THE LOW TEMPERATURE MICROFLORA OF YOUNG CHEDDAR CHEESE[1]

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Summary

A plating procedure simulating conditions in ripening Cheddar cheese was used to isolate microorganisms in young cheese that grow at ordinary ripening temperatures. Forty-one commercial cheese samples, most 7 days old, were obtained from ten Iowa manufacturing plants. Samples were plated in special Trypticase-soy agar, covered with 50 ml of 1.5% (w/v) agar, and incubated at 7.2 C for 3 months. Following incubation, 967 microorganisms were isolated, purified, and characterized. Numbers and percentages of microorganisms were: 475 enterococci, 49.5%; 148 micrococci, 15%; 126 lactic streptococci, 13%, 108 miscellaneous gram-positive rods, 11%; 48 associate bacteria (resembling species of *Leuconostoc*), 5%; 33 miscellaneous gram-negative rods, 3.5%; 24 lactobacilli, 2.5%; and 5 other miscellaneous microorganisms, 0.5%.

Forty-five commercial lactic starter cultures were examined by the same plating procedure. Duplicate plates of each culture were incubated at 7.2 and 21 C. Although agar plate counts were slightly higher at 21 C, they were within the same range, at both incubation temperatures, for most samples. No enterococci were recovered from commercial lactic starter cultures.

The bacteriology and chemistry of Cheddar cheese ripening have been studied extensively. Most bacteriological investigations have dealt with attempts to isolate and identify microorganisms of importance in flavor production and body breakdown.

One of the earliest reports of the bacterial flora of cheese was made by Russell *(21)*, who stated that the bacterial content of cheese differed markedly from that of milk. Harding and Prucha *(10)* studied the microflora of normal cheeses representing first-class factories. More than 300 cultures were isolated from agar plates, 167 of which were studied extensively and divided into 33 different groups. Hastings, Evans, and Hart *(12, 13)* found only two groups of bacteria constantly present in large numbers in Cheddar cheese, while Evans, Hastings, and Hart *(6)*, from a study of 21 raw-milk cheeses, reported that all microorganisms constantly found in cheese were included in four groups.

Hucker's results *(14)* indicated that better grades of Cheddar cheese contained a microflora distinctly different from the microflora of poorer grades. Tittsler et al. *(25)* confirmed the correlation between bacterial flora and cheese quality reported by Sherwood *(24)* that cheese manufactured from poor and very poor milk, either raw or pasteurized, contained many more bacterial types than did cheese manufactured from fair and good milk.

Feagan and Dawson *(7)*, Irvine and Beach *(15)*, and Kelly *(16)* have reported the results of their investigations of the microflora of young Cheddar cheese. However, a search of the literature has failed to reveal reports of any previous attempts to study the Cheddar cheese microflora that grow at low temperatures.

This study was undertaken to determine the low-temperature microflora in young Cheddar cheese. Because Cheddar cheese ordinarily is cured at low temperatures, this approach is commercially important.

Experimental

Collection, handling, and treatment of samples.

Forty-one commercial Cheddar cheese samples from ten Iowa cheese manufacturing plants were obtained for study. All samples were collected and handled according to procedures described in *Standard Methods for the Examination of Dairy Products (3).*

Bacteriological examination of samples.

Samples were plated immediately upon arrival at the laboratory. The procedure given in *Standard Methods for the Examination of Dairy Products (3)* for the yeast and mold count of cheese, other than Cottage, was used to prepare samples for plating, except that emulsification was obtained by high-speed agitation in a Waring Blendor (Waring Product Corporation, New York, N. Y.) for 2 min. Plates were prepared in duplicate and poured with 15 ml of special Trypticase-soy agar composed of:

	% (w/v)
Trypticase, BBL	1.5
Phytone, BBL	0.5
NaCl	0.4
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.1
L-cystine	0.02
Dextrose	0.5
Bacto-agar, Difco	1.5

The medium was adjusted to pH 6.7 before autoclaving at 121 C for 20 min. After solidification, 50 ml of 1.5% (w/v) agar were added to each petri dish. Following solidification

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of the cover-layer, plates were sealed tightly with 1¾-inch-wide rubber bands and incubated at 7.2 C for 3 months. All cheese samples were between 4 and 8 days old when plated.

Isolation and purification of microorganisms.

An attempt was made to pick 30 colonies from one agar plate of each cheese sample. All 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, the colonies were picked according to the random sampling method suggested by Harrison *(11)* until at least 30 isolates were obtained to represent each cheese sample.

