Assessing the yield, microstructure, and texture properties of miniature Chihuahua-type cheese manufactured with a phospholipase A₁ and exopolysaccharide-producing bacteria

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ABSTRACT

Chihuahua cheese or Mennonite cheese is one of the most popular and consumed cheeses in Mexico and by the Hispanic community in the United States. According to local producers the yield of Chihuahua cheese ranges from 9 to 9.5 kg of cheese from 100 kg of milk. Cheese yield is a crucial determinant of profitability in cheese-manufacturing plants; therefore, different methods have been developed to increase it. In this work, a miniature Chihuahua-type cheese model was used to assess the effect of a phospholipase A₁ (PL-A₁) and exopolysaccharide (EPS)-producing bacteria (separately and in combination) on the yield, microstructure, and texture of cheese. Four different cheeses were manufactured: cheese made with PL-A₁, cheese made with EPS-producing bacteria, cheese with both PL-A₁ and EPS-producing bacteria, and a cheese control without PL-A₁ or EPS-producing bacteria. The compositional analysis of cheese was carried out using methods of AOAC International (Washington, DC). The actual yield and moisture-adjusted yield were calculated for all cheese treatments. Texture profile analyses of cheeses were performed using a texture analyzer. Micrographs were obtained by electron scanning microscopy. Fifty panelists carried out sensorial analysis using ranking tests. Incorporation of EPS-producing bacteria in the manufacture of cheese increased the moisture content and water activity. In contrast, the addition of PL-A₁ did not increase fat retention or cheese yield. The use of EPS alone improved the cheese yield by increasing water and fat retention, but also caused a negative effect on the texture and flavor of Chihuahua cheese. The use of EPS-producing bacteria in combination with PL-A₁ improved the cheese yield and increased the moisture and fat content. The cheeses with the best flavor and texture were those manufactured with PL-A₁ and the

cheeses manufactured with the combination of PL-A₁ and EPS-producing culture.

Key words: Chihuahua cheese, cheese yield, phospholipase, exopolysaccharide-producing bacteria

INTRODUCTION

Cheese yield is a crucial determinant of profitability in cheese-manufacturing plants. This process variable is affected by several different factors, such as the characteristics of milk (i.e., composition, milk quality, and SCC, among others) and by the cheese-making conditions (i.e., homogenization, standardization, pasteurization, coagulant type, starter cultures, vat design, and curd firmness at cutting, among others). Due to its economic impact, several different methods have been developed through the years to increase yield (Barbano et al., 1991; Guinee et al., 2006; Banks, 2007; Fagan et al., 2007). These methods include the incorporation of whey proteins, UF of cheese milk (Abd El-Gawad and Ahmed, 2011), increment of the protein-to-fat ratio (Guinee et al., 2007), use of ultrasound on cheese milk (Bermúdez-Aguirre and Barbosa-Cánovas, 2010), treatment of cheese milk with phospholipase A₁ (PL-A₁; De Maria et al., 2007), and use of exopolysaccharide (EPS)-producing bacteria (Costa et al., 2012).

The use of phospholipases is common in the production of vegetable oil, starch, and baking ingredients, but their use in the dairy industry had not been considered until recently (De Maria et al., 2007). Nielsen (2001) patented a process to produce Mozzarella cheese from milk treated with phospholipase A₁, A₂, and B. Five years later, Patkar et al. (2006) patented a fungal phospholipase that was modified to alter its substrate specificity, obtaining an enzyme with phospholipase activity and reduced activity on triglycerides. In the same year, Lilbæk et al. (2006) used a PL-A₁ from *Fusarium venenatum* in the manufacture of Mozzarella cheese, reporting an increase in the cheese yield (3.2%) due to moisture and fat retention. Some indications exist that phospholipases detach bilayer fragments from the milk fat globule membrane (MFGM), where about 60% of

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milk phospholipids occur, modifying the membrane surface properties and decreasing the ζ -potential (Keenan and Mather, 2006; Lilbæk et al., 2007; Bourlieu et al., 2012). However, the effect of PL-A₁ on the MFGM and its influence during the manufacture of cheese is not yet fully understood.

On the other hand, a growing interest exists nowadays on the use of EPS-producing bacteria to increase cheese yield, mainly because of the generally recognized as safe (GRAS) status of these microorganisms, and because its industrial use is easy and inexpensive (Lin and Chang Chien, 2007). The use of EPS-producing bacteria to increase cheese yield has been explored in different types of cheeses, such as half-fat Cheddar cheese (Dabour et al., 2006; Costa et al., 2010), Mozzarella (Petersen et al., 2000), and fresh cheese (Jiménez-Guzmán et al., 2009). It is believed that bacterial polysaccharides increase the cheese yield by trapping the fat and non-fat milk solids into the web of EPS and the casein matrix (Petersen et al., 2000; Dabour et al., 2006; Jiménez-Guzmán et al., 2009; Costa et al., 2010). Bacterial polysaccharides also increase the moisture content of the cheese, although the chemical interaction between water and polysaccharides is very weak; therefore, water is just physically retained into the web of polysaccharides (Costa et al., 2012). It is known that the properties of microbial EPS depend on their composition, structure, charge, linkage between units, and presence of repeated side chains (Duboc and Mollet, 2001). However, it is still not clear how these polysaccharides interact with certain milk components and the kind of interactions that occur between EPS and cheese components.

