3-1970

The effect of heat stable bacterial lipase (S) on the flavor and fat degradation of pasteurized milk cheddar cheese

Daniel Nelson Hamilton

To the Graduate Council:

I am submitting herewith a thesis written by Daniel Nelson Hamilton entitled "The effect of heat stable bacterial lipase (S) on the flavor and fat degradation of pasteurized milk cheddar cheese." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Husbandry.

W. W. Overcast, Major Professor

We have read this thesis and recommend its acceptance:

J. T. Miles, M. R. Johnson

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

March 1, 1970

To the Graduate Council:

I am submitting herewith a thesis written by Daniel Nelson Hamilton entitled "The Effect of Heat Stable Bacterial Lipase(s) on the Flavor and Fat Degradation of Pasteurized Milk Cheddar Cheese." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Dairying.

W. W. Overcast
Major Professor

We have read this thesis
and recommend its acceptance:

J. T. Miles

M. R. Johnston

Accepted for the Council:

Hilton A. Smith
Vice Chancellor for
Graduate Studies and Research

THE EFFECT OF HEAT STABLE BACTERIAL LIPASE(S) ON THE FLAVOR AND FAT DEGRADATION OF PASTEURIZED MILK CHEDDAR CHEESE

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Daniel Nelson Hamilton

March 1970

ACKNOWLEDGMENTS

The author wishes to express appreciation to Dr. W. W. Overcast for his patient guidance in the conducting of the research and the preparation of this thesis.

Appreciation is expressed to Dr. J. T. Miles and Dr. M. R. Johnson for help and encouragement as well as for serving on the committee.

Appreciation is also expressed to Dr. B. J. Demott, Mr. Stanley Snowden, Mr. Brient Mayfield, Mr. David Effler and Mr. Benton Jones for their efforts on the taste panel. And to Mr. Henry Dowlen and Mr. Larrie Harlan for their help in manufacturing the cheese samples.

I am deeply indebted to my wife, Nancy, for her help and consideration.

ABSTRACT

Two species of psychrophilic bacteria, Pseudomonas fragi 169 and Pseudomonas fluorescens 31, chosen for their high production of heat stable lipase(s) in a milk medium, were each inoculated into five lots of raw milk. The samples were incubated until counts were from 3 to 5 million cells per ml. as determined by the Direct Microscopic Count and were then pasteurized at 63°C for 30 minutes. Lots of the same milk not inoculated were pasteurized at 63°C for 30 minutes and used as controls. After pasteurization, inoculated and control lots of milk were made into Cheddar cheese.

Acid Degree Values were determined on extracted fat after two weeks and again at successive three month intervals for one year. Flavor differences between treated and control samples were determined by a triangle taste test.

The Acid Degree Values of fat from inoculated lots of cheese increased significantly over those from controls in the first three months of curing. Significant differences in Acid Degree Values were noted at every curing period. The Acid Degree Values produced by the lipases of Pseudomonas fragi 169 were significantly higher than those produced by Pseudomonas fluorescens 31.

No significant difference in the flavor of cheese made from milk inoculated with Pseudomonas fragi 169 or its controls was noted until after nine months of curing. A significant difference was noted between cheese lots made from milk inoculated with Pseudomonas

fluorescens 31 and control lots after twelve months curing. Inoculated and control lots of cheese scored very close in flavor.

TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

TABLE PAGE

CHAPTER I

INTRODUCTION

The importance of psychrophilic bacteria to the dairy industry is linked to their ability to grow and produce heat stable lipase(s) in milk at refrigeration temperatures. The presence of heat stable bacterial lipase(s) in pasteurized milk used for manufacture of Cheddar cheese is directly related to the growth of psychrophilic bacteria present in the raw milk prior to pasteurization and can be linked to increased usage of holding times under refrigeration.

The psychrophiles are of importance in this study because of their ability to grow and produce lipase(s) in milk at refrigeration temperatures near 4°C. These lipases have been shown to produce undesirable flavor changes in dairy products. Further, some of the species of the genus Pseudomonas produce lipase which has been found to be heat stable at pasteurization temperatures of 63°C and above for 30 minutes (59).

This study was designed to determine the effects of heat stable lipase(s), produced by psychrophiles, on Cheddar cheese flavor development and fat degradation when the organisms were grown in milk at populations that equal or exceed the maximum limits of class 2 milk. The range set for this study was 3 to 5 million cells per ml. in raw milk before pasteurization.

CHAPTER II

REVIEW OF LITERATURE

As early as 1891, Van Slyke (81) recognized that the fat of cheese changed in flavor during ripening. It was his opinion that the change in the fat was primarily hydrolytic, releasing free fatty acids. Suzuki et al. (79) in 1910 showed that non-nitrogenous products formed during the ripening of Cheddar cheese contributed appreciably to its flavor. It has been observed that no typical flavor develops in Cheddar cheese made from skim milk (38). This suggests that milk fat is the substrate for typical cheese flavor formation. In addition the typical cheese flavor has been found to be soluble in fat solvents and to be concentrated in the fat phase of cheese (11, 38).

Early work on the ripening of hard rennet cheese established that enzymes developed from one or more of the following sources: milk, rennet extract, or the bacterial flora of the cheese.

Milk Lipase

Rice and Markley (63) first suggested in 1922 that milk lipase was one of the causes of rancidity in cheese. They assumed that rancidity in cheese was due to the production of lower fatty acids such as butyric by the action of milk lipase on natural milk fats. Hood et al. (28) and Hlynka et al. (23, 24) indicated that the milk lipase activated by agitation at temperatures from 45° to 86°F for 5 to 15 minutes can cause rancidity in the resulting cheese made

from the milk. When the activation of lipase was less, flavors normally termed "unclean" were produced, indicating that certain types of unclean flavors were related to rancidity. As the length of agitation time and temperature of the milk increased within the range of 45° to 86°F, the flavor score of the resulting cheese was progressively lower. In a related study on the keeping quality of cheese with rancid flavors produced by agitation, Hlynka and Hood (21) reported that cheese flavor did not improve with age and milk lipase was not the agent promoting rancid and unclean flavors in stored cheese. Hlynka et al. (25) reported that milk lipase activated by homogenization at 86°F could be inhibited or its activity confined to the glycerides of non-volatile fatty acids. This inhibition was produced by increasing the acidity of the milk with hydrochloric acid to a titratable acidity of 0.25 percent or by the addition of 1 percent lactic culture 24 hours prior to cheesemaking. Cheese made from acidified milk, which inactivated milk lipase, scored slightly higher than cheese made from milk not acidified. Hlynka and Hood (22) later credited the increased fat acidity of a large number of Canadian cheese to milk lipase.

Peterson et al. (56) have shown that the stability and pH activity of milk lipases are such that this enzyme can play no continuing role in the ripening of Cheddar cheese. Analyses made at intervals during the making and ripening of raw milk Cheddar cheese show that milk lipase disappears during the making and is completely absent in young cheese.

Rennet extract added during the making of Cheddar cheese

causes an increase in lipolytic activity (56). Acetic and normal butyric acids were the only free fatty acids produced in significant amounts from the lipolytic activity of the milk, rennet extract, and starter culture used during cheesemaking. There was, however, negligible carry-over of these acids into the finished cheese since they can be accounted for completely in the whey at the time of dipping (57).

A later study by Albrecht and Jaynes (1) on the lipolytic activity (hydrolysis of tributyrin) in skim milk at various acidities showed the existence of two lipase systems with optimum activities at pH 5.4 and 6.3 respectively. Both systems exhibited a secondary optimum activity at pH 8.5. They believed that in raw skim milk there was a complex system of lipases with optimum activity at pH 5.7 to 6.0. These lipases were linked with the casein (71) and remained with the curd after rennet-or-acid-coagulation. They also reported that raw milk Cheddar cheese may contain milk lipases active at the pH of cheese which may play a part in the ripening of the cheese.

Stadhouders (73) has confirmed milk lipase destruction at 55°C. However, the bulk of factory processed Cheddar cheese is now made from milk treated with heat, which destroys much of the lipolytic activity of the milk lipase active at pH 5 (31, 69). Since the lipases are intimately associated with milk casein, a concentrating process occurs during cheesemaking.

Added Lipase

Babel and Hammer (4) studied the fat acidities of Cheddar cheese when lipase in the form of rennet paste or mulberry juice was added

to milk pasteurized at 143°F for 30 minutes. Variations both in the original fat acidities and in the increases during ripening were demonstrated in cheese made from different lots of milk. Rancidity occurred somewhat sooner where rennet paste was used than with the use of mulberry juice. There was no correlation between the degree of rancidity and the fat acidities of cheese made with added lipase. Further studies by the same workers (5) showed a slow, steady rise in the titratable acidity of the fat of control cheese during nine months ripening. When lipase was added as rennet paste replacing part of the rennet extract, the fat acidities increased to a greater though variable extent. Larger lipase additions caused rancidity which was increasingly noticeable in the early stages of ripening but tended to decrease later; moderate additions gave cheese of better flavor than the controls. Mulberry juice as a source of lipase caused still greater and more variable increases in fat acidity and produced a rancidity which did not decrease with storage. Addition of a proportion of rancid cheese was noted to improve the flavor of processed cheese.

