

# Agar Medium for Differential Enumeration of Lactic Streptococci<sup>1</sup>

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Received for publication 12 June 1972

An agar medium containing arginine and calcium citrate as specific substrates, diffusible ( $K_2HPO_4$ ) and undiffusible ( $CaCO_3$ ) buffer systems, and bromocresol purple as the pH indicator was developed to differentiate among lactic streptococci in pure and mixed cultures. Milk was added as the sole source of carbohydrate (lactose) and to provide growth-stimulating factors. Production of acid from lactose caused developing bacterial colonies to seem yellow. Subsequent arginine utilization by *Streptococcus lactis* and *S. diacetylactis* liberated ammonia, resulting in a localized pH shift back toward neutrality and a return of the original purple indicator hue. The effects of production of acid from lactose and ammonia were fixed around individual colonies by the buffering capacity of  $CaCO_3$ . After 36 hr at 32 C in a candle oats jar, colonies of *S. cremoris* were yellow, whereas colonies of *S. lactis* and *S. diacetylactis* were white. *S. diacetylactis*, on further incubation, utilized suspended calcium citrate, and, after 6 days, the citrate-degrading colonies exhibited clear zoning against a turbid background, making them easily distinguishable from the colonies of the other two species. The medium proved suitable for quantitative differential enumeration when compared with another widely used general agar medium for lactic streptococci.

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The 7th edition of *Bergey's Manual of Determinative Bacteriology* (2) recognizes two species of lactic group streptococci: *Streptococcus lactis* and *S. cremoris*. Matuszewski et al. (7) were the first to isolate and describe the closely related citric acid-fermenting species, *S. diacetylactis*. Sandine et al. (13) in 1962 made a comprehensive study of lactic streptococci, including several *S. diacetylactis* strains, and suggested that the latter should be included as a third species in this group.

These three species frequently are grown in association with one another in mixed lactic starter cultures used in the manufacture of several dairy products (3). Presently, no simple technique for direct differential enumeration of the individual species in a given mixture is available. *S. cremoris* physiologically differs from *S. lactis* (9, 14) and *S. diacetylactis* by its inability to hydrolyze arginine and liberate ammonia. *S. diacetylactis* is separated from the

other two species by its ability to produce diacetyl and its reduction products from citrate (13). Reddy et al. (11) exploited the ability of these species to utilize arginine in order to develop a medium for qualitative and quantitative differentiation of *S. cremoris* and *S. lactis* in mixtures. On this solid medium, colonies of *S. diacetylactis* and *S. lactis* were indistinguishable.

The first attempt at devising a differential agar containing citrate for use in identifying the citrate-fermenting lactic streptococci and *Leuconostoc* species in mixed lactic starters was made by Galesloot et al. (4). They used uniformly suspended, poorly soluble calcium citrate as the specific substrate, thereby imparting pronounced turbidity to the solid medium. Citrate-utilizing colonies developing on this agar exhibited clear zoning through degradation of the relatively insoluble tricarboxylic acid salt; colonies formed by non-citrate-fermenting species failed to show zoning. Nickels and Leesment (8) improved the medium by providing for distinction between slow and fast citrate-fermenting microorganisms. Their technique afforded differential enumeration of not

<sup>1</sup> Journal Paper J-7273 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project 1838.

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only non-citrate-fermenting lactic streptococci and *S. diacetilactis*, but also the slow-growing and slow-citrate-fermenting *Leuconostoc* species.

Our medium is an extension of the differential agar developed earlier (11) for the separation of *S. lactis* and *S. cremoris*. With this medium, colonies of the three species could be distinguished. This was accomplished by inclusion of relatively insoluble calcium citrate as an additional substrate and by providing suitable pH adjustment of the original *S. lactis*-*S. cremoris* differential agar.

#### MATERIALS AND METHODS

**Cultures.** Fourteen *S. cremoris*, 10 *S. lactis*, and 13 *S. diacetilactis* strains were used in this investigation. The cultures were obtained from the culture collection of the Department of Food Technology, Iowa State University.

Cultures were maintained by twice-a-week transfer in reconstituted milk (11% solids). The inoculated cultures were incubated at 21 C for 18 hr. Between transfers, they were stored at 5 C.

