LIPOLYTIC AND PROTEOLYTIC ACTIVITY OF ENTEROCOCCI AND LACTIC GROUP STREPTOCOCCI ISOLATED FROM YOUNG CHEDDAR CHEESE[1]

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Abstract

Lipolytic and proteolytic screening techniques were applied to cultures isolated from young Cheddar cheese manufactured in 10 Iowa cheese plants. Twenty-one cultures were selected for study. These included 16 enterococci and 5 lactic group streptococci. These strains were examined for lipolytic activity when grown in skimmilk, cream, and skimmilk containing tributyrin; changes in proteolysis index, plate counts, and pH in skimmilk incubated at 7, 15, 21, and 32 C also were determined. And, combinations of enterococci and lactic streptococci were studied.

One-half of the *Streptococcus durans* strains frequently produced as much as 10 times more acetic acid than the others; the five strains of lactic streptococci consistently produced the lowest quantities of acetic acid. Compared with enterococci, except for *Streptococcus faecalis* var. *liquefaciens*, the lactic streptococci were more proteolytic, produced lower pH values, and had less viability at 15, 21, and 32 C. Enterococci other than S. *faecalis* var. *liquefaciens* were not proteolytic. All cultures showed tributyrinase activity; enterococci were the most active. Combining enterococci and lactic streptococci produced anomalous results.

After lactose degradation, lipolysis and proteolysis are presumed to be the principal changes responsible for developing Cheddar cheese flavor. Peterson et al. *(18)* have shown that lipolytic and proteolytic enzymes present during cheese ripening probably are of bacterial origin. The number and specificity of bacteria in the milk and curd should, therefore, predetermine kinds and degree of lipolysis and proteolysis subsequently occurring.

Clark and Reinbold *(4)* showed enterococci may constitute about one-half of the low-temperature flora of young Cheddar cheese; 56% of the enterococci they found were *Streptococcus durans*. Only 13.0% of the low-temperature flora were lactic group streptococci.

The ubiquity, wide growth-temperature range, and high tolerance of heat, salt, and acid of enterococci could render them important in Cheddar cheese flavor formation. Indeed, *Streptococcus faecalis* was used as a supplemental starter in Cheddar cheese manufacture by Tittsler et al. *(20)*, although no benefit was reported. On the other hand, Dahlberg and Kosikowsky *(7)* attributed improvement in Cheddar cheese body and flavor to use of S. *faecalis*. *Streptococcus durans* has been used in short-time Cheddar cheese making *(5, 22)*, although Czulak and Hammond *(6)* later recommended use of *Streptococcus thermophilus* for faster acid production. These investigations and other unreported commercial trials were made with single strain S. *faecalis* or S. *durans* selected largely on ability to produce acidity during cheese making.

Breed et al. *(3)* have not included lipolytic properties of the streptococci in their descriptions. Long and Hammer *(16)*, however, noticed strain specificity in a study of *Streptococcus liquefaciens*. Milk fat and cottonseed oil were not hydrolyzed, but 64 cultures attacked tripropionin and tributyrin. Wolf *(23)* and Oterholm et al. *(17)* also have demonstrated lipolysis by streptococci.

Other workers *(2, 9, 10, 16, 19)* have studied the proteolytic activity of enterococci, lactic streptococci, or both.

Our investigation was designed to study the proteolytic and lipolytic characteristics of enterococcus and lactic strains of streptococci in pure and mixed cultures. It was believed that cultures could be selected for Cheddar cheese manufacture on the basis of these characteristics.

Methods and Materials

General procedure

Cultures. One hundred and seven cultures of enterococcus and lactic group streptococci isolated from 41 young Cheddar cheeses from 10 Iowa cheese plants *(4)* were used in this study.

Single strain studies. Sixty-two enterococcus and lactic group streptococci were screened for lipolytic activity on 6 different substrata. Nile blue sulfate agar plates *(14)* were incubated at 21 C; readings were made at 8 and 32 days.

Simultaneously, 107 cultures were tested for proteolysis in skimmilk using an Orange G dye-binding technique *(13)*. Skimmilk cultures were incubated at 32 C; duplicate readings were made after 2, 6, 14, and 30 days.

