Associative Growth of *Lactococcus lactis* and *Leuconostoc mesenteroides* Strains in Milk

PASCALE BELLENGIER, JEAN RICHARD, and CATHERINE FOUCAUD[1]
Institut National de la Recherche Agronomique, Unité de Recherches Laitières,
78352 Jouy-en-Josas Cedex, France

ABSTRACT

The associative growth of a proteinase-positive strain of *Lactococcus lactis* ssp. *lactis* CNRZ 1076 and *Leuconostoc mesenteroides* ssp. *mesenteroides* UM10 in reconstituted skim milk was studied in detail. The maximum population and the growth rate of *Lc. lactis* CNRZ 1076 were not affected by the association between strains. The growth rate of *Leuc. mesenteroides* UM10 was lower in mixed-strain cultures than in pure culture (0.40 vs. 0.63/h, respectively), and the maximum population was 10-fold lower. Acidification was essentially due to the action of *Lc. lactis* CNRZ 1076, and the acidification rate in mixed-strain cultures and in pure cultures were comparable except when the percentage of *Lc. lactis* cells was lower than 12%. Inhibition of *Leuc. mesenteroides* growth was less when the percentage of *Lc. lactis* cells was lower in the inoculum. Inhibition was almost suppressed when reconstituted skim milk was supplemented with amino acids, Mn^{2+}, and Mg^{2+}. Twenty-four paired associative cultures of 3 *Lc. lactis* strains and 8 *Leuc. mesenteroides* strains in reconstituted skim milk confirmed that competition between the two species for nitrogenous nutrients is a common feature of mixed-strain cultures, regardless of the subspecies of *Leuc. mesenteroides* or *Lc. lactis* strains used.
(**Key words**: *Leuconostoc mesenteroides*, *Lactococcus lactis*, mixed-strain culture, milk)

Abbreviation key: **Prt$^+$** = proteolytic, **RSM** = reconstituted skim milk, $\mathbf{V_{mar}}$ = mean acidification rate.

INTRODUCTION

Mixed-strain cultures of lactic acid bacteria are commonly used as a starter in the manufacture of cheese. These mixed-strain cultures are complex for several reasons. First, various species of lactic acid bacteria are contained in mixed-strain cultures. Different strains of each species vary in several properties, including growth rate, acid production, aroma production, proteolytic activity, bacteriocin sensitivity and production, and phage sensitivity. Possible microbial interactions, either beneficial (cooperation) or unfavorable (inhibition), usually generate uncontrollable changes in the composition of the bacterial flora during milk fermentation (11, 17, 25). These variations should be avoided to prevent slower acidification and to prevent the modification of texture and organoleptic properties of the cheese.

Leuconostoc mesenteroides ssp. *mesenteroides* or *Leuconostoc mesenteroides* ssp. *dextranicum* are the leuconostocs most frequently isolated from French cheese prepared from raw milk (8). These strains grow associatively with acid-producing lactococci and are employed for their technological properties (mainly aroma and texture) (5, 25). The associative growth between these two groups of bacteria has been studied with respect to citrate metabolism and aroma formation (3, 15, 18, 25, 26) and has been described as a synergistic functional relationship. Far less attention has been devoted to the growth rate, acid production, and final population of these bacteria, which also reflect the interactions occurring in mixed-strain cultures of *Leuconostoc* and *Lactococcus* (3). Inhibition of *Leuc. cremoris* growth in mixed-strain cultures with *Lactococcus lactis* ssp. *cremoris* was reported by Pack et al. (20). The improved maximum growth of *Leuc. lactis* CNRZ 1091 in mixed-strain cultures with *Lc. lactis* ssp. *cremoris* AM2 in milk illustrated microbial cooperation between the two species (3). Also, various types of interaction may occur because the final population of *Leuc. mesenteroides* varied depending on the *Lc. lactis* strain used in the associative growth in milk (15).

