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The low temperature microflora of young cheddar cheese

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THE LOW TEMPERATURE MICROFLORA OF YOUNG CHEDDAR CHEESE

by

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INTRODUCTION

Recently, a comment was made by Dr. P. Swartling, of Sweden, regarding the microflora of young Cheddar cheese. Dr. Swartling, in a discussion at the 16th International Dairy Congress held in Denmark during September, 1962, stated:

> It is thus evident that the study of the dominating flora of young cheeses under normal and abnormal conditions is an important field for investigation. On this occasion it would be of great interest to hear from those present what information is now available concerning the flora of various cheese varieties when fresh. (73, p. 198)

Cheese has been made for thousands of years either by recipe or by use of empirical methods. Increasing knowledge in the field of dairy technology, however, has developed many important facets of learning within the past century. This new fund of information has helped to explain situations and conditions taken for granted in the past, as well as to open new and challenging areas of research for the future. Certainly, the subject of the bacteriology of Cheddar cheese fits into both of these areas. For example, prior to the time that the presence of bacteria had been demonstrated in milk, successful cheese makers actually encouraged growth of desirable organisms by establishing conditions favorable for microbial growth and activity. Many traditional methods of cheese making were instituted in this way. The practice of saving whey from cheese made one day for addition to cheese milk the next day is only one example. By the end of the nineteenth cen-

tury, when bacteria had been recognized and were valued for their beneficial role to mankind, this practice had led to the use of specific starter cultures. For the future, the problem of bacteriophage and its responsibility for starter failures will continue to receive widespread attention. Continuing accrual of knowledge in dairy bacteriology, specifically in the area of the bacteriology of Cheddar cheese, will aid investigations into this problem.

Bacteriologists have been studying the bacteriology and chemistry of Cheddar cheese for years. Particular interest has been shown in the area of cheese ripening as evidenced by increasing attention to the use of plant enzymes to replace rennet, flexible and impermeable packaging materials and the constant efforts regarding development, handling and preservation of cultures used for cheese making. Most specific investigations into the bacteriology of Cheddar cheese have dealt with attempts to isolate and identify microorganisms of importance in flavor production and rapid body break-down.

This study was undertaken to determine the low temperature microflora of young Cheddar cheese.

A search of the literature has failed to reveal any previous attempts to study the Cheddar cheese microflora capable of growth at low temperatures. This approach was felt to be important in view of the fact that Cheddar cheese most commonly is cured at low temperatures. It was hoped that a study

of these microorganisms might reveal characteristics of importance in cheese ripening. In addition, numerous possibilities exist for further application of this study, either in the manufacture or ripening of natural cheese.

LITERATURE REVIEW

The bacteriology of Cheddar and other varieties of cheese has been studied by a large number of investigators. These workers have been concerned either with the entire flora or with a specific group of bacteria present in the cheese. Because considerable literature pertaining to both types of studies is available, only the more pertinent investigations regarding the microflora of Cheddar cheese will be reviewed.

The Bacterial Flora of Cheddar Cheese

One of the earliest reports of the bacterial flora of cheese was made by Russell (61), who stated that the bacterial content of cheese differed markedly from that of milk. He found that in milk the lactic acid bacteria predominated, but always were accompanied by liquefying or peptonizing organisms, and, as a rule, bacteria capable of developing gaseous by-products. In the ripening cheese, peptonizing or casein digesting bacteria were eliminated quickly while the gas-producing bacteria disappeared more slowly, sometimes persisting in very small numbers for a long time. The lactic acid bacteria, on the other hand, developed rapidly for a time until the cheese was partially ripened. At this time they too began to diminish in numbers.

Harding and Prucha (29) studied the microflora of nine normal cheeses representing four first-class commercial fac-

tories. Over 300 cultures were isolated from agar plates incubated at room temperature (which ranged from 21 to 31 C depending upon the season of the year) for 10 days. Pure cultures were studied both morphologically and culturally, duplicates being eliminated from further examinations. A total of 167 cultures were studied extensively and divided into 33 different groups. Results of the qualitative study of the cheeses showed that Bacterium lactis acidi was the only form constantly found in all Cheddar cheeses. This organism practically always composed over 99% of the total bacterial count. In addition to Bacterium lactis acidi, four other groups of microorganisms were found. These were: acid liquefiers, gas-producing forms, an inert group and yeasts. The acid liquefiers occurred sufficiently often to suggest that they might play some part in ripening. The gas-producing forms made little or no growth in cheese. Bacterium coli aerogenes was isolated from cheese only through the first day of ripening. Bacterium lactis aerogenes was found in considerable numbers in some cheeses and was very resistant to acidity. The inert group consisted of organisms lacking apparent action in milk. This group was fairly numerous, at times, in milk and fresh curd. These organisms usually disappeared quickly and no evidence was found to indicate that they increased in the cheese. Very few species of yeasts were found although an effort was made to prepare plates especially suitable to

their growth habits.

In 1912, Hastings et al. (30, 31) reported finding only two groups of bacteria constantly in large numbers in Cheddar cheese. These were Bacterium lactis acidi and Bacillus bulgaricus. The former grew until the sugar was completely fermented while the latter, although reaching numbers comparable to the former, grew after the fermentation of sugar. During the early part of the ripening period, Bacterium lactis acidi comprised over 90%, and, sometimes, even 100% of the lactic acid bacteria. In the late ripening period, Bacillus bulgaricus predominated, and in many cheeses consisted of over 90% of the lactic acid flora. Coccus forms producing small amounts of acid occasionally were found to be the commonest type of organism. This type was found to predominate at some time or other in 11 of the 13 cheeses sampled. The chromogenic cocci and liquefying forms found were considered unimportant in ripening of the cheese as they were not consistently present in large numbers.

Evans et al. (22), from a study of 21 raw-milk cheeses, reported that all organisms constantly found in cheese were included in four groups: Bacterium lactis acidi, Streptococcus, Micrococcus and Bacterium casei. Variations in number and flora were observed; however, all good cheeses contained the four groups listed. Spore-forming organisms and liquefying types occurred in very small numbers or were absent en-

tirely. Whenever these forms were present in sufficient numbers to have an appreciable influence on the ripening, it was to the detriment of good flavor. The bacterial flora of pasteurized-milk cheese was found to be dependent upon the starter used.

In a study of the streptococci concerned in cheese ripening, Evans (21) frequently encountered three streptococcus strains. These were termed S. lacticus, S. kefir and S. X. The three strains were described culturally and biochemically and their role in the ripening of Cheddar cheese was discussed.

Hucker (34) examined 37 samples of Cheddar cheese obtained from approximately 25 factories. Results indicated that the better grades of cheese contained a distinctly different flora than that of the poorer grades. In the better grades, S. lactis and the lactobacilli predominated while in the poorer grades, spore-forming rods and gram-negative rods were present in the largest numbers. Cocci, and streptococci other than S. lactis, varied little in numbers between different quality cheeses. Emphasis was placed on the finding of a large number of spore-forming and gram-negative rods in the poorer grade cheeses as being indicative that these organisms are undesirable for the production of a high-grade Cheddar cheese. In this investigation, 265 organisms were isolated from agar plates incubated at 25 C for 5 days. The organisms were studied and placed into the following groups in decreasing order of frequency of occurrence: spore-formers, gram-

negative rods, lactobacilli, S. lactis, cocci, streptococci other than S. lactis and yeasts, only three of which were recovered. Observation of the forms of gelatin liquefiers revealed that in poorer grades of Cheddar cheese, the majority were rod-shaped. In the good grades of Cheddar, the majority of gelatin liquefiers were cocci.

In 1932, Kelly (37) reported the findings of his study of 200 isolates obtained from normal Cheddar cheese during the first 3 weeks of ripening. Isolations were made from agar plates incubated at 37 and 25 C. Classification was determined by morphology and carbohydrate fermentation. Approximately one-half of the isolates, 102, were S. lactis; another 20 were S. cremoris. Other organisms found were Micrococcus sp., Leuconostoc sp. and gram-positive rods. In both commercial Cheddar cheese made without starter, and in cheese made using pure cultures of S. lactis and S. cremoris, S. lactis was found to comprise about 50% of the flora. Streptococcus cremoris constituted 9% of the flora in the commercial cheese and 26% of the flora of starter-made cheese.

Allen and Knowles (3), using various selective media to enumerate different groups of bacteria in Cheddar cheese, reported that in cheeses made from milk of a very low bacterial count, the predominant flora consisted of lactic acid streptococci for at least the first 19 to 20 weeks. They also reported that some atypical organisms isolated from

cheese possibly were derived from more typical strains of lactic acid streptococci whose characters had become modified in the course of cheese ripening. These workers emphasized the fact that the flora of Cheddar cheese tends to develop in stages; one set of microorganisms having persisted through conditions adverse to their active multiplication subsequently take the place of another set when growth conditions have altered.

Cheeses were plated at 28 C for 10 days by Davis (14). He incubated the agar plates with a second layer of the medium added over the first to insure microaerophilic conditions. Of the 248 colonies picked and studied, representing 18 Cheddar cheeses at different stages of curing from Cheddaring to 18 months of age, 247 were true lactic acid bacteria. Streptococci made up 117 and lactobacilli 130 of this total. The lactic acid bacteria were placed into four groups: S. lactis, S. cremoris, Streptobacterium plantarum and Sbm. casei. Streptococci were found which differed from the two recognized groups, however, the differences were not felt to be great enough to justify separate species. Some S. lactis strains grew in litmus milk at 50 C, which indicated to this worker the possible relationship of these strains to S. faecalis. The streptococci were found during the first month of ripening, but seldom thereafter. Lactobacilli were found starting at 7 days; these predominated after a month.

The bacterial flora of New Zealand Cheddar cheese was investigated by Sherwood (70). He isolated 720 strains from 36 Cheddar cheeses ranging in age from 1 to 80 weeks. Isolates were made from agar plates incubated at 30 C for 14 days. The dominant organism was found to be Sbm. plantarum, comprising 62% of the total count. Other organisms and their incidence were: Sbm. casei, 16%; intermediates between these two species, 12%; Betabacterium brevis, 6%; Betacoccus sp., 3% and other lactic acid bacteria, 1%. After the relatively short interval immediately following manufacture, during which the starter streptococci predominated, lactobacilli, and less commonly leuconostocs, were reported to flourish over the major portion of the ripening period. An attempt also was made to correlate flora with cheese quality. In this respect, it was noted that with good quality cheese, two or three varieties of lactic acid bacteria (lactobacilli) appeared in large numbers. In poor quality cheese, a larger number of types of lactic acid bacteria occurred.

Rogers (60) studied cheese made from raw milk and reported that bacteria growing in numbers large enough to be significant included various species of the lactobacillus type and a number of species of micrococci. He stressed the necessity of differentiating between bacteria that grow in milk and those that grow in cheese in the curing room, since conditions in a cheese are not favorable to the growth of most

bacteria because of low temperature, high salt concentration and high degree of anaerobiosis.

In comparative studies on Cheddar cheese made with normal starter and with certain pure cultures, Deane and Anderson (16) reported that S. lactis constituted 90 to 99% of the streptococci which they isolated. Cheese curing temperatures of 43 and 63 F were used. In another study, Deane et al. (17) reported isolating an "acidoproteolytic" coccus from 4-year old Cheddar cheese. This particular organism resembled S. faecalis var. liquefaciens, although positive identification was not made.

