# ASSOCIATIVE GROWTH RELATIONSHIPS IN TWO STRAIN MIXTURES OF *STREPTOCOCCUS LACTIS* AND *STREPTOCOCCUS CREMORIS*[1]

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Abstract

A recently described differential agar medium was used to study strain interactions in two-strain mixtures of *Streptococcus lactis* and *Streptococcus cremoris*. Two *S. cremoris* strains (ML4 and DR7) exhibited marked dominance over four *S. lactis* cultures. One *S. cremoris* strain, designated 1, showed excellent compatibility in all combinations. *Streptococcus cremoris* HP was progressively suppressed by all *S. lactis* strains. The associative growth patterns at 32 C and 21 C were similar irrespective of the initial cell numbers of the component strains.

The technique described in this paper could be used in conjunction with phage tracer methods to investigate growth relationships among mixed strain lactic starters containing more than one strain each of *S. lactis* and *S. cremoris*.

Several investigators have examined associative growth relationships among lactic streptococci that are widely used as starters in the dairy industry. The earliest work in this area was done by Nichols and Ineson *(10)*, employing phage-tracer techniques to identify component strains in mixtures. Using similar techniques, Czulak and Hammond *(1)*, Collins *(2)*, and Lightbody and Meanwell *(7)* investigated strain-interactions among lactic streptococci. In a later investigation, Collins *(3)* studied the role of antibiotic production *(8, 11)* in dominance observed among mixed strain lactic starters. Recent studies on associative growth patterns in starter mixtures containing heterologous species *(6, 13)* also were conducted using strain-specific bacteriophages.

In all the foregoing investigations, the dominance and (or) compatibility among component strains in lactic starters were studied by indirect techniques using strain-specific phages and activity tests. Direct evidence through counting procedures could not be obtained because of the close relatedness of individual strains within lactic *Streptococcus* species and between species themselves, making it impossible to differentiate even colonies of heterologous species. Recently, Reddy et al. *(12)* described a differential medium for qualitative and quantitative determinations of individual components in two strain mixtures of *Streptococcus lactis* and *Streptococcus cremoris*. This paper describes the application of the differential medium to study inter-relationships among several two-strain mixtures of *S lactis* and *S. cremoris*.

## Materials and Methods

### *Cultures*

Four strains of *S. cremoris*, designated as HP, ML4, 1, and DR7, and 4 strains of *S. lactis*, designated as C2, E, 10, and 7963, were used. These strains were selected from the culture collection at the Department of Food Technology, Iowa State University on the basis of satisfacory acid-producing activity determined by the method of Horrall and Elliker *(4)*.

### *Propagation*

The selected cultures were routinely grown in 11% Matrix milk medium (Galloway-West, Fond du Lac, Wisconsin) by inoculating a 1% milk culture and incubating it at 32 C for 14 to 16 hr.

### *Counting medium and technique*

The differential medium described by Reddy et al. *(12)* was used, closely adhering to the specific technique recommended. The incubation period in the candle oats jar was, however, reduced to 36 hr from 48 hr. This modification allowed clearcut differentiation of *S. cremoris* colonies when their numbers relative to those of *S. lactis* colonies were very small.

### *Arginine hydrolysis test*

To confirm the presence of *S. lactis* in two strain mixtures of lactis-cremoris, the arginine hydrolysis test described by Niven et al. *(9)* was employed. Tubes of medium were tested for $NH_3$ at 24 hr intervals for 5 days.

