streptococci reached. This would indicate that the staphylococci were competing somewhat successfully with the streptococci. The temperature of this experiment (30° C.) was significant in that the only time in the manufacture of Cottage cheese where the temperature would be this high would be in the short set method. In this case, a holding temperature of 29 to 32° C. is used, and during the cooking a temperature of 43 to 54° is reached. George et al. (20) have shown that S. aureus is capable of growth at 45 to 47° C. but not at 48° C. In fact there is rapid death of the culture at 48° C. Growth of these bacteria at 21° C is slower and this temperature would give a much greater advantage to the streptococci than they have at 30° C. A comparison of the degree of inhibition in this experiment and the next one (Table 11) shows quite clearly that the lower levels of S. aureus were inhibited much more readily than the high levels. The use of milk instead of trypticase broth could also make a difference, however, the growth rates of S. diacetilactis in Trypticase-Soy broth and in milk were very much the same, and the maximum numbers reached

Table 11. Effect of pH on Growth of S. aureus in Presence of S. diacetilactis 4R5 in Milk at 30° C.

Time (hours)	A		B		C		D	
	S. aureus & S. diacetilactis		S. aureus		S. aureus		S. aureus & S. diacetilactis	
	pH	count[a]	pH[b]	count	pH	count	pH[c]	count
0	6.40	6.3	6.40	6.5	6.40	7.1	6.40	5.6
3	6.30	13	6.30	27	6.35	21	6.35	16
6	6.00	34	6.00	555	6.37	905	6.37	27
9	5.75	60	5.75	34,500	6.30	30,000	6.30	52
12	5.55	55	5.55	48,000	6.30	64,000	6.30	66
24	4.75	84	4.75	137,000	6.20	273,000	6.20	54

[a] Plate count of S. aureus only; all counts x 10^3.

[b] pH lowered with sterile lactic acid to equal pH of culture "A" which contains a fast acid producing strain of S. diacetilactis.

[c] Acid produced by S. diacetilactis 4R5 was neutralized with sterile NaOH to give a pH equal to the low-acid producing culture of S. aureus, "C."

were also the same.

Effect of pH on S. diacetilactis and S. aureus in Milk at 30° C.

Table 11 shows the results of the controlled pH experiment where the culture of S. aureus was acidified with 10 percent lactic acid to the same level as a mixed culture of S. aureus and S. diacetilactis. The strain of S. diacetilactis used was 4R5, a fast acid producer which will produce sufficient acid to coagulate milk within 24 hours; the pH of the milk after incubation was 4.75. It might be expected that this low pH would inhibit the growth of the pure culture of S. aureus kept at the same pH as the culture of S. diacetilactis. The plate counts on these cultures (B & C), however, were essentially the same; the difference, 1.37×10^8 and 2.73×10^8, was not significant and did not indicate inhibition since this small difference between them could be attributed to plating error. The difference between "A" and "B" was very significant and showed a percent inhibition of 99.94 percent. The difference between "C" and "D" revealed an even greater difference with a percent inhibition of 99.98 percent. This experiment therefore provided convincing evidence that the pH of the culture did not have any bearing on the inhibition caused by the active culture of S. diacetilactis.

This same experiment was repeated using a culture of S. diacetilactis 18-16, a low acid producer which required up to 48

hours to coagulate milk at 30° C. The lowest pH reached by the mixed culture "A" in this experiment was 5.50 and the inhibition was 99.0 percent. In this case the starting numbers of S. aureus were about five times higher than in the preceding experiment using S. diacetilactis 4R5. The difference in starting numbers could very well account for the higher numbers that were achieved in this experiment, and subsequently the lower percent inhibition. The inhibition however was still very much in evidence.

Effect of pH on S. diacetilactis 18-16 and P. fragi in Milk at 21° C.

This experiment was similar to the last one except the organism used was P. fragi and consequently the test temperature was lowered to 21° C. An interesting aspect of this experiment was that the P. fragi, even though it was an active culture that had been grown in milk at 21° C., showed a lag period of over nine hours when grown alone. This lag may also have occurred in the mixed culture but it is difficult to say with certainty because of the inhibition of the culture. The milk used in the experiment was not autoclaved however and had only been steamed (to 100° C.) and the difference in the heat treatment could cause this lag. Why the lag should be so long is not clearly understood.

The results of this experiment, shown in Table 12, are very

Table 12. Effect of pH on Growth of P. fragi in Presence of S. diacetilactis 18-16 in Milk at 21° C.

Time (hours)	A P. fragi & S. diacetilactis pH	A Count[a]	B P. fragi pH[b]	B Count	C P. fragi pH	C Count	D P. fragi & S. diacetilactis pH[c]	D Count
0	6.50	2.5	6.50	3.1	6.50	2.9	6.50	4.6
3	6.50	6.1	6.50	1.9	6.50	1.1	6.50	6.9
6	6.40	22	6.40	1.0	6.55	1.0	6.55	14
9	6.30	33	6.30	2.5	6.55	2.5	6.55	17
12	6.10	12	6.10	100	6.55	150	6.55	12
24	5.70	7.0	5.70	15,000	6.50	30,000	6.50	45

[a] Plate count of P. fragi only: all counts x 10^3.

[b] pH lowered with sterile lactic acid to equal pH of culture "A" which contains a slow acid producing strain of S. diacetilactis.

[c] Acid produced by S. diacetilactis 18-16 was neutralized with sterile NaOH to give a pH equal to the non-acid producing culture of P. fragi, "C."

similar to the experiment with S. aureus under the same conditions. The percent inhibition was 99.95 between "A" and "B" and 99.85 percent between "C" and "D." As can be seen by the data, there was an actual drop in numbers in the count of "A" from a high of 33×10^3 to 7×10^3.

