

Cell lysis induced by nisin in sensitive strains of *Lactococcus lactis*: *in vitro* and *in situ* studies

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Abstract

Nisin is a bacteriocin that produces pores in the cell wall, and releases the intracellular material. The present work aimed to evaluate the cell lysis of *Lactococcus lactis* subsp. *lactis*, by either adding nisin directly in the medium or by adding a nisin-producing bacteria, developed in two studies. The *in vitro* study assessed 19 strains of *L. lactis* for their ability to metabolise citrate and diacetyl, and their sensitivity to nisin and NaCl. In the *in situ* study, the effect of nisin on the cellular lysis of *L. lactis* during the ripening of Chihuahua cheese was analysed. Three different types of cheese were manufactured and monitored for 90 days: a control cheese made with a commercial dairy starter culture, a cheese made with a nisin-producing bacteria and nisin, and a cheese without nisin but made with nisin-producing bacteria. The *in vitro* study showed that ten out of 19 strains tested were resistant to the action of nisin, and nine were sensitive. It also revealed that different concentrations of NaCl influenced the lytic effect of nisin in *L. lactis*. The *in situ* study showed that cell lysis remained constant during the ripening of all cheeses.

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Introduction

Chihuahua cheese is a traditional Mexican cheese with a texture ranging from semi-hard to soft, typically undergoes maturation by microorganisms under controlled conditions of time, temperature, and humidity (NOM, 1994a). The formation of flavour in cheese is the result of a complex balance between hundreds of volatile and non-volatile compounds originating mainly during its maturation (Fernández-García *et al.*, 2004; Smit *et al.*, 2005).

Lactic acid bacteria (LAB) are starter culture widely used to enhance desired metabolites (Kadyan *et al.*, 2021) as they are the primary source of enzymes involved in the biochemical transformation of milk components into flavour and odour compounds (Yvon and Rijnen, 2001; Garde *et al.*, 2003), mainly those involved in proteolysis (Rojas-González *et al.*, 2022). The capacity for lysis, acidification, proteolytic activity, and production of flavour compounds (Eman *et al.*, 2000) such as diacetyl (the smell of butter) resulting from the

degradation of citrate (Smit *et al.*, 2005), are the parameters to be taken into account for the industrial selection of LAB strains for cheese production (Boutrou *et al.*, 1998).

Cheese maturation is a long and expensive biochemical process, during which lysis occurs in LAB, releasing its intracellular enzymes. LAB are the source of proteinases, peptidases, esterases, and carbohydrate-catabolising enzymes (Garde *et al.*, 2003). Cell lysis by the action of autolysins and the activity of lytic bacteriocins exposes the intracellular material allowing a higher number of reactions to be carried out (Da Silva Oliveira *et al.*, 2024). The bacteriocins produced by LAB are natural compounds with great potential as food biopreservatives (Villarreal *et al.*, 2024) such as nisin (Rajan *et al.*, 2025). Bacteriocins are biologically active proteins or protein fractions that function as antimicrobials with a very narrow spectrum of action, primarily against Gram-positive bacteria, to which the producing strains are immune (Centeno *et al.*, 2002; Lorestanizadeh *et al.*, 2025). The bacteriocins

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of LAB have a molecular weight of 3 - 10 kDa, and are very diverse in their range of inhibitory activity, biochemical properties, and genetic determinants. The mechanisms of action of bacteriocins include permeabilisation and disruption of the cytoplasmic membrane, leading to cell lysis. This effect promotes the rapid effluent of small metabolites, ions, and cytoplasmic solutes to the outside of the cell (De Martinis *et al.*, 2002).

