Vol. 20, No. 3(2021) Bio2483
Revista Mexicana de Ingeniería Química

Exopolysaccharide-producing bacteria improves survival and proteolytic profile of *Lactobacillus rhamnosus* GG added to semi-ripened cheese

Bacteria productora de exopolisacárido mejora la sobrevivencia y el perfil proteolítico de *Lactobacillus rhamnosus* GG adicionado a un queso semimadurado

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Received: May 14, 2021; Accepted: July 20, 2021

Abstract

Currently, the interest of the food industry on the use of probiotics in order to provide beneficial effects is increasing. However, one limitation is getting microorganisms to survive in food matrices. In this work, protective effect of exopolysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 on *Lactobacillus rhamnosus* GG survival during semi-ripening of cheese, was analyzed. Ripening was carried out at 14 ° C for 28 days in a controlled RH chamber. Scanning electron microscopy (SEM) was performed to determine interactions of probiotic with the exopolysaccharide. During ripening process, the proteolytic activity was determined through TNBS, SDS-PAGE and size exclusion-HPLC techniques. Viability of probiotic was measured by plate count. Cheese inoculated with both strains showed an improvement in probiotic survival. Exopolysaccharide was shown to have an effect on protein aggregation and cheese structural uniformity. Micrographs revealed a direct interaction between probiotic and exopolysaccharide. Proteolytic capacity did not decrease, and the generation and decrease of low molecular weight peptides (less than 3 kDa) was verified throughout the ripening process. Thus, using an exopolysaccharide-producing strain during cheese ripening has a positive effect on probiotic survival when they are used as starter cultures.

Keywords: probiotic, exopolysaccharide, semi-ripened cheese, proteolytic activity.

Resumen

Actualmente, está aumentando el interés de la industria alimentaria por el uso de probióticos con el fin de proporcionar efectos beneficicos. Sin embargo, una limitación es lograr que los microorganismos sobrevivan en las matrices alimentarias. En este trabajo se analizó el efecto protector del exopolisacárido producido por *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 sobre la supervivencia de *Lactobacillus rhamnosus* GG durante la semi-maduración del queso. La maduración se llevó a cabo a 14 ° C durante 28 días en una cámara de HR controlada. Se realizó microscopía electrónica de barrido (SEM) para determinar las interacciones del probiótico con el exopolisacárido. Durante el proceso de maduración, la actividad proteolítica se determinó mediante técnicas TNBS, SDS-PAGE y HPLC de exclusión por tamaño. La viabilidad del probiótico se midió mediante recuento en placa. El queso inoculado con ambas cepas mostró una mejora en la supervivencia de los probióticos. Se demostró que el exopolisacárido tiene un efecto sobre la agregación de proteínas y la uniformidad estructural del queso. Las micrografías revelaron una interacción directa entre probióticos y exopolisacáridos. La capacidad proteolítica no disminuyó, y se verificó la generación y disminución de péptidos de bajo peso molecular (menos de 3 kDa) durante todo el proceso de maduración. Por tanto, se demostró que el uso de una cepa productora de exopolisacáridos durante la maduración del queso tiene un efecto positivo sobre la supervivencia de los probióticos cuando se utilizan como cultivos iniciadores.

Palabras clave: probiótico, exopolisacárido, queso semi-madurado, actividad proteolítica.

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https://doi.org/10.24275/rmiq/Bio2483
ISSN:1665-2738, *issn-e*: 2395-8472

1 Introduction

The word probiotic derives from two Greek words that are: pro (in favor of) and biotic (life). Fuller (1989) defined probiotic microorganisms as "food supplement with live microorganisms that beneficially affects the host, improving the intestinal microbial balance". However, recently, the definition of probiotic has been extended to true probiotic, pseudoprobiotic, and phantom probiotic (Zendeboodi *et al*., 2020). Among the most important probiotic microorganisms are lactic acid bacteria (LAB), mainly lactobacilli and bifidobacteria. Due to their ability to hydrolyze lactose, these microorganisms can be used as starters in production of many dairy products, such as fermented milk and ripened cheeses (Bintsis, 2018). When a probiotic is used as starter in these kinds of products, several factors must be considered, which influence survival of the microorganism in the fermentation process and during storage (Melgar-Lalanne *et al*., 2014; Gomand *et al*., 2019). These factors include inoculum status, storage conditions and chemical composition of food (acidity, available carbohydrates, nitrogen source, available oxygen, salt concentration and water activity). The recommended amount of probiotic microorganisms that a food must contain should be 10^7 to 10^9 CFU / mL of viable microorganisms at the time of consumption to confer a benefit to consumers health (Farnworth, 2008, Chung & Kamal-Eldin, 2020).