In the present work, the interaction between *Leuc. mesenteroides* UM10 and *Lc. lactis* CNRZ 1076 in mixed-strain cultures in reconstituted skim milk (**RSM**) was investigated in detail. Growth rate, acidification rate, and maximum population were determined in mixed-strain cultures and compared with those obtained in pure cultures of each strain. The relationship between the two strains was analyzed for nutrient competition. To extend our observations, 3 strains of the genus *Lactococcus* and 8 strains of the

Received May 24, 1996.
Accepted February 3, 1997.
[1]Corresponding author.

genus *Leuconostoc* were then studied as mixed-strain cultures.

MATERIALS AND METHODS

Bacterial Strains

Bacterial strains were obtained from the CNRZ collection (Institut National de la Recherche Agronomique, Jouy-en-Josas, France); 6 strains were *Leuc. mesenteroides* ssp. *mesenteroides* (UM6, UM9, UM10, UM12, UM13, and UM14), and 2 strains were *Leuc. mesenteroides* ssp. *dextranicum* (UD17 and UD23). All of these strains metabolized lactose and citrate (2). Three proteolytic (**Prt**$^+$) strains—*Lactococcus lactis* ssp. *lactis* CNRZ 1076 and CNRZ 1124 and *Lc. lactis* ssp. *cremoris* Wg2—could grow on lactose (13).

Strains were stored at –20°C in skim milk containing 0.07% Bacto-litmus (Difco, Detroit, MI) and were supplemented with 0.5% yeast extract and 0.5% glucose.

Culture Medium and Culture Conditions

Lactococcus and *Leuconostoc* cells were precultured at 30°C in RSM [10% (wt/vol), low heat, spray-dried skim milk powder (Nilac®, Netherlands Institute for Dairy Research, Ede, The Netherlands)] that had been supplemented with 0.5% yeast extract and 0.5% glucose (1). Cultures were grown in RSM that had been inoculated with ca. 10^7 cfu/ml cells in the exponential phase of growth. Mixed-strain cultures contained 50% *Lc. lactis* cells, unless otherwise indicated. In some experiments, the pH was maintained by the automatic addition of 3*N* sodium hydroxide.

Bacterial Enumeration and pH Measurement

After the chains of cells were mechanically disrupted (Ultra-Turrax model T25; Janke and Kunkel GmbH and Co. KG, Staufen, Germany) for 30 s at 20,000 rpm (16), cell populations were estimated by plating appropriate dilutions in 0.1% peptone water, using a spiral plater (Spiral System, Cincinnati, OH).

Cell populations of pure cultures were estimated on MRS agar (6). Lactose was sterilized by filtration and added to MRS. Cell populations of mixed-strain culture were distinguished on MRS agar overlaid with 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N′-dimethyl-formamide (22). *Leuconostoc mesenteroides* strains that were positive for β-galactosidase developed blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside containing MRS agar, and *Lc. lactis* strains that were positive for phospho-β-galactosidase developed uncolored colonies.

pH was monitored during bacterial growth using a combination electrode (U402-M6-S7/100; Ingold Messtechnick AG, Urdorf, Switzerland) connected to a pH meter (pHN 81; Radiometer, Copenhagen, Sweden).

Production of Inhibitory Substances

The possible production of an inhibitory substance by *Lactococcus* or *Leuconostoc* strains toward the other associated strains was assessed as described previously (13).

Curve and Statistical Analysis

The values for $\log_{10}$ colony-forming unit per milliliter were plotted against time. Linear regression analysis was performed on experimental data showing alignment. According to Hassan et al. (10), linearity was assumed when the standard deviation of the slope was <0.07 $\log_{10}$ cfu/ml. Growth rate (μ) was calculated as follows: μ (per hour) = [slope (log colony-forming units per milliliter per hour)]/$\log_{10}2$.

The mean acidification rate ($\mathbf{V_{mar}}$; pH units per hour] during the phase of rapid pH decrease (between 6.2 and 4.8) was also determined by regression analysis (7). The V_{mar} was assumed to be linear if the standard deviation of the slope was < 0.02 pH unit.

Mean values for growth parameters (growth rate, V_{mar}, and maximum population) with confidence limits (P = 0.95) were calculated according to Snedecor and Cochran (23) on the basis of three independent experiments.