Nisin is a lantibiotic bacteriocin produced by certain strains of *Lactococcus lactis* subs. *lactis* (Sen *et al.*, 2023; Cui *et al.*, 2025), a peptide of 34 amino acids of amphipathic character, with a molecular weight of 3,510 D, and with activity against Gram-positive bacteria (Mitra *et al.*, 2005). Nisin is a post translationally modified antimicrobial peptide that can induce its own biosynthesis (Khusainov *et al.*, 2015). There are many types of nisin (A, E, F, G, H, L, M, O, P, Q, R, and Z) that differ in structure; nisin A and its natural variant, and nisin Z are the two most commonly used. These variants differ by the substitution of histidine by asparagine at position 27 of its structure (Da Silva Oliveira *et al.*, 2024). Nisin was first discovered in 1928 by Rogers and Whittier (Reiners *et al.*, 2020), and since 1960 has been used as an antimicrobial (Gill and Holley, 1999). It was the first FDA-approved antimicrobial peptide (Zhang *et al.*, 2024), and a natural food preservative generally recognised as safe (GRAS) since 1988 (Reiners *et al.*, 2020). The Joint Food and Agriculture Organisation / World Health Organisation (FAO/WHO) allow the use of nisin in cheese at a concentration maximum of 0.0025%, and it is currently the most studied (Fernández-Pérez *et al.*, 2018).

Therefore, the present work aimed to evaluate the cell lysis of *Lactococcus lactis* subsp. *lactis*, by either adding nisin directly in the media or by adding a nisin-producing bacteria.

Materials and methods

Nineteen strains of *Lactococcus lactis* subsp. *lactis* were isolated and identified in previous studies (Gutiérrez-Méndez *et al.*, 2008; Montes and Rodríguez-González, 2010). The codes and the description of each strain are presented next: (i) eleven strains from commercial starter cultures: CZ01ca, CZ02a, EZ02a, EZ03b, KK05, KK01, Li, Lp, PK04, MA16, and MM10; (ii) two reference strains: ATCC 11454 and FCVSBS-10; (iii) one strain isolated from alfalfa (*Medicago sativa*), Alf-2;

(iv) one isolated from beets (*Beta vulgaris*), BB07; (v) one isolated from green beans (*Phaseolus vulgaris*), EJ06; (vi) one isolated from requesón (fresh cheese), RQ07; and (vii) two isolated from commercial Chihuahua cheese, C272 and De01ba. All strains were maintained in M17 medium supplemented with 40% glycerol (v/v) at -20°C before their corresponding analysis.

In vitro study

i) Evaluation of citrate fermentation

The biochemical test of citrate fermentation in each strain of *Lactococcus lactis* subsp. *lactis* was performed using the medium developed by Kempler and McKay (1980). Once the medium was prepared, each strain (preincubated) was inoculated under carboxyphilic conditions at 37°C for 48 h. After incubation, the growth of the colonies was observed; colonies showing a Prussian blue colour were considered citrate positive, while white colonies were considered citrate negative; and the brown colonies were considered as poor citrate fermentation.

ii) Evaluation of diacetyl production

The capacity to produce diacetyl was evaluated following the Franciosi *et al.* (2009) methods. The inoculated commercial UHT milk was incubated for 24 h at 37°C under carboxyphilic conditions with each strain preincubated. After the milk was fermented, 0.5 mL of α -naphthol (1%, w/v) and 0.5 mL of KOH (16%, w/v) were added. Finally, the tubes were incubated at 37°C for 30 min. The formation of a red ring on the liquid surface was considered indicative of diacetyl production; while the absence of the red ring indicated that the strain was not a producer of diacetyl.

iii) Sensitivity to nisin action

The sensitivity to nisin was preliminarily evaluated using the spectrophotometric method of Piraino *et al.* (2008). Two concentrations of nisin (0.00125 and 0.0025%) were chosen, these represent the maximum allowed by the Mexican standard (NOM, 1994a) and by the United States Food and Drug Administration (FDA). Each of the strains was inoculated in 10 mL of M17 broth, and incubated for 24 h at 32°C under carboxyphilic conditions. The broths were then centrifuged at 2,000 g at 4°C for 10 min. The supernatant was decanted, and the cells were suspended in sterile phosphate buffer (pH 7), and twice washed. From the suspension of cells, 240

μL was deposited in the wells of a microplate. Subsequently, 10 μL of a concentrated solution of nisin was added so that it reached the desired concentration. Each strain was incubated at 30°C in constant agitation, and evaluated in quintuplicate at 610 nm for 18 h (BIOTEK EL-808, USA).

