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ACE- Inhibitory and metal-binding activity produced during milk fermentation by three probiotic potential LAB strains isolated from Chiapas double cream cheese

Actividad inhibidora de la ECA y fijadora de metales producida durante la fermentación de la leche con tres cepas BAL potencialmente probióticas aisladas del queso doble crema de Chiapas

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Abstract

During the fermentation of milk with lactic acid bacteria (LAB), bioactive compounds can be generated, including bioactive peptides. The aim of this work was to evaluate the inhibitory activity of the angiotensin converting enzyme (ACE) and metal (calcium and iron) binding activity during milk fermentation with each of the three potentially probiotic LAB strains (*Lactobacillus plantarum*, *Lactobacillus pentosus* and *Lactobacillus acidipiscis*) as well as mixed fermentation. These strains were previously isolated from Chiapas double cream cheese. Fermented milks with *Lb. acidipiscis*, *Lb. pentosus* and mixed culture exhibited the highest ACE-inhibitory activity (97%). Concerning the iron-binding activity, the fermented milks with *Lb. plantarum* and the mixed culture showed the highest activities with 99 and 92%, respectively. For calcium, mixed and *Lb. acidipiscis* fermentation had the highest calcium-binding activity. These results showed that the microorganisms isolated from double cream cheese, especially when used as co-culture at a ratio of 1:1:1, have a great potential to be used in the production of functional fermented foods.

Keywords: Lactic acid bacteria, ACE-inhibitory activity, metal-binding activity, double cream cheese, probiotics potential.

Resumen

Durante la fermentación de la leche con bacterias ácido lácticas (BAL) se pueden generar compuestos bioactivos, incluyendo a los péptidos bioactivos. El objetivo de este trabajo fue evaluar la actividad inhibidora de la Enzima convertidora de la angiotensina (ECA) y fijadora de metales (calcio y hierro) durante la fermentación de la leche con cada una de las tres cepas de BAL potencialmente probióticas (*Lactobacillus plantarum*, *Lactobacillus pentosus* y *Lactobacillus acidipiscis*) así como la fermentación mixta. Estas cepas fueron previamente aisladas del queso doble crema de Chiapas. Las leches fermentadas con *Lb. acidispiscis*, *Lb. pentosus* y el cultivo mixto fueron las que presentaron una mayor actividad inhibidora de la ECA (97%). Con respecto a la actividad de fijadora de hierro, las leches fermentadas con *Lb. plantarum* y el cultivo mixto fueron las que presentaron una mayor actividad con 99 y 92%, respectivamente. Para el caso del calcio, la fermentación mixta y la realizada con *Lb. acidispicis* fueron las que presentaron una mayor actividad fijadora. Estos resultados muestran que los microorganismos aislados del queso doble crema, sobre todo empleados como co-cultivo en una proporción 1:1:1, presentan un gran potencial para ser utilizados en la producción de alimentos fermentados funcionales.

Palabras clave: Bacterias ácido lácticas, actividad inhibidora de la ECA, actividad fijadora de metales, queso doble crema, probióticos potenciales.

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1 Introduction

Lactic acid bacteria (LAB) have been used as starter cultures for fermented food production due to their good antimicrobial compounds production capacity (organic acids, bacteriocins, hydrogen peroxide, among others). In addition, LAB produce compounds that positively impact the texture and flavor characteristics of foods produced by lactic fermentation (Melgar-Lalanne *et al*., 2013; Reyes *et al*., 2018; Mendoza-Avendaño *et al*., 2019). Fermented dairy products are one of the most important kinds of fermented food due to their health-enhancing effects such as anti-inflammatory activities, memory-enhancing, cholesterol reduction, and enhanced absorption of nutrients, including minerals (Buttriss, 1997; Xiang *et al*., 2019). However, there are also other activities related to the production of compounds with biological activity by different LAB strains including the following: antioxidant (Abubakr *et al*., 2012; Aguilar-Toalá *et al*., 2016; Taha *et al*., 2017; Ibrahim *et al*., 2018; Soleymanzadeh *et al*., 2019), antihypertensive (Abdel-Hamid *et al*., 2017; Shu *et al*., 2017; Gonzalez-Gonzalez *et al*., 2019; Soleymanzadeh *et al*., 2019; Mazorra-Manzano *et al*., 2020), antidiabetic (Ayyash *et al*., 2018), immunomodulatory (Aguilar-Toalá *et al*., 2016), metal-chelating (Figueroa-Hernández *et al*., 2012; Guzmán-Rodríguez *et al*., 2019; García-Gamboa *et al*., 2020), opioid (Fernández-Tomé *et al*., 2016), antithrombotic (Rojas-Ronquillo *et al*., 2012; Guzmán-Rodríguez *et al*., 2019), and antimicrobial (Aguilar-Toalá *et al*., 2016; Taha *et al*., 2017). These bioactivities are mainly attributed to the production of specific peptide sequences (bioactive peptides) derived from milk proteins during fermentation by several LAB strains (Rojas-Ronquillo *et al*., 2012; Rodríguez *et al*., 2019; Soleymanzadeh *et al*., 2019; Tagliazucchi *et al*., 2019).