### *Experimental designs for associative growth studies*

A graphical representation of the experimental design for associative growth studies is shown in Fig. 1. All platings for counts represented in Fig. 1 were made at dilutions of $10^{-7}$ and $10^{-8}$. *At lower dilutions, differentiation and counting efficiencies were very poor.*

Whenever *S. cremoris* dominated the mixture, the extent of suppression of the corresponding *S. lactis* was determined by inoculating lower dilutions of the lactis-cremoris mixtures into Niven's broth *(9)*. After incubation, the cultures were

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TABLE 1. POPULATION TRENDS[a] OF COMPONENT STRAINS IN MIXED CULTURES OF LACTIC STREPTOCOCCI WITH SUCCESSIVE TRANSFERS IN MILK.

| | | | *S. cremoris* HP | | | *S. cremoris* ML4 | | | *S. cremoris* 1 | | | *S. cremoris* DR7 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Pure culture | Pure culture | Mixed culture | | Pure culture | Mixed culture | | Pure culture | Mixed culture | | Pure culture | Mixed culture | |
| *S. lactis* strains | Transfers | *S. lactis* counts | *S. cremoris* counts | *S. lactis* counts | *S. cremoris* counts | *S. cremoris* counts | *S. lactis* counts | *S. cremoris* counts | *S. cremoris* counts | *S. lactis* counts | *S. cremoris* counts | *S. cremoris* counts | *S. lactis* counts | *S. cremoris* counts |
| | PC[b] | 200 | 150 | — | — | 130 | — | — | 95 | — | — | 95 | — | — |
| | 0[c] | 200 | 130 | 130 | 46 | 100 | NC[d] | 90 | 91 | 90 | 27 | 85 | 10 | 11 |
| C2 | 1 | 200 | 150 | 230 | 19 | 110 | NC | 130 | 80 | 62 | 41 | 81 | NC | 13 |
| | 2 | 190 | 130 | 170 | 13 | 95 | NC | 120 | 88 | 40 | 44 | 82 | NC | 17 |
| | 3 | 210 | 130 | 170 | 11 | 91 | NC | 100 | 82 | 25 | 50 | 86 | NC | 38 |
| | PC[b] | 260 | 150 | — | — | 130 | — | — | 95 | — | — | 95 | — | — |
| | 0[c] | 250 | 130 | 190 | 62 | 100 | NC | 150 | 91 | 116 | 42 | 85 | NC | 32 |
| E | 1 | 230 | 150 | 270 | 39 | 110 | NC | 170 | 80 | 153 | 41 | 81 | NC | 94 |
| | 2 | 220 | 130 | 220 | 13 | 95 | NC | 110 | 88 | 95 | 41 | 82 | NC | 49 |
| | 3 | 230 | 130 | 220 | 4 | 91 | NC | 110 | 82 | 77 | 58 | 86 | NC | 23 |
| | PC[b] | 170 | 150 | — | — | 130 | — | — | 95 | — | — | 95 | — | — |
| | 0[c] | 200 | 130 | 180 | 61 | 100 | NC | 92 | 91 | 89 | 55 | 85 | NC | 57 |
| 10 | 1 | 180 | 150 | 230 | 19 | 110 | NC | 140 | 80 | 130 | 46 | 81 | NC | 78 |
| | 2 | 180 | 130 | 290 | 7 | 95 | NC | 66 | 88 | 150 | 40 | 82 | NC | 66 |
| | 3 | 180 | 130 | 240 | 3 | 91 | NC | 130 | 82 | 150 | 36 | 86 | NC | 22 |
| | PC[b] | 260 | 150 | — | — | 130 | — | — | 95 | — | — | 95 | — | — |
| | 0[c] | 230 | 130 | 150 | 29 | 100 | 8 | 90 | 91 | 130 | 39 | 85 | 46 | 8 |
| 7963 | 1 | 230 | 150 | 220 | 18 | 110 | NC | 130 | 80 | 120 | 14 | 81 | 1 | 35 |
| | 2 | 230 | 130 | 190 | 10 | 95 | NC | 120 | 88 | 73 | 20 | 82 | NC | 42 |
| | 3 | 240 | 130 | 220 | NC | 91 | NC | 130 | 82 | 53 | 53 | 86 | NC | 38 |

[a] (count/ml) × $10^{-7}$

[b] PC - Pure culture counts when blending.

[c] Initial propagation.

[d] No colonies at $10^{-7}$ dilution.

tested for $NH_3$. This procedure was applied only when no S. *lactis* colonies were detected at $10^{-7}$ dilution of the specific lactis-cremoris mixture cultured in milk. The maximum dilution of the two strain milk culture that gave a positive test for arginine hydrolysis provided the most probable numbers (MPN) of the S. *lactis* strain in the mixture. This procedure also is graphically shown in Fig. 2.