Tittsler et al. (76) reported that cocci from the lactic starter predominated in young cheese, regardless of milk quality or pasteurization. After 1 month, however, the flora of pasteurized-milk cheese consisted almost entirely of enterococci, and that of raw-milk cheese of lactobacilli, enterococci and a few diversified types. The results of these investigators confirmed the correlation between bacterial flora and cheese quality reported by Sherwood (70), that cheese made from poor and very poor milk, either raw or pasteurized, contained many more bacterial types than did cheese made from fair and good milks.

Dahlberg and Kosikowsky (13) used a strain of S. faecalis, which rapidly fermented lactose, as the starter for Cheddar cheese. They reported that a normal cheese having more flavor

and better quality and body could be made using S. faecalis rather than a lactic starter. The S. faecalis also hastened the ripening of cheese at curing temperatures of 50 and 60 F. In conjunction with this study, Kosikowsky and Dahlberg (39) reported that S. faecalis was able to grow and survive in Cheddar cheese in large numbers for a considerable period of time when the cheese was ripened at 50 or 60 F.

A Cheddar cheese made using S. lactis, S. cremoris and S. diacetilactis as the starter organisms was used by Dawson and Feagan (15) to study the fate of starter organisms during manufacturing and maturing. Streptococcus cremoris was found to attain lower maximum counts than both S. lactis and S. diacetilactis. A difference in the time required to reach maximum count also was observed. Streptococcus cremoris reached this point at milling, whereas S. lactis and S. diacetilactis attained it at half-Cheddar. In maturing cheese, S. lactis gave the highest initial count, S. cremoris the lowest. During curing, S. cremoris strains died out more rapidly than the other two. Robertson and Perry (59) and Perry (53) made identical observations regarding maximum numbers and survival in cheese in a comparison of cheeses made with S. lactis and S. cremoris starters. The non-starter Cheddar cheese population reported by Dawson and Feagan (15) consisted of 70% lactobacilli and 30% micrococci. All bacteriological counts in this study were made at 30 C for 4 days.

Dacre (12) examined the microflora of ten cheeses made from flash-pasteurized milk. He classified isolates as streptococci, lactobacilli and pediococci. Month-old cheese yielded 6% streptococci, 61% lactobacilli and 33% pediococci. No streptococci were found in cheese ripened for 2 months at room temperature. By the third month, the cheese flora consisted of 75% lactobacilli and 25% pediococci. This flora persisted through the ninth month of ripening. Times were given indicating when the microorganisms found first appeared, and, also, when they ceased to be present.

Feagan and Dawson (23) studied the bacteriology of 12 cheeses. In nine of these, at 8 days, the starter organism count was equal to the total count. In the other three samples, the starter organisms constituted 98, 92 and 20% respectively of the total count. In the latter sample, the following bacterial populations were found: 40% micrococci, 30% lactobacilli and 10% other organisms. Coliforms were detected in some samples in low numbers, but disappeared prior to the second month of curing.

Recently, Rašić (57) reported on trends of bacterial population during the manufacture and ripening of white cheese. He found that streptococci comprised 96% of the bacterial flora on the first day after manufacture, 80% after 4 weeks and 50% after 6 weeks. The lactobacilli were found to reach maximum numbers after 4 weeks, when they constituted 30% of

the bacterial flora. After 6 weeks, the lactobacilli comprised 50% of the flora. Coliform organisms multiplied during manufacture of the cheese, but, like the micrococci, disappeared rapidly during ripening. The following species were isolated and identified in the study: S. lactis, S. cremoris, S. diacetilactis, S. faecalis, Leuconostoc lactis, Pediococcus cerevisiae, L. casei, L. plantarum, L. brevis, E. coli, A. aerogenes, M. caseolyticus, M. freudenreichii, M. varians and M. luteus.

Franklin and Sharpe (25, 26) attempted to determine the bacterial flora of cheeses made from milks subjected to different heat treatments, and the effect of the residual flora on cheese flavor. Gram-positive cocci constituted 54% of the microflora, gram-positive bacilli 38% and gram-negative bacilli 7%. No Leuconostoc sp. were found. Lactobacillus casei was the most important organism numerically. Other microorganisms isolated were: L. brevis, L. plantarum, Pediococcus sp. and S. faecium. A positive correlation was observed between numbers of lipolytic bacteria and desirable flavor. Lipolytic organisms, however, were not found to increase during storage at 50 F. Rather, they constituted a static population. Group D streptococci nearly always were present in commercial cheese. The most commonly occurring species was S. faecium. Towards the end of the experiment, these workers used a selective medium technique to determine the presence of

gram-negative bacteria. On occasion, these organisms were present in considerable numbers in commercial cheese. It was felt that the influence or possible influence of these micro-organisms on flavor and additional ripening must be considered.

Irvine and Beach (35) used Standard Plate Count agar, to which was added 3.75 ppm brom cresol purple, to enumerate non-lactic organisms in fresh Cheddar cheese. The brom cresol purple was inhibitory to starter bacteria and allowed the contaminating organisms in month-old Cheddar cheese to grow. A 0.1 g sample of each cheese was examined, plates being incubated at 32 C for 24 to 36 hours. The majority of colonies appearing in the plates were small, round, acid-producing colonies of the Micrococcus genus. Numbers per gram of cheese ranged from practically zero in cheese made from pasteurized and heat-treated milk to above 500 million in raw-milk cheese with fruity and similar undesirable flavors. Larger, alkaline-producing colonies of small rod-shaped organisms also were frequently observed. High counts of these organisms were believed to be indicative of unhygienic factory conditions.

Specific Groups of Microorganisms in Cheddar Cheese

Lactobacilli

Lactobacilli may be found wherever sugars, protein breakdown products, vitamins and low oxygen tension occur (63).

They can grow over a wide temperature range, some developing at a temperature just above freezing, others having an optimum growth temperature near 45 C. The large quantities of lactic acid produced by lactobacilli act as a preservative, suppressing the growth of proteolytic organisms, while metabolic products of lactobacilli growing on certain substrates often impart characteristic flavors. This has been widely reported in Cheddar cheese ripening.

Mabbitt and Zielinska (42) described a modified Rogosa agar (Acetate agar) for use in detecting lactobacilli in Cheddar cheese. With this medium, they were able to demonstrate that lactobacilli grew slowly and continuously from the beginning of the ripening period. Naylor and Sharpe (47) reported that this medium was satisfactory for the isolation and enumeration of lactobacilli from Cheddar cheese during ripening, as large numbers of starter streptococci were completely suppressed and lactobacilli were isolated during the early stages of ripening. In this investigation, the cheese was ripened at 55 F. Sampling was begun at 3 days of age and continued at intervals for 172 days. Lactobacilli were isolated from the first sampling. Initially, high bacterial counts were due to streptococci, although these diminished and finally disappeared. At about 50 days, the numbers of lactobacilli rose above those of streptococci and later in ripening, lactobacilli comprised the entire population. Of

the lactobacilli isolated and identified, 17% were L. brevis, 8% were L. plantarum and the remainder were low temperature, homofermentative lactobacilli.

Naylor and Sharpe (48) found large numbers of lactobacilli in Cheddar cheese during ripening. The predominant species was L. brevis. Other lactobacilli, including L. plantarum and L. casei also were found.

Serological typing was used by Naylor and Sharpe (49) to trace the source of lactobacilli in Cheddar cheese. The main sources of these organisms were the milk used in cheese making and the air of the dairy. Most of the lactobacilli from these two sources were low temperature, homofermentative strains. Sharpe and Mattick (64) found that 100% of the raw milks examined by them contained lactobacilli. Counts ranged from less than 100/ml to 1,000,000/ml. No seasonal trend in counts was observed. Perry et al. (55) took air samples from the cheese-making room of seven creameries and from three stages of processing in one processed cheese plant. They found lactobacilli in all samples. Lactobacillus casei occurred most frequently, followed by L. plantarum and L. brevis in decreasing order of occurrence. Lactobacillus acidophilus was found once, however, it was considered of no importance since it will not grow in cheese because of the low ripening temperature.

Hill and Thornton (33) reported unexpectedly low counts

of lactobacilli during the curing and storage of some Cheddar cheeses which they examined. They also found a very uneven distribution of these organisms throughout the cheese. Johns and Cole (36), however, found large numbers of lactobacilli present in Canadian Cheddar cheese when 38 cheeses were examined at intervals for over a year. They found that lactobacilli commonly increased sharply during the first 2 weeks, then more slowly until the third to sixth month. By the end of 1 year, a pronounced drop in the lactobacillus count was noted. Hill and Thornton (33) were openly criticized, by these workers, for the experimental methods used in their investigation.

Perry and Sharpe (54) studied the lactobacillus flora of single-herd milk and of the Cheddar cheese made from this milk. They found that L. plantarum and L. casei increased more rapidly than did L. brevis in cheese. The cheeses were ripened at 55 F. From the results obtained, these workers concluded that the lactobacillus flora of Cheddar cheese was derived mainly from milk.

A recent study by Smith and Cunningham (71) showed the frequency of occurrence of lactobacilli in Canadian Cheddar cheese. A total of 245 cultures were characterized and the following distribution was found: L. plantarum, 138; L. casei, 35; L. brevis, 19; L. helveticus, 16; L. fermenti, 16 and unclassified, 21.

Staphylococci

The common occurrence of coagulase-positive staphylococci in raw milk has been reported by many workers (6, 7, 20, 45, 78). Therefore, the high incidence of these organisms in Cheddar cheese (19, 74, 75, 77) is not surprising. The survival of Staphylococcus aureus in cheese has been reported by Mattick et al. (43) and McLeod et al. (46). Staphylococcus food poisoning has been attributed to the consumption of raw-milk Cheddar cheese (32).

Alford and Frazier (2) examined cheeses ripened at 5 to 7 C to determine if any of the organisms found in the cheese, other than lactic acid bacteria, occurred in sufficient numbers to be of importance in the development of flavor. Cultures were divided into groups on the basis of morphology, pigmentation, action in litmus milk, proteolysis in milk agar, lipolysis in tributyrin and butterfat agar, growth in 6.5% sodium chloride and growth at pH 5.2. These characteristics were selected as it was felt they would give a better indication of the possible importance of the isolated organisms in cheese ripening. Of 600 cultures isolated, 78% were micrococci closely related to M. freudenreichii, M. caseolyticus and M. conglomeratus. The remaining 22% of the isolates were coliforms and miscellaneous types.

Coliforms

Literature pertaining to the incidence of coliform organisms in Cheddar cheese and other dairy products is too extensive to completely review here. Therefore, only a few of the more pertinent references have been included.

Crossley (10) studied the coliform organisms associated with commercial manufacture and storage of Cheddar cheese. He isolated 116 cultures from cheeses 2 days to 12 months old representing three factories. All factories used pasteurized milk. Of these 116 cultures, 99 were E. coli types, 13 were A. aerogenes types and four were intermediate types.

Yale and Marquardt (79) made coliform counts on 35 lots of Cheddar cheese. Cheeses were ripened at 50 F. Coliforms in cheese made with poor milk (20,000 to 2,100,000 coli/ml) survived for 6 to 12 months. With better quality milk, containing fewer coliform organisms, these bacteria survived for 3 to 6 months. It was reported that coliforms grew and died at such variable rates in the different lots of cheese that the coliform count is not a reliable index for comparing the quality of different lots of cheese, even at the same age.

Based on the incidence of presumptive positive tests, Crossley (9) found coliforms in 100% of the 44 blown Cheddars examined as well as in 100% of the normal Cheddars examined when sampled up to 1 week of age. That coliforms rapidly decrease was clearly evident by results showing 58.8% of 3-month

old normal Cheddar containing coliforms while only 20% of normal Cheddar over 3 months of age contained these organisms.

