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Original article

Production of carbon dioxide by *Lactococcus lactis* strains with attenuated lactate dehydrogenase activity, in pure cultures and in mixed cultures with an acidifying strain

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Abstract – Lactic acid bacteria that produce CO_2 are used in the dairy industry to favor the formation of openings in some types of cheeses. This work was undertaken to characterize the gas producing activity of *Lactococcus lactis* strains that over-produce CO_2 as a result of their attenuated lactate dehydrogenase activity. The method used involved following the concentration of dissolved CO_2 and the quantity of CO_2 released into the gas phase in standard conditions. Five mutants with attenuated lactate dehydrogenase activity were tested in pure culture in milk. Total CO_2 production after 24 h of culture varied between 47 and 88 mmol·L^{-1}, while that of the parental strain was only 17 mmol·L^{-1}. CO_2 was released into the gas phase only after the concentration of dissolved CO_2 reached the saturation point in the culture medium, i.e. about 29 mmol·L^{-1}. Its release was then linear vs. pH. Co-culturing these mutants with an acidifying strain of *L. lactis* increased the rate of acidification of cultures and reduced CO_2 production and the pH below which CO_2 was released into the gas phase. This effect increased with the size of the inoculum of the acidifying strain. These results show that very different changes in pH and CO_2 production can be obtained by using strains *L. lactis* strains with attenuated lactate dehydrogenase activity and by the conditions of combining these strains with an acidifying *Lactococcus*. For a given cheese technology, this could lead to a more precise determination of the optimal characteristics of acidification and CO_2 production for the formation of openings.

***Lactococcus lactis* / CO_2 / lactate dehydrogenase / cheese**

Résumé – **Production de dioxyde de carbone par des souches de *Lactococcus lactis* ayant une activité lactate déshydrogénase atténuée, en cultures pures et en cultures mixtes avec une souche acidifiante.** Des bactéries lactiques productrices de CO_2 sont utilisées dans l'industrie laitière pour favoriser la formation d'ouvertures dans certains types de fromages. L'objectif de cette étude était de

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caractériser l'activité gazogène de souches de *Lactococcus lactis* capables de surproduire du CO_2 du fait de l'atténuation de leur activité lactate déshydrogénase. Pour cela, une méthode permettant de suivre, dans des conditions standardisées, la concentration en CO_2 dissous et la quantité de CO_2 libéré en phase gazeuse, a été utilisée. Cinq mutants ayant une activité lactate déshydrogénase atténuée ont été testés en culture pure dans du lait. Leur production totale de CO_2 après 24 h de culture était variable, et comprise entre 47 et 88 mmol·L^{-1}, alors que celle de la souche parentale n'était que de 17 mmol·L^{-1}. Le CO_2 n'était libéré dans la phase gazeuse qu'à partir du moment où la concentration en CO_2 dissous atteignait la saturation du milieu de culture, soit approximativement 29 mmol·L^{-1}. Sa libération évoluait ensuite de façon linéaire par rapport au pH. L'association de ces mutants avec une souche de *L. lactis* acidifiante augmentait la vitesse d'acidification des cultures et diminuait la production de CO_2 ainsi que le pH à partir duquel le CO_2 est libéré dans la phase gazeuse. Cet effet était d'autant plus important que le niveau d'ensemencement de la souche acidifiante était élevé. Ces résultats montrent qu'en agissant sur le choix des souches de *L. lactis* ayant une activité lactate déshydrogénase atténuée, ainsi que sur les conditions d'association de ces souches avec un lactocoque acidifiant, il est possible d'obtenir des évolutions de pH et de production de CO_2 très différentes. Ceci pourra servir à déterminer plus précisément, pour une technologie fromagère donnée, quelles sont les caractéristiques d'acidification et de production de CO_2 optimales pour la formation des ouvertures.

***Lactococcus lactis* / CO_2 / lactate déshydrogénase / fromage**

1. INTRODUCTION

During the manufacture of cheeses and fermented milks, the production of CO_2 by microorganisms present may be a default or a desired characteristic, e.g. to favor the formation of openings of certain types of cheeses. Mesophilic lactic acid bacteria such as *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* and *Leuconostoc* sp. participate in the formation of openings of blue-veined cheeses and cheeses such as Gouda and Cheddar [4]. They can produce CO_2 from citrate in the milk, but only the genus *Leuconostoc* can also produce the gas from lactose. McKay and Baldwin [5] isolated a mutant of *L. lactis,* however, that produced high quantities of CO_2 from glucose. This mutant lacked lactate dehydrogenase (LDH), resulting in the partial detour of the carbon flux towards acetoin formation with the simultaneous synthesis of CO_2 by α-acetolactate synthase and α-acetolactate decarboxylase.