In order to maintain the probiotic viability in dairy matrices, different strategies have been implemented. Some authors propose the use of the addition of prebiotics; others further propose the addition of encapsulated probiotics with different techniques (Costa *et al*., 2019; Jaimez-Ordaz *et al*., 2018; Ningtyas *et al*., 2019; Ceja-Medina, *et al*., 2021). However, technologically this type of action affects the sensory and rheological characteristics of the food. Currently, the use of filamentous microorganisms has been used as starter cultures (Xu *et al*., 2019).

Some LAB are capable of deriving a proportion of fermentable sugars towards the biosynthesis of exopolysaccharides (EPS), which are long chain polysaccharides with branches of repeating sugar units (Bancalari *et al*., 2019; Zhou *et al*., 2019). These sugar units are mainly glucose, galactose and rhamnose in different proportions (Parra-Huertas, 2010). They are associated with cell surface in form of a capsule and are secreted to environment. EPS in their natural environment play an important role in microbial cell protection against desiccation, phagocytosis, action of antibiotics and toxic compounds, predation by protozoa, osmotic pressure, adherence to surfaces, film formation, and cell recognition, but they are not food for the same producing bacteria (Leroy & De Vuyst, 2016). On the other hand, it has been observed that these compounds can favor the retention of nutrients in the EPS matrix that can later be used as a source of carbon and energy (Patel *et al*. 2012). EPS can be produced by *Lc. lactis* subsp. lactis, *Lc. lactis* subsp. *cremoris*, *Lb. casei*, *Lb. sake*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. helveticus* and *S. thermophilus* (Lynch *et al*., 2018).

Thus, the addition of probiotics in the production of ripening cheeses could be accompanied by exopolysaccharide-producing LAB to contain its viability (Badel *et al*., 2011). The technological benefit for using this type of microorganisms has been demonstrated in several studies. However, the health benefits and protective effect of EPS on probiotics viability in semi-ripening cheeses is a topic that raises expectations and determines new horizons in food science (Mathur *et al*., 2020). That is why the objective of this research was to determine the protective effect of EPS produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 on viability and proteolytic activity of *Lactobacillus rhamnosus* GG monitored during 28 days of ripened of a semi-ripening cheese.

2 Materials and methods

2.1 Starter preparation

Lactobacillus delbrueckii subsp. *bulgaricus* NCFB 2772 and *Lactobacillus rhamnosus* GG were provided by the Food Biotechnology Laboratory of the Biotechnology department of the Universidad Autónoma Metropolitana-Iztapalapa. Microorganisms were propagated in 10 mL of MRS broth (DifcoTM). *Lb. delbrueckii* was incubated at 42 ºC and *Lb. rhamnosus* at 37 °C, both for 24 h. Purity was checked through cell morphology observed by Gram stain. Each microorganism was conditioned by bringing a scraping of the propagated inoculum to 10 mL of 10% pasteurized skim milk powder solution (90 °C for 10 min) (Svelty, Nestlé ®). Incubation conditions were the same. Viable cells analysis was performed by plate count and 10^9 CFU / mL of each microorganism was inoculated in 600 mL of 10 % skim milk powder

previously pasteurized. Incubation conditions were the same. Starters were prepared just prior to cheese making.

2.2 Manufacture of semi-ripened cheese

Semi-ripened cheeses were made at the Universidad Autónoma del Estado de Hidalgo (UAEH). 70 L of pasteurized milk from the UAEH Dairy Products PROUNILAC, were used. Milk was standardized to 3.6% fat using a skimmer (Elecrem 315L) and it was pasteurized. Milk was kept at 36 ºC and 60 L were inoculated with 1% of each adapted culture of *Lb. rhamnosus* GG and *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 (CH-RB) and 10 L were inoculated at 1% only with the probiotic bacteria (CH-R). After 40 min at 36 °C (until 18.5 °D), 7.2% (v / v) of rennet (Fromase strength 1: 10000) and 1.2% (p / v) of calcium chloride, were added. It was to stand for 1 h to allow total flocculation of the caseins. The curd was cut into 1 cm cubes. It was stirred constantly without heat for 40 min to stimulate whey release until reaching 11.5 °D. Whey was separated and flocculated casein was deposited in 1 kg molds. Cheese units were pressed (3 kg / cm^2) for 2 h and turned over to continue pressing under the same conditions for a further 24 h. At the end of pressing, cheeses were unmold and vacuum packed (MultivacC100). They were stored for four weeks at 14 ºC.

One experimental unit of cheese was sampled at day 0, 7, 14, 21 and 28, under sterile conditions and each analysis was performed in duplicate. In order to determine viable count, 10 g of cheese core and edge were taken and placed in sterile bags for microbiological samples. To analyze the proteolytic profile, 20 g of cheese were taken and placed in sampling flasks. At the end of each sampling, cheeses were vacuum packed again and returned to storage conditions.

