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ORIGINAL ARTICLE

Effect of pH on the growth of three lactic acid bacteria strains isolated from sour cream

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Abstract

Lactic acid bacteria (LAB) have an important role in the food industry because they are used in the production of fermented foods. To use these microorganisms in the food industry, it is necessary to obtain a high amount of biomass. One of the most important environmental factors in the growth of LAB is pH. Most of LAB species can tolerate a pH below 5.0, however, a suboptimal pH is expected to limit LAB growth. For this reason, the LAB strains *Leuconostoc mesenteroides* 67-1, *Lactobacillus plantarum* 60-1, and *Streptococcus infantarius* 46-3, isolated from sour cream, were grown in culture media under four different intial pH values to determine their optimal growth pH. Growth was assesed via colony-forming unit (CFU/ml) determination. We found that the growth of each LAB was affected by culture medium pH. We determined that the setpoints of pH for *Leuconostoc mesenteroides* 67-1, *Streptococcus infantarius* 46-3, and *Lactobacillus plantarum* 60-1 were of 4.5 ($\pm$ 0.5), 5.5 ($\pm$ 0.5), and 6.0 ($\pm$ 0.5), respectively. We thus conclude that the growth of these LAB strains is pH-dependent ($p < 0.05$).

Keywords: *Leuconostoc mesenteroides*; *Lactobacillus plantarum*; *Streptococcus infantarius*; pH; bacterial growth; lactic acid bacteria.

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Introduction

Lactic acid bacteria (LAB) are facultative anaerobes, acid-tolerant, non-sporulating, gram-positive microorganisms with either rod (bacilli) or spherical (cocci) shapes. In addition, LAB share common metabolic and physiological characteristics. The families in which LAB are arrange are *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae,* and *Streptococcaceae* [1, 2].

LAB are boradly employed in the food industry. LAB are required in the production of food items, such as dairy products, cheese, sourdoughs, pickles, fermented sausages, fermented vegetables, soy-fermented foods, and fermented cereal products. LAB can also enhance shelf-life, safety, functionality, sensory, and nutritional properties [3]. Furthermore, certain species of these bacteria have been regarded as probiotics [4]. The Food and Agriculture Organization of the United Nations and the World Health Organization define probiotics as live microorganisms that confer a health benefit on the host when they are administered in adequate amounts [5].

Currently, industrial microbiology has focused on developing methods for obtaining biomass that can be used directly in the transformation of food (Direct vat set-DVS) [6]. Consequently, it is important to study the different environmental factors that directly affect biomass growth in bioreactors. [7, 8]. One of the most important environmental factors for growth and survival of LAB is pH. Although most LAB species are frequently isolated from acid habitats and can tolerate pH values below 5.0, it is necessary to determine their optimum growth pH prior to applying them in fermented food production [9, 10]. An inadequate pH during the growth of LAB leads to alterations in the stability of their membranes, hinders their capacity to exchange of hydrogenions, and can inhibit their enzymatic activity, resulting in a negative effect on yield.

In this work we evaluated the growth of three LAB strains in culture media with four different pH values (from 4.0 and 7.0). The three strains evaluated were isolated from sour cream called "Suero costeño" [7]. We altered the pH of conventional culture media following an experiment design for each microorganism and compared them using ANOVA. We used different conventional culture media for each microorganism according to a previous investigation [11]. This is the first report of pH kinetics and attempt to set points to control pH for LAB isolated from traditional Colombian sour cream.

Materials and Methods

Lactic Acid Bacteria (LAB) Strains

The LAB strains used in this work were *Lactobacillus plantarum* 60-1, *Streptococcus infantarius* 46-3, and *Leuconostoc mesenteroides* 67-1 obtained from the culture collection of the Biotransformation Research Group at Universidad de Antioquia, Colombia. The strains were isolated from sour cream and were identified by morphological, physiological, biochemical, and

molecular (16s rDNA sequence analysis) methods, as previously published [7]. The strains were preserved at -20 °C in de Man Rogosa and Sharpe broth (MRS; Oxoid, United Kingdom) or M17 broth (Oxoid, United Kingdom) with 30 % (v/v) glycerol (2 ml cryovials) [12].