In previous work, we selected a collection of *L.l.l. diacetylactis* RD311 mutants with attenuated LDH activities by nitrosoguanidine and UV mutagenesis and spontaneous mutation [2]. Production of CO_2 and lactate by these mutants was variable and only a minority was stable after repeated transfers [1]. The use of stable mutants could lead to the better control of gas production by mesophilic lactic starters used to produce certain cheeses and fermented milks. For example, in the case of Roquefort, which is a blue-veined cheese having large irregularly shaped openings, many manufacturing runs produce cheeses with insufficient opening, or with oval shaped openings. Among the factors affecting the formation of openings, there is one set related to CO_2 production by microorganisms and another that is not, for example, the mechanical work of curd grains [4]. For the first set, it should be pointed out that the formation of openings is influenced not only by the total quantity of CO_2 produced, but also by the production profile of this gas as a function of time and pH. The pH affects the solubility of CO_2 and the deformation and fracture properties of curd grains. Experimentally it is very difficult to study CO_2 production profiles directly on cheeses. Methods are required to determine the quantities of dissolved CO_2 and of free gaseous CO_2 throughout the entire manufacturing process. Furthermore, the pH and CO_2 concentration in cheeses are often

heterogeneous [6]. For these reasons, we have developed a simplified experimental device in which strains are cultured in sealed sachets that are constantly mixed. This device enables the quantities of dissolved CO_2 and CO_2 released into the gas phase to be followed during cultures in milk at constant pressure. It was used in this work to compare the gas producing properties of 5 stable *L.l.l. diacetylactis* mutants with attenuated LDH activity and to determine the effects of their combination with an acidifying strain of *L.l. lactis*.

2. MATERIALS AND METHODS

2.1. Bacterial strains used

Lactococcus lactis subsp. *lactis* biovar *diacetylactis* RD311 and *L.l. lactis* RD525 were obtained from Rhodia Food (Dangé-Saint-Romain, France). Strains A110, A141, A216, A402 and A550 are mutants with attenuated LDH activity. They were isolated from the parental strain RD311 [2]. Mutant A216 was obtained after UV treatment, mutants A141, A110 and A402 after nitrosoguanidine treatment and A550 was a spontaneous mutant. LDH activity of the parental strain was 12.2 $U \cdot mg^{-1}$ protein and was only 1.09, 0.54, 0.78, 0.0053 and 1.18 $U \cdot mg^{-1}$ protein for mutants A216, A141, A110, A402 and A550, respectively.

2.2. Determination of biomass

The absorbance of milk precultures was carried out after clarification of the medium [3]. The sample was first diluted 1/10 with a solution of EDTA at 2 $g \cdot L^{-1}$ whose pH was adjusted to 12.5 with 10 $mol \cdot L^{-1}$ sodium hydroxide, and absorbance was then read at 405 nm. The difference between the absorbance of the sample and that of non-inoculated milk treated in the same conditions was calculated. Bacterial populations in cultures were determined by enumerating colonies after dilution in tryptone-salt solution and inoculation with a spiral inoculator (Spiral System, Interscience, St-Nom-la-Bretèche, France) on LDHA-20 agar [2]. Bacterial suspensions were diluted 1/10 and mixed vigorously on a vortex mixer for 90 s in order to reduce the length of lactococci chains. Each dilution was inoculated in triplicate in Petri dishes, that were counted after incubating for 48 h at 30 °C. Mutants from strain RD311 formed brown colonies on LDHA-20 medium, while the parental strain and strain RD525 formed clear colonies. A correlation between absorbance of milk precultures and the corresponding bacterial populations was determined for all strains in order to standardize the initial population of cultures by measuring the absorbance of precultures. In the case of strain RD525, one absorbance unit was equivalent to 1.2×10^9 $cfu \cdot mL^{-1}$ and to 5.9×10^8 $cfu \cdot mL^{-1}$ for the other strains.

2.3. Culture conditions

The strains were inoculated in 5 mL of reconstituted skimmed ewes' milk (130 $g \cdot L^{-1}$; Société des Caves, Roquefort-sur-Soulzon, France) that had been autoclaved for 15 min at 110 °C. Cultures were incubated for 24 h at 25 °C and were then used to inoculate at 1% 60 mL bottles containing 20 mL of reconstituted skimmed ewes' milk, that were closed with cellulose caps. After incubating for 24 h at 25 °C, the absorbance of cultures was determined as described above. These cultures were then used to inoculate a 1 L flask containing 600 mL of reconstituted skimmed ewes' milk (130 $g \cdot L^{-1}$) that had been autoclaved for 15 min at 110 °C. Strain RD311 and its mutants were inoculated at a concentration of 3.0×10^7 $cfu \cdot mL^{-1}$. The inoculation concentrations of acidifying strain RD525 are indicated in the figure legends. After mixing, 20 mL of culture were transferred to 25 gas-tight polyethylene sachets

(Bernhardt, Boulogne-sur-mer, France). The sachets were 58 mm wide and 220 mm long and were delivered sealed on three sides, the fourth being sealed with a heat-sealer (Impulse Sealer Tish-100; TEW Electric Heating Equipment Co., Taiwan) after adding the culture to the sachets and being careful to chase off bubbles. The sachets were then placed on a support connected to a motor that imparted a continuous rotation at 10 rpm around an axis perpendicular to the surface of the sachets. Cultures were incubated at 25 °C and samples were taken hourly for 24 h, using one sachet per sample.