**Agar composition.** The differential medium contained 0.5% tryptone, 0.5% yeast extract, 0.25% Casamino Acids, 0.5% L-arginine-hydrochloride, 0.125%  $K_2HPO_4$ , 1% calcium citrate, 1.5% carboxy methyl cellulose (CMC, Cekol MV, Uddeholm, Sweden), and 1.5% agar. DuPont CMC grade P-754 at the rate of 0.6% may be substituted for CMC, Cekol MV. Just before the pouring of plates, 5.0 ml of sterile, reconstituted, nonfat milk (11% solids), 10 ml of sterile 3% (w/v)  $CaCO_3$  in distilled water, and 2.0 ml of sterile 0.1% bromocresol purple in distilled water were added to every 100 ml of sterile agar (melted and tempered to 55 C) and mixed to obtain homogeneity. After these additions, the medium pH was  $5.9 \pm 0.1$ .

**Agar preparation.** The amount of agar required for 1 liter of the medium was suspended in 500 ml of distilled water and steamed until dissolution. In another glass beaker containing 500 ml of distilled water, 10 g of calcium citrate and 15 g of CMC were suspended and heated while being stirred until a homogeneous, white, turbid suspension was formed. The two portions were mixed together in a separate stainless-steel vessel containing the required quantities of tryptone, yeast extract, Casamino Acids,  $K_2HPO_4$ , and arginine. The mixture was covered and steamed for 15 min. The pH of the medium after steaming was adjusted to 5.6 with 6 N HCl. The agar was then dispensed into bottles in 100-ml quantities and sterilized at 121 C for 15 min.

**Media comparison.** Eugonagar (BBL, Bioquest, Cockeysville, Md.) was used to test the efficiency of the differential medium for the quantitative recovery of fastidious streptococci.

**Plating techniques.** The spread plate procedure on previously poured agar surfaces as described by Reddy et al. (11) was followed. At the time of plating, the pH of the medium was  $6.35 \pm 0.05$ .

Wherever more than one species was plated

together in any one combination, 1.0 ml from each culture in the combination was mixed together in a sterile screw-cap test tube. Serial dilutions of the mixture were then made, and 0.1 ml of the dilution was spread on the agar surface. Conventional pour platings were made according to *Standard Methods for the Examination of Dairy Products* (1). The plates were incubated in a candle oats jar at 32 C and were examined after 36 to 40 hr and after day 6 of incubation.

**Counting.** After 36 to 40 hr of incubation at 32 C, the plates were removed from the candle oats jar and counted. First, the total count was taken, and then the counts of yellow *S. cremoris* colonies were taken. The plates then were returned to the candle oats jar and incubated for an additional 4 days. At the end of incubation, the plates were removed and exposed to the air for 1 hr, and the colonies were counted. Again, the total count was taken first. Then, all colonies showing zones of clearing of the turbid suspension of calcium citrate were counted. The latter figure represented numbers of *S. diacetilactis*. The sum of *S. cremoris* (taken after 36 to 40 hr) and *S. diacetilactis* counts was subtracted from the total count to obtain the *S. lactis* population in the mixture.

An alternate method of counting was used for culture mixtures containing slow-arginine-hydrolyzing or nonhydrolyzing strains of *S. diacetilactis*. These strains sometimes produced yellow colonies similar to *S. cremoris* after 36 to 40 hr. In such instances, when the first counts were made, all the yellow colonies were marked with an indelible felt pen (Carter's Marks-A-Lot, Carter's Ink Co., New York). When the final count was taken, if some marked colonies showed clearing, they were counted as *S. diacetilactis*, and the corresponding number was subtracted from the original yellow colony count to obtain the accurate value for *S. cremoris* numbers.

**Test for rates of citrate utilization in a simulated system.** To substantiate the need for pH adjustment of the agar to obtain sufficient clearing of the suspended calcium citrate within 6 days at 32 C in a candle oats jar, an experiment in a broth system that would simulate the conditions in the agar was designed. Agar and CMC were deleted, and insoluble calcium citrate was replaced by an equivalent amount of citric acid; other ingredients and their concentrations remained unaltered. Broth pH was adjusted to  $6.3 \pm 0.05$  and  $7.00 \pm 0.05$ .

One-milliliter portions of a  $10^{-5}$  dilution of fresh 18-hr milk cultures of *S. diacetilactis* 18-16 and 26-2 were inoculated into broth and incubated at 32 C for 24 hr. Samples of the cultures were aseptically removed at various intervals, and the amount of citrate was determined by the method of Marier and Boulet (6). The pH of the samples also was determined simultaneously. The bottle contents were mixed at intervals to enable the settled  $CaCO_3$  to neutralize the developing acidity more readily and uniformly.