Strain activation. The strains were activated as follows: i- The strains were thawed at room temperature, ii- then, 50 μl were plated in two different types of agar, MRS or M17 agar. *Lactobacillus plantarum* 60-1 and *Leuconostoc mesenteroides* 67-1 were plated in MRS at 32 °C, and *Streptococcus infantarius* 46-3 was plated in M17 at 37 °C. iii- All three strains were grown for 48 h in a WTC incubator (Binder, United Kingdom) under anaerobic conditions using a GasPak system (Becton Dickinson, USA). iv- A single colony of each strain was picked up from the plate and inoculated into 5 ml of broth (MRS or M17 broth as per Table 1) and incubated at the specific temperature for 20-24 hours until stationary phase was reached (1st generation). v- Next, 300 μl of cell culture (1st generation) were subcultured in 15 mL of fresh broth (MRS or M17 broth as per table 1) and incubated at the temperature indicated for each microorganism until the cells reached the early stationary phase of its growth curve (2nd generation). The second generation was used as inoculum for the experiments. **Table 1** presents the conditions [7, 12]. The optical density (OD600) concentration of the second generation of all cells was standardized at 0,8 ± 0,1 (approx. 1×10^8 UFC/ml) in a Spectronic spectrophotometer 3w2R179001 genesys 2PC (Thermo Fisher, USA).

LAB kinetic growth with varying initial pH values

To determine the kinetic growth of each microorganism during batch fermentations, the initial pH from the cell culture medium (MRS or M17) were adjusted to 4, 5, 6, or 7. Afterward, aliquots (5 ml) of each inoculum (obtained as described in the last section) were placed into glass flasks with 45 ml of MRS or M17 broth. After incubation in microaerophilic atmosphere, at the corresponding temperature (see table 1), cells growing in an incubator shaker (Model G24, New Brunswick Scientific, USA) for 24 hours, the time when the fermentation was over.

Microbial growth was assesed at 24 hours by optical density and Colony-forming unit (CFU) methods [13]. Optical densities were measured at a wavelength of 600 nm, using a Spectronic Genesys 2 PC spectrophotometer. To determinate colony-forming units, serial decimal dilutions were made in PBS solution and plated in duplicate onto selective

Table 1. LAB strain growth conditions.

Strain	Culture medium	Temperature °C
Leuconostoc mesenteroides 67-1	MRS	32
Streptococcus infantarius 46-3	M17	37
Lactobacillus plantarum 60-1	MRS	32

media (see table 1). The LAB were enumerated by the drop count technique and reported CFU/ml. Microbial counts were transformed to logarithmic reduction using Eq. 1:

$$CFU/ml = log\frac{N \cdot DF}{V} \quad (1)$$

$log(N/N_0)$, where, N is the number of colonies, DF is the dilution factor and V is the volume of the culture plate.

pH measurement during fermentation

Variations in pH value were measured every 2 hours after inoculation (at 0 hours) during the first 12 hours. Also pH were recorded at the end of the fermentation (at 24 hours). The pH meter used was a PH-20II multi-parameter pH meter and HANNA electrodes.

All of the LAB strains were exposed to different initial pH values. The pH evaluated were 4.0, 5.0, 6.0, and 7.0. To adjust the pH two different buffer solutions were employed. To achieve intitial pH values of 4.0 and 5.0 an acetate buffer was prepared; and for initial pH values of 6.0 and 7.0 a phosphate buffer was prepared.