Windlan and Kosikowski (83) studied the influence of milk-coagulating enzymes of nonanimal origin upon the flavor and constituents of Cheddar cheese. When rennet-like enzymes from microbial sources were used at optimum concentrations, smooth curd resulted and bitter flavors in either curd or cheese were relatively insignificant. However, deviation above optimum enzyme concentration resulted in some bitterness in flavor, which was associated with the appearance of a peptide in the cheese.

Measuring Lipase in Milk and Cheese

The early methods most often used to determine milk lipase were those of Rice and Markley (63), Hlynka and Hood (20), and Reder (60). None of these workers studied the quantitative relation between the amount of lipase and the degree of hydrolysis. Peterson et al. (54), using the method of Reder (60) as a basis, developed a quantitative method for the determination of milk lipase. With tributyrin as the substrate, they found milk lipase was most active at pH 8.5 when 5 ml. aliquots were incubated at 40°C for 15 to 120 minutes. Peterson et al. (55) later modified this method for the determination of cheese lipase. By means of a composite buffer, pH was controlled throughout the range desired for cheese. They found that tributyrin was split most rapidly at pH 5, with a secondary optimum occurring at pH 6.5 to 7.0. They also found hydrolysis to be directly proportional to the quantity of cheese lipase present, up to the point when 0.40 ml. of 0.1 normal butyric acid was present in a 5 ml. aliquot. Hydrolysis of tributyrin by cheese lipase at pH 5 was linear to time when 0.40 ml. of 0.1 normal butyric acid was present in 5 ml. aliquots. A later study (56) revealed that most of the lipase active at pH 5 was set free between the fifth and hundredth day of ripening. Larger amounts of lipolytic activity were detected in pasteurized milk Cheddar cheese between the fifth and twenty-fifth day of the ripening period than in corresponding raw milk Cheddar cheese. At any time after 30 days of age, however, the total lipolytic activity of pasteurized milk cheese was less than half that of corresponding raw milk cheese.

Lubert et al. (37) reported that they could not reproduce the hydrolysis method of Peterson et al. (55).

Scott (68) reports a method of measuring lipase activity in Cheddar cheese. The butyric acid liberated from a protein suspension by hydrolysis of tributyrin at pH 7.5 incubated at 30°C for 24 hours was used to determine lipase activity.

Chemical Changes Brought About by Lipolysis and Methods to Measure the Change

The determination of chemical changes occurring in the fat of Cheddar cheese during ripening has been the object of numerous investigations. In 1910, Suzuki et al. (79) reported the results of partitioning by the Duclaux technique, the volatile fatty acids: formic, acetic, propionic, butyric and valeric, which were obtained from steam distillation of acidified suspensions of Cheddar cheese.

Many early investigators measured the free fatty acids of Cheddar cheese as a group and not as individual components. Among the methods most often used were the direct determination of cheese fat acidities (5, 35, 36), the steam or direct distillation of acidified cheese suspensions (17, 32, 39) or extraction (18, 19, 72) followed by distillation and titration of the distillate. Babel and Hammer (5), Irvin (30) and Hlynka and Hood (22) reported simpler more arbitrary methods of titrating fat acidities. Undoubtedly the lack of accurate qualitative or quantitative data with regard to Cheddar cheese free fatty acid determination methods has contributed to the failure of many investigators to find a relationship between

Cheddar cheese flavor and free fatty acid content (5, 12, 13, 19, 36, 69).

Peterson et al. (52) reported a rapid partition chromatographic method for the quantitative estimation of formic, acetic, propionic, normal butyric, caproic, caprylic and capric acids in biological materials. A study of both raw and pasteurized milk Cheddar cheese (57), using the method previously described (52), showed that during the first 30 days of ripening, caporic, caprylic, and capric acids were absent, while normal butyric was present at slightly lower levels than those in the same cheese at 420 days. Acetic acid levels after 30 days of ripening for both types of cheese were approximately one half of that at 420 days. Raw milk cheese at 420 days had considerably higher levels of normal butyric and acetic acids than corresponding pasteurized milk cheese. Caporic acid levels for the two types were quite similar.

Dacre (11) identified the compounds which contain the flavor principles as ethanol, butyraldehyde, ethyl acetate, and ethyl butyrate; the volatile fatty acid fraction was shown by gas-liquid chromatographic analysis to consist of acetic acid together with lesser amounts of both butyric and caproic acids. None of these substances, either singly or in combination, had the odor or taste of Cheddar cheese. The results of this investigation were not in agreement with the observations of Silverman and Kosikowski (70), which indicated that the volatile fatty acids are important flavor constituents of Cheddar cheese.

Stadhouders and Mulder (74) found all saturated fatty acids

present in cheese milk to be present in the same ratios in the corresponding cheese.

Day and Kenney (14) identified the carbonyl compound, 3-methylthiopropanal as contributing more to Cheddar cheese flavor than other carbonyls. They did not identify a number of other carbonyls of lesser importance.

Kristoffersen and Gould (33) suggest a possible realtionship between the rate of appearance and actual concentration of both free fatty acids and hydrogen sulfide and characteristic Cheddar flavor development. Evidence presented indicated that optimum flavor results only through simultaneous action of the agents responsible for fat and protein degradation.

Scarpellino (65) and Scarpellino and Kosikowski (67) report the application of gas chromatographic techniques to the study of volatile compounds in Cheddar cheese. The major volatile compounds (67) were acetic acid, butyric acid, ethanol, methyl ethyl ketone, and secondary butyl alcohol. Acetylmethylcarbinol and diacetyl were present at trace levels. This was the first time secondary butyl alcohol had ever been reported in the literature while methyl ethyl ketone was reported earlier (14, 66). Patton (51) indicated that the volatile acids, acetic, butyric, caproic and caprylic are of primary importance in Cheddar cheese aroma; in the absence of acetic acid the characteristic of the volatile fatty acid mixture was found to be one of hydrolytic rancidity of milk fat.

Bills and Day (6) reported that percentages by weight of individual acids from caproic to linolenic were similar to values

reported for the same esterified acids of milk fat, but the concentration of free butyric acid was twice that for esterified butyric acid in milk fat. Normal valeric, iso-valeric, formic and propionic acids were not detected in Cheddar cheese.

Ohren and Tuckey (48) indicated that free fat acidity increases with increases in fat and the bacterial count of the raw milk but the quality of flavor decreased. Fermented, unclean and whey-taint flavors were associated with high contents of C_{10}, C_{12}, and C_{14} fatty acids. More recent work by these same authors (47, 49) confirms the contribution of C_{10}, C_{12} and C_{14} fatty acids to off flavors. This work also indicated that typical Cheddar cheese flavor was related to a balance of free fatty acids to acetate. They reported that only Cheddar cheese containing 50 percent fat or more in the dry matter developed a typical flavor, whereas cheese with less than 50 percent fat did not. As fat in the cheese decreased, the concentration of fatty acids decreased, but the acetate increased so the ratio between free fatty acids and acetate became undesirable.

Lipolytic Activity of Bacteria Found in Cheese Milk

Several workers have indicated they believed the action of intercellular bacterial lipases to be responsible for part of the increase in free fatty acids and for flavor development in Cheddar cheese (26, 27, 53, 54, 55, 75, 76, 77).

Hood et al. (26, 27) found that when they added pure cultures of lipolytic bacteria to the cheese milk in the vat just prior to making cheese, higher fat acidity values and consistently lower flavor

scores were produced. The chief flavor defects encountered were "unclean" and "rancid." The fat acidity in the cheese increased with the amount of inoculum of lipolytic bacteria added to the milk.

Peterson and Johnson (53) found certain lactobacilli and micrococci isolated from Cheddar cheese to be lypolytic to butterfat when inoculated for 60 days and that autolysis occurred in a media adjusted to pH 5 to 6. They indicated that these intercellular enzymes may be concerned in the development of volatile free fatty acids during late stages of ripening Cheddar cheese.

Stadhouders (73) and Stadhouders and Muller (75, 76, 77) have indicated that heat stable lipase producing organisms present in the milk belonging to the genera Alcaligenes, Achromobacter, Pseudomonas, Flavobacterium and Serratia were the most active lipolytic enzyme producing agents in Cheddar cheese. Their lipase, except for Alcaligenes and Flavobacterium was resistant to 74°C for 4 seconds and a relationship between the count of raw milk and the quality of cheese made from its was demonstrated.

