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Carbon Dioxide Production by *Leuconostoc mesenteroides* Grown in Single and Mixed Culture with *Lactococcus lactis* in Skimmed Milk

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INTRODUCTION

Lactic acid bacteria are now used extensively as starter cultures in the dairy industry and therefore the optimization of growth conditions appears to be essential for successful industrial applications. Furthermore, studying the effects of some environmental parameters on growth kinetics should provide useful information concerning the physiology of the microorganisms [1-3]. Strains belonging to the species *Lactococcus lactis* and *Leuconostoc* sp. are the most important organism in the manufacture of these products at a moderate temperature [4]. Mixed cultures of these bacteria are commonly used as a starter in manufacture of cheese and fermented milk [5]. Large-scale industrial processes rely on the use of starter cultures that have been selected for their performance during milk fermentation and product formation.

Leuconostoc strains grow associatively with acid producing *Lactococcus* strains and are employed for their technological properties (mainly aroma and texture). The associative growth between these two groups of bacteria has been studied with respect to citrate metabolism and aroma formation and has been described as a synergistic functional relationship. Thus 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* [5, 6].

The importance of *Leuconostoc* sp. in dairy fermentation is related to their ability to produce carbon dioxide from lactose and citrate. Various methods have been used to measure CO_2 production by microorganisms [7-11]. Holmes *et al.* [12] demonstrated that cell extract of *Lactococcus lactis* and *Lactobacillus* enhanced gas production *of Leuconostoc mesenteroides* subsp. *cremoris*. During the fermentation of milk by *Leuconostoc* the ratio of CO_2 is critical as it affects not only the flavor and aroma of the fermented product but also on the texture of blue cheese.

The shift in metabolic pathways in response to environmental conditions is well documented in the literature in the case of homofermentative species [13], but little information is available about the behavior of heterofermentative species such as *Leuconostoc mesenteroides*. The quantitative aspects and kinetics of evolved CO_2 in mixed culture of *Leuconostoc-Lactococcus* under different culturing conditions have been fairly studied [14]. In point of view of the recent report demonstrating the importance of evolved CO_2 to the texture of dairy products, a study of this matter seemed appropriate.

The present study was undertaken to determine whether *Leuconostoc mesenteroides* plays a role in carbon dioxide production in pure and mixed culture with *Lactococcus lactis* in milk.

MATERIALS AND METHODS

Bacterial strains: *Leuconostoc mesenteroides* subsp. *dextranicum* (L4), *Leuconostoc mesenteroides* subsp. *mesenteroides* (19D) and *Lactococcus lactis* subsp. *lactis* (SLO_3), which is known for its proteolytic activity, were obtained from Laboratoire de Microbilogie-biotechnologie, ENSBANA, Dijon, France. The identity of species was confirmed by the use of physiological, biochemical and sugar test by API 50 CHL system according to the manufacturer's instructions (API System, Bio-Merieux France) [15-17].

The strains were stored as frozen stock at - 20°C in fortified skimmed milk (10% skim milk, 0.25% yeast extract, 0.5 % glucose) containing 30% glycerol as

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appropriate. Working cultures were prepared from stock cultures by two consecutive transfers in fresh MRS and M17 broth for *Leuconostoc* and *Lactococcus* respectively [18].

Media and conditions cultures: *Leuconostoc* strains were cultured in MRS broth [19] and *Lactococcus lactis* in M17 broth [20] . Milk medium (reconstituted skim milk 10%) was also used for the preparation of single and mixed cultures. Skimmed-milk medium was prepared from reconstituted skim milk powder 10% (w/v) and sterilized by autoclaving at 110°C for 10 min. All cultures were incubated at 30°C for 48 hours.

Kinetic growth on milk: The growth rate, lactic acid production and evolved CO_2 were studied on reconstituted skim milk. The reconstituted milk was sterilized at 110°C for 10 min, cooled at room temperature (20°C) and inoculated with the strains at 30°C. All the mother culture of *Leuconostoc* and *Lactococcus lactis* were grown separately in sterile skim milk at 30°C.

Freshly prepared starter culture of *Leuconostoc* was inoculated at 5% initially yielded about 5 10^7 cfu ml^{-1}, however, in the case of *Lactococcus lactis*, the yield was about 2.5 10^5 cfu ml^{-1} at 0.1% of inoculums. Cultures were incubated at 30°C for 24 hours. Every hour, samples were aseptically withdrawn from tubes to determine pH, titratable acidity and evolved CO_2, the experiments were repeated three times.

