Scientific Research and Essay Vol. 4 (11), pp. 1348-1353, November, 2009
Available online at http://www.academicjournals.org/SRE
ISSN 1992-2248 © 2009 Academic Journals

Full Length research Paper

Carbon dioxide production by *Leuconostoc mesenteroîdes* grown in single and mixed culture with *Lactococcus lactis* in skim milk

M. Kihal[1]*, H. Prevost[2], D. E. Henni[1], Z. Benmechernene and C. Diviès[2]

[1]Laboratoire de Microbiologie Appliquée, Département de Biologie, Faculté des Sciences, Université Oran, Essenia, Bp 16, 31000, Oran, Algérie.
[2]Laboratoire de Microbiologie-Biotechnologie, ENSBANA, 1 Esplanade Erasme Université de Bourgogne 21000, Dijon, France.

Accepted 25 September, 2009

The effect of mixed culture of *Leuconostoc mesenteroîdes* subsp. dextranicum and *Lactococcus lactis* subsp. *lactis (prot+)* was investigated to achieve an optimal production of carbon dioxide. Only the strain of *L. mesenteroîdes* 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 studied. When the initial inoculum of *L. lactis* was 2.5 x 10^5 cfu/ml, the growth and evolved CO_2 by *L. mesenteroîdes* (3 x 10^7 cfu/ml) increased, whereas high inoculum of *L. lactis* induced a decrease of growth and CO_2 production by *L. mesenteroides.* In mixed culture, CO_2 production continued after growth stopped, a partial uncoupling can be observed between growth and CO_2 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 *L. mesenteroîdes*.

Key words: *Leuconostoc mesenteroîdes*, *Lactococcus lactis,* milk, lactic acid, acetic acid, citric acid, growth kinetics, fermented milk.

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., 2002; Ayad et al., 2004). 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 (Drosinos et al., 2005). Mixed cultures of these bacteria are commonly used as a starter in manufacture of cheese and fermented milk (Bellangier et al., 1999). 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* (Bellangier et al., 1999; Padmanabhan and Kim, 2002).

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 (Gibson and Abdelmalek, 1945; Kneifel and Gretner, 1992; Mohr et al., 1993; Girard and Boyaval, 1994; Kihal et al., 2006). Holmes et al. (1968) demonstrated that cell extract of *L. lactis* and *Lactobacillus* enhanced gas production *of Leuconostoc mesenteroîdes* subsp. cre-

*Corresponding author. E-mail: Kihalm@hotmail.com.

moris. 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 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 (Bobillo and Marshall, 1992; Kandler, 1983), but little information is available about the behavior of heterofermentative species such as *L. mesenteroîdes*. The quantitative aspects and kinetics of evolved CO_2 in mixed culture of *Leuconostoc-Lactococcus* under different culturing conditions have been fairly studied (Beal and Corrieu, 1991; Kihal et al., 1996). 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 *L. mesenteroîdes* plays a role in carbon dioxide production in pure and mixed culture with *L. lactis* in milk.

MATERIALS AND METHODS

Bacterial strains

L. mesenteroîdes subsp. *dextranicum* (L4), *L. mesenteroîdes* subsp. *mesenteroîdes* (19D) and *L. 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) (Huang et al., 1994; Thapa et al., 2006; Perea et al., 2007).

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 (Kihal, 1996).

Media and conditions cultures

Leuconostoc strains were cultured in MRS broth (De Man et al., 1960) and *L .lactis* in M17 broth (Terzaghi and Sandine, 1975). 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 h.

Growth kinetic in 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 *L. lactis* were grown separately in sterile skim milk at 30°C.

Freshly prepared starter culture of *Leuconostoc* inoculated at 5% initially yielded about 5 x 10^7 cfu/ml, however, in the case of *L. lactis*, the yield was about 2.5 x 10^5 cfu/ml at 0.1% of inoculums. Cultures were incubated at 30°C for 24 h. 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 mM of lactic acid (Huang et al., 1994; Badis et al., 2004).

