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Improved Isolation and Differentiation of Enterococci in Cheese

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Further documentation of an enterococcus selective differential (ESD) medium was obtained in isolations from eight different cheeses. An improved differentiation of tetrazolium salt (2, 3, 5-triphenyl tetrazolium hydrochloride [TTC])-reducing strains of *Streptococcus faecalis* from TTC-nonreducing or TTC-faintly-reducing *Streptococcus faecium* was attained. The sensitivity of the medium was evaluated in comparison with that of KF streptococcal, Pfizer selective enterococcus (PSE), the medium of Reinbold, Swern, and Hussong (RSH), and the medium of Saraswat, Clark, and Reinbold (SCR). Selective counts, rate of colony formation, and ease of isolation and differentiation of colonies were examined. The specificity of the medium was also investigated. ESD supported the fastest rate of growth and the maximum size of colonies; counts in this medium were in most cases possible within 17 h of incubation, whereas the other media required 24 to 48 h. A presumptive identification of 1,077 isolates by four biochemical tests disclosed that SCR, RSH, and ESD selected high, comparable percentages of strains that approximated most closely the typical description of enterococci (66, 60.1, and 58%, respectively). Low percentages (21.1 and 30.7%) were yielded by KF and PSE. The utility of ESD for a rapid, presumptive identification of enterococci was confirmed by serological and biochemical testing of color TTC-differentiated colonies isolated from 18 cheeses.

A selective medium suitable for the isolation of enterococci was previously developed by incorporation of a low sodium azide concentration (0.01%) and a high alkaline (pH 9.6) into a manganese-deficient nutrient base (5). In the initial evaluation of this medium, known pure strains of bacteria were used. In the present study, we extended the utility of the medium by including in its composition a tetrazolium salt indicator effecting presumptive speciation of the enterococcal isolates. We also tested the selective and differential capacities of the medium on various cheeses. Comparative isolations of enterococci were conducted by using several standard media. We made observations on the sensitivity of enterococcal recovery; selective counts, rate of colony formation, ease of isolation, and differentiation were considered. For an evaluation of specificity of the medium, we carried out a comparatively presumptive identification of cheese isolates. Finally, we examined the practical features of the medium in a few parallel applications of its solid and liquid form.

MATERIALS AND METHODS

Culture media. The medium tested in this study, designated enterococcus selective differential (ESD), was that which was described in a previous study (5), modified to include a tetrazolium salt indicator. After addition of sterile agar, the temperature of the medium was decreased to 50 C, and 5 ml of a 2% 2,3,5-triphenyl tetrazolium hydrochloride (TTC), sterilized previously in flowing steam for 30 min, was added. This medium was also tested as ESD broth (agar omitted). In this case, the sugar was autoclaved separately as a concentrated (10%) solution and its final concentration was reduced to 1% (the glucose level of Barnes TG (1) medium with which ESD was compared).

The following agar media were used in addition to ESD: KF-streptococcal (BBL), Pfizer selective enterococcus (PSE), the medium of Reinhold, Swern, and Hussong (RSH;14), and the medium of Saraswat, Clark, and Reinbold (SCR;15). Standard methods agar (SMA;BBL) was used as a nonselective control. Yeast extract (YE) agar was used as recovery and maintenance medium for all isolates from cheese. The composition of this medium was: yeast extract (20 g), K_2HPO_4 (2 g), $MgSO_4 \cdot 7H_2O$ (0.1 g), glucose (2 g), agar (15 g), and distilled water to 1,000 ml. The final pH was adjusted to 6.8. The same composition, but without agar, was used as YE broth for preparation of inocula in the presumptive identification of cheese isolates.

