Vol. 16, No. 2 (2017) 425-434
Revista Mexicana de Ingeniería Química

Listeria monocytogenes GROWTH INHIBITION IN INOCULATED FRESH PANELA CHEESE BY THE ADDITION OF *Rhoeo discolor* AQUEOUS EXTRACTS COMBINED WITH POTASSIUM SORBATE

INHIBICIÓN EN EL CRECIMIENTO DE *Listeria monocytogenes* EN QUESO PANELA SUPLEMENTADO CON EXTRACTOS ACUOSOS DE *Rhoeo discolor* CON COMBINACIÓN CON SORBATO DE POTASIO

R. Benítez-Azaola[1,a], R. García-Varela[1,2,a], R. M. García-García[3], A. Suárez-Jacobo[2], G.A. Cardineau[1] , J. Altamirano[4], S.O. Serna-Saldívar[1]*

[1]*Centro de Biotecnología FEMSA, Escuela de Ingeniería y Ciencias, Tecnológico de Monterrey, Campus Monterrey, Av. Eugenio Garza Sada 2501 Sur, C.P. 64849 Monterrey, NL, México.*

[2]*Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C. Unidad Noreste, Autopista Mty-Aeropuerto, Km 10. Parque PIIT. Vía de Innovación 404, Apodaca, NL 66629.*

[3]*Departamento de Ingeniería de Alimentos, Tecnológico de Monterrey, Campus Monterrey, NL, México.*

[4]*Instituto de Cardiología y Medicina Vascular, Escuela de Medicina, Tecnológico de Monterrey, Campus Monterrey, Batallón San Patricio 112 Col. Valle de San Agustín CP 66278, San Pedro Garza García, Nuevo León, México.*

Received November 10, 2016; Accepted January 4, 2017

Abstract

Natural resources have been exploited in the search for new bioactive compounds. *Rhoeo discolor* is a plant commonly employed in traditional folk medicine throughout Latin America. The antimicrobial effect of extracts derived from this plant, have proven effective against a variety of microorganisms. The inhibitory effect against *Listeria monocytogenes* growth of two aqueous extracts obtained from dry *Rhoeo discolor* leaves at 25°C and 90°C and containing 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.3, 3.1 or 1.6 solids/mL was evaluated.. These extracts were then combined with 50, 25, 12.5, 6.3, 3.1 and 1.6 mg/kg of potassium sorbate. The extracts that showed better inhibitory effect by the reduction of two full logarithmic growth cycles were those containing 800 or 400 μg/mL of *R. discolor* extract and 6.3, 3.1 or 1.6 mg/kg of potassium sorbate, these were tested in inoculated *Panela* cheese and where later evaluated in terms of *L. monocytogenes* growth inhibition. Acceptance analyses were performed to determine alteration in the organoleptic properties in control and experimental (non-inoculated) cheese. Results revealed that the additions of *R. discolor* extracts and potassium sorbate in these concentrations did not affect organoleptic properties. Experimental cheese samples were in occasions ranked better than the control cheese regarding organoleptic parameters.

Keywords: *Listeria monocytogenes*, *Rhoeo discolor*, *Panela* cheese, aqueous extracts, shelf life.

Resumen

La ciencia ha recurrido a la naturaleza en busca de nuevos compuestos bioactivos. Extractos de *Rhoeo discolor*, una planta comúnmente empleada en medicina tradicional en América Latina, ha mostrado efectos antimicrobianos efectivos contra numerosos microorganismos. Dos extractos acuosos, hojas previamente secadas hirviéndolas y no hervidas, a concentraciones de 3200, 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.3, 3.1 o 1.6 μg/mL; combinados con 50, 25, 12.5, 6.3, 3.1 y 1.6 mg/kg de sorbato de potasio fueron probados contra *Listeria monocytogenes*. Las combinaciones entre 800 y 400 μg/mL de extracto de *R. discolor* y 6.3, 3.1 o 1.6 mg / kg de sorbato de potasio fueron agregados en la formulación de queso *Panela* inoculado con *L. monocytogenes*, logrando una disminución significativa por dos ciclos logarítmicos el crecimiento de la población microbiana. Adicionalmente, se realizaron análisis de aceptación para determinar la alteración de las propiedades organolépticas en el queso experimental (no inoculado). Los resultados revelaron que la adición de extractos de *R. discolor* y sorbato de potasio en estas concentraciones no afectaron las propiedades organolépticas. Las muestras experimentales de queso fueron en ocasiones mejor clasificadas que el queso de control en cuanto a sus parámetros organolépticos.