Determination of pH and total acidity: Measurement of pH was carried out by pH-meter. The total acidity was determined by titrating 10 ml of culture with 0.1 N NaOH, phenolphthalein was used as indicator and total acidity was expressed as millimole of lactic acid [15, 21].

Growth kinetic: Cultures samples were collected aseptically at 0 hours and every 2 hours post inoculation until 24 hours. Culture sample of 1 ml were submitted to decimal dilutions in sterile tryptone salt solution and agar plate were performed to assess cell count. *Leuconostoc mesenteroides* and *Lactococcus lactis* were enumerated in MRSv and M17 respectively according to the method of Mathot *et al.* [22]. Plates were incubated at 30°C for 48 h. Generation times were calculated in the logarithmic phase of growth.

CO_2 Production: The evolved CO_2 was measured by a technique based on the pressure which is created by CO_2 production by culture in tubes. Evolved CO_2 was trapped in burette in which it was measured [11]. The total amount of CO_2 produced was released by acidifying with 0.5 ml of HCl 2N [9].

In order to evaluate the linearity of the method, solution of sodium carbonate 50 mM were used to liberate CO_2 in the tube by addition of sulfuric acid (2N). The blank contains 10 ml of sterile milk.

Analytical methods: The ability to produce different lactic acid isomers (L-lactate and D-lactate) was tested by an enzymatic method utilizing Boehringer Mannheim GmbH (Mannheim, Germany) Also citric acid and acetic acid were carried out by enzymatic methods (Boehringer) [23].

RESULTS AND DISCUSSION

Strains characteristics: All the used strains were cocci associated in diplococci and chain, Gram+, catalase- and were can grow under both aerobic and anaerobic conditions. All the strains form on solid medium identical lentil colonies.

The two *Leuconostoc* strains were heterofermentatives and produce CO2 and D-lactic acid from glucose. The production of dextrane from saccharose was observed. However, *Leuconostoc mersenteroides* subsp. *mesenteroides* (19D) was arabinose+ and *Leuconostoc mersenteroides* subsp. *dextranicum* (L4) was arabinose-.

In the case of *Lactococcus lactis* (SLO_3) was homofermentative and produce the isomer L-lactate from glucose. This strain can not use citrate and hydrolyze arginine [17].

All this characteristics are in accordance with Carr *et al.* [2], Stiles and Holzapfel, [24] and Klein *et al*. [25].

Limite of detection: A high correlation was observed when the volume of CO_2 measured is lower than 80 mM. The method has a good linearity between 0 to 80 mM of CO_2, but the application of the method to the determination of CO_2 content in the sample needs a blank which must be prepared and measured before each culture sample.

The lower value of coefficient of variation observed was caused by the preparation of sample than by the measurement itself (Table 1).

Table 1: Illustrate the response of the complete device to the liberation of CO_2 from a solution of sodium carbonate; Mean and standard deviation were obtained from ten measurements

ml of Na_2CO_3 50 mM	Mean (ml)	Standard Deviation	Coefficient of variance %
5	6.61	0.180	2.72
8	9.70	0.380	3.20
10	10.91	0.099	0.90
12	14.50	0.510	3.50

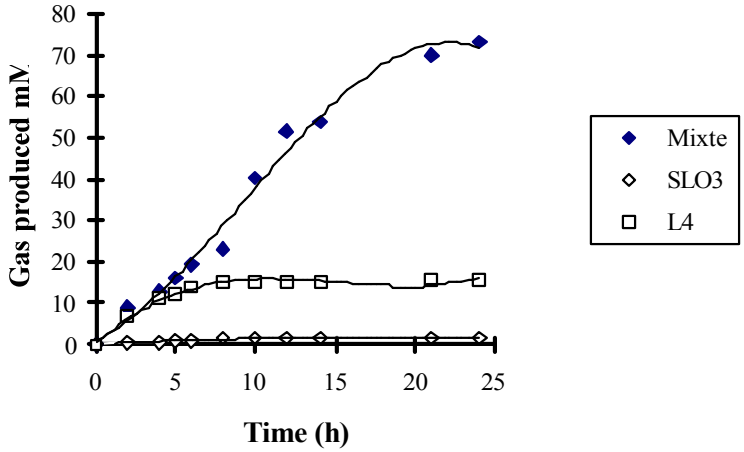

Fig. 1: Kinetics of CO_2 production by *Leuconostoc mesenteroides* subsp *dextranicum* (□), *Lactococcus lactis* subsp. lactis (✧) in pure culture in milk and in mixed culture of the two strains (■)