General Conclusions

The main conclusion that can be reached from these experiments is that the acid produced by the streptococci and subsequently the lowering of the pH of the medium is not a factor in the inhibition of the test organisms. The inhibition takes place to the same extent when the culture is allowed to produce enough acid to lower the pH below 5.0 as when the acid is neutralized as it is produced, and the pH is kept at 7.0.

The experiment with the P. fragi and S. diacetilactis held at 7.5° C. suggests that cell multiplication is not necessary for the S. diacetilactis to exert its inhibitory effect, but the fact that the cells are metabolizing is sufficient. The presence of the heat-killed cells of S. diacetilactis does not cause any inhibition on the growth of P. fragi either, suggesting that some factor in the active metabolism of the cell is the causitive agent.

It is possible that some enzyme produced by the streptococci could result in this inhibition, but that the enzyme is so unstable that

it constantly is being inactivated, possibly by the streptococci themselves. This would explain the necessity for the continued active metabolism of the streptococci cells to produce the inhibitory effect.

PART III

INHIBITION OF SPOILAGE ORGANISMS IN CONTAMINATED COTTAGE CHEESE BY USE OF CREAM CULTURED WITH STREPTOCOCCUS DIACETILACTIS 18-16

Introduction

These experiments were undertaken to show the possible practical application of the inhibitory action of viable cells of S. diacetilactis 18-16. The first phase of this part of the work was done to determine whether or not the numbers of contaminating organisms increase, remain static, or decrease in Cottage cheese under different storage conditions. The second phase of the work was to demonstrate the effect that culturing Cottage cheese cream with S. diacetilactis on the odor and appearance of grossly contaminated product.

Experimental Methods

Inhibition of P. fragi in Cottage Cheese With Cultured Dressing

An active culture of P. fragi was grown in tryptone-glucose-yeast-extract (TGY) broth at 21° C. for 24 hours. A direct count was made of the 24 hour culture to determine the approximate numbers of organisms present; a Petroff-Hausser counting chamber was used

to make the direct count. The culture was diluted out in sterile buffered water and added to commercially prepared, dry curd Cottage cheese to give a final concentration in the creamed cheese (200 grams of dry curd and 100 milliliters of 12 percent cream) of 1×10^2 cells per gram, 1×10^4 cells per gram, 1×10^6 cells per gram or 1×10^8 cells per gram. Duplicate sets of each concentration of P. fragi were made; one set was creamed with pasteurized cream (12 percent butterfat) and the other set was creamed with pasteurized cream that had been cultured with S. diacetilactis 18-16 at 21° C. for six hours. The creamed Cottage cheese was stored in plastic cartons at 7° C. (45° F.).

At five-day intervals, beginning with the first day, the cheese was sampled and a differential count was made to determine the numbers of P. fragi and lactic organisms present. To accomplish this, each carton of Cottage cheese was thoroughly mixed and an 11-gram sample was then placed in a sterile mortar. Three grams of sterile sodium citrate were added and mixed, using a sterile pestile, into the cheese curd in order to emulsify the curd and achieve a uniform mixture with the dilution water. Ninety nine milliliters of sterile water was then mixed into the emulsified curd and the whole mixture poured back into the dilution bottle. This constituted a one to ten dilution; standard diluting procedure was used to obtain the other necessary dilutions.

Each sample was plated out on lactic agar to get a total count of the numbers of lactic organisms; lactic agar with two parts per million of crystal violet added was used to count P. fragi; TGY agar with two parts per million crystal violet added was used as another measure of the numbers of P. fragi present. The odor and appearance of each of the samples was also noted.

Inhibition of Spoilage Organisms in Cottage Cheese by S. diacetilactis

In this experiment the following organisms were used: P. fragi, P. putrifaciens, P. fluorescens, P. viscosa, A. metalcaligenes, C. lividum, A. aerogenes, and P. vulgaris. An active culture of each organism was grown in milk for 24 hours at 21° C. Two milliliters of a culture were added to 75 milliliters of sterile buffered water. This was then mixed well into 300 grams of dry Cottage cheese curd. The mixture was allowed to soak for 10 minutes then the excess liquid was drained off. Then 100 milliliters of pasteurized 12 percent cream was added to the contaminated curd and mixed in well. The creamed Cottage cheese was divided into two equal parts and put into clean plastic cartons. One of the cartons was held at 21° C. and the other at 10° C. A duplicate set for each organism was made using the same method except that the cream was inoculated with S. diacetilactis 18-16 at the rate of one percent and incubated at

21° C. for six hours before being added to the contaminated curd. Control sets also were made in which no spoilage organisms were intentionally added; one carton of plain curd with pasteurized cream held at 21° C. and one at 10° C.; one carton of plain curd with cultured cream held at 21° C. and one at 10° C. A total of 36 cartons were prepared; four cartons contaminated with each of the eight organisms and four cartons, uncontaminated, serving as controls. All of the cartons were examined every two days for twelve days. Color pictures were taken to show the changes that occurred and the odor and appearance of each carton was also noted. The set that was held at 21° C. spoiled within six days and so data on these samples was not collected thereafter.

Results and Discussion

Preliminary Experiment

A preliminary experiment was carried out to measure the enhanced keeping quality effect of the cultured dressing when Cottage cheese was stored for 30 days at 7.5° C. Figure 9 shows clearly the inhibition of *P. putrefaciens* which occurred as a result of using cream containing live cells of *S. diacetilactis*.