RESULTS

Growth and Acidification Parameters of Pure and Mixed-Strain Cultures

Pure cultures of *Lc. lactis* ssp. *lactis* CNRZ 1076 in milk displayed two exponential growth phases (1.69 ± 0.08 and 1.13 ± 0.05/h, respectively; Figure 1a) as described previously (13). The population level at which the shift in the exponential growth phases occurred was calculated at the intersection of the two regression lines and was equal to 1.20 (± 0.20) × 10^8 cfu/ml. A maximum population of 2.10 (± 0.20) × 10^9 cfu/ml was reached after ca. 7 h of growth. Growth of *Leuc. mesenteroides* ssp. *mesenteroides* UM10 as pure cultures in milk began immediately and lasted about 7 h. Growth rate was 0.63 ± 0.07/h, and the maximum population was 2.30 (± 0.20) × 10^8 cfu/ml (Figure 1a).

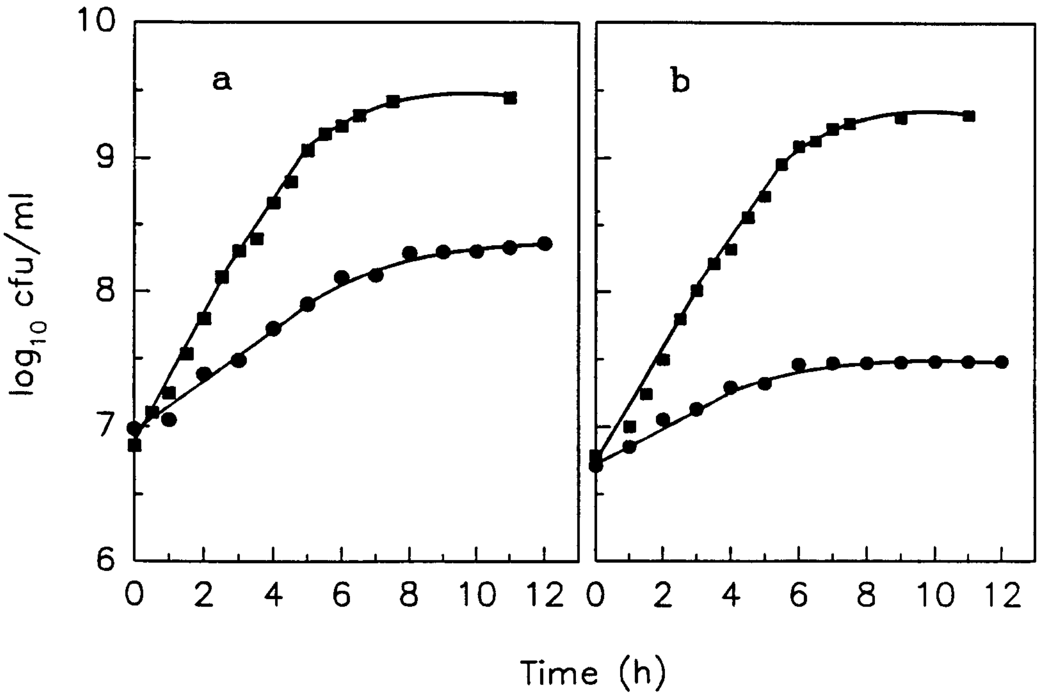

Figure 1. Growth of *Leuconostoc mesenteroides* ssp. *mesenteroides* UM10 (●) and *Lactococcus lactis* ssp. *lactis* CNRZ 1076 (■) in pure (a) or mixed-strain cultures (b).

The growth patterns of the mixed-strain cultures of *Lc. lactis* CNRZ 1076 and *Leuc. mesenteroides* UM10 are illustrated in Figure 1b. The initial population was 1.10 (± 0.10) × 10^7 cfu/ml with 55% *Lc. lactis* cells. Growth of the Prt^+ strain CNRZ 1076 was unaffected in mixed-strain cultures. In contrast, *Leuc. mesenteroides* UM10 displayed a shorter exponential growth phase and a lower growth rate than in the pure culture (0.40 ± 0.07 vs. 0.63 ± 0.07/h). Moreover, the maximum population was 10-fold lower [2.51 (± 0.20) × 10^7 cfu/ml].