iv) Effect of salt concentration on nisin action

The percentage of lysis was calculated according to Piraino *et al.* (2008). Two resistant and two sensitive strains to nisin were selected to carry out studies on the salt concentration with the following treatment: M17 broth was inoculated with the corresponding strain for 24 h under carboxyphilic conditions. It was centrifuged at 4°C, 2,000 g for 10 min, two washes were performed with citrate buffer (50 Mm) pH = 5.4 sterile (the pH was adjusted with lactic acid) (Boutrou *et al.*, 1998). The obtained cells were suspended in the same buffer using four times less volume of the broth to concentrate. NaCl at 0.17 mol/L or 0.51 mol/L was added as appropriate and homogenised manually; this cell suspension was considered as crude cell extract (CCE). Subsequently, the microplate was filled with 336 μL of the CCE of each strain, also adding to each well 14 μL of a concentrated nisin solution in such a way that the final volume of each well was 350 μL, with a nisin concentration of 0.00125%. As a target, 50 mM citrate buffer pH 5.4 was used. The microplate was incubated for 48 h at 37°C, and optical density readings were taken at 610 nm every 1.5 h. Additionally, the degree of cell lysis in the CCEs was determined according to Wittenberg and Angelo (1970), by measuring the changes in the activity of the intracellular enzyme lactate dehydrogenase, E.C. 1.1.1. 27 (LDH) for 48 h of incubation at 37°C. LDH catalyses the reduction of pyruvate by NADH (nicotinamide adenine dinucleotide in reduced form), obtaining lactate and NAD⁺, with the following reaction: Pyruvate + NADH + H⁺ → LDH → Lactate + NAD⁺. The catalytic concentration was determined from the disappearance rate of NADH at 340 nm for 5 min using a spectrophotometer (Perkin Elmer UV/VIS Lambda 25, USA). The molar extinction coefficient of NADH is 6,220 M⁻¹ cm⁻¹. The activity obtained was expressed as μmol/mL/min for mL of crude cell extract; or as U/mL of extract (U = international unit of activity, defined as the amount that catalyses the formation of 1 μmol of product per minute under defined temperature conditions).

In situ study

i) Preparation of cultures and reagents

The strains selected for the *in situ* study included two nisin-sensitive strains (EZ02 and RQ07) and two resistant strains (BB07 and FCVSBS-10, the last a producer of nisin). Before the use of the cultures in the production of cheese, the cultures were kept at -20°C in M17 broth with 40% glycerol. Before cheesemaking, 100 mL of reconstituted milk powder 10% (v/v) was sterilised, cooled to 37°C, and each of the strains was incubated for 24 h. In the case of the control cheese, it was added with the commercial culture of Danisco MM101, with *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar *diacetylactis*, and *L. lactis* subsp. *cremoris*.

ii) Production of Chihuahua cheeses

Three types of Chihuahua cheeses were produced, two of which were added with dairy starter by the *L. lactis* strains selected in the *in vitro* study, (two sensitive to nisin and two resistant, including a nisin-producing bacterium). The first cheese was added only with the culture selected (CS), another added with the same culture and nisin (Sigma Aldrich) at 0.00125% (CSN), and finally the control cheese (CC) with a commercial culture of *L. lactis* (Danisco MM101). The production of the cheeses was carried out in triplicate. As a first step, the milk was pasteurised at 65°C for 30 min. Subsequently, the milk was cooled to 37°C and calcium chloride (CaCl₂) was added at a rate of 0.2 g/L. Once the milk had been inoculated with the respective culture, the renin (strength 1: 35,000) was added, and it was left to rest for 30 min so that the clot formed, and it was then cut into cubes of 1 cm³. The cut curd was heated with agitation until reaching 37°C, once the desired texture was obtained, the whey was drained, and the curd was stacked; this procedure was repeated until reaching at pH of 6.0 - 5.8. At this point, the blocks of curd were cut into cubes, and added with NaCl 3%. The salted curd was placed in moulds of 1 kg, and pressed for 12 h, then the cheeses were taken out of the moulds, and a repose was carried out for 1 h at 8°C and 43% relative humidity. The cheeses were finally vacuum packed and stored at 8°C.