Studies of the selective release of volatile acids from butterfat by microbial lipases indicate that they differ in their ability to release fatty acids. Some strain variations were also evident (44, 82).

Morrison and Hammer (42) noted the wide distribution of Pseudomonas fragi in milk, dust and equipment used in milking operations. They also noted its proteolytic, lipolytic and psychrophylic properties.

Nashif and Nelson (43, 44, 45) made detailed studies of the

lipase of Pseudomonas fragi. They found that lipase was produced at temperatures below 30°C. Once produced, the lipase had a temperature optimum of 40°C, had a pH optimum at 7.0 to 7.2, exhibited a specificity for tricaprylin over other triglycerides, was heat stable at 61.6 to 71.6°C for 30 minutes requiring heating to 99°C for 20 minutes for complete inactivation. The lipase of Pseudomonas fragi in cream was active between pH 4.9 and 8.2 with over 50 percent not inactivated by pasteurizing at 71.5°C for 30 minutes. A continued study (46) of other Gram-negative non-sporeforming rod-shaped bacteria confirmed that temperature of growth had an important effect on lipase production. All Pseudomonas species studied produced the greatest amounts of lipase at 21°C or below. Peptone broth and nutrient broth were found to give the maximum cell and lipase production (2, 44).

Mencher and Alford (40) have reported the purification and characterization of the lipase of Pseudomonas fragi. The enzyme was found to exist in heavy and light forms exhibiting the same position specificity for triglycerides. The light form appeared present in the heavy one, probably in a complexed state. The purified lipase was found to hydrolyze only glycerol esters of fatty acids; it required a water fat interface; exhibited a 1-3 position specificity for triglycerides and was found to be stable in the pH range 6.6 to 7.8.

Nashif and Nelson (46) reported that two cultures of Pseudomonas fluorescens studies produced appreciable lipase which retained its activity at 71.6°C for 30 minutes. This agreed with the early work of Söhngen, reported by these same authors, who observed the production

by Bacillus fluorescens liquefeciens (Pseudomonas fluorescens) of lipase that could withstand heating at 100°C for 5 minutes.

Csiszar and Romlehner (9) reported higher percentages of inactivation of lipase produced by different strains of Pseodomonas fluorescens, in cream when the temperature was raised from 75° to 95°C. There were marked variations noted in the heat resistance of lipases produced by different strains of Pseodumonas fluorescens. Pinheiro et al. (58) determined the heat stability of lipases of selected psychrophilic bacteria in milk and Purdue Swiss-type cheese and found that the enzyme produced by Pseudomonas fluorescens was inactivated by the commercial heat treatment applied to milk, whereas the enzymes produced by Pseudomonas fragi and Pseudomonas mucidolens were more heat stable.

Cutchins et al. (10) reported that the carbon and nitrogen sources of the medium affect the production of lipase by Pseudomonas fluorescens. Several investigators have concluded that the number of lipolytic Gram-negative bacteria do not increase in numbers during cheese ripening, but in fact decrease in numbers during the first few weeks and are replaced by lactobacilli (15, 16, 61, 62). From this they suggested that lipolytic activity produced by these organisms would have to result from lipase produced in the milk prior to death of the cells although no other evidence was presented.

CHAPTER III

EXPERIMENTAL METHODS

Selection of Cultures

Six cultures were chosen from a group of 23 psychrophilic cultures in the culture collection of the Dairy Microbiology Laboratory of the University of Tennessee on the basis of their history of lipase activity (50) and heat stable lipase production (64).

Each of the six cultures was tested for heat stable lipase production in milk by inoculating approximately 0.01 ml. of a 24 hour Nutrient Broth culture of the organism into 100 ml. of whole milk previously sterilized by autoclaving at 15 pounds pressure for 10 minutes. The inoculated milk samples were incubated at 5°C until the count reached 5 to 5 million per ml. as determined by the Direct Microscopic Count (DMC) (3). The inoculated milk samples and uninoculated controls of the same sterile milk, held at the same temperature and conditions as the inoculated, were pasteurized at 63°C for 30 minutes in a E. H. Sargent circulating water bath thermostatically controlled by a Sargent model S-W thermonitor sensitive to ±0.01°C and immediately cooled in ice water.

The two highest lipase-producing organisms of the six tested (*Pseudomonas fragi* 169 and *Pseudomonas fluorescens* 31) were characterized by the methods described in *Bergey's Manual of Determinative Bacteriology* (7) and were used in this study.

Heat stable lipase activity was determined by making the

following modifications in the method of lipase determinations of Peterson et al. (54).

1. Phosphate barbitol buffered milk samples were adjusted to pH 7.0 with 5 normal sodium hydroxide or 5 normal acetia acid, instead of pH 8.5.
2. The time of incubation was increased from 120 minutes to 18 hours to allow more hydrolysis of tributyrin and butterfat by small amounts of lipase(s).
3. Titrations were made with 0.05 normal sodium hydroxide, instead of 0.1 normal sodium hydroxide, to allow more accurate measurement of smaller amounts of acid.
4. Tributyrin was added to the control sample to allow a measurement of a difference in hydrolysis of substrates in both inoculated and control samples.

The first two modifications of Peterson's et al. (54) methods were methods of favorable environmental control, while the third was a modification of measurement. These modifications were adapted from those used by Nashif and Nelson (44) in their studies of heat stable lipase activity produced by Pseudomonas species.

The organisms were prepared for inoculating the milk by growing them at 21°C for 24 hours on Nutrient agar slants. The cells produced were washed from the slant with 50 ml. of Nutrient Broth and were added to the raw milk at a rate sufficient to produce a total count of 75 to 150 thousand cells per ml. as estimated by the DMC. The rate of culture addition was determined for each set of Nutrient Broth cultures by adding 0.1 ml. portions of the culture

to a series of 100 ml. samples of the raw milk used in the lots of cheese. The ratio of culture to milk that produced 75 to 150 thousand total cell count per ml. was then used for the bulk of cheese milk of each inoculated lot.

Preparing Cheese Milk

Six hundred sixty pounds of whole fresh raw milk of low bacteria content were obtained from the University Creamery and split into 330 pound lots. One of the lots of milk was pasteurized at 63°C for 30 minutes in a spray pasteurization vat at the University Creamery. This lot was designated as control (C) and stored at 5 to 7°C until manufactured into Cheddar cheese. The remaining lot of raw milk was inoculated with a culture of either Pseudomonas fragi 169 or Pseudomonas fluorescens 31 at the rate described earlier. This lot of milk was designated as inoculated (I) and stored at 5 to 7°C until the DMC of a representative sample was estimated to be between 3 and 5 million per ml. After the desired DMC per ml. was reached the milk was pasteurized at 63°C for 30 minutes in the same vat used for pasteurizing the control lot. Five lots of inoculated and control cheese were made using each of the two heat stable lipase producing organisms.

Standard Plate Count (SPC) per ml. and Psychrophilic Bacteria Count (PBC) per ml. were determined on the raw milk, pasteurized control milk, inoculated milk, inoculated milk after storage at 5 to 7°C, and the inoculated milk lots after pasteurization by the methods described in the Standard Methods for the Examination of Dairy Products (3).

Manufacturing Cheese

Each inoculated lot and its corresponding control lot of milk was manufactured into Cheddar cheese by the procedures of Price and Calbert (59) with a milling acid of 0.45 percent as the goal. Hansen's number H-44 cheese culture and Hansen's cheese rennet were used for curd production. Eight ml. of cheese color was added to each lot of cheese milk for color uniformity. A typical record form of the manufacturing procedure used in this study is shown in the appendix.

Each lot of milk resulted in four 8.5 to 9.0 pound young American hoops of cheese. Each cheese was labeled with the culture number appropriately as either inoculated or control, dated, waxed, and cured at 7 to 8°C.