2.4. Measure of the quantity of CO_2 released into the gas phase

Cultures were grown in sealed sachets and so the quantity of CO_2 released in the gas phase was calculated by measuring sachet volume. The sachet was weighed and then placed in a 500 mL cylinder with a 5 cm inner diameter and was maintained with a blocking system. Water was added up to a reference mark near the top of the cylinder so that the sachet was entirely submersed. The final water level was precisely adjusted after chasing off bubbles on the surface of the cylinder and the sachet. The device was then weighed. All these operations were carried out at 25 °C and volumes of the liquid and gas phases were determined using the following equations:

$$V_{liq} = (a - b) / \rho_{milk}$$

$$V_{gas} = c / \rho_{water} + b \cdot ((\rho_{sachet} / \rho_{milk}) - 1) / \rho_{sachet} + a \cdot (1 / \rho_{milk} - 1 / \rho_{water}) - d / \rho_{water}$$

V_{liq}: volume of the liquid phase (dm^3)
V_{gas}: volume of the gas phase (dm^3)
a: weight of the sachet and the culture (g)
b: weight of an empty sachet (g)
c: weight of the cylinder without the sachet and whose water level was adjusted to the reference mark (g)
d: weight of the cylinder with the sachet and whose water level was adjusted to the reference mark (g)
ρ_{water}: density of water at 25 °C (1.010×10^3 g·dm^{-3})
ρ_{milk}: density of milk at 25 °C (1.051×10^3 g·dm^{-3})
ρ_{sachet}: density of the sachet at 25 °C (1.039×10^3 g·dm^{-3}).

For the calculation of the quantity of CO_2 present in the gas phase, it was taken that the gas phase contained only water and CO_2 and that the compounds behaved as ideal gases. The following equation could thus be applied:

$$n_{CO2} = (P - P_{H2O}) \cdot V_{gas} / (R \cdot T)$$

n_{CO2}: quantity of CO_2 in the gas phase (mol)

V_{gas}: volume of the gas phase (dm^3)
P: atmospheric pressure (1 atm)
P_{H2O}: water vapor pressure at 25 °C (0.0313 atm)
R: ideal gas constant (0.082 atm·dm^3·mol^{-1}·K^{-1})
T: temperature (298 K).

2.5. Assay of dissolved CO_2

The quantity of CO_2 present in the liquid phase of cultures in sealed sachets was determined with an enzymatic method. A procedure was developed to prevent part of the CO_2 from escaping from the liquid when sampling. About 2.5 mL of Tris-HCl buffer (500 mmol·L^{-1}, pH 9.4) were taken up with a syringe and needle. The culture sachet was pierced and about 1 mL of medium was removed. The needle was then closed off with a flat clip. After mixing the content of the syringe, the sample was stored at –20 °C. Adding the Tris-HCl buffer increased the pH of the milk, thereby converting all CO_2 present to HCO_3^- and CO_3^{2-}, two non-volatile ions. It was verified that

samples thus prepared remained stable for at least one week at –20 °C in terms of the CO_2 assay. The dilution factor of each sample was calculated by weighing the empty syringe, the syringe containing about 2.5 mL of buffer and the syringe containing the buffer and the milk.

Samples were thawed and centrifuged for 30 min at 21 000 *g* and +4 °C. The supernatant was assayed with an enzymatic assay kit (Sigma 132-A; St-Quentin-Fallavier, France). The assay was linear between 0 and 10 mmol·L^{-1}. If necessary, samples were diluted with ultra-pure water. The concentration of CO_2 in the culture medium was determined by factoring in the dilution in Tris-HCl buffer when sampling, and any dilution in ultra-pure water done before the assay. The water and Tris-HCl buffer were degassed before use by mixing under vacuum for 30 min. In spite of this treatment, the Tris-HCl buffer still contained residual quantities of CO_2, of the order of 0.5 to 1 mmol·L^{-1}. This concentration was included in the calculation of CO_2 concentrations of samples.

2.6. High performance liquid chromatography assays

Acetoin, 2,3 butanediol and citrate were assayed by high performance liquid chromatography as described elsewhere [1].

2.7. Statistical analysis

The difference between the quantities of CO_2 calculated from high performance liquid chromatography assay and those obtained by assaying dissolved and free CO_2 were determined by calculating a relative error as follows:

$$\text{Relative error (\%)} = \sqrt{\sum((CO_{2calc} - CO_{2meas}) / CO_{2meas})^2 / n} \cdot 100$$

where CO_{2calc} is the total production of CO_2 calculated from high performance liquid chromatography assays, CO_{2meas} the sum of the quantities of dissolved and free CO_2 and n the number of experimental points.