Lightbody (40) showed that coliform organisms were present (<10/g) in 97% of 30 samples from 19 cheese factories. E. coli type I organisms were present in 70% of the samples. Also, the majority of first-grade cheese had coliform counts of less than 1,000/g.

Pediococci

Although pediococci apparently never have been isolated and reported to occur in Cheddar cheese in this country, their occurrence has been reported by workers in other countries. Dacre (11) isolated over 4,000 cultures of lactic acid-producing bacteria during a study of the microflora of ripening New Zealand Cheddar cheese. Cultures were isolated from agar plates incubated at 30 C. Although the majority of cultures isolated were rod-shaped species of lactobacilli, large cocci with a predominant and characteristic diplococcoid form appeared from all 20 of the cheese samples examined. These cocci initially were thought to be Leuconostoc sp., but further investigations showed them to be pediococci. Sharpe (63) and Dacre (12) also reported that pediococci occurred in many Cheddar cheeses.

EXPERIMENTAL METHODS

Collection, Handling and Treatment of Samples

Fifty commercial cheese samples from 11 Iowa cheese manufacturing plants were obtained for study. Samples were taken during February, March, April, May and August, 1961. All samples were collected and handled according to the procedures described in Standard Methods for the Examination of Dairy Products (4).

Bacteriological Examination of Samples

All samples were plated immediately upon their return to the laboratory. The procedure given in Standard Methods for the Examination of Dairy Products (4) for the yeast and mold count of cheese other than cottage was used to prepare samples for plating. The single deviation from this procedure was that emulsification was effected by high-speed agitation in a Waring Blendor for 2 minutes.

Plates were prepared in duplicate and poured with 15 ml of special Trypticase-soy agar (Appendix). After solidification, a 50 ml cover-layer of sterile 1.5% agar was added. Following solidification of the cover-layer, the plates were sealed tightly with 1 3/4 inch wide rubber bands and incubated at 7 C for 3 months. In addition, three cheese samples (Table 1) were incubated at this temperature, in a desiccator in

Table 1. Source and identification of cheese samples

Sample number	Cheese plant	Vat code	Heat treatment of milk	Date cheese made	Date cheese plated	Age at plating	Agar plate count/g[a]
1	Independence	A	162 F; 20 sec	2-22-61	3-1-61	7 days	6 x 10^5
2	"	B	"	"	"	"	10 x 10^5
3	"	C	"	"	"	"	10 x 10^5
4[b]	Pioneer	-[c]	162 F; 20 sec	2-6-61	2-25-61	19 days	110 x 10^5
5[b]	"	-	"	2-8-61	"	17 days	160 x 10^4
6[b]	"	-	"	2-22-61	"	3 days	110 x 10^4
7	Dows	A	162 F; 15 sec	2-22-61	3-1-61	7 days	10 x 10^5
8	Hampton	1	160 F (pasteurized)	2-19-61	2-24-61	5 days	72 x 10^4
9	"	2	"	"	"	"	45 x 10^4
10	"	3	"	"	"	"	97 x 10^4
11	Wadena	A	152-156 F; 15 sec	2-21-61	3-1-61	8 days	25 x 10^5
11A[d]	"	A	"	"	"	"	30 x 10^5
12	"	A	"	2-22-61	"	7 days	31 x 10^5
12A[d]	"	A	"	"	"	"	20 x 10^5
13	"	B	"	"	"	"	21 x 10^5
13A[d]	"	B	"	"	"	"	24 x 10^5
14	Wadena	A	152-156 F; 15 sec	2-3-61	2-10-61	7 days	16 x 10^5
15	"	B	"	"	"	"	32 x 10^5
16	"	C	"	"	"	"	11 x 10^5
17	Westgate	B	150-162 F; 15 sec	2-21-61	3-1-61	8 days	54 x 10^5

[a]Plate count after incubation at 7 C for 3 months.

[b]Colby cheese.

[c]Single vat; no code used.

[d]Duplicate sample; agar plates incubated in an increased carbon dioxide atmosphere.

Table 1. (Continued)

Sample number	Cheese plant	Vat code	Heat treatment of milk	Date cheese made	Date cheese plated	Age at plating	Agar plate count/g
18	Westgate	A	150-162 F; 15 sec	2-22-61	3-1-61	7 days	76×10^6
19	"	B	"	"	"	"	100×10^6
20	Zearing	4	155 F; 15 sec	2-22-61	3-1-61	7 days	55×10^5
21	"	2	"	2-23-61	"	6 days	55×10^5
22	"	3	"	"	"	"	49×10^6
23	Hopkinton	3	151 F; 15 sec	8-3-61	8-10-61	7 days	50×10^5
24	"	5	"	"	"	"	130×10^5
25	"	6	"	"	"	"	34×10^5
26	"	8	"	"	"	"	11×10^5
27	"	9	"	"	"	"	53×10^5
28	"	10	"	"	"	"	21×10^5
29	Renwick	B	149-152 F; 17 sec	2-19-61	2-24-61	5 days	250×10^5
30	"	C	"	"	"	"	69×10^5
31	"	B	"	2-20-61	"	4 days	150×10^5
32	Wilton Jn.	1	147 F; 20 sec	2-28-61	3-7-61	7 days	110×10^5
33	"	2	"	"	"	"	110×10^5
34	"	3	"	"	"	"	85×10^5
35	"	4	"	"	"	"	16×10^6
36	"	1	"	3-1-61	3-8-61	"	47×10^5
37	"	2	"	"	"	"	10×10^6
38	"	3	"	"	"	"	10×10^6
39	Goldfield	2	147-148 F; 16 sec	4-7-61	4-14-61	7 days	40×10^5
40	"	4	"	5-3-61	5-10-61	"	35×10^5
41	"	2	"	5-4-61	5-11-61	"	34×10^5
42	"	2	"	5-29-61	6-5-61	"	38×10^5

Table 1. (Continued)

Sample number	Cheese plant	Vat code	Heat treatment of milk	Date cheese made	Date cheese plated	Age at plating	Agar plate count/g
43	Goldfield	3	147-148 F; 16 sec	5-29-61	6-5-61	7 days	63×10^5
44	"	1	"	5-30-61	6-6-61	"	85×10^5
45	"	2	"	4-7-61	7-5-61	3 mo.	78×10^4
46	"	4	"	5-3-61	8-1-61	"	80×10^4
47	"	2	"	5-4-61	"	"	17×10^5
48	"	2	"	5-29-61	9-14-61	"	23×10^5
49	"	3	"	"	"	"	71×10^5
50	"	1	"	5-30-61	9-13-61	"	28×10^5

which candles had been ignited, in an increased carbon dioxide atmosphere. This was undertaken to determine whether this change in the atmospheric condition during incubation would affect the numbers or types of microorganisms developing. After incubation, colonies appearing in the agar plates were counted using a Quebec colony counter.

Three samples of Colby cheese were obtained from one Iowa plant. These samples were 3, 17 and 19 days old when plated. All other cheese samples were between 4 and 8 days old when plated. In addition, six samples, which were obtained in sealed cans, were re-plated after being ripened at a temperature of 7 C for 3 months (Table 1).

Isolation and Purification of Microorganisms

An attempt was made to pick 30 colonies from one agar plate of each cheese sample. All the colonies present were picked when plates contained between 20 and 40 colonies. Colonies were picked from duplicate plates when fewer than 20 colonies appeared in a plate. If plates contained more than 40 colonies, all colonies within a predetermined area were picked so that approximately 30 isolates would be obtained from the sample.

To facilitate picking, the thick, two-layer agar plaque was inverted into the petri dish cover with the aid of a sterile spatula. Appearance of each colony was recorded

before the colony was picked and inoculated into sterile litmus milk. The litmus milk used in this study was prepared from fresh skim milk autoclaved at 121 C for 12 minutes.

Litmus milk cultures were incubated at 21 C until a reaction was observed. Next, they were streaked on special Trypticase-soy agar plates for purification. These plates were incubated at 21 C until colonies formed. A single, characteristic colony from each plate was picked to litmus milk again, grown at 21 C, examined for Gram stain reaction and morphological characteristics and frozen. If more than one type of colony was observed, a colony representing each type was picked.

Isolates not producing visible changes in litmus milk after 5 days were streaked and treated as stated previously.

After all samples had been collected and the isolates obtained, purified and frozen, characterization and identification of the isolates was undertaken.

Characterization and Identification of Isolates

Frozen cultures were rapidly thawed in a 40 C water bath and immediately inoculated into tubes of litmus milk for incubation at 21 C. As soon as a litmus reaction was observed (usually 24 hours) and not longer than 3 days if no change in litmus was produced, a drop of the fresh transfer was inoculated into Trypticase-soy broth (Appendix). This broth was

incubated at 21 C until abundant growth was observed (usually 24 hours) and not longer than 3 days for isolates not showing abundant growth.

The following determinations were made on each culture using Trypticase-soy broth for subculturing: Gram stain for verification of purity, catalase reaction, litmus milk reaction at 21, 32 and 37 C, methyl red and Voges-Proskauer tests, salt tolerance, gelatin liquefaction and carbohydrate fermentation. In addition, an agar slant was made of every catalase-positive organism. All cultures of catalase-negative cocci were examined for ammonia production from arginine, reduction of methylene blue milk, initiation of growth at high pH and growth in litmus milk at 45 C. Those growing at 45 C were streaked on bovine blood agar to determine their hemolytic reaction. Isolates not growing at 45 C were checked for growth in litmus milk at 40 C. All catalase-positive organisms and all cultures of rod-shaped organisms were tested for nitrate reduction. All gram-negative rods were tested for formation of indole from tryptophan. Lipolytic and proteolytic ability was determined for all catalase-positive isolates. Except when stated otherwise, characterization of isolates was carried out at 21 C. All media were stored under refrigeration, but were removed and properly tempered before inoculation and subsequent incubation.

Gram stain

The Hucker modification of Gram's stain was used. Preparation of reagents for the stain and the staining method used were in accordance with the Manual of Microbiological Methods (72).

Catalase reaction

The slide catalase test was used. One drop of culture was mixed with a drop of 30% hydrogen peroxide on a clean glass slide. In a few instances, small air bubbles became entrapped between the slide and the hydrogen peroxide. These were eliminated by rubbing a sterile loop across the area before adding the culture.

Litmus milk reaction

Every culture was inoculated into tubes of litmus milk for incubation at 21, 32 and 37 C. As previously mentioned, some cultures also were inoculated into litmus milk for incubation at 40 or 45 C, or both. The litmus reaction of these cultures was recorded daily until no additional changes were observed.

Methyl red and Voges-Proskauer tests

The basal medium for these tests was as shown in the Appendix. Reactions were determined as recommended in the

Manual of Microbiological Methods (72).

Salt tolerance

All isolates were tested for their ability to initiate growth in salt broths containing 4.0 and 6.5% sodium chloride. The basal medium was Trypticase-soy broth, in which sodium chloride concentrations had been increased to the desired levels. Presence or absence of growth was recorded daily for a period of 7 days, if necessary, before the test cultures were discarded.

Gelatin liquefaction

Each culture was inoculated into a tube of Nutrient gelatin. The composition and preparation of Nutrient gelatin was as specified in the Difco Manual of Dehydrated Culture Media and Reagents (18). Non-liquefying isolates were incubated for 1 month before being discarded.