Although bioactive peptides have been identified from several animal and plant sources; milk proteins are the primary source (Fajardo-Espinoza *et al*., 2020). These peptide sequences are produced during milk fermentation by the LAB strains because they have a proteolytic system that allows them to obtain the nitrogen from the milk proteins, mainly casein (Pessione and Cirrincione, 2016; Raveschot *et al*., 2018). The bioactivity produced during the milk fermentation is related to the concentration and sequence of the peptides released, which depends on LAB strain used, fermentation time and fermentation conditions, like pH and temperature (Agyei *et al*., 2016).

On the other hand, among bioactivities related to the production of bioactive peptides during milk fermentation by LAB strains, two of them have a great potential for the production of functional foods and nutraceuticals, antihypertensive and the metal-chelating activities. Antihypertensive activity in dairy products is well-documented and is attributed to the production of milk derived peptide sequences that inhibit the Angiotensin-Converting Enzyme (ACE) activity. This enzyme has an effect on the renin-angiotensin (RAS) and kallikrein-kinin (KKS) systems. These pathways are involved in the production of angiotensin II, a powerful vasoconstrictor and the hydrolysis of bradykinin, a potent vasodilator, leading to a rise in blood pressure. Therefore, inhibiting their activity can have a blood pressure-lowering effect (Chen *et al*., 2010; Shu *et al*., 2017; Panatoya *et al*., 2018; Tagliazucchi *et al*., 2019). Concerning the metal-chelating activity, this is attributed to the release of peptides that contains phosphorylated serine, therefore, they contain a large concentration of negative charges that allow them bind thigh with several divalent minerals, such as calcium, iron, and zinc enhancing their bioavailability (Guzmán-Rodríguez *et al*., 2019). Iron is an essential mineral in human nutrition due it is involvement in several biochemical reactions for electron transfer, cell differentiation and oxygen binding and transportation. The iron deficiency in the human diet can cause immune system dysfunctions (Figueroa-Hernández *et al*., 2012; Hassan *et al*., 2016). On the other hand, calcium is a crucial element for bone structure, muscle contraction, protein structure, and is an enzymatic co-factor. Its deficiency in the human diet can lead to the development of osteoporosis and some types of cancer (Figueroa-Hernández *et al*., 2012; Huang *et al*., 2015).

For the aforementioned and the fact that it can contain health-promoting microorganisms (probiotic potential), cheeses and fermented milks have been extensively studied in recent years (Melgar-Lalanne *et al*., 2019; Xiang *et al*., 2019; Mazorra-Manzano *et al*., 2020). One of the most representative traditional cheeses from southern Mexico is double cream which is produced in the State of Chiapas, Mexico. This cheese has an acidic taste, a creamy sensory attribute and a friable texture (Gonzalez-Gonzalez *et al*., 2019). Morales *et al*. (2011) isolated three strains of LAB (*Lactobacillus plantarum*, *Lb. pentosus*, and *Lb. acidipiscis*) from this cheese. These *Lactobacillus*

strains isolated by Morales *et al*. (2011) from the double cream cheese produced in Chiapas, have also been found in other fermented foods. It has been reported that *Lb. plantarum* can be found in a wide variety of fermented foods, cheeses, and during cocoa fermentation (dos Santos *et al*., 2015; Oguntoyinbo and Narbad, 2015; Behera *et al*., 2018; Figueroa-Hernández *et al*., 2019). In the case of *Lb. pentosus*, it was previously reported as a constituent of different fermented foods microbiota (Argyri *et al*., 2013; Oh and Jung, 2015; Vasilev *et al*., 2015) and it has also been used to produce lactic acid from sugar cane bagasse hydrolysates (González-Leo *et al*., 2020). *Lb. acidipiscis* had previously been found in the microbiota of Thai- fermented fish (Tanasupawat *et al*., 2000); however, in recent years, it has also been reported as part of some traditional Greek cheeses microbiota (Kazou *et al*., 2017).

The probiotic potential of the three BAL strains isolated from the cheese (*Lb. plantarum*, *Lb. pentosus*, and *Lb. acidipiscis*) were evaluated by Melgar-Lalanne *et al*. (2013). In this study the BAL strains isolated from the cheese had compared them with two commercial probiotic strains, *Lb. plantarum* 299v and Lb. casei Shirota. More recently, three highly proteolytic strains (s6-HTCH, s10-AVCH, and s12-AVCH) isolated from double cream cheese showed ACE and high antioxidant activities during milk fermentation (Gonzalez-Gonzalez *et al*., 2019). However, the production of biologically active compounds by *Lactobacillus plantarum*, *Lb. pentosus*, and *Lb. acidipiscis* has not been evaluated. For this reason, the objective of this work is to assess the antihypertensive activity (ACE inhibitory activity) and metal-chelating (calcium and iron) in vitro activity during milk fermentation by these probiotic strains in single and mixed culture fermentations.

2 Materials and methods

2.1 Microorganisms

The three potential probiotic *Lactobacillus* strains: *Lb. pentosus*, *Lb. plantarum* and *Lb. acidipiscis* were previously isolated and identified from Chiapas double cream cheese by Morales *et al*. (2011). Stock cultures were prepared and then conserved by storage in glycerol 30% (v/v) at -20 ºC. LAB strains were grown in MRS broth (Dibico, Mexico) and were incubated at 37 ºC for 24 hours in order to activate the microorganisms. Inoculum for the fermentation was prepared with a culture of 24 hours of each of the strains in 10% (w/v) reconstituted whole milk (Alpura, Cuautitlán Izcalli, Mexico) at 37 ºC. Proximate composition of milk powder for each 100 g was carbohydrate 39 g, protein 26 g, fat 26 g of which 16.9 g corresponded to saturated fat, and approximately 3.5 g of ash.