On the basis of the first screening, 21 cultures were selected to adequately represent different taxonomic groups for study. The subsequent intensive study included determina-

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tion of volatile fatty acids (VFA) produced by each of the 21 cultures in skimmilk, cream, and skimmilk containing 4% tributyrin. Incubation was at 21 C; VFA measurements were made at 4, 16, and 32 days. Plate counts (PC) and pH determinations were made on all cultures at 4 and 16 days, and repeated at 32 days only on the skimmilk-tributyrin medium.

In addition, proteolysis indices (PI), PC, and pH changes of skimmilk cultures incubated at 7, 15, 21, and 32 C after 4, 16, and 32 days of incubation were determined.

Mixed strain studies. After the single strain studies, 7 culture combinations were tested for VFA, PC, and pH in skimmilk, cream, and skimmilk plus tributyrin incubated at 21 C for 4, 16, and 32 days.

Temperature effect studies. To simulate temperature changes during cheese making and to determine the possible effect of temperature changes, skimmilk cultures of the same 7 combinations were incubated, consecutively, at 32 C for 1 day, 21 C for 1 day, and at 7 C for 30 days. Proteolysis indices, PC, and pH were determined at the end of 4 days and the total 32-day incubation period.

Lipolytic activity tests

(a) Screening technique on Nile blue sulfate agar. Tripropionin, tributyrin, tricaproin, tricaprylin, triolein, and milk fat were stained with Nile blue sulfate according to the method of Knaysi *(14)*. One ml each of the sterile, stained substrate was mixed with 100 ml of special Trypticase-soy agar *(4)*. Immediately before each mixture attained gelation, plates were poured to obtain better fat dispersion. After streaking with culture, the plates were sealed with 1.75-inch wide rubber bands to prevent contamination and moisture loss and were incubated at 21 C. Changes in color, shape, and disappearance of fat globules were recorded.

(b) Determination of volatile fatty acids. Portions of sterile, reconstituted skimmilk containing 11% nonfat milk solids and 1% Tween 80, cream containing 12% milk fat, and reconstituted skimmilk containing 1% Tween 80 and 4% tributyrin were inoculated with fresh litmus milk transfers of the cultures. Five grams of each well-mixed, incubated culture were weighed into a test tube, and 0.05 ml of 10% aqueous methyl cellosolve was added as an internal standard. The culture was made acidic with 0.25 ml concentrated HCl, mixed, and centrifuged. The aqueous layer was used to measure acetic, propionic, and butyric acids by gas chromatography with a hydrogen flame detector as described by Hammond and Reinbold *(12)*. Peak areas were measured with the disc chart integrator and corrected for background. In each step, an uninoculated control with each of the 3 media was included. Amounts of the VFA were calculated by the formula:

$$C_x = \frac{M_s A_x}{M_x A_s} C_s$$

where M is the area of methyl cellosolve peak, A is the area of the acid peak, x refers to the unknown, C is concentration in mg/100 g, and s refers to the standard; and:

$$C^1_x = C_x - C_c$$

where C^1 is the corrected concentration and c refers to the uninoculated controls.

When examining the entire chromatogram, ghost peaks that appeared in a chromatogram following a bigger peak were discarded. In each trial, the skimmilk control was first chromatographed, followed by the cream, and, finally, the skimmilk plus tributyrin. After the introduction of the standards, or following a chromatogram with large peaks, water was injected to reduce the remaining amounts of VFA in the column.

Proteolytic activity test

Orange G dye solution was prepared and used according to the procedure of Hammond, Seals, and Reinbold *(13)*, except that 6.3020 g of oxalic acid dihydrate per liter was used. A single batch of 25 liters was prepared and used exclusively throughout the experiment.

Prescription bottles containing 100 ml of skimmilk autoclaved at 121 C for 13 min were inoculated with 0.5 ml of a fresh litmus milk transfer. From this, 1-ml aliquots were aseptically pipetted into sterile 20 x 125 mm screw-cap tubes. Uninoculated controls were made for each batch of skimmilk. Enough tubes were prepared to obtain quadruplicate readings at 4 days of incubation and triplicate readings thereafter. Additional culture was made for PC and pH measurements. Tests were made to determine if contamination had occurred during inoculation and transfer of the skimmilk to the tubes. If contaminants appeared on special Trypticase-soy agar plates, the corresponding series of tubes were discarded.