One of the most popular and consumed cheeses in Mexico is the Chihuahua cheese or Mennonite cheese (Tunick et al., 2008). The history of Chihuahua cheese is closely related to Mennonite communities who migrated from Canada to Chihuahua, Mexico, in 1922 (Hansen, 2005). The manufacturing process of Chihuahua cheese resembles that of Cheddar cheese, although its texture is more similar to fresh Colby cheese. Chihuahua cheese is typically yellow and turns golden yellow when ripe, it has a semi-hard and sliceable consistency, and its sensory characteristics are similar to that of 1-mo-old Cheddar cheese (Gutiérrez-Méndez and Nevárez-Moorillon, 2009). Little information exists about the yield of Chihuahua cheese, but according to local producers, it ranges from 9 to 9.5 kg of cheese from 100 kg of milk.

The State of Chihuahua is located in a desert region in Mexico, where milk production is expensive and, thus, the profitability of cheese production is low. For this reason, a major concern for local cheese makers is the cheese yield and the alternatives to increase it. In

this work, the effect of 2 combined methods (cheese milk treated with PL-A₁ and the incorporation of an EPS-producing culture) on the yield, texture, and microstructure of cheese was explored. The research was carried out using miniature versions of Chihuahua cheese to improve the reproducibility of experimental cheeses. Miniature model cheeses are similar to pilot plant cheeses and can be manufactured in the laboratory under controlled conditions, minimizing manufacturing variations and reducing microbiological contamination (Shakeel-Ur-Rehman et al., 1998, 1999, 2001; Hynes et al., 2000, 2003; Milesi et al., 2007, 2008).

MATERIALS AND METHODS

Microorganisms and Phospholipase

Two different cultures of lactic acid bacteria (**LAB**) were used in this study: a starter culture and an EPS-producing culture. The starter culture (MM101-LY025; Danisco Deutschland GmbH, Niebüll, Germany) used for the manufacture of Chihuahua cheese included strains of *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *cremoris*, and *Lactococcus lactis* ssp. *lactis* var. *diacetylactis*. The EPS-producing culture (Y 470E; Sacco International, Cadorago, Italy) had a strain of *Streptococcus thermophilus* (ropy EPS-producing streptococci) and a strain of *Lactobacillus delbrueckii*. The phospholipase used in this study was PL-A₁ (phosphatidylcholine 1-acylhydrolase; EC 3.1.1.32) from *Aspergillus oryzae*. The PL-A₁ solution (YieldMAX; Chr. Hansen A/S, Hørsholm, Denmark) had the strength of 2,300 Lecitase units (**LEU**)/mL and was added into the milk at a rate of 5 LEU per gram of milk fat, according to the specifications of the manufacturer.

Determination of the Effect of PL-A₁ on Bacterial Growth and EPS Production

Eight conical tubes (Falcon; BD, Franklin Lakes, NJ) with 50 mL of sterile M17 broth were inoculated with EPS-producing bacteria, and then 3.25 μ L of PL-A₁ (YieldMAX; Chr. Hansen A/S) was added to 4 of these tubes. The final concentration of PL-A₁ in the broths was 15 LEU/100 mL of M17 broth. Broths were poured into wells of sterile microplates (96-well U-bottom shape), adding 250 μ L of broth in each well. Microplates were incubated at 37°C in a microplate reader (ELx808; BioTek Instruments Inc., Winooski, VT), and changes in optical density at a wavelength of 630 nm (OD₆₃₀) were monitored every 60 min for 24 h. Data collected were subjected to nonlinear regression analysis using the Baranyi model (Baranyi and Roberts, 1994) with the software DMFit (version 2.1; Institute of Food Re-

search, Norwich, UK) to obtain the growth parameters of the EPS-producing culture.

Production of EPS was measured in the 8 M17 broths immediately after inoculation (initial polysaccharide concentration) and after incubation at 37°C for 24 h (final concentration). Five milliliters from each broth was heated at 80°C for 15 min and then cooled and centrifuged at $1,700 \times g$ for 15 min at 20°C. Ten milliliters of ethanol (80%) was added to the supernatants obtained, which were centrifuged again at $1,700 \times g$ for 15 min at 20°C. These supernatants were analyzed for their sugar content according to the method described by DuBois et al. (1956) and expressed as glucose equivalents. The polysaccharide yield was calculated by subtracting the initial polysaccharide concentration from the final concentration.