Growth kinetic

Cultures samples were collected aseptically at 0 h and every 2 h post inoculation until 24 h. Culture sample of 1 ml was submitted to decimal dilutions in sterile tryptone salt solution and agar plate was performed to assess cell count. *L. mesenteroîdes* and *L. lactis* were enumerated in MRSv and M17, respectively, according to the method of Mathot et al. (1994). 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 (Kihal et al., 2006). The total amount of CO_2 produced was released by acidifying with 0.5 ml of HCl 2N (Mohr et al., 1993).

In order to evaluate the linearity of the method, solution of sodium carbonate 50 mM was used to liberate CO_2 in the tube by addition of sulfuric acid (2 N). 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) (Bjokroth et al., 2000).

RESULTS AND DISCUSSION

All the used strains were cocci associated in diplococci and chain, Gram+, catalase-, and 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 CO_2 and D-lactic acid from glucose. The production of dextrane from saccharose was observed. However, *L. mesenteroîdes* subsp. *mesenteroîdes* (19D) was arabinose+, and *L. mesenteroîdes* subsp. *dextranicum* (L4) was arabinose-. In the case of *L. lactis* (SLO_3), it was homofermentative and produce the isomer L-lactate from glucose. This strain cannot use citrate but hydrolyzes arginine (Perea et al., 2007). All these characteristics are

Table 1. Response of the complete device to the liberation of CO_2 from a solution of sodium carbonate;

ml of Na_2CO_3 50 mM	Mean (ml)	Standard deviation	Coefficient of variance %
5	6.61	0.18	2.72
8	9.70	0.38	3.2
10	10.91	0.099	0.9
12	14.5	0.51	3.5

Mean and standard deviation were obtained from ten measurements.

in accordance with Carr et al. (2002), Stiles and Holzapfel (1997), and Klein et al. (1998).

A high correlation was observed when the volume of CO_2 measured was 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).

By definition, heterofermentative lactic acid bacteria ferment glucose to produce equimolecular amounts of lactate, carbon dioxide and acetate or ethanol (Kandler, 1983; Kihal et al., 1996). 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 Figures 1 and 2.

When *L. mesenteroîdes* was cultured with *L. lactis* subsp. *lactis prot(+)* in milk, several differences in the fermentation products were observed. No evolved CO_2 was observed in pure culture of *L. lactis*. A proportion of 2.5 x 10^6 cfu/ml of *L. 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 x 10^5 cfu/ml of *L. lactis* enhanced CO_2 production in mixed culture (Figure 1). Good growth of *L. mesenteroîdes* was observed by Todorov and Dicks (2005) 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 *L. mesenteroîdes* subsp *dextranicum* and *L. lactis,* respectively. A volume of 72.86 mM of CO_2 was observed when *L. lactis* was used (2.5 x 10^5 cfu/ml) with *Leuconostoc* (3 x 10^7 cfu/ml) (Figure 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 *L. lactis*. Bellangier et al. (1999) 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 was presented. A shift between CO_2 production rate in pure and mixed culture could be appreciated. In pure culture of *L. mesenteroîdes,* 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*.

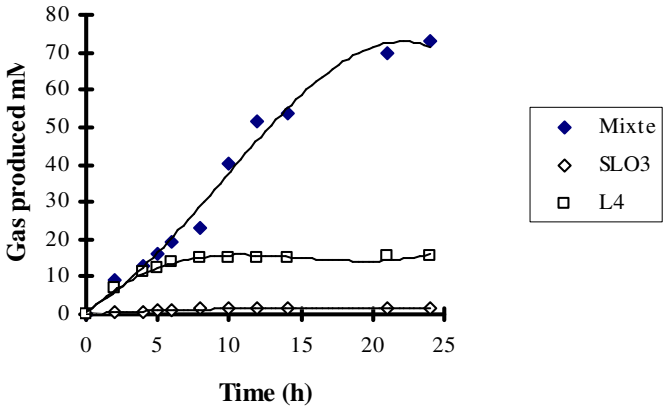

Figure 1. Kinetics of CO_2 production by *Leuconostoc mesenteroîdes* subsp *dextranicum (□), Lactococcus lactis* subsp. lactis (✧) in pure culture in milk and in mixed culture of the two strains (•).