TTC reduction in ESD media. The kinetics of TTC reduction by enteroccocci were studied comparatively in the standard TG medium of Barnes (1) and ESD broth. For this purpose, the same concentration of TTC (75 μg/ml) was added to both media. Each tube, containing 5 ml of TTC medium, was

inoculated with 3 drops of a 24-h culture. The reducing activity of known strains of enterococci, representing different physiological types, was examined visually every hour between 0 and 8 h, and at 24 h. Reduction was demonstrated by the appearance of magenta-colored triphenyl tetrazolium formazan. The reduced TTC was extracted with *n*-butanol. The optical density of the extracts was measured at 575 nm. Several concentrations of TTC were added to the selective ESD broth and were tested for a determination of the optimal concentration, i.e., the amount that was nontoxic and produced the best visible results. The same TTC concentrations were also incorporated in ESD agar plates which were then surface inoculated with the test organisms. At 0.01%, added to both liquid and solid media, no inhibition of cultures was noted, and the color of positive reactions was intense. Afterwards, this concentration was used routinely with both forms of ESD.

Cheese samples analyzed. Eight different samples of cheese were used for the comparative isolation, enumeration, and presumptive identification of enterococci. An additional collection of 18 cheeses was used for the isolation, presumptive, and confirmed identification of isolates from the ESD medium only. All cheeses were obtained in retail packages from the local New York market. They represented cheese varieties differing as to country of origin, manufacturing procedures, species of animal that produced the milk manufactured into cheese, and environmental conditions prevailing at the time of manufacture and early ripening. All samples had been ripened for at least 60 days; some samples had been ripened for more than 6 months before they were marketed.

Isolation and enumeration of enterococci. The handling, preparation, and analysis of the cheese samples were performed according to recommended methods (17). Serial, 10-fold dilutions were plated in SMA to determine the total viable count and in the selective media for comparative counting and isolation of enterococci. In the selective agar media, the size of inoculum was 0.1 ml; it was spread evenly on the surface of the agar using a bent glass rod. All cheese dilutions were inoculated in triplicate. The plates were incubated aerobically at 37 C. Counts were determined at 17, 24, and 48 h. Individual colonies were isolated by using the random sampling method of Harrison (9) after 48 h of incubation, and 30 to 60 colonies were picked per sample and medium. The counts were compared by the F test.

For an assessment of sensitivity, the average size of 10 representative colonies, developed on the surface of each medium, was determined. The size or diameter of each colony was measured in 0.1-mm units on a Quanti Plate viewer (Kallestad Lab., Minneapolis, Minn.) apparatus utilizing dark-field lighting and a magnifying optical comparator, ordinarily used to measure immunoprecipitation patterns in agar gels. The relative sensitivity of the media was established by statistical analysis of the mean diameter of the enterococcus colonies by the Student-Newman-Keuls test. This is a test of multiple comparisons among means based on equal sample sizes (in this study, 10 colonies per plate), and it is derived from the principles of analysis of variance (16).

Presumptive identification of enterococci. The cheese isolates obtained from the five selective media were inoculated in YE agar stabs. When growth was obtained, the cultures were placed in a refrigerator, where they were kept until further testing. For a presumptive identification of enterococci, the following five tests were performed: catalase activity, ability to grow on bile esculin medium (BEM), SF (Difco) medium, 0.1% methylene blue milk (MBM), and 6.5% NaCl broth. The latter four tests were performed and interpreted as a battery of tests. They were carried out according to the methods of Facklam and Moody (7). Catalase activity was tested in YE broth cultures. Inocula for all five tests were also grown in YE broth. The tests were adapted to utilize the multipoint inoculation system of Lighthart (12) and were read after 24 h of incubation at 37 C. The evaluation of relative specificity of the five media for enterococci was based on a statistical analysis (chi-square method) of the comparative identification data.

Confirmed identification of enterococci. Colonies from 18 different samples of cheese, upon isolation from ESD agar, were transferred into serological tubes containing 2.5 ml of ESD broth. These cultures were incubated for 4 h at 37 C and then were examined for TTC reduction. The identification tests included the following. The morphology of isolates was examined by the gram stain. Presumptive verification was according to Sherman's criteria, the catalase reaction, and appearance on BEM (Difco). A screening for group D antigen was carried out by the Lancefield precipitation method in capillary tubes; for this test, a grouping antiserum was prepared in rabbits using the strain Lancefield D76 (*S. faecalis* var. *zymogenes* ATCC12958). Antigenic extracts for precipitation were produced from cells cultured in glucose Lemco broth according to Medrek and Barnes (13). Differentiation into species was determined in representative TTC-reducing and TTC-nonreducing strains, by testing for tolerance to potassium tellurite, fermentation of sorbitol, mannitol, arabinose, melibiose and raffinose, determination of folic acid requirement, substrate utilization of pyruvate, malate, and serine, and ability to grow in broth at 50 C. These tests were essentially performed according to Deibel (2, 3).