Manufacture of Miniature Chihuahua-Type Cheese

Four different types of cheeses were manufactured in this study: (a) control cheese without addition of PL-A₁ and (or) EPS-producing bacteria, (b) cheese made with milk treated with PL-A₁ (YieldMAX; Chr. Hansen A/S), (c) cheese manufactured with an EPS-producing culture (Y 470E; Sacco International), and (d) cheese made with milk treated with PL-A₁ and inoculated with an EPS-producing culture. The PL-A₁ was added in a concentration of 15 LEU/100 g of milk, whereas EPS-producing bacteria were inoculated by adding 1% (vol/vol) of milk previously fermented with this culture. Each treatment was repeated 4 times in different batches on different days, using a similar procedure to the one described by Shakeel-Ur-Rehman et al. (1998). Portions of 250 g of standardized (3% fat) milk were placed in four 500-mL glass beakers and pasteurized at 63°C for 30 min. Milk portions were cooled to 32°C and inoculated with freeze-dried starter culture (MM101-LY025; Danisco Deutschland GmbH), the EPS culture, and (or) the PL-A₁. The milk was incubated for 1 h at 32°C; then, 100 µL of 4.6 M CaCl₂ was added (to obtain a final concentration in the milk of 1.84 mM) and 13.75 µL of chymosin [strength 1,000 international milk-clotting units (IMCU)/mL; CHY-MAX; Chr. Hansen A/S] was added. After 1 h of incubation at 32°C, curds were cut with a stainless steel spatula into small cubes of 0.8 cm³, held for 10 min, and then stirred at 150 rpm for 60 min in an orbital shaker (DS-500; VWR Scientific Products, Thorofare, NJ) at 32°C. Afterward, whey was removed using a strainer and the curd was warmed to 38°C in a water bath until the pH reached 5.6. The curd was then salted with 1% NaCl (wt/wt) and transferred into polypropylene tubes (4.3-cm diameter and 9-cm height). The tubes were centrifuged for 30 min at $1,700 \times g$ at room temperature. The expelled whey was

drained, and the curd was again centrifuged for 60 min at $1,700 \times g$ at room temperature. Mini-cheeses were removed from the tubes and refrigerated (4°C at 75% relative humidity) without packing for 48 h to adjust the moisture content. Finally, mini-cheeses were wiped with tissue paper (to remove excess moisture), packed in hermetic polyethylene bags (16.5 × 14.9 cm), and stored at 3°C for 1 wk before their analysis.

Compositional Analysis of Cheeses

Whey and cheese samples were analyzed (in triplicate) for moisture, protein, fat, and ash content using AOAC International (1998) methods (method 926.08, 991.22, 905.02, and 935.42, respectively). The pH was obtained using AOAC International (1998) method 981.12 with a pH meter (Pinnacle; Corning Inc., Corning, NY). The measurement of ionic calcium in whey samples was performed using an ion-selective electrode (HI 4104; Hanna Instruments Inc., Woonsocket, RI), as described by Nielsen (2010).

Determination of Cheese Yield

The weight of the milk, curd, and cheese was measured during manufacture of the miniature cheeses using an analytical balance (0.01-mg resolution). The weight of the whey recovered from the drainage of curd plus the weight of the whey obtained from the 2 centrifugation steps was considered as the total weight of whey. Miniature cheeses were weighed before their packaging in plastic bags and 10 d after their manufacture. Actual yield (Y_a) was calculated using the weight of the cheeses after 10 d of manufacture.

The percentage of milk fat lost in whey (%FL) was calculated with the following formula:

$$\%FL = \frac{\text{weight of whey} \times \text{proportion of fat in whey} \times 100}{\text{weight of milk} \times \text{proportion of fat in milk}}.$$

The Y_a was calculated using the following formula:

$$Y_a = \frac{\text{weight of cheese} \times 100}{\text{weight of milk}}.$$

The moisture-adjusted cheese yield (Y_{ma}) was calculated using the formula

$$Y_{ma} = Y_a \times \left(\frac{100 - Ma}{100 - Mr} \right),$$

where Ma corresponds to the moisture observed in the cheese samples and Mr to the reference moisture

(Guinee et al., 2006). The *Mr* used (40%) was obtained from the analysis of 12 different commercial Chihuahua cheese brands.

Texture Analysis

Texture profile analysis of miniature cheeses was performed 10 d after their manufacture using a texture analyzer (TA.XT*plus*; Stable Micro Systems Ltd., London, UK), with a cell load of 50 kg and a cylinder flat-ended probe (P/2 SL) of 2.5-cm diameter. Three cylindrical sections (1.2-cm diameter and 1.5-cm height) from each miniature cheese were compressed (75% of their original height) twice using a crosshead speed of 0.05 cm/s. The second compression was delayed 5 s from the first compression. Cheese samples were allowed to equilibrate for 1 h at room temperature (25°C) before testing. Hardness, fracturability, adhesiveness, springiness, cohesiveness, and apparent Young's modulus were calculated according to Bourne (1978, 2002) using the instrument's software (Texture Exponent; Stable Micro Systems Ltd.).