Cheese Testing Procedure

After two weeks and again at successive three month intervals for one year the surface of the waxed cheese was cleaned with 95 percent alcohol and allowed to dry. Representative samples were withdrawn from the hoops of cheese with a trier dipped in 95 percent alcohol and air dried. Methods of aseptic transfer, testing and recording of results were observed for cheese samples during sampling (3). Acid Degree Values (ADV) (the milliliters of 1 normal potassium hydroxide required to titrate 100 grams of fat) (34), pH, and taste evaluations were made on each sample after bacterial sampling was completed. Acid Degree Values were determined on a water-cheese suspension of a 20 g. representative sample of each lot of

cheese reconstituted to a water to total solids ratio, equivalent to 3.5 percent butterfat milk, based upon an average moisture content of 37.5 percent in cheese. The water-cheese suspension was made by blending a 20 g. representative sample of cheese in 74.2 ml. of distilled water at 60°C for 3 minutes in a Virtis Homogenizer. Thirty ml. of the resulting water-cheese suspension was pipetted into a Tesa fat test bottle. Twelve ml. of Bureau Dairy Industry (BDI) reagent were added and the fat separated and titrated by the method of Thomas et al. (80) except that the 0.02 normal alcoholic potassium hydroxide was added from a 0.2 ml. burette calibrated in 0.001 ml. Four replicate titrations were made of the fat of each cheese sample. The ADV was calculated from the average of the four titrations. The BDI reagent consisted of 30 g. Triton-X-100 and 70 g. sodium tetra-phosphate (quadrofos detergent) dissolved in distilled water and made up to one liter (34). The quantity of BDI reagent used to separate the fat and solids of the water-cheese suspension was calculated from the quantity of BDI reagent needed to separate the fats and solids of 3.5 percent butterfat milk. When calculated on the same basis, the water-cheese suspension had a fat to solids ratio slightly higher than 3.5 percent milk. The water-cheese suspension required 0.372 ml. BDI reagent per 1.0 ml. of suspension for total fat separation. The 30 ml. of water-cheese suspension required 11.2 ml. BDI reagent. After some preliminary trials an extra 0.8 ml. BDI reagent was found to insure total fat and solids separation in the water-cheese suspension. The usage of the extra BDI reagent was in accord with the findings of Hunter et al. (29). They found that no

significant change in ADV of milk occurred as a result of increasing the quantity of BDI reagent beyond the volume needed for complete fat separation.

Taste evaluations were made by the triangle taste test method using a panel of five experienced cheese judges. The judges were also asked to score their preference for different or matching samples on the hedonic scale (41). A sample form used by each judge on the taste panel for recording triangle test and preference results is shown in the appendix.

At the end of the one year aging period moisture and fat determinations were made and the taste panel judges scored the flavor of each lot of cheese. A sample scoring guide used is given in the appendix.

Statistical Analysis

Statistical analyses of the ADV data were based on an analysis of variance for factorial experiments outlined by Steel and Torrie (78). The statistical analysis of the taste panel data was determined by the methods described in Merck Technical Bulletin (41).

CHAPTER IV

RESULTS AND DISCUSSION

Milk or cheese made from milk inoculated with a culture of either Pseudomonas fragi 169 or Pseudomonas fluorescens 31 prior to pasteurization will be referred to as inoculated. Reference to control milk or cheese indicates that the milk was from the same lot as the inoculated milk but was not inoculated with any culture.

Heat Stable Lipase Activity in Milk

Data in Table 1 and Table 2 show higher levels of hydrolysis of tributyrin and butterfat in inoculated milk containing either Pseudomonas fragi 169 or Pseudomonas fluorescens 31 than in the corresponding control milk. The net mean increases of 0.30 ml. of 0.05 normal sodium hydroxide used to titrate the free fatty acids produced by Pseudomonas fragi 169 and 0.27 ml. produced from Pseudomonas fluorescens 31 represents the heat stable lipase activity of the cultures. A standard deviation of ±0.02 ml. sodium hydroxide for titration of 10 replicate pasteurized control samples shown in Table 3 confirms that increases in titration values of inoculated milk represent real increase in substrate hydrolysis.

Bacterial Quality of the Milk Used to Manufacture Cheese

Data in Table 4 and Table 5 indicate that raw milk of a relatively low bacterial count was used for manufacturing the cheese. The Standard Plate Count was 30,000 per ml. or less and the Psychrophilic

Table 1. Hydrolysis of tributyrin and butterfat in five milliliter aliquots of milk containing Pseudomonas fragi 169 and controls after pasteurization

Milk sample replicates (N)	Milliliters of .05 normal NaOH					
	Pseudomonas fragi 169			Control		
	0 hr.	18 hr.	Increase	0 hr.	18 hr.	Increase
1	3.74	4.25	.51	3.75	3.85	.10
2	3.75	4.22	.47	3.75	3.89	.14
3	3.74	4.19	.45	3.75	4.00	.25
4	3.74	4.20	.46	3.75	4.00	.25
5	3.74	4.25	.46	3.75	3.90	.15
6	3.74	4.25	.50	3.75	3.92	.17
7	3.74	4.25	.51	3.74	3.94	.20
			ΣX 3.41			ΣX 1.26
			$\bar{X}^a$ .48			$\bar{X}^a$ .18

[a]Difference in $\bar{X}$ = .30 ml.

Table 2. Hydrolysis of tributyrin and butterfat in five milliliter aliquots of milk containing Pseudomonas fluorescens 31 and corresponding controls after pasteurization

Milk sample replicates (N)	Milliliters of .05 normal NaOH					
	Pseudomonas fluorescens 31			Control		
	0 hr.	18 hr.	Increase	0 hr.	18 hr.	Increase
1	4.66	5.02	.36	4.64	4.76	.12
2	4.66	4.98	.32	4.68	4.78	.10
3	4.68	5.02	.34	4.68	4.78	.10
4	4.66	5.00	.34	4.68	4.72	.04
5	4.70	5.04	.34	4.70	4.72	.02
6	4.68	4.98	.30	4.70	4.72	.04
7	4.68	5.02	.34	4.70	4.76	.06
			ΣX 2.34			ΣX .48
			$\bar{X}^a$ .33			$\bar{X}^a$ .06

[a]Difference in $\bar{X}$ = .27 ml.

Table 3. Hydrolysis of 10 replicate five milliliter aliquots of pasteurized control milk incubated at 40°C for 18 hours

Milk sample replicates (N)	Milliliters of .05 normal NaOH[a]	$(X)^2$
1	4.76	22.6576
2	4.78	22.8484
3	4.78	22.8484
4	4.72	22.2784
5	4.72	22.2784
6	4.74	22.4676
7	4.76	22.6576
8	4.75	22.5625
9	4.76	22.6576
10	4.74	22.4676
	ΣX 47.51	ΣX^2 225.7241

$$\text{Standard deviation} = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N}} = \sqrt{\frac{225.7241 - 225.7200}{10}} = \pm .02.$$

[a]Values expressed as ml. .05N NaOH represent the hydrolysis of substrate.

Table 4. Bacterial quality of the five lots of milk inoculated with Pseudomonas fragi 169 and corresponding controls

Lot	Raw		Pasteurized control		Inoculated raw		Inoculated raw after growth		Inoculated after pasteurization	
	SPC[a]	PBC[b]	SPC	PBC	SPC	PBC	SPC	PBC	SPC	PBC
					x 10^5					
I	26,000	1,100	<300	<300	1.4	1.2	44	40	<30	90
II	20,000	<300	2,800	<300	1.5	1.3	39	32	3,100	2,800
III	2,100	<300	<300	<300	.92	.80	41	40	<300	<300
IV	19,000	7,000	<300	<300	1.4	1.1	43	38	<300	<300
V	13,000	9,000	<300	<300	1.6	1.2	58	49	<300	<300

[a]Standard Plate Count per ml.

[b]Psychrophilic Bacterial Count per ml.

Table 5. Bacterial quality of the five lots of milk inoculated with Pseudomonas fluorescens 31 and corresponding controls

Lot	Raw		Pasteurized control		Inoculated raw		Inoculated raw after growth		Inoculated after pasteurization	
	SPC[a]	PBC[b]	SPC	PBC	SPC	PBC	SPC	PBC	SPC	PBC
					x 10^5					
I	30,000	1,400	<300	<300	1.4	.90	38	32	<300	<300
II	10,000	840	<300	<300	1.0	.82	42	37	<300	<300
III	6,100	600	77	<30	.78	.70	40	34	125	<30
IV	18,000	1,100	459	<30	1.1	.97	52	47	<300	450
V	5,100	760	1,200	65	1.6	1.0	55	48	<30	<30

[a]Standard Plate Count per ml.

[b]Psychrophilic Bacterial Count per ml.

Bacterial Count was 9,000 per ml. or less in the raw milk. Pasteurization of control lots of milk resulted in PBC of less than 300 per ml. in all lots. Standard Plate Counts were less than 300 per ml. in all lots except II in Table 4 and IV and V in Table 5. The data in Table 4 and Table 5 show that the SPC were within the desired limits after inoculation, except for lot V in each case. Standard Plate Counts per ml. of inoculated milk after growth exceeded the desired maximum of 5 million per ml. by 800,000 in lot V (Table 4) and by 200,000 and 500,000 in lots IV and V respectively (Table 5). Pasteurization of the inoculated lots of milk after growth resulted in SPC of less than 300 per ml. in nine of the ten lots and a PBC of less than 300 per ml. in eight of the ten lots of milk. The low psychrophilic counts on the pasteurized lots of milk would indicate that the Pseudomonas cultures could play no further role in flavor or fat degradation in the resulting cheese except for their heat stable enzymes.