#### RESULTS AND DISCUSSION

Preliminary studies were made to modify the medium described by Reddy et al. (11) to

accommodate additional differentiation of *S. diacetilactis*. When calcium citrate was added to the medium and *S. diacetilactis* was spread on it, no clearing was observed even after prolonged incubation in a candle oats jar. To increase citrate uptake by *S. diacetilactis*, the pH was lowered to 6.3. Rapid utilization of calcium citrate by *S. diacetilactis* then occurred within 2 to 6 days of incubation in a candle oats jar. Lowering of the initial pH in the medium, however, changed the medium color from violet to light yellow and drastically affected the

efficiency of differentiation between *S. lactis* and *S. cremoris* because the differentiation was based on acid-base reactions of the pH indicator. To offset this loss of efficiency, an increase in arginine level was required.

Graphs showing rates of citrate utilization by *S. diacetilactis* strains 18-16 and 26-2 in broth systems adjusted to pH 6.3 and 7.0 are presented in Fig. 1 and 2. The pH values at the various sampling intervals also are shown. The citrate utilization patterns of these two strains were similar. No appreciable change in pH was

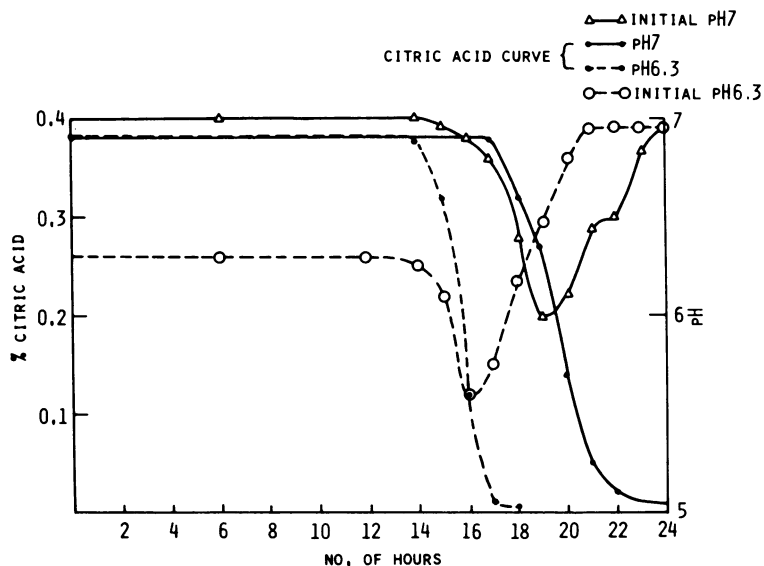


FIG. 1. Effect of initial pH of broth medium on citric acid utilization by *S. diacetilactis* 26-2.

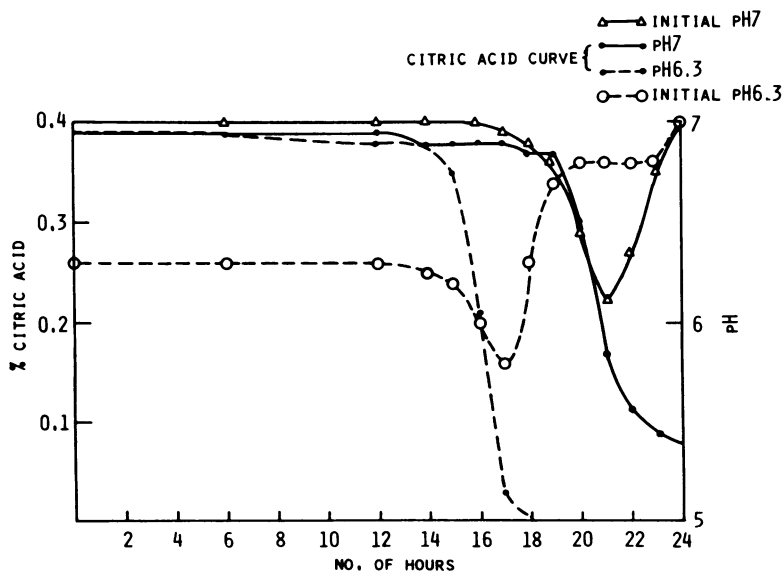


FIG. 2. Effect of initial pH of broth medium on citric acid utilization by *S. diacetilactis* 18-16.

observed within 14 hr of incubation; correspondingly, no utilization of citrate occurred within 14 hr. Beyond this time, a correlation was observed between decrease in pH and citrate uptake. When the broth pH was initially adjusted to 6.3, citrate utilization was complete within 18 hr of incubation, and the pH of the broth registered a rapid decrease after 14 hr. Delayed citrate utilization was noticed in the broth preadjusted to pH 7.0. Similarly, pH depression in the neutral broth system also commenced later, i.e., only after 16 hr of incubation.