RESULTS

TTC reduction in ESD. A summary of results is presented in Table 1. In the TG medium, the differentiation of typical *S. faecalis* from *S. faecium* by TTC reduction required 24 h of incubation. Intermediate enterococci, such as the epiphytic strains T-15, FMA 2, and FMA-11 (5), developed a positive reaction. These atypical *S. faecium* strains were confused with some of the slowly reducing strains of *S. faecalis*.

The reduction time of all known *S. faecalis* strains in ESD broth was less than 4 h. The optical density values, obtained from formazan extracts of cultures at 4 h, ranged approximately between 0.35 (strong TTC reducers, e.g., strain ATCC11700) and 0.12 (moderate

TABLE 1. *Comparative reduction of TTC in TG and ESD broth medium by enterococci*

Strain	TG medium reaction[a]		ESD medium reaction	
	4 h	24 h	4 h	24 h
S. faecalis				
ATCC 11700	W	+	+	+
19-1	−	+	+	+
S. faecium				
ATCC 6057	−	−	−	+
SJU-15	−	−	−	+
T-15	−	+	−	+
FMA-2	−	+	−	+
FMA-11	−	+	−	+

[a] Appearance of cultures against uninoculated controls: +, pronounced TTC reduction (formazan production); W, weak color change; −, no color change.

TTC reducers, e.g., strain 19-1). Within the same interval, *S. faecium* produced traces of formazan. The intermediate enterococci did not show appreciable formazan production within 4 h; thus, they could be distinguished from weakly reducing *S. faecalis* strains, which did so within 4 h.

The incorporation of TTC into ESD agar resulted in a relatively rapid differentiation of enterococci since the reduction of TTC coincided with the early development of colonies on this medium. Colonies of *S. faecalis* assumed a magenta color, whereas those of *S. faecium* appeared white. The intermediate strains yielded pink colonies. All magenta-colored colonies, isolated from the various cheeses on ESD agar, when transferred into ESD broth reduced TTC strongly within 4 h.

Sensitivity of ESD for enterococci. The development of selective counts on KF, RSH, and SCR media required between 24 and 48 h of incubation. Four out of eight cheese samples plated on PSE yielded ample growth at 17 h, whereas seven out of eight samples plated on ESD produced readable counts at 17 h (Table 2). Between 17 and 48 h, the average increase of these counts was 1.6 times on ESD and 3.2 times on PSE. The highest average counts per gram of cheese at 48 h were obtained in KF and PSE (61×10^5). The counts were lower in RSH, SCR, and ESD (23×10^5, 19×10^5, and 17×10^5, respectively). A statistical comparison of the enterococcus recovery rates on the various media could not be made because of the significant variance of the counts (F test). For a valid comparison, multiple samples of a particular type of cheese, with similar distributions of bacterial taxa, should be included.

Some of the colonies that developed on PSE failed to show the typical black halo of enterococci. The percentage of such atypical colonies varied from cheese to cheese; the lowest proportion that could be determined was 0% in the Kefalotyri sample and highest (51%) in the blue cheese sample. The recognition and enumeration of these atypical colonies on PSE, quite easy early in the incubation, became progressively difficult after 21 h as the black salts of hydrolyzed esculin surrounding typical colonies diffused through the agar. A meaningful comparison of colony sizes on all five selective media became possible after 48 h of incubation, when measurements could be made on KF, RSH, and SCR media. Table 2 indicates the average diameters of colonies that developed on each medium. Statistical analysis of the means (Student-Newman-Keuls test) disclosed that the colonies grown on KF, PSE, RSH, and SCR media did not differ in size significantly. However, the size differences between colonies grown on ESD and all other media were significant at the 95% level.