3. RESULTS

3.1. Validation of the experimental device

The experimental device used to study gas production by pure and mixed cultures is described in detail in Section 2. Cultures were grown in sealed sachets that guaranteed maintenance of constant pressure, equal to 1 atmosphere. It may be considered that continuous agitation rendered the composition of gas and liquid phases homogeneous and that the two phases were in equilibrium. At the start of incubation the medium was not in contact with a gas phase. Once bubbles formed, it was considered that the composition of the gas phase was constant and composed primarily of CO_2 and water vapor.

The relative errors of enzymatic assays of dissolved CO_2 in the same sample were included between 5 and 10%. For the determinations of CO_2 released into the gas phase, the standard deviations of the results were constant regardless of the quantities of gas produced and were close to 1 mmol·L^{-1} (with respect to the volume of the liquid medium), corresponding to a volume of 0.5 mL. Total quantities of CO_2 produced could be determined indirectly from high performance liquid chromatography assays of acetoin, 2,3 butanediol and citrate [1]. These quantities were compared to those obtained by adding the results of assays of dissolved and free CO_2. Twelve pure cultures of mutants with attenuated LDH activity were prepared with variable incubation times (Fig. 1). The relative error of the data in Figure 1, calculated as described in Section 2, was somewhat low (10%). The results of the assay of dissolved and free CO_2 in the gas phase are thus in agreement with the expected figures.

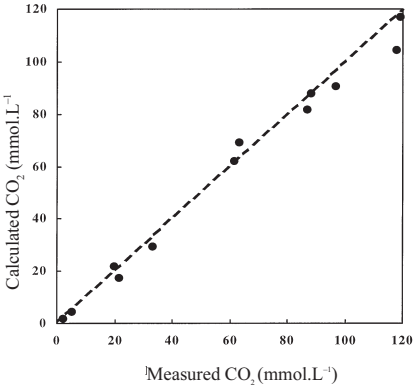

Figure 1. Quantity of CO_2 calculated from high performance liquid chromatography assays (calculated CO_2) vs. the quantity of dissolved CO_2, plus the quantity of CO_2 released (measured CO_2). Experiments were performed with cultures of mutants with attenuated LDH activity incubated for variable times at 25 °C. Equivalence line (-----).

3.2. Pure cultures

Parental strain RD311 and five stable mutants obtained from it were grown in ewes' milk for 24 h at 25 °C. The growth of mutants A550, A141 and A216 was similar to that of the parental strain (Fig. 2). The growth of mutant A402 was slower than that of the parental strain, but the final population was the same, whereas the final population of mutant A110 was lower. The pH of the culture medium decreased less with the mutants than with the parental strain (Fig. 2).

The concentration of dissolved CO_2 reached a plateau close to 29 ± 3 mmol·L^{-1} in the five mutant cultures, while the parental strain produced only about 17 mmol·L^{-1} (Fig. 3). CO_2 appeared in the gas phase of mutant cultures only after 8 h, while the parental strain culture exhibited no CO_2 release into the gas phase. In contrast to the results for dissolved CO_2, the quantities

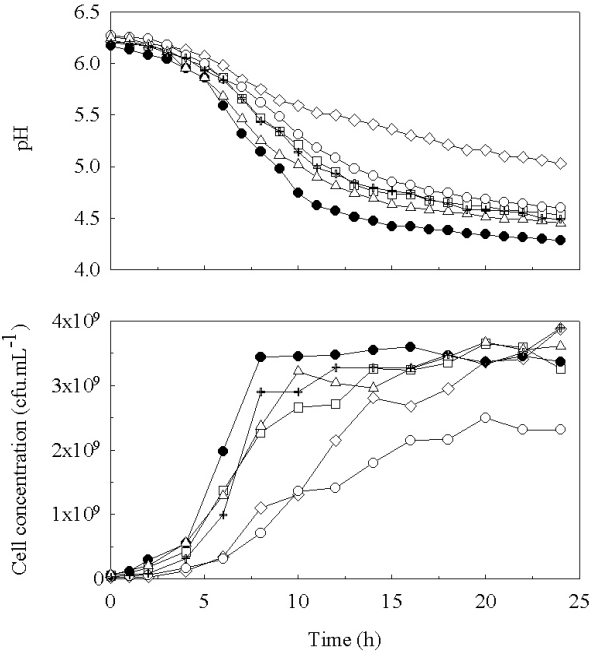

Figure 2. Changes of pH and cell concentration in pure cultures of the parental strain RD311 (●) and mutants A110 (○), A141 (△), A216 (□), A402 (◇) and A550 (+) in ewes' milk at 25 °C.