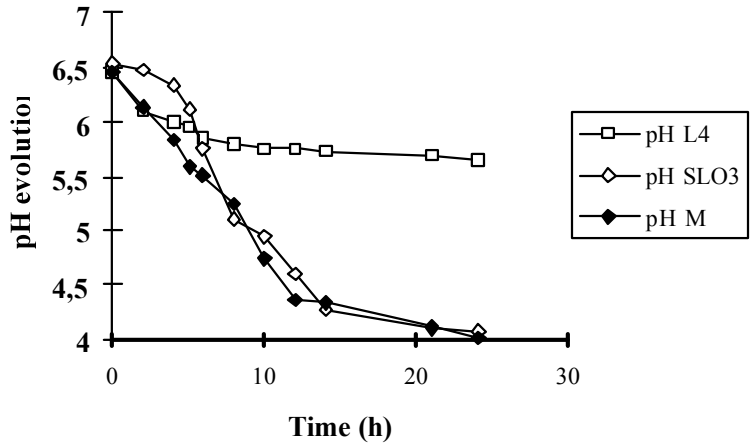

Fig. 2: Kinetics of pH evolution by *Leuconostoc mesenteroides* subsp *dextranicum* (□), *Lactococcus lactis* subsp. lactis (✧) in pure culture in milk and in mixed culture of the two strains (■)

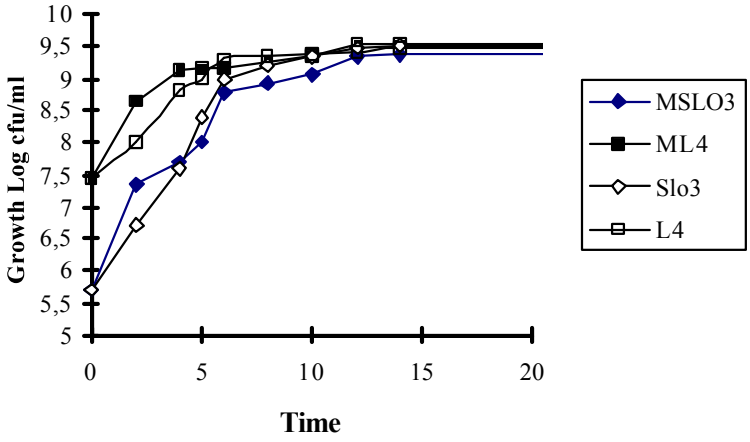

Fig. 3: Growth kinetics of *Leuconostoc mesenteroides* subsp *dextranicum* (□,■), *Lactococcus lactis* subsp. lactis (◊, ▪) in pure culture in milk and in mixed culture of the two strains

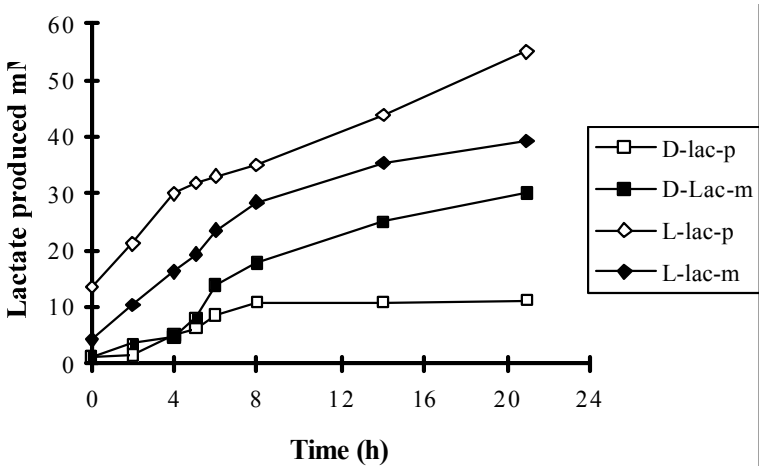

Fig. 4: Kinetics of lactic acid production during incubation of *Leuconostoc mesenteroides* subsp *dextranicum* (□), *Lactococcus lactis* (◊) in pure culture in milk and D-lactic acid (■) and L-lactic acid (◆) production in mixed culture