2.3 Viability

Viability was performed through plate count technique. To 10 g of sample, 9 mL of sterile 1% peptone water (DifcoTM) were added. It was homogenized (Stomacher ® 400 circulator Seward) for 3 min and 1 mL of sample was diluted with 9 mL of 1% peptone water. Subsequent dilutions were made to a final dilution of $10^{-6} \cdot 100\mu$L of each dilution were taken and placed in Petri dishes with MRS agar (DifcoTM) adjusted to pH 5.20 with 0.1 N acetic acid and 0.15% of bile salts, favoring the growth of probiotic lactobacilli. Petri plates were incubated at 37 °C, under anaerobic conditions for 72 h. Plate count was performed for each test.

2.4 Scanning Electron Microscopy (SEM)

To observe exopolysaccharide presence produced by *Lb. delbrueckii* subsp. *bulgaricus* NCBF 2772 and its interaction with the probiotic, an SEM analysis was performed in the scanning electron microscopy laboratory of UAM-I using a JEOL JSM-5900LV Scanning Electron Microscope equipment. Sample fixation was carried out taking a small fraction from the edge and center of each cheese. Samples were placed in a bottle with 3% glutaraldehyde, for 36 h at 4 °C. Subsequently, 3 washes were carried out every 10 min with a 0.2 M phosphate buffer. Then, samples were placed in 1% osmium tetroxide, to carry out a post-fixation process for 1 h at 4 °C. After this time, the samples were dehydrated with ethyl alcohol at different percentages (30%, 40%, 50%, 70%, 80%, 90% and 100%), carrying out two washes every 15 min with each solution during the change period. Dehydrated cheeses were placed in microporous boxes and dried to a critical point (PO 1040 lb / in^2, 31 ºC) (Sambri 780-B, Sample Drying at the critical Point Tousimis Research corporation, Japan) using carbon dioxide as medium of transition. Finally, they were covered with a layer of gold to give it conductive properties.

2.5 Proteolytic profile analysis

Three different studies were carried out to analyze proteolytic profile characterization of cheese during ripening. Free amino groups determination was performed through TNBS technique to know the concentration of the peptides produced. Polyacrylamide gel electrophoresis (SDS-PAGE) was used to observe protein hydrolysis and peptide derived fractions. Finally, to observe changes in peptide fractions concentration, especially those of low molecular weight during cheese ripening, an HPLC analysis by size exclusion column was carried out.

2.5.1 Sample preparation

Samples were prepared by adding 50 mL of deionized water to 10 g of cheese and then they were homogenized at 260 rpm for 3 min in stomacher (Stomacher® 400 circulator Seward). Then, 10 mL of

the sample was placed in centrifuge tubes (HERMLE Labnet Z323K). Samples were centrifuged at 7, 200 x g for 5 min at 4 °C and supernatant was filtered through Whatman 4 paper under vacuum. Filtrate was centrifuged again at 7, 200 x g during 10 min at 4 °C and supernatant was decanted. Samples were stored at -18 °C until their analysis.

2.5.2 *Free amino groups analysis*

Free amino groups derived from ripening process were measured with 2,4,6-trinitrobenzenesulphonic acid (TNBS). A volume of 125 μL of sample was mixed with 1 mL of phosphate buffer solution of 0.21 M, pH 8.2 in test tubes wrapped with aluminum foil. After that, 1 mL of TNBS (0.10%) was added to phosphate buffer 0.21 M, pH 8.2, and each tube was agitated in vortex. Tubes were incubated for 1 hour at 50 ºC in darkness. Reaction was interrupted after 60 minutes adding 2 mL of hydrochloric acid 0.1 N. It was read in a spectrophotometer at 340 nm of wavelength against control. Control was prepared with deionized water and a glycine concentration curve (0.05 to 0.25 mg/mL) was used.

2.5.3 *Polyacrylamide gel electrophoresis (SDS-PAGE)*

Denaturing SDS-PAGE at T=15% (w/v) was carried out to separate low molecular weight peptides. A broad range (Bio-Rad, USA) molecular weight standard was used. Gels were dyed with Comassie G-250 (Bio-Rad, USA). Peptides molecular weight was analyzed using the electrophoretic profiles determined by GelDoc software by BioRad. The methodology reported by Laemmli (1970) with modification of González-Olivares *et al*. (2011), was used.

2.5.4 *Proteolytic profile analyzed by size exclusion HPLC*

Separation was performed according to the methodology by González-Olivares *et al*. (2011) with some modifications. Samples were filtered through 0.22 μm PTFE Millex® and they were injected on a High Performance Liquid Chromatograph (HPLC) Perkin Elmer Series 200. A size exclusion column (Biosep-SEC 2000 Phenomenex ®, Torrance, CA, USA, particle size 5 mm, pore size 145 Å, 300 x 7.8 mm), was used. A phosphate buffer solution at pH 6.8 was used as a mobile phase. The volume of injected sample was 20 μL and the analysis was performed at room temperature using a flow of 0.25 mL/min for 60 min. Detection was performed at 220 nm using a diode array detector (Spectra System UV 6000 LP, Providence, RI, USA).