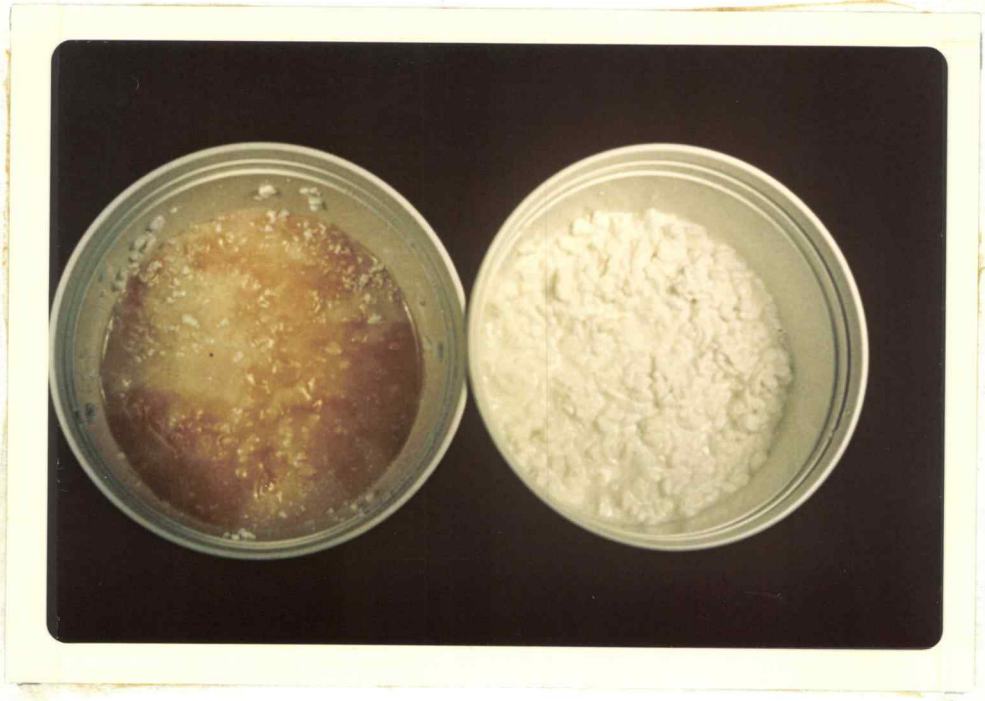

Figure 9. Preliminary experiment showing effect of cultured dressing on growth of P. putrifaciens after 30 days storage at 7.5° C.

LEFT: Creamed with plain cream
RIGHT: Creamed with cultured dressing

Inhibition of P. fragi in Cottage Cheese with Cultured Dressing

The next part of this phase of the work was carried out to determine the growth curve of P. fragi in competition with S. diacetilactis 18-16 using Cottage cheese as the growth medium. It was also necessary to determine whether or not the starting concentration of the spoilage organisms would affect the growth curve. The results of this experiment were indecisive in that counts on some duplicate samples taken from the same carton did not agree within one log number. The initial counts of the numbers of P. fragi also did not come close to the numbers that were added to the carton, indicating that many were killed off either by the diluting procedure or by some factor in the Cottage cheese. However, the counts in the Cottage cheese with the cultured cream were always lower than counts on cheese creamed with the plain pasteurized cream. This indicated that the competition was too great for many of the P. fragi cells and they died off, possibly because of lack of essential nutrients, or by some extremely labile, inhibitory substance secreted by the S. diacetilactis.

When it was seen that experiments with the cheese were not going to provide definitive data, similar studies using sterile milk as the growth medium were conducted. The procedures and results can be seen in Part II under the Section "Growth of S. diacetilactis

18-16 and P. fragi in milk at 7.5° C.," pages 52-53, 60-63.

Inhibition of Spoilage Organisms in Cottage Cheese by S. diacetilactis

The third part of this experimental phase concerned with the quality of Cottage cheese that had been grossly contaminated with different spoilage organisms. The purpose was to determine whether or not the cultured cream dressing used to cream half of the samples would prevent or retard the growth of the spoilage organisms. If so, this would prevent the product from deteriorating as rapidly as control samples creamed with only uncultured, pasteurized cream.

Table 13 shows the results of this experiment in tabular form. The odor of the product was rated +++, which indicated a desirable product with the odor of diacetyl present; ±, which indicated that the product lacked diacetyl aroma but did not possess any off flavors; or ---, which indicated that the product was spoiled and had a putrid odor. A rating of - or -- indicated progressive deterioration and the presence of strong, undesirable odors. A rating of ++ or + indicated relatively lesser amounts of diacetyl aroma than +++ samples.

The column indicating the appearance of the cheese is self explanatory. Some effort was made to note the degree of change in appearance by using "very slimy" and "very moldy" to show that the

Table 13. Effect of Cultured and Uncultured Dressing on the Odor and Appearance of Cottage Cheese Contaminated with Various Spoilage Organisms.

Organism	Cultured cream	2 days @ 21° C. odor[a]	2 days @ 21° C. appear.[b]	6 days @ 21° C. odor	6 days @ 21° C. appear.	4 days @ 10° C. odor	4 days @ 10° C. appear.	12 days @ 10° C. odor	12 days @ 10° C. appear.
P. putrifaciens	+	+++	NC	++	NC	+++	NC	++	M
	-	+	M	--	VS;M	+	NC	--	VS;M
P. fluorescens	+	+++	NC	++	NC	+++	NC	+	M
	-	-	S;M	---	VS;M	---	VS	---	VS
P. viscosa	+	+++	NC	+	M	+++	NC	+	NC
	-	±	M	---	VS;VM	-	NC	--	VS
P. fragi	+	+++	NC	-	M	+++	NC	--	S;M
	-	--	S	---	VS;M	---	VS	---	VS;M
A. metalcaligenes	+	+++	NC	++	NC	+++	NC	++	NC
	-	±	S	--	VS	-	S	--	S;M
C. lividum	+	+++	NC	++	NC	+++	NC	+	M
	-	±	S	--	S;M	±	NC	--	S;M
A. aerogenes	+	+++	NC	++	NC	+++	NC	+	NC
	-	±	S	--	S	±	NC	-	VS;M
P. vulgaris	+	+++	NC	++	NC	+++	NC	±	S;M
	-	+	NC	--	S;M	+	NC	--	S;M
Control	+	+++	NC	+	M	+++	NC	±	M
	-	+	NC	--	S;M	++	NC	--	VM

[a] Odor: +++ (very good; diacetyl present) to (not objectionable) to --- (rotten or putrid)
[b] Appearance: NC = No Change; S = Slimy; M = Moldy; VS = Very Slimy; VM = Very Moldy.

product was completely spoiled.