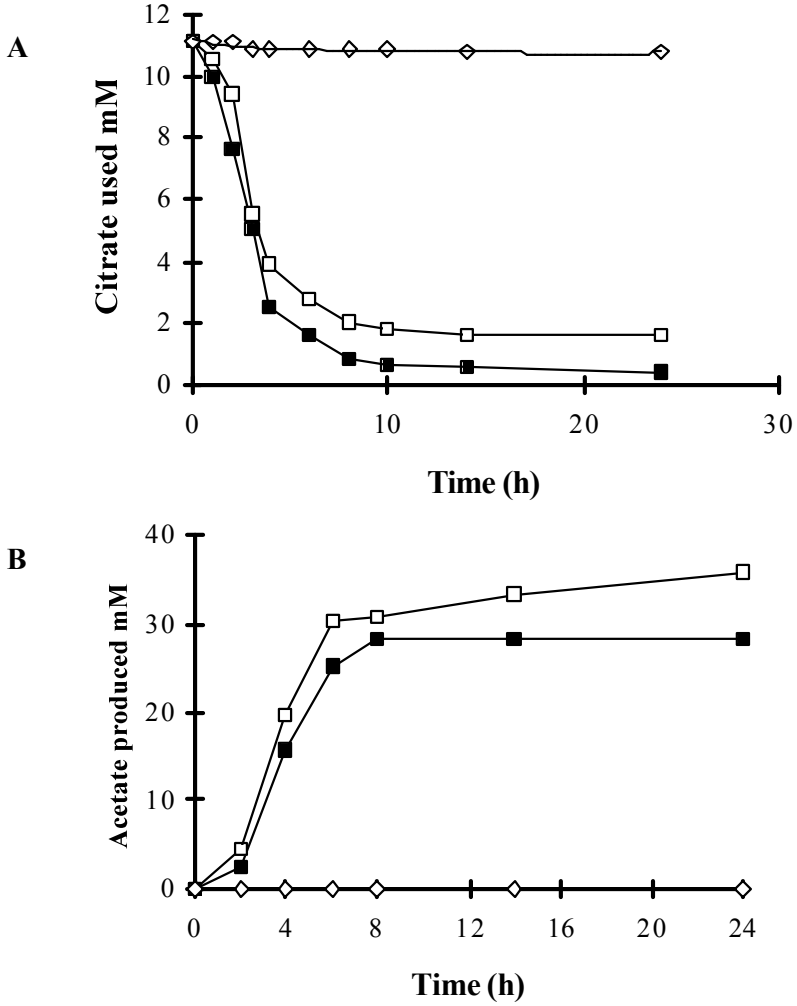

Fig. 5: Kinetic of citrate consumption (A) and acetate production (B) in culture pure in milk by *Leuconostoc mesenteroides* subsp *dextranicum* (□) *Lactococcus lactis* subsp *lactis* (◊) and in mixed culture (■)

By definition, heterofermentative lactic acid bacteria, ferment glucose to produce equimolecular amounts of lactate, carbon dioxide and acetate or ethanol [26] However, certain modifications in the conditions of culture may result in the prevalence of one of these products. The kinetics of both evolved CO_2 and pH evolution are shown in figure 1 and 2. When *Leuconostoc mesenteroides* was cultured with *Lactococcus lactis* subsp. *lactis prot(+)* in milk, several differences in the fermentation products were observed. No evolved CO_2 was observed in pure culture of *Lactococcus lactis*. A proportion of $2.5\ 10^6$ cfu ml^{-1} of *Lactococcus lactis* decreases the CO_2 production and gives only 12.5 mM of CO_2 in mixed culture after 24 h of incubation, while $2.5\ 10^5$ cfu ml^{-1} of *Lactococcus lactis* enhanced CO_2 production in mixed culture (Fig. 1). Good growth of *Leuconostoc mesenteroides* was observed by Todorov and Dicks [27] in the presence of 10% soy milk or molasses. The final volume of CO_2 was 17.4 and 1.5 mM in pure culture of *Leuconostoc mesenteroides* subsp *dextranicum* and *Lactococcus lactis* respectively. A volume of 72.86 mM of CO_2 was observed when *Lactococcus lactis* was used ($2.5\ 10^5$ cfu ml^{-1}) with *Leuconostoc* ($3\ 10^7$ cfu ml^{-1}) (Fig. 3). A greater inhibition of evolved CO_2 coming from the contribution of total lactic acid in mixed culture was caused by high inoculums of *Lactococcus lactis*. Bellangier *et al.,* [5] demonstrated that the proteolytic *Lactococcus* inhibit the gas production of *Leuconostoc* sp. In figure 1, the estimation of results coming from the pure culture relationships applied to a mixed culture experiment are presented. A shift between CO_2 production rate in pure and mixed culture could be appreciated. In pure culture of *Leuconostoc mesenteroides* the production of lactic acid was correlated with evolved CO_2 and the coefficient of correlation was high (r = 0,996). The evolution of the curves of CO_2 and lactic acid production were almost identical in pure culture of *Leuconostoc*. Kinetics of pH evolution by culture was presented in Fig. 2. The lowest titrable acidity and pH evolution were given by the pure culture of *Leuconostoc*. As starter *cultures Leuconostoc mesenteroides* may offer the potential to carry out fermentations that have a less acidic character which is mentioned earlier and may be desirable from a sensory standpoint. In mixed culture the final acidity could be substantially reduced by partial conversion of sugar to ethanol and CO_2. A high ratio of volatile/ non volatile acids might result in better flavor quality. The lactic acid production in skim milk was chosen as an index of starter performance by Beal and Corrieu [14].