2.2 Reagents and material

All the reagents employed in this work were analytical grade unless otherwise indicated. Material used in this experimental work commonly found in a microbiology laboratory.

2.3 Milk fermentation conditions

Lactobacillus strains were grown in 10% (w/v) reconstituted whole milk at 37 ºC for 24 hours to inoculate the fermentations subsequently. Fermentations were conducted in 500 mL Erlenmeyer flasks with 300 mL of 10% (w/v) reconstituted whole milk. The milk was sterilized at 120 ºC for 10 minutes. Fermentation media were inoculated with 5% (v/v) of the 24-hours culture of each *Lactobacillus* strains, and for the mixed fermentation, it was inoculated at 1:1:1 ratio of each LAB strain. The concentration of microorganisms in the inoculums are 6.7 ± 0.1 log CFU/mL. Fermentations were performed in duplicate for 48 hours at 37 °C. During the fermentation process, samples were collected every eight hours. Microbial growth was measured by the Miles *et al*. (1938) method and by measurement of the pH decrease. The pH of fermentation samples were adjusted to 9 with addition NaOH (2N), to allow casein solubilization to prevent the loss of casein-derived peptide during the centrifugation. Fermentation samples were centrifuged at 14,000xg for 30 minutes at 4 ºC. The protein concentration of cell-free supernatants (CFS) were determined by the Lowry *et al*. (1951) method with some modifications as reported by Figueroa-Hernández *et al*. (2012), using a standard curve of bovine serum-albumin (1mg mL^{-1}). Results of the protein concentration in the samples of CFS fermentations samples are not shown in the manuscript.

2.4 Determination of the proteolytic activity of LAB strains during milk fermentation

To quantify the release of peptide fragments, the methodology proposed by Adler-Nissen (1979) was used. This spectrophotometric method measures the released free amino groups by the 2, 4, 6-trinitrobenzene sulphonic acid (TNBS) reagent. One milliliter of 0.2125 M phosphate buffer pH 8.2 was mixed with 125 μL of CFS samples, and then the mixture was stirred vigorously and mixed with one milliliter of the 0.1% (v/v) solution of TNBS (Sigma-Aldrich). Reaction mixture was stirred and dark-incubated at 50 ºC for one hour. Two milliliters of HCl (J. T. Baker, Mexico) 0.1N was added to each tube to stop the reaction. The tubes with reaction mixture were allowed to cool, and then their absorbances were read at 340 nm employing a GENESYS 10S Vis Spectrophotometer (Thermo Scientific, USA). A stock solution of L-Leucine (Sigma-Aldrich) 3mM was used to make a standard curve to measure the concentration of free amino groups in the fermentation samples.

2.5 Determination of ACE-inhibitory activity of LAB strains during milk fermentation

To measure the inhibition of the ACE-inhibitory activity of the CFS samples, the methodology proposed by Cushman and Cheung (1971) was used. The pH of each CFS sample was adjusted to 8.3, which is the optimal pH of ACE. An aliquot of 80 μL of CFS samples was mixed with 200 μL of 5mM Hippuryl-Histidyl-Leucine substrate (Sigma-Aldrich, St. Louis, USA) dissolved in borate buffer 0.1 M at pH 8.3 with NaCl 0.3 M, prepared before being used. This mixture was pre-incubated at 37 ºC for 3 minutes, and the reaction was started by the addition of 20 μL of ACE from porcine kidney (0.2 U/mL, Sigma-Aldrich) in phosphate buffer 0.1M (pH 8.3 with NaCl 0.3M). This mixture was shaken for 30 seconds and then incubated at 37 ºC for 30 minutes. The reaction was stopped by the addition of 250 μL of HCl 1N. An aliquot of 1.7 milliliters of ethyl acetate was added to the reaction tubes to extract the formed hippuric acid. The tubes were gently shaken and then centrifuged at 2,490xg for 5 minutes. Four hundred milliliters of the organic phase of each tube was taken and heated in a water bath at 92 ºC for 30 minutes to evaporate the ethyl acetate. The content of each tube was resuspended in one milliliter of distilled water just before reading absorbance at 230 nm. A solution 1:10 of captopril (1mg/mL) was used for comparison purposes with the ACE-inhibition activity of CFS fermentation samples. The inhibition percentage was calculated with the Eq. 1.

$$ACE - Inhibition\ (\%) = \frac{B-A}{B-C}100 \qquad (1)$$

where:
A= absorbance of the reaction mixture with CFS sample
B= absorbance of the reaction mixture without CFS (100% activity)
C= absorbance of the reaction mixture without ACE (0% activity)

2.6 Determination of iron-binding capacity of LAB strains during milk fermentation

Iron-binding capacity of the CFS fermentation samples was determined by the methodology reported by Hwang *et al*. (2001) with some modifications. An aliquot of 0.1 milliliter of CFS sample (diluted 1:10 with deionized water) was mixed with 0.6 milliliters of deionized water and 0.1 milliliters of $FeCl_2 \bullet 4H_2O$ 0.2 mM. Samples were incubated for 30 seconds at room temperature and then mixed with 0.2 milliliters of ferrozine 1mM (Sigma-Aldrich). This mixture was stirred and incubated for 10 minutes at room temperature. Absorbance was read at 520 nm. A blank was also prepared in the same way but using water instead of the CFS sample.