Carbohydrate fermentation

Determination of the fermentative pattern of cultures was accomplished using eight compounds: arabinose, glucose, glycerin, lactose, maltose, mannitol, sucrose and xylose. A 10% solution of each was prepared and sterilized by Seitz filtration. One ml of the concentrated, sterile solution was added aseptically to 9.0 ml of the previously heat-sterilized

basal medium. Composition of the basal medium was as shown in the Appendix. This medium was tubed in 9.1 ml amounts in cotton-stoppered test tubes containing Durham tubes. Incubation was continued to 1 week for cultures giving immediate fermentative patterns, and to 2 weeks for those having weak or no fermentative ability. In no instances were fermentation tubes discarded until no change in the medium had been observed for 5 consecutive days.

Agar slants

Standard Methods agar (4) was used for preparation of the slants.

Ammonia production from arginine

Determination of the ability of organisms to hydrolyze arginine to form ammonia was performed according to the method of Niven et al. (51). Cultures giving a negative test after 2 days were re-incubated and tested periodically up to 7 days, if necessary.

Reduction of methylene blue milk

All cultures of catalase-negative cocci were subcultured in 0.1% and 0.3% methylene blue milk. To prepare the milk, a 10% stock solution of methylene blue was made from methylene blue chloride. The 0.1% methylene blue milk was prepared by

adding the proper quantity of dye solution directly to the milk, mixing, tubing and autoclaving at 121 C for 12 minutes. To avoid an interaction between the dye and milk, which resulted in the precipitation of milk protein upon heating, it was necessary to sterilize the dye solution separately from the milk when preparing 0.3% methylene blue milk. After both the dye solution and the skim milk had been sterilized and cooled, they were mixed and dispensed aseptically into previously sterilized test tubes. Fresh skim milk was used in the preparation of methylene blue milk. Cultures were incubated for periods up to 30 days before methylene blue reduction was considered negative.

pH broth

All cultures of catalase-negative cocci were tested for their ability to initiate growth at both pH 9.2 and pH 9.6. Nutrient broth adjusted to these high pH levels was used as recommended by Lord (41). The broth was prepared in small quantities which would be utilized rapidly and was meted into screw-cap test tubes. The medium was autoclaved at 121 C for 15 minutes. Cultures were incubated up to 7 days, being examined daily for evidence of growth.

Blood agar

This medium was used to determine the hemolytic reaction of the cultures. It was prepared by adding 10% citrated bovine blood to previously sterilized and cooled Blood agar base (18). Plates were prepared for streaking by pouring approximately 20 ml of the medium into each petri dish. After the plates had solidified, they were incubated at 35 C for 24 hours to check for sterility and to dry the agar surface for streaking. Cultures streaked on Blood agar were incubated at 37 C and examined after 18, 24 and 48 hours.

Nitrate reduction

The procedure recommended in the Manual of Microbiological Methods (72) was used to determine reduction of nitrates to nitrites.

Indole from tryptophan

The medium employed for this test was as shown in the Appendix. Kovács reagent, as recommended in the Manual of Microbiological Methods (72), was used to test for the presence of indole. Determinations usually were made on 48-hour cultures, unless growth appeared scanty. In these cases, the test was performed when adequate growth was observed.

Lipolytic action

The medium used to detect the lipolytic ability of isolates contained Nile blue as recommended by Knaysi (38), with Standard Methods agar (4) as the basal medium. Cultures were streaked on the agar surface and plates were incubated and examined daily up to 7 days, if necessary.

Proteolytic action

Proteolysis was determined by adding 10% sterile skim milk to previously sterilized and cooled Standard Methods agar (4) as recommended by Foster et al. (24). Cultures were streaked on the agar surface and plates were incubated for as long as 7 days before cultures were considered non-proteolytic.

Plating Lactic Starter Cultures

To ascertain that the plating procedure used to isolate organisms from the young cheese samples was not inhibitory to lactic starter organisms, 45 commercial lactic starter cultures, obtained from five different sources, were plated in quadruplicate according to the procedure recommended in Standard Methods for the Examination of Dairy Products (4). All cultures, except those from the Food Products Analysis Laboratory, were obtained lyophilized. Samples from the Food Products Analysis Laboratory were taken from mother cultures

being transferred routinely three times a week. Cultures were transferred for 3 consecutive days before plating. The plating medium was as given for cheese plating. Duplicate plates of each starter culture were incubated at 21 C for 10 days and at 7 C for 3 months to obtain a comparison between the counts at each incubation temperature. Bacteriological counts at 7 C were estimated after 1 and 2 months. Following 3 months of incubation, the plates were counted and agar colonies from each sample were picked to litmus milk. After one transfer in litmus milk at 21 C, transfers were made to litmus milk, previously tempered to 45 C, for incubation at 45 C. Table 2 shows the designation and source of each culture plated.

Table 2. Source and identification of commercial lactic starter cultures

Culture designation		Source
CH 2	CH 12	Hansen's Dri-Vac Cultures
CH 3	CH 15	Chris Hansen's Laboratory, Inc.
CH 4	CH 18	9015 W. Maple St.
CH 5	CH 40	Milwaukee 14, Wisconsin
CH 6	CH 41	
CH 8	CH 42	
CH 9	CH 43	
CH 10	CH 60	
CH 11	CH 60X	
LC 10		Cultures, Inc.
LC 11		3868 E. Washington St.
LC 12		Indianapolis 1, Indiana
LC 21		
LC 26		
LC 28		
LC 29		
K 11	Pf 3	Chas. Pfizer & Co., Inc.
K 22	Pf 3A	4253 N. Port Washington Ave.
K 33	Pf 4	Milwaukee 12, Wisconsin
K 44	Pf 4A	
Pf 1	Pf 51	
Pf 1A	Pf 52	
Pf 2		
FD		Food Products Analysis Laboratory
FL		Dept. Dairy and Food Industry
M 9		Iowa State University
Xi		Ames, Iowa
122		
W 26		
B 6		

EXPERIMENTAL RESULTS

Bacteriological Examination of Cheese Samples

Table 1 shows the source and identification of cheese samples, the heat treatment of milk used in their manufacture and the agar plate counts of each. Bacteriological counts of the cheese samples ranged from 450,000/g to 100,000,000/g. The cheeses made from pasteurized milk tended to have slightly lower counts than those made from heat-treated milk. Even considering the heat treatment given the milk, no large differences in counts were observed between cheeses from different plants.

The three samples of Colby cheese gave counts within the range obtained for the Cheddar cheeses. No difference in count was obtained between plates incubated in an increased carbon dioxide atmosphere and the duplicate plates incubated under regular atmospheric conditions. Only one of the six cheeses examined at 7 days, and again at 3 months, did not show a decreased count on the second plating. Bacterial numbers remained the same in this sample.

Characterization and Identification of Microorganisms Isolated from Cheese

From the 50 different cheese samples examined, 1162 microorganisms were isolated and characterized. An additional 67 isolates, from duplicate samples, also were characterized.

Organisms were placed into eight groups based upon their morphological and biochemical characteristics. These groups were: enterococcus group streptococci, lactic group streptococci, miscellaneous associated bacteria (bacteria resembling and commonly associated with lactic streptococci, such as Leuconostoc sp.), lactobacilli, micrococci, miscellaneous gram-positive rods, miscellaneous gram-negative rods and other miscellaneous microorganisms (Table 3). The three samples which were plated in quadruplicate, duplicate plates being incubated under different atmospheric conditions, are included in Table 3. However, because the microflora was not altered by incubation in the carbon dioxide atmosphere, isolates from these samples (11A, 12A and 13A) are not included in the total or percentage figures.

The following numbers and percentages of microorganisms were found: 578 enterococci, 50%; 149 micrococci, 13%; 126 lactic streptococci, 10.5%; 110 miscellaneous gram-positive rods, 9%; 93 miscellaneous associated bacteria, 8%; 67 lactobacilli, 6%; 34 miscellaneous gram-negative rods, 3% and five other miscellaneous microorganisms, 0.5%.

Although comprising only 50% of the total microbial flora, enterococci constituted the entire population recovered from ten of the 50 samples and over 70% of the population in 11 other samples. Enterococci were found in all but eight samples, occurring much more frequently than any other group

Table 3. Classification of microorganisms isolated from young Cheddar cheese

Sample number	Streptococci, Enterococcus group		Streptococci, Lactic group		Miscellaneous associated bacteria		Lactobacilli		Micrococc	
	No.	%	No.	%	No.	%	No.	%	No.	%
1	1	17	0	0	0	0	1	17	4	66
2	1	14	0	0	0	0	0	0	5	72
3	8	73	0	0	0	0	0	0	3	27
4	0	0	0	0	12	92	1	8	0	0
5	0	0	0	0	18	100	0	0	0	0
6	0	0	0	0	14	100	0	0	0	0
7	0	0	2	22	0	0	3	34	2	22
8	0	0	9	28	1	3	0	0	9	28
9	3	12	0	0	0	0	1	4	15	57
10	9	50	9	50	0	0	0	0	0	0
11	18	75	0	0	0	0	0	0	2	8
11A[a]	24	92	0	0	0	0	0	0	0	0
12	1	3	1	3	0	0	0	0	8	25
12A[a]	1	4	0	0	0	0	0	0	11	48
13	10	43	0	0	0	0	0	0	5	22
13A[a]	16	89	0	0	0	0	0	0	2	11
14	14	93	0	0	0	0	1	7	0	0
15	0	0	27	88	0	0	0	0	2	6
16	3	21	0	0	0	0	0	0	10	72
17	25	93	0	0	0	0	2	7	0	0
18	0	0	26	50	0	0	0	0	0	0
19	4	21	1	5	0	0	14	74	0	0
20	9	30	6	20	14	47	0	0	1	3
21	21	95	0	0	0	0	1	5	0	0
22	19	100	0	0	0	0	0	0	0	0
23	12	39	0	0	0	0	0	0	6	19
24	27	69	0	0	7	18	0	0	3	8
25	1	4	0	0	0	0	0	0	11	46
26	10	59	0	0	0	0	0	0	5	29
27	22	82	0	0	0	0	0	0	2	7

[a]Duplicate sample; agar plates incubated in an increased carbon dioxide atmosphere. Re:

;rococci		Miscellaneous gram-positive rods		Miscellaneous gram-negative rods		Other miscellaneous microorganisms		
).	%	No.	%	No.	%	No.	%	Total
4	66	0	0	0	0	0	0	6
5	72	1	14	0	0	0	0	7
3	27	0	0	0	0	0	0	11
)	0	0	0	0	0	0	0	13
)	0	0	0	0	0	0	0	18
)	0	0	0	0	0	0	0	14
2	22	0	0	1	11	1	11	9
9	28	10	32	2	6	1	3	32
5	57	7	27	0	0	0	0	26
)	0	0	0	0	0	0	0	18
2	8	3	13	1	4	0	0	24
)	0	2	8	0	0	0	0	26
3	25	12	38	10	31	0	0	32
1	48	2	9	8	35	1	4	23
5	22	2	9	5	22	1	4	23
2	11	0	0	0	0	0	0	18
)	0	0	0	0	0	0	0	15
2	6	0	0	2	6	0	0	31
)	72	0	0	1	7	0	0	14
)	0	0	0	0	0	0	0	27
)	0	26	50	0	0	0	0	52
)	0	0	0	0	0	0	0	19
1	3	0	0	0	0	0	0	30
)	0	0	0	0	0	0	0	22
)	0	0	0	0	0	0	0	19
6	19	8	26	3	10	2	6	31
3	8	2	5	0	0	0	0	39
1	46	11	46	1	4	0	0	24
5	29	2	12	0	0	0	0	17
2	7	0	0	3	11	0	0	27

e. Results not included in cumulative total or average figures.