Little acid was produced by the pure culture of *Leuc. mesenteroides* UM10 in RSM; the pH decreased 0.35 unit in 12 h (Figure 2). The acidification rate of mixed-strain cultures was similar to that of the pure culture of *Lc. lactis* Prt^+ strain (–0.51 ± 0.02 and –0.49 ± 0.03 pH unit/h, respectively) because the acidification was primarily due to the action of *Lc. lactis* CNRZ 1076. However, the RSM containing the mixed-strain culture coagulated 30 ± 5 min earlier than did the pure culture of *Lc. lactis* CNRZ 1076.

Effect of Lactate and pH on Growth Parameters of the Mixed-Strain Culture

Growth of *Leuc. mesenteroides* UM10 in RSM at pH 6.8 was not affected by the presence of 2.2 g/L of L-lactate (equivalent to lactic acid produced until pH 5.5).

When pH was controlled at specific values between 7.0 and 5.5, growth of *Lc. lactis* CNRZ 1076 and *Leuc. mesenteroides* UM10 was similar to that at uncontrolled pH. When pH was maintained at 5.1, growth of both strains was lower than when pH was uncontrolled. *Lactococcus lactis* grew with two exponential growth phases (1.36 ± 0.05 and 0.70 ± 0.05/h, respectively) up to a maximum population of 7.90 (±0.20) × 10^8 cfu/ml. *Leuconostoc mesenteroides* reached a maximum population of 0.83 (± 0.20) × 10^8 cfu/ml with a growth rate of 0.58 ± 0.02/h. When pH was maintained at 8.4, *Lc. lactis* displayed only one exponential phase of growth (0.56 ± 0.02/h), and the maximum population was lower than at uncontrolled pH [1.20 (± 0.20) × 10^8 vs. 2.10 (± 0.20) × 10^9 cfu/ml]. *Leuconostoc mesenteroides* UM10 did not grow at pH 8.4.

When the mixed-strain culture was grown in RSM, the maximum population and the growth rate of *Leuc. mesenteroides* were lower than in pure culture, regardless of whether or not pH was controlled, showing that acidification was not involved in the inhibition of *Leuc. mesenteroides* growth (data not shown).

Effect of the Percentage of *Lc. lactis* Cells in the Inoculum

The growth rate and the maximum population of Prt^+ *Lc. lactis* CNRZ 1076 were similar in mixed-

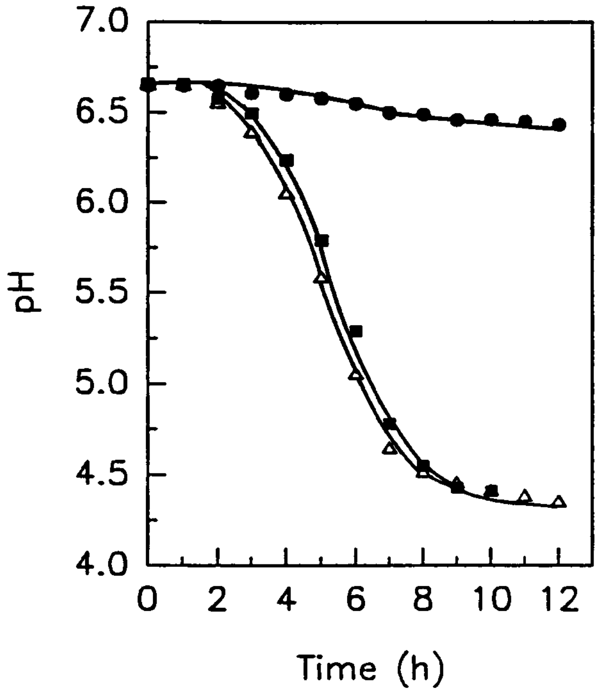

Figure 2. pH changes in pure culture of *Leuconostoc mesenteroides* UM10 (●) or *Lactococcus lactis* CNRZ 1076 (■) and in mixed-strain cultures (△).