Ethylenediaminetetraacetic acid (EDTA-disodium salt, Baker, Mexico) was used for comparison. The bound iron was determined with Eq.2

$$Bound\ iron\ (\%) = \frac{100(C0 - C1)}{C0} \qquad (2)$$

where:

$C0$ = total iron concentration
$C1$ = unbound iron concentration

2.7 Determination of calcium-binding capacity of LAB strains during milk fermentation

Calcium-binding assay of CFS samples was carried out by the method reported by Jung and Kim (2007) with modifications. Two milliliters of $CaCl_2$ (5 mM,

prepared in deionized water) was mixed with one mL of diluted CFS sample (1:10 in deionized water). The mixture was incubated at 25 ºC for 30 minutes under constant agitation. Sample was filtered through a Whatman 1 filter paper, and the content of CFS was determined with a calcium-ion selective electrode (LAQUAtwin, Horiba Scientific, Japan). The amount of calcium-binding was established with the following Eq. 3:

$$Bound\ Ca = C0 - C1 \tag{3}$$

where:

$C0$ = the total calcium concentration of the blank ($CaCl_2$ solution plus deionized water)
$C1$= unfixed calcium concentration (with CFS samples and after incubation)

2.8 *Statistical analysis*

A two-way analysis of variance (MANOVA) was performed for microbial growth, pH, free amino groups, ACE-inhibitory activity, and metal-chelating activity (iron and calcium) of CFS fermentation samples. The difference between groups was determined by the mean of Tukey-Kramer Multiple-Comparison (α =0.05) using NCSS 11 Statistical Software (NCSS, LLC, Kaysville, Utah, USA).

3 Results and discussion

3.1 *Microbial growth and pH variation during milk fermentation with potential probiotic LAB strains isolated from double cream cheese*

Fermentation of the milk was carried out with each of the potential probiotic *Lactobacillus* strains (*Lb. plantarum*, *Lb. pentosus*, and *Lb. acidipiscis*), that were previously isolated from the double cream cheese as well as a mixed culture fermentation (inoculated with the three *Lactobacillus* strains in the same proportion). The microbial growth and pH variation during milk fermentation by LAB strains at 37 ºC are shown in Figure 1. These results showed that the *Lb. plantarum* fermentation had a significant higher microbial growth (11.07 ± 0.02 log CFU mL^{-1} after 24 hours of fermentation) while the *Lb. pentosus* fermentation had a significant lower microbial growth (8.2 ± 0.12 log CFU mL^{-1} after 32 hours of fermentation).

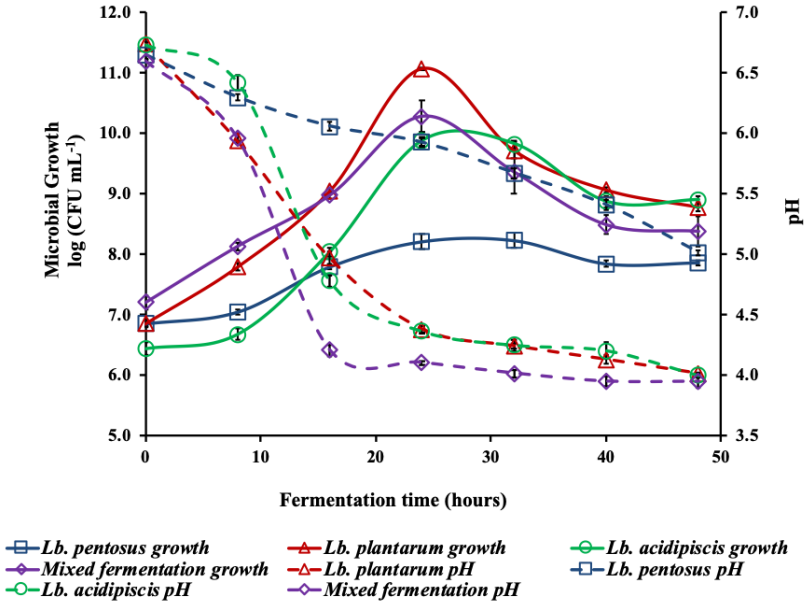

Fig. 1. Microbial growth and pH variation during milk fermentations with potential probiotic LAB strains (*Lb. pentosus, Lb. plantarum* and *Lb. acidipiscis*) isolated from double cream cheese. Values are means of two independent fermentations by duplicates (n=12).

The maximum cell population reached by mixed fermentation and *Lb. acidipiscis* fermentations were 10.28 ± 0.26 log CFU mL^{-1} and 9.87 ± 0.07 log CFU mL^{-1}, respectively, after 24 hours of fermentation, also showed a significant difference on microbial growth in both fermentations. In a study, performed by Matejčeková *et al*. (2016), to evaluate the temperature dependence of the growth of *Lb. plantarum* in UHT milk between 8 and 40 ºC. They were found that there was a significant increase of microbial growth at 37 °C, which ranged from about 3.0 log CFU mL^{-1} at start of fermentation to 7.2 log CFU mL^{-1} at 24 hours of fermentation. This microbial increase represents 4 log CFU mL^{-1}, as observed in this study for the growth of *Lb. plantarum* at the same incubation temperature. According to some research, it has been demonstrated that the pH contributes significantly to the proteolytic activity and/or the production of bioactive peptides by LAB strains. More specifically, it has been found that during the fermentation of milk with *Lb. helveticus*, the concentration of peptides and its bioactivity decreases significantly when the pH of the fermentations decreases from 4.3 to 3.5 (Nielsen *et al*., 2009). Subsequently, it was observed that the proteolytic activity of *Lb. helveticus* decreased dramatically at a pH below 6.5, however at a pH of 7, the highest ACE-inhibitory activity in milk fermented by this LAB was found (Pan and Guo, 2010).