Figures 10 thru 20 are photographs showing the end results of these tests. Figure 10 shows the two control cartons (one with cultured cream and one with pasteurized cream) and two of the cartons that were contaminated with C. lividum (one with cultured cream and one with pasteurized cream) at the beginning of the experiment. There is virtually no difference between the four samples at this point.

Figures 11 thru 15 show the cartons that were stored at 21° C. for six days. The two cartons at the top, in each case, are the ones that were creamed with the cultured cream, and the two at the bottom were creamed with pasteurized, non-cultured cream. The samples that were creamed with the cultured cream and held at 21° C. had a tendency to show cream separation and coagulation due to the continued activity of the S. diacetilactis culture. In some cases (Figure 12), the two samples that were contaminated with A. aerogenes appeared to be quite similar, but in the bottom carton the cheese is covered with slime while the cheese in the top carton shows very little change from when it was made. In Figure 14, the upper left carton, which was the one contaminated with P. viscosa and creamed with the cultured dressing, shows some mold spots, but it still looks pretty good when compared to its companion which was creamed with the plain pasteurized cream (bottom left). Figure 15

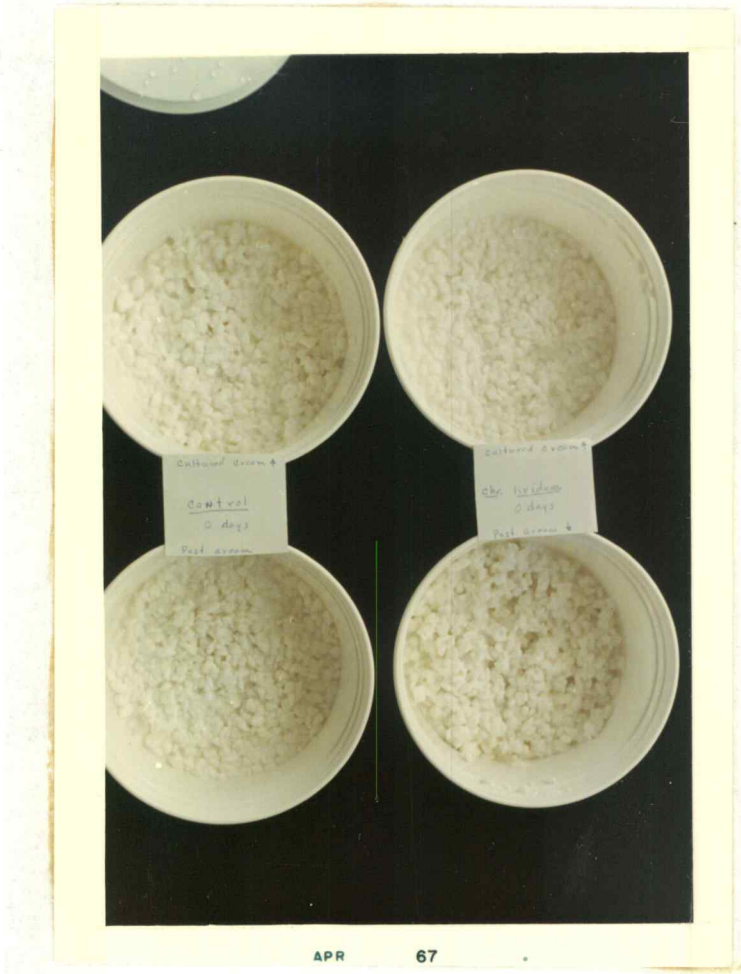

Figure 10. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Beginning of experiment
LEFT: Control (no organisms added)
RIGHT: Contaminated with C. lividum
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