The SPC per g. of cheese made from milk inoculated with Pseudomonas fragi 169 and controls are presented in Table 6. Counts made on inoculated and control cheese in lots I, II and IV declined from two weeks to three months of curing while the count of lot III increased. Counts on lots I, III, V and inoculated IV declined between three and six months of curing. Counts on control lot I, inoculated lot IV, and both inoculated and control lots III and V increased between six and nine months of curing. Counts increased from nine to twelve months on inoculated lot III and inoculated and control lots IV and V while the counts on the other lots declined.

Table 6. Standard Plate Count per gram of five lots of Cheddar cheese made from milk inoculated with Pseudomonas fragi 169 and corresponding controls

Lot	2 weeks		3 months		6 months		9 months		12 months	
	I[a]	C[b]	I	C	I	C	I	C	I	C
	x 10^5									
I	5.9	3.5	2.20	0.60	0.86	0.38	0.19	0.69	0.09	0.08
II	0.7	7.5	0.40	0.30	0.46	0.90	0.04	0.10	0.03	0.01
III	1.4	1.4	2.2	4.1	0.02	0.23	0.18	0.35	0.98	0.10
IV	9.2	4.1	0.63	0.28	0.01	0.58	0.16	0.10	0.25	0.17
V	---	---	16.	15.	0.59	0.01	2.10	2.20	230.	220.

[a]Inoculated.

[b]Control.

Data in Table 7 show the SPC per g. of five lots of cheese made from milk inoculated with Pseudomonas fluorescens 31 and controls. The counts on all lots except controls of lots II and III declined through six months of curing. Inoculated and control lots III and IV and inoculated lot V increased from six to nine months. Counts on inoculated and control lots I, IV, V and inoculated lot III increased from nine to twelve months while inoculated and control of lot II and control lot III declined. Inoculated and control lot V in Table 6 and inoculated lots III and V in Table 7 had the largest increases between nine and twelve months curing. Large numbers of organisms in aged cheese are not uncommon and reportedly do not detract from a good quality cheese unless they are of spoilage or pathogenic types (8).

Data presented in Table 8 and Table 9 indicate that psychrophilic bacteria were present in only limited numbers in inoculated and control cheese after three months curing and remained low through six months. Data in Table 9 indicates that psychrophiles decline rapidly in the first weeks of the curing period. Increases in psychrophiles were noted in lots III, IV and V in Table 8 and lots III and IV in Table 9, at nine and twelve months of curing. Examinations of these organisms were not made, but counts after pasteurization and the rapid decline of psychrophilic bacteria in early curing periods indicate they were not the psychrophiles used for inoculation of the milk. High psychrophilic counts appeared in the same lots of cheese that had high SPC.

Table 7. Standard Plate Count per gram of five lots of Cheddar cheese made from milk inoculated with Pseudomonas fluorescens 31 and corresponding controls

Lot	2 weeks		3 months		6 months		9 months		12 months	
	I[a]	C[b]	I	C	I	C	I	C	I	C
	x 10^5									
I	7.0	4.2	---	---	0.03	0.01	0.012	0.009	0.026	0.027
II	52.	11.	41.	14.	3.0	2.1	1.2	0.029	1.1	0.20
III	4.1	2.2	0.36	9.9	0.33	0.08	4.5	0.55	110.	0.13
IV	370.	90.	210.	6.0	1.2	0.03	3.2	0.38	5.0	0.52
V	300.	220.	280.	150.	2.0	3.0	5.1	1.60	32.	3.0

[a]Inoculated.

[b]Control.

Table 8. Psychrophilic Bacterial Count per gram of five lots of Cheddar cheese made from milk inoculated with Pseudomonas fragi 169 and corresponding controls

Lot	2 weeks		3 months		6 months		9 months		12 months	
	I[a]	C[b]	I	C	I	C	I	C	I	C
I	--	--	<300	<300	<300	<300	<300	<300	<300	<300
II	--	--	<300	<300	<300	<300	<300	<300	<300	<300
III	--	--	<300	<300	<300	<300	<300	1,000	<300	650
IV	--	--	<300	<300	<300	<300	<300	<300	770	1,900
V	--	--	<300	<300	<300	<300	700	400	10,000	5,600

[a]Inoculated.

[b]Control.

Table 9. Psychrophilic Bacterial Count per gram of five lots of Cheddar cheese made from milk inoculated with Pseudomonas fluorescens 31 and corresponding controls

Lot	2 weeks		3 months		6 months		9 months		12 months	
	I[a]	C[b]	I	C	I	C	I	C	I	C
I	2,100	<300	<300	<300	<300	<300	<300	<300	<300	<300
II	900	<300	<300	<300	<300	<300	<300	<300	<300	3,100
III	1,700	<300	<300	<300	<300	<300	1,100	900	4,100	9,700
IV	9,000	<300	<300	<300	<300	<300	650	<300	7,000	3,400
V	7,200	<300	<300	<300	<300	<300	440	<300	<300	<300

[a]Inoculated.

[b]Control.

Acid Degree Values of Cheese

Data in Table 10 show the ADV of the five lots of cheese made from milk inoculated with Pseudomonas fragi 169 and controls. Data in Table 11 show the ADV of five lots of cheese made from milk inoculated with Pseudomonas fluorescens 31 and controls. A higher ADV was noted for all lots of inoculated cheese than for controls at every testing period. The largest ADV increase was noted between two weeks and three months of curing. Inoculated lot III and inoculated and control lots IV in Table 10 and inoculated lots II, III and IV in Table 11 had ADV peaks at six months of curing. Inoculated lots II and V in Table 10 and control lot II in Table 11 had secondary ADV peaks at nine months curing. Inoculated and control lots V in Table 11 had their highest ADV at twelve months curing.

The analyses of variance of ADV from Table 10 and Table 11 is shown in Table 12. A significant difference occurred in the ADV between treatments, inoculated and control. This indicates that heat stable lipases produced by the culture increased the ADV in cheese made from inoculated milk. The data also show a significant difference between cultures, Pseudomonas fragi 169 and Pseudomonas fluorescens 31. Significant differences were also shown in the same lots of cheese at different curing ages. No significant difference was found in the interaction of cultures and treatments, indicating that the same relationship of higher ADV in inoculated over control cheese exist with both cultures. This lends confidence to the significant difference between inoculated and control cheese and further indicates that the higher ADV in the inoculated cheese were the result of the action

Table 10. Acid Degree Values of five lots of Cheddar cheese inoculated with Pseudomonas fragi 169 and corresponding controls

Age	Lot I		Lot II		Lot III		Lot IV		Lot V	
	I[a]	C[b]	I	C	I	C	I	C	I	C
2 wk.	1.571	1.432	1.575	1.440	1.441	1.336	1.311	1.255	1.472	1.313
3 mo.	2.284	1.932	2.000	1.684	2.040	1.713	1.933	1.912	2.015	1.971
6 mo.	2.051	1.862	1.902	1.708	2.215	1.727	2.100	1.975	1.862	1.586
9 mo.	1.911	1.708	1.997	1.897	1.967	1.662	1.784	1.708	1.949	1.584
12 mo.	1.713	1.491	1.851	1.566	1.886	1.655	1.953	1.680	1.857	1.673

[a]Inoculated.

[b]Control.

Table 11. Acid Degree Values of five lots of Cheddar cheese inoculated with Pseudomonas fluorescens 31 and corresponding controls

Age	Lot I		Lot II		Lot III		Lot IV		Lot V	
	I[a]	C[b]	I	C	I	C	I	C	I	C
2 wk.	1.462	1.385	1.450	1.365	1.400	1.320	1.367	1.320	1.401	1.301
3 mo.	2.006	1.840	2.044	1.706	1.913	1.762	1.657	1.564	1.757	1.411
6 mo.	1.855	1.651	2.157	1.688	1.986	1.584	1.666	1.327	1.682	1.333
9 mo.	1.773	1.638	1.720	1.368	1.815	1.627	1.644	1.535	1.749	1.432
12 mo.	1.918	1.611	1.786	1.455	1.757	1.755	1.748	1.544	1.855	1.600

[a]Inoculated.

[b]Control.

Table 12. Analysis of variance of acid degree values of ten lots of inoculated and control Cheddar cheese when Pseudomonas fragi 169 and Pseudomonas fluorescens 31 were used

Source	df	SS	MS	F
Total	99	5,560,661		
Lots	4	203,846	50,961	5.291**
Treatments (I & C)	1	1,129,583	1,129,583	117.421**
Culture	1	413,449	413,449	42.978**
Age	4	2,544,438	636,101	66.122**
C x T	1	353	353	.036^{ns}
C x A	4	155,330	38,832	4.036**
T x A	4	107,681	26,920	2.798**
C x T x A	4	274,798	68,699	7.141**
Error	76	731,183	9,620	

ns = No significant difference.