3.2 Proteolytic activity of LAB strains during milk fermentation

During milk fermentation with proteolytic LAB, casein-derived peptides could be released (Gobbetti *et al*., 2002). The release of peptides by LAB is a consequence of their having a proteolytic system that allows them to hydrolyze the casein in order to obtain the essential amino acids to support their growth in milk (Tagliazucchi *et al*., 2019). The TNBS method is a widely used approach to determine proteolytic activity during fermentation or enzymatic hydrolysis (Zotta *et al*., 2006; Carballo-Sánchez *et al*., 2016). This spectrophotometric method is based on the reaction of primary amino groups with the TNBS reagent forming a complex whose absorbance can be quantified at 340 nm under alkaline conditions (Adler-Nissen, 1979). The concentrations of free amino groups that were found in the CFS fermentation samples are shown in Table 1. It was observed amino groups a significant increase of the concentration of free amino groups during the fermentation until the maximum concentration has obtained, which is between 24 and 40 hours of fermentation, depending on the strain of LAB used. These differences found on proteolysis related to BAL strain used and fermentation time have also been reported by other researchers (Donkor *et al*., 2007; Mazorra-Manzano *et al*., 2020).

Table 1. Free amino groups concentration of CFS fermentation samples performed by potential probiotic LAB strains isolated from double cream cheese.

Fermentation time (hours)	Free amino groups concentration (μg of Leucine mL^{-1})			
	Strain			
	Lb. pentosus	*Lb. plantarum*	*Lb. acidipiscis*	Mixed fermentation
0	209.9 ± 13.8 $^{a\,AB}$	203.7 ± 11.2 $^{a\,CD}$	192.5 ± 15.0 $^{a\,C}$	199.1 ± 17.7 $^{a\,C}$
8	198.6 ± 7.4 $^{b\,BC}$	221.5 ± 12.7 $^{a\,BC}$	170.8 ± 12.2 $^{c\,D}$	208.1 ± 13.7 $^{b\,C}$
16	211.6 ± 9.2 $^{b\,AB}$	153.7 ± 8.4 $^{d\,D}$	199.0 ± 10.2 $^{c\,C}$	275.5 ± 12.4 $^{a\,B}$
24	218.3 ± 12.7 $^{b\,A}$	271.0 ± 20.3 $^{a\,A}$	221.8 ± 15.0 $^{b\,B}$	267.6 ± 15.7 $^{a\,B}$
32	209.6 ± 16.3 $^{c\,AB}$	209.5 ± 8.3 $^{c\,BC}$	257.3 ± 9.8 $^{b\,A}$	289.1 ± 20.6 $^{a\,B}$
40	173.5 ± 17.7 $^{d\,D}$	279.0 ± 11.6 $^{b\,A}$	227.3 ± 8.6 $^{c\,B}$	381.1 ± 23.1 $^{a\,A}$
48	189.0 ± 10.9 $^{c\,CD}$	227.1 ± 12.0 $^{b\,B}$	155.7 ± 9.6 $^{d\,E}$	356.8 ± 35.3 $^{a\,A}$

Values are means of two independent fermentations by duplicates (n=12). Different letter within a same row (lower) and/or within a same column (upper) are significant different (Tukey-Kramer, $P \leq 0.05$).

Hence, in this paper, it was found that mixed fermentation had the highest content of free amino groups whereas the *Lb. pentosus* fermentation had the lowest content of free amino groups and, therefore, lower proteolysis. This LAB strain also showed the lowest microbial growth and pH variation during fermentation whereas mixed fermentation exhibited the most significant decrease on pH and higher microbial growth, only surpassed by the growth of *Lb. plantarum* fermentation.

Mazorra-Manzano *et al*. (2020) reported that the concentration of free amino groups for a 120-hour fermentation at 37 °C of cheese whey with native microbiota ranged from 36 to 360-456 μg of Glycine mL^{-1}, and that the o-phthalaldehyde (OPA) method was used for quantification of free amino groups.

Donkor *et al*. (2007) were found that microbial growth and the proteolytic pattern of LAB strains during fermentation are strongly related. Furthermore, it has been found that pH variation during fermentation can affect the proteolysis of the LAB strain (Nielsen *et al*., 2009; Pan and Guo, 2010). However, the results of this study, showed that at least the effect of pH on proteolysis during mixed and *Lb. pentosus* fermentations were contrary to the aforementioned studies. This effect could be explained by differences in the enzymatic activity of the proteolytic system of the BAL strains, mainly proteinases, caused by the variation of the pH during fermentation (Nielsen *et al*., 2009; Lim *et al*., 2019). Furthermore, these differences on enzyme activity can be explained by the alteration of the hydrogen ion balance, which changes the active structure of the enzyme and/or the protonation state of the substrate (Lim *et al*., 2019).