Table 3. (Continued)

Sample number	Streptococci, Enterococcus group No.	%	Streptococci, Lactic group No.	%	Miscellaneous associated bacteria No.	%	Lactobacilli No.	%	Microc No.
28	2	8	0	0	0	0	0	0	14
29	2	8	6	23	18	69	0	0	0
30	3	8	26	70	6	16	1	3	0
31	1	33	2	67	0	0	0	0	0
32	29	91	0	0	1	3	0	0	1
33	25	100	0	0	0	0	0	0	0
34	21	100	0	0	0	0	0	0	0
35	13	87	0	0	0	0	0	0	0
36	26	100	0	0	0	0	0	0	0
37	6	100	0	0	0	0	0	0	0
38	9	100	0	0	0	0	0	0	0
39	26	72	3	8	0	0	0	0	6
40	17	46	0	0	0	0	0	0	18
41	27	85	0	0	0	0	0	0	1
42	13	40	7	21	1	3	0	0	5
43	20	63	1	3	0	0	0	0	10
44	17	100	0	0	0	0	0	0	0
45	34	100	0	0	0	0	0	0	0
46	28	94	0	0	1	3	1	3	0
47	16	100	0	0	0	0	0	0	0
48	2	9	0	0	0	0	19	83	0
49	0	0	0	0	0	0	22	92	1
50	23	100	0	0	0	0	0	0	0
Total	578		126		93		67		149
Average		50%		10.5%		8%		6%	

Micrococci		Miscellaneous gram-positive rods		Miscellaneous gram-negative rods		Other miscellaneous microorganisms		Total
No.	%	No.	%	No.	%	No.	%	
14	56	8	32	1	4	0	0	25
0	0	0	0	0	0	0	0	26
0	0	0	0	1	3	0	0	37
0	0	0	0	0	0	0	0	3
1	3	1	3	0	0	0	0	32
0	0	0	0	0	0	0	0	25
0	0	0	0	0	0	0	0	21
0	0	2	13	0	0	0	0	15
0	0	0	0	0	0	0	0	26
0	0	0	0	0	0	0	0	6
0	0	0	0	0	0	0	0	9
6	17	0	0	1	3	0	0	36
18	49	2	5	0	0	0	0	37
1	3	3	9	1	3	0	0	32
5	15	7	21	0	0	0	0	33
10	31	1	3	0	0	0	0	32
0	0	0	0	0	0	0	0	17
0	0	0	0	0	0	0	0	34
0	0	0	0	0	0	0	0	30
0	0	0	0	0	0	0	0	16
0	0	1	4	1	4	0	0	23
1	4	1	4	0	0	0	0	24
0	0	0	0	0	0	0	0	23
149		110		34		5		1162
	13%		9%		3%		0.5%	

of microorganisms. Table 4 shows the type of enterococci isolated. Of these 578, 345 (60%) belonged to the S. durans group. In addition, 159 (27%) were identified as S. faecalis. Fifty-nine isolates (10%) were S. faecalis var. liquefaciens and 15 (3%) were S. faecalis var. zymogenes.

Micrococci were the second most frequent group of microorganisms found. This group constituted 13% of the isolates and they were found in 25 of the cheese samples. The highest level of micrococci was in samples 2 and 16 where they contributed 72% of the total flora. In seven samples these microorganisms comprised about 50% of the bacterial population.

Streptococci of the lactic group were recovered from only 14 of the 50 samples. In five of these, they comprised 50% or more of the flora. The highest level of lactic streptococci found in a sample was 88%. Enterococci were not found in this sample.

Miscellaneous gram-positive rods were recovered from 20 cheese samples. Except in two samples, where they constituted 50 and 46% of the flora, these organisms occurred in small numbers contributing one-third or less of the microbial population in all samples. One-hundred-ten isolates of this type were recovered.

Only 93 miscellaneous associated bacteria were isolated. Approximately one-half of these, 44, were from the three

Table 4. Identification of enterococci isolated from young Cheddar cheese

Sample number	Streptococcus durans group No.	%	Streptococcus faecalis No.	%	Streptococcus faecalis var. zymogenes No.	%	Streptococcus faecalis var. liquefaciens No.	%	Total
1	0	0	1	100	0	0	0	0	1
2	1	100	0	0	0	0	0	0	1
3	6	75	0	0	0	0	2	25	8
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	3	100	0	0	0	0	0	0	3
10	9	100	0	0	0	0	0	0	9
11	3	17	14	77	1	6	0	0	18
11A[a]	24	100	0	0	0	0	0	0	24
12	1	100	0	0	0	0	0	0	1
12A[a]	1	100	0	0	0	0	0	0	1
13	1	10	8	80	1	10	0	0	10
13A[a]	1	6	6	38	9	56	0	0	16
14	13	93	0	0	0	0	1	7	14
15	0	0	0	0	0	0	0	0	0
16	3	100	0	0	0	0	0	0	3
17	19	76	4	16	0	0	2	8	25
18	0	0	0	0	0	0	0	0	0
19	4	100	0	0	0	0	0	0	4
20	9	100	0	0	0	0	0	0	9
21	17	81	4	19	0	0	0	0	21
22	17	89	0	0	0	0	2	11	19
23	2	17	3	25	0	0	7	58	12
24	13	48	8	30	2	7	4	15	27
25	0	0	0	0	0	0	1	100	1
26	10	100	0	0	0	0	0	0	10
27	21	95	1	5	0	0	0	0	22

[a]Duplicate sample; agar plates incubated in an increased carbon dioxide atmosphere. Results not included in cumulative total or average figures.

Table 4. (Continued)

Sample number	Streptococcus durans group No.	%	Streptococcus faecalis No.	%	Streptococcus faecalis var. zymogenes No.	%	Streptococcus faecalis var. liquefaciens No.	%	Total
28	1	50	0	0	1	50	0	0	2
29	2	100	0	0	0	0	0	0	2
30	2	67	1	33	0	0	0	0	3
31	1	100	0	0	0	0	0	0	1
32	0	0	25	86	0	0	4	14	29
33	1	4	24	96	0	0	0	0	25
34	9	43	12	57	0	0	0	0	21
35	2	15	8	62	0	0	3	23	13
36	22	84	3	12	1	4	0	0	26
37	3	50	3	50	0	0	0	0	6
38	0	0	9	100	0	0	0	0	9
39	26	100	0	0	0	0	0	0	26
40	2	12	0	0	0	0	15	88	17
41	4	15	14	52	9	33	0	0	27
42	13	100	0	0	0	0	0	0	13
43	9	45	11	55	0	0	0	0	20
44	17	100	0	0	0	0	0	0	17
45	29	85	5	15	0	0	0	0	34
46	10	36	1	4	0	0	17	60	28
47	15	94	0	0	0	0	1	6	16
48	2	100	0	0	0	0	0	0	2
49	0	0	0	0	0	0	0	0	0
50	23	100	0	0	0	0	0	0	23
Total	345		159		15		59		578
Average		60%		27%		3%		10%	

samples of Colby cheese. This type of microorganism constituted the entire flora of two of the Colby cheese samples and 92% of the population of the third Colby sample. The infrequent occurrence of this group of bacteria in young Cheddar cheese was demonstrated by their presence in only eight of 47 Cheddar cheeses.

Lactobacillus sp. occurred infrequently in the cheeses used in this study. These organisms were isolated in 12 of the 50 samples. Lactobacilli were isolated from three of the six 3-month old Cheddar cheese samples. In two of these samples they contributed 83 and 92% of the total bacterial flora.

Overall, gram-negative rods were found infrequently. In one sample, however, they constituted one-third of the flora. This group contributed 3% of the total bacterial population and was recovered in only 15 samples.

Only five isolates were categorized as other miscellaneous microorganisms. Of these, three closely resembled the Arthrobacter, one was a yeast and one was a mold.

Proteolytic and lipolytic action was determined on 265 isolates. Only two lipolytic isolates were observed, however, 204 isolates were proteolytic while 61 were not.

Gas-forming organisms were recovered from only ten samples. Of the 75 isolates which did produce gas, 39 produced only trace amounts. Organisms in this group included

26 miscellaneous associated bacteria, ten S. durans and three S. faecalis. In one sample, number 18, 26 gram-positive rods were isolated. All of these produced large amounts of gas in all carbohydrates, and, also, in Nutrient gelatin.

Plating Lactic Starter Cultures

Table 5 shows the counts obtained when 45 commercial lactic starter cultures were plated using the medium and techniques employed in plating cheese samples. To further verify the growth of lactic cultures under the conditions used, counts were determined at 21 C for comparison with the counts obtained at 7 C. A t test analysis showed that the counts obtained at 21 C were significantly higher at the 1% level than those at 7 C.

A total of 379 colonies were picked to litmus milk, from the agar plates incubated at 7 C, to determine if growth would occur at 45 C. None of the isolates grew at this temperature as determined by the absence of a change in the litmus milk.

Table 5. Agar plate counts of commercial lactic starter cultures

Culture designation	Agar plate count/ml 21 C - 10 days	Agar plate count/ml 7 C - 3 months
CH 2	48 x 10^7	91 x 10^6
CH 3	130 x 10^7	130 x 10^7
CH 4	96 x 10^7	110 x 10^7
CH 5	35 x 10^6	37 x 10^6
CH 6	220 x 10^6	190 x 10^6
CH 8	54 x 10^7	44 x 10^7
CH 9	40 x 10^7	120 x 10^6
CH 10	22 x 10^6	18 x 10^6
CH 11	100 x 10^7	99 x 10^7
CH 12	84 x 10^6	53 x 10^6
CH 15	58 x 10^7	200 x 10^6
CH 18	110 x 10^6	130 x 10^6
CH 40	290 x 10^6	33 x 10^7
CH 41	210 x 10^6	13 x 10^6
CH 42	4 x 10^6	<1 x 10^6
CH 43	200 x 10^6	<1 x 10^6
CH 60	110 x 10^6	35 x 10^6
CH 60X	68 x 10^7	48 x 10^7
LC 10	120 x 10^6	32 x 10^6
LC 11	59 x 10^7	63 x 10^7
LC 12	29 x 10^7	19 x 10^6
LC 21	89 x 10^6	35 x 10^6
LC 26	80 x 10^7	87 x 10^7
LC 28	160 x 10^7	22 x 10^6
LC 29	80 x 10^7	76 x 10^7
K 11	90 x 10^6	5 x 10^6
K 22	110 x 10^6	5 x 10^6
K 33	6 x 10^6	2 x 10^6
K 44	2 x 10^6	<1 x 10^6
Pf 1	7 x 10^6	<1 x 10^6
Pf 1A	29 x 10^6	11 x 10^6
Pf 2	65 x 10^6	44 x 10^7
Pf 3	160 x 10^6	20 x 10^6
Pf 3A	2 x 10^6	8 x 10^6
Pf 4	38 x 10^6	92 x 10^6

Table 5. (Continued)

Culture designation	Agar plate count/ml 21 C - 10 days	Agar plate count/ml 7 C - 3 months
Pf 4A	2×10^6	24×10^6
Pf 51	190×10^6	63×10^6
Pf 52	5×10^6	20×10^6
FD	47×10^7	73×10^6
FL	130×10^6	27×10^6
M 9	280×10^6	100×10^6
Xi	190×10^6	53×10^6
122	270×10^6	58×10^6
W 26	73×10^6	31×10^6
B 6	200×10^6	20×10^6

DISCUSSION

Many workers have shown interest in the microflora of young Cheddar cheese. Robertson (58) demonstrated his interest by development of an agar medium which would inhibit starter streptococci so that during the first few weeks of ripening the predominance of lactic streptococci would not obscure the remainder of the bacterial flora present. Robertson stated:

> Yet a knowledge of the incidence of bacterial strains and species apart from those derived from the starter is desirable because they may play an important part in the development of cheese flavour. They may act by means of extra- or intra-cellular enzymes even if they survive for only a short time in the ripening cheese. A short-lived species in the cheese may be completely concealed and thus escape isolation. (58, p. 1)

Robertson and Perry (59) stated that the results presented in their paper clearly demonstrated the fallacy of assuming that it is necessarily the organisms present in mature cheese which are responsible for Cheddar flavor. In their paper they drew attention to the possibility that bacterial action in the milk and in the young cheese may play an important part in flavor development.