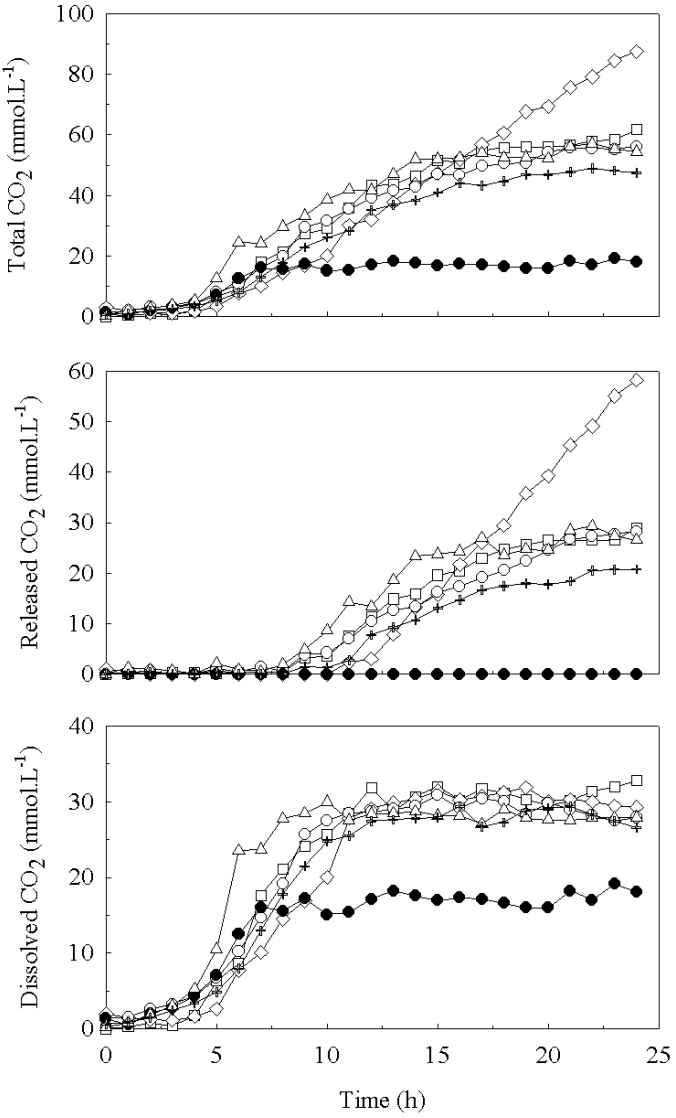

Figure 3. Production of CO_2 in pure cultures of the parental strain RD311 (●) and mutants A110 (○), A141 (△), A216 (□), A402 (◇) and A550 (+) in ewes' milk at 25 ºC.

of CO_2 released into the gas phase were highly variable. Mutant A550 produced 21 mmol·L^{-1}, while mutant A402 produced 58 mmol·L^{-1}. A significant release of CO_2 into the gas phase occurred only when the dissolved CO_2 concentration reached a value close to 29 mmol·L^{-1}.

In another experiment, two flasks containing milk acidified with lactic acid to pH values of 4.6 and 5.2 were saturated with CO_2 by mixing and providing a constant supply of the gas. In both cases, the concentration of dissolved CO_2 was 33 mmol·L^{-1} (S.D. = 1 mmol·L^{-1}). This saturation value

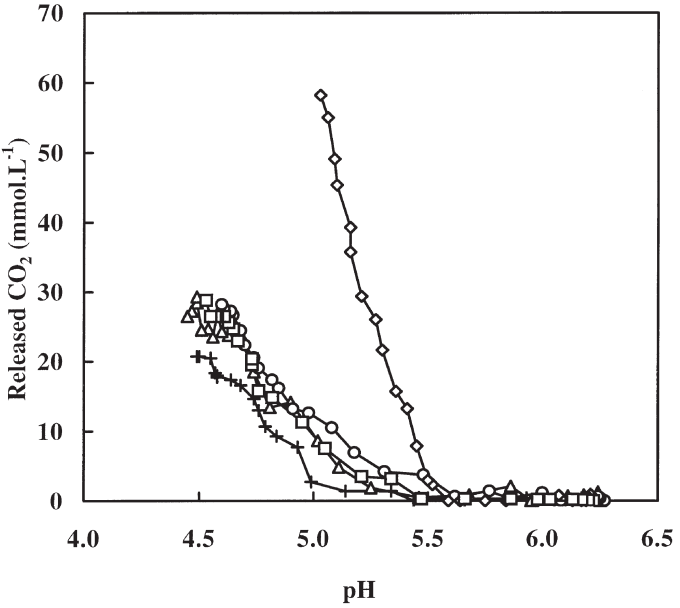

Figure 4. Quantity of CO_2 released vs. the pH in pure cultures of mutants A110 (○), A141 (△), A216 (□), A402 (◇) and A550 (+) in ewes' milk at 25 °C.

is slightly higher than the mean value of the concentration of dissolved CO_2 in the stationary phase of mutant cultures (29 mmol·L^{-1}). It is possible that CO_2 is somewhat more soluble in milk acidified with lactic acid than in the cultures. This difference can also be explained, at least partially, by the fact that the gas phase is saturated with water in mutant cultures, which was not the case in the experiments of saturating acidified milk in which the gas phase was uniquely CO_2.