Sensory Analysis

Chihuahua cheeses (2-kg blocks) were manufactured in a local dairy pilot plant using the conventional procedure but adding PL-A₁ or EPS-producing culture, or both, as done with the miniature cheeses. Twenty-five liters of pasteurized milk (63°C for 30 min) with 3% fat was inoculated with freeze-dried starter culture (MM101-LY025; Danisco Deutschland GmbH). Depending on the treatment, cheese milk was inoculated with 1% (vol/vol) milk previously fermented with the EPS culture (Y 470E; Sacco International) and (or) PL-A₁ (YieldMAX; Chr. Hansen A/S) at 15 LEU per 100 g of milk. The milk was incubated for 1 h at 32°C; then, a solution of 4.6 M CaCl₂ was added (to obtain a final concentration in the milk of 1.84 mM) and chymosin (5.5 mL/100 L; CHY-MAX; Chr. Hansen A/S) was added. After 1 h, the curd was cut with harps into cubes of 0.8 cm³, held for 10 min, and then stirred manually for 1 h. The curd was warmed up to 38°C (increasing 1°C every 5 min) and kept at this temperature for 30 min. After this period of time, whey was drained, and curds were salted (1% NaCl, wt/wt) when curds reached a pH of 5.6. Finally, the curd was placed in rectangular molds of 2 kg and pressed at 150 kPa overnight using a pneumatic press (150 kPa). Cheeses were stored unpacked in refrigeration (4°C) at 75% relative humidity for 48 h and then packed using polyethylene bags and stored at 3°C for 1 wk before being used for sensory analyses. All experimental cheeses were manufactured the same day.

Cheese blocks (10 d old) were cut into small cubes of 1 cm³ and stored at 10°C until sensory tests were

conducted. Cheese samples were evaluated by staff and students of the University Autonomous of Chihuahua using a ranking test (Meilgaard et al., 1999). Sensory tests were carried out by 50 panelists (25 males and 25 females) with an age range of 18 to 39 years old (mean = 24; median = 21). Each trial included 4 cubes of cheese (corresponding to the 4 treatments) identified with a 3-digit code and presented randomly. Panelists were instructed to taste the cheeses and rank them in order of increasing preference in flavor and texture.

Electron Scanning Microscopy

Preparation of cheese samples was performed according to the procedure described by Dykstra (Dykstra, 1993). Samples from miniature cheeses were finely sliced (1-mm thick) and submerged immediately in a solution with (2.5%, wt/vol) glutaraldehyde and sodium cacodylate (0.1 M). Samples were held for 8 h at 10°C to allow glutaraldehyde fixation. Afterward, cheese samples were dehydrated in increasing concentrations of ethanol (55, 65, 95, and 100%), washing the cheese samples for 20 min with each ethanol solution. Finally, samples were dried with CO₂ (critical point drying) and coated with a thin layer of Au:Pb (60:40). The cheese samples were observed with a scanning microscope (JSM-5800LV; Jeol Ltd., Tokyo, Japan) using an acceleration voltage of 10 kV.

Statistical Analysis

Comparison of polysaccharide production by the EPS-producing culture with and without the presence of PL-A₁ was performed using a Student's *t*-test (2-tailed) with a significance level of 0.05. Cheeses were manufactured 4 times with each treatment in different batches and each batch was manufactured on different days. Data collected from the compositional analysis, texture analysis, and cheese yields were analyzed in a 2-level factorial design with the software Minitab 16 (Minitab Inc., State College, PA). Two (categorical) factors were included in this design: the PL-A₁ and the EPS-producing culture. Both factors were applied in 2 levels: presence (+) and absence (−). Data collected from the sensorial analyses were first analyzed using a Friedman analysis (Meilgaard et al., 1999) and then subjected to a multiple comparison test according to the procedure described by Christensen et al. (2006).

RESULTS AND DISCUSSION

Microbial Growth and EPS Production

Phospholipids are present in all biological membranes, including the microbial cell membranes (Mus-

tranta et al., 1995). The hydrolysis of phospholipids by phospholipases generates tensoactive products. The release of these products from the cellular membrane can alter the supramolecular structure (Bourlieu et al., 2012). In this regard, some phospholipases have shown the ability to kill a broad spectrum of gram-positive bacteria (Qu and Lehrer, 1998). For this reason, one concern in this study was the possible effect of PL-A₁ against the growth of the EPS-producing bacteria or polysaccharide production. However, the presence of PL-A₁ in the media did not affect the growth of the EPS-producing culture (Table 1) or the growth of the starter culture used in the manufacture of cheese (data not shown). The presence of this enzyme did not produce any change on the lag time or growth rate of the EPS-producing culture (Table 1).

The production of polysaccharides by the EPS-producing bacteria was not affected by the addition of PL-A₁ in the media (Table 1), obtaining the same polysaccharide yield with or without the addition of PL-A₁ (≈ 200 mg/L). It has been reported that the yield of EPS produced by LAB varies depending on the strain, medium, and growing conditions from 150 to 600 mg/L (Degeest et al., 2001).

Compositional Analysis and Cheese Yield

Addition of PL-A₁ into the cheese milk for the manufacture of Chihuahua cheese significantly increased protein retention (in DM). Nevertheless, the cheeses manufactured with PL-A₁ did not increase the retention of solids or fat in DM (Table 2). Incorporation of EPS-producing bacteria in the manufacture of Chihuahua cheese increased the water activity, protein content (in DM), and moisture content. Additionally, the manufac-

ture of cheese with EPS-producing culture significantly decreased fat loss in whey and increased fat retention (in DM) in the cheese. Cheeses manufactured with both PL-A₁ and EPS-producing cultures had higher water activity, protein content (in DM), and moisture content than control cheeses, but lower pH and released whey compared with control cheeses. Despite these differences from the control cheeses, it is worth highlighting that no significant differences existed in the pH and whey released among all the cheese treatments (PL-A₁, EPS, and EPS + PL-A₁).