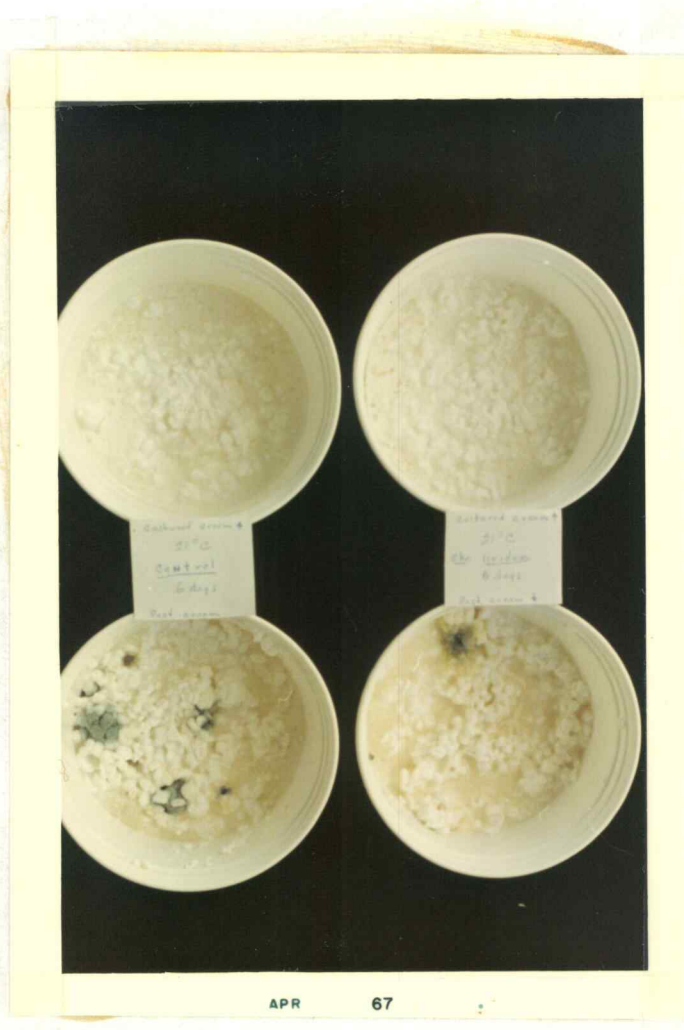

Figure 11. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Six days at 21° C.
LEFT: Control
RIGHT: Contaminated with C. lividum
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

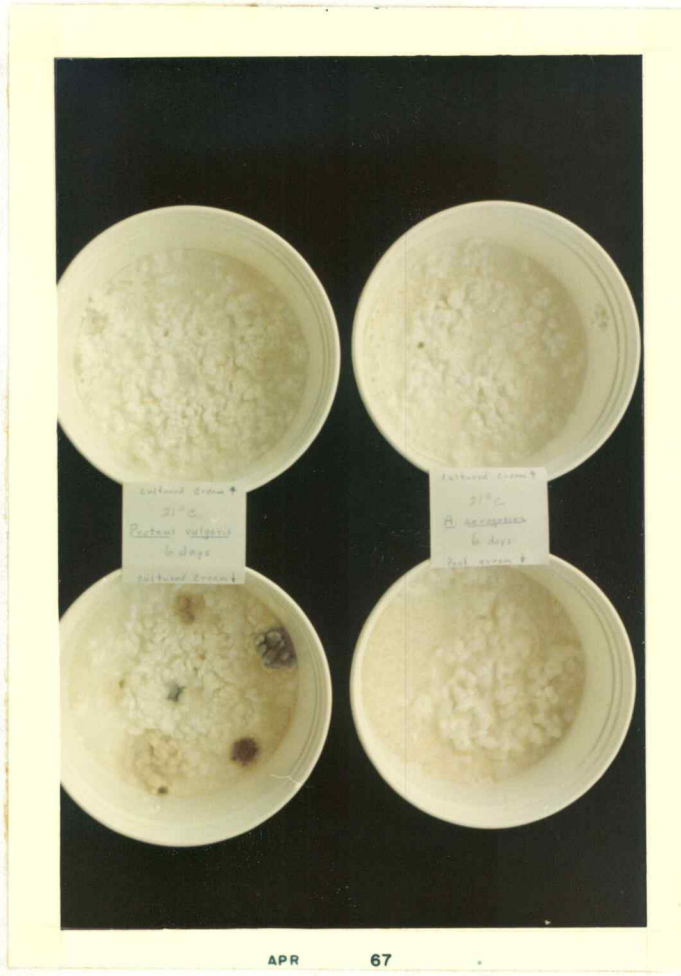

Figure 12. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Six days at 21° C.
LEFT: Contaminated with P. vulgaris
RIGHT: Contaminated with A. aerogenes
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

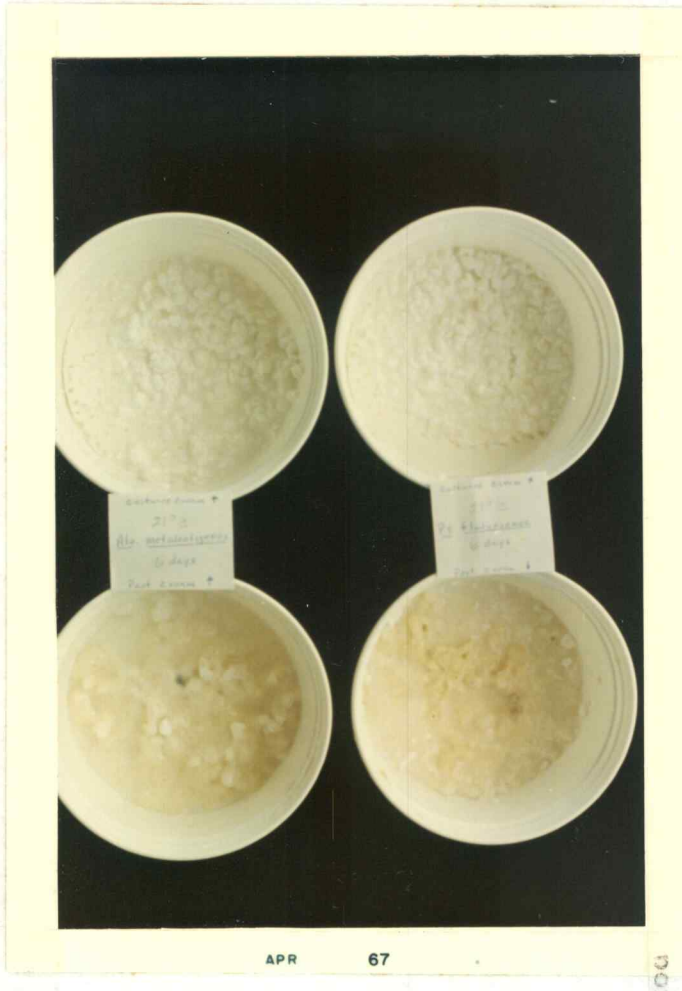

Figure 13. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Six days at 21° C.
LEFT: Contaminated with A. metalcaligenes
RIGHT: Contaminated with P. fluorescens
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

