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Effect of Fermentation Conditions on Growth of *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ 1091 in Pure and Mixed Cultures

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Two strains of mesophilic lactic acid bacteria, *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ 1091, were grown in pure and mixed cultures in the presence or absence of citrate (15 mM) and at controlled (pH 6.5) or uncontrolled pH. Microbial cell densities at the end of growth, maximum growth rates, the pH decrease of the medium resulting from growth, and the corresponding acidification rates were determined to establish comparisons. The control of pH in pure cultures had no effect on *L. lactis* CNRZ 1091 populations. The final populations of *S. cremoris* AM2, however, were at least five times higher than when the pH was not controlled (4×10^8 vs. 2×10^9 CFU · ml^{-1}). The pH had no effect on the growth rate of either strain. That of *S. cremoris* AM2 (0.8 h^{-1}) was about twice that of *L. lactis* CNRZ 1091. When the pH fell below 5, the growth of both strains decreased or stopped altogether. Citrate had no effect on *S. cremoris* AM2, while final populations of *L. lactis* CNRZ 1091 were two to three times higher (3×10^8 CFU · ml^{-1}); it had no effect on the maximum growth rates of the two strains. Citrate attenuated the pH decrease of the medium and reduced the maximum acidification rate of the culture by 50%, due to the growth of *S. cremoris* AM2. Acidification due to *L. lactis* CNRZ 1091, however, was very slight. Regardless of the conditions of pH and citrate, the total bacterial population in mixed culture was lower (by 39%) than that of the sum of each pure culture. Mixed culture improved the maximum growth rate of *L. lactis* CNRZ 1091 (0.6 h^{-1}) by 50%, while that of *S. cremoris* AM2 was unaffected. The acidification rate of the growth medium in mixed culture, affected by the presence of citrate, resulted from the development and activity of *S. cremoris* AM2.

Mesophilic lactic acid bacterial starters used in the dairy industry are mixtures of genera, species, strains, and even different variants (10). Their composition is thus not always known, particularly in the case of natural starters. In addition, microbial interactions, either beneficial (cooperation) or deleterious (inhibition), generally lead to uncontrollable changes in the composition of the starter. Thus, a strain may be eliminated because of a lower maximal growth rate (10, 12) or because of its sensitivity to bacteriocins produced by other strains (5, 14). Inversely, growth may be stimulated by activators produced by the other microorganisms in the mixture (8, 22). Interactions occurring in mixed cultures are generally reflected by final bacterial population levels (19), by acid production (1, 11, 19), and by concentrations of secondary metabolites (9) which differ from those obtained in pure cultures. The results of these variations in the composition of starters may be a disadvantage for lactic fermentation: slower acidification and modification in the texture of the curd and of the organoleptic properties of the cheese.

One means of controlling bacterial populations is to follow their changes in mixed cultures. This was done in the present work by growing two strains of mesophilic lactic acid bacteria, *Streptococcus cremoris* AM2 and *Leuconostoc lactis* CNRZ 1091. This type of combination is frequently encountered in the cheese industry in France, since strains of *S. cremoris* and *L. lactis* are responsible for acidification and flavor development, respectively. Cultures were grown at controlled pH (similar to the production of starters) and at uncontrolled pH (similar to cheese manufacture). Quantitative (maximum populations reached, total pH variation) and kinetic (maximum growth rate, maximum acidification rate) parameters were calculated. The results obtained in mixed cultures were compared with those in pure cultures for each strain.

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MATERIALS AND METHODS

Microorganisms. The strains used were *S. cremoris* AM2 (also called CNRZ 380) and *L. lactis* CNRZ 1091, obtained from the culture collection of the Centre National de Recherches Zootechniques (CNRZ, Jouy-en-Josas, France). They were stored frozen at −18°C in litmus milk (1 g of Bacto litmus [Difco] per liter of skim milk) and in MRS medium (6), respectively.

Culture media. The medium of Desmazeaud and Vassal (7) in deionized water was used for all fermentations. Citrate (15 mM) was added to some cultures. Citrate and lactose were sterilized at 110°C for 10 min, separately from the Bactotryptone and from the yeast extract (120°C for 20 min).