Phosphorous and calcium are the most abundant components of salts in milk. Part of these salts is attached to casein micelles as colloidal calcium phosphate (CCP). The pH of the curd when whey is drained and at the moment of pressing determines how much CCP will not be incorporated in the cheese, because acidification leads to dissolution of CCP. Furthermore, the pH of the curd also may affect the retention of NaCl added in the manufacture of cheese. Cheeses with low pH lose more salt in the expressed whey than cheeses with high pH (Walstra et al., 2006). In the current work, it was observed that the mineral content of cheeses (including the NaCl added) decreased markedly in those manufactured with EPS-producing bacteria (alone or in combination with PL-A₁). The reduction of mineral content in the cheeses manufactured with EPS-producing bacteria was not attributed to pH variations, because all the cheese treatments had similar pH in the curds during their manufacture (data not shown) and 10 d after manufacture (Table 2). For this reason, the reduction in mineral content in the cheeses manufactured with EPS-producing bacteria was attributed to changes in the water retention of these cheeses (Table 2) rather than to differences in pH. On the other hand,

Table 1. Influence of phospholipase A₁ on exopolysaccharide production and growth of *Streptococcus thermophilus*¹

Item	Treatment ²	
	M17 broth (control)	M17 broth with added phospholipase A ₁
Growth rate ³	0.110	0.129
Lag time ³ (h)	Not observed	Not observed
SE	0.029	0.035
R ²	0.92	0.94
Glucose equivalents (mg/L); $t = 0^4$	163.70 $\pm$ 0.21 ^a	156.90 $\pm$ 1.30 ^b
Glucose equivalents (mg/L); $t = 24$	371.88 $\pm$ 5.83 ^a	363.44 $\pm$ 1.59 ^a
Polysaccharide yield (mg/L)	208.18 $\pm$ 3.28 ^a	206.54 $\pm$ 1.43 ^a

^{a,b}Means within a row with different superscript letters differ ($P < 0.05$); $n = 4$.

¹Results are presented as means $\pm$ SD.

²Treatment with phospholipase A₁ included addition of 15 Lecitase units/100 g of M17 broth.

³Growth parameters were obtained by fitting 3 repetitions of each treatment to the Baranyi and Roberts (1994) equation.

⁴ t = incubation time in hours.

Table 2. Effect of adding phospholipase A₁ (PL-A₁) or exopolysaccharide (EPS)-producing bacteria, or both, to cheese milk on the chemical composition of miniature Chihuahua cheeses 10 d after their manufacture

Item ¹	Treatment ²				SEM
	Control (n = 4)	PL-A ₁ (n = 4)	EPS (n = 4)	EPS + PL-A ₁ (n = 4)	
Cheese					
Cheese weight (g)	20.61 ^c	25.50 ^b	26.25 ^a	25.82 ^{ab}	0.242
pH	5.44 ^b	5.32 ^{ab}	5.34 ^{ab}	5.20 ^a	0.042
a _w	0.970 ^a	0.972 ^{ab}	0.978 ^b	0.975 ^{ab}	0.002
Moisture (g/100 g)	43.09 ^a	43.42 ^{ab}	48.28 ^c	45.72 ^{bc}	0.614
Fat (g/100 g)	30.56 ^{ab}	29.43 ^a	30.45 ^{ab}	31.32 ^b	0.296
Protein (g/100 g)	21.87 ^a	25.67 ^b	22.84 ^a	23.62 ^{ab}	0.589
Ash (g/100 g)	3.04 ^{ab}	3.19 ^a	2.71 ^c	2.73 ^{bc}	0.081
TS (g/100 g)	56.91 ^a	56.58 ^{ab}	51.72 ^c	54.28 ^{bc}	0.614
FDM (%)	53.79 ^b	52.03 ^b	58.90 ^a	57.71 ^a	0.832
PDM (%)	38.47 ^b	45.39 ^a	44.17 ^a	43.50 ^a	1.102
Whey					
Weight of bulk whey (g)	217.36 ^a	212.66 ^b	211.76 ^b	213.26 ^b	0.560
Ionic calcium (mM × 10 ⁻³)	26.63 ^a	17.87 ^b	25.55 ^a	14.84 ^b	2.472
Fat (g/100 g)	0.350 ^a	0.192 ^{ab}	0.097 ^b	0.227 ^{ab}	0.056
Fat lost in whey (FL%)	10.89 ^a	5.23 ^{ab}	2.75 ^b	3.40 ^{ab}	1.86

^{a-c}Means within a row with different superscript letters differ ($P < 0.05$).

¹a_w = water activity; FDM = fat in DM; PDM = protein in DM.