Plate counts and pH measurement

Procedures listed in the 11th edition of *Standard Methods for the Examination of Dairy Products (1)* were followed. Duplicate plates were poured with special Trypticase-soy agar and were incubated at 21 C for 5 days. When PCs were made along with VFA determinations, test aliquots were withdrawn directly from the flasks of incubated milk. When PCs were made to correspond with PIs, 9 ml of sterile, buffered distilled water were added to 1-ml aliquots described in the earlier sections; successive dilutions were made as required from these aliquots.

All pH measurements were made with a Beckman Zeromatic pH meter. Readings to correspond with VFA determinations were made (as with PCs) by aseptic withdrawal of test material from the bulk culture. Readings to correlate with PIs were made from separate flasks of inoculated media prepared and inoculated simultaneously with the 1-ml aliquots.

RESULTS AND DISCUSSION

Lipolytic tests on Nile blue sulfate agar

Results of the screening tests on Nile blue sulfate agar are given in Table 1. Although all cultures were able to hydrolyze tripropionin, the clear zones around the enterococcus colonies were larger after prolonged incubation. Tributyrin was less susceptible to hydrolysis but, again, the enterococci were more active than the lactic streptococci. Tricaproin and tricaprylin were less frequently hydrolyzed and triolein was not attacked at all. Table 1 shows that a few cultures seemingly hydrolyzed milk fat, but the readings are subject to question. In quite a few instances, the bacterial colonies absorbed the blue dye, this could have masked changes in the underlying material. Some of the "positive" readings resulted from fat globule distortion, but in no instances were the "positive" reactions absolutely clear. The shorter chained the triglyceride, the more distinct the readings became. Further, readings were recorded only as

TABLE 1. RESULTS OF LIPOLYSIS SCREENING TEST ON NILE BLUE SULFATE AGAR PLATES INCUBATED AT 21 C

Species	Reaction observed[a]	Substrate: Tripropionin (days) 8	Tripropionin 32	Tributyrin 8	Tributyrin 32	Tricaproin 8	Tricaproin 32	Tricaprylin 8	Tricaprylin 32	Triolein 8	Triolein 32	Milk fat 8	Milk fat 32
S. durans	−			15	1	20	16	20	22	11	11	32	28
	+	25		18	24	3	7	3	1				2
	++	7	22		6								2
	+++	1	11		2								
		33[b]	33	33	33	23	23	23	23	11	11	32	32
S. faecalis	−			4		1	1	2	2			5	5
	+	4		1	5	1							
	++	1	3				1						
	+++		2										
		5[b]	5	5	5	2	2	2	2			5	5
S. faecalis var. *liquefaciens*	−			3		4	1	3	3	1	1	5	5
	+	3		2	3		3						
	++	3	3		2								
	+++		3										
		6[b]	6	5	5	4	4	3	3	1	1	5	5
S. faecalis var. *zymogenes*	−			1	1	1		3	2	2	2	4	4
	+	4		3	3		1		1				
	++		2										
	+++		2										
		4[b]	4	4	4	1	1	3	3	2	2	4	4
Lactic group streptococci	−	2		8	2	7	5	7	7	3	3	13	12
	+	12	1	4	9	1	3					1	1
	++		10		1								1
	+++		3										
		14[b]	14	12	12	8	8	7	7	3	3	14	14

[a]Symbols: − = no lipolysis or questionable reaction.
+ = slight positive reaction, disappearance or change in shape of colonies in immediate vicinity of bacterial growth.
++ = fat globules disappeared up to 2 mm around bacterial growth; color change.
+++ = more than 2 mm of cleared zone; distinct color change.

[b]Total number of cultures studied.

positive or negative when sufficient bacterial surface growth to produce changes had occurred. Adequate growth in the presence of longer-chained triglycerides was not always obtained. Consequently, Table 1 records progressively fewer readings from tripropionin through triolein. The Knaysi modification of the Nile blue sulfate tests thus lacked sensitivity, but did indicate that enterococci are more able to hydrolyze short-chained triglycerides than are lactic streptococci. To substantiate this, the more sensitive gas chromatographic procedure was adopted.