strain and pure cultures, regardless of the percentage of *Lc. lactis* cells in the inoculum (data not shown). However, V_{mar} increased significantly when the percentage of *Lc. lactis* CNRZ 1076 cells in the inoculum increased from 2 to 50% (from –0.39 ± 0.02 to –0.49 ± 0.03 pH unit/h, respectively), suggesting a possible uncoupling of growth and acid production of *Lc. lactis* in mixed-strain cultures (Table 1). When *Lc. lactis* CNRZ 1076 cells constituted 2% of the inoculum, the growth rate of *Leuc. mesenteroides* UM10 in mixed-strain cultures was not significantly different from that in pure culture, but the maximum population decreased by 2.3-fold. When the percentage of *Lc. lactis* CNRZ 1076 cells increased from 2 to 12%, both the growth rate and the maximum population markedly decreased. A higher percentage of *Lc. lactis* CNRZ 1076 cells in the inoculum did not further modify either parameter.

Effect of Nutrient Addition

The addition of Mg^{2+} and Mn^{2+}, amino acids, or both to RSM stimulated growth of *Leuc. mesenteroides* in pure culture as previously reported (1) and in mixed-strain cultures (Table 2). The Mg^{2+} and Mn^{2+} were more efficient than the amino acids in stimulating the growth rate and increasing the maximum population. The V_{mar} remained unchanged in the presence of Mg^{2+} and Mn^{2+} but was enhanced upon addition of amino acids. Addition of Mg^{2+}, Mn^{2+}, and amino acids displayed an additive effect only for the maximum population, which was stimulated in pure and in mixed-strain cultures. Upon addition of Mg^{2+} and Mn^{2+}, or amino acids, or Mg^{2+}, Mn^{2+}, and amino acids to RSM, growth rates in mixed-strain cultures were similar to those in pure culture. However, the maximum population in mixed-strain cultures remained lower than in pure culture, showing that inhibition of *Leuc. mesenteroides* UM10 in mixed-strain cultures was only partially suppressed by RSM supplementation. Stimulation of growth parameters was also obtained upon the addition of a pancreatic digest of casein (5 g/L of Bacto-tryptone; Difco) to RSM (data not shown).

In pure culture, growth of *Lc. lactis* CNRZ 1076 was unchanged when Mg^{2+} and Mn^{2+} were added to

TABLE 1. Effect of the inoculum composition on growth parameters of *Leuconostoc mesenteroides* ssp. *mesenteroides* UM10 grown in reconstituted skim milk as mixed-strain cultures with *Lactococcus lactis* CNRZ 1076.[1]

Lc. lactis CNRZ 1076 cells	*Leuc. mesenteroides* growth parameters[1]					
	Growth rate		Maximum population		V_{mar}[2]	
(%)	(/h)		(10^8 cfu/ml)		(pH unit/h)	
	$\overline{X}$	CL	$\overline{X}$	CL	$\overline{X}$	CL
0	0.63	0.07	2.30	0.20	–0.03	0.02
2	0.73	0.07	0.93	0.02	–0.39	0.02
12	0.43	0.07	0.26	0.20	–0.45	0.02
50	0.40	0.07	0.23	0.15	–0.49	0.03
55	0.40	0.07	0.25	0.20	–0.51	0.02

[1]Means of three independent experiments with 95% confidence limits.

[2]Mean acidification rate.

TABLE 2. Effect of the addition of Mn^{2+}, Mg^{2+}, amino acids, or all to reconstituted skim milk (RSM) on the growth parameters of *Leuconostoc mesenteroides* ssp. *mesenteroides* UM10 in pure or mixed-strain cultures with *Lactococcus lactis* CNRZ 1076.[1,2]

RSM Supplement	Growth rate, Pure (/h) $\bar{X}$	CL	Growth rate, Mixed (/h) $\bar{X}$	CL	Maximum population, Pure (10^8 cfu/ml) $\bar{X}$	CL	Maximum population, Mixed (10^8 cfu/ml) $\bar{X}$	CL	V_{mar}[3], Mixed (pH unit/h) $\bar{X}$	CL
Unsupplemented	0.63	0.07	0.40	0.07	2.30	0.20	0.25	0.02	–0.49	0.03
Mn^{2+} and Mg^{2+}	1.00	0.07	0.83	0.10	4.27	0.17	2.88	0.15	–0.46	0.02
Amino acids	0.70	0.07	0.66	0.10	3.55	0.20	1.48	0.16	–0.59	0.03
Mn^{2+}, Mg^{2+}, and amino acids	0.93	0.07	0.86	0.13	5.50	0.10	3.72	0.15	–0.57	0.03

[1]Means of three independent experiments with 95% confidence limits.