Once the release of CO_2 was observed, there was a practically linear relationship between CO_2 released and the decrease in pH (Fig. 4). In cultures of mutants A402 and A550, CO_2 was released at a higher (close to 5.5) and lower pH value (close to 5.0), respectively, than in cultures of the other three mutants. The total quantities of CO_2 produced by the different strains was variable (Fig. 3). For a given strain, the variation of total CO_2 production was not proportional to the quantity of CO_2 released. For example, total CO_2 production by mutant A402 was 1.4 times higher than that of mutant A216, while the ratio was 2 when gaseous CO_2 is taken into account.

3.3. Mixed cultures

In mesophilic lactic starters that can produce CO_2, gas producing strains are systematically combined with acidifying lactococci. These starters are required to have a given acidifying activity that would be difficult to obtain with pure cultures of gas producing strains.

The effect of the inoculation ratio between the gas producing strain A141 and the acidifying strain RD525 was investigated. RD525 does not metabolize citrate and does not produce CO_2 when grown in ewes' milk. The experiments were conducted with a constant inoculum of the gas producing strain (3×10^7 cfu·mL^{-1}), and the quantity of the acidifying strain inoculated varied from 0.75×10^7 to 6×10^7 cfu·mL^{-1}. When the acidifying inoculum increased, the population of the gas producing strain decreased, especially visible after 8 h of culture

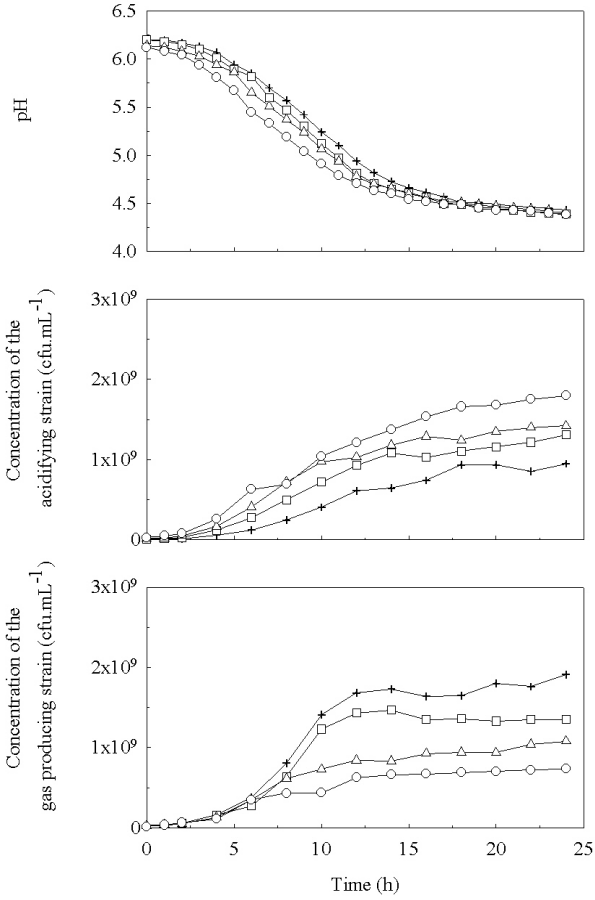

Figure 5. Changes in pH and cell concentration of gas producing strain A141 and acidifying strain RD525 in mixed cultures inoculated with 3×10^7 cfu·mL^{-1} of the gas producing strain and 0.75×10^7 (+), 1.5×10^7 (□), 3×10^7 (△) and 6×10^7 (○) cfu·mL^{-1} of the acidifying strain. Cultures in ewes' milk at 25 ºC.

(Fig. 5). The final population of the acidifying strain increased as its inoculation level increased, but by a much lower proportion. Increasing the acidifying strain inoculum also resulted in a more rapid acidification of the cultures (Fig. 5).

As in the case of pure cultures, the concentration of dissolved CO_2 reached a plateau close to 29 mmol·L^{-1} (Fig. 6) and the release of CO_2 into the gas phase started only when the quantity of dissolved CO_2 reached a value close to this plateau. Increasing the inoculation quantity of the acidifying strain reduced the quantity of CO_2 released into the gas phase. Thus, after 24 h of growth, gaseous CO_2 decreased from 17.5 to 5.7 mmol·L^{-1} when the acidifying strain inoculum was increased from 0.75×10^7 to 6×10^7 cfu·mL^{-1}. Increasing the quantity of the acidifying strain also decreased the pH after which CO_2 was released (Fig. 7). In the four mixed cultures examined, CO_2 production was lower (between 34.0 and 46.9 mmol·L^{-1}) than that observed in pure culture (54.4 mmol·L^{-1}).