To facilitate picking, the thick, two-layer agar plaque was inverted with the aid of a sterile spatula into the petri dish cover. The appearance of each colony was recorded before it was picked for inoculation into sterile litmus milk. The litmus milk used in this study was prepared from fresh skim-milk and was autoclaved at 121 C for 12 min.

Litmus milk cultures were incubated at 21 C until a reaction was observed. They were streaked for purification on special Trypticase-soy agar plates. Plates were incubated at 21 C until colonies formed. A single, characteristic colony from each plate was picked into litmus milk, grown at 21 C, and examined for Gram stain reaction and morphological characteristics of the microorganism. The litmus milk culture then was frozen. If more than one type of colony was observed on the purification agar plates, a colony representing each type was picked.

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

Characterization and identification of isolates.

Frozen cultures were thawed rapidly in a 40 C water bath and immediately inoculated into tubes of litmus milk for incubation at 21 C. As soon as a litmus milk reaction was observed (usually 24 hr), a drop of the fresh transfer was inoculated into special Trypticase-soy broth. This broth was incubated at 21 C until abundant growth was observed. The broth composition was the same as that for special Trypticase-soy agar, except that Bacto-agar was not added.

The following characteristics were determined for each culture by using the special 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 reactions, ability to grow in 4.0 and 6.5% NaCl, gelatin liquefaction, and carbohydrate fermentation. Determination of the fermentative pattern was accomplished by using eight compounds: arabinose, dextrose, glycerin, lactose, maltose, mannitol, sucrose, and xylose. In addition, all catalase-negative cocci were examined for ammonia production from arginine, reduction of 0.1 and 0.3% methylene blue milk, initiation of growth at pH 9.2 and 9.6, and growth in litmus milk at 45 C. Isolates growing at 45 C were streaked on bovine blood agar to determine hemolytic reactions. Those not growing at 45 C were tested for growth in litmus milk at 40 C. All catalase-positive microorganisms were tested for nitrate reduction; all gram-negative rods were tested for the formation of indole from tryptophan. Lipolytic and proteolytic actions were determined for all catalase-positive isolates by streaking the cultures on Nile blue sulfate agar *(17)* and Standard methods agar plus 10% (v/v) added sterile skimmilk *(3)*, respectively. Except when stated otherwise, characterization of isolates was accomplished at 21 C. All media were stored under refrigeration but were removed and properly tempered before inoculation and subsequent incubation.

Plating lactic starter cultures.

To ascertain that the plating procedure used to isolate microorganisms from the young cheese samples did not inhibit lactic starter cultures, 45 commercial starter cultures, obtained from four different sources, were plated in quadruplicate *(3)*. Duplicate plates of each starter culture were incubated at 7.2 C for 3 months and 21 C for 10 days to obtain a comparison between the counts at each incubation temperature. Following the 3-month incubation period, colonies were counted, and 379 were picked into litmus milk. After one transfer in litmus milk at 21 C, transfers were made into litmus milk, previously tempered to 45 C, for incubation at that temperature to insure that the lactic starter cultures were not a source of enterococci.

RESULTS AND DISCUSSION

Bacteriological examination of samples.

Bacterial counts of the cheese samples ranged from 45 x 10^4/g to 100 x 10^6/g (Table 1). Cheeses made from pasteurized milk tended to have slightly lower counts than those made from heat-treated milk. These results are in accord with the findings of Franklin and Sharpe *(8, 9)* as well as most other workers. Even considering the heat treatment given the milk, no large differences in counts were observed between cheeses manufactured at different plants.

Characterization and identification of microorganisms isolated from cheese.

Microorganisms isolated from Cheddar cheese were classified into eight groups based upon morphological and biochemical characteristics. The data presented in Table 2 summarize the types and incidence of microorganisms isolated from the cheese samples studied.

Enterococci constituted the entire population recovered from seven samples and more than 70% of the population in ten others. Enterococci were present in all but four samples, occurring more frequently and in much larger numbers than any other group. Fifty-six per cent of the enterococci belonged in the *Streptococcus durans* group, 32% were identified as *S. faecalis*, 9% as *S. faecalis* var. *liquefaciens*, and 3% as *S. faecalis* var. *zymogenes*. Several workers *(8, 9, 22, 25)* have reported the frequent occurrence of enterococci in young cheese. Others *(5, 7)* have isolated and characterized microorganisms only as lactic acid bacteria. Enterococci could have constituted a high percentage of this group of bacteria. In addition, early workers *(6, 10, 21)* reported the occurrence of lactic acid bacteria without clearly specifying what was included in this group. To these investigators, enterococci, as we know them, were not defined and, therefore, could not be reported separately.