Palabras clave: *Listeria monocytogenes*, *Rhoeo discolor*, queso *Panela*, extractos acusosos, vida de anaquel.

* *Corresponding author. E-mail*: sserna@itesm.mx

[a] *These two authors contributed equally to this research*

1 Introduction

The excessive incorporation of chemical additives, in our daily food consumption, has been a source of controversy because several of these chemicals used as food preservatives are under revision due to their possible side-effects, such as toxicity or long-term health damage. This fact has required food industries to either completely remove these non-GRAS chemicals or to apply natural alternatives to extend the shelf-life of food products. The interest of these natural preservatives is focused on preventing foodborne pathogen growth or to delay food spoilage while reducing synthetic preservatives (Gould, 2012; Jimenez *et al*., 2011). Many natural compounds, such as phenolic compounds and organic acids, have been considered for these applications. Several plant extracts have proven to possess antimicrobial activity, this property could be applied in food preservation, with little to no effects in human health and without damaging the overall quality of food products (Smith-Palmer *et al*., 1998).

In Mexico, traditional medicine has led to the use of a plant named *Rhoeo discolor* (*R. discolor*), commonly known as "purple maguey". This plant has been submitted to rigorous research because of the medical benefits claimed from popular knowledge. The leaves have been used by regional native cultures, mostly consumed as infusions or by direct topical use. The plant has been used to treat several pathologies, such as cancer, and also as an antimicrobial agent (García-Varela *et al*., 2016; González-Avila *et al*., 2003). These properties have been attributed to the bioactive phytochemicals profile mainly constituted by secondary metabolites, especially phenolic compounds (Idaka *et al*., 1987). Although it has been used for antimicrobial purposes among folk knowledge, extracts have never been incorporated in to food matrixes as a natural preservative.

Phenolic compounds are ubiquitous to plant products and are widely consumed all over the world without toxic effects; they have also been shown to inhibit many types of microorganisms; for this reason it is important to investigate their potential application to replace chemical preservatives. Addition of these natural compounds could extend the product quality and also enhance its functional food properties. Many phenolic compounds exert antioxidant activities and prevent oxidative stress, which exacerbates chronic diseases and cancer. Plants such as *R. discolor* are poorly exploited; for this reason further research is necessary to study their potential as sources of bioactive extracts (Kabara, 1991; Cardenas-Sandoval, *et al*., 2012)

Panela cheese is a fresh, artisan Mexican cheese, with high moisture content, commonly formulated from raw cow milk. This sort of cheese is widely consumed in Mexico; however, it may often be contaminated by *Listeria monocytogenes*, a pathogenic bacterium associated with this and other cheeses and dairy products (Alvarado *et al*., 2005; Domínguez-Niño *et al*., 2016). The addition of other bacteria cultures, such as lactic acid bacteria and probiotics, to the formulation may decrease *L. monocytogenes* outbreak; nevertheless, because of the high moisture content of this cheese and its pH, other preservatives must be incorporated to reduce or prevent the risk of pathogen growth (McAuliffe *et al*., 1999).

In order to decrease spoilage, food products are required to have longer shelf-lives; however, the maximum allowed concentration of non-GRAS chemical additives are regulated in domestic and export markets. In order to approve new chemical preservatives and their maximum permissible levels in foods, the additives have to be thoroughly tested with time consuming and expensive toxicological studies. These obstacles provide an important opportunity to introduce natural food preservatives as alternative or complementary additives that may not only increase shelf-life but also improve functional properties to these foods (Gould, 2012). Therefore, the aim of this study was centered in achieving a significant antimicrobial effects against *Listeria monocytogenes* by adding natural *R. discolor* extracts in combination applied as complementary preservative along with potassium sorbate in a fresh *Panela* cheese matrix. Findings may lead to their application not only in health related issues, but also as natural food preservatives or antimicrobial agents.