Reactor. The reactor was composed of a 2.5-liter useful-volume round glass flask. Temperature was maintained at 30°C in a water bath. The medium was stirred at low speed (200 rpm). Sterile nitrogen was blown across the surface of the medium to maintain an anaerobic atmosphere.

In some experiments, the pH was maintained constant at 6.5 by the automatic addition of 10 N sodium hydroxide. Cultures at uncontrolled pH were all started at a pH close to 7 (pH of the medium after sterilization).

Preparation of inocula. Inocula were prepared in sodium phosphate buffer (100 mM, pH 6.5) and incubated for 9 h (*S. cremoris* AM2) or 12 h (*L. lactis* CNRZ 1091). They were

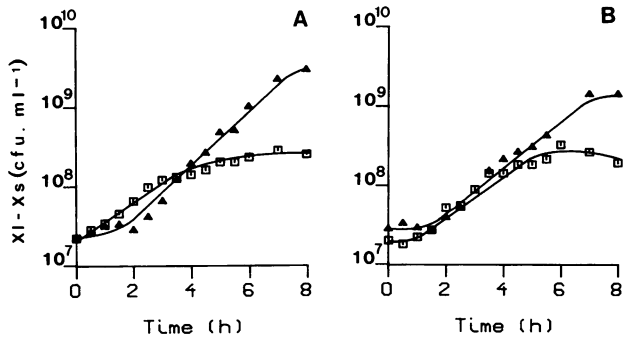

FIG. 1. Cultures at controlled pH in the presence of citrate. (A) Pure cultures of *L. lactis* CNRZ 1091 and *S. cremoris* AM2. (B) Mixed culture. Symbols: □, population of *L. lactis* CNRZ 1091 (*Xl*); ▲, population of *S. cremoris* AM2 (*Xs*).

used to inoculate the reactor at 3% (vol/vol) (*S. cremoris* AM2) and 1% (*L. lactis* CNRZ 1091).

Experimental cultures. Each strain was grown in pure culture. Mixed cultures were also grown. The following fermentation conditions were tested in duplicate in pure and mixed cultures: (i) pH controlled, with citrate; (ii) pH not controlled, with citrate; (iii) pH controlled, without citrate; (iv) pH not controlled, without citrate. Fermentation lasted 8 h. Samples were taken every 30 min during the first 6 h and then every hour.

Bacterial counts. Each sample was diluted 10-fold and treated with a TURRAX disperser for 15 s (18). The bacterial suspension was then diluted in peptone water.

Each bacterial population was counted by plating on selective medium: Kempler and McKay medium (16) for *L. lactis* CNRZ 1091, and that of Huggins and Sandine (13) for *S. cremoris* AM2. Petri dishes were inoculated with a Spiral inoculator (Spiral Systems Marketing, Inc.) and incubated anaerobically for 48 h (GasPak; BBL Microbiology Systems).

Determination of maximal and total bacterial populations. Bacterial counts at the beginning and end of the culture furnished the maximum population by difference: Xl_{max} represents *L. lactis* CNRZ 1091 and Xs_{max} represents *S. cremoris* AM2. In mixed culture, the total population Xt_{max} was the sum of Xl_{max} and Xs_{max}.

Determination of the maximum growth rate. During exponential growth, the logarithm of the bacterial population varies linearly with time. Thus, the maximum growth rate, μ_{max}, is the slope of this line. It was determined by linear regression applied to the experimental points of the line: $\ln X = \mu_{max}t + b$. The observed μ_{max} values were compared with the Fisher test described below.

Determination of maximum rates of acidification. It was observed that the pH of cultures decreased linearly with time during exponential growth. The corresponding regression lines were calculated. The absolute value of their slope is a reflection of the maximum acidification rate, Va_{max} (pH = $Va_{max}t + b$). The observed maximum acidification values were compared with the Fisher test (20, 23).