Chromatographic measurement of lipolysis

The production of VFA by the 21 selected cultures in skimmilk, cream, and skimmilk containing 4% tributyrin was then studied. To assure dispersion of the tributyrin, 1% Tween 80 was added to the last medium. The first medium was also fortified with Tween 80 to serve as a control. Careful testing showed that Tween 80 was neither inhibitory nor stimulatory to growth or VFA production.

Chromatographic studies (Table 2) showed that most enterococci produced more acetic acid than lactic streptococci. Strain differences with species were apparent as 50% of the *S. durans* cultures frequently produced as much as ten times more acetic acid than the others. There was little or no difference in amounts of acetic acid produced in the three media, although the presence of 4% tributyrin seemed to suppress this activity to a minor degree. The cultures that normally formed more acetic acid did not necessarily produce more butyric acid. The greater amount of milk fat in the cream medium did not lead to more acetic, propionic, or butyric acid production than in the skimmilk medium. This casts

TABLE 2. VOLATILE FATTY ACIDS, PLATE COUNTS, AND pH IN VARIOUS GROWTH MEDIA AFTER INCUBATION FOR 32 DAYS AT 21 C

Species	Culture no.	Growth medium: Skimmilk + 1% Tween 80 — Acetic	Propionic	Butyric	Cream (12% milk fat) — Acetic	Propionic	Butyric	Skimmilk + 1% Tween 80 + 4% tributyrin — Acetic	Propionic	Butyric	PC[a]	pH
		(mg/100 g of culture)										
S. *durans*	1	36(41)[b]	3	——[c]	8(17)	——	——	29(36)	——	114(145)	11,000	4.55
	2	256(567)	t[d]	——	348(773)	——	——	151(427)	——	57(139)	210,000	4.60
	3	35(112)	——	——	36(36)	t (2t)	——	73(87)	——	164(220)	240,000	4.90
	4	276(583)	——	——	378(722)	——	——	277(576)	7	215(260)	370,000	4.70
	5	19(92)	——	——	——(27)[e]	——	——	13(13)	t	182(213)	340,000	4.50
	6	245(601)	——	——	245(601)	——	——	227(407)	4	207(344)	290,000	4.62
	7	——(70)	——	——	——(71)	——	——	19(19)	——(t)	211(361)	96,000	4.58
	8	266(710)	t	——	266(710)	——	——	264(570)	——	219(277)	200,000	4.65
S. *faecalis*	9	——(131)	——	——	54(84)	——	——	77(172)	——	70(218)	180,000	4.72
	10	140(369)	——	——	107(368)	——	——	78(283)	——	82(194)	330,000	4.72
	11	89(187)	——	——	60(153)	——	——	33(216)	——	13(138)	350,000	4.70
	12	82(290)	——	——	24(75)	——	——	115(231)	——	53(116)	650,000	4.85
S. *faecalis*	13	50(466)	——	t	234(679)	——	t	69(307)	——	74(145)	420,000	4.75
var. *liquefaciens*	14	100(161)	——	t	48(155)	——	t	49(141)	——(55)	19(176)	97,000	4.50
S. *faecalis*	15	102(551)	——	——	183(688)	——	——	63(453)	t	23(187)	240,000	5.10
var. *zymogenes*	16	48(250)	——	——	64(270)	——	——	117(215)	——	18(183)	180,000	4.80
Lactic group streptococci	17	——(47)	——	——	40(116)	——	——	32(115)	——	——(123)	45	4.40
	18	——(36)	——	——	——(36)	——	——	25(56)	——	——(61)	4	4.22
	19	10(44)	——	——	14(56)	11(11)	——	60(103)	t	——(14)	<1	4.30
	20	32(73)	——	——	32(73)	——	——	24(50)	——	——(15)	18	4.12
	21	——(59)	——	——	4(51)	——	——	42(58)	——(6)	37(127)	<100	4.25

[a]Plate count/ml x 10^3.
[b]Figures within parentheses represent total amount of volatile fatty acids from determinations made after 4, 16, and 32 days incubation expressed as mg/100 g of culture.
[c]Amounts present equal to or less than in uninoculated control.
[d]Trace quantities.
[e]——(27) indicates volatile fatty acid not present at 32-day determination although measurable amounts had been present earlier.