2.6 *Statistical analysis*

Experimental results of survival and quantification of free amino groups of the strains at different storage temperatures and during different days of experimentation were carried out in duplicate. They were analyzed by analysis of variance (ANOVA) by Tuckey's method with a significance level of 0.05. The program used for the analysis was NCSS-2007 (version: 07-1-15).

3 Results and discussion

3.1 *Viability*

Table 1 shows viability values of probiotic lactobacili in cheeses. According to the results, cheese inoculated only with *Lb. rhamnosus* (CH-R) has a decrease during ripening with significant differences since week 1 until the ending time. One logarithmic cycle was the difference between week 0 and 4. In contrast, a significant increase in cheese with probiotic *Lb. delbrueckii* (CH-RB), was observed. During ripening time, the probiotic concentration increased since week 2. Between week 2 and 3 a non-significant decrease was presented, increasing at the end of the ripening at the same concentration of week 1.

One of the factors that cause probiotics death in a dairy matrix such as cheese is mainly associated with the lack of starter cultures that stimulate the growth of these probiotics. This effect is due in part to the production of free amino acids (Farnworth, 2008). Therefore, the production

Table 1. Probiotic lactobacili survival during cheese ripening without (CH-R) and with *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 (CH-RB).

Week	Log CFU / mL	
	CH-R	CH-RB
0	6.67 ± 0.07	6.93± 0.16
1	5.85± 0.06	7.97± 0.08
2	5.80± 0.03	7.35± 0.08
3	5.57± 0.14	7.46± 0.15
4	5.45± 0.02	7.98± 0.11

of some flavors and aromas during ripening exerts an inhibitory effect on cell growth. However, the addition of probiotics along with starter cultures in semi-mature cheeses has been successful to maintain probiotic survival (Dinkçi *et al*., 2019; Sadaghdar *et al*., 2012).

The viability of the probiotic when it is not mixed with the starter culture could be favored by other factors, such as the composition of the cheese and the presence of other microorganisms that exert a protective effect (Lucatto *et al*., 2020, Pivetta *et al*., 2020). This protective effect may be due to exopolysaccharides production from the starter culture and the release of growth factors necessary for the probiotic (Karimi *et al*., 2011).

Comparing the viability of cheese inoculated only with probiotic and the one inoculated with both microorganisms, it is observed that there was a symbiosis in the second case. This symbiosis is exerted since *Lb. delbrueckii* subsp. *bulgaricus* releases amino acids and peptides that the probiotic needs for its growth (Farnworth, 2008). Additionally, *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 produces an exopolysaccharides, which favors nutrients retention, but it is not for feeding the cell. Probiotics such as *Lb. rhamnosus* GG use these exopolysaccharides for its easy availability, as a source of carbon and nitrogen for its survival (Badel *et al*., 2011).

3.2 Cheese structure and probiotic-EPS interaction analyzed by SEM

Different micrographs were made of the cheeses CH-R (Fig. 1). These images show the fat globules trapped in the protein matrix. López *et al*. (2007) observed during the maturation of a cheese that, in the microstructure of the interfacial area there are interactions between the casein structure and the fat globule, which is located within the casein matrix. These results are consistent with micrograph 1B. Furthermore, pressing a cheese is known to cause very small cavities and higher fat content. This type of cavities are observed in Figures 1B and 1C, in which the union of casein agglomerates was also observed (Figure 1A).