Fisher test. It was considered that each experiment constituted a group of data (at least five data per experiment). The regression lines used to determine the maximum growth rates or maximum rates of acidification define a general model characterized for each line by a slope and a y intercept. For this model, the residual sum of squares (SS_0) and the residual variance (MS_0) were calculated. Then, the hypothesis of equality of the slopes of all the regression lines was tested. Finally, the residual sum of squares (SS_i) was calculated, and the value of F (Fisher statistics) was determined: $F = [(SS_i - SS_0)/(df_i - df_0)]/MS_0$, where df_i and df_0 are the degrees of freedom for the hypothesis tested and for the general model, respectively. A threshold was set in order to interpret these tests, defined as the probability of erroneously rejecting a true hypothesis. The hypothesis was tested at the 5% threshold. It was accepted if the calculated value of F was lower than the value read off the Fisher table, with $df_i - df_0$ the degrees of freedom in the numerator and df_0 those in the denominator. In this case, the slopes of the regression lines (maximum growth rates, maximum rates of acidification) are considered equal.

RESULTS

Effect of culture conditions on population changes. Growth of *L. lactis* CNRZ 1091 in pure culture lasted about 6 h (Fig. 1A and 2A). This period was shortened slightly (from 4.5 to 5 h) in mixed culture (Fig. 1B and 2C). At uncontrolled pH and without citrate, growth stopped after about 4 h, when the pH reached 5 (Fig. 2C).

The maximal populations of *L. lactis* CNRZ 1091 (Table 1) differed as a function of the presence or absence of citrate; in its presence, the maximal population was always greater than 1.9×10^8 CFU · ml^{-1}, but never reached this value in its absence. These values were unchanged in mixed culture and were independent of the control of pH.

The lag phase of *S. cremoris* AM2 was 1 to 2 h in all conditions (Fig. 1A and B and 2B and C). At uncontrolled pH, the exponential phase of *S. cremoris* AM2 growth ended after 4.5 h of fermentation without citrate (Fig. 2B and C) and after 5.5 h in its presence (results not shown), at which time the pH was between 4.7 and 5.3. Final population levels were practically unchanged under conditions of uncontrolled pH. When pH was controlled at 6.5, the exponential phase of *S. cremoris* AM2 growth ended after 7 h of culture (Fig. 1A and B), since practically no more growth was noted after longer times of culture (24 h).

The maximum populations of *S. cremoris* AM2 differed in pure and mixed cultures depending on whether the pH was controlled (Table 1). In the case of controlled pH, maximum population density was between 1.3×10^9 and 2.2×10^9 CFU · ml^{-1}, while it never exceeded 4.1×10^8 CFU · ml^{-1} in uncontrolled conditions.

TABLE 1. Maximum populations in pure and mixed cultures[a]

Culture conditions	Population (10^8 CFU · ml^{-1})					
	Xl_{max}		Xs_{max}		Xt_{max}	
	Pure	Mixed	Pure	Mixed	Sum of pure cultures	Mixed
With citrate						
pH controlled	3.4	2.4	19	14	22	16
	2.3	3.3	22	16	24	19
pH not controlled	4.2	2.0	3.7	2.8	7.9	4.8
	3.9	1.9	3.3	2.5	7.2	4.4
Without citrate						
pH controlled	0.8	1.3	20	19	21	20
	1.1	1.8	20	13	21	15
pH not controlled	1.1	1.6	4.1	3.3	5.2	4.9
	1.1	0.9	3.1	3.5	4.2	4.4

[a] Two experiments were carried out for each culture condition. Xl_{max}, Maximal population of *L. lactis* CNRZ 1091; Xs_{max}, maximal population of *S. cremoris* AM2; $Xt_{max} = Xl_{max} + Xs_{max}$.