Specificity of ESD for enterococci. A presumptive identification was carried out on 1,077 isolates. This total represented strains from all selective media and most of the cheese samples included (Table 2). Thirteen strains (1.2%) showed a positive catalase test. Table 3 gives the distribution of strains that reacted positively in 4, 3, 2, 1, or none of the presumptive enterococcus tests. The results indicate that SCR, RSH, and ESD media selected the highest percentages of strains that reacted positively in all four presumptive tests (66, 60.1, and 58% respectively). In contrast, KF and PSE yielded low percentages (21.1 and 30.7, respectively). Substantially similar comparisions were made when the statistical evaluation was based on the results of only two of the four tests performed, i.e., reaction on BEM and tolerance to 6.5% NaCl; the combination of the 2 tests was found recently to be a reliable criterion for a presumptive identification of group D streptococci (6, 8).

A total of 936 other isolates from 18 cheese samples were obtained on ESD agar (Table 4). These strains were subjected to verification of identity. They were all gram-positive cocci and catalase negative; with some minor variations, they conformed to the Sherman's criteria. They could grow in the presence of bile salts and hydrolyzed esculin, and they demonstrated the group D antigen. Strains that reduced TTC on the ESD agar plates and showed TTC reduction in less than 4 h in ESD broth were confirmed as *S. faecalis* by the following pattern of biochemical characteristics: they could grow in the

TABLE 2. *Comparative isolation of enterococci from eight cheeses by five selective media*

Description of cheese	Medium							
	SMA (48 h)	KF (48 h)	PSE		ESD		RSH (48 h)	SCR (48 h)
			17 h	48 h	17 h	48 h		
Gouda	40×10^5	46×10^3	0	44×10^3	VSN[a]	14×10^3	23×10^2	0
Kefalotyri	86×10^6	22×10^6	26×10^6	43×10^6	46×10^5	12×10^6	16×10^6	15×10^6
Cheddar, extra sharp	21×10^6	12×10^3	21×10^2	16×10^3	23×10^2	33×10^2	47×10^2	19×10^2
Cheddar, sharp	31×10^7	87×10^3	VSN	17×10^4	26×10^2	59×10^2	50×10^2	60×10^1
Fontina	91×10^6	69×10^5	VSN	30×10^5	30×10^3	42×10^3	75×10^2	60×10^2
Port Salut	18×10^7	17×10^6	12×10^3	24×10^3	11×10^3	16×10^3	21×10^2	55×10^2
Cheddar, mild	16×10^5	73×10^4	VSN	25×10^4	59×10^2	85×10^2	13×10^3	13×10^2
Blue cheese	15×10^6	23×10^5	14×10^5	24×10^5	15×10^5	17×10^5	24×10^5	79×10^4
Mean diameter of colonies ($mm \times 10^{-1}$)		5.3	7.8		18.6		8.3	5.4

[a] VSN, Very small number.

TABLE 3. *Comparison of specificity of five enterococcus selective media*

Medium	No. of strains tested	Positive responses (%)[a]					χ^{2b}	
		0	1	2	3	4	A[c]	B[d]
ESD	239	0.8	8.0	9.6	23.6	58.0		
KF	222	7.0	20.7	21.6	28.8	21.1	81.53	69.10
PSE	228	7.0	9.6	28.9	23.7	30.7	35.44	27.50
RSH	223	3.0	4.0	9.4	23.3	60.1	0.17	.35
SCR	165	0.6	3.6	9.1	20.6	66.0	2.63	3.79

[a] Total number of positive reactions on: BEM, SF, MBM, and 6.5% NaCl broth; 0, 1, 2, 3, 4 refer to specific number of tests in which the listed % of strains reacted positively.

[b] Significant χ^2 values were those greater than 3.84 at the 95% level and 1 degree of freedom.

[c] Comparison of ESD with the other 4 media on the basis of all 4 presumptive tests.