3.3 ACE-inhibitory activity of LAB strains during milk fermentation

The ACE-inhibitory activity found in the CFS fermentation samples is shown in Figure 2. It was observed that the supernatants of unfermented milk had an ACE-inhibitory activity close to 35%. The high ACE inhibitory activity found in the unfermented milk sample (t=0), could be caused by some peptides (such as those derived from the plasmin hydrolysis of β-casein) present in the milk that can exert this activity) or by the release of some casein-derived peptides with ACE-inhibitory activity during the BAL activation for fermentations (pre-culture). This was also observed in other studies, where ACE inhibition activities for unfermented milk ranged from 20 to 40% (Pihlanto *et al*., 2010; Solieri *et al*., 2015; Kliche *et al*., 2017). Nevertheless, it was observed a significant increase in the ACE-inhibitory activity in all CFS fermentation samples during milk fermentation until the maximal ACE- inhibitory activity was reached between 16 and 24 hours of fermentation. Fermentation with *Lb. plantarum* had the lowest degree of ACE inhibition (~80%) while the other fermentations reached up to 97% inhibition.

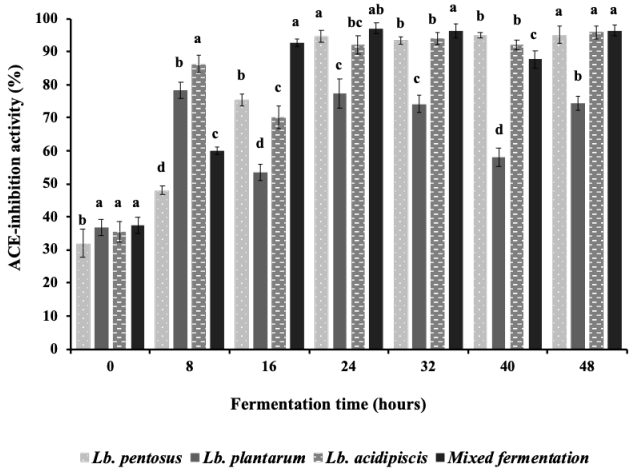

Fig. 2. ACE-inhibitory activity of CFS fermentation samples performed by potential probiotic LAB strains (*Lb. pentosus, Lb. plantarum* and *Lb. acidipiscis*) isolated from double cream cheese. Values are means of two independent fermentations by duplicate (n=12). Different letters indicate significant differences (Tukey-Kramer, $P \leq 0.05$).

Table 2. Inhibitory efficiency ratio (ACE-inhibitory activity/total protein (mg mL^{-1}) of CFS fermentation samples performed by potential probiotic LAB strains isolated from double cream cheese.

Fermentation time (hours)	ACE-inhibitory activity/total protein concentration (mg mL^{-1})			
	Strain			
	Lb. pentosus	*Lb. plantarum*	*Lb. acidipiscis*	Mixed fermentation
24	75.2 ± 1.4 [a]	55.3 ± 1.6 [b]	56.8 ± 1.6 [b]	73.4 ± 1.2 [a]
48	70.5 ± 1.7 [a]	59.3 ± 1.6 [c]	62.9 ± 1.3[b]	71.0 ± 1.1 [a]

Values are means of two independent fermentations by duplicate (n=12). Means in the same row with different superscript letter indicate significant differences (Tukey-Kramer, $P \leq 0.05$).

ACE-inhibitory activity of the supernatants of the fermentations was equivalent to a 100 μg mL^{-1} solution of captopril, a potent vasodilator drug used to control high blood pressure (Antonios and MacGregor, 1995). The ACE-inhibitory activity of this captopril was 94.48 ± 1.80%.

The significant increase in ACE inhibitory activity can be explained by the production of milk derived-bioactive peptides produced by the proteolytic system of the BAL strains during fermentation. This effect has been observed in several studies (Pihlanto *et al*., 2010; Gonzalez-Gonzalez *et al*., 2011; Solieri *et al*., 2015; Mazorra-Manzano *et al*., 2020). Some researchers have suggested that proteolytic activity, BAL strain, microbial growth, and fermentation conditions (pH, temperature), are some of the factors that influence the production of ACE-inhibiting peptides (Nielsen *et al*., 2009; Pan and Guo, 2010; Pihlanto *et al*., 2010; Gonzalez-Gonzalez *et al*., 2011; Mazorra-Manzano *et al*., 2020).

Therefore, it was observed that the mixed fermentation which was one of the fermentations that exhibited a higher ACE-inhibitory activity also had a higher proteolysis and most pronounced decrease in pH whereas the *Lb. plantarum* fermentation had the lowest ACE with a higher microbial growth and lower degree of proteolysis. This combination could be provoked the lower ACE-inhibition activity, because some of the peptides produced by this BAL strain were consumed in order to support its growth.

In a research performed by Gonzalez-Gonzalez *et al*. (2011), the inhibitory activity of supernatants of fermented milks with different LAB strains were quantified, including a strain of *Lb. plantarum* that showed an ACE inhibitory activity of 40% and 92% at 24 and 48 hours of fermentation, respectively. The ACE inhibitory activity of 48 hours-fermented milk by *Lb. plantarum* was higher than that found in this study, whose maximum inhibitory activity was 80%. Later, Chaves-López *et al*. (2014) found that the ACE-inhibitory activity of milk CFS samples of 36 hours of fermentation by two strains of *Lb. plantarum* KLATI and LAT3 were 65.2% and 50.4%, respectively. This result shows that the *Lb. plantarum* probiotic strain isolated from the double cream cheese has a similar ACE-inhibitory activity compared to the *Lb. plantarum* strains analyzed in that study. In 2019, Gonzalez-Gonzalez *et al*. proposed the use of the inhibitory efficiency ratio (IER), to provide a better approach to the inhibitory activity presented by the sample. The IER is the quotient of ACE-inhibitory activity divided by the concentration of total protein. Therefore, this value represents the ACE-inhibitory activity per mg mL^{-1} of protein, so the higher the value the more ACE- inhibitory potency the fermentation supernatant samples will have. The IER values of the 24 and 48 hours of fermentation CFS samples are shown in Table 2.