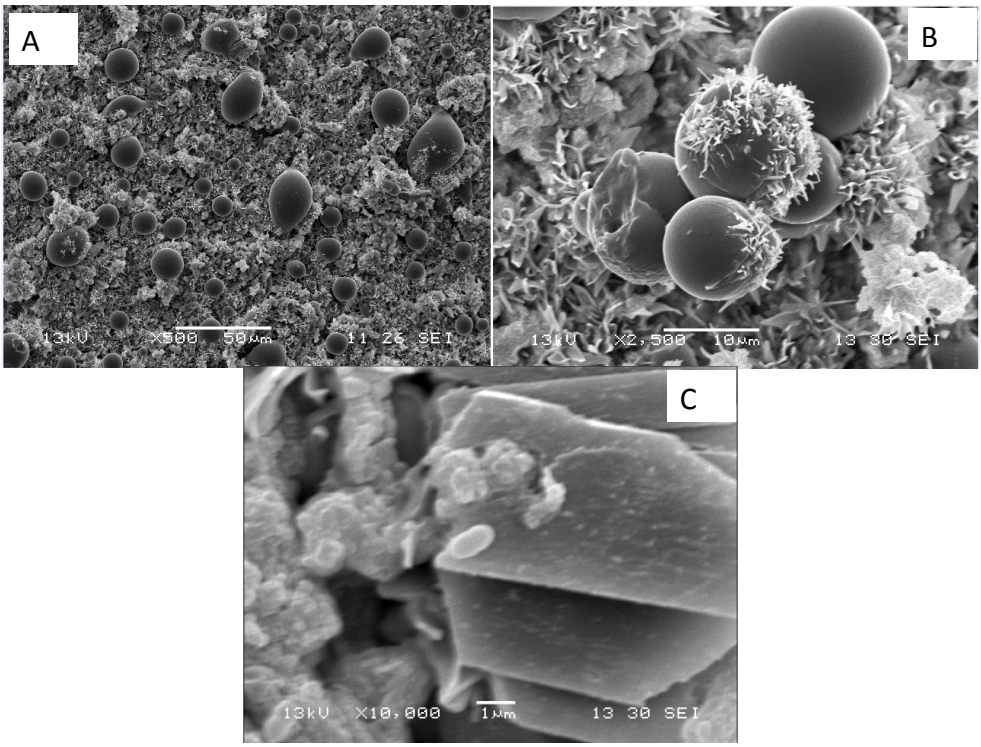


Fig. 1. Micrographs of cheese inoculated with *Lb. rhamnossus* GG (A) fat globule and protein interaction in cheese edge (500X); (B) fat globule and protein interaction in cheese core (2,500X) and (C) *Lb. rhamnossus* GG in interaction with proteins in cheese core (10,000X).

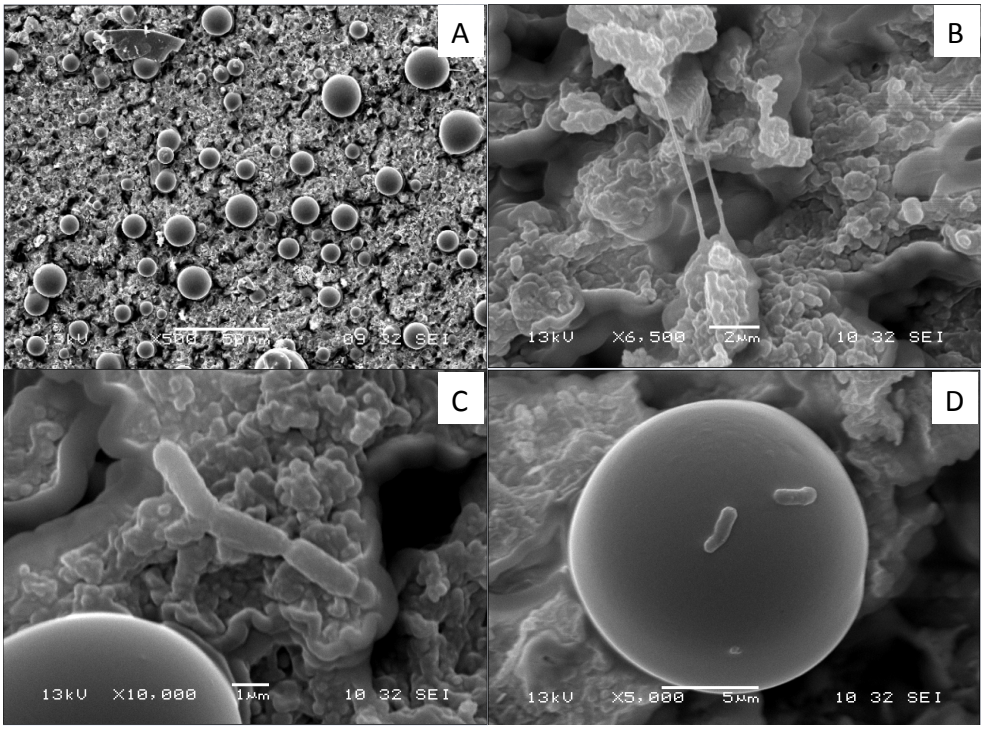


Fig. 2. Micrographs of cheese inoculated with *Lb. rhamnossus* GG and *Lb. delbruekii* subsp. *bulgaricus* NCFB 2772 (A) fat globule and casein interaction; (B) interaction between protein and exopolysaccharide cheese edge (6,500X) and (10,500X); (C) and (D) *Lb. rhamnossus* GG in interaction with proteins, EPS and fat globule in cheese core (10,000X) and (5,000X).

When zooming in on the image up to 2500 x (Figure 1B), in addition to the interactions, indeterminate aggregates were observed which might be due to protein fractions released from casein micelles and may be interacting directly with the membrane of the fat globule. On the other hand, Figure 1C shows the presence of bacilli probably related with *Lb. rhamnosus* GG interacting with the protein matrix. Due to the concentration of viable microorganisms in the cheese, the presence of the probiotic was very dispersed. This micrograph is consistent with the one reported by Kiekens *et al* (2019).

The photomicrographs obtained by SEM of the cheese CH-RB, are shown in Figure 2. In Figure 2A, the interaction between the fat molecules dispersed in the cheese, surrounded by the protein matrix, is observed. The casein agglomerates in this system are better fused than those observed in the cheese inoculated only with the probiotic. Hassan *et al*. (2002) also observed that in a Feta cheese, EPS producing-culture induces large agglomerates of casein, which are not found in cheeses manufactured with common starters. The characteristics of the fat globule observed in the cheese inoculated with both microorganisms, reflected a higher concentration of globules than in the cheese inoculated only with the probiotic.