In both broth systems (pH 6.3 and 7.0), the two strains of *S. diacetilactis*, after identical incubation periods, exhibited an abrupt shift in pH towards neutrality. In the medium adjusted first to pH 7.0, this phenomenon was noticed after 21 hr, and in the broth preadjusted to pH 6.3 this shift occurred after 17 hr.

These results suggest two possible explanations in regard to the relationship of citrate utilization to pH of the medium. It is possible that once citrate utilization is initiated pH does not play a major role in the uptake of the tricarboxylic acid. The other possibility is that a major portion of citrate, if not all, is utilized when the pH is depressed below 6.0. The shift in pH from the acid range toward alkalinity possibly was caused by the liberation of ammonia from arginine. Although these broth systems do not completely simulate the conditions in a differential agar plate, the results obtained showed the need for pH adjustment in the agar medium to obtain rapid clearing of calcium citrate. The requirement for pH adjustment to obtain rapid utilization of citrate by *S. diacetilactis* was encountered in our earlier investigation in the development of a differential broth for lactic streptococci (10). The optimal pH range for citrate permease probably is involved in this phenomenon.

The completed medium gave excellent growth and differentiation for all possible combinations of the 14 *S. cremoris*, 10 *S. lactis*, and 13 *S. diacetilactis* strains. *S. cremoris* produced yellow colonies with yellow zones (indicative of acid production alone), whereas *S. lactis* and *S. diacetilactis* exhibited white colonies (indicative of neutralization of acid by the liberated  $\text{NH}_3$ ) after 36 to 40 hr of incubation in a candle oats jar at 32 C. The acid-induced yellow color of the indicator in and around *S. cremoris* colonies tended to diffuse out rapidly beyond 40 hr of incubation; past this stage, clarity of differentiation was lost. Incubation of plates for 4 additional days in the candle oats jar permitted *S. diacetilactis* to exhibit clear zones around individual colonies (Fig. 3 and 4). A  $\text{CO}_2$  incubator could be substituted for the candle oats jar.

No clearing was observed, even after prolonged incubation, when the plates were incubated in air at 32 C. The increased rate of citrate utilization in candle oats jar probably is caused by the pH-depressing effect of the high  $\text{CO}_2$  tension of the enclosed environment. The pH of the medium was lowered from  $6.35 \pm 0.05$  to  $6.05 \pm 0.05$  in a candle oats jar as compared with  $6.1 \pm 0.05$  in a  $\text{CO}_2$  incubator. The pH of sterile agar in a corresponding poured plate incubated at 32 C in air for the same period was  $6.35 \pm 0.05$ . By conventional pour plating, distinct differentiation was not achieved. Maximal differentiating efficiency was obtained only when the counts on the individual plates did not exceed 250 colonies and fresh medium was employed.

The efficiency of recovery of the fastidious lactic streptococci by the differential medium as compared with Eugonagar is shown in Table 1. The data in Table 1 show that the differential agar provided as good recovery as did Eugonagar. Statistical analysis by the chi-square test

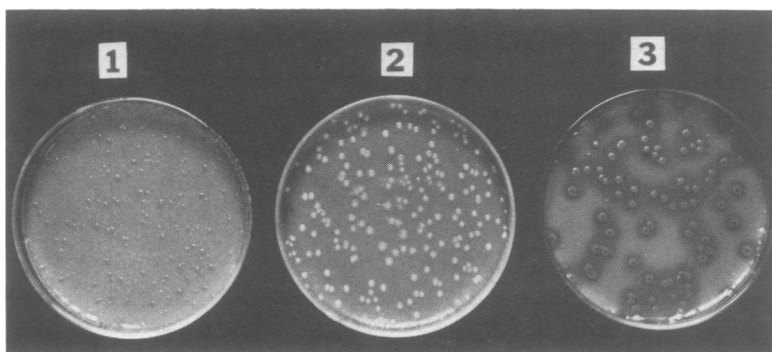


FIG. 3. Colonial growth on differential agar of *S. cremoris* HP—plate 1 (yellow color, no clearing); *S. lactis* 7963—plate 2 (white color, no clearing); and *S. diacetilactis* 26-2—plate 3 (white color, cleared zones).