TABLE 3. CHANGES IN PROTEOLYSIS INDEX, PLATE COUNTS[a] AND pH IN SKIMMILK INCUBATED FOR 32 DAYS AT 7, 15, 21, AND 38 C

Species	Culture No.	Temperature 7C PI	7C PC	7C pH	15C PI	15C PC	15C pH	21C PI	21C PC	21C pH	32C PI	32C PC	32C pH
S. durans	1	--[b]	310,000	4.95	--	510,000	4.50	0.01	350,000	4.50	0.02	<10	4.60
	2	0.02	480,000	5.07	0.02	1,300,000	4.60	0.01	280,000	4.42	0.02	<10	4.55
	3	--	120,000	5.65	--	370,000	4.80	--	270,000	4.70	0.02	240,000	4.70
	4	0.02	450,000	5.10	0.01	100,000	4.70	0.01	400,000	4.55	0.02	300	4.55
	5	0.01	440,000	5.10	0.03	1,200,000	4.65	--	20,000	4.52	0.04	<10	4.50
	6	--	230,000	5.65	--	560,000	4.80	0.01	510,000	4.52	--	8	4.88
	7	0.01	280,000	5.25	0.02	250,000	4.62	--	450,000	4.42	--	19	4.52
	8	0.02	320,000	5.00	0.01	720,000	4.73	--	600,000	4.60	0.01	11	4.52
S. faecalis	9	0.01	180,000	5.32	0.01	570,000	4.92	--	700,000	4.65	--	49	4.70
	10	--	110,000	5.40	--	160,000	4.95	0.02	400,000	4.82	0.03	610,000	4.80
	11	--	57,000	5.85	--	260,000	4.85	--	2,900,000	4.82	--	1	4.75
	12	0.01	200,000	5.47	--	610,000	5.22	--	61,000	4.75	--	900,000	4.85
S. faecalis	13	0.52	600,000	5.00	0.81	970,000	4.80	0.83	650,000	4.62	0.87	75	4.75
var. *liquefaciens*	14	0.27	53,000	4.45	0.52	1,500,000	4.48	0.61	530,000	4.42	0.61	30	4.42
S. faecalis	15	0.02	230,000	6.30	--	550,000	5.62	--	500,000	4.97	0.01	5,900	4.96
var. *zymogenes*	16	0.03	250,000	5.55	0.02	520,000	5.18	--	370,000	4.82	0.02	2,200	4.82
Lactic group	17	--	360,000	5.00	0.01	70,000	4.35	0.02	15,000	4.25	0.03	<1	4.45
streptococci	18	0.05	63,000	4.25	0.09	30	4.45	0.12	<1	4.15	0.14	<1	4.22
	19	--	440,000	4.98	0.01	120,000	4.32	0.03	190,000	4.28	0.03	<10	4.32
	20	--	21,000	4.85	0.08	270,000	4.28	0.12	260,000	4.10	0.11	<10	4.20
	21	0.03	280,000	4.65	0.03	290,000	4.28	0.04	<10	4.25	0.02	<10	4.45

[a]Plate count/ml x 10^3.

[b]Amount of proteolysis present equal to or less than in uninoculated control.

TABLE 4. VOLATILE FATTY ACIDS, PLATE COUNTS, AND pH PRODUCED BY MIXED CULTURES OF ENTEROCOCCI AND LACTIC GROUP STREPTOCOCCI IN VARIOUS GROWTH MEDIA AFTER INCUBATION FOR 32 DAYS AT 21 C