[2]Mn^{2+} as $MnSO_4·H_2O$, 0.75 mg/L; Mg^{2+} as $MgSO_4·7H_2O$, 500 mg/L; amino acids as described by Bellengier et al. (1).

[3]Mean acidification rate.

RSM (data not shown). In the presence of amino acids, *Lc. lactis* CNRZ 1076 displayed only one exponential growth phase, and growth rate was 1.53 ± 0.08/h. In the presence of Mg^{2+}, Mn^{2+}, and amino acids in RSM, growth of *Lc. lactis* CNRZ 1076 was similar to that in the RSM that was supplemented with amino acids. The maximum population and V_{mar} were higher in RSM supplemented with amino acids and Mg^{2+} and Mn^{2+} than in RSM [3.43 ± (0.16) × 10^9 vs. 2.10 (±0.20) × 10^9 cfu/ml, –0.59 ± 0.03 vs. –0.49 ± 0.02 pH unit/h, respectively]. In mixed-strain cultures, growth and acidification parameters of *Lc. lactis* CNRZ 1076 were not significantly different from those of pure cultures.

Mixed-Strain Cultures of *Leuc. mesenteroides* and *Lc. lactis* Strains

Eight strains of *Leuc. mesenteroides* ssp. *mesenteroides* or ssp. *dextranicum* and 3 strains of *Lc. lactis* ssp. *lactis* or ssp. *cremoris* were grown as mixed-strain cultures in RSM that was unsupplemented or supplemented with Mn^{2+}, Mg^{2+}, and amino acids as described. We first determined that no interaction in mixed-strain cultures between bacteria of the two species could have been attributed to the production of an inhibitory substance. The ratios of the bacterial population after 3 or 12 h in mixed-strain cultures to that in pure cultures were used to determine the effect of growing *Leuc. mesenteroides* strains and *Lc. lactis* strains as mixed-strain cultures on the growth rate and the maximum population of *Leuc. mesenteroides*, respectively (Figure 3).

As shown in Figure 3a, most ratios were <1 for mixed-strain cultures in RSM, indicating that the growth rate in mixed-strain cultures of *Leuc. mesenteroides* was lower than that in pure cultures. *Leuconostoc mesenteroides* UM10 was the most susceptible strain, regardless of the *Lc. lactis* strains with which it was associated. In contrast, *Leuconostoc mesenteroides* UM12 seemed indifferent to the association. It is worth noting that the effect of the association on growth rate of *Leuc. mesenteroides* depended more on *Leuc. mesenteroides* strains than on the *Lc. lactis* strains with which they were grown. The maximum population of *Leuc. mesenteroides* strains, including strain UM12, was even more negatively affected by the association than was the growth rate because few ratios were >0.5 (Figure 3b). Because the association did not affect the growth rate of *Lc. lactis* or V_{mar}, the growth of *Leuc. mesenteroides* strains was probably inhibited completely by depletion of growth nutrients in RSM. As in the detailed study, the addition of Mn^{2+}, Mg^{2+}, and amino acids to RSM partially suppressed the inhibitory effect of growing *Leuc. mesenteroides* as a mixed-strain culture with *Lc. lactis* both in the exponential (Figure 3c) and the stationary phases (Figure 3d).