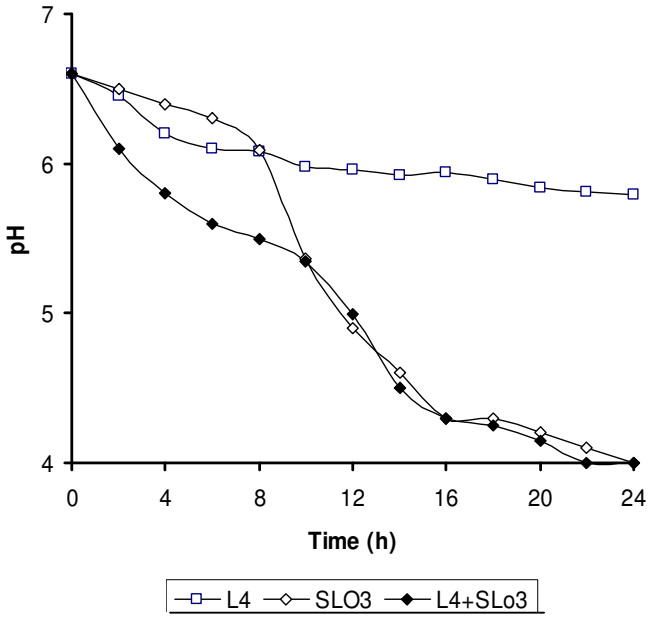

Figure 2. Kinetics of pH evolution by *Leuconostoc mesenteroîdes* subsp *dextranicum (□), Lactococcus lactis* subsp. lactis (✧) in pure culture in milk and in mixed culture of the two strains (•).

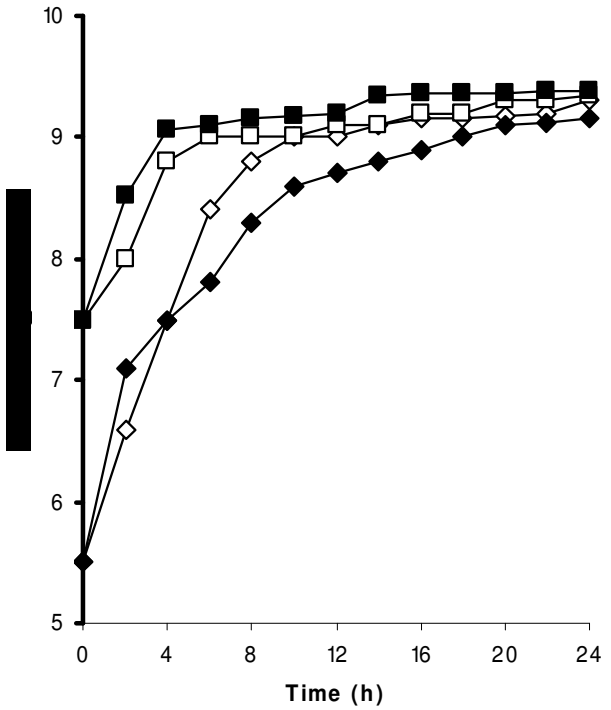

Figure 3. Growth kinetics of *Leuconostoc mesen-teroîdes* subsp *dextranicum* (□, ■), *Lactococcus lactis* subsp. lactis (◊,•) in pure culture in milk and in mixed culture of the two strains.

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 L. mesenteroîdes* 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 (1991).

In the first phase, the specific growth rate, lactic acid production rate and evolved CO_2 rate exhibited a constant relationship. After the first ten hours, an inhibition of growth, lactic acid production and evolved CO_2 were observed. Maximum CO_2 production rate (Vmax) showed a decrease when the inoculum level of *L. lactis* increases by a factor of 10 from 2.5 x 10^5 to 2.5 x 10^6 cfu/ml in mixed culture with *L. mesenteroîdes* (3 x 10^7 cfu/lm). This parameter of Vmax could be used to compare different mixed cultures. *L. mesenteroîdes* subsp. *dextranicum* had a maximum rate of CO_2 production of 3 mM/h in pure culture. In the presence of 2,5 x 10^5 cfu/ml of *L. 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*. *L. mesenteroîdes* did not produce 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 (Cogan, 1987). The pH value of pure culture of *L. mesenteroîdes* decreased from 6.7 to 5.6 after 10 h, whereas in mixed culture and pure culture of *L. 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 (Figure 2).