TABLE 1. AGAR PLATE COUNTS OF CHEESE SAMPLES

Manufacturing plant	Sample number	Heat treatment of milk	Age of cheese	Agar plate count/g[a]
			(Days)	(X 10^9)
A	1	162 F; 20 sec	7	6
	2			10
	3			10
B	4	162 F; 15 sec	7	10
C	5	160 F; (Pasteurized)	5	7.2
	6			4.5
	7			9.7
D	8	152-156 F; 15 sec	8	25
	9		7	31
	10			21
	11			16
	12			32
	13			11
E	14	150-162 F; 15 sec	8	54
	15		7	760
	16			1000
F	17	155 F; 15 sec	7	55
	18		6	55
	19			490
G	20	151 F; 15 sec	7	50
	21			130
	22			34
	23			11
	24			53
	25			21
H	26	149-152 F; 17 sec	5	250
	27			69
	28		4	150
I	29	147 F; 20 sec	7	110
	30			110
	31			85
	32			160
	33			47
	34			100
	35			100
J	36	147-148 F; 16 sec	7	40
	37			35
	38			34
	39			38
	40			63
	41			85

[a]Plate count in special Trypticase-soy agar with cover layer after incubation at 7.2 C for 3 months.

Micrococci, the second most frequent group of microorganisms recovered, constituted 15% of the isolates and were found in 24 of the cheese samples. They contributed 72% of the total flora in two samples and composed about 50% of the microbial population in seven others. Alford and Frazier *(1)* reported that micrococci constituted 78% of the microbial flora, other than lactic acid bacteria, in cheeses (ripened at 5 to 7 C) that they studied. Other workers *(5, 7)* also have reported finding large numbers of micrococci in some cheese samples.

Lactic group streptococci were recovered from only 14 of the 41 samples. In five, the lactic streptococci contributed 50% or more of the flora. It was difficult to classify these isolates to typical *S. lactis* and *S. cremoris* species. Therefore, these microorganisms were reported only as members of the lactic group. The common occurrence of variants of *S. lactis* and *S. cremoris* strains has been reported frequently *(4, 20, 23, 26)*. Allen and Knowles *(2)* reported that some atypical microorganisms isolated from cheese possibly were derived from more typical strains of lactic acid streptococci that had become modified during cheese ripening. Nichols and Hoyle *(19)* reported difficulty in the classification of "wild" lactic strains and grouped these strains as intermediates between *S. lactis* and *S. cremoris*.

Miscellaneous gram-positive rods were recovered from 18 cheeses. Except in two samples, in which they constituted 46 and 50% of the flora, these microorganisms occurred in small numbers, contributing one-third or less of the microbial population in all samples.

Associate bacteria (resembling species of *Leuconostoc*), miscellaneous gram-negative rods, lactobacilli, and other miscellaneous microorganisms, contributing the remaining 11.5% of the microflora, were found infrequently and in low numbers when re-

TABLE 2. GROUPS AND FREQUENCY OF OCCURRENCE OF MICROORGANISMS ISOLATED FROM YOUNG CHEDDAR CHEESE

Microorganisms isolated	Incidence[a]	Range in % of sample flora[b]	% of total isolates
1. Enterococci	37/41	3-100	49.5
2. Micrococci	24/41	3- 72	15
3. Lactic streptococci	14/41	3- 88	13
4. Miscellaneous gram-positive rods	18/41	3- 50	11
5. Associate bacteria[c]	7/41	3- 69	5
6. Miscellaneous gram-negative rods	14/41	3- 35	3.5
7. Lactobacilli	8/41	3- 74	2.5
8. Other miscellaneous microorganisms	4/41	3- 11	0.5

[a]Number of samples recovered from total number of samples studied.

[b]Percentages based upon those samples where recovery was made.

[c]Organisms resembling *Leuconostoc* species.

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

Gas-forming microorganisms, determined by detection of gas in Durham tubes placed in the carbohydrate media, were recovered from only seven samples. Of the 50 isolates that did produce gas, 39 produced only trace amounts. Half of the gas-forming microorganisms isolated were obtained from a single sample, comprising 50% of the sample flora. However, gas formation was not observed in the cheese sample.

Of the 265 isolates tested for proteolysis and lipolysis, 77% were proteolytic, while less than 1% were lipolytic. Because degradation of protein has been firmly established as one of the major changes occurring in ripening cheese, the isolation of proteolytic microorganisms is not unusual. Proteolytic microorganisms capable of growth at temperatures common to those used for ripening cheese may play a role in protein breakdown. The proteolytic action of streptococcal and lactobacillus isolates was not determined. However, these microorganisms also are known to influence protein degradation in ripening Cheddar cheese. The significance of lipolytic microorganisms in Cheddar cheese is unknown because the role of fat hydrolysis during ripening has not been fully explained.