Δ pH measurement. In order to calculate "delta" pH, we used Eq. 2:

$$\Delta pH = pH_{initial} - pH_{final} \quad (2)$$

where, pH initial is the pH value at 0 hours, and pH final is the pH value at 24 hours

Statistical analysis

The effects of the initial pH and types of microorganisms on growth, expressed as colony-forming units (CFU/ml), were evaluated by analysis of variance (one-way ANOVA) and Tukey's multiple comparison test. Moreover, the tests of Shapiro-wilk and Barllet were permormed on the dataset, in davance, in order to ensure an accurated parametrical statistical analisys. All test was run with 95 % of statistical confidence. The tests were performed using R Software (version 3.4.4- 2018). The data were obtained from two independent trials and each analysis was made triplicate.

Results

The growth of the three LAB strains evaluated at four different pH conditions is shown in **Fig. 1**. Growth was expressed as colony forming units (CFU/ml). For *L. mesenteroides* 67-1, the highest growth was between pH 4 and 5 with values of 10.7 and 11 CFU/ml respectively, while at pH 6 and 7 the growth was lesser, with values of 9.18 and 9.02, respectively. The growth for *S. infantarius* 46-3 was similar at pH 5.0 (10.26 CFU/ml) and 6.0 (10.21 CFU/ml), but different at pH 4.0 (9.17 CFU/ml) and 7.0

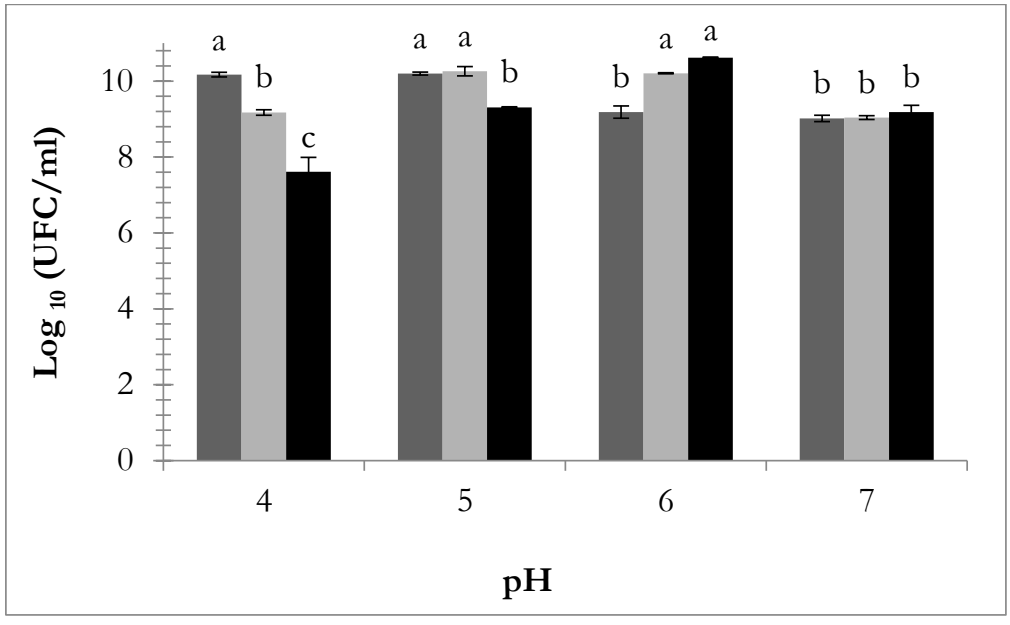

Figure 1. Changes in cell viability of three LAB strains at different pH values. Bar graph showing *Leuconostoc mesenteroides* 67-1 (dark gray), *Streptococcus infantarius* 46-3 (ligth gray) and *Lactobacillus plantarum* 60-1 (black). The letters (a, b and c) represent the different statistical groups.