## RESULTS AND DISCUSSION

Results of associative growth patterns in 16 possible lactis-cremoris combinations of cultures chosen for this investigation are shown in Table 1. In this table, all values shown were calculated from colony counts at $10^{-7}$ dilution (see footnotes for Table 1). As explained under Experimental Methods, the most reliable differential enumeration of lactis-cremoris milk cultures was possible at this dilution. From data in this table, it is evident that pure culture colony counts at $10^{-7}$ dilution for all the strains at the time of blending were quite high, ranging from 95 to 260. Hence, the initial count of the component strains (soon after mixing) at $10^{-7}$ dilution in the various blends would have been one-half this value; i.e., ranging from 48 to 130, which could be accurately enumerated on the differential agar. In this experiment, no attempt was made to ensure equal or nearly equal numerical counts of the component strains in the two strain mixtures.

Pure culture counts for all strains remained stable through initial propagation and three successive transfers, indicating that differences in the populations of individual strains in the mixtures were caused by strain interactions and not by fluctuations in the cell numbers of pure cultures themselves from day to day.

In all combinations containing S. *cremoris* strains ML4 and DR7, there was a definite suppression of

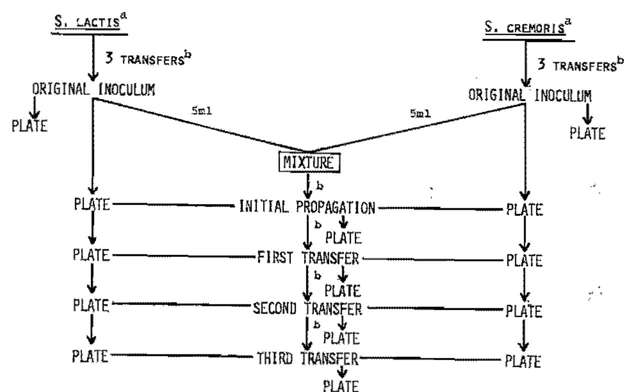


Figure 1. Procedure used for determining population trends of component strains in mixed cultures of lactic streptococci with successive transfers in milk.

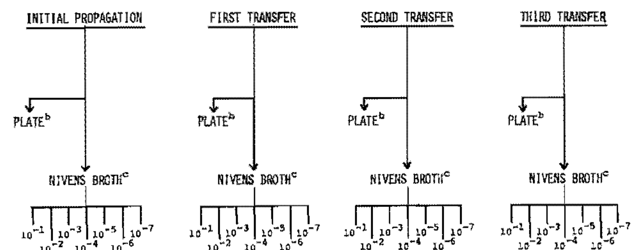


Figure 2. Procedure for determining the extent of domination by S. *cremoris* strains in two strains lactis - cremoris mixtures.

the corresponding S. *lactis* strains. This is evident from counts obtained after the initial propagation. Whereas the population of each S. *lactis* strain in the blends soon after mixing with either S. *cremoris* strain (namely ML4 or DR7) would have been at least $80 \times 10^{-7}$/ml (approximately, one-half the pure culture count at the time of blending, Table 1), after merely propagating once, the colony counts were considerably reduced or were not obtainable at a culture dilution of $10^{-7}$. There were, however, differences in the extent of inhibition with different S. *lactis* cultures (Fig. 3 and 4). Each bar in these figures represents the maximum dilution of the various mixtures at each stage of propagation in milk (shown in Fig. 1 and 2), at which a positive Niven's test (indicative of the presence of S. *lactis*) is obtained. *Streptococcus lactis* E was almost completely suppressed, whereas the other S. *lactis* strains were reduced in count by 2 to 5 logarithms by the third successive transfer.

There was a progressive domination of S. *cremoris* HP by all strains of S. *lactis*. *Streptococcus cremoris* 1 exhibited excellent "compatibility" with all four strains of S. *lactis*. This strain, or cultures with similar associative growth patterns, would be the choice for use in cheese starters.