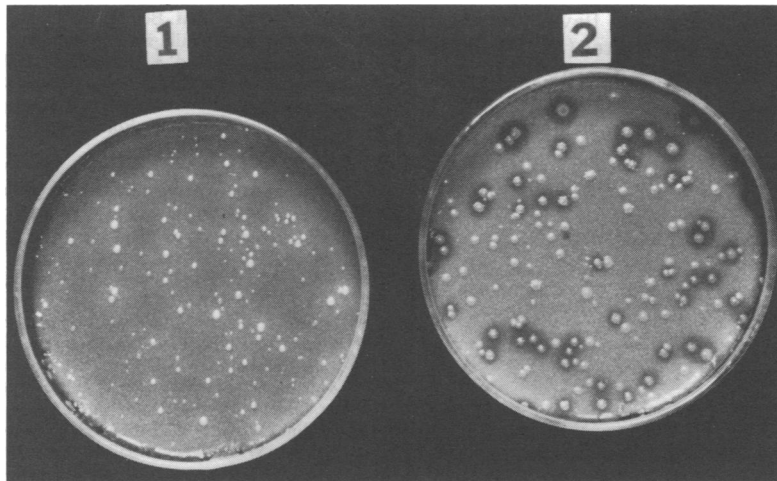


FIG. 4. Differences in appearance of colonial growth of a *S. lactis*-*S. cremoris*-*S. diacetilactis* mixture plated on the differential agar and incubated in a candle oats jar at 32 C. Plate 1 was photographed on the 2nd day; plate 2 was photographed on the 6th day.

TABLE 1. Efficiency of recovery of *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* in pure and mixed cultures on the differential medium and Eugonagar

Species	Strain	Counts/ml × 10 <sup>8</sup>	
		Differential agar	Eugonagar
<i>Streptococcus lactis</i>	7963	24	22
	C2	23	22
	10	15	15
	E	23	24
<i>S. diacetilactis</i>	31-2	12	11
	26-2	14	13
	18-16	13	12
	11007	16	14
	DRC-3	18	19
<i>S. cremoris</i>	HP	16	15
	ML4	14	14
	DR7	12	13
	SC1	16	14
Mixed <sup>a</sup>	A	17	18
	B	(7 + 5 + 5) <sup>b, c</sup>	19
	C	(7 + 8 + 4)	17
		(7 + 4 + 5)	

<sup>a</sup> A, *S. lactis* C2, *S. diacetilactis* 11007, and *S. cremoris* HP. B, *S. lactis* 7963, *S. diacetilactis* DRC3, and *S. cremoris* SC1. C, *S. lactis* C2, *S. diacetilactis* 26-2, and *S. cremoris* ML4.

<sup>b</sup> Figures in parentheses indicate counts of individual strains in three strain mixtures. The first figure is the count of *S. lactis*, the second is *S. cremoris*, and the third is the *S. diacetilactis* strain in the mixture.

<sup>c</sup> 0.750 < P < 0.900.

(15) revealed insignificant differences (see footnote c in Table 1). These results indicate that the proposed medium and technique could be used for both qualitative and quantitative differentiation of mixtures of *S. cremoris*, *S. lactis*, and *S. diacetilactis* strains.

In addition to the 37 strains of lactic streptococci used, two strains each of *S. faecalis* and *S. faecium* were plated on this medium. Yellowish-white colonies with distinct zones of cleared calcium citrate were formed by *S. faecalis* when incubated at 32 C in air. *S. faecium* produced white colonies and did not clear the turbid calcium citrate even after prolonged incubation (10 days). *S. faecalis* produced larger zones of clearing when incubated aerobically rather than in candle oats jars at 32, 37, and 45 C. These findings raise two points worthy of comment. The medium is not selective and must only be used in pure culture studies; natural products such as cheese or contaminated cultures would give erroneous results. Secondly, incorporation of selective factors in this medium with additional refinement might produce a selective and differential medium for the study of enterococcus or group D streptococci.

From a practical standpoint, the triple-species, differential agar described here could be used for studying associative growth relationships in the triple-species, starter mixtures involving strains of *S. lactis*, *S. cremoris*, and *S. diacetilactis*. This agar greatly reduced the tedium of picking colonies from a general-purpose agar into different test media to identify members of lactic group streptococci. *S. diacetilactis* strains could cause the slit defect of cheddar cheese (16), floating curd in cottage

cheese (12), and the green flavor defect in cultured sour cream and butter (5). Therefore, our medium could be used to verify the composition of mixed starter culture in the manufacturing plant. The culture manufacturer also could use our medium for screening single strains for compatibility in mixed cultures.

#### ACKNOWLEDGMENTS

C. J. W. was a postdoctoral research associate under Public Health Service training grant FD 00005-04 from the Food and Drug Administration.

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