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

H Kihal, D Prevost, D Henni, Z Benmechernene

Abstract
The effect of mixed culture of Leuconostoc mesenteroides subsp. dextranicum and Lactococcus lactis subsp. lactis (prot+) was investigated to achieve an optimal production of carbon dioxide. Only the strain of Leuconostoc mesenteroides can produce carbon dioxide from lactose and citrate in milk. The influence of the initial concentration ration between the two strains on growth, carbon dioxide, L-lactate, acetic acid production and citrate used was displayed. When the initial inoculum of Lactococcus lactis was 2.5 10^5 cfu/ml, the growth and evolved CO2 by Leuconostoc mesenteroides (3 10^7 cfu/ml) increased. Whereas, high inoculum of Lactococcus lactis induce a decrease of growth and CO2 production by Leuconostoc mesenteroides. In mixed culture CO2 production continued after growth stopped, a partial uncoupling can be observed between growth and CO2 production. A shift of acetate production was observed in mixed culture and 25.6 mM was obtained, whereas 30.18 mM was obtained at the same time in pure culture of Leuconostoc mesenteroides.

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 (Carr et al., [16] and Ayad et al., [1]. 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 [16]. Mixed cultures of these bacteria are commonly used as a starter in manufacture of cheese and fermented milk [1]. 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 [12].

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 CO2 production by microorganisms [12,22,23,19,13,20]. Holmes et al. [13] 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 CO2 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 [15,1], but little information is available about the behavior of heterofermentative species such as Leuconostoc mesenteroides. The quantitative aspects and kinetics of evolved CO2 in mixed culture of Leuconostoc-Lactococcus under different culturing conditions have been fairly studied [17]. In point of view of the recent report demonstrating the
importance of evolved CO₂ 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 (SLO³), which is known for its proteolytic activity, were obtained from Laboratoire de Microbiologie-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)

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

**MEDIA AND CONDITIONS CULTURES**

Leuconostoc strains were cultured in MRS broth and Lactococcus lactis in M17 broth. 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 CO₂ were studied on reconstituted skim milk (10 % skim milk, 0.25 % yeast extract, 0.5 % glucose) containing 30% glycerol as appropriate. Working cultures were prepared from stock cultures by two consecutive transfers in fresh MRS and M17 broth for Leuconostoc and Lactococcus respectively.

**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 mM of lactic acid.

**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. Plates were incubated at 30°C for 48 h. Generation times were calculated in the logarithmic phase of growth.

**CO PRODUCTION**

The evolved CO₂ was measured by a technique based on the pressure which is created by CO₂ production by culture in tubes. Evolved CO₂ was trapped in burette in which it was measured (Kihal et al., 2006). The total amount of CO₂ produced was released by acidifying with 0.5 ml of HCl 2N.

**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. Also citric acid and acetic acid were carried out by enzymatic methods (Boehringer). The results were evaluated by a dilution technique.

**RESULTS AND DISCUSSION**

The two Leuconostoc strains were heterofermentatives and able to produce different lactic acid isomers (L-lactate and D-lactate) was tested by an enzymatic method utilizing Boehringer Mannheim GmbH.
produce CO2 and D-lactic acid from glucose. The production of dextrane from saccharose was observed. However, Leuconostoc mesenteroides subsp. mesenteroides (19D) was arabinose+, and Leuconostoc mesenteroides subsp. dextranicum (L4) was arabinose-.

In the case of Lactococcus lactis (SLO) was homofermentative and produce the isomer L-lactate from glucose. This strain can not use citrate and hydrolyze arginine [27].

All this characteristics are in accordance with Carr et al., [7], Stiles and Holzapfel. [30], and Klein et al. [21].

**LIMITE OF DETECTION**

A high correlation was observed when the volume of CO2 measured is lower than 80 mM. The method has a good linearity between 0 to 80 mM of CO2, but the application of the method to the determination of CO2 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 (Tab.1).