[d] Comparison of ESD with the other 4 media on the basis of reaction on BEM and tolerance to 6.5% NaCl only.

presence of 0.05% potassium tellurite; they could produce acid from sorbitol and mannitol, whereas they failed to produce acid from arabinose, melibiose, and raffinose; they grew well in folic acid assay medium without the addition of the cofactor; they utilized pyruvate, malate, and serine as energy sources; and they were unable to grow at 50 C. Strains that did not reduce TTC were confirmed at *S. faecium* by their intolerance to potassium tellurite, their production of acid from mannitol, arabinose and, with some exceptions, from melibiose. These strains required folic acid for growth. They could not utilize pyruvate, malate, and serine, and most of them were able to grow at 50 C. A few TTC-nonreducing strains showed these reactions but failed to ferment mannitol and arabinose. They were considered *S. faecium* var. *durans* (3).

The enterococci of 8 of the 18 cheeses (Table 4) were also enumerated by using the membrane filter technique and ESD broth. The counts by this method were slightly higher than those obtained in the surface-inoculated ESD agar plates. This was probably due to some cell loss that occurred during the spreading of inocula on the surface of the agar by means of glass rods.

DISCUSSION

S. faecalis shows strong TTC reduction, whereas *S. faecium* reduces TTC weakly (1). However, this presumptive differentiation can be equivocal due to variation in the intensity of TTC reduction among biotypes of *S. faecalis* and the occasional faint reactivity of *S. faecium* variants. Langston et al. (11), using Barnes' TG medium and method, were not able to distinguish atypical *S. faecium* strains, reducing TTC faintly, from *S. faecalis*. After 8 h of incubation the appearance of both types of cultures was similar. The same authors noticed that some differentiation could be made between the two biotypes of enterococci when observations of TTC reduction were made earlier than 8 h. In our study, the kinetics of reduction of ESD broth also provided a better criterion for differentiation than the mere ability to reduce TTC. The distinction between the two species (including atypical varieties of *S. faecium*) was attained in less than 4 h (Table 1). Differentiation by ESD proved useful in the isolation and identification of enterococci from various cheeses (Tables 2 and 4).

Of the five selective media used, ESD sup-

ported the fastest rate of growth and the maximum size of colonies. Differential counts on this medium could be determined in 25 out of 26 cheeses analyzed as early as 17 h, whereas the relatively large size of the colonies made their enumeration and isolation convenient. Quantitatively, the selective counts on ESD at 48 h appeared roughly comparable to those on RSH and SCR, media well suited for isolating and enumerating enterococci in milk, cheese, and other dairy products (10, 14, 15).

The previous observation that ESD allows good growth of a wide range of physiological types of enterococci (5) was coupled in this study with demonstration of a high degree of selectivity for this group of bacteria (Table 3). Two of the selective media used, KF and PSE, showed too broad specificity, since they allowed the selection of large percentages of strains with a combination of characteristics atypical for enterococci. Both PSE and KF were found to lack in specificity towards enterococci previously (6, 7). The present data indicate that the selectivity of KF and PSE is downgraded appreciably when enterococci are isolated from habitats such as cheese containing preponderant numbers of lactobacilli and related bacteria. In contrast, the suitability of ESD for such isolations was demonstrated in this study; the specificity of ESD for enterococci was shown to be equivalent to that of RSH and SCR media (Table 3).

TABLE 4. *Selective enumeration of enterococci in 18 cheeses using ESD medium*

Cheese	Counts/g		
	SMA	ESD	Evidence of TTC reduction[a]
Kasseri	32×10^5	29×10^4	−
Kefalotyri	31×10^4	12×10^4	−
Cheddar	28×10^6	13×10^3	+
Tilsit	29×10^6	16×10^4	+
Roquefort	30×10^8	21×10^5	−
Gouda	28×10^5	12×10^3	−
Gruyere	13×10^4	30×10^1	±
Emmenthaler	20×10^6	81×10^5	−
Cheddar	14×10^7	13×10^4	±
Fontina	86×10^8	18×10^5	+
Parmesan	32×10^3	35×10^3	−
Blue cheese	13×10^9	35×10^3	−
Feta	81×10^8	30×10^8	−
Kaskaval	13×10^4	11×10^2	−
Provolone	13×10^3	75×10^1	−
Port Salut	32×10^8	54×10^4	−
Kasseri	50×10^7	48×10^6	−
Feta	33×10^9	73×10^7	−

[a] Symbols: +, More than 80% of colonies reduced TTC; −, more than 80% of colonies did not reduce TTC; ±, both reducing and nonreducing types were present in substantial numbers.