²Control = cheese milk without EPS-producing culture or PL-A₁; PL-A₁ = cheese milk with added PL-A₁ (15 Lecitase units/100 g of milk); EPS = cheese milk inoculated at 1% (vol/vol) with an EPS-producing culture; EPS + PL-A₁ = cheese milk with an added EPS-producing culture and PL-A₁.

the whey obtained from the cheeses manufactures with PL-A₁ (alone or in combination) presented the lowest concentration of ionic calcium. This suggests that ionic calcium was retained in the cheese matrix. Nevertheless, it is still not clear how the PL-A₁ promotes the retention of ionic calcium.

Cheeses manufactured with PL-A₁ and the cheeses made with EPS-producing bacteria, as well as those manufactured with both PL-A₁ and EPS-producing bacteria showed an increase in their final weight and a significant reduction in the amount of whey released during their manufacture, compared with control cheeses (Table 2). These changes in the weight of the cheeses and the release of whey affected the yields of these cheese processes. The yield of control cheese was 9.21%, and the incorporation of EPS-producing bacteria in the manufacture of Chihuahua cheese significantly increased the yield to 10.53% (Figure 1). The addition of PL-A₁ into the cheese-milk did not cause a significant increase in cheese yield (9.66%) compared with control cheese. In contrast, the combination of PL-A₁ and EPS-producing bacteria in the manufacture of Chihuahua cheese produced the highest actual yield (10.81%). The moisture-adjusted cheese yield (Y_{ma}) of cheeses manufactured with PL-A₁ was 9.39%, whereas cheeses made with EPS-producing bacteria obtained a Y_{ma} of 9.07%. These yields were not significantly higher ($P > 0.05$) than the Y_{ma} observed in control cheeses (8.73%). Cheeses manufactured with both PL-

A₁ and EPS-producing bacteria significantly increased their Y_{ma} (9.78%) compared with control cheeses. The Y_{ma} was calculated to eliminate the direct effect of differences in cheese moisture. The cheeses manufactured with PL-A₁ or with EPS-producing bacteria did not significantly increase the Y_{ma} compared with control cheeses because these treatments mainly improved the water retention. In contrast, the cheeses manufactured with both PL-A₁ and EPS-producing culture increased the Y_{ma} by improving the retention of water, fat, and protein in the cheese.

The idea of using phospholipases to increase cheese yield had not been considered until recently, and the effect of these enzymes on the MFGM remains unclear (De Maria et al., 2007). Bovine milk typically contains $>10^{10}$ fat globules per milliliter and about 60% of phospholipids occur in the MFGM (Keenan and Mather, 2006; Walstra et al., 2006). Some authors have reported that phospholipase radically changes the surface of the MFGM, detaching bilayer fragments (lysophospholipids and FA) in the aqueous phase (Lilbæk et al., 2007; Bourlieu et al., 2012). Modification of the surface of the MFGM also reduces the ζ -potential on the surface of fat globules due to intensification of negative charges from carboxylate anions (Bourlieu et al., 2012). The change in the ζ -potential avoids the coalescence of fat globules, as was observed by Bourlieu et al. (2012) and Lilbæk et al. (2007), because the aggregation of milk globules occurs only if the van der Waals attraction and repulsive

steric forces are larger than the electrostatic repulsion. Nevertheless, the increase in negative charges at the surface of the MFGM also improves the solubility of fat globules in the serum phase and might promote their loss during whey drainage. This may explain the poor retention of fat in the Chihuahua cheeses manufactured with PL-A₁. In contradiction with this finding, it has been reported that the use of PL-A₁ (from *Fusarium venenatum*) in the manufacture of Mozzarella cheese increases fat retention, moisture content, and cheese yield by 3.2% (Lilbæk et al., 2006). This discrepancy may be due to differences in the manufacture procedures between Mozzarella and Chihuahua cheese. In the manufacture of Mozzarella cheese, the curd is stretched and kneaded in hot water. During the plasticizing of the curd, some fat is lost in the hot water and probably the effect of PL-A₁ on fat retention becomes more evident.

On the other hand, protein content increased in Chihuahua cheeses manufactured with PL-A₁ (alone or in combination with the EPS-producing bacteria). Some authors have suggested that lysophospholipids and FA derived from PL-A₁ action interact with milk proteins, including whey proteins and caseins. Binding of lysophospholipids to milk proteins prevents protein-protein interactions and modifies their surface properties (Lilbæk et al., 2007). Therefore, the formation of lysophospholipid-protein complexes might improve the retention of some additional proteins in the cheese matrix.