iii) Proximate composition analysis

The determination of the contents of fat, protein, lactose, cryoscopic point, non-fat solids, and density in raw milk was carried out in triplicate, using

a milkotester ultrasound system (Master mini, Bulgaria). The acidity of the milk was measured by the alalinometric method (NOM, 2012). The pH of the milk and cheeses was determined according to AOAC (1998) using an electronic potentiometer (Corning, Pinnacle 542, Ny, USA). In the cheeses, the percentage of humidity due to water loss was determined (NOM, 1994b): percent of salt with a salinometer (ATAGO, Japan), fat by the Gerbers butirometric method (NOM, 2010); and proteins by the micro Kjeldahl method of the AOAC (2000).

iv) Determination of LDH activity in cheese

The preparation of the cheese for the determination of LDH was carried out according to Wilkinson *et al.* (1993); by adding 5 g of each cheese in 10 mL of phosphate buffer (pH 7.0, 0.05 M) with sucrose (0.2 M), mixed in the stomacher (Bag Mixer 400, Interscience, France) for 5 min, then 4 mL of the homogenate was taken and centrifuged (Eppendorf 5702R, Alemania) at 10°C for 15 min at 3,000 g. From the supernatant, 2 mL were taken and centrifuged for 10 min at 3,000 g at 10°C. Then, 1 mL of the supernatant was transferred to a tube, and centrifuged again in the same conditions. The supernatant obtained was quantified for the activity of the LDH enzyme as described in the *in vitro* study.

Statistical analysis

The means between the different treatments or samples were compared using One-way analysis of variance (ANOVA) and Tukey's *post hoc* test using 0.05 as *p*-value in the *in vitro* study (evaluation of citrate fermentation and diacetyl production, sensitivity to the action of nisin, and effect of salt concentration on the action of nisin), and in the *in situ* study (proximate composition analysis and determination of LDH activity in cheese). The statistical results were reported as mean and standard deviation of the mean (STD). Minitab^R package (2003 - version 14.12, USA), was used for data processing.

Results and discussion

In vitro study

i) Fermentation of citrate and diacetyl production

From the strains tested, 18 out of 19 were able to metabolise citrate (only Alf2 was negative), while 16 out of 19 strains were diacetyl producers (only CZ02a, EZ02a, and ATCC 11454 were negative). Both diacetyl production and the fermentation of

citrate are relevant to choose as a lactic acid bacteria for cheesemaking. Diacetyl is the compound that is responsible for butter flavour, and it is considered as a pleasant flavour in Chihuahua cheese (Oumer *et al.*, 2001; Van Hekken *et al.*, 2006). Citrate is an intermediate for the generation of volatile compounds that contribute to the pleasant taste and aroma of cheese (Smit *et al.*, 2004). Some *L. lactis* strains are citrate or diacetyl negative since they do not possess the enzymes required for carrying out the following route: the citrate is mediated by the citrate permease system, this compound is then cleaved by the enzyme citritase to oxalacetate and acetic acid, the oxaloacetate is converted to pyruvate, which when in excess, is converted by a series of reactions to diacetyl (Kempler and McKey, 1980).

ii) Sensitivity to nisin action

From the 19 strains analysed, nine were identified as resistant to nisin and ten as sensitive to nisin. Table 1 shows each of the strains with their respective percentages of lysis (after subtracting the autolysis corresponding to each strain monitored at 0% nisin); that is to say, the lysis that is not due to the action of nisin. The strains are presented in increasing order of the lysis presented with 0.00125% nisin; since it was the one that had the most lytic effect on most cells. The strain EJ06 showed the lowest lysis of all strains, even negative values were recorded. This occurred probably because the cells multiplied forming long strings of cocci which increased the optical density instead of reducing it. Similarly, negative lysis values have been reported by other authors in *L. lactis* strains with similar conditions (Boutrou *et al.*, 1998).

On the other hand, from the 19 strains of *L. lactis*, 16 showed maximum lysis at a concentration of 0.00125%; only three strains increased their percentage of lysis with double the nisin concentration (0.0025%). To date, no explanation has been reported for this behaviour, as a higher bacteriocin concentration would be expected to enhance lysis. However, most of the strains evaluated in the present work showed 5 to 20% of lysis due to the action of nisin. However, some strains like the EJ06, MM101, and FCVSBS-10 had less than 5% lysis. Resistance to the action of nisin was expected in strain FCVSBS-10, because this strain is a bacteriocin-producing bacteria. The bacteriocin producing strains are usually resistant to the same bacteriocin (Chen and Hoover, 2003). The