(9.04 CFU/ml). Finally, the best growth for *L. plantarum* 60-1 was obtained at pH 6.0 (10.62 CFU/ml), whereas at pH 4, 5 and 7 the growth obtained was 7.61 CFU/ml, 9.31 CFU/ml and 9.18 CFU/ml respectively. We run four test on the obtained dasaet, sumarized in Fig. 1. The data was normally distrubuted (Shapiro-Wilk, p-value of 0.2909). The Bartlett test of homogeneity of variances revelaed variance homogeneity with p-value of 0.1862. Both results allowed us to proceed with ANOVA and Tukey tests. The p-value obtained from the ANOVA test was 4.02e-9, indicating significant diffenrences between treatments. The Tukey test revelaed three statistical groups.

The pH kinetics of the three LAB strains were evaluated during 12 hours (**Fig. 2**). In the case of *L. mesenteroides* 67-1 (Fig. 2A), a rapid drop in pH within the first 12 hours for all initial pH values was observed. In contrast to *L. mesenteroides* 67-1, for *S. infantarius* 46-3 not all of the initials pH values dropped rapidly; it took 12 hours for the initial pH of 7.0 to decrease in about one unit (Fig. 2B). Finally, the pH kinetics of *L. plantarum* 60-1 were similar to those of *L. mesenteroides* 67-1; all the initial pH values had decreased rapidly at 12 hours (Fig. 2C).

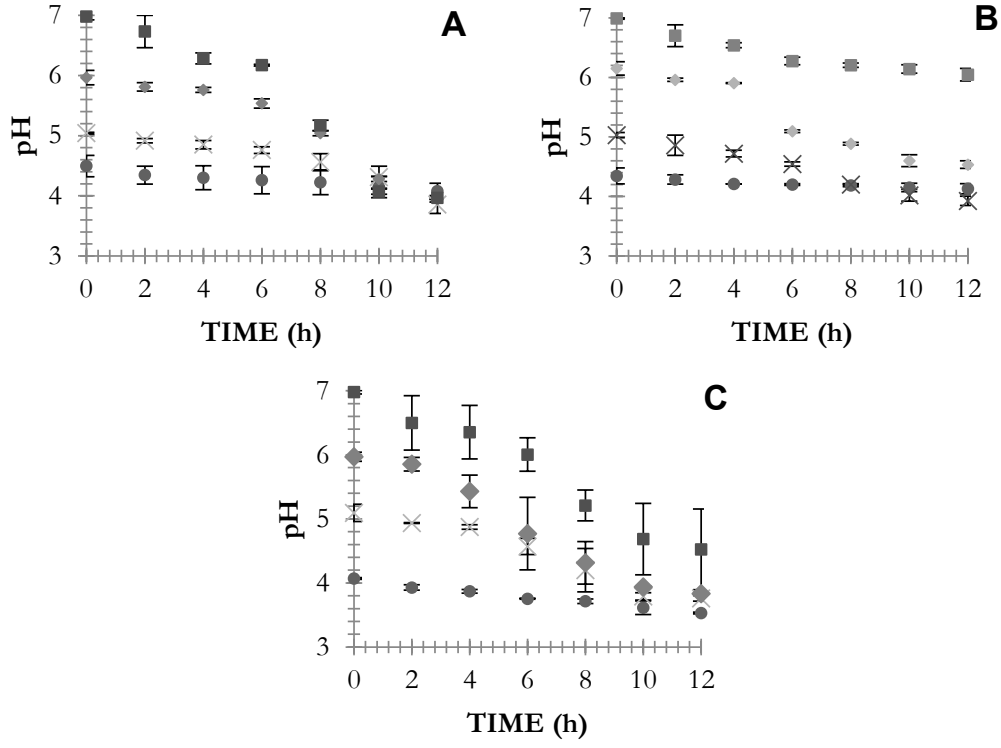

Figure 2. Changes in pH throughout 12 hours for different initial pH values: pH 4 (•), pH 5 (×), pH 6 (♦), and pH 7 (■) for three lactic acid bacteria strains. A) *Leuconostoc mesenteroides* 67-1; B) *Streptococcus infantarius* 46-3; and C) *Lactobacillus plantarum* 60-1.