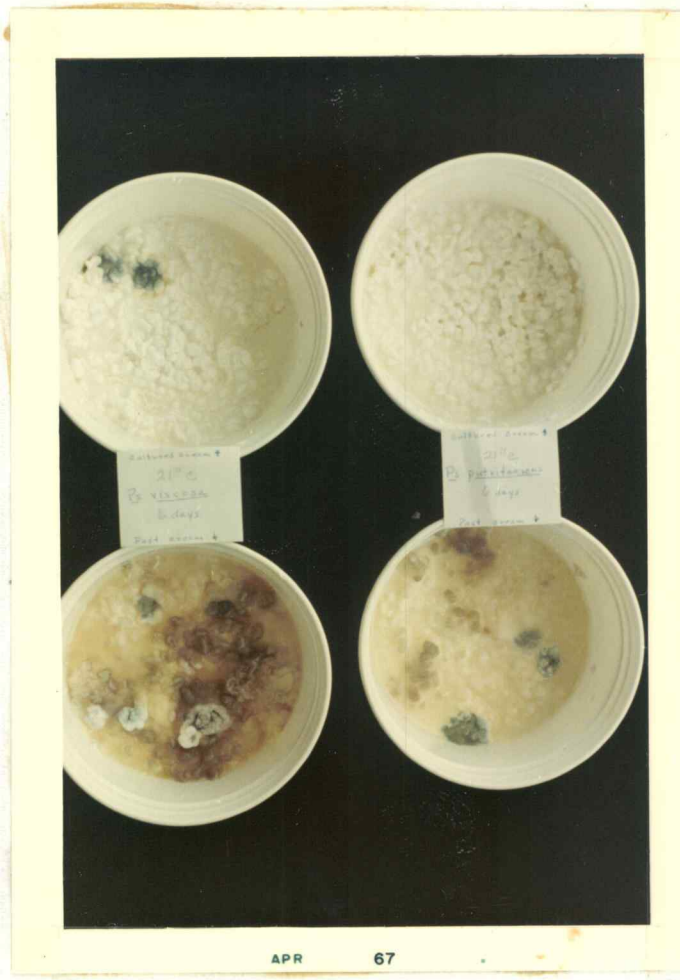

Figure 14. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Six days at 21° C.
LEFT: Contaminated with P. viscosa
RIGHT: Contaminated with P. putrifaciens
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

Figure 15. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Six days at 21° C.
BOTH: Contaminated with P. fragi
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

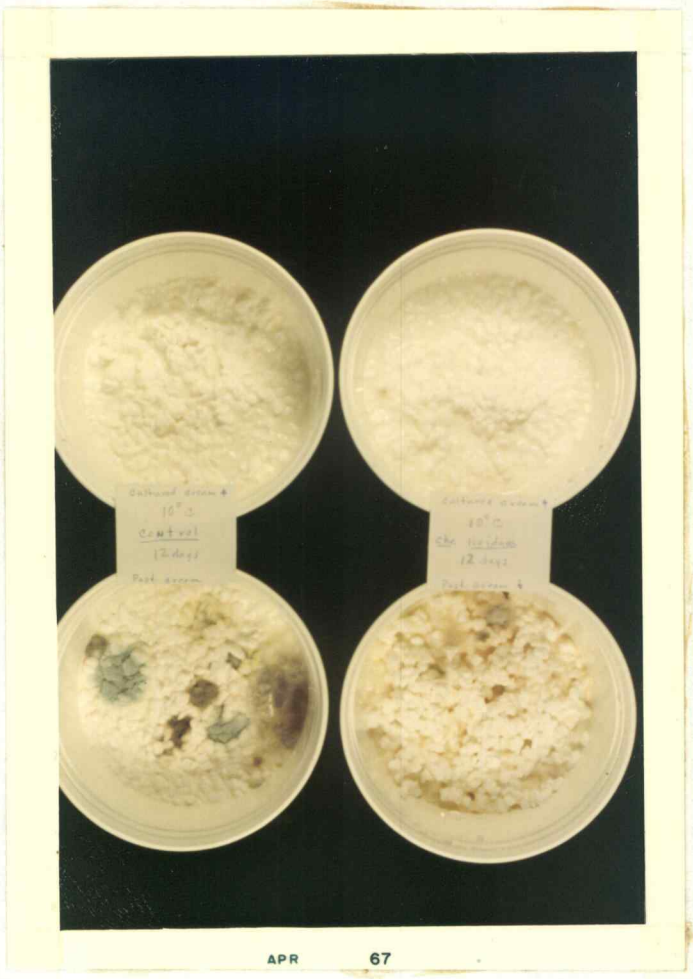

Figure 16. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Twelve days at 10° C.
LEFT: Control
RIGHT: Contaminated with C. lividum
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