The IER values of the *Lb. pentosus* and the mixed fermentation at 24 hours of fermentation were higher than the ones obtained in the other fermentations. The IER in Lb. acidipisicis and *Lb. pentosus* samples increased after 48 hours of fermentation, suggesting that peptide sequences with higher ACE-inhibitory potential are generated if the incubation times exceed 24 hours. All IER values found in this study are higher than those found by Gonzalez-Gonzalez *et al*. (2019) for fermented milks produced by LAB strains isolated from Chiapas double cream cheese, where the maximum IER value was 9.11 found in the 48-hours milk fermentation by LAB strain identified as s12-AVCH. However, it should be noted that the methodology used in this study for the measurement of ACE inhibitory activity was different because it was used FAPGG as a substrate instead of HHL, which makes the comparison, not the most accurate one.

3.4 Determination of iron-binding capacity of LAB strains during milk fermentation

The release of peptide sequences with iron-binding capacity during milk fermentation by some LAB strains has been previously reported (Abubakr *et al*., 2012). The iron-binding activity of the CFS fermentation samples performed by LAB strains isolated from double cream cheese was shown in Figure 3. A good iron-binding capacity of the milk at the beginning of fermentation was observed (68%). This result is explained by the fact that some milk proteins can bind iron, such as lactoferrin, α-lactalbumin, β-lactoglobulin and the casein fraction, which could be present in CFS samples. Figueroa-Hernández *et al*. (2012) had already reported a good initial iron-binding capacity in unfermented milk, however, this capacity (30%) was lower than the one in this study.

During fermentation, the CFS fermentation samples had different iron-binding capacities. In the case of *Lb. plantarum* fermentation, a value of up to 99% of iron-binding capacity was found. In the case of the mixed culture fermentation, a high iron-binding capacity (92%) was also obtained. The samples of fermentations performed by *Lb. pentosus* and *Lb. acidipiscis* showed a lower capacity of binding iron than the other fermentations. However, an increase of up to 19% was achieved with respect to the initial value (t=0) in some fermentation times. These results showed that LAB-CFS samples have a high potential for iron-binding which is similar to that of EDTA (80% at 2 mg mL^{-1}). It has been suggested that this potential in CFS fermentation samples is caused by the presence of phosphoseryl groups (derived from caseins), which have a high affinity for iron, as well as the carboxylic group of aspartic and glutamic acids, which could bind iron as well and milk proteins with iron-binding activity such as lactoferrin and α-lactalbumin (Wong and Kitts, 2003).

Abubakr *et al*. (2012) found that the iron-binding capacity present in FS using several *Lb. plantarum* strains isolated from fruits were from 76 to 97.6% after 24 hours of fermentation. They also reported that the iron-binding capacity of CFS using *Lb. pentosus* isolated from fruits was in the range of 82 to 97%, after 24 hours of fermentation. These values are similar to those found in this work. In another study (Figueroa-Hernández *et al*., 2012), the iron-binding capacity of CFS samples from a pH-controlled fermentation by *Lactococcus lactis* NCFB 712 reached a value of 60% after 24 hours of incubation at 30 ºC. This iron-binding value, however, was lower than the ones reported in this work.

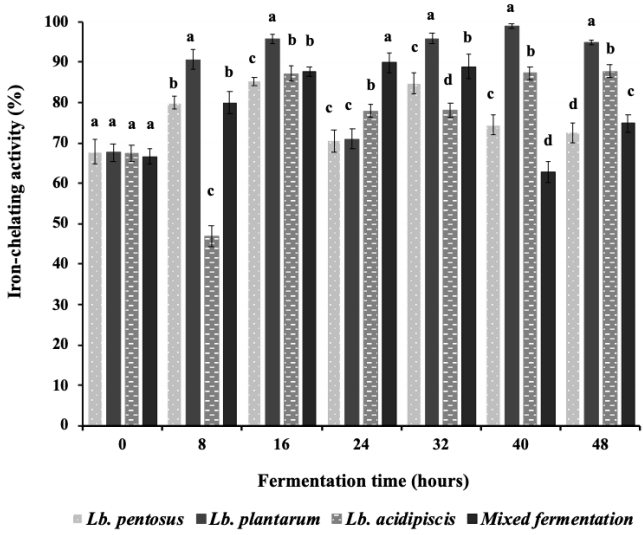

Fig. 3. Iron-binding capacity of CFS samples (1:10) fermented by potential probiotic BAL (*Lb. pentosus, Lb. plantarum* and *Lb. acidipiscis*) isolated from double cream cheese. Values are means of two independent fermentations by duplicates (n=12). Different letters indicate significant differences (Tukey-Kramer, P≤0.05).