Robertson stated:

> Information about the non-starter bacteria which are not sufficiently numerically important to be detected by isolating colonies from starter-free plates can only be obtained by the use of appropriate selective media. (58, p. 6)

Perhaps the reason this attitude has prevailed among research

workers in the past is because no reports were available of the microbial flora capable of growth in Cheddar cheese at the common, low ripening temperatures. The primary importance of this research study is that it is the first known report of the microorganisms, found in young Cheddar cheese, which are capable of multiplication at the common cheese ripening temperature.

Bacteriological Examination of Cheese Samples

The slightly lower agar plate counts of the cheese samples made from pasteurized milk is in accord with the findings of Franklin and Sharpe (25, 26), as well as most other workers. If this trend in count did not agree with the trends reported by other workers, it probably could be supported by the differences in plating and incubation, because the techniques used here were considerably different from any others previously reported. Decreases observed in the bacterial count of Cheddar cheese with increased ripening also have been reported commonly by many workers, including Franklin and Sharpe (25, 26).

Characterization and Identification of Microorganisms Isolated from Cheese

Table 6 shows the theoretical number of each type of microorganism isolated from the cheese samples. These numbers were calculated from the percentage of each group of organ-

Table 6. Theoretical number of microorganisms in each sample[a,b]

Sample number	Streptococci, Enterococcus group	Streptococci, Lactic group	Miscellaneous associated bacteria	Lactobacilli	Micrococc
1	102	--[c]	--	102	396
2	140	--	--	--	720
3	730	--	--	--	270
4	--	--	10,120	880	--
5	--	--	1,600	--	--
6	--	--	1,100	--	--
7	--	220	--	340	220
8	--	202	22	--	202
9	54	--	--	18	256
10	485	485	--	--	--
11	1,875	--	--	--	200
11A[d]	2,760	--	--	--	--
12	93	93	--	--	775
12A[d]	80	--	--	--	960
13	903	--	--	--	462
13A[d]	2,136	--	--	--	264
14	1,488	--	--	112	--
15	--	2,816	--	--	192
16	231	--	--	--	792
17	5,022	--	--	378	--
18	--	38,000	--	--	--
19	21,000	5,000	--	74,000	--
20	1,650	1,100	2,585	--	165
21	5,225	--	--	275	--
22	49,000	--	--	--	--

[a]Based on percentage of isolates and total agar plate count/g at 7 C.

[b]Numbers expressed in thousands/g.

[c]None at the dilution studied.

[d]Duplicate sample; agar plates incubated in an increased carbon dioxide atmosphere.

rococci	Miscellaneous gram-positive rods	Miscellaneous gram-negative rods	Other miscellaneous microorganisms	Total
396	--	--	--	600
720	140	--	--	1,000
270	--	--	--	1,000
--	--	--	--	11,000
--	--	--	--	1,600
--	--	--	--	1,100
220	--	110	110	1,000
202	229	43	22	720
256	122	--	--	450
--	--	--	--	970
200	325	100	--	2,500
--	240	--	--	3,000
775	1,178	961	--	3,100
960	180	700	80	2,000
462	189	462	84	2,100
264	--	--	--	2,400
--	--	--	--	1,600
192	--	192	--	3,200
792	--	77	--	1,100
--	--	--	--	5,400
--	38,000	--	--	76,000
--	--	--	--	100,000
165	--	--	--	5,500
--	--	--	--	5,500
--	--	--	--	49,000

Table 6. (Continued)

Sample number	Streptococci, Enterococcus group	Streptococci, Lactic group	Miscellaneous associated bacteria	Lactobacilli	Micrococci	g
23	1,950	--	--	--	950	
24	8,970	--	2,340	--	1,040	
25	136	--	--	--	1,564	
26	649	--	--	--	319	
27	4,346	--	--	--	371	
28	168	--	--	--	1,176	
29	2,000	5,750	17,250	--	--	
30	552	4,830	1,104	207	--	
31	4,950	10,050	--	--	--	
32	10,010	--	330	--	330	
33	11,000	--	--	--	--	
34	8,500	--	--	--	--	
35	13,920	--	--	--	--	
36	4,700	--	--	--	--	
37	10,000	--	--	--	--	
38	10,000	--	--	--	--	
39	2,880	320	--	--	680	
40	1,610	--	--	--	1,715	
41	2,890	--	--	--	102	
42	1,520	798	114	--	570	
43	3,969	189	--	--	1,953	
44	8,500	--	--	--	--	
45	780	--	--	--	--	
46	752	--	24	24	--	
47	1,700	--	--	--	--	
48	207	--	--	1,909	--	
49	--	--	--	6,532	284	
50	2,800	--	--	--	--	

ococci	Miscellaneous gram-positive rods	Miscellaneous gram-negative rods	Other miscellaneous microorganisms	Total
950	1,300	500	300	5,000
,040	650	--	--	13,000
,564	1,564	136	--	3,400
319	132	--	--	1,100
371	--	583	--	5,300
,176	672	84	--	2,100
--	--	--	--	25,000
--	--	207	--	6,900
--	--	--	--	15,000
330	330	--	--	11,000
--	--	--	--	11,000
--	--	--	--	8,500
--	2,080	--	--	16,000
--	--	--	--	4,700
--	--	--	--	10,000
--	--	--	--	10,000
680	--	120	--	4,000
,715	175	--	--	3,500
102	306	102	--	3,400
570	798	--	--	3,800
,953	189	--	--	6,300
--	--	--	--	8,500
--	--	--	--	780
--	--	--	--	800
--	--	--	--	1,700
--	92	92	--	2,300
284	284	--	--	7,100
--	--	--	--	2,800

isms, based upon the total agar plate count at 7 C. The figures are of interest from either viewpoint; that of Hastings et al. (30) that the constant presence of microorganisms in large numbers is the only certain proof that the organism is of importance in the ripening of cheese, or that of Robertson (58) that a short-lived, minority species or group of organisms may be important in cheese ripening. Although the figures are only theoretical, they serve as a better source of comparison with other reports on levels of organisms found in cheese.

The large numbers and high percentages of enterococci recovered from cheese are not in complete accord with previous reports on the levels of these organisms. However, several workers (25, 26, 28, 29, 44, 62, 76) have reported the frequent occurrence of enterococci in young cheese. All of these reports have been published since 1942. Some research workers (15, 23) have isolated and characterized microorganisms only as lactic acid bacteria. Enterococci could have comprised high percentages of this group of bacteria, although complete identification was not made. Many early workers (3, 14, 21, 22, 61) also reported the occurrence of lactic acid bacteria without additional information as to what constituted this group. To these workers, enterococci, as we know them today, were not defined and therefore could not be reported separately.

In this study, the classification of streptococci as given in Bergey's Manual of Determinative Bacteriology (5) was used, and only those organisms included in the enterococcus group were regarded as enterococci. In characterization and classification of the enterococci, two similar, yet uniformly distinct groups of partial-reducing organisms were found. Both of these were included in the S. durans group. Therefore, this group consists of 273 typical and 72 atypical S. durans species. Isolates were considered atypical only if they exhibited more than two properties not characteristic of S. durans.

Certain observations made of the two types of organisms included in the S. durans group are worthy of further consideration. Usually, hemolysis, carbohydrate fermentation pattern, litmus reaction and reduction of methylene blue milk distinguished between the two types. Typical S. durans was beta-hemolytic, produced acid from three carbohydrates (glucose, lactose and maltose), always coagulated litmus milk prior to partial reduction and was delayed in reducing methylene blue milk. Atypical S. durans varied from alpha- or beta-hemolytic, to alpha- and beta-hemolytic, to non-hemolytic. These strains usually produced acid from six carbohydrates. They seldom coagulated litmus milk at 45 C although showing a definite acid reaction with a completely reduced butt and partial reduction throughout the tube. They also rapidly

reduced methylene blue milk.

Non-hemolytic strains of S. durans have been reported by Gibson and Abd-El-Malek (27) and Shattock (68). Shattock (67) cautioned against the use of hemolysis as a suitable character for differentiation of group D streptococci. She also acknowledged (67, 68) that not all group D streptococci can be assigned a species name and that many strains from dairy products, as well as from other sources, are so divergent in their biochemical and cultural reactions that for the present time they must remain anonymous.

Rather than record atypical isolates as species variants, or as new species, thus burdening the already confused literature on this subject, typical and atypical S. durans types were recognized as a "physiological division within the enterococci" (66), and were considered to constitute the S. durans group.

An interesting property of the majority of both typical and atypical S. durans isolates was their inability to produce a positive Voges-Proskauer reaction. Only one of 273 typical S. durans strains was Voges-Proskauer positive. Fifteen of the 72 atypical S. durans strains gave positive Voges-Proskauer tests. The low incidence (16 of 345) of isolates within this group producing a positive Voges-Proskauer test was felt to be significant. Abd-El-Malek and Gibson (1) reported that the formation of acetoin from citrate and glucose was common,

but not a universal property, among organisms included in the enterococcus group.

Reduction of 0.3% methylene blue milk was found to be a characteristic common to most enterococci. Except in a few instances, all enterococci reduced 0.3% methylene blue milk. Although this property is not utilized for primary identification of the enterococci, it is, nevertheless, an important characteristic because certain lactic streptococci also have this property. The partial-reducing enterococci (S. durans group) varied in their ability to reduce 0.3% methylene blue milk, some showing only reduction of the butt.

Only eight of the cheese samples examined did not contain enterococci, and five of these were made from fully pasteurized milk. These findings appear to be contrary to those reported by Tittsler et al. (76), that after 1 month the flora of pasteurized-milk cheese consisted almost entirely of enterococci. However, because of the few pasteurized-milk cheese samples studied, as well as the difference in age of cheese samples in this investigation, it is not possible to base definite conclusions on these results.

Some correlation appeared to exist between good Cheddar cheese and the level of enterococci recovered from the samples. For instance, in 19 samples, from two cheese plants making high quality Cheddar cheese, 13 contained in excess of 85% enterococci. Dahlberg and Kosikowsky (13) reported that

they obtained Cheddar cheese with more flavor and better quality using a strain of S. faecalis for the starter rather than a lactic starter culture.

Continuously throughout this experiment, one type of microorganism was found to constitute 50% or more of the bacterial flora in a sample. In 42 samples, one group of organisms comprised the majority of the flora. In addition, in four of the eight remaining samples, one group constituted greater than 45% of the sample flora. This associates a particular type of microorganism with each cheese sample. An attempt to correlate a particular flora with each cheese plant was difficult, primarily because of the high incidence of enterococci. However, as previously stated, 13 of 19 samples from two factories contained over 85% enterococci. In these plants, the characteristic, dominant flora was enterococci.

The significance of enterococci in Cheddar cheese is still unknown. Various workers (52, 62) have reported the presence of these organisms in cheese, and their fate upon ripening, without being able to place any specific significance on them.