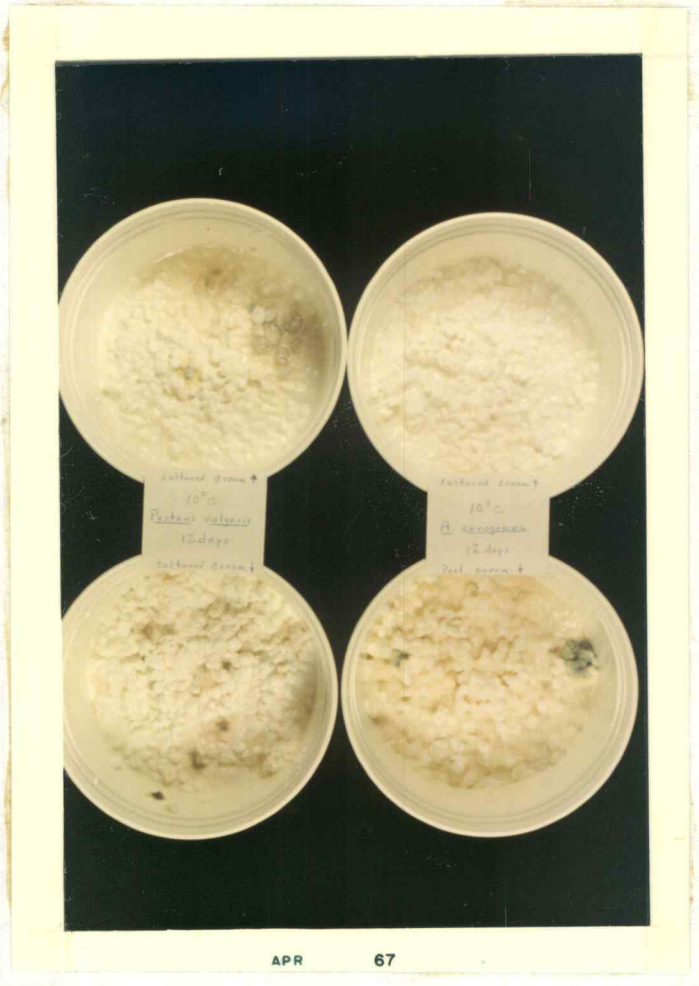

Figure 17. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Twelve days at 10° C.
LEFT: Contaminated with P. vulgaris
RIGHT: Contaminated with A. aerogenes
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

Figure 18. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Twelve days at 10° C.
LEFT: Contaminated with A. metalcaligenes
RIGHT: Contaminated with P. fluorescens
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

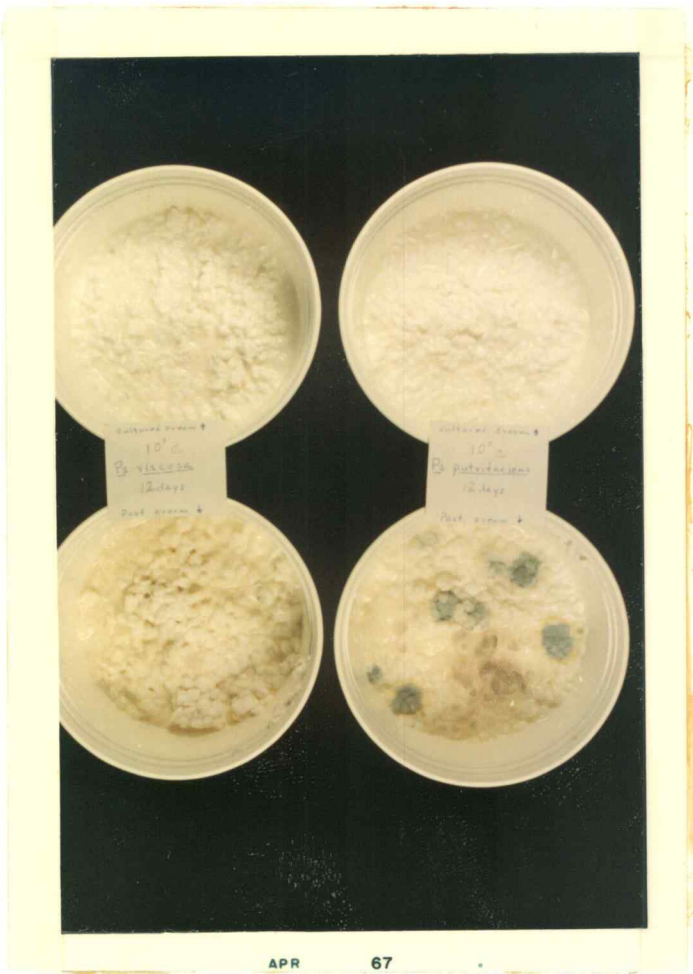

Figure 19. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Twelve days at 10° C.
LEFT: Contaminated with P. viscosa
RIGHT: Contaminated with P. putrifaciens
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

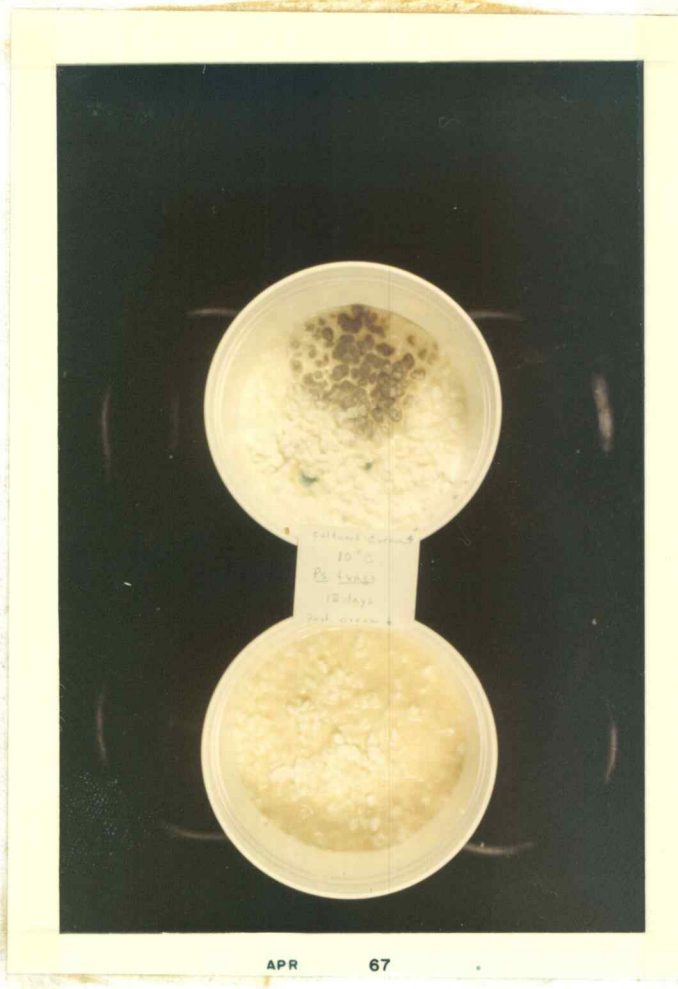

Figure 20. Contamination of Cottage cheese with spoilage organisms

TIME & TEMP: Twelve days at 10° C.
BOTH: Contaminated with P. fragi
UPPER: Creamed with cultured dressing
LOWER: Creamed with plain cream