** = Significance at .05 level.

of heat stable lipase(s) upon the fat of the cheese. The standard deviation ± .010 ml. for eight replicate titrations of control cheese fat with 0.02 normal potassium hydroxide is presented in Table 13. This standard deviation indicates that increases in titrations of inoculated over control represent real increases in the ADV.

Taste Panel Evaluation of Cheese

The results of the triangle taste panel for five lots of cheese inoculated with Pseudomonas fragi 169 and controls are given in Table 14. The number of correct responses in the triangle test were plotted against the number of judgements made on a graph in the Merck Technical Bulletin (41). Points falling on or above the lower curve indicated significance at the ($P \leq 0.05$) level. Points on or above the upper curve indicated significance at the ($P \leq 0.01$) level. When the five lots of cheese and controls were considered, the taste panel found no significant difference between inoculated and controls until the ninth month of curing. A difference at the ($P \leq .01$) level was found in the five lots after twelve months curing.

Data in Table 15 show the triangle taste panel results of the five lots of cheese made from milk inoculated with Pseudomonas fluorescens 31 and controls. A significant difference between all five lots of inoculated and control samples could be detected only after twelve months of curing.

The results of the preference test scored on the hedonic scale were not considered valid for use in a statistical analysis due to the low number of correct answers and very small differences in

Table 13. Titration of 8 replicate one milliliter cheese fat samples with 0.02 normal alcoholic potassium hydroxide

Cheese fat replicates (N)	Milliliters of 0.02 normal KOH	$(X)^2$
1	0.756	.571536
2	0.775	.600625
3	0.784	.614656
4	0.775	.600625
5	0.768	.589824
6	0.776	.602176
7	0.757	.573049
8	0.766	.586756
	ΣX 6.157	ΣX^2 4.739247

$$\text{Standard deviation} = \sqrt{\frac{\Sigma X^2 - \frac{\Sigma (X)^2}{N}}{N}} = \sqrt{\frac{4.739247 - 4.738581}{8}} = \pm .010.$$

Table 14. Triangle taste panel results comparing five lots of Cheddar cheese inoculated with Pseudomonas fragi 169 and controls

Lot	Age							
	3 months		6 months		9 months		12 months	
	Right	Wrong	Right	Wrong	Right	Wrong	Right	Wrong
I	4	6	2	6	7*	3	3	6
II	3	7	4	4	3	7	3	6
III	4	6	2	7	4	5	3	6
IV	5	5	7*	2	4	5	9**	0
V	4	6	3	7	4	4	4	4
Total	20^{ns}	30	18^{ns}	26	22*	24	22**	22
No. of judgments	50		44		46		44	

ns = No significance.

* = Significance at .05 level.

** = Significance at .01 level.

Table 15. Triangle taste panel results comparing five lots of Cheddar cheese inoculated with Pseudomonas fluorescens 31 and controls

Lot	Age							
	3 months		6 months		9 months		12 months	
	Right	Wrong	Right	Wrong	Right	Wrong	Right	Wrong
I	0	10	2	8	1	7	4	4
II	1	9	3	6	4	4	5*	3
III	4	5	3	6	4	4	5*	3
IV	5	5	4	4	0	8	1	7
V	4	6	5	5	6*	2	3	5
Total	14^{ns}	35	17^{ns}	29	15^{ns}	25	18*	22
No. of judgments	49		46		40		40	

ns = No significance.

* = Significance at .05 level.

preference recorded by the judges. No correlation was attempted between the ADV and the period of curing when flavor differences appeared, due to early increase in the ADV and late appearance of flavor differences.

The average flavor scores for the five lots of inoculated and control cheese for each of the cultures Pseudomonas fragi 169 and Pseudomonas fluorescens 31 are given in Table 16 and Table 17 in the appendix. A very small difference in flavor score between any inoculated or control cheese was noted. The scores ranged from 37.5 to 39.5 indicating that all lots of cheese were of good flavor quality.

Data in Table 18 and Table 19 in the appendix show the butterfat content of each lot of milk used and the butterfat and moisture content of each inoculated and control lot of cheese. The data presented in these tables have little bearing on the ADV of the cheese but were added to further complete the records of all lots of cheese.

In summary, cultures of psychrophilic organisms growing in milk at populations of 3 to 5 million and producing heat stable lipase(s) can significantly increase the ADV of pasteurized milk Cheddar cheese. Significant flavor differences could be detected between inoculated cheese and control cheese after aging for nine to twelve months. Flavor differences appeared after the ADV had declined except for one lot of cheese.

The results indicate that if several heat stable lipase-producing psychrophiles were present in milk at or above the maximum limit for class 2 milk at the time of pick-up from the farm, the flavor of pasteurized milk Cheddar cheese would not be greatly effected.

However, if this milk were stored for several hours or days at temperatures of 5 to 7°C before pasteurization, the psychrophilic bacteria could increase in number beyond those studied. The flavors of cheese produced from such high count milk might become more pronounced than those observed at the level of this study.

CHAPTER V

SUMMARY AND CONCLUSION

The influence of heat stable bacterial lipase(s) on the flavor and fat degradation in pasteurized milk Cheddar cheese was studied. Acid Degree Values increased more between two weeks and three months curing than in any subsequent three month curing period. However, the ADV of some lots of cheese did not reach a peak until six or nine months of curing while one lot was still increasing at twelve months. Several lots of cheese had secondary peaks of ADV appearing later in the curing period. Significant differences in ADV were found between lots of cheese made from milk inoculated with Pseudomonas fragi 169 or Pseudomonas fluorescens 31 and cheese made from uninoculated control lots of milk. The inoculated lots had higher ADV than control lots at every testing period.

A taste panel using the triangle taste test method found significant differences in the flavor of inoculated and control samples of cheese made from milk inoculated with Pseudomonas fragi 169 after nine and twelve months curing. The taste panel did not find a significant difference in inoculated and control lots of cheese inoculated with Pseudomonas fluorescens 31 until after twelve months curing. Flavor scores of all cheese lots after twelve months curing indicate that increased ADV in inoculated lots did not detract from the cheese flavor although a difference could be detected between inoculated and control lots.

The results of this study indicate that heat stable lipase(s) produced by psychrophilic bacteria of the Pseudomonas species present within the range of 3 to 5 million per ml. can cause increased lipolysis of pasteurized milk Cheddar cheese fat. Also, minor flavor effects may result but probably will not detract noticeably from the flavor of aged Cheddar cheese.

LIST OF REFERENCES

LIST OF REFERENCES

(1) Albrecht, T. W., and H. O. Jaynes. 1955. Milk lipases. J. Dairy Sci., 38:137.

(2) Alford, J. A., and D. A. Pierce. 1963. Production of lipase by Pseudomonas fragi in a synthetic medium. J. Bacteriol., 86:24.

(3) American Public Health Association. 1967. Standard Methods for the Examination of Dairy Products. 12th ed. American Public Health Association, Inc. New York, New York.

(4) Babel, F. J., and B. W. Hammer. 1943. Fat acidities of Cheddar cheese made with added lipase. J. Dairy Sci., 26:773.

(5) Babel, F. J., and B. W. Hammer. 1945. Fat degradation in Cheddar cheese made from pasteurized milk without and with added lipase. J. Dairy Sci., 28:201.

(6) Bills, D. D., and E. A. Day. 1964. Determination of the major free fatty acids of Cheddar cheese. J. Dairy Sci., 47:733.

(7) Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's Manual of Determinative Bacteriology. 7th ed. The Williams and Wilkins Company, Baltimore, Md.

(8) Charrington, V. A., and B. W. Hammer. 1948. Relationship of micro-organisms to the disappearance of rancidity in Cheddar cheese. Res. Bull. Idaho Agr. Exp. Sta. 14.

(9) Csiszar, J., and A. Romlehner-Bakos. 1956. The heat resistance of lipase produced by Pseudomonas fluorescens. Int. Dairy Congr., 2(1):48.

(10) Cutchins, E. C., R. N. Doetsch, and M. J. Pelczar. 1952. The influence of medium composition on the production of bacterial lipase. J. Bacteriol., 63:269.

(11) Dacre, J. C. 1955. A chemical investigation of the volatile flavor principle of Cheddar cheese. J. Dairy Res., 22:219.

(12) Dahlberg, A. C., and F. V. Kosikoski. 1947. The flavor, volatile acidity, and soluble protein of Cheddar and other cheeses. J. Dairy Sci., 30:165.

(13) Dahlberg, A. C., and F. V. Kosikoski. 1947. Flavor development in Cheddar cheese. Natl. Butter and Cheese J., 38:44.

(14) Day, E. A., and M. Kenney. 1958. Identification of volatile carbonyls from Cheddar cheese. J. Dairy Sci., 41:718.

(15) Franklin, J. G., and M. E. Sharpe. 1963. The incidence of bacteria in cheese milk and Cheddar cheese and their association with flavor. J. Dairy Res., 30:87.