To verify if similar associative growth patterns could be obtained in the same mixtures when initial populations of the component strains at the time of blending were adjusted to equal or nearly equal numbers, another experimental design was adopted. Pure cultures were grown through several successive transfers under strictly controlled incubation conditions (time-temperature) and accuracy of inocula. After each transfer, single-strain milk cultures were plated on the differential agar, and counts were taken. Counts were quite stable for each strain after the second successive transfer. By using these data, dilution factors for each high count culture for each specific combination were calculated. At the time

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TABLE 2. POPULATION TRENDS[a] OF COMPONENT STRAINS PREADJUUSTED TO APPROXIMATELY "EQUAL NUMBERS MIXED CULTURES OF LACTIC STREPTOCOCCI WITH SUCCESSIVE TRANSFERS IN RECONSTITUTED SKIM MILK.

| *S. lactis* strains | Transfers | Differential counts | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *S. cremoris* HP | | *S. cremoris* ML4 | | *S. cremoris* 1 | | *S. cremoris* DR7 | |
| | | SL[b] | SC[b] | SL | SC | SL | SC | SL | SC |
| | PC[c] | 49 | 48 | 120 | 110 | 120 | 100 | 50 | 70 |
| | 0[d] | 63 | 18 | NC[e] | 99 | 79 | 57 | 6 | 90 |
| C2 | 1 | 48 | 14 | NC | 110 | 88 | 69 | NC | 92 |
| | 2 | 55 | 8 | NC | 110 | 16 | 110 | NC | 32 |
| | 3 | 77 | 10 | NC | 120 | 6 | 90 | NC | 27 |
| | PC | 45 | 48 | 84 | 99 | 84 | 86 | 45 | 70 |
| | 0 | 59 | 23 | NC | 160 | 91 | 86 | NC | 140 |
| E | 1 | 40 | 8 | NC | 120 | 45 | 71 | NC | 130 |
| | 2 | 41 | 17 | NC | 120 | 8 | 89 | NC | 33 |
| | 3 | 72 | 16 | NC | 130 | 6 | 86 | NC | 38 |
| | PC | 51 | 48 | 120 | 110 | 120 | 100 | 51 | 71 |
| | 0 | 110 | 18 | NC | 120 | 130 | 74 | 2 | 99 |
| 10 | 1 | 100 | 9 | NC | 150 | 110 | 46 | NC | 160 |
| | 2 | 170 | 7 | NC | 140 | 83 | 54 | NC | 110 |
| | 3 | 220 | 3 | NC | 120 | 93 | 82 | NC | 120 |
| | PC | 55 | 48 | 100 | 110 | 100 | 99 | 55 | 70 |
| | 0 | 53 | 21 | NC | 110 | 62 | 70 | 4 | 78 |
| 7963 | 1 | 69 | 21 | NC | 120 | 29 | 75 | 1 | 78 |
| | 2 | 58 | 6 | NC | 130 | 14 | 81 | NC | 24 |
| | 3 | 110 | 9 | NC | 140 | 6 | 86 | NC | 29 |

[a](count/ml) × $10^{-7}$
[b]SL - S. *lactis*
SC - S. *cremoris*
[c]Pure culture counts before mixing.
[d]Initial propagation.
[e]No colonies at $10^{-7}$ dilution.

of blending, dilutions were made in sterile Matrix medium, and diluted cultures were then used as inocula in the blends. Results of the experiment using such blends are summarized in Table 2. Associative growth patterns in mixtures initially containing near-equal cell numbers of the component strains were similar to those found in blends made up without prior numerical adjustments. Hoyle and Nichols *(5)* and Collins *(2)* also reported that wide differences in inocula did not determine ultimate strain dominance.