DISCUSSION

When *Leuc. mesenteroides* UM10 was grown associatively with *Lc. lactis* CNRZ 1076 in RSM, the growth rate and the maximum population of the former were always lower than in pure culture. Growth of *Lc. lactis* was not modified, as has been previously observed (3) with other *Lactococcus* and *Leuconostoc* strains. The lower growth rate of *Leuc. mesenteroides* UM10 undoubtedly reflects a phenomenon of interaction in which only *Leuconostoc* is affected negatively. Neither inhibitory substances nor low pH was involved in the reduction of *Leuc. mesenteroides* growth. Because *Leuc. mesenteroides* and *Lc.*

lactis were fastidious organisms that require the addition of amino acids to grow to high concentrations in milk (1), possible nutrient competition was considered. Competition was first evidenced by altering the inoculum composition of the mixed-strain culture (i.e., the percentage of *Lc. lactis* cells) as had been done for the Prt^+ *Lc. lactis* strain and its nonproteolytic variant (14) or *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (19). We reasoned, and observations confirmed, that the higher the percentage of *Lc. lactis* CNRZ 1076 cells was, the stronger the inhibition of *Leuc. mesenteroides* UM10 would be. Addition of amino acids, peptides, or Mg^{2+} and Mn^{2+}, alone or in combination, to RSM partially suppressed