Different lactic acid bacteria produce EPS and its composition is dependent of the nitrogen and carbon sources (Hernández-Rosas, *et al*., 2021). The exopolysaccharide produced by the filant strain was observed to be interacting with the protein matrix of the cheese (Fig. 2B). EPS increase moisture retention by links with water or by entrapment of its three-dimensional structure (Hassan, 2008). According to the observed in the scanning electron microscopy the interactions between components with water, generated empty spaces when evaporating during the preparation of the samples (Fig. 2B). Guzel-Seydim *et al*. (2005) reported that EPS strongly bind to water within the matrix of string cheese and retain it for longer than in cheese without string culture. In the case of the EPS produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772, it was proved that this EPS binds efficiently water in cheese matrix. This effect could be due to its composition. The EPS is structured

with different carbohydrates chains composed by glucose, galactose, and rhamnose (Grobben *et al*., 1994).

Lactobacilli were located on the protein matrix of the cheese (Figure 2C) and on a fat globule (Figure 2D). Hassan *et al*. (1995) and Guerin *et al*. (2017) used scanning electron microscopy and observed that the protein matrix is interconnected and the liquid phase and the LAB are immobilized in the interstitial space. Furthermore, these authors observed that the filamentous strains become part of the microstructure, due to the connections that occur between the bacterial surface and the matrix that surrounds the EPS. This information agrees with what was observed. Lactobacilli apparently of the genus *Lb. rhamnosus* GG are immobilized in the protein matrix. Thus, a protective effect of EPS on the probiotic was observed due to interactions between cheese components and microorganism.

3.3 Proteolytic profile

3.3.1 Free amino groups by TNBS

Free amino groups determination of each cheese analyzed during the ripening was carried out by the TNBS method (Table 2). It was observed that on day 0 there was presence of free amino groups, 0.283 ± 0.012 mg / L in CH-R and 0.322 ± 0.016 mg / L in CH-RB.

Free amino groups presence at the beginning of the study is attributed to the pre-ripening process. In that moment, enzymes of the proteolytic system of lactic acid bacteria promote the first proteolysis of caseins in milk. A higher initial activation was observed in cheese inoculated with *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 and the probiotic. It is known that in protocooperation systems there is a greater proteolytic activity mainly due to the presence of two microorganisms that carry out the immediate hydrolysis of the protein chains (Vinderola *et al*., 2002; Chen *et al*., 2017).

Significant differences of free amino groups concentration were observed in CH-RB during the ripening process. At the end of the study, an increase in the concentration of peptides produced during ripening was found from 0.339 ± 0.046 to 0.597 ± 0.016 mg / L). In CH-R, a significant increase in the concentration was not found until week 3, reaching a final concentration of 0.511 ± 0.039 mg / L.

Table 2. Free amino groups concentration during cheese ripening without EPS (CH-R) and with EPS (CH-RB) produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772.

Week	mg / L	
	CH-R	CH-RB
0	0.283 ± 0.012^{a}	0.322 ± 0.016^{a}
1	0.312 ± 0.006^{a}	0.364 ± 0.003^{b}
2	0.399 ± 0.032^{b}	0.417 ± 0.003^{c}
3	0.451 ± 0.014^{c}	0.490 ± 0.017^{d}
4	0.511 ± 0.001^{d}	0.597 ± 0.021^{e}

The proteolytic activity of lactic bacteria begins with degradation of caseins by action of the proteolytic system and consequently there is a generation of low molecular weight peptides (Rojas-Ronquillo *et al*. 2012, Ramírez-Godínez *et al*. 2021). Likewise, degradation of free amino acids and small peptides is due to the proteinase-peptidase system of starter culture (*Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772). This protein degradation is what supports the bacteria growth, since the amount of free amino acids found mainly in milk is insufficient for the microorganism to grow optimally (Juillard, 1996). In addition, it has been shown that the contribution of non-initiating BAL enzymes in cheese proteolysis is low in terms of the production of short chain peptides and free amino acids (McSweeney *et al*. 1994; Lane and Fox, 1996).

The use of exopolysaccharide-producing starter cultures in manufacture of cheeses has been reported as a factor that increases the metabolic activity of microorganisms, causing an increase in the release of free amino groups (Awad, *et al*. 2005, Oluk *et al*., 2013). However, Coehlo-Nepomuceno *et al*. (2016) found that despite the fact that during the first days of ripening there is a greater production of free amino groups, they determined that after 30 days this production does not show significant difference. Due to this, the sensory aspects of cheese are not affected, but cheese yield is higher in cheeses with exopolysaccharide, because of the amount of linked water.