mechanism by which a strain becomes resistant to the action of bacteriocins is not yet clear, but it is believed that this involves: the production of proteases that hydrolyse the bacteriocin; alteration of the bacteriocin by membrane compounds; or modification of membrane receptors, preventing adherence (De Martinis *et al.*, 2002). Additionally, the strains that showed greater sensitivity to nisin

were PK04 and EZ02a. Finally, the selected strains formed the selected culture which consisted of two resistant strains (FCVSBS-10 and BB07), the first a nisin producer and two sensitive strains (EZ02a and RQ07). The selection of these strains considered their stability and overall behaviour observed during kinetics study.

Table 1. Percentage of lysis in strains of *Lactococcus lactis* treated with two different concentrations of nisin and incubated for 48 h at 37°C.

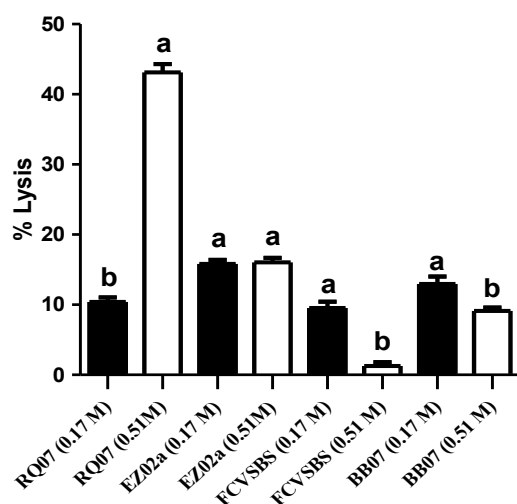
	0.00125% Nisin	0.0025% Nisin
Nisin-resistant strain		
EJ06	-5.63 ± 16.74 ^a	-14.15 ± 11.900 ^b
FCVSBS-10	0.08 ± 1.72 ^a	0.57 ± 2.89 ^b
MM101	1.96 ± 11.93 ^a	2.23 ± 12.93 ^a
ALF-2	7.20 ± 12.31 ^a	5.49 ± 14.54 ^b
MA16	7.40 ± 12.77 ^a	4.46 ± 9.80 ^a
ATCC 11454	7.87 ± 1.72 ^a	6.67 ± 2.89 ^a
KK01	10.41 ± 8.87 ^a	2.34 ± 4.68 ^b
BB07	12.12 ± 4.75 ^a	9.80 ± 4.50 ^b
EZ03b	13.32 ± 16.03 ^a	1.39 ± 12.19 ^a
Nisin-sensitive strain		
Lp	14.45 ± 17.30 ^a	23.43 ± 12.20 ^a
CZ02a	14.95 ± 9.70 ^a	-6.06 ± 6.60 ^a
C272	18.68 ± 7.67 ^a	15.68 ± 4.13 ^a
CZ01ca	18.95 ± 9.53 ^a	8.31 ± 7.23 ^a
Rq07	22.75 ± 11.09 ^a	16.01 ± 7.16 ^a
Li	24.06 ± 1.14 ^a	15.02 ± 11.04 ^b
KK05	24.86 ± 6.67 ^a	15.87 ± 9.22 ^b
DE01b	26.23 ± 3.59 ^a	7.84 ± 12.21 ^b
EZ02a	31.02 ± 3.77 ^a	25.79 ± 8.38 ^b
PK04	36.90 ± 12.29 ^a	32.11 ± 7.92 ^b

Different lowercase superscripts in same row indicate significant differences between concentrations ($p < 0.05$).