In comparison with other available media, the preparation of ESD agar presents the average laboratory with some inconvenience. Several component solutions are made up and sterilized separately. Their mixing requires aseptic conditions. The addition of hot agar solution and the lability of TTC demand attention and control of temperature. For adequate exclusion of cations, it is preferable to use deionized water and purified agar. Surface inoculation is more time consuming than preparation of pour plates. Most of these limitations, however, can be eliminated with the liquid version of the medium. Used in conjunction with the membrane filter technique, ESD broth presents the same convenience as other standard media utilized in this manner.

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LITERATURE CITED

1. Barnes, E. M. 1956. Tetrazolium reduction as a means of differentiating *Streptococcus faecalis* from *Streptococcus faecium*. J. Gen. Microbiol. **14**:57-68.
2. Deibel, R. H. 1964. The group D streptococci. Bacteriol. Rev. **28**:330-366.
3. Deibel, R. H., D. E. Lake, and C. F. Niven, Jr. 1963. Physiology of the enterococci as related to their taxonomy. J. Bacteriol. **86**:1275-1282.
4. Efthymiou, C. J., and S. W. Joseph. 1972. Difference between manganese ion requirements of pediococci and enterococci. J. Bacteriol. **112**:627-628.
5. Efthymiou, C. J., and S. W. Joseph. 1974. Development of a selective enterococcus medium based on manganese ion deficiency, sodium azide and alkaline pH. Appl. Microbiol. **28**:411-416.
6. Facklam, R. R. 1973. Comparison of several laboratory media for presumptive identification of enterococcal and group D streptococci. Appl. Microbiol. **26**:138-145.
7. Facklam, R. R., and M. D. Moody. 1970. Presumptive identification of group D streptococci: the bile-esculin test. Appl. Microbiol. **20**:245-250.
8. Facklam, R. R., J. F. Padula, L. G. Thacker, E. C. Wortham, and B. J. Sconyers. 1974. Presumptive identification of Group A, B, and D streptococci. Appl. Microbiol. **27**:107-113.
9. Harrison, J. 1938. Numbers and types of bacteria in cheese. J. Appl. Bacteriol. **1**:12-14.
10. Hartman, P. A., G. W. Reinbold, and D. S. Saraswat. 1966. Media and methods for isolation and enumeration of the enterococci. Advan. Appl. Microbiol. **8**:253-289.
11. Langston, C. W., J. Gutierrez, and C. Bouma. 1960. Motile enterococci (*Streptoccocus faecium* var. *mobilis* var. n.) isolated from grass silage. J. Bacteriol. **80**:714-717.

12. Lighthart, B. 1968. Multipoint inoculator system. Appl. Microbiol. **16**:1797-1798.
13. Medrek, T. F., and E. M. Barnes. 1962. The influence of the growth medium on the demonstration of a Group D antigen in faecal streptococci. J. Gen. Microbiol. **28**:701-709.
14. Reinbold, G. W., M. Swern, and R. V. Hussong. 1953. A plating medium for the isolation and enumeration of enterococci. J. Dairy Sci. **36**:1-6.
15. Saraswat, D. S., W. S. Clark, Jr., and G. W. Reinbold. 1963. Selection of a medium for the isolation and enumeration of enterococci in dairy products. J. Milk Food Technol. **26**:114-117.
16. Sokal, R. R., and F. J. Rohlf. 1969. Single classification analysis of variance, p. 240-241. *In* Biometry, the principle and practice of statistics in biological research, W. H. Freeman and Co., San Francisco.
17. Walter, W. G. (ed.) 1967. Standard methods for the examination of dairy products, 12th ed. American Public Health Association, Inc., New York.