3.3.2 Peptide separation by SDS-PAGE

The proteolytic activity determined by SDS-PAGE of *Lb. rhamnosus* GG in CH-R and CH-RB is observed in Figures 3a and 3b, respectively. The metabolic activity in both experiments was demonstrated by caseins

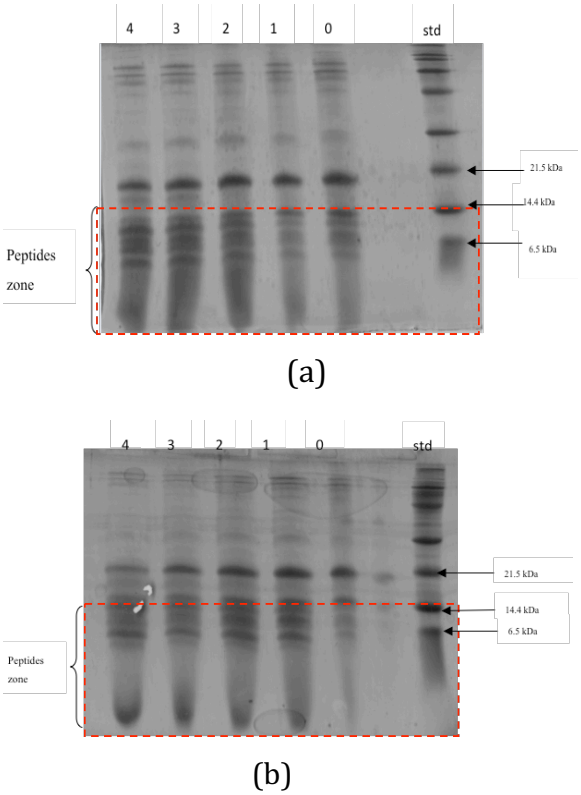


Fig. 3. Gel of SDS-PAGE (T=15%) of peptide profiles of semi-ripened Mexican Cheese compared with an molecular weight standard (std) A) without EPS (CH-R) and B) with EPS (CH-RB) produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 at 0, 1, 2, 3 and 4 weeks of ripening.

hydrolysis, which was observed from the first week of ripening. The decrease in caseins concentration was evident while the accumulation of peptides of molecular weight lower than the weight of caseins was observed throughout the ripening process. However, peptides accumulation of molecular weight less than 6.5 kDa was higher in CH-RB. In the case of CH-R, no well-defined bands were observed below 6.5 kDa in contrast to those that were separated in CH-RB.

Accumulation of low molecular weight peptides is directly related to the proteolytic system of the bacteria presents in cheese. It is known that peptide sequences related to flavors characteristic of ripened cheeses are released from protein degradation. Likewise, these peptides are the result of the taking of essential amino acids for the growth of microorganisms, which are usually different due to the auxotrophies of each strain. Protein degradation is also a factor related to the metabolic activity of lactic acid bacteria during the ripening process. As it is an unfavorable environment for the viable maintenance of lactic acid bacteria, it is observed that the presence of the exopolysaccharide, in addition to keeping the bacteria viable, also stimulates their development.

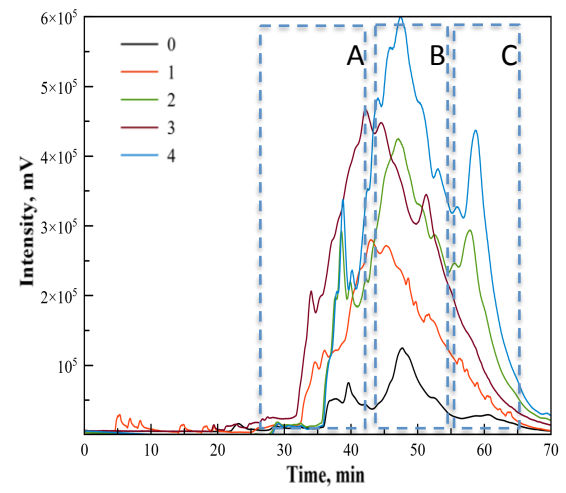
When calculating molecular weights with the Bio-Rad Gel-Doc program, a peptide of 1.4 kDa was identified from the first week of ripening in CH-RB, while in CH-R the smallest detectable peptide was 0.6 kDa, however, it was only observed in the first week and after this time it was not detectable.

It has been observed that the presence of exopolysaccharide during cheese ripening favors proteins hydrolysis due to the higher metabolic activity of lactic acid bacteria. Oluk *et al*. (2013) determined that in the 90-day maturation of a low-fat Tulum cheese, the production of low molecular weight peptides is favored by the presence of the exopolysaccharide produced by a starter culture. Wang *et al*. (2018) determined that the proteolytic profiles change and the concentration of low molecular weight peptides increases during the production of a cheddar cheese matured for 8 weeks.