iii) Effect of salt concentration on nisin action

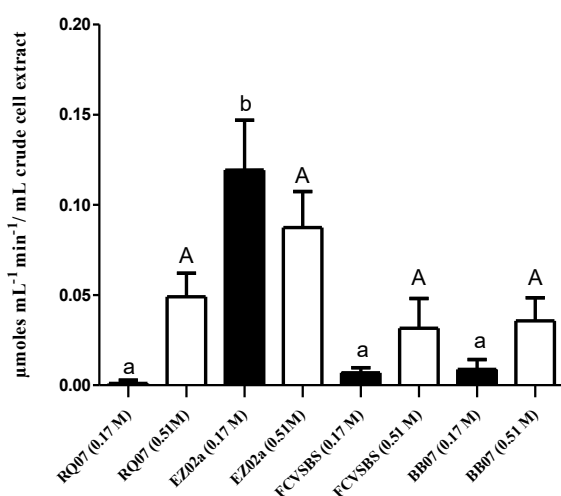
The decrease in the optical density (OD₆₁₀) was used to observe the lysis by effect of the addition of two concentrations of NaCl after 48 h (Figure 1a). On the two nisin-resistant strains (FCVSBS-10 and BB07), a higher percentage of lysis was observed with the lower concentration of NaCl. On the other hand, only one of the sensitive strains (RQ07) increased the percentage of lysis with less NaCl. However, when this strain was exposed to low salt concentration, it took 8 h to initiate cell lysis. This

delay in cell lysis could also be observed in strain EZ02a, but less obviously. So, the addition of NaCl not only increased the amount of lysis by the addition of nisin, but also accelerated the lysis process. Due to the variability typically observed in lysis measurements using the optical density method, cell lysis was also assessed indirectly by evaluating LDH enzyme activity (Figure 1b). It was also established that a higher NaCl concentration increased the lysis effect of nisin on *L. lactis*, probably because Na⁺ and Cl⁻ ions enhanced nisin adherence to the cell wall.



Lactococcus lactis strains (NaCl Concentration)

Figure 1a. Percentage of lysis (OD₆₁₀) observed in four strains of *Lactococcus lactis* after being suspended in 50 mM citrate buffer (pH 5.4), added with nisin (0.00125%) and NaCl, and incubated at 37°C for 48 h; black column treatments are added with 0.17 mol/L NaCl; white column treatments are added with 0.51 mol/L of NaCl. Different lowercase letters indicate significant differences between NaCl concentrations ($p < 0.05$).



Lactococcus lactis strains (NaCl concentration)

Figure 1b. Activity of intracellular lactate dehydrogenase (LDH) enzyme observed in four strains of *Lactococcus lactis* after being suspended in 50 mM citrate buffer (pH 5.4) added with nisin (0.00125%) and NaCl, and incubated at 37°C for 48 h, with significant differences between concentrations ($p < 0.05$). Different lowercase letters indicate significant differences between strains at 0.17 mol/L of NaCl ($p < 0.05$); different uppercase letters indicate significant differences between concentrations at 0.51 mol/L of NaCl ($p > 0.05$).

Similar to the results obtained with OD₆₁₀, the measurement of an intracellular enzyme was evidence of cell lysis demonstrating that increasing the NaCl concentration enhanced cell lysis. The enzymatic activity of LDH was expressed in U/mL of crude cell extract, where 1 unit is the amount of enzyme needed to catalyse the oxidation of 1 μ M of NADH/min per millilitre of supernatant (O'Sullivan *et al.*, 2002). The LDH activity observed in the four strains showed a pattern similar to that observed with changes in OD₆₁₀. Notably, the EZ02a strain did not show a significant difference in enzyme activity with increasing salt concentration. However, the other three strains increased their activity with increasing the salt concentration. The enzymatic activity values of the LDH found in this work were like those reported by other authors such as O'Sullivan *et al.* (2002). These authors evaluated the lysis induced by a bacteriocin in different strains of *L. lactis*, finding enzymatic levels of LDH between 0.3 - 0.76 U/mL in the supernatant of M17 broth. Therefore, based on the results from OD₆₁₀ and LDH release, we can conclude that increasing NaCl concentration enhanced the effectiveness of nisin in inducing cell lysis in *L. lactis*. The anionic lipids of the cytoplasmic membrane (in Gram-positive bacteria) are the main receptors of bacteriocins in the formation of pores. However, other anionic polymers on the surface of the cell, such as the teichoic and lipoteichoic acids, also intervene in the interaction with the cationic bacteriocins (Moll *et al.*, 1999). Changes in the electrochemical properties of the membrane and cell wall by an increase in the ionic strength of the medium can alter the interaction between phospholipids (anionic) and nisin (cationic). It is known, for example, that the change in pH and NaCl concentration influences the degree of autolysis of *L. lactis*. This is mainly due to changes in the electrochemical properties of the cell wall, which promotes an expansion-contraction of the cell wall (Ramírez and Romero, 2010). In addition, the pH reduction of 6.0 to 5.5 increases the effectiveness of nisin to inhibit the production of *Clostridium botulinum* spores (Somers and Taylor, 1972). Although the mode of action is not yet clear, it is possible that the increase in Na⁺ and Cl⁻ ions in the medium improves the solubility of nisin and facilitates the adhesion of the molecule by altering the electrochemical properties of the mobile membrane. Therefore, the effectiveness of the bacteriocin was increased to produce lysis in the cells of *L. lactis*.