Fig. 3 shows the final values of three LAB strains from different initial pH values evaluated. The end pH values were mainly similar for each microorganism. According to the Shapiro-Wilk normality test (p-value 0.4387) and the Bartlett test of homogeneity of variances (p-value 0.9616), the data exhibited a normal distribution and variance homogeneity. The subsequent ANOVA and Tukey test (95 %) revealed significant diffenrences between treatments (ANOVA, p-value = 3.92 e-12) and five statistical groups, as idiecated by the letters a – d on top of the bars in fig. 3.

The pH values at 24 hours for *L. mesenteroides* 67-1 were not statistically different aming the four intial pH treatment; all share the c-d-e group label. For this strain, the initial pH values of 4, 5, 6, and 7 dropped by 9.34 %, 23.51 %, 33.95 %, and 43.19 % at 24 hours, respectively. In the case of *S. infantarius* 46-3, the observed pH change at 24 hours was similar for bacteria grown at the intial pH values of 4 and 6 (statistical group b). The pH drop at 24 hours for the bacteria grown at an initial pH of 7 (statistical group a) stood out from all comparisons in Fig. 3; for this LAB strian, the initial pH values of 4, 5 6, and 7 dropped by 5.75 %, 23.56 %, 33.25 %, and 14.23 % at 24 hours, respectively. For *L. plantarum* 60-1 the different statistical groups were at pH 4 (e), pH 5 (d-e), pH 6 (c-d-e), and pH 7 (b-c) (Fig. 3). For this LAB strain, the initial values for pH of 4, 5, 6, and 7 dropped by 13.88 %, 28.07 %, 38.11 %, and 43.62 % at 24 hours, respectively.

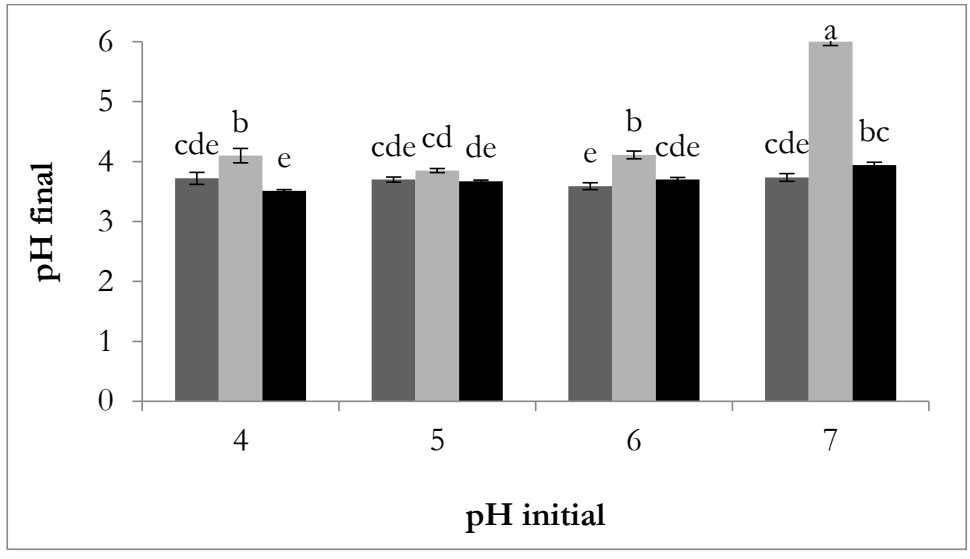

Figure 3. Final (at 24 hours) pH values for three LAB strains grown with initial pH values of 4, 5, 6, and 7. *Leuconostoc mesenteroides* 67-1 (dark gray), *Streptococcus infantarius* 46-3 (ligth gray), and *Lactobacillus plantarum* 60-1 (black). The letters (a, b, c, d and e) represent the different statistical groups.