The use of an EPS-producing culture enhanced the moisture content and cheese yield of Chihuahua cheese. Several authors have reported the improvement of water retention and cheese yield by the use of EPS-producing bacteria. In low-fat Cheddar cheese, an increase in water retention from 1.24 to 4.80% and an improvement of the cheese yield of 0.58 to 0.60% has been observed (Dabour et al., 2006; Costa et al., 2010, 2012). Previous research on Mozzarella cheese has reported a 6.1% increase in the moisture content (Petersen et al., 2000) and research conducted on fresh cheese showed the ability of EPS-producing bacteria to increase yield by 2.21%, the moisture by 3.90%, and fat content by 7.64% (Jiménez-Guzmán et al., 2009). In general, it has been observed that the use of an EPS-producing culture in the manufacture of cheese increases yield mainly through higher water retention. Such results may vary, depending on the cheese variety and the nature of the EPS produced by the LAB. Some authors have suggested that EPS filaments are attached to the protein matrix (by electrostatic interactions), creating a web of filaments (Ayala-Hernandez et al., 2008). The additional water retained in the cheese matrix is weakly bound to the EPS filaments and is just mechanically trapped in the web of filaments (Laws and Marshall,

Figure 1. Actual cheese yield (Y_a) and moisture-adjusted cheese yield (Y_{ma}) of Chihuahua cheese manufactured with an exopolysaccharide (EPS)-producing bacteria or phospholipase A₁ (PL-A₁), or both. Bars of the same color with different letters (a–c, or A and B) are significantly different (multiple mean comparison, Tukey-Kramer test, $\alpha = 0.05$).

2001; Ayala-Hernandez et al., 2008; Costa et al., 2010). This may explain the significant increase in the water activity of the Chihuahua cheeses manufactured with an EPS-producing culture.

In the present study, the mini-cheeses manufactured with PL-A₁ in combination with EPS-producing bacteria had the highest Y_{ma} and the smallest loss of ionic calcium into the whey. Interestingly, the micrographs (Figure 2) from these cheeses did not show a web of filamentous polysaccharides attached to the cheese matrix, as was observed in those cheeses manufactured only with the EPS-producing bacteria. The interaction of bacterial EPS with the casein matrix has been widely recognized, but little attention has been paid to the relation of EPS with the lipid fraction or other molecules. Results from this study suggest that some EPS bind to components of the MFGM and that modification of the MFGM by action of the PL-A₁ might alter these interactions. However, more research is required to understand more fully the interaction of polysaccharides with the components of the MFGM surface.

Texture Analysis

Texture profile analysis showed that control cheese was significantly harder than cheeses manufactured with a PL-A₁ or EPS-producing bacteria, or both (Table 3). Incorporation of an EPS-producing culture or PL-A₁, or both, in the manufacture of Chihuahua cheese also reduced the force required to fracture the cheese samples (fracturability), producing crumblier

Figure 2. Scanning electron microscopy of Chihuahua cheeses. I = control Chihuahua cheese without phospholipase or exopolysaccharide-producing bacteria; II = Chihuahua cheese with phospholipase A₁; III = Chihuahua cheese made with exopolysaccharide-producing bacteria; IV = Chihuahua cheese made with phospholipase A₁ and exopolysaccharide-producing bacteria. The white line represents a length of 10 μm .

cheeses. Similarly, Dabour et al. (2006) reported a decrement of hardness in low-fat Cheddar cheese manufactured with ropy EPS streptococci. The decrement in hardness of Chihuahua cheese by addition of PL-A₁ or EPS-producing bacteria, or both, is probably related to changes in the components of the cheese; chiefly to enhanced moisture content in cheese manufactured with EPS-producing bacteria and the protein-to-fat ratio in cheese with PL-A₁. Nevertheless, other factors such as the pH and ash content might have affected the cheese texture. Cheeses with a less acidic pH and high ash content (such as the control cheese) were harder than cheeses with a more acidic pH and low ash content (see Table 2). The solubilization of colloidal calcium increases at pH 5, decreasing the interactions between proteins and improving the solvation of caseins and hydration of the protein matrix (Lucey et al., 2003). It has been reported that reducing the pH of Cheddar cheese from 5.4 to 5.0 increases the calcium solubilization and moisture content, producing a decrease in the hardness of the cheese (Pastorino et al., 2003).

Springiness and cohesiveness of Chihuahua cheese were not significantly modified by the addition of the

PL-A₁ or the EPS-producing bacteria, or both. However, the chewiness (energy required to masticate a solid food product) of Chihuahua cheeses was significantly reduced when PL-A₁ was incorporated in the process, possibly caused by changes in the proportion of protein and fat in the cheeses manufactured with PL-A₁ (Table 2). These changes in chewiness were expected, as this parameter is related to hardness, springiness, and cohesiveness of the cheeses (Bourne, 2002). The cohesiveness and springiness did not change significantly in any of the cheese treatments and thus chewiness became approximately proportional to the hardness of the cheeses.