Both S. *cremoris* ML4 and DR7 exerted an inhibitory effect against all strains of S. *lactis* in mixtures with and without numerical adjustment at the time of blending. Lightbody and Meanwell *(7)* also found that S. *cremoris* dominated in every instance when grown in association with S. *lactis.* Similar trends were observed by Hoyle and Nichols *(5)* in lactis-cremoris mixtures made up with "starter strains." In mixtures containing either of these S. *cremoris* strains, dominance was exhibited *rather abruptly* after the initial propagation. It is probable that the abrupt manifestation of dominance could be caused by elaboration of an antibiotic that acts immediately and effectively against susceptible strain in the mixture. In this connection, the inhibitory effect of S. *cremoris* ML4 against S. *lactis* C2, found in these mixtures, is in agreement with results of Collins *(3)* who showed that this S. *cremoris* culture produced an antibiotic active against S. *lactis* C2.

The *gradual* domination observed in mixtures containing S. *cremoris* HP does not seem to be because of antibiotic production by the corresponding S. *lactis* cultures. Dominations of this nature could be caused either by differences in acid tolerance and (or) competitive growth abilities as suggested by Czulak and Hammond *(1)* and Collins *(2)*.

In later experiments, when the cultures were treated exactly alike with the exception of incubation temperature, which was lowered to 21 C (the routine propagation temperature of starter), trends similar to those observed at 32 C were obtained (data not presented here). Hence, we concluded that, contrary to the findings of Nichols and Ineson *(10)*, temperature of incubation had little or no effect on the associative growth patterns in mixed strain lactic starters. Collins *(2)* also found no correlation between temperatures of incubation and associative growth patterns. These experiments were repeated twice, and in every instance, trends in strain interaction were similar.

We have demonstrated the suitability of the dif-

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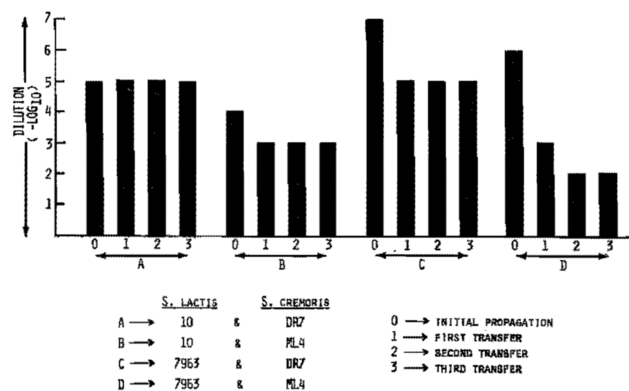


Figure 3. Results of arginine hydrolysis tests showing the extent of domination by S. *cremoris* strains in mixtures containing S. *lactis* strains.

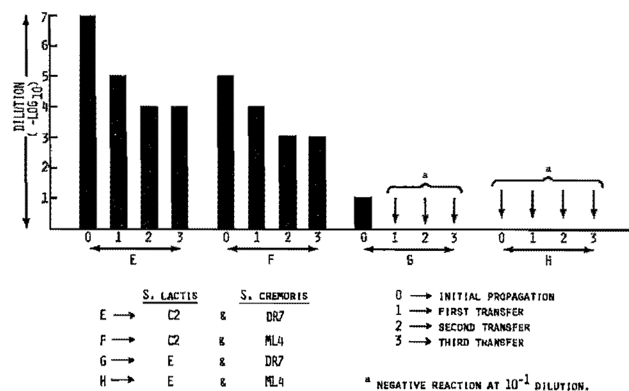


Figure 4. Results of arginine hydrolysis test showing the extent of domination by S. *cremoris* strains in mixtures containing S. *lactis* strains.

ferential agar of Reddy et al. *(12)* to study strain interactions in lactis-cremoris mixtures. The phage typing technique used by earlier workers could be used in conjunction with our procedure to study associative growth patterns in starter mixtures containing more than one strain each of these two species. Colonies from the differential agar could be picked and tested against specific phages to trace the identity of the component strains. In addition, extent of domination also could be demonstrated by checking milk or broth tubes inoculated and incubated with serial dilutions of the specific mixture against specific phage types. Such a procedure would simplify the methods previously used for similar studies.

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