**Figure 1**

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

<table>
<thead>
<tr>
<th>ml of Na2CO3 50 mM</th>
<th>Mean (ml)</th>
<th>Standard Deviation</th>
<th>Coefficient of variation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6.61</td>
<td>0.18</td>
<td>2.72</td>
</tr>
<tr>
<td>8</td>
<td>9.70</td>
<td>0.38</td>
<td>3.2</td>
</tr>
<tr>
<td>10</td>
<td>10.91</td>
<td>0.099</td>
<td>0.9</td>
</tr>
<tr>
<td>12</td>
<td>14.5</td>
<td>0.51</td>
<td>3.5</td>
</tr>
</tbody>
</table>

By definition, heterofermentative lactic acid bacteria, ferment glucose to produce equimolecular amounts of lactate, carbon dioxide and acetate or ethanol [17,18]. However, certain modifications in the conditions of culture may result in the prevalence of one of these products. The kinetics of both evolved CO2 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 CO2 was observed in pure culture of Lactococcus lactis.

By definition, heterofermentative lactic acid bacteria, ferment glucose to produce equimolecular amounts of lactate, carbon dioxide and acetate or ethanol [17,18].

A proportion of 2.5 $10^6$ cfu/ml of Lactococcus lactis decreases the CO2 production and gives only 12.5 mM of CO2 in mixed culture after 24 h of incubation, while 2.5 $10^5$ cfu/ml of Lactococcus lactis enhanced CO2 production in mixed culture (Fig 1). Good growth of Leuconostoc mesenteroides was observed by Todorov and Dicks [33] in the presence of 10% soy milk or molasses. The final volume of CO2 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 CO2 was observed when Lactococcus lactis was used (2.5 $10^7$ cfu/ml) with Leuconostoc (3 $10^7$ cfu/ml) (Fig. 3). A greater inhibition of evolved CO2 coming from the contribution of total lactic acid in mixed culture was caused by high inoculums of Lactococcus lactis. Bellangier et al., [4] 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 CO2 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 CO2 and the coefficient of correlation was high ($r = 0.996$). The evolution of the curves of CO2 and lactic acid production were almost identical in pure culture of Leuconostoc. Kinetics of pH evolution by culture was presented in figure 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 [3].

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 in mixed culture with Leuconostoc mesenteroides (3 \(10^7\) cfu/ml). 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 of Lactococcus lactis Vmax of CO\(_2\) production was higher (4 mM/h) 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 synthese by several intermediates of sugar metabolism [4].

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).

**Figure 3**
Figure 2: Kinetics of pH evolution by subsp. lactis (*) in pure culture in milk and in mixed culture of the two strains (o)

**Figure 4**
Figure 3: Growth kinetics of subsp. lactis (◊,·) in pure culture in milk and in mixed culture of the two strains

**Figure 5**
Figure 4: Kinetics of lactic acid production during incubation of subsp (‖), (◊) in pure culture in milk and D-lactic acid (‖) and L-lactic acid (‖) production in mixed culture.

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 \( \text{CO}_2 \) and \( \text{D(-)} \) 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 \( \mu_{\text{max}} \) 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 \([14, 28]\). *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 \([23]\) has suggested that mixed culturing of Lactobacilli may be more effective than single culturing of Lactobacillus for improving lactic acid production.

**Figure 6**

Figure 5: Kinetic of citrate consumption (○) and acetate production (♂) in culture pure in milk by subsp (♀) subsp (♂) and in mixed culture (□).

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 explained 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., \([29]\) and Zurera-Cosano et al., \([34]\).

In conclusion a greater inhibition of evolved \( \text{CO}_2 \) in *Leuconostoc mesenteroides* coming from the high production of lactic acid in mixed culture by high inoculums of *Lactococcus lactis* (SLO \( \text{prot } + \) ) was observed. The growth and \( \text{CO}_2 \) production by *Leuconostoc mesenteroides* was related to know the precise ratio of initial inoculum of two strains milk for good performance in dairy industries.

**References**

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**Author Information**

**H. M. Kihal**  
Laboratoire de Microbiologie Appliquée Département de Biologie, Faculté des Sciences, Université Oran

**DE Prevost**  
Laboratoire de microbiologie-biotechnologie, ENSBANA, 1 Esplanade Erasme Université de Bourgogne

**DE Henni**  
Laboratoire de microbiologie-biotechnologie, ENSBANA, 1 Esplanade Erasme Université de Bourgogne

**Z. Benmechernene**  
Laboratoire de microbiologie-biotechnologie, ENSBANA, 1 Esplanade Erasme Université de Bourgogne