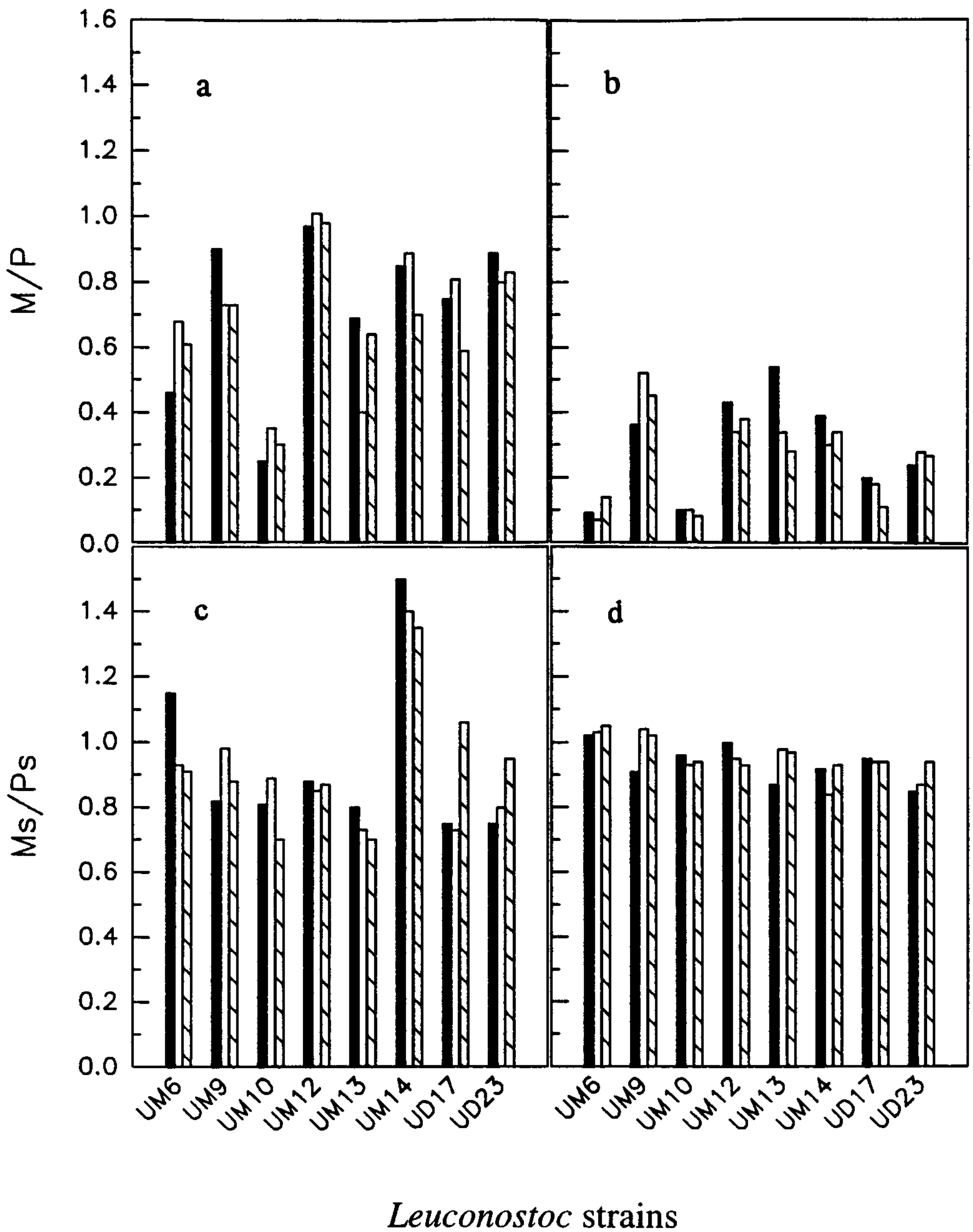

Figure 3. Mixed-strain cultures of *Leuconostoc mesenteroides* and *Lactococcus lactis* CNRZ 1076 (black bar), CNRZ 1124 (white bar), or Wg2 (striped bar). The ratio of the population of a *Leuc. mesenteroides* strain in mixed-strain cultures with a *Lc. lactis* strain (M) to the population of the same *Leuc. mesenteroides* strain as a pure culture (P) after 3 h (a and c) or 12 h (b and d) of growth was used to determine the effect of the mixed-strain culture on the growth rate (3 h) and the maximum population (12 h) of the *Leuc. mesenteroides* strain. Ratios are represented as a function of *Leuc. mesenteroides* strains. Cultures were grown at 30°C in reconstituted skim milk (M/P) or in reconstituted skim milk supplemented with amino acids, $MnSO_4{\cdot}H_2O$ (0.75 mg/L), and $MgSO_4{\cdot}7H_2O$ (500 mg/L) (M_S/P_S).

the inhibition, suggesting that some competition for these nutrients occurred in mixed-strain cultures. Growth of *Lc. lactis* was modified by the addition of amino acids or peptides to RSM but not by that of Mn^{2+} and Mg^{2+}, which was unlike the response of *Leuc. mesenteroides*. Therefore, competition essentially concerned nitrogenous nutrients during mixed-strain cultures and not Mn^{2+} and Mg^{2+}, for which only *Leuconostoc* displayed a high requirement. Similar requirements for amino acids (1, 4) and differences in the affinities of transport systems for amino acids (20, 27) and peptides (Foucaud et al., 1996, unpublished results) between *Leuc. mesenteroides* and *Lc. lactis* may contribute to inhibition. Moreover, *Lc. lactis* grow much faster than *Leuc. mesenteroides*, which may be inhibited by the competitive and rapid utilization of nitrogenous nutrients [this study; (3, 24)] by *Lc. lactis* to the detriment of *Leuc. mesenteroides*.

Our results showed that the competition for nitrogenous nutrients was a general feature of mixed-strain cultures of *Leuc. mesenteroides* and *Lc. lactis* in RSM. *Leuconostoc* growth was either unchanged or was inhibited during the exponential phase and was systematically inhibited in the stationary phase. Inhibition was usually suppressed upon addition of amino acids, peptides, Mn^{2+} and Mg^{2+}, regardless of the strains of *Lc. lactis* and *Leuc. mesenteroides* used. Proteolytic activities of *Lc. lactis* strains, the requirements for amino acids, and the utilization of amino acids and peptides (e.g., nature, rate, and affinity of the transport systems) varied with the strains used and may have contributed to differences between strains [(1, 12); Foucaud et al., 1996, unpublished results].

Differences in the growth pattern of the various strains constituting a mixed-strain starter can lead to unavoidable and important changes in its composition in a relatively short cultivation period. Thus, the use of *Leuconostoc* mixed-strain concentrates would avoid the imbalance of population and the rapid dominance of *Lc. lactis* over *Leuc. mesenteroides* as reported here and elsewhere (9, 11, 20, 21).

ACKNOWLEDGMENTS

Part of this work was financially supported by the Ministry of Research and Education (Contract Number R91-61). P. Bellengier is a beneficiary of a grant from the Institut National de la Recherche Agronomique.

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