Culture	Growth medium: Skimmilk + 1% Tween 80			Cream (12% milk fat)			Skimmilk + 1% Tween 80 + 4% tributyrin				
	Acetic	Propionic	Butyric	Acetic	Propionic	Butyric	Acetic	Propionic	Butyric	PC[a]	pH
	(mg/100 g of culture)										
S. *durans* 6 + Lactic gp. strep. 19	45(180)[b]	t[c](1)	--[d]	57(91)	t	--	61(175)	t(7)	19(107)	2,800	4.28
S. *faecalis* 9 + Lactic gp. strep. 19	59(137)	--(1)[e]	--	29(59)	--(t)	--	19(63)	--	13(44)	1,300	4.28
S. *f. var. liquef.* 13 + Lactic gp. strep. 19	117(328)	--(t)	--	40(133)	--(t)	--	40(198)	--(t)	15(114)	1,900	4.30
S. *durans* 6 + S. *faecalis* 9	35(153)	--(t)	--	71(138)	--(t)	--	84(195)	--(12)	28(28)	310,000	4.68
S. *durans* 6 + S. *f.* var. *liquef.* 13	174(281)	--	--	171(387)	--	--	148(320)	--	70(111)	490,000	4.80
S. *durans* 6 + Commercial starter	52(219)	--(4)	t	103(290)	--(t)	t	137(316)	--(1)	12(97)	4,900	4.22
S. *faecalis* 9 + Commercial starter	116(256)	--(t)	t	118(232)	--(t)	t	99(284)	--(1)	41(77)	650	4.25

[a]Plate count/ml x 10^3.
[b]Figures within parentheses represent total amount of volatile fatty acids from determinations made after 4, 16, and 32 days incubation expressed as mg/100 g of culture.
[c]Trace quantities.
[d]Amounts present equal to or less than in uninoculated control.
[e]—(1) indicates volatile fatty acid not present at 32-day determination although measurable amounts had been present earlier.

doubt upon the true lipolytic activity of the enterococci. The acetic acid formed may result from normal fermentative processes although supporting data were not obtained. The presence of tributyrin, however, greatly enhanced the liberation of butyric acid, thereby reemphasizing the greater tributyrinase activity of the enterococci and, especially, of S. *durans*. According to Forss and Patton *(11)*, acetic, butyric, and caproic acids appear to be indispensable for the flavor and aroma of Cheddar cheese. Possibly, the optimum ratio is 8:1:0.3, or the amounts 900, 110, and 35 ppm. A bacterial culture that could produce these compounds in approximately this ratio would be of value in cheese ripening provided it was not strongly proteolytic or produced undesirable flavors from other metabolic activities and large quantities of carbon dioxide. The amounts of acetic acid shown here were found in a fluid medium with properties radically different from cheese curd. Nevertheless, continuing growth of enterococci could conceivably perform this function although Dahlberg and Kosikowsky *(7)* indicated that their cheese made with S. *faecalis* contained less VFA than the negative controls. Their culture had been selected for its ability to produce lactic acid and not VFAs.

Table 2 also shows that enterococci remain viable under unfavorable culture conditions much longer than do lactic streptococci. This ability of enterococci which may be of value in Cheddar cheese ripening, has already been noted by Kosikowsky and Dahlberg *(15)*. The difference in 32-day pH values (enterococci, pH 4.5-5.10 and lactic streptococci, pH 4.12-4.40) undoubtedly is of some importance in this respect. Only the PC and pH readings at 32 days in skimmilk plus tributyrin are given in Table 2, since earlier tests showed the same trends.

Within the 4 temperatures and 3 times studied (only 32-day values are recorded in Table 3, however), there were general differences in growth and viability between the two groups of streptococci. Although not evident in Table 3, the enterococci grew only slightly better at 7 C than did the lactic streptococci. There was little difference in numbers between the groups after 4 days at 15 C, but the lactic streptococci then declined more rapidly during prolonged incubation. At 21 and 32 C, the lactic streptococci had reached maximum numbers and had declined rapidly before the first count was made at 4 days. Under the test conditions it was not evident whether the enterococci were continuing to grow or whether they had reached a near maximum level and persisted at that level under the unfavorable growth conditions. This differentiation could be important because continuing metabolic activity could be of value in cheese ripening if it would contribute to an increase of flavor compounds or even deter the growth of less desirable or deleterious microorganisms.