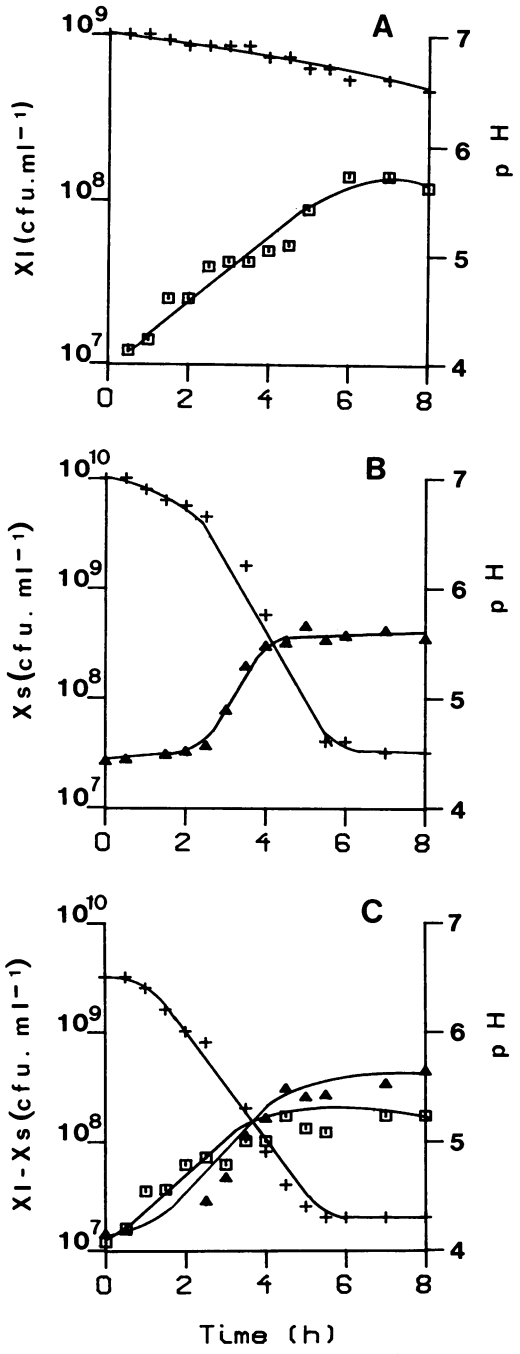

FIG. 2. Cultures at uncontrolled pH in the absence of citrate. (A) Pure culture of *L. lactis* CNRZ 1091. (B) Pure culture of *S. cremoris* AM2. (C) Mixed culture. Symbols: +, pH; □, population of *L. lactis* CNRZ 1091 (*Xl*); ▲, population of S. *cremoris* AM2 (*Xs*).

The sum of the maximum populations of *L. lactis* CNRZ 1091 and *S. cremoris* AM2 is designated Xt_{max} in Table 1. In mixed cultures, Xt_{max} was lower (by up to 39%) than the sum of the two maximum populations measured in pure cultures (Table 1). The combination of the two strains thus does not favor biomass production.

Effect of culture conditions on maximum growth rates. In spite of the difference in growth rates between repeated experiments (13%), it was observed (Table 2) that the mean growth rate of *L. lactis* CNRZ 1091 in pure culture and independently of other culture conditions (0.41 ± 0.06 h^{-1}) was lower than that obtained in mixed culture (0.62 ± 0.11 h^{-1}). The results of the Fisher test (Table 3) indicate that the hypothesis of equality of regression line slopes was acceptable at the 5% threshold for pure cultures and for mixed cultures. However, this hypothesis was no longer acceptable for both types of cultures tested together. The maximum growth rate of *L. lactis* CNRZ 1091 established with the Fisher test was 0.41 ± 0.01 h^{-1} in pure cultures, significantly different from that obtained in mixed cultures (0.61 ± 0.02 h^{-1}).

TABLE 2. Maximum growth rates in pure and mixed cultures[a]

Culture conditions	μ_{max} (h^{-1})			
	L. lactis CNRZ 1091		*S. cremoris* AM2	
	Pure	Mixed	Pure	Mixed
With citrate				
pH controlled	0.40	0.62	0.83	0.80
	0.45	0.48	0.95	0.83
pH not controlled	0.41	0.66	0.71	0.80
	0.45	0.58	0.84	0.86
Without citrate				
pH controlled	0.46	0.69	1.05	0.89
	0.34	0.66	0.92	0.90
pH not controlled	0.35	0.58	0.96	0.96
	0.43	0.67	0.99	0.89

[a] Two experiments were carried out for each culture condition.

The results in Table 2 show that the mean maximum growth rate of *S. cremoris* AM2 was similar in pure and mixed cultures (0.89 ± 0.17 h^{-1}). The results of the Fisher test for *S. cremoris* AM2 showed that the hypothesis of equality of regression line slopes was acceptable in all culture conditions (Table 3). The maximum growth rate for all fermentations established with the Fisher test was 0.78 ± 0.05 h^{-1}. This value was somewhat lower than the algebraic mean.