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

The estimated results coming from the pure culture relationships and applied to mixed culture experiment were presented in (Figure 4). A shift between Vmax for *Leuconostoc* and *Lactococcus* could be appreciated. This shows that *L. lactis* grew faster than *L. mesenteroîdes* due to a more rapid assimilation of nutrients and their proteolytic activity. The same phenomenon was reflected by the specific lactic acid production curves (Gonzalo et al., 1994; Robert et al., 2006). *L. lactis* cannot consume citric acid (Figure 5). In mixed culture and pure culture of *L. mesenteroîdes*, citric acid began to be consumed at the beginning of fermentation, but a 12 mM was entirely consumed within 7 h as shown in Figure 5. Whereas, Lee (2005) had 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 *L. mesenteroîdes*. 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 *L. mesenteroîdes* on lactose was stimulated by citrate but no growth on citrate alone was observed. The growth stimulation of *L. mesenteroîdes* 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. (1990) and Zurera-Cosano et al. (2006).

In conclusion, a greater inhibition of evolved CO_2 in *L. mesenteroîdes* coming from the high production of lactic acid in mixed culture by high inoculums of *L. lactis (SLO$_3$ prot$^+$)* was observed. The growth and CO_2 production by *L. mesenteroîdes* was related to know the precise ratio of initial inoculum of two strains milk for good performance

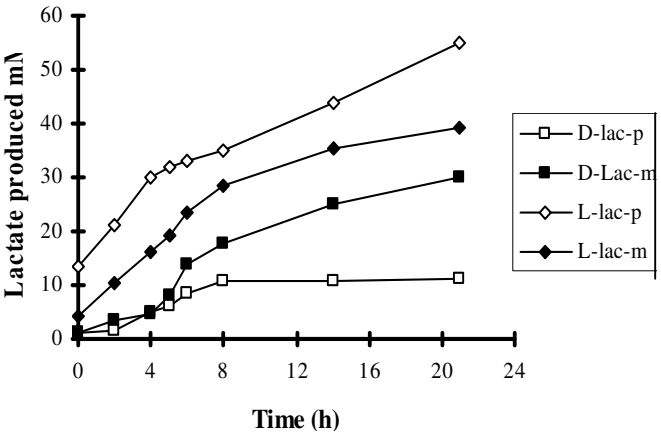

Figure 4. Kinetics of lactic acid production during incubation of *Leuconostoc mesenteroîdes* subsp. *dextranicum* (□), *Lactococcus lactis* (◊) in pure culture in milk and D-lactic acid (■) and L-lactic acid (◆) production in mixed culture.