Streptococcus durans and S. *faecalis* showed barely measurable increases in PI, with essentially no changes at 7, 15, and 21 C and only an exceedingly minor increase at 32 C. *Streptococcus faecalis* var. *zymogenes* (only 2 cultures tested) showed no differences in proteolytic activity at the different incubation temperatures; PI changes for S. *faecalis* var. *liquefaciens* were related directly to temperature, with growth at 32 C producing earlier and greater proteolysis. Temperature increase affected lactic streptococcus PIs; higher temperatures produced higher PIs. Strain differences were evident; those cultures that produced lower pH readings were more proteolytic, except with the enterococcus cultures. In general, the enterococci neither produced as low pH values at any temperature nor did the pH drop occur as quickly.

Mixing enterococci and lactic group streptococci produced the results given in Table 4. When three different enterococci were separately combined with a low acetic-acid-producing and weak tributyrinolytic lactic streptococcus, acetic acid and tributyrinase production by the enterococci were invariably inhibited. Final pH values were representative of lactic group streptococci, and the TCs were greatly reduced. Mixing two enterococcus cultures did not reduce either viability or final pH, but did reduce anticipated acetic and butyric acid production. The use of a commercial Cheddar cheese starter also reduced PC, pH, and VFA content of the 32-day-old cultures. Trace quantities of propionic and butyric acid increased in the cream and skimmilk media. Cheesy flavors were never noticed in this or any other of our experiments.

The same mixed cultures of enterococci and lactic group streptococci were used to study effect upon PI (Table 5). To simulate cheese ripening conditions, cultures were grown at 32 C for the first day, transferred to 21 C for the second day, and were then held at 7 C until tested at 4 and 32 days after inoculation. Single-strain enterococcus and lactic group streptococcus combinations had little effect upon PI except where S. *faecalis* var. *liquefaciens* was included. In all seven trials, greatest proteolysis occurred within the first 4 days; indeed, during the remaining 28 days at 7 C, 5 cultures showed a negligible decrease in PI, meaning that activity had been only at the lower limit of testing sensitivity. The S. *faecalis* var. *liquefaciens* culture, greatly proteolytic after 4 days, continued to raise the PIs by 0.10 and 0.08 unit up to 32-day readings of 0.80 and 0.81. Changes of these magnitudes would, of course, be

TABLE 5. DIFFERENCES IN PROTEOLYSIS INDEX, PLATE COUNTS[a], AND pH PRODUCED BY MIXED CULTURES OF ENTEROCOCCI AND LACTI GROUP STREPTOCOCCI IN SKIMMILK INCUBATED FOR 1 DAY AT 32 C, 1 DAY AT 21 C, AND FOR 2 AND 30 DAYS AT 7 C

Culture	PI		PC		pH	
	days		days		days	
	4	32	4	32	4	32
S. *durans* 6 + Lactic gp. strep. 19	0.03	--[b]	2,200	140	4.30	4.28
S. faecalis 9 + Lactic gp. strep. 19	0.02	--	1,500	730	4.25	4.25
S.*f.* var. *liquef.* 13 + Lactic gp. strep. 19	0.70	0.80	2,900	2,000	4.65	4.70
S. *durans* 6 + S. faecalis 9	0.03	0.01	1,400	1,500	4.52	4.50
S. *durans* 6 + S.*f.* var. *liquef.* 13	0.73	0.81	2,400	1,800	4.67	4.75
S. *durans* 6 + Commercial starter	0.06	0.05	1,200	21	4.50	4.55
S. faecalis 9 + Commercial starter	0.05	0.05	290	40	4.42	4.60

[a]Plate count/ml x 10^{-6}.
[b]Amount of proteolysis equal to or less than in uninoculated control.

undesirable in Cheddar cheese ripening. Deane *(8)* has reported that the addition of 0.5% S. *faecalis* var. *liquefaciens* to a commercial starter caused cheese to develop an intense, bitter flavor and a soft, pasty body. The temperature sequence used produced notably high PCs after 4 days, and the subsequent decline in viability at 7 C was less than would have been anticipated at the unfavorable pH levels. There was little change in pH from 4 to 32 days. Three of the culture combinations stayed essentially the same, 2 increased by only 0.05 unit, and the remaining cultures increased 0.08 and 0.18 unit.

By studying a selected group of enterococcus and lactic group streptococci in pure and combined culture, we have been able to note differences between proteolytic and lipolytic characteristics of strains of the same species. Some of these strains will be used to manufacture Cheddar cheese which will be compared for differences in body, flavor, and texture.

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