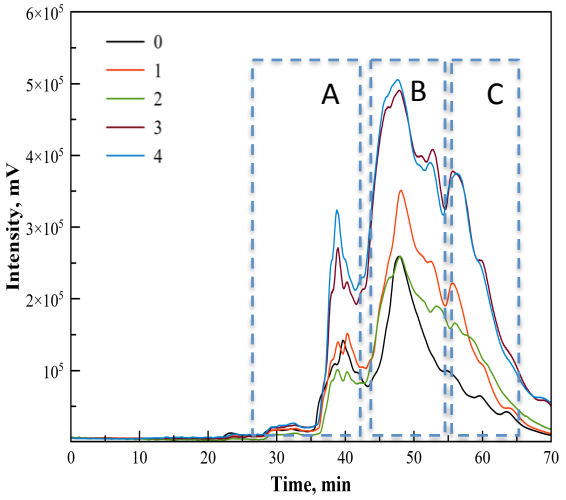
3.3.3 Proteolytic profile analyzed by size-exclusion HPLC

Chromatograms of cheeses during ripening are shown in Figure 4, which are divided into three zones (A, B, and C), according to the calculated molecular weight. Zone A delimited by the molecular weight of the caseins, zone B by peptides of intermediate molecular weight (up to 6 kDa) and zone C with those peptides with molecular weights lower than 6 kDa. Chromatograms of both type of cheeses showed accelerated changes and distribution of different peaks in the area of each zones. These changes suggest the progressive breakdown of proteins (specially caseins) and high molecular weight peptides, resulting in peptides formation of different molecular masses and free amino acids. In addition, the accumulation of low molecular weight peptides is more evident in CH-RB (Fig. 4-b), which accumulation of this kind of peptides increases at the end of the ripening. Results indicated the favored metabolism due to exopolysaccharide by the presence of proteolytic products and increasing contribution of bacterial enzymes with ripening time.

Due characteristics of appear and disappear of peptidic fractions; it was found differences during CH-BR ripening time, mainly in zone B and C. This behavior corresponds to the characteristic cascade breakdown system of the proteolytic system, where proteins are the substrate for obtaining high molecular



a. Cheese without EPS (CH-R)



b. Cheese with EPS (CH-RB)

Fig. 4. Size-exclusion-HPLC chromatograms of proteolytic profiles changes of semi-ripened Mexican Cheese a) without EPS (CH-R) and b) with EPS (CH-RB) produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 at 0, 1, 2, 3 and 4 weeks of ripening. The chromatograms were compiled from the mean data taken from 3 replicate trials.

weight peptides, which in turn are the initiators in the generation of intermediate and low molecular weight peptides. The latter can come from both primary protein hydrolysis and subsequent peptide hydrolysis (Gasson and De Vos, 1994). González-Olivares *et al*., 2011, performed peptide separations through size exclusion chromatography and they determined that accumulation of low molecular weight and its correlation with diminish of proteins and intermediate molecular weight peptides concentration, could be analyzed with this technique.

The results obtained are consistent with some that have shown that exopolysaccharide presence has a positive effect on cheese ripening. Awad *et al*. (2005) determined that the use of exopolysaccharide-producing cultures has an effect on both the yield and the metabolic activation during the ripening process of cheddar cheese for 6 months. Actually, production of hydrophobic peptides is increased at the end of the process when the exopolysaccharide is present. Additionally, it is known that the use of this type of culture results in a higher extent of proteolysis measured through HPLC-RP for the manufacture of low Tulum cheese ripened for 3 months (Oluk *et al*., 2013). The main changes observed in the proteolytic profile were derived from the accumulation of hydrophobic low molecular weight peptides. Recently, Wang *et al*. (2019) demonstrated that exopolysaccharide presence during Cheddar cheese ripening for 90 days is related to a higher bioactivity (ACE-inhibitory, antitumor, antioxidant, and inhibition of both *alpha*-Amylase and α-Glucosidase) of peptide fractions obtained.

Conclusions

Exopolysaccharide produced by *Lb. delbrueckii* subsp. *bulgaricus* NCFB 2772 is a promising material to be used in production of semi-ripened Mexican cheese added with the probiotic *Lb. rhamnosus* GG, due to the improvement of its survival and proteolytic activity. Moreover, the higher concentration of low molecular weight peptides released by the probiotic during the ripening process is an advantage in food technology diversifying the inclusion of probiotics in dairy process. The results of this paper highlight that semi-ripened cheese represents an opportunity to develop functional food, which could maintain the probiotic viability to benefit human health.

Acknowledgements

The project was developed with any financial support in Biotechnology laboratory in Centro de Investigaciones Químicas and PROUNILAC of Universidad Autónoma del Estado de Hidalgo. SEM analysis was performed in Universidad Autónoma Metropolitana campus Iztapalapa in Food

Biotechnology Laboratory. The authors thank Food Engineering Zaira Liliana López Cuellar for her excellent technical support. The authors have not stated any other conflicts of interest.

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