Mixed cultures with four different inoculation levels of the acidifying strain were prepared with the four other gas producing mutants. Figure 8 shows the results after 24 h of culture for all gas producing mutants. For each, there was a practically linear relationship between the pH of the

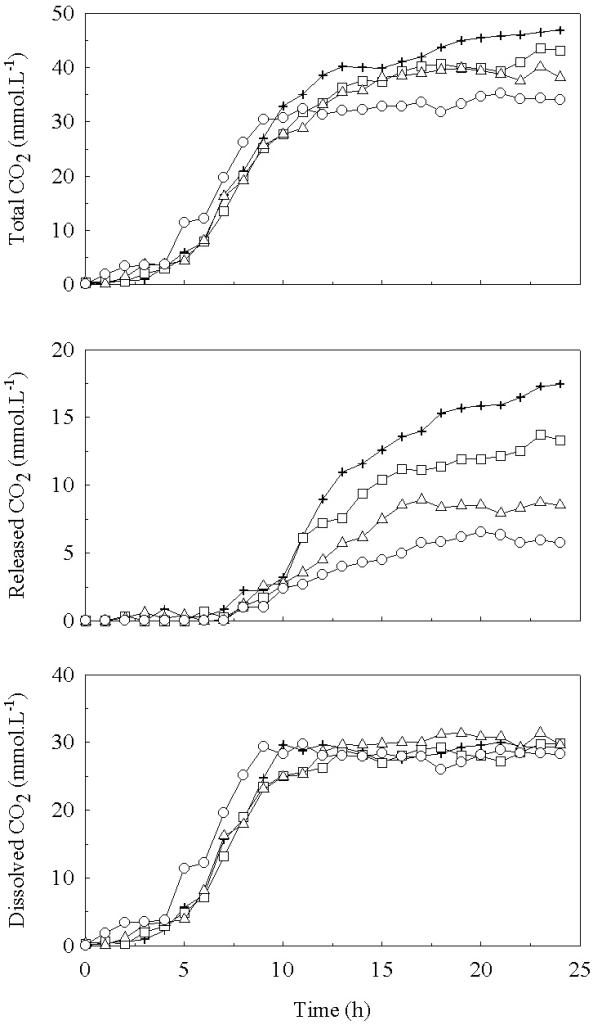

Figure 6. Production of CO_2 in mixed cultures inoculated with 3×10^7 cfu·mL^{-1} of the gas producing strain A141 and 0.75×10^7 (+), 1.5×10^7 (□), 3×10^7 (△) and 6×10^7 (○) cfu·mL^{-1} of the acidifying strain RD525. Cultures in ewes' milk at 25 ºC.

medium after 24 h of culture and the population of the gas producing strain. The same type of relationship was obtained between the quantity of CO_2 released and the population of the gas producing strain.

4. DISCUSSION

The aim of this work was to characterize the gas producing activity of *L. lactis* mutants with attenuated LDH activity. Industrial conditions of using gas producing lactic starters are incompatible with easily monitoring their production of CO_2. This is due primarily to the fact that this gas is partially released to the exterior of the cheese and that CO_2 concentration gradients form inside the cheese. The experimental device we used enabled the gas producing activity of lactic starters to be studied in standardized conditions. Pressure was constant at 1 atmosphere, the liquid and gas phases were homogeneous and once the gas phase

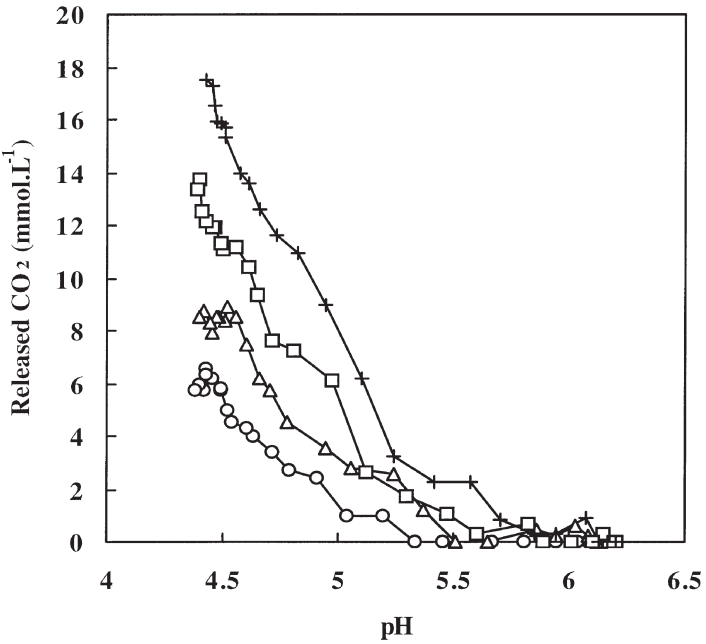

Figure 7. Quantity of CO_2 released vs. pH in mixed cultures of the gas producing strain A141 and the acidifying strain RD525 in ewes' milk at 25 ºC. Cultures were inoculated with 3.10^7 cfu·mL^{-1} of the gas producing strain and 0.75×10^7 (+), 1.5×10^7 (□), 3×10^7 (△) and 6×10^7 (○) cfu·mL^{-1} of the acidifying strain.

