Associative Growth Studies in Three-Strain Mixtures of Lactic Streptococci

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A recently developed differential agar medium was used to study associative growth patterns in 17 different heterologous, three-strain mixtures of *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* grown in milk. Mixtures were made by combining equal volumes of 18-hr milk cultures of the three species. Relative populations of component species were followed through three successive transfers in milk after the initial mixed propagation. Direct evidence for strain dominance and compatibility was obtained. A procedure also was developed to estimate the extent of suppression of *S. lactis* and *S. diacetilactis* in a mixture containing a dominant *S. cremoris* strain. The technique described could be successfully applied in quality-control work in the dairy-starter manufacturing industry.

Several investigators have examined associative growth relationships among the lactic group streptococci widely used as starters in the dairy industry. Hammer and Babel (4) and Knudsen (8) were the first to point out that, when lactic acid and flavor microorganisms are grown together in butter cultures, each type undoubtedly has an influence on the other. In 1954, Czulak and Hammond (3) determined "compatibility" among strains of lactic streptococci in triple-species, starter mixtures containing *Streptococcus lactis*, *S. cremoris*, and *S. diacetilactis* by applying phage tracer techniques. Their data indicated that mixtures of *S. lactis*, *S. cremoris*, and *S. diacetilactis* retained their near-original composition longer than did starters made exclusively of different *S. cremoris* strains. Later, Henning et al. (5) and Vedamuthu et al. (13) studied associative growth patterns in mixed cultures containing the three *Streptococcus* species also by using phage tracer techniques. They observed that *S. diacetilactis* invariably became dominant after even a few successive subcultures in milk.

In our study, we attempted to obtain direct evidence for domination or compatibility by application of differential enumeration techniques (M. S. Reddy, M. S. thesis, Iowa State University, Ames, 1971; 12).

**MATERIALS AND METHODS**

* Cultures. Three strains of *S. lactis* (C, 10, and 7963), four strains of *S. cremoris* (1, DR, HP, and ML), and four of *S. diacetilactis* (DRC, 18-16, 26-2, and 31-2) were included in this investigation. These strains were obtained from the culture collection of the Department of Food Technology, Iowa State University. The *S. lactis* and *S. cremoris* strains were selected on the basis of satisfactory acid-producing activity in the Horral and Elliker test (6).

* Cultures were maintained by transferring three times a week into reconstituted, pretested, nonfat dry milk (11% solids) and incubating at 21 C for 18 hr. Between transfers, the cultures were stored at 5 C.

* Counting medium and plating technique. The differential agar medium and the specific plating technique described by Reddy and his associates (M. S. Reddy, M. S. thesis, Iowa State University, Ames, 1971; 12) were used.

* Associative growth relationships in threestrain mixtures. The experimental design shown in Fig. 1 was followed. All platings for counts represented in this figure were made at culture dilutions of 10^-1 and 10^-4. These dilutions provided the best differential counting efficiency.

* Whenever an *S. cremoris* strain entirely dominated in a given mixture (as determined by counts at the 10^-4 dilution), the extent of suppression of the corresponding *S. lactis* and *S. diacetilactis* strains was determined by inoculating lower dilutions (<10^-7) of the culture mixtures into Niven broth (9) and sterile, reconstituted, nonfat dried milk. After sufficient incubation, the broth cultures were tested for NH₃ (9), and the milk cultures were tested for diacetyl-acetoin (7). The maximal dilution of a three-
strain mixture yielding a positive test for arginine hydrolysis and a negative King test (7) provided the most probable numbers of the *S. lactis* culture in the mixture; in a parallel series, a positive test for both arginine hydrolysis and diacetyl-acetoin production revealed the most probable numbers of the *S. diacetilactis* strain in the mixture. Graphical representation of this procedure is shown in Fig. 2.

**RESULTS AND DISCUSSION**

Associative growth patterns in four representative *S. lactis*- *S. cremoris*- *S. diacetilactis* combinations from a total of 17 different mixtures are shown in Tables 1 and 2. All counts reported in these tables represent the numbers of colonies developing on the agar at the 10^-7 dilution of the mixed culture. Statistical analyses of the data were not made because of the obvious differences in the counts of the component strains for several of these mixtures.

Associative growth responses shown in Tables 1 and 2 represent possibly four different types of growth relationships. In the combination consisting of *S. cremoris* ML4, *S. lactis* 7963, and *S. diacetilactis* 18-16, *S. cremoris* abruptly and totally inhibited *S. lactis* and *S. diacetilactis*. In such instances, dominance could be directly attributed to specific antibiotic production as suggested by Collins (2). We reported earlier on similar observations with two-strain mixtures (11).