The apparent Young's modulus represents the stiffness of the material to an applied load. A Young's modulus with larger value denotes that higher stress (force per unit area) is needed to generate a given strain (deformation). Cheese manufactured with PL-A₁ obtained the lowest Young's modulus value, whereas cheese manufactured with EPS obtained the highest Young's modulus value. The enhancement of the stiffness in the cheese manufactured with the EPS-producing bacteria may be explained by the increase in water content and the formation of a filamentous web of polysaccharides

Table 3. Effects of adding phospholipase A₁ (PL-A₁) or exopolysaccharide (EPS)-producing bacteria, or both, in cheese milk on the texture properties of miniature Chihuahua cheeses 10 d after their manufacture

Item	Treatment ¹				SEM
	Control (n = 4)	PL-A ₁ (n = 4)	EPS (n = 4)	EPS + PL-A ₁ (n = 4)	
Hardness (N)	5.81 ^b	4.07 ^a	4.10 ^a	3.83 ^a	0.331
Fracturability (N)	4.38 ^b	2.08 ^a	2.71 ^a	2.12 ^a	0.339
Springiness (mm)	4.55 ^a	3.64 ^a	3.99 ^a	2.89 ^a	0.463
Chewiness (mJ)	5.79 ^a	2.06 ^b	3.77 ^{ab}	1.74 ^b	0.771
Cohesiveness	0.222 ^a	0.205 ^a	0.207 ^a	0.171 ^a	0.026
Apparent Young's modulus (kPa)	5.2 ^b	4.7 ^c	5.9 ^a	4.4 ^c	0.280

^{a-c}Means within a row with different superscript letters differ ($P < 0.05$).

¹Control = cheese milk without EPS-producing culture or PL-A₁; PL-A₁ = cheese milk with added PL-A₁ (15 Lecitase units/100 g of milk); EPS = cheese-milk inoculated at 1% (vol/vol) with an EPS-producing culture; EPS + PL-A₁ = cheese milk with an added EPS-producing culture and PL-A₁.

in the cheese matrix. The filamentous polysaccharides produced by EPS-producing bacteria were observed intertwined in the protein matrix of the cheese (Figure 2), producing larger whey pockets than those observed in control cheeses or cheeses manufactured with PL-A₁. Other authors have observed similar characteristics in the matrix of cheeses manufactured with EPS-producing bacteria (Dabour et al., 2006; Ayala-Hernandez et al., 2008). The structure observed in the cheeses manufactured with the EPS-producing culture explains the increase in water content, but also provides more evidence supporting the idea that water is just trapped and probably weakly bound.

Sensory Analysis

Commercial Chihuahua cheese usually displays mild to tangy flavor and has a firm but yet not hard texture. This cheese is slightly sticky in the mouth and quite soft when chewed (Van Hekken et al., 2006). All the participants in the sensory evaluation expressed to be regular consumers of Chihuahua cheese. All cheeses were equally preferred in flavor, with the exception of the cheese manufactured with both PL-A₁ and EPS-producing bacteria, which had highest preference in flavor (Table 4). One concern in the case of cheeses manufactured with phospholipase was the possible development of rancid flavors produced by hydrolysis of triglycerides. Nevertheless, and according to the comments of the judges, these cheeses did not showed rancid flavor. On the other hand, the texture of the cheese manufactured with EPS-producing bacteria was perceived as unpleasant by most of the judges. This may have been caused by the excessive increase in water retention and modification of rheological properties (Table 3). Similarly, Dabour et al. (2006) reported that the use of ropy EPS streptococci induced the formation of a less compact texture, which is undesirable in

aged Cheddar cheese, usually characterized by harder texture.

CONCLUSIONS

The presence of PL-A₁ in the growth medium did not affect the growth and polysaccharide production by the EPS-producing bacteria. The use of PL-A₁ in the manufacture of Chihuahua cheese did not increase fat retention or cheese yield as was expected. The use of EPS improved cheese yield by increasing water and fat retention, but caused a negative effect on the texture and flavor of Chihuahua cheese. The combination of PL-A₁ with the EPS-producing culture improved cheese yield and increased the moisture and fat content. The cheeses with the best flavor and texture were those manufactured with PL-A₁ and the cheeses manufactured with the combination of PL-A₁ and EPS-producing culture. On the other hand, it is still unclear how PL-A₁ produces modifications in the milk fat, particularly on the MFGM and the effect of these changes on the quality of the cheese. Another

Table 4. Sensory analysis of Chihuahua cheese manufactured with an exopolysaccharide (EPS)-producing bacteria or phospholipase A₁ (PL-A₁), or both¹

Treatment	Rank sum ²	
	Flavor	Texture
Control cheese	98 ^a	110 ^{ab}
Cheese made with PL-A ₁	134 ^{ab}	140 ^b
Cheese made with EPS-producing culture	103 ^a	95 ^a
Cheese made with EPS + PL-A ₁	156 ^b	145 ^b

^{a,b}Rank sums within a column with different superscript letters differ, according to the critical difference value between samples (Christensen et al., 2006).

¹The significance level was $P = 0.01$ and the critical difference was 39 for both flavor and texture.

²Rank sum obtained from the responses of 50 nontrained judges.

issue derived from this study that would be important to address would be the residual phospholipase activity and its effect during the ripening of cheese. More study is also required regarding the interaction of bacterial polysaccharides with cheese components, such as the surface of the MFGM. Despite the lack of a better understanding of the intimate mechanisms involved, clear evidence exists that cheese yield is increased when PL-A₁ and EPS-producing culture are used in combination in the manufacture of cheese. These findings need still to be successfully extrapolated and proven in large-scale production. Additionally, more research is required to understand the interactions established by PL-A₁ and the bacterial polysaccharides with the cheese components.

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