(16) Fryer, T. E., M. E. Sharpe, and B. Reiter. 1966. The microflora of Cheddar cheese made aseptically from milk containing a standard raw milk reference flora. Proc. XVII Int. Dairy Cong., D 61. (Dairy Sci. Abstracts, 3605, 1966.)

(17) Hiscox, E. R., and J. Harrison. 1938. Volatile acids of cheese. I. Retentive power of cheese and its constituents. J. Dairy Res., 9:227.

(18) Hiscox, E. R., J. Harrison, and J. Z. Volf. 1938. Volatile acids of cheese. II. Methods of extraction. J. Dairy Res., 9:215.

(19) Hiscox, E. R., J. Harrison, and J. Z. Volf. 1938. Volatile acids of cheese. III. Application of the extraction method. J. Dairy Res., 12:165.

(20) Hlynka, I., and E. G. Hood. 1942. Lipase in relation to flavor in cheese. J. Dairy Sci., 25:111.

(21) Hlynka, I., and E. G. Hood. 1944. Keeping quality of cheese with rancid and unclean flavors. Canad. Dairy Ice Cr. J., 23(2):35.

(22) Hlynka, I., and E. G. Hood. 1947. The application of fat-acidity determination to a study of lipolytic flavor defects in Cheddar cheese. J. Dairy Res., 15:94.

(23) Hlynka, I., E. G. Hood, and C. A. Gibson. 1943. Agitation and temperature of cheese milk and the development of rancid unclean flavors in Cheddar cheese. J. Dairy Sci., 26:1111.

(24) Hylnka, I., E. G. Hood, and C. A. Gibson. 1944. Further studies on the effect of agitation and temperature as a cause of rancidity in cheese. Canad. Dairy Ice Cr. J., 23(3):26.

(25) Hlynka, I., E. G. Hood, and C. A. Gibson. 1947. The influence of acidity on the development of lipolytic defects in Cheddar cheese. Sci. Agr., 27:50.

(26) Hood, E. G., C. A. Gibson, and J. F. Bowen. 1949. Lipolytic bacteria a cause of rancidity in Cheddar cheese. Canad. Dairy Ice Cr. J., 28(2):27.

(27) Hood, E. G., C. A. Gibson, and J. F. Bowen. 1949. Further studies with lipolytic bacteria and rancid flavours in Cheddar cheese. Canad. Dairy Ice Cr. J., 28(6):27.

(28) Hood, E. G., I. Hlynka, and C. A. Gibson. 1941. Rancidity in Cheddar cheese--An analysis of the problem. Canad. Dairy Ice Cr. J., 20:26.

(29) Hunter, A. C., J. M. Wilson, and W. B. Grace. 1968. A modification of the Acid Degree Value Test for lipolytic rancidity in milk. J. Dairy Res. 35:19.

(30) Irvin, O. R. 1946. Contributions to the study of rancidity in Canadian Cheddar cheese. III. A comparison of the lipolytic activity of slight rancid and first grade samples. Sci. Agr., 26(1):7.

(31) Irvin, O. R., D. H. Bullock, and W. H. Sproule. 1954. Flavor development in pasteurized milk Cheddar cheese. I. The effect of inoculating milk with Geotrichum Candidum. J. Dairy Sci., 37:637.

(32) Kosikowski, F. V., and A. C. Dahlberg. 1946. A rapid direct-distillation method for determining the volatile fatty acids of cheese. J. Dairy Sci., 29:861.

(33) Kristoffersen, I., and I. A. Gould. 1960. Cheddar cheese flavor. II. Changes in flavor quality and ripening products of commercial Cheddar cheese during controlled curing. J. Dairy Sci., 43:1202.

(34) Laboratory Manual. Methods of Analysis of Milk and Its Products. 1959. 3rd ed. Milk Industry Foundation. Washington, D.C.

(35) Lane, C. B., and B. W. Hammer. 1939. Studies on the fat of Cheddar cheese. Iowa State Col. J. Sci., 13:149.

(36) Lane, C. B., and B. W. Hammer. 1941. Bacteriology of cheese. VI. Relationship of fat hydrolysis to the ripening of Cheddar cheese. Iowa Agr. Exp. Sta. Bull. 291.

(37) Lubert, D. J., L. M. Smith, and H. R. Thornton. 1949. Estimation of lipase in dairy products. III. Lipase activity in cultures of microorganisms and in cheese. Canad. J. Res., F 27:499.

(38) Mabbitt, L. A., and M. Zielinska. 1956. Flavour production in Cheddar cheese. Int. Dairy Congr., 2(2):323.

(39) Marquardt, J. C., and A. C. Dahlberg. 1941. Determining volatile acids in cheese. Natl. Butter and Cheese J. 32(8):40.

(40) Mencher, J. R., and J. A. Alford. 1967. Purification and characterization of the lipase of Pseudomonas fragi. J. Gen. Microbiol., 48:317.

(41) Merck Technical Bulletin. 1963. An Introduction to Taste Testing of Foods. Food Products Department, Merck Chemical Division. Merck and Co., Inc. Rahway, New Jersey.

(42) Morrison, H. B., and B. W. Hammer. 1941. Distribution of Pseudomonas fragi. J. Dairy Sci., 24:9.

(43) Nashif, S. A., and F. E. Nelson. 1953. The lipase of Pseudomonas fragi. I. Characterization of the enzyme. J. Dairy Sci., 36:459.

(44) Nashif, S. A., and F. E. Nelson. 1953. The lipase of Pseudomonas fragi. II. Factors affecting lipase production. J. Dairy Sci., 36:471.

(45) Nashif, S. A., and F. E. Nelson. 1953. The lipase of Pseudomonas fragi. III. Enzyme action in cream and butter. J. Dairy Sci., 36:481.

(46) Nashif, S. A., and F. E. Nelson. 1953. The extracellular lipases of some gram-negative non-sporeforming rod-shaped bacteria. J. Dairy Sci., 36:698.

(47) Ohren, J. A. 1966. The relation of flavor development in Cheddar cheese to chemical changes in the fat of cheese. Diss. Abstr. 26:7252. (Univ., Urbana, Illinois.)

(48) Ohren, J. A., and S. L. Tuckey. 1965. Relation of fat hydrolysis to flavor development in Cheddar cheese. J. Dairy Sci., 48:765.

(49) Ohren, J. A., and S. L. Tuckey. 1969. Relation of flavor development in Cheddar cheese to chemical changes in the fat of cheese. J. Dairy Sci., 52:598.

(50) Overcast, W. W., and J. D. Skean. 1970. Unpublished data.

(51) Patton, S. 1963. Volatile acids and the aroma of Cheddar cheese. J. Dairy Sci., 46:856.

(52) Peterson, M. H., and M. J. Johnson. 1948. The estimation of fatty acids of intermediate chain length by partition chromatography. J. Biol. Chem., 174:775.

(53) Peterson, M. H., and M. J. Johnson. 1949. Delayed hydrolysis of butterfat by certain lactobacilli and micrococci isolated from cheese. J. Bacteriol., 58(5):701.

(54) Peterson, M. H., M. J. Johnson, and W. V. Price. 1943. Determination of milk lipase. J. Dairy Sci., 26:233.

(55) Peterson, M. H., M. J. Johnson, and W. V. Price. 1948. Determination of cheese lipase. J. Dairy Sci., 31:31.

(56) Peterson, M. H., M. J. Johnson, and W. V. Price. 1948. Lipase activity during making and ripening of Cheddar cheese. J. Dairy Sci., 31:39.

(57) Peterson, M. H., M. J. Johnson, and W. V. Price. 1949. Liberation of fatty acids during making and ripening of Cheddar cheese. J. Dairy Sci., 32:862.

(58) Pinheiro, A. J. R., B. J. Liska, and C. E. Parmelee. 1965. Heat stability of lipases of selected psychrophilic bacteria in milk and Purdue Swiss-type cheese. J. Dairy Sci., 48:983.

(59) Price, W. V., and H. E. Calbert. 1953. Cheddar cheese from pasteurized milk. Wisc. Agr. Exp. Sta. Bull., 464 (revised).

(60) Reder, R. 1938. The chemical composition and properties of normal and rancid Jersey milk. J. Dairy Sci., 21:369.

(61) Reiter, B., T. F. Fryer, A. Pickering, H. R. Chapman, R. C. Lawrence, and M. E. Sharpe. 1967. The effect of the microbial flora on the flavour and free fatty acid composition of Cheddar cheese. J. Dairy Res., 34:257.

(62) Reiter, B., T. F. Fryer, M. E. Sharpe, and R. C. Lawrence. 1966. Studies on cheese flavour. J. Appl. Bacteriol., 29:231.