*In situ study**i) Proximate composition analysis*

The proximate composition analysis for the milk was: fat, $3.16 \pm 0.2\%$; non-fat solids, $8.37 \pm 0.1\%$; density, 1.028 ± 0.0 g/ml; cryoscopic point, $0.51 \pm 0.0^\circ\text{C}$; protein (soluble nitrogen), $3.25 \pm 0.0\%$; lactose, $4.4 \pm 0.1\%$; and insoluble salts, $0.7 \pm 0.0\%$. On the other hand, the proximal characterisation of all cheeses was performed in triplicate (Table 2), and all the parameters obtained were in accordance with the Mexican standard (NOM, 2010).

It was observed that among the different types of cheese, there was no significant difference in the contents of fat, protein, salt, and moisture. There was only difference in the pH of the different cheeses after 24 h of production. In other words, the bacteria of the commercial culture produced lactic acid more quickly, derived from the fermentation of lactose; the result was a cheese with a more acidic pH. The

addition of nisin in the milk delayed the growth of LAB which caused the cheeses to reach a pH of only 5.94. When the nisin was omitted, the bacteria managed to develop further, which was reflected in a less acid pH (5.77); although this value remained statistically similar to that of the cheese made with nisin. During the processing stage, the cheeses added with nisin or with the nisin-producing bacteria obtained a less acidic pH during the Cheddarisation stage. After the first day of cheese production, it was observed that the control treatment had a more acidic pH than the other two treatments (Figure 2a). However, during the next 90 days of cheese storage at 4°C , all treatments had a similar pH. With respect to the moisture content recorded in each of the cheeses for 90 days (Figure 2b), it was observed that the humidity did not show a significant change during the maturation of the cheese; mainly because the cheeses were vacuum-packed with a plastic film

Table 2. Proximate analysis of Chihuahua cheese made with commercial culture (CC), selected *L. lactis* strains (CS), and selected strains plus nisin (CSN).

	Control cheese (CC)	Cheese with selected culture and nisin (CSN)	Cheese with selected strains (CS)
Fat (%)	19.33 ± 1.15^a	19.00 ± 1.32^a	19.50 ± 1.73^a
Protein* (%)	21.25 ± 0.63^a	21.08 ± 0.28^a	21.16 ± 0.34^a
NaCl (%)	0.80 ± 0.11^a	0.82 ± 0.01^a	0.797 ± 0.06^a
pH**	5.23 ± 0.12^a	5.94 ± 0.16^b	5.77 ± 0.07^b
Humidity (%)	46.19 ± 3.42^a	48.63 ± 0.12^a	47.97 ± 0.39^a

*Soluble nitrogen; **pH 24 h after processing; different lowercase superscripts in same row indicate significant differences between types of cheese ($p < 0.05$).

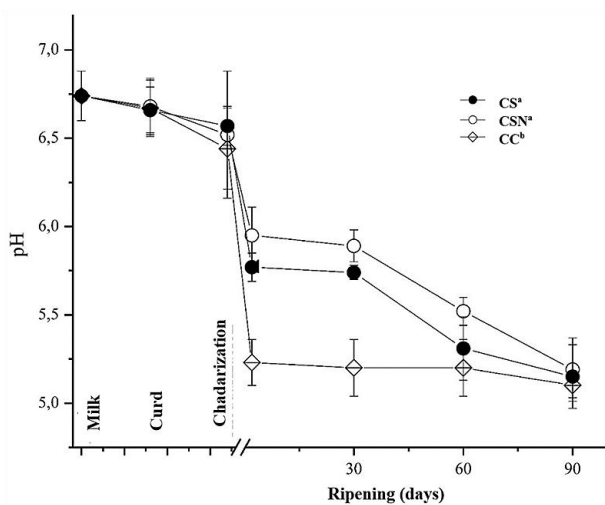


Figure 2a. pH observed of three types of Chihuahua cheese made with commercial culture (CC), selected strains of *L. lactis* (CS), and selected strains plus nisin (CSN), during 90 days of ripening at 4°C .