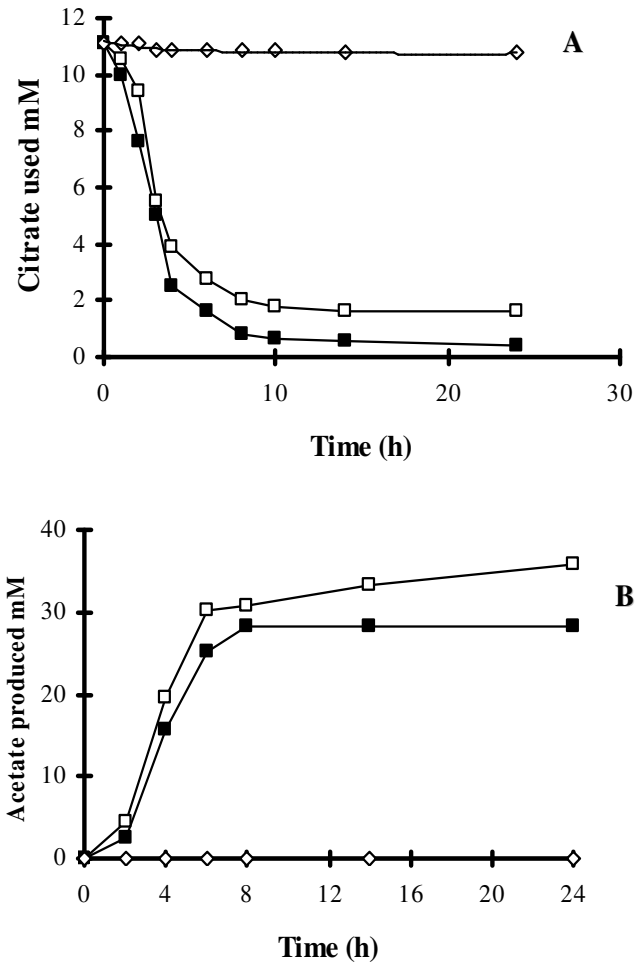

Figure 5. Kinetic of citrate consumption (**A**) and acetate production (**B**) in culture pure in milk by *Leuconostoc mesenteroîdes* subsp *dextranicum* (□) *Lactococcus lactis* subsp *lactis* (◊) and in mixed culture (■).

in dairy industries.

REFERENCES

Ayad EHE, Nashat S, El-Sadek N, Metwaly H, El-Soda M (2004). Selection of wild lactic acid bacteria isolated from traditional Egyptian dairy products according to production and technological. Criteria Food Microbiol. 21: 715–725.

Badis A, Guetarni D, Moussa BB, Henni DE, Kihal M (2004). Identification and technological properties of lactic acid bacteria isolated from raw goat milk of four Algerian races. Food Microbiol. 21: 579–588.

Beal C, Corrieu G (1991). Influence of pH, temperature, and inoculum composition on mixed cultures of *Streptococcus thermophilus* 404 and *Lactobacillus bulgaricus* 398. Biotechnol. Bioeng. 38: 90-98.

Bellangier P, Richard J, Foucaud C (1999). Associative growth of lactococcus lactis and Leuconostoc mesenteroides strains in milk. J. Dairy Sci. 80: 1520-1527.

Bjokroth KJ, Geisen R, Schillinger U, Weiss N, DeVos P, Holzapfel WH, Korkeala JH, Vandamme P (2000). Characterization of *Leuconostoc gasicomitatum* sp. associated with spoiled raw tomato Marinated Broiler meat strips packaged under modified atmosphere conditions. Appl. Environ. Microbiol. 66(9): 3764-3772.

Bobillo M, Marshall VM (1992). Effect of acidic pH and salt on acid end-products by Lactobacillus plantarum in aerated, glucose-limited continuous culture. J. Appl. Bacteriol. 73: 67-70.

Carr FJ, Chill D, Maida.N (2002). The Lactic Acid Bacteria: A Literature Survey. Crit. Rev. Microbiol. 28(4): 281–370.

Cogan TM (1987). Co-metabolism of citrate and glucose by Leuconostcoc spp.: effet on growth, substrates and products. J. Appl. Bacteriol. 63: 551-558.

De Man MC, Rogosa M, Sharpe ME (1960). A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. 23 : 130-135.

Drosinos EH, Mataragas M, Nasis P, Galiotou M, Metaxopoulos J (2005). Growth and bacteriocin production kinetics of *Leuconostoc mesenteroides* E131. J. Appl. Microbiol. 99: 1314–1323.

Gibson T, Abdelmalek Y (1945). The formation of carbon dioxide by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. J. Dairy Res. 14: 35-44.

Girard F, Boyaval P (1994). Carbon dioxide measurement in swiss-types cheeses by coupling extraction and gas chromatography. Le lait 74 : 389-398.

Gonzalo A, Latrille E, Beal C, Corrieu G (1994). On line estimation of biological variables during pH controlled lactic acid fermentations. Biotechnol. Bioeng. 44: 1168-1176.