Effect of culture conditions on acidification of the medium. The pH of pure *L. lactis* CNRZ 1091 cultures decreased by about 0.3 U in 8 h, reaching a value close to 6.5 at the end of growth. The total decrease in pH during growth of *S. cremoris* AM2 was 1.8 to 2.7 U. In mixed cultures, the pH decreased by the same amount as in a pure culture of *S. cremoris* AM2 (final pH, 4.3 to 5.2).

The calculation of the maximum acidification rate of *L. lactis* CNRZ 1091 was rendered difficult by the very low pH

TABLE 3. Fisher test with maximum growth rates[a]

Strain	Culture	$df_i - df_0$	df_0	F	Result[b]
L. lactis CNRZ 1091	Pure	7	65	1.69	A
	Mixed	7	48	1.26	A
	Pure and mixed	1	141	6.76	R
S. cremoris AM2	Pure	7	34	1.03	A
	Mixed	7	30	0.49	A
	Pure and mixed	1	92	1.00	A

[a] Hypothesis of equality of regression line slopes was tested.
[b] A, Hypothesis accepted at the 5% threshold; R, hypothesis rejected at the 5% threshold.

TABLE 4. Maximum acidification rates in pure and mixed cultures

Fermentation condition	Maximal acidification rate[a] (pH units · ml^{-1})	
	With citrate	Without citrate
Pure *S. cremoris* AM2 cultures	0.35	0.79
	0.48	0.77
Mixed cultures	0.39	0.66
	0.44	0.78

[a] Two experiments were carried out for each fermentation condition.

changes observed. These results were thus not included in Table 4.

Maximum rates of acidification were calculated with a precision on the order of 14% (Table 4). In light of this error, acidification results were not significantly different between pure *S. cremoris* AM2 cultures and mixed cultures. This confirms that acidification is primarily due to the action of *S. cremoris* AM2.

In the presence of citrate, the final pH of *S. cremoris* AM2 cultures or of mixed cultures was always higher than when citrate was absent, the observed difference being about 0.7 pH unit. The maximal acidification rate in this case was lower (about 50% according to Table 4).

The results of the Fisher test (Table 5) indicate that the hypothesis of equality of regression line slopes was acceptable in the presence and in the absence of citrate. But this hypothesis was no longer acceptable if both conditions held concomitantly: in the presence of citrate, the maximum acidification rate established with the Fisher test was 0.41 ± 0.02 pH unit · h^{-1}. This value was significantly different from that obtained in the absence of citrate (0.76 ± 0.03 pH unit · h^{-1}).

DISCUSSION

L. lactis CNRZ 1091 is a strain which metabolizes citrate to acetate. Citrate catabolism results in the synthesis of additional pyruvate, which becomes available for anabolic pathways (4). It is thus possible that the presence of citrate favors *L. lactis* CNRZ 1091 growth, explaining why the final microbial population is twice as great.

The final populations of *S. cremoris* AM2 were lower than 4.1×10^8 CFU · ml^{-1} in cultures at uncontrolled pH and on the order of 2×10^9 CFU · ml^{-1} at controlled pH. Control of the pH at 6.5 thus favors the growth of *S. cremoris* AM2. Higher values of final populations (7×10^9 to 8×10^9 CFU · ml^{-1}) were reported by Law et al. (17) for two strains of *S. cremoris* grown at the controlled pH of 6.6 on reconstituted skimmed milk, but those values were obtained for culture periods twice as long as those of the present work (17 h).

When the pH of the medium reached 5, growth of *S. cremoris* AM2 decreased. This has been observed for other lactic streptococcus strains. Thus, Bergère (2) showed that the growth of a strain of *Streptococcus lactis* was very low at pH 5.2 and stopped altogether at pH 4.5.

TABLE 5. Fisher test with maximum acidification rates[a]

Culture	$df_i - df_0$	df_0	F	Result[b]
Pure	3	13	3.09	A
Mixed	3	12	1.05	A
Pure and mixed	1	37	16.58	R

[a] Hypothesis of equality of regression line slopes was tested.
[b] See Table 3, footnote *b*.