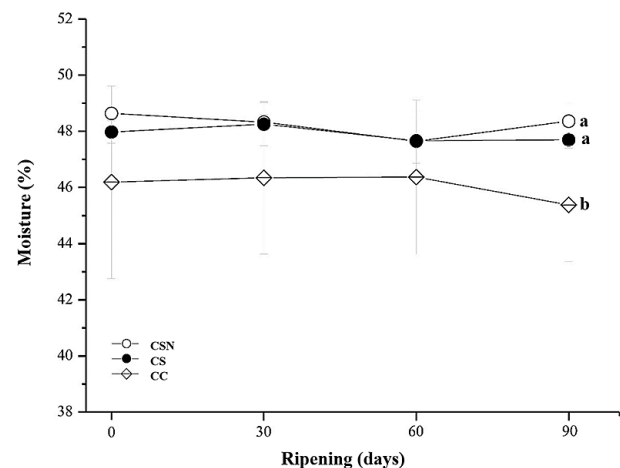


Figure 2b. Percentages of moisture observed of three types of Chihuahua cheese made with commercial culture (CC), selected strains of *L. lactis* (CS), and selected strains plus nisin (CSN), during 90 days of ripening at 4°C . Different lowercase letters indicate significant differences between treatments ($p < 0.05$).

impervious to water vapour. In this way, a significant loss of water was not allowed during the maturation of the cheeses. It was also observed that the control cheese showed slightly lower percentages of moisture than the other two cheeses, although statistically these values were not significant.

ii) Determination of LDH activity in cheeses

Monitoring cell lysis in the cheeses showed that there was no increase in LDH activity during cheese maturation (Table 3). The LDH values observed in Chihuahua cheeses were within the range

reported by Wilkinson *et al.* (1993) for Cheddar cheese with different NaCl treatments (0, 1.2, 2.7, and 5%), where values ranged from 0.0 to 0.15 U/mL of extract during the cell lysis of *L. lactis* subsp. *cremoris*. Additionally, Morgan *et al.* (1997) reported LDH activity of *L. lactis* ranging from 0.0 to 0.15 U/g in Cheddar cheese. On the other hand, the addition of nisin directly into the milk is not recommended, as a large portion of it is lost to the whey. This likely explains why there was no substantial increase in LDH activity.

Table 3. Enzymatic activity of lactate dehydrogenase (LDH) observed in three types of Chihuahua cheese made with commercial culture (CC), selected *L. lactis* strains (CS), and selected strains plus nisin (CSN) during ripening.

Ripening period (d)	$\mu\text{mol/mL/min/mL}$ crude cell extract		
	Control cheese (CC)	Cheese with selected culture (CS)	Cheese with selected culture with nisin (CSN)
1	0.023 $\pm$ 0.05	0.007 $\pm$ 0.01	0.082 $\pm$ 0.13
30	0.024 $\pm$ 0.00	0.012 $\pm$ 0.00	0.013 $\pm$ 0.01
60	0.049 $\pm$ 0.00	0.024 $\pm$ 0.02	0.025 $\pm$ 0.01
90	0.018 $\pm$ 0.01	0.053 $\pm$ 0.02	0.013 $\pm$ 0.03

No significant differences are detected between treatments or ripening periods ($p > 0.05$).

Conclusion

The percentage of lysis in *L. lactis* varied based on the concentration of nisin used for resistant and sensitive strains. Also, when NaCl was added, lysis was enhanced in a variable way based on the concentration of salt, specifically, it was observed that in resistant strains, a greater lytic effect was obtained when 0.17 mol/L was applied. When evaluating the addition of nisin to cheeses, it was observed that the degree of cell lysis remained constant throughout cheese maturation, and no significant differences were found among the *L. lactis* cultures. Therefore, it is recommended to add nisin directly to the curd rather than to the milk.

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