Difficulty was encountered in classification of the lactic streptococci to typical S. lactis and S. cremoris species. For this reason, these organisms were included together in the lactic group. The common occurrence of variants of S. lactis and S. cremoris strains has been

reported by Yawger and Sherman (80), Sherman and Hussong (69), Davis (14) and Rašić (56), as well as many other workers. Allen and Knowles (3) reported that some atypical organisms isolated from cheese possibly were derived from more typical strains of lactic acid streptococci whose characters had become modified in the course of cheese ripening. Nichols and Hoyle (50) reported difficulty in the classification of "wild" lactic strains and grouped the isolates as intermediates between S. lactis and S. cremoris.

Except for the large percentage of enterococci, and the low incidence of lactic streptococci, the bacterial flora recovered in this experiment did not differ extensively from the flora reported by most other research workers. Micrococci, which constituted 13% of the isolates, were the predominant flora in seven samples. This result is in agreement with other workers (15, 23) who have reported large numbers of micrococci in some cheese samples. Specific tests were not used to determine whether any of the organisms included in the micrococcus group were staphylococci.

Costilow and Humphreys (8) reported that certain strains of L. plantarum reduced nitrates. Bergey's Manual of Determinative Bacteriology (5) indicates that nitrates are not reduced, except under certain conditions, with L. plantarum. Of the 67 lactobacilli isolated in this study, 64 reduced nitrates, two did not reduce nitrates and one was not tested

for this property. The lactobacillus isolates were not identified to species. These results indicate that nitrate reduction, at least among isolates from Cheddar cheese, may be a more common characteristic of the lactobacilli than previously believed, since special conditions for determining nitrate reduction were not utilized.

Reports that low levels of coliform organisms commonly are found in Cheddar cheese (23, 40, 62) were supported by this investigation. Based upon the definition of coliform organisms presently in use (4) no coliform organisms were recovered. In fact, gas-producing microorganisms in general were recovered infrequently.

One-third of the gas-forming organisms recovered were isolated from a single sample, number 18. These 26 isolates were gram-positive rods and comprised 50% of the sample flora. However, abnormalities were not observed in the cheese sample. Twenty-five, or another one-third, of the gas-producing organisms were isolated from the three Colby cheese samples. These isolates were characterized as miscellaneous associated bacteria. The remaining gas-producing organisms were recovered sporadically from the remaining 46 samples. Depending upon the circumstances and conditions existing, gas-producing microorganisms might be responsible for the gassy defect or slit-openings which may occur in Cheddar cheese.

Of the microorganisms tested for proteolysis, 77% gave

positive results. This cannot be considered unusual because degradation of protein has been firmly established as one of the changes occurring in ripening cheese. Certainly, proteolytic microorganisms capable of growth at temperatures common to those used for ripening cheese may play a role in this process. The proteolytic action of streptococci and lactobacilli was not determined. However, these organisms also are known to influence protein degradation in ripening Cheddar cheese.

Less than 1% of the microorganisms tested for lipolysis gave positive results under the test conditions used. It is not surprising to find fewer lipolytic than proteolytic microorganisms, since the role of fat hydrolysis has not yet been fully explained in Cheddar cheese ripening.

Perhaps this discussion of the characterization of microorganisms isolated from cheese best can be summarized by reference to the comments and observations made by Alford and Frazier on a study they made of the organisms found in raw-milk cheese:

> Some of the characteristics that were used to indicate the possible importance of the micrococci in cheese ripening were not necessary for the differentiation of species. This emphasizes the difficulty of classification of cultures for specific purposes or showing unusual characteristics on the basis of conventional differential tests. However, the present knowledge of bacterial taxonomy has not clearly indicated what constitutes a fundamental characteristic for differentiation of species and what should be considered as a characteristic of incidental value; therefore, any additional arbitrary designation of new species would only add to the present confusion. (2, p. 113)

In this investigation, it is important to realize that only a brief segment of time was included. This stage was early in the ripening period of the cheese and an attempt was not made to determine whether levels of the microorganisms recovered were increasing, decreasing or remaining stationary. Specific delineation of the role of these organisms in cheese ripening would depend upon their ability to survive, at least in moderate numbers, for a time to leave sufficient levels of metabolic products to effect later changes. Also, additional information regarding the biochemical reactions of these microorganisms would be necessary for establishing their role in the ripening process. Before either of these areas could be studied, it was necessary to find out what organisms usually are present in young Cheddar cheese under selected conditions. It is felt that the present investigation fulfilled this need.

Plating Lactic Starter Cultures

Although the agar plate count of starter cultures was significantly higher at 21 than at 7 C, counts were within the same general range for most samples. In view of this, it can be said that the plating procedure used in this study was not unduly inhibitory to lactic starter microorganisms. Cognizance is given to the fact that in spite of the differences in cultural procedures used in this investigation, microorgan-

isms have optimum growth temperatures. A temperature of 21 C is more favorable for the growth of lactic streptococci than is 7 C. Therefore, even the prolonged, 3-month incubation period may not have permitted maximum growth by the lactic streptococci.

The failure to recover enterococci from any of the commercial lactic starter cultures studied indicates that these cultures are not the source of enterococci in Cheddar cheese. Although Sharpe et al. (65) found that lactobacilli were common in cheese starter cultures, Kosikowsky and Dahlberg (39) and Nichols and Hoyle (50) reported that no enterococci could be isolated from the commercial starter cultures which they examined.

SUMMARY

Fifty, young, commercial Cheddar cheese samples, from 11 Iowa manufacturing plants, were plated to determine their low temperature microflora. The plating medium used was special Trypticase-soy agar. After the pour plates had been prepared and allowed to solidify, 50 ml of 1.5% agar was used as a cover-layer on each. Plates were sealed with wide rubber bands and incubated at 7 C for 3 months.

Following incubation, colonies were picked from the agar plates and transferred to litmus milk. Purification was accomplished by streaking special Trypticase-soy agar plates from the litmus milk culture and picking a representative, well isolated colony. Purified isolates were Gram stained and then frozen until all isolations had been made.

Isolates were characterized using the following tests: Gram stain, catalase reaction, litmus milk reaction at 21, 32, 37 and 45 C, methyl red and Voges-Proskauer tests, salt tolerance, gelatin liquefaction, carbohydrate fermentation, ammonia production from arginine, reduction of methylene blue milk, initiation of growth at high pH, hemolytic reaction, nitrate reduction, formation of indole from tryptophan, growth on an agar slant and proteolytic and lipolytic action.

From the cheese samples examined, 1162 microorganisms were isolated and characterized. An additional 67 isolates, from agar plates of three duplicate samples, incubated in an

increased carbon dioxide atmosphere, also were characterized. The following numbers and percentages of microorganisms were found: 578 enterococci, 50%; 149 micrococci, 13%; 126 lactic streptococci, 10.5%; 110 miscellaneous gram-positive rods, 9%; 93 miscellaneous associated bacteria, 8%; 67 lactobacilli, 6%; 34 miscellaneous gram-negative rods, 3% and five other miscellaneous microorganisms, 0.5%.

To ascertain that the plating procedure used to isolate microorganisms from the young Cheddar cheese samples was not inhibitory to lactic starter organisms, 45 commercial lactic starter cultures, obtained from five different sources, were plated using the procedure followed for examination of the cheese samples. Counts were made at 21 and 7 C for comparative purposes. The counts obtained at 21 C were significantly higher than those obtained at 7 C. However, for most samples, the counts at both temperatures were within the same range. Of 379 colonies picked to litmus milk, from the agar plates incubated at 7 C, none initiated growth at 45 C. This indicated that commercial lactic starter cultures are not a source of enterococci in Cheddar cheese.

LITERATURE CITED

1. Abd-El-Malek, Y. and Gibson, T. Studies in the bacteriology of milk. I. The streptococci of milk. J. Dairy Res. 15: 233. 1948.

2. Alford, J. A. and Frazier, W. C. Occurrence of micrococci in Cheddar cheese made from raw and from pasteurized milk. J. Dairy Sci. 33: 107. 1950.

3. Allen, L. A. and Knowles, N. R. Studies in the ripening of Cheddar cheese. IV. The bacterial flora of cheese made from milk of a very low bacterial count. V. The influence of the rennet flora on the flora of the cheese. VI. The influence of the starter on the ripening process. J. Dairy Res. 5: 185. 1934.

4. American Public Health Association. Standard methods for the examination of dairy products. 11th ed. New York, N.Y., Author. 1960.

5. Breed, R. S., Murray, E. G. D. and Smith, N. R. Bergey's manual of determinative bacteriology. 7th ed. Baltimore, Md., Williams and Wilkins Co. 1957.

6. Clark, W. S., Jr., Moore, T. D. and Nelson, F. E. Characterization of coagulase-positive staphylococci isolated from raw milk. Appl. Microbiol. 9: 195. 1961.

7. Clark, W. S., Jr. and Nelson, F. E. Multiplication of coagulase-positive staphylococci in grade A raw milk samples. J. Dairy Sci. 44: 232. 1961.

8. Costilow, R. N. and Humphreys, T. W. Nitrate reduction by certain strains of Lactobacillus plantarum. Sci. 121: 168. 1955.

9. Crossley, E. L. The coliform flora of milk and dairy products. J. Dairy Res. 14: 233. 1945.

10. Crossley, E. L. Coliform organisms in Cheddar cheese. Soc. Agr. Bacteriol. Proc. (Abstr.) 1942: 16. 1942.

11. Dacre, J. C. Characteristics of a presumptive pediococcus occurring in New Zealand Cheddar cheese. J. Dairy Res. 25: 409. 1958.

12. Dacre, J. C. A note on the pediococci in New Zealand Cheddar cheese. J. Dairy Res. 25: 414. 1958.

13. Dahlberg, A. C. and Kosikowsky, F. V. The development of flavor in American Cheddar cheese made from pasteurized milk with Streptococcus faecalis starter. J. Dairy Sci. 31: 275. 1948.

14. Davis, J. G. Studies in Cheddar cheese. IV. Observations on the lactic acid flora of Cheddar cheese made from clean milk. J. Dairy Res. 6: 175. 1935.

15. Dawson, D. J. and Feagan, J. T. Bacteriology of Cheddar cheese. A study of starter organisms in manufacture and maturing. J. Dairy Res. 24: 210. 1957.

16. Deane, D. D. and Anderson, T. G. Comparative studies on Cheddar cheese prepared with starter and with certain pure cultures. J. Dairy Sci. 25: 729. 1942.

17. Deane, D. D., Anderson, T. G., Warren, F. G. and Dahle, C. D. Use of pure cultures in the manufacture of Cheddar cheese. Natl. Butter Cheese J. 36: 25. 1945.

18. Difco Laboratories, Inc. Difco manual of dehydrated culture media and reagents. 9th ed. Detroit, Mich., Author. 1953.

19. Donnelly, C. B., Black, L. A. and Lewis, K. H. The occurrence of coagulase-positive staphylococci in Cheddar cheese. A preliminary report. Bacteriol. Proc. 26: 23. 1962.

20. Evans, A. C. The bacteria of milk freshly drawn from normal udders. J. Infectious Diseases 18: 437. 1916.

21. Evans, A. C. A study of the streptococci concerned in cheese ripening. J. Agr. Res. 13: 235. 1918.

22. Evans, A. C., Hastings, E. G. and Hart, E. B. Bacteria concerned in the production of the characteristic flavor in cheese of the Cheddar type. J. Agr. Res. 2: 167. 1914.