Only microorganisms present in large numbers during the early ripening period of the cheese were studied in this investigation. No attempt was made to determine if numbers of microorganisms recovered were increasing, decreasing, or remaining stationary. Specific delineation of the role of these microorganisms in cheese ripening would depend upon their ability to survive, at least in moderate numbers, for a time to leave sufficient quantities of metabolic products to affect 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 what microorganisms usually are present in young Cheddar cheese under selected conditions – the purpose of the present investigation.

Commercial lactic starter cultures.

Although the counts obtained by incubating commercial lactic starter cultures at 7.2 and 21 C average higher, arithmetically, at 21 C than at 7.2 C, statistical analysis showed that this difference was not significant. Thus, the plating procedure used in this study was not unduly inhibitory to lactic starter microorganisms. However, microorganisms have optimum growth temperatures, and a temperature of 21 C is more favorable for the growth of lactic streptococci than is 7.2 C. Therefore, even the prolonged, 3-month incubation period may not have permitted maximum growth of the lactic streptococci.

None of the 379 isolates representing the 45 commercial lactic starter cultures grew in litmus milk incubated at 45 C. Failure to recover enterococci from any of the commercial lactic starter cultures studied indicates that starter cultures commonly used for the manufacture of Cheddar cheese are not a source of enterococci in cheese. Kosikowsky and Dahlberg *(18)* and Nichols and Hoyle *(19)* also reported that no enterococci could be isolated from the commercial starter cultures they examined.

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NATIONAL CONFERENCE ON INTERSTATE MILK SHIPMENTS

Plans for the 1967 meeting of the National Conference on Interstate Milk Shipments to be held at the Deauville Hotel, Miami Beach, April 2-6, have about reached completion. Don Race, Chairman of the Program Committee, promised to have the programs in the mail by the end of February.

HOTEL RESERVATIONS

About February 1, the management of the Deauville Hotel will mail a hotel reservation card to each person who registered at the 1965 Conference in Louisville.

The management of the Deauville Hotel is offering special convention rates of $6, $7, $8 and $9 per person double occupancy, or $10 single occupancy on the European Plan. Those who wish the Modified American Plan (breakfast and dinner nightly) will be offered a special rate of $4 per person per day extra.

Any persons who wish to arrive up to 3 days in advance, or remain 3 days after the official convention has adjourned, will still enjoy the same convention rates if rooms are available. Room reservations, to be assured of confirmation at the special rate, must be made at least 4 weeks in advance of the convention dates.

RECEPTION

The management of the Deauville Hotel will provide a complimentary hour-long reception on Tuesday evening, April 4, during which time hot and cold hors d'oeuvres and liquid refreshments will be served.

LADIES ACTIVITIES

The Deauville Hotel will sponsor several activities of particular interest to the ladies, such as bingo games, quiz games and scenic tours as well as fashion shows and dance instructions. If a sufficient number of ladies attend the meeting, the hotel chef will put on a demonstration on the preparation of hors d'oeuvres and fancy food items. Miss Alyce Conti, hotel hostess, will assist the ladies by answering questions concerning shopping, various hotel facilities, etc. It would be appreciated if all men who intend to bring their wives and/or families to the convention would notify the Secretary-Treasurer at least two weeks in advance of the meeting so that suitable arrangement can be made for a coffee-get-together on Monday afternoon, April 3.

TENTATIVE PROGRAM

Sunday, April 2

2-5 P.M.	Registration
2-5 P.M.	Committee meetings—Committee chairmen wishing to hold meetings on Sunday afternoon or Monday morning must contact the Secretary-Treasurer not later than March 15.

Monday, April 3

8 A.M.-5 P.M.	Registration
9 A.M.	Executive Board Meeting
9 A.M.-Noon	Committee meetings
1:30 P.M.	General Session—Governor Kirk is expected to give the welcoming address. Orlo Osten of Minnesota will speak on "Rules of the Road." Dr. K. G. Weckel will review the activities and accomplishments of the National Conference from the time of its inception in 1950 through the 1965 meeting.

Tuesday, April 4

9 A.M.-Noon	Registration
9 A.M.-Noon	Continuation of general session
1:30-5 P.M.	Task Force meetings

Wednesday, April 5

9 A.M.-Noon	Task Force meetings
Afternoon	No meetings—Time available to enjoy the many scenic beauties of Miami Beach and southern Florida.

Thursday, April 6

8:30 A.M.-Noon	General Session
1:30 P.M.	Executive Board Meeting