The second type of dominance involving a progressive suppression of component strains is represented by the mixture comprising *S. cre-

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**Fig. 1.** Procedure used for determining population trends of component strains in mixed cultures of lactic streptococci, with successive transfers in reconstituted skim milk.
The extent of domination by *S. cremoris* DR7 and ML4, in mixtures containing *S. lactis* C2 and 7963, and *S. diacetilactis* 18-16, as determined by the Niven and King tests, is shown in Fig. 3. In the three-strain mixture containing *S. cremoris* DR7, *S. lactis* C2, and *S. diacetilactis* 18-16, both *S. lactis* and *S. diacetilactis* were suppressed progressively until the second transfer. *S. diacetilactis* 18-16 totally disappeared from the mixture at the third transfer. On the other hand, when *S. cremoris* ML4 was combined with *S. lactis* 7963 and *S. diacetilactis* 18-16, the heterofermentative strain was not detectable even after the first transfer. These results also were confirmed by parallel inoculations of the various dilutions into the differential broth described by Reddy et al. (10).

These observations again emphasize the need for discarding the practice of day-to-day transfer or "carrying" of mixed lactic cultures used in the dairy industry unless the mixtures in question have been pretested for compatibility and maintenance of the near-original proportions of the component strains through several transfers.

In the combination involving *S. cremoris* ML4, *S. lactis* C2, and *S. diacetilactis* 26-2,

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**Fig. 2.** Procedure for determining the extent of domination by *S. cremoris* strains in three-strain *S. lactis*- *S. cremoris*- *S. diacetilactis* mixtures.

**Table 1.** Population trends of component species in three-strain mixtures of lactic streptococci with successive transfers in milk

| *S. lactis* strain | Transfers | Pure culture *S. lactis* counts | Counts of *S. cremoris*- *S. diacetilactis* combinations ([counts/ml] × 10⁷)* |
|-------------------|-----------------|-----------------------------|---------------------------------------------------------------------------------|
|                   | Pure culture   | SC HP-SD 31-2 | SC ML₄, SD 18-16 | SC HP-SD 31-2 | SC ML₄, SD 18-16 |
|                   | HP | SD | SL | HP | SD | SC | SD | SC | SD | SC | SD |
| 7963              | PC  | 280 | 150 | 120 | 150 | 150 | 190 | NC | 190 | NC | 190 |
|                   | 0°  | 290 | 150 | 120 | 67  | 49  | 78  | 160 | 130 | NC | 190 |
|                   | 1   | 300 | 140 | 130 | 140 | 28  | 130 | 160 | 150 | NC | 190 |
|                   | 2   | 270 | 140 | 120 | 56  | 28  | 220 | 160 | 150 | NC | 180 |
|                   | 3   | 290 | 160 | 130 | 36  | 22  | 200 | 140 | 140 | NC | 190 |

*SC, *S. cremoris*; SD, *S. diacetilactis*; SL, *S. lactis*.

ªPC, Pure culture counts when blending.

ªInitial propagation.

ªNo colonies at 10⁻⁷ dilution.
TABLE 2. Population trends of component species in three-strain mixtures of lactic streptococci with successive transfers in milk*

| S. lactis strain | Transfers | Pure culture S. lactis counts | Counts of S. cremoris-S. diacetilactis combinations ([counts/ml] \times 10^7) |
|------------------|-----------|-----------------------------|------------------------------------------------------------------|
|                  |           |                             | SC DR, SD 18-16                                                   |
|                  |           |                             | Pure culture | Mixed culture | Pure culture | Mixed culture |
|                  |           | SC | SD | SL | SC | DR | SD | SL | SC | ML | SD | SL | SC | ML | SD |
| C₄               | PC        | 120 | 150 | 56 | 90 | 24 | 150 | 130 | NC | 96 | 8  |
|                  | 0         | 120 | 150 | 4  | 120 | NC | 160 | 120 | NC | 11 | 11 |
|                  | 1         | 140 | 150 | NC | 170 | NC | 160 | 130 | NC | NC | 210|
|                  | 3         | 140 | 140 | NC | 170 | NC | 140 | 120 | NC | NC | 120|

*See Table 1 for abbreviations.

![ARGinine HYDROlysis](image)

**Kings Test**

**CULTure COMBINATIONS**

0 - INITIAL PROPAGATION 1 - FIRST TRANSFER 2 - SECOND TRANSFER 3 - THIRD TRANSFER

**NO POSITIVE REACTION AT 1/10 DILUTION**

**FIG. 3.** Results of arginine hydrolysis and King test showing the extent of domination by S. cremoris strains in mixtures containing S. lactis and S. diacetilactis strains.

the extent of inhibition of S. cremoris by the S. diacetilactis culture could not be ascertained with this technique. Also, it is not feasible to determine the degree of suppression of S. lactis (if it were to be inhibited below the detectable level at the 10⁻⁷ dilution) in a mixture where the dominant strain happens to be a strain of S. diacetilactis. Such problems could be solved if this technique is used in conjunction with phage tracer methods. Combinations of these two techniques also would facilitate examination of associative growth patterns in mixtures containing multiple strains of each of the three lactic group Streptococcus species.

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