S. cremoris AM2 growth was unaffected by the presence of *L. lactis* CNRZ 1091 and by the citrate content in the culture medium. The μ_{max} of *S. cremoris* AM2 was similar regardless of whether the pH was controlled. Nevertheless, the *S. cremoris* AM2 population was lower at uncontrolled pH. This is explained primarily by the shorter period of growth. The μ_{max} values obtained in pure culture of *S. cremoris* AM2 (about 0.78 h^{-1}) were higher than those reported by Cogan (3) for the other strains of *S. lactis* and *S. cremoris* grown on skim milk (about 0.6 h^{-1}). Hugenholtz and Veldkamp (12) reported values between 0.6 and 0.8 h^{-1} for strains for *S. cremoris* on MRS medium.

The maximum growth rate of *L. lactis* CNRZ 1091 was 0.4 h^{-1} in pure cultures and 0.6 h^{-1} in mixed cultures. The latter value is usually given for mesophilic lactic acid bacteria. The improved maximal growth rate of *L. lactis* CNRZ 1091 in mixed culture showed a phenomenon of microbial cooperation between the two strains (15). Since this interaction was beneficial only for *L. lactis* CNRZ 1091, it is thus one of commensalism. This type of interaction has rarely been quantitated in mesophilic lactic acid bacteria. The comparison of growth rates in pure and mixed cultures is thus a good means of quantitating these phenomena.

L. lactis CNRZ 1091 behaved like a protease-negative variant on Huggins and Sandine differentiation medium (13). This medium contains only milk as the carbon and nitrogen source. This strain thus probably does not possess a cell wall-bound proteolytic system enabling it to hydrolyze milk proteins. It may be supposed that in mixed culture, *S. cremoris* AM2 (protease positive) furnishes the essential amino acids to *L. lactis* CNRZ 1091. This synergy would thus be comparable to that exerted by a protease-positive variant on a protease-negative one (11, 22).

The results of Hugenholtz et al. (10, 12) showed how one strain with a low maximum growth rate could be eliminated from a mixture after several transfers. In the present case and at growth at uncontrolled pH, neither of the two strains was eliminated, since the final populations contained 2.1×10^8 and 3.3×10^8 CFU of *L. lactis* CNRZ 1091 and *S. cremoris* AM2, respectively, per ml.

At controlled pH, however, the growth of *S. cremoris* AM2 was greater than that of *L. lactis* CNRZ 1091. This imbalance may be corrected by (i) the size of the inoculum, the balance of a mixture being highly dependent on the adjustment of the inoculum (1) or (ii) the physicochemical parameters of fermentation. This may be done by defining an incubation temperature (21) or a culture medium favoring the growth of *L. lactis* CNRZ 1091 at the expense of *S. cremoris* AM2.

The acidification of the medium in growth at uncontrolled pH was a good reflection of bacterial growth, especially during the exponential phase. This is why the measurement of pH is sometimes used to follow growth (3).

In the presence of citrate, the decrease in pH resulting from the growth of *S. cremoris* AM2 was less than that in the absence of citrate on both the quantitative and kinetic levels. The hypothesis of the inhibition of *S. cremoris* AM2 growth and thus a decreased acidification rate by citrate is hardly justified, since the maximum growth rate of the strain did not decrease. Citric acid is a tricarboxylic acid which is a buffer at pH values close to its pK_a values (3.1, 4.74, and 6.40). Its addition thus reinforces the buffering power of the medium used (7), which is already buffered by Bacto-tryptone at 20

g/liter and by yeast extract at 7 g/liter. As in the case of all fermentations including citrate characterized by relatively high final concentrations (10 to 15 mM at the end of growth), it is normal that the pH remained higher than 5.2. On the other hand, in the absence of citrate, the pH of the medium could descend to about 4.3 to 4.5.

A starter is composed of strains with properties defined by their acidifying and flavor-developing properties, their sensitivity to bacteriophages, and their potential production of bacteriocins and bitter peptides. It can now be seen that these characteristics are no longer sufficient to characterize the starter: it is necessary to study the behavior of individual strains in mixed culture under different fermentation conditions to determine final populations, μ_{max}, and acidification rates. We have shown that this can be accomplished by using a reactor in well-controlled culture conditions. The same methods applied to other strains should contribute to better control of the compositions of cultures involved in cheese production.

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