(63) Rice, F. E., and A. L. Markley. 1922. Proof of the presence of lipase in milk and a new method for the detection of the enzyme. J. Dairy Sci., 5:64.

(64) Sandhu, S. M. M. 1967. Contributions of the heat-resistant lipase of psychrophilic bacteria to off-flavors in pasteurized milk. M.S. Thesis. University of Tennessee, Knoxville.

(65) Scarpellino, R. J. 1961. The application of gas chromatographic techniques to the study of volatile compounds in Cheddar cheese. Diss. Abstr., 22(2):421. (Cornell Univ., Ithaca, N.Y.)

(66) Scarpellino, R. J., and F. V. Kosikowski. 1958. Methyl ethyl ketone and other carbonyl compounds in Cheddar cheese. J. Dairy Sci., 41:718.

(67) Scarpellino, R., and F. V. Kosikowski. 1962. Evolution of volatile compounds in ripening raw and pasteurized milk Cheddar cheese observed by gas-chromatography. J. Dairy Sci., 45:343.

(68) Scott, K. 1962. A method for measuring the lipase activity of Cheddar cheese. Queenland J. Agr. Sci., 19:101.

(69) Sheuring, J. J., and S. L. Tuckey. 1947. A study of the changes occurring in the fat constants of milkfat of Cheddar cheese during the ripening process. J. Dairy Sci., 30:803.

(70) Silverman, G. J., and F. V. Kosikowski. 1953. Observations on cheese flavor production by pure chemical compounds. J. Dairy Sci., 36:574.

(71) Skean, J. D., and W. W. Overcast. 1961. Apparent location of lipase in casein. J. Dairy Sci., 44:823.

(72) Smiley, K. L., F. V. Kosikowski, and A. C. Dahlberg. 1946. A simplified extraction-distillation method for the determination of the volatile fatty acids of cheese. J. Dairy Sci., 29:307.

(73) Stadhouders, J. J. 1956. The hydrolysis of cheese fat in relation to the flavour of cheese. Landbouwhogeschool, Wageningen:Thesis. (Dairy Sci. Abstracts, 19, II, 592.)

(74) Stadhouders, J., and H. Mulder. 1956. The hydrolysis of fat during the ripening of hard cheese. Int. Dairy Congr., 2(2):907.

(75) Stadhouders, J., and H. Mulder. 1957. Fat hydrolysis and cheese flavours. I. The enzymes responsible for the hydrolysis of fat in cheese. Neth. Milk Dairy J., 11(2):164.

(76) Stadhouders, J., and H. Mulder. 1959. Fat hydrolysis and cheese flavours. III. Surface organisms associated with fat hydrolysis in cheese. Neth. Milk Dairy J., 13:291.

(77) Stadhouders, J., and H. Mulder. 1960. Fat hydrolysis and cheese flavours. IV. Fat hydrolysis in cheese from pasteurized milk. Neth. Milk Dairy J., 14(2):141.

(78) Steel, R. G. D., and J. H. Torrie. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Co., Inc., New York.

(79) Suzuki, S. K., E. G. Hastings, and E. B. Hart. 1910. The production of volatile fatty acids and esters in Cheddar cheese and their relation to the development of flavor. Wis. Agr. Exp. Sta. Res. Bull. 11.

(80) Thomas, E. L., A. J. Nielson, and J. C. Olson, Jr. 1955. Hydrolytic rancidity in milk--A simplified method for estimating the extent of its development. American Milk Review, 17(1):50.

(81) Van Slyke, L. L. 1891. Investigation of cheese. Experiments in the manufacture of cheese. Influence of composition of milk on composition and yield of cheese. A study of the process of ripening of cheese. N.Y. (State) Agr. Exp. Sta. Bull. 37.

(82) Wilcox, J. C., W. O. Nelson, and W. A. Wood. 1955. The selective release of volatile acids from butterfat by microbial lipases. J. Dairy Sci., 38:775.

(83) Windlan, H., and F. V. Kosikowski. 1956. The influence of milk-coagulating enzymes of nonanimal origin upon the flavor and constituents of Cheddar cheese. J. Dairy Sci., 39:917.

APPENDIX

TYPICAL CHEDDAR CHEESE MANUFACTURING RECORD

Maker	Vat	Date
Milk lbs.	Plate count estimate or Methylene blue of raw milk	hrs.
Starter lbs.	Flavor of Milk	
Total lbs.	Starter acid %	Age hrs.
Original milk fat %	lbs. Milk snf. %	lbs.
Standardized milk fat %	lbs. Whey snf. %	lbs.

Operation	Time	Temperature	Acid %	Rennet test	Comments
Pasteurization					Date of pasteurization
Added starter					Kind %
Added color					Kind Amt.
Added rennet					Amount
Coagulation				Hot Iron	pH
Cutting					Size of knives
Steam on					
Steam off					
Settling					
Dipping					pH
Packing					
Piling 2 high					
Piling 3 high					
Milling					pH
Salting					Amount
Pressing					
Dressing					

TYPICAL TRIANGLE TASTE PANEL SCORE CARD

Name_____ Code_____

Sample_____ Sample_____

Different 1_____ 2_____ 3_____ Different 1_____ 2_____ 3_____

Preference Preference

Different sample		Matching sample
Like	9	
	8	
	7	
	6	
	5	
	4	
	3	
	2	
Dislike	1	

Different sample		Matching sample
Like	9	
	8	
	7	
	6	
	5	
	4	
	3	
	2	
Dislike	1	

Sample_____ Sample_____

Different 1_____ 2_____ 3_____ Different 1_____ 2_____ 3_____

Preference Preference

Different sample		Matching sample
Like	9	
	8	
	7	
	6	
	5	
	4	
	3	
	2	
Dislike	1	

Different sample		Matching sample
Like	9	
	8	
	7	
	6	
	5	
	4	
	3	
	2	
Dislike	1	

CHEDDAR CHEESE FLAVOR SCORE GUIDE

Flavor criticisms	Intensity of defect		
	Slight	Definite	Pronounced
Acid	39	37	35
Bitter	39	37	34
Feed	39	38	36
Fermented/Fruity	38	36	35
Flat	39.5	38.5	37
Garlic/Onion	36	34	31
Heated	39	38	37
Moldy	37	35	33
Rancid	36	34	31
Sulfide	39	37	34
Unclean	38	36	35
Whey taint	38	37	35
Yeasty	36	34	31

Range of score for each class of flavor quality for Cheddar cheese:

Excellent	40 (No criticism)
Good	38-39.5
Fair	36-37.5
Poor	35.5 or less
Normal range	31-40

Table 16. Average flavor scores of five lots of Cheddar cheese inoculated with Pseudomonas fragi 169 and corresponding controls

Lot	Inoculated	Control
I	38.5	38.0
II	38.5	39.0
III	38.5	38.0
IV	38.0	38.0
V	39.5	38.5

Table 17. Average flavor scores of five lots of Cheddar cheese inoculated with Pseudomonas fluorescens 31 and corresponding controls

Lot	Inoculated	Control
I	37.5	38.0
II	38.0	38.5
III	37.5	38.0
IV	39.0	38.5
V	38.0	39.0

Table 18. Butterfat of cheese milk and butterfat and moisture of cheese inoculated with Pseudomonas fragi 169, and corresponding controls

Lot	Butterfat of milk	Cheese butterfat		Cheese moisture	
		I[a]	C[b]	I	C
I	3.7	34.5	32.5	34.59	33.06
II	3.6	37.0	36.0	30.15	36.72
III	3.6	34.0	34.0	33.31	34.49
IV	3.7	34.5	34.0	32.57	32.60
V	3.9	35.5	34.0	34.42	35.60

[a]Inoculated.

[b]Control.

Table 19. Butterfat of cheese milk and butterfat and moisture of cheese inoculated with Pseudomonas fluorescens 31, and corresponding controls

Lot	Butterfat of milk	Cheese butterfat		Cheese moisture	
		I[a]	C[b]	I	C
I	3.9	38.5	36.5	32.32	33.52
II	3.9	35.0	34.5	35.03	34.01
III	3.9	34.5	34.5	34.31	35.95
IV	3.9	34.0	34.0	37.64	36.86
V	3.9	34.5	34.0	33.72	34.76

[a]Inoculated.

[b]Control.

VITA

Daniel Nelson Hamilton was born in Athens, Tennessee, on June 15, 1942. He was educated in the elementary schools of McMinn County and was graduated from McMinn County High School in 1960. The following September he entered Tennessee Wesleyan College for a period of part-time study and work. He transferred to the University of Tennessee in September 1964, and received a Bachelor of Science degree in Dairy Manufacturing in March 1967. He entered the graduate school of the University of Tennessee on assistantship in March 1967, for advanced study in Dairy Manufacturing.

He is married to the former Nancy Keene of Edison, New Jersey.