2 Materials and methods

2.1 Rhoeo discolor aqueous extract preparation

Fresh leaves of the *Rhoeo discolor* plant were harvested from the location of Antón Lizardo in the state of Veracruz, Mexico during the month of December (botanical reference voucher No. CIB 14425). Leaves were washed with clean tap water and cut in to 1.5x3 trips before controlled dehydration in a

pilot plant Shel Lab oven set at 40°C for 24h (García-Varela *et al*., 2016). Two aqueous extracts were prepared; the first extract (E1) obtained from fresh dry leaves (5 g) which were submerged in 500 mL Milli-Q water at room temperature and stirred overnight; whereas the second extract (E2) from dry leaves which were boiled (5 g of dry leaves boiled for 30 min in 500 mL of Milli-Q water) (Rosales-Reyes *et al*., 2007). The organic solid matter was mechanically separated with a metal strainer and resulting extracts filtered through a 0.22 μm membrane filter (Corning vacuum filtering system, NY, USA). Subsequently, extracts were frozen, then freeze-dried (Labconco FreeZone 12, Kansas city, MO, USA) and stored at -20°C until further use. Extracts were assayed for phenolics by the Folin- Ciocalteu assay and further characterized by HPLC and HPLC-MS-TOF, data previously published by García-Varela *et al*. (2015).

2.2 *Listeria monocytogenes growth*

Initially, a 24 h *L. monocytogenes* (ATCC 90714) growth kinetics was performed in order to determine growth rate; this provides information regarding de amount of CFU in a determined period of time and exponential growth. Bacteria were grown under optimal conditions in Trypticasein Soy Broth (TSB) at 37°C for 24 h. Bacteria were incubated under aerobic conditions until exponential growth was reached (Al-Zeyara *et al*., 2011; García-Varela *et al*., 2015).

2.3 *Determination of the minimal inhibitory concentration (MIC)*

Optimal concentrations of bioactive extracts were tested in a 96-well plate to determine the MIC. For this purpose, Nutrient broth (BD Bioxon, Estado de Mexico, Mexico) was prepared and sterilized; in parallel, 3.2 mg/mL stock solutions of E1 and E2 were also prepared. These initial stock solutions were subsequently serially diluted (1:1) with sterilized broth to yield concentrations from 1600, 800, 400, 200, 100, 50, 25, 12.5, 6.3, 3.1 to 1.6 μg/mL. These concentrations tested beforehand (García-Varela *et al*., 2015) were reported effective against microbial growth. Once concentrations were set, 200 μL of each concentration was placed into the plate wells (Soni *et al*., 2010). Then, 15 μL of broth containing 106 CFU/mL *L. monocytogenes* were added (Su *et al*., 2014). The negative control consisted of sole inoculum, while the positive control contained 5% of penicillin-streptomycin (v/v) (Su *et al*., 2014). Also, combinations of the *R. discolor* extracts and potassium sorbate (50, 25, 12.5, 6.3, 3.1 and 1.6 mg/kg) were tested to determine the most effective antimicrobial activity. The plate was incubated at 37°C for 24 h. The absence or presence of turbidity, indicating bacterial growth, was determined with a microplate reader (BioTek Instruments, Winooski, VT) at a wavelength of 630 nm (Min *et al*., 2014).

2.4 *Production of Panela fresh cheese with R. discolor extracts*

To produce 100 g of cheese, one liter of whole milk (pasteurized commercial brand, Mexico) was weighed and heated to 50°C. After heat was discontinued, 300 mg of calcium chloride ($CaCl_2$)/liter previously dissolved in 15 mL of distilled water were added and stirred for 1 minute. Once the milk temperature dropped to 40°C, 100 μL of rennin/L of milk was added, mixed for one minute, and coagulation proceeded in a stainless steel pot. The curd was manually cut with a knife in 1 cm3 pieces and incubated in a water bath set at 37.7°C for 30 min. The curd was transferred to a strainer to discard excess whey and 1.5% of salt was added (w/w). The curd was then wrapped in cheesecloth, placed in a cheese mold and pressure was applied (for 3 h) to remove the remaining whey. The resulting cheeses were placed in sealed sterile 24 oz Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA).

2.5 *Microbial challenge test: inoculation of the cheese with L. monocytogenes and bioactive R. discolor extracts*

For the microbial challenge purposes *Panela* cheeses were produced using the same process stated in section 2.4. Both E1 and E2 *R. discolor* extracts and potassium sorbate were dissolved in sterile water and added at the effective MIC, they were then mixed and homogenized after the salt addition in the previously stated protocol. Afterwards, 20 mL of *L. monocytogenes* at 10^8 CFU/g was added and incorporated simultaneously (Solomakos *et al*., 2008). Inoculated *Panela* cheeses were divided (10 g), placed in individual sterile Whirl-Pak bags and maintained at room temperature for 12 h, to simulate commercial distribution conditions, and finally stored at 4°C and analyzed at 0, 0.5, 2, 4, 7, 14, 21 or 28 days (Gadotti *et al*., 2014). For each time based analysis, individual bags were withdrawn from incubation and 90 mL of peptone

water (BD Bioxon, Estado de Mexico, Mexico) were added, followed by homogenization in a Stomacher (IUL Instruments, Barcelona, Spain) for 20 seconds. Serial dilutions were prepared as necessary. The pour plate method was applied using Modified Oxford agar (BD Difco, Pont de Claix, France) specific for *Listeria spp*. growth. The plates were incubated at 37 °C for 24 hours as standard protocol; all samples were prepared in triplicate. Colonies were counted and data was reported as CFU/g (Gadotti *et al*., 2014; De Oliveira *et al*., 2013). Non-inoculated control samples were also evaluated.