3.5 Determination of the calcium-binding capacity of LAB strains during milk fermentation

Similarly, to iron, it was previously reported that calcium-binding sequences could be released during the milk fermentation (Dimitrov, 2009; Figueroa-Hernández *et al*., 2012). It has been reported that the biological activity related to these peptide sequences is caused by the occurrence of clusters composed of three phosphorylated serine and two glutamic acid residues (Ser-Ser-Ser-Glu-Glu). This highly polar acidic motif is a binding site for calcium, forming soluble and stable complexes that promote calcium absorption (Sun *et al*., 2016).

The calcium-binding activity of CFS samples of milk fermented by double cream cheese probiotic LAB strains is shown in Fig 4. It can be observed that the non-fermented milk (t=0) has a calcium-binding capacity of 0.5 mM of Ca^{2+} mg protein^{-1}. It was also observed that, after 8 hours of fermentation, the calcium-binding capacity of all the CFS fermentation samples increased until they reached maximal values after 48 hours of fermentation. The *Lb. acidipiscis* and mixed CFS fermentation samples showed the higher calcium-binding capacity (1.9 mM of Ca^{+2} mg protein^{-1}).

The calcium-binding activities produced during the milk fermentation by LAB strains were compared with that of an EDTA solution (2mg/mL), which has a binding activity of 4.91 mM of Ca^{2+}. This value is almost twice the higher value obtained for the calcium-binding activities found during the fermentations.

The potential of the production of peptides with calcium binding capacity was studied by Dimitrov in (2009) using 210 strains of LAB to ferment the milk. It was found that only twenty LAB showed significant activity, including *Lb. helveticus*, *Lactococcus lactis*, *Lb. plantarum*, and *Lb. casei*, among others. It was found that the LAB strain with the highest calcium-binding activity was *Lb. casei* (6.4 mM of Ca^{2+}) whereas a *Lb. plantarum* strain had an activity of just 0.2 mM of Ca^{2+}, a 10-fold lower value compared with those found in this study. In another study conducted by Figueroa-Hernández *et al*. (2012), the calcium-binding capacity of a fermented milk by *Lactococcus lactis* subsp. cremoris NCFB 712 with and without pH control were tested. A higher calcium-binding activity was observed when the pH was controlled during fermentation, with a value of (0.28 mM of Ca^{2+} mg protein^{-1}) whereas when pH was not controlled the activity was 0.23 mM of Ca^{2+} mg protein^{-1}. These activities are 10-fold lower than those reported in this work.

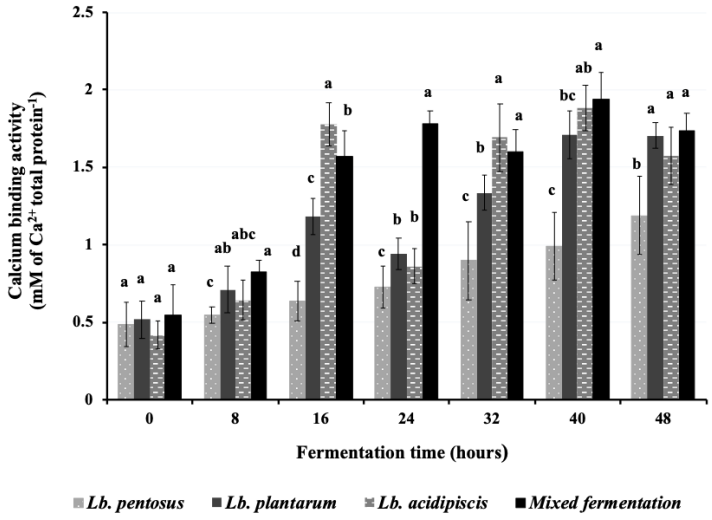

Fig. 4. Calcium-binding capacity of CFS samples fermented by potential probiotic LAB strains (Lb. pentosus, Lb. plantarum and Lb. acidipiscis) isolated from double cream cheese. Total protein concentration is expressed in mg mL^{-1}. Values are means of two independent fermentations by duplicate (n=12). Different letters indicate significant difference (Tukey-Kramer, P≤0.05).

Conclusions

The samples of fermented milks by potential probiotic LAB strains (*Lb. plantarum*, *Lb. pentosus*, and *Lb. acidipiscis*) isolated from the double cream cheese showed an ACE- inhibitory and metal-binding activities *in vitro*. These bioactivities could be closely related to the production of bioactive peptides during milk fermentation with the BAL strains used in this study, because during all fermentations an significant increase of free amino groups were found (proteolysis), however, to corroborate the production of bioactive peptides by these LAB strains during milk fermentations, are needed proteomic or electrophoretic studies, that will also allow the quantification and identification of the peptides produced during fermentation. Among the biological activities found, *Lb. pentosus* and the mixed fermentation showed a higher Inhibitory efficiency ratio (IER) for ACE-inhibitory activity and therefore, peptides with higher ACE-inhibitory potential could have been produced in these fermentations. Also, these two fermentations showed the highest iron-binding activity. Whereas *Lb. plantarum* and mixed fermentation had the highest calcium-binding activity. As the mixed fermentation by a mixed culture composed of the three potential probiotic LAB strains isolated from the Chiapas double cream cheese, at a ratio of 1:1:1, were showed a higher ACE-inhibitory or mineral-binding activities, it is suggested that this co-culture can be used for the production of functional fermented foods. Nevertheless, it is necessary to investigate the effect of this mixed culture in the generation of flavor and aromatic compounds, mainly if it is to be used as a cheese starter culture, to guarantee that the cheese not only has a functional potential but also retains the characteristic sensory profile of this cheese.

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