23. Feagan, J. T. and Dawson, D. J. Bacteriology of Cheddar cheese. Some observations on the microflora during maturing. Australian J. Dairy Technol. 14: 59. 1959.

24. Foster, E. M., Nelson, F. E., Speck, M. L., Doetsch, R. N. and Olson, J. C., Jr. Dairy microbiology. Englewood Cliffs, N. J., Prentice-Hall, Inc. 1957.

25. Franklin, J. G. and Sharpe, M. E. The bacterial content of milk used for making Cheddar cheese and its effect on the flavor and quality of the cheeses produced. Intern. Dairy Congr. 16, Vol. C: 305. 1962.

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

27. Gibson, T. and Abd-El-Malek, Y. The formation of carbon dioxide by lactic acid bacteria and Bacillus licheniformis and a cultural method of detecting the process. J. Dairy Res. 14: 35. 1945.

28. Hannay, C. L. and Newland, L. G. Haemolytic streptococci in cheese. J. Dairy Res. 17: 206. 1950.

29. Harding, H. A. and Prucha, M. J. The bacterial flora of Cheddar cheese. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 8. 1908.

30. Hastings, E. G., Evans, A. C. and Hart, E. B. The bacteriology of Cheddar cheese. U. S. Dept. Agr. Bur. Animal Industry Bull. 150. 1912.

31. Hastings, E. G., Evans, A. C. and Hart, E. B. Studies on the factors concerned in the ripening of Cheddar cheese. Wisc. Agr. Expt. Sta. Res. Bull. 25. 1912.

32. Hendricks, S. L., Belknap, R. A. and Hausler, W. J., Jr. Staphylococcal food intoxication due to Cheddar cheese. I. Epidemiology. J. Milk and Food Technol. 22: 313. 1959.

33. Hill, D. A. and Thornton, H. R. Lactobacilli in Edmonton dairy products. Can. J. Microbiol. 4: 215. 1958.

34. Hucker, G. J. The types of bacteria found in commercial Cheddar cheese. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 90. 1922.

35. Irvine, O. R. and Beach, M. E. Method for enumerating nonlactic organisms in fresh Cheddar cheese. J. Dairy Sci. 45: 648. 1962.

36. Johns, C. K. and Cole, S. E. Lactobacilli in Cheddar cheese. J. Dairy Res. 26: 157. 1959.

37. Kelly, C. D. Lactic acid streptococci associated with the early stages of Cheddar cheese ripening. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 201. 1932.

38. Knaysi, G. On the use of basic dyes for the demonstration of the hydrolysis of fat. J. Bacteriol. 42: 587. 1941.

39. Kosikowsky, F. V. and Dahlberg, A. C. The growth and survival of Streptococcus faecalis in pasteurized milk American Cheddar cheese. J. Dairy Sci. 31: 285. 1948.

40. Lightbody, L. G. Coliform organisms in Queensland Cheddar cheese. Qld. J. Agr. Sci. 19: 305. 1962.

41. Lord, T. H. Determinative bacteriology laboratory manual. Minneapolis, Minn., Burgess Publishing Co. c1959.

42. Mabbitt, L. A. and Zielinska, M. The use of a selective medium for the enumeration of lactobacilli in Cheddar cheese. J. Appl. Bacteriol. 19: 95. 1956.

43. Mattick, A. T. R., Neave, F. K., Chapman, H. R. and Berridge, N. J. The fate of Staphylococcus aureus in Cheddar cheese. J. Appl. Bacteriol. 21: ii. 1958.

44. Mattick, A. T. R. and Shattock, P. M. F. Group D streptococci in English hard cheese. Monthly Bull. Ministry Health and Public Health Lab. Serv. 2: 73. 1943. Original not available; cited in Hannay, C. L. and Newland, L. G. Haemolytic streptococci in cheese. J. Dairy Res. 17: 206. 1950.

45. McCoy, E. Staphylococcal problems in the dairy industry. Milk Products J. 50, No. 12: 14. Dec. 1959.

46. McLeod, R. W., Roughley, R. J. and Richards, J. Staphylococcus aureus in Cheddar cheese. Effect of numbers on survival. Australian J. Dairy Technol. 17: 54. 1962.

47. Naylor, J. and Sharpe, M. E. Lactobacilli in Cheddar cheese. I. The use of a selective media for isolation and of serological typing for identification. J. Dairy Res. 25: 92. 1958.

48. Naylor, J. and Sharpe, M. E. Lactobacilli in Cheddar cheese. II. Duplicate cheeses. J. Dairy Res. 25: 421. 1958.

49. Naylor, J. and Sharpe, M. E. Lactobacilli in Cheddar cheese. III. The source of lactobacilli in cheese. J. Dairy Res. 25: 431. 1958.

50. Nichols, A. A. and Hoyle, M. The identity of streptococci from starter and of streptococci, suitable for use as starter, isolated from sour milk. J. Dairy Res. 15: 409. 1948.

51. Niven, C. F., Jr., Smiley, K. L. and Sherman, J. M. The hydrolysis of arginine by streptococci. J. Bacteriol. 43: 651. 1942.

52. Nogle patogene bakteriers termoresistens ved pasteurisering af ostemaelk og disse bakteriers resistens ved fremstilling og lagring af danask ost. (In Danish, English summary.) Beretning fra Statens Forsøgsmejeri. 124. 1960.

53. Perry, K. D. A comparison of the influence of Streptococcus lactis and Str. cremoris starters on the flavour of Cheddar cheese. J. Dairy Res. 28: 221. 1961.

54. Perry, K. D. and Sharpe, M. E. Lactobacilli in raw milk and in Cheddar cheese. J. Dairy Res. 27: 267. 1960.

55. Perry, K. D., Sharpe, M. E. and Mattick, A. T. R. Lactobacilli in the air of creameries. J. Dairy Res. 25: 407. 1958.

56. Rašić, J. A study of the resistance of lactic acid bacteria to sodium chloride. Intern. Dairy Congr. 16, Vol. B: 881. 1962.

57. Rašić, J. Trends of bacterial population during the manufacture and ripening of white cheese. Intern. Dairy Congr. 16, Vol. B: 840. 1962.

58. Robertson, P. S. Methods for investigating the bacteria in young Cheddar cheese: inhibition of starter streptococci with bacteriophage and α-bromopropionic acid. J. Dairy Res. 27: 1. 1960.

59. Robertson, P. S. and Perry, K. D. Enhancement of the flavour of Cheddar cheese by adding a strain of micrococcus to the milk. J. Dairy Res. 28: 245. 1961.

60. Rogers, L. A. Cheese investigations. U. S. Dept. Agr. Bur. Dairy Industry Rept. 1942: 17. 1942.

61. Russell, H. L. The rise and fall of bacteria in Cheddar cheese. Wisc. Agr. Expt. Sta. Ann. Rept. 13: 95. 1896.

62. Saraswat, D. S. Detection and significance of enterococci in dairy products. Unpublished Ph.D. thesis. Ames, Iowa, Library, Iowa State University of Science and Technology. 1963.

63. Sharpe, M. E. Enumeration and studies of lactobacilli in food products. Dairy Sci. Abstr. 24: 165. 1962.

64. Sharpe, M. E. and Mattick, A. T. R. Lactobacilli in milk used for commercial cheesemaking. J. Dairy Res. 27: 277. 1960.

65. Sharpe, M. E., Perry, K. D., Mattick, A. T. R., Adams, R. P., Druce, R. G., Egdell, J. W., Thomas, S. B. and Crawford, R. J. M. Lactobacilli in cheese starter cultures. J. Dairy Res. 25: 418. 1958.

66. Shattock, P. M. F. Enterococci. In Ayres, J. C., Kraft, A. A., Snyder, H. E. and Walker, H. W., eds. Chemical and biological hazards in food. pp. 303-319. Ames, Iowa, Iowa State University Press. c1962.

67. Shattock, P. M. F. The identification and classification of Streptococcus faecalis and some associated streptococci. Ann. Inst. Pasteur, Lille 7: 95. 1955.

68. Shattock, P. M. F. Streptococcus durans. Soc. Appl. Bacteriol. Proc. (Abstr.) 1945: 18. 1945.

69. Sherman, J. M. and Hussong, R. V. Fermentative variability among substrains of Streptococcus cremoris and Streptococcus lactis obtained from pure cultures. J. Dairy Sci. 20: 101. 1937.

70. Sherwood, I. R. The bacterial flora of New Zealand Cheddar cheese. J. Dairy Res. 10: 426. 1939.

71. Smith, R. E. and Cunningham, J. D. The characterization of lactobacilli from Cheddar cheese. I. An evaluation of physiological and biochemical tests. Can. J. Microbiol. 8: 727. 1962.

72. Society of American Bacteriologists. Committee on Bacteriological Technic. Manual of microbiological methods. New York, N. Y., McGraw-Hill Book Co., Inc. 1957.

73. Solberg, P. The effect of the acid development on properties of cheese. Intern. Dairy Congr. 16, Vol. D: 191. 1962.

74. Takahashi, I. and Johns, C. K. Staphylococcus aureus in Cheddar cheese. J. Dairy Sci. 42: 1032. 1959.

75. Thatcher, F. S., Simon, W. and Walters, C. Extraneous matter and bacteria of public health significance in cheese. Can. J. Public Health 47: 234. 1956.

76. Tittsler, R. P., Geib, D. S., Sanders, G. P., Walter, H. E., Sager, O. S. and Lochry, H. R. The effects of quality and pasteurization of milk on the bacterial flora and quality of Cheddar cheese. J. Bacteriol. 51: 590. 1946.

77. Walker, G. C., Harmon, L. G. and Stine, C. M. Staphylococci in Colby cheese. J. Dairy Sci. 44: 1272. 1961.

78. Walters, A. H. The isolation of coagulase-positive staphylococci from routine composite milk samples. J. Appl. Bacteriol. 22: 248. 1959.

79. Yale, M. W. and Marquardt, J. C. Coliform bacteria in Cheddar cheese. N. Y. (Geneva) Agr. Expt. Sta. Tech. Bull. 270. 1943.

80. Yawger, E. S. and Sherman, J. M. Variants of Streptococcus lactis which do not ferment lactose. J. Dairy Sci. 20: 83. 1937.

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APPENDIX

Composition of Bacteriological Media Expressed as Grams per Liter of Distilled Water

1. Special Trypticase-soy agar

Trypticase, BBL	15.0
Phytone, BBL	5.0
Sodium chloride	4.0
Sodium citrate	1.0
L-cystine	0.2
Dextrose	5.0
Bacto-agar	15.0

Adjust to pH 6.7 before autoclaving.
Autoclave at 121 C for 20 min.

2. Trypticase-soy broth

Trypticase, BBL	15.0
Phytone, BBL	5.0
Sodium chloride	4.0
Sodium citrate	1.0
Potassium phosphate, dibasic	2.0
Dextrose	5.0

Adjust to pH 7.0 before autoclaving.
Autoclave at 121 C for 15 min.

3. Methyl red Voges-Proskauer medium

Bacto-Peptone	7.0
Dextrose	5.0
Potassium phosphate, dibasic	5.0

Adjust to pH 7.2 before autoclaving.
Autoclave at 121 C for 15 min.

4. Basal medium for carbohydrate fermentation

Bacto-Yeast extract	2.0
Bacto-Proteose peptone, Difco	20.0
Phenol red	0.036

Adjust to pH 7.5 before autoclaving.
Autoclave at 121 C for 15 min.

5. Indole medium

Bacto-Tryptone	10.0
Bacto-Beef extract	10.0

Autoclave at 121 C for 15 min.