shows the two cartons that were contaminated with P. fragi; the top carton (creamed with cultured dressing) shows quite a bit of mold growth and had developed a definite off odor. However, the other carton (creamed with plain pasteurized cream) was completely spoiled. In all cases, the difference between the two methods of creaming the curd was very marked, with the cheese dressed with cultured cream being much better than that creamed with the plain pasteurized cream. With the single exception of the cheese contaminated with the P. fragi (Figure 15), all of the cheese that had been creamed with the cultured cream was still eatable after six days at 21° C. and was considered superior to the cheese that had been creamed with the plain pasteurized cream when it was fresh.

Samples of the dressed and undressed Cottage cheese held at 10° C. (Figures 16 thru 20) showed results similar to those that were held at 21° C. It is interesting to note that the sample that was contaminated with P. vulgaris and creamed with the cultured cream developed some slime even though this organism is not supposed to be able to grow at this low temperature. The same sample held at 21° C. did not develop any slime in six days which might indicate that the higher temperature gave the S. diacetilactis a growth advantage that the P. vulgaris was not able to overcome. The growth of the sample held at 10° C. could be due to natural contamination and not to the growth of the P. vulgaris. As was the case with

cartons held at 21° C., the sample that was contaminated with P. fragi and creamed with the cultured cream developed a very definite off odor.

These results show that the competitive action of S. diacetilactis 18-16 can be of significant benefit in preventing deterioration of the Cottage cheese by preventing growth of spoilage organisms. This would be of particular benefit under conditions where the product might be subjected to temperatures above the normal storage temperatures, which might occur during transportation to a retail market or subsequent storage in the display cases where the temperature is not always constant.

The growth of considerable amounts of mold on the Cottage cheese during this experiment could be avoided by incorporating an antimycotic agent such as potassium sorbate in the Cottage cheese during manufacture, or by limiting the air space in the carton by filling it as full as possible or by putting a tight fitting inner cover that fits down over the surface of the cheese. The cartons used in this experiment were a little over half full and were opened every two days for examination which provided opportunities for mold contamination and growth.

The fact that P. fragi was able to grow and cause spoilage in spite of the competition with S. diacetilactis indicates that this method of increasing the shelf-life of Cottage cheese is not a

substitute for good sanitation in the plant. This organism is widely distributed and if allowed to build up on the processing equipment so as to contaminate the product, the cultured cream process would retard the spoilage but would not extend the shelf-life as long as it would if the product were not as heavily contaminated.

General Conclusions

One conclusion that can be drawn from the first phase of this experiment is that Cottage cheese is not uniform enough in composition to be used for plate count experiments. The very nature of the curd makes it difficult if not impossible to extract a uniform and representative sample from the larger quantity.

The second phase of these experiments showed that the cultured creaming mixture was effective against a variety of organisms, especially those that might be a problem at the low storage temperatures that are encountered after the product is packed and distributed for sale. The cultured dressing was also shown to improve the organoleptic qualities of the cheese as well as its appearance and keeping quality.

SUMMARY

An investigation of the inhibition of spoilage organisms in Cottage cheese due to the presence of S. diacetilactis 18-16 in the creaming mixture was undertaken with the following objectives:

1. To show the presence or absence of an antibiotic agent secreted by the lactic streptococci that would be effective against a wide range of spoilage bacteria and potential pathogens.
2. To show the effect of metabolic by-products, such as lactic acid and pH on the growth of spoilage bacteria and potential pathogens.
3. To show the effect of the active metabolism of the cells of lactic streptococci against the spoilage bacteria.

All efforts to isolate or show evidence of an antibiotic agent produced by S. diacetilactis or other lactic streptococci that would be effective against spoilage bacteria were unsuccessful. Disc assay experiments showed that whenever the viable cells of S. diacetilactis were present, inhibition would take place; however, if the cells were sonicly disrupted there was no inhibition due to the cellular material alone.

The disc assay method was also used to determine if any other by-products of metabolism such as lactic acid would inhibit the

spoilage organisms. It was found that the supernatant from a broth culture of S. diacetilactis was ineffective against these organisms even when it was concentrated ten times. Sterile lactic acid at a pH of 4.5 was found to produce a slight inhibition of these organisms, however, none of the cultures tested except S. diacetilactis 4R5 was able to reach this low a pH in 24 hours, showing that the pH of the medium was not a major contributing factor. This was also shown in experiments where the pH of pure cultures of S. aureus and P. fragi was lowered with sterile lactic acid.

The minimum growth temperature of S. diacetilactis 18-16 was found to be about 7.5° C.; the cells were still metabolically active at this temperature and were able to exert an inhibitory effect on P. fragi even though the streptococcal cells were not able to multiply to any great extent. This suggested that cells of S. diacetilactis (and perhaps other streptococci) may exert an inhibitory effect on other organisms by exhausting essential nutrients that are not present in high concentrations.

The inhibitory effect of S. diacetilactis creaming mixture on the Cottage cheese spoilage bacteria was shown to be very great even when the cheese was grossly contaminated. This was true even when product was stored at 21° C., emphasizing the practical importance of this process in extending shelf life of Cottage cheese.

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