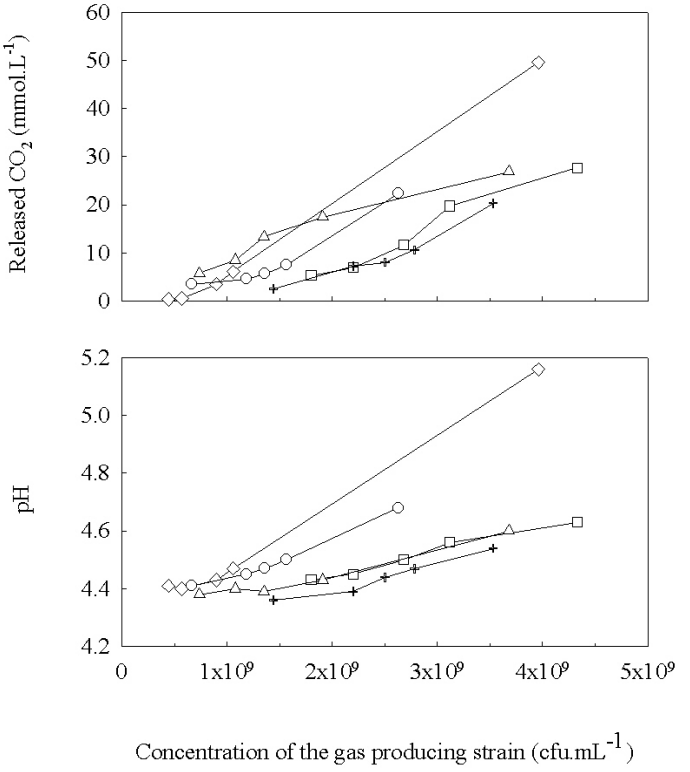

Figure 8. pH and quantity of CO_2 released after 24 h of culture vs. the population of the gas producing strain. Cultures in ewes' milk at 25 ºC, inoculated with 0, 0.75×10^7, 1.5×10^7, 3×10^7 or 6×10^7 cfu·mL^{-1} of the acidifying strain RD525 and with 3×10^7 cfu·mL^{-1} of the gas producing strain A110 (○), A141 (△), A216 (□), A402 (◇) and A550 (+).

formed it was composed uniquely of CO_2 and water vapor. This device can be used to compare the gas producing activity of lactic starters or to determine the effect of certain operating conditions such as temperature.

Five *L. lactis* mutants with attenuated LDH activity were examined in pure culture in ewes' milk. Acidifying activity was variable but consistently lower than that of the parental strain RD311. Total CO_2 production after 24 h of culture was between 47 and 88 mmol·L^{-1}, while that of the parental strain was only 17 mmol·L^{-1}. In the experimental conditions used, CO_2 was released into the gas phase starting from the time when the concentration of dissolved CO_2 reached a plateau close to 29 mmol·L^{-1}. When CO_2 was released into the gas phase, the gas present in the liquid phase was primarily non-ionized, since the pH of the cultures was much lower than the pKa of H_2CO_3, equal to 6.3. It can thus be considered that the release of CO_2 into the gas phase is due to the fact that CO_2 production by gas producing strains continued when the medium was saturated in H_2CO_3. Another possibility, albeit not verified in our experiments, is that gas producing strains produced CO_2 before the pH became acid. CO_2 would thus remain in the liquid phase, partially ionized, and its release into the gas phase would be triggered by the acidification of the medium. This would convert HCO_3^- and CO_3^{2-} ions into H_2CO_3. The fact that CO_2 was released into the gas phase only after its concentration in the liquid phase reached a value close to 29 mmol·L^{-1} explains why this release occurred late. This also implies that in relative value a slight increase in total CO_2 production can result in a considerable increase in the quantity of CO_2 released into the gas phase. In addition, once the release of CO_2 into the gas phase was observed, there was a practically linear relationship between gas released and pH. Nevertheless, the five mutants examined differed with respect to the pH after which CO_2 was released, as well as by the slope of the curve of CO_2 released vs. the decrease in pH.

The co-culture of mutants with attenuated LDH activity with an acidifying strain of *L. lactis* increased the rate of acidification of the cultures and reduced CO_2 production, as well as the pH after which CO_2 was released into the gas phase. This effect increased with the inoculation level of the gas producing strain. In the experimental conditions used, there was a linear relationship between the population of the gas producing strain after 24 h of culture and the quantity of CO_2 released.

In conclusion, these results show that it is possible to obtain highly variable changes in pH and CO_2 production depending on the choice of gas producing lactic acid bacteria strains, as well as the conditions of combining these strains with an acidifying *L. lactis* strain. An interesting path for continuing this work would be the selection of a given number of conditions having generated different results in terms of acidification and CO_2 production, and to carry out the corresponding cheese manufacturing tests. This would be followed by the determination of correlations between the characteristics of cheese opening and the experimental conditions used. For example, this would lead to understanding if obtaining the desired opening requires being in conditions where the production of CO_2 occurs early or late. This type of approach should lead to a better understanding of the relationships between the formation of openings and gas producing and acidifying activities of lactic starters.

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