The magnitudes of observed ΔpH values were independent of the intial pH values but tended to be starin-related. Results of the ΔpH assesment revealed *S. infantarius* 46-3 as the microorganism experienceing the least pH change compared to *L. mesenteroides* 67-1 and *L. plantarum* 60-1 (**Table 2**). For the initial pH values of 4.0, 6.0, and 7.0, the largest ΔpH were observed in *L. mesenteroides* 67-1, whereas for an intial pH of 5.0 the largest pH change was observed in *L. plantarum* 60-1.

Table 2. Values of pH change (ΔpH) for the three tested LAB strains.

Strain	Δ pH			
	4	5	6	7
Leuconostoc mesenteroides 67-1	0.8	1.3	2.4	3.2
Streptococcus infantarius 46-3	0.3	1.2	2.0	1.0
Lactobacillus plantarum 60-1	0.6	1.4	2.3	3.0

Discussion

All of the three LB strains experienced growth differently under the four pH conditions tested. *Leuconostoc. mesenteroides* 67-1 grew best at initial pH values of 4.0 and 5.0, and at initial pH values of 6.0 and 7.0 this strain grew less (Fig. 1). These results differ from that reported in the Bergey's manual where the optimum pH for *L. mesenteroides* 67-1 is 6.5 [8]. Similarly to the Bergey's manual, [14] found that the best growth for *L. mesenteroides* 67-1 occurs at a pH of 6.5. However, in Drosino and colleagues' work the best yield of biomass and substrate took place at a pH of 5.5. The species *L. mesenteroides* is present in several fermented foods having applications in the dairy industry [15].

The growth of *Streptococcus infantarius* 46-3 differed from that of *L. mesenteroides* 67-1. The largest growth occurred at pH of 5.0 and 6.0, whereas at pH 4.0 and 7.0 the growth was lesser. Similarly to our results,

Yuwono *et al*. [16] found that the productivity of l-lactic acid and the specific growth rate for *Streptococcus bovis* was at an optimum pH of 5.5. Contrary to our results, Beal *et al*. [17] found that *Streptococcus thermophilus* grows best at a pH of 6.5 and 40 °C.

The genus *Streptococcus* includes several species with variable behavior, applications, and safety. *Streptococcus* species (and strains) are chiefly reported as pathogenic, and a method to differentiate pathogenic from nonpathogenic strains is still to be developd [18]. In recent studies, *Streptococcus* strains have been isolated from fermented foods. Campanero *et al*. [19] isolated *Streptococcus infantarius* subsp. *infantarius* LP90 from Venezuelan water-buffalo milk and characterized it as avirulent and with broad and strong anti-pneumococcal spectrum. Similarly, Jimenez *et al.* [20] used a strain of *Streptococcus infantarius* isolated from Pozol, a traditional fermented Mexican beverage, to produce a pectin-gellan film against *Escherichia coli*, *Staphylococcus aureus*, and *Listeria monocytogenes*. Dominguez *et al.* [21] disolated the strain *Streptococcus infantarius* subsp. *infantarius* 25124 also from Pozol in order to evaluate the effect of acid and alkali stresses on the strain in commercial APT broth. *Streptococcus infantarius* has been found in traditional fermented dairy products from Africa such as fènè, mala, roab (cow milk), gariss, and suusac (camel milk) [22]. In this study, we evaluated an isolated *S. infantarius* 46-3 from a Colombian sort of sour cream called "Suero costeño" based on their potential as starter culture [7].

Finally, the best growth pH for *Lactobacillus plantarum* 60-1 was at an initial pH of 6.0, at initial pH values of 5.0 and 7.0 no statistically significant differences were detected, and at an initial pH of 4.0 cell growth was inhibited. Fu *et al.* [23] studied the effect of pH on the growth of *L. plantarun*, their results also found that at a pH of 4.0 cell growth was inhibited, while at pH values between 5.0 and 7.0 *L. plantarum* 60-1 had a rapid growth. According to the Bergey's manual *L. plantarum* is aciduric and its optimum pH for growth is between 5.5 and 6.2 [24].