In the first phase, the specific growth rate, lactic acid production rate and evolved CO_2 rate exhibited a constant relationship. After the ten first hours an inhibition of growth, lactic acide production and evolved CO_2, were observed. Maximum CO_2 production rate (Vmax) showed a decrease when the inoculum level of *Lactococcus lactis* increases by a factor of 10 from $2.5\ 10^5$ to $2.5\ 10^6$ cfu ml^{-1} in mixed culture with *Leuconostoc mesenteroides* ($3\ 10^7$ cfu lm^{-1}). This parameter of Vmax could be used to compare different mixed cultures. *Leuconostoc mesenteroides* subsp. *dextranicum* had a maximum rate of CO_2 production of 3 mM/h in pure culture. In the presence of $2.5\ 10^5$ cfu ml^{-1} of *Lactococcus lactis* Vmax of CO_2 production was higher (4 mM h^{-1}) and was obtained quicker in mixed culture than in pure culture, although the higher rate of CO_2 production was maintained for a shorter period in pure culture of *Leuconostoc*. *Leuconostoc mesenteroides* did not produced acetoine and diacetyl, the lack of acetoine production at neutral pH is thought to be due to inhibition of acetolactate synthase by several intermediates of sugar metabolism [28]. The pH value of pure culture of *Leuconostoc mesenteroides* decreased from 6.7 to 5.6 after 10 h, whereas, in mixed culture and pure culture of *Lactococcus lactis*, the pH progressively dropped to 4.4 after 14 h, a value at which growth of both strains ceased. This resulted in the reduction of CO_2 production (Fig. 2).

An interesting way to estimate independently *Leuconostoc mesenteroides and Lactococcus lactis* concentration during mixed cultures is to use the relationships obtained for pure culture with the associated D(-)lactic acid for *Leuconostoc mesenteroides* and L(+)lactic acid for *Lactococcus lactis*. Using curves measurements of D(-) and L(+) lactic acid concentration indicate the evolution of the concentration of each strain in mixed cultures which was obtained from the relationships. A linear correlation were established between evolved CO_2 and D(-) lactic acid production by *Leuconostoc mesenteroides*

The estimated results coming from the pure culture relationships applied to mixed culture experiment are presented in (Fig.4). A shift between μmax for *Leuconostoc* and *Lactococcus* could be appreciated. This shows that *Lactococcus lactis* grew faster than *Leuconostoc mesenteroides* due to a more rapid assimilation of nutrients and their proteolytic activity. The same phenomenon is reflected by the specific lactic acid production curves [29, 30]. *Lactococcus lactis* can not consume citric acid (Fig. 5). In mixed culture and pure culture of *Leuconostoc mesenteroides*, citric acid began

to be consumed at the beginning of fermentation, but a 12 mM were entirely consumed within 7 h. As shown in (Fig. 5). Whereas, Lee [31] has suggested that mixed culturing of Lactobacilli may be more effective than single culturing of Lactobacillus for improving lactic acid production The amount of acetic acid formed from citrate was higher in pure and mixed culture of *Leuconostoc mesenteroides*. The kinetics of acetate production and citric acid consumption were similar and a peak of production at early sampling times followed by deceleration of acetate production rate.

Growth of *Leuconostoc mesenteroides* on lactose was stimulated by citrate but no growth on citrate alone was observed. The growth stimulation of *Leuconostoc mesenteroides* in the presence of citrate can be expained by the action of citrate as an external electron acceptor, resulting in more acetate (and ATP) production and less ethanol production during the heterofermentative lactose conversion. This phenomenon has been described by Schmitt *et al*. [32] and Zurera-Cosano *et al*. [33].

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