Holmes B, Sandine WE, Elliker PR (1968). Some factors influencing carbon dioxide production by Leuconostoc citrovorum. Appl. Microbiol. 1: 56-61.

Huang DQ, Prevost H, Kihal M, Diviès C (1994). Instability of plasmid encoding for B-galactosidase in *Leuconostoc mesenteroides* subsp *mesenteroides*. J. Basic Microbiol. 34. (1): 23-30.

Kandler O (1983). Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leewenhoek 49: 209-224.

Kihal M (1996). Etude de la production du dioxyde de carbone par *Leuconostoc mesenteroides*, élément d'application en technologie fromagère type fromage bleu. Thèse de Doctorat d'état. Université d'Oran Algérie. p. 167.

Kihal M, Henni DE, Prevost H, Diviès C (2006). New manometric method for measuring carbon dioxide production by dairy starter culture : a case of *Leuconostoc mesenteroides*. Afr. J. Biotechnol. 5 (4): 378-383.

Kihal M, Prevost H, Lhotte ME, Huang DQ, Diviès C (1996). Instability of plasmid-encoded citrate permease in *Leuconostoc*. Lett. Appl . Microbiol. (Mars) 22 : 219-223.

Klein G, Pack A, Bonaparte C, Reuter G (1998). Taxonomy and physiology of probiotic lactic acid bacteria. Int. J. Food Microbial. 41 : 103-125.

Kneifel W, Gretner R (1992). A simple method for estimating the carbon dioxide product of mesophilic starter cultures. Milchwissenschaft 11: 108-111.

Lee K (2005). Comparison of fermentative capacities of lactobacilli in single and mixed culture in industrial media. Process Biochem. 40: 1559-1564.

Mathot AG, Kihal M, Prevost H, Diviès C (1994). Selective Enumeration of *Leuconostoc* on Vancomycine Agar Media. Int. Dairy. J. 4(5): 459-469.

Mohr SA, Zottola WE, Reineccius GA (1993). The use of gas chromatography to measure carbon dioxide production by starter cultures. J. Dairy Sci. 76: 3350-3353.

Padmanabhan PA, Kim DS (2002). Production of insoluble dextran using cell-bound dextransucrase of *Leuconostoc mesenteroides* NRRL B-523. Carbohydr. Res. 337: 1529–1533.

Perea VM, Hermans K, Verhoeven TLA, Lebeer SE, Vanderleyden J, De Keersmaecker SCJ (2007). Identification and characterization of starter lactic acid bacteria and probiotics from Columbian dairy products. J. Appl. Microbiol. 32: 1-9

Robert H, Gabriel V, Lefebvre D, Rabier P, Vayssier Y, Fontagne CF (2006). Study of the behaviour of *Lactobacillus plantarum* and *Leuconostoc* starters during a complete wheat sourdough breadmaking process. LWT 39: 256–265.

Schmitt P, Diviès C, Merlot C (1990). Origin of end products from the metabolism of glucose and citrate by *Leuconostoc mesenteroides* subsp. *cremoris.* Appl. Microbiol. Biotechnol. 36: 679-683.

Stiles ME, Holzapfel WH (1997). Lactic acid bacteria of foods and their current taxonomy. Int. J. Food Microbiol. 36: 1-29

Terzaghi BE, Sandine WE (1975). Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. 29: 807-813.

Thapa N, Pal J, Tamang JP (2006). Phenotypic identification and technological properties of lactic acid bacteria isolated from traditionally processed fish products of the Eastern Himalayas. Inter J. Food Microbiol. 107: 33 – 38.

Todorov SD, Dicks LMT (2005). Production of bacteriocin ST33LD, produced by *Leuconostoc mesenteroides* subsp. *mesenteroides*, as recorded in the presence of different medium components. World J. Microbiol. Biotechnol. 21: 1585–1590.

Zurera-Cosano G, Garcı´a-Gimeno RM, Rodrıguez-Perez R, Hervas-Martınez C (2006). Performance of response surface model for prediction of *Leuconostoc mesenteroides* growth parameters under different experimental conditions. Food Control 17: 429–438.

.