We found differences in the pH kinetics for each strain evaluated. However, at 12 hours of fermentation the three LAB strains dropped the pH of the culture medium below 4.5 (except for *S. infantarius* 46-3 at pH 7.0). The main product of LAB growth is lactic acid. Lactic acid accumulating during a fermentation lowers the pH of the culture medium [25]. If a bacterial isolate is studied as a potential starter culture, a drop in pH, after inoculation, must be rapid. This inhibitings other bacterial growth [26]. This characteristic was observed in the three LAB strains evaluated. Therefore they can be

considered for further investigations as possible candidates for industrial and gastronomic processes, other than the current production of sour cream "Suero costeño".

In the present work, eventhough almost all of the intial pH values dropped, final cell counts varied. At an initial pH of 4.0, the three isolates showed statistically significant differences in growth; while at pH values of 5.0 and 6.0, growth was optimal in *L. plantarum* 60-1 and *L. mesenteroides* 67-1, respectively. Finally, at a pH of 7.0 there were no growth differences among strains. These variable outcomes are due to LAB ability to tolerate stress conditions given the differences between their physiological pH and the pH of the culture medium [27]. Cell growth could be affected by product inhibition. Lactic acid produced during fermentation could be present in the dissociated or undissociated forms. When internal pH is farther away from that of the medium, product inhibition effect increases. [23]. However, by controlling the culture medium pH for each microorganism, the inhibition for growth could be minimized [16].

Our uncontrolled pH kinetic study for 24 hours indicates that, in all experimnets but one, regardless of the initial pH, the strains evaluated can reach a specific final pH close to the pKa of lactic acid (3.86), which is the main product of fermentation [23]. This final pH obtained is due to the release of lactic acid [26], [28]. Lactic acid production is desirable because it can help preserve foods and could inhibit the growth of other [29]–[31].

The tolerance of LAB studied at different pH and the high cellular concentration found, indicate a great potential for the use of *L. mesenteroides* 67-1, *S. infantarius* 46-3, and *L. plantarum* 60-1 in lactic acid fermentations for industrial applications.

We estimated pH change (ΔpH) values. As we expected, when the initial pH was lower, the Δ pH value was also small. Larger Δ pH values indicate that there was a higher production of lactic acid. Moreover, product inhibition on LAB growth could be due to changes in the culture's pH. Lactic acid could be present in dissociated and undissociated forms, however, undissociated organic acids can inhibit growth in a fermentation process [23].

Conclusions

This is the first report in Colombia about the effect of different culture pH values on the growth of lactic acid bacteria strains from sour cream. The pH is a key environmental parameter that affects the growth of the LAB strains

studied. We found, with 95 % confidence, that growth is pH-dependent for the three LAB strains evaluated (Fig 1). The Optimum pH for cell growth was found at 4.5 (± 0.5) for *L. mesenteroides* 67-1, at 5.5 (± 0.5) for *S. infantarius* 46-3, and 6.0 (± 0.5) for *L. plantarum* 60-1. In almost all pH kinetics experiments the final pH was below 4.11 except for *S. infantarius* 46-3 at initial pH 7.0, this value is far away from the pKa of lactic acid (3.86).

The ability to grow at different pH values differed between the tested LAB strains. This may condition strain dependence relative to acid adaptation. Acid stress influences cells in a dynamic manner, namely limiting or boosting cell growth and lactic acid production. Fermentation times should all be taken into account when investigating the effects of pH on growth [32]. A low growth pH is desirable since the maintenance coefficient is positively related to pH. In addition, if bacteria grow better at a low pH, this minimizes the risk of contamination with other microorganisms. Another advantage of growing at low pH is in the application of LAB in foods, which are usually prepared at acidic pH values.

Our results constitute ground to develop a bioprocess to enhance biomass production of the three LAB strains tested, closely controlling pH. Additionally, the lactic acid bacteria strains evaluated have a good potential as starter cultures in fermented food products, other than the sour cream called “Suero costeño”.

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Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent arrangements), or non (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Efecto del pH en el crecimiento de tres cepas de bacterias acidolácticas aisladas de crema agria

Resumen: Las bacterias acidolácticas (BAL) tienen un importante papel en la industria alimenticia, dado que se utilizan en la producción de alimentos fermentados. Para poder utilizar estos microorganismos en la industria, es necesario obtener una gran cantidad de biomasa. Uno de los factores ambientales más importantes en el crecimiento de BAL es el pH. Muchas especies de BAL pueden tolerar un pH inferior a 5.0. Sin embargo, es de esperarse que un pH subóptimo limite el crecimiento de las BAL. Por esta razón, las cepas de BAL *Leuconostoc mesenteroides* 67-1, *Lactobacillus plantarum* 60-1 y *Streptococcus infantarius* 46-3, aisladas de crema agria, se hicieron crecer en medio de cultivo bajo cuatro valores diferentes de pH inicial con el fin de determinar el valor óptimo de pH para su crecimiento. El crecimiento se determinó por medio de unidades formadoras de colonia (UFC/ml). Se encontró que el crecimiento de cada cepa de BAL se afectó por el pH del medio de cultivo. Se determinó que los pH óptimos para *Leuconostoc mesenteroides* 67-1, *Streptococcus infantarius* 46-3, and *Lactobacillus plantarum* 60-1 fueron de 4.5 (± 0.5), 5.5 (± 0.5) y 6.0 (± 0.5), respectivamente. De esta manera, se concluyó que el crecimiento de estas cepas de BAL depende del pH ($p < 0.05$).

Palabras clave: *Leuconostoc mesenteroides*; *Lactobacillus plantarum*; *Streptococcus infantarius*; pH; crecimiento bacteriano; bacterias acidolácticas.

Efeito do pH no crescimento de três cepas de bactérias ácido láticas isoladas de creme azedo

Resumo: As bactérias ácido láticas (BAL) tem um papel importante na indústria de alimentos, dado que são utilizadas na produção de alimentos fermentados. Para poder utilizar estes microrganismos na indústria, é necessário obter uma grande quantidade de biomassa. Um dos fatores ambientais mais importante no crescimento de BAL é o pH. Muitas espécies de BAL podem tolerar um pH inferior a 5,0. Não obstante, é de se esperar que um pH subótimo limite o crescimento das BAL. Por este motivo, as cepas de BAL *Leuconostoc mesenteroides* 67-1, *Lactobacillus plantarum* 60-1 e *Streptococcus infantarius* 46-3, isoladas de creme azedo, foram cultivadas em meio de cultura em quatro valores diferentes de pH inicial, com a finalidade de determinar um valor ótimo de pH para seu crescimento. O crescimento se determinou por meio da unidades formadoras de colônia (UFC/mL). Se encontrou que o crescimento de cada cepa de BAL foi afetado pelo pH do meio de cultivo. Determinou-se que os pH ótimos para *Leuconostoc mesenteroides* 67-1, *Streptococcus infantarius* 46-3, e *Lactobacillus plantarum* 60-1 foram 4,5 (± 0,5), 5,5 (± 0,5) e 6,0 (± 0,5), respectivamente. De esta maneira, se concluiu que o crescimento dessas cepas de BAL depende do pH ($p < 0,05$).

Palavras-chave: *Leuconostoc mesenteroides*; *Lactobacillus plantarum*; *Streptococcus infantarius*; pH; crescimento bacteriano; bactérias ácido láticas.

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