2.6 Sensory quality

The formulated *Panela* cheese, treated with the bioactive *R. discolor* leaf extracts and potassium sorbate at different concentrations, were subjected to overall quality assessment by forty untrained tasters (27 females and 13 males, 25-35 years old). The samples were kept at 10°C, cut into pieces (about 1.5 × 1.5 × 1.5 cm in size), and placed in small dish coded with three digit random numbers. Before evaluation, panelists were instructed to eat a plain biscuit and drank cold, filtered tap water. A standard cheese (without additives), was used as control to compare the different formulations. Evaluation sheets consisted of a 9-point hedonic scale that were used to score the attributes, where 1 and 9 corresponded to dislike immensely and like immensely, respectively (Bouton *et al*., 2009; De Oliveira *et al*., 2013) . The untrained member panel also evaluated the acceptance according to the following parameters: appearance, texture, odor and flavor (Dantas *et al*., 2016).

2.7 Statistical analysis

For *in vitro* assays, experiments were performed in triplicate and replicated 3 times; for cheese microbial challenge experiments triplicates were also performed. A repetition Block ANOVA statistical analysis was applied to determine the optimal extract and extract:potassium sorbate interaction concentrations. The inoculated cheeses were analyzed via one-way ANOVA with repeated measures. Statistical analyses were performed using Minitab v.17 software (State College, PA). Differences were considered significant at $p < 0.05$.

3 Results and discussion

Microorganism growth is greatly impacted by the features of the food, including pH, aw, redox potential, natural antimicrobial agents, among others (Viuda-Martos *et al*., 2008). It has been proven that *Panela* cheese is an adequate environment for putrefactive and pathogenic microorganism growth (Felicio *et al*., 2015). An important decrease in bacterial proliferation in cheese samples that contained the different mixture of both aqueous extracts and potassium sorbate was observed; however, preliminary studies depicted that the use of potassium sorbate alone did not produce significant growth inhibition as compared to the combinations presented herein. It is important to highlight that the salt concentration used in the cheese formulation was kept to the minimum in order to discard the possibility of a false positive inhibitory effect.

3.1 Determination of the antimicrobial composition for Panela cheese production

An initial screening was performed to determine the optimal concentrations of aqueous extract that would reduce *L. monocytogenes* population. The optimal MIC for each extract was applied for *Panela* cheese production. Preliminary results indicated that the optimal concentrations for both E1 and E2 aqueous extracts were set between 100 and 800 μg/mL (Table 1). The aqueous extract-concentration interaction reduced bacterial population significantly; however, they were unable to totally eliminate the microbial load. When comparing both E1 (8.5 ± 3.7 μg GAE/mg) and E2 (16.9 ± 3.7 μg GAE/mg) results, a somewhat better antimicrobial performance was observed in the case of E2 possible due to the higher phenolic compound concentration; however, it was not statistically significant. This could be affected by the boiling process, which enhanced lixiviation of natural antimicrobial compounds associated to *R. discolor* (Bankar *et al*., 2010).

Previous studies reported a high content of phenolic compounds in extracts from *R. discolor*; including carotenoids, anthocyanins, flavonoids, saponins, terpenoids, vanillic acid and also p-coumaric, ferulic and chlorogenic acids, and steroidal compounds among others (Arriaga-Alba *et al*., 2011; García-Varela *et al*., 2015; Rosales-Reyes *et al*., 2007). The majority of these compounds have been reported as successful antimicrobials

(Mujeeb *et al*., 2014). These compounds are known to affect microorganisms viability by means of several mechanisms; e.g. disrupting of Gram-positive bacteria cell wall and cytoplasmic membrane (Patra *et al*., 2014). This rupture produces the leakage of cellular content followed by death (Akagawa *et al*., 2003; Min *et al*., 2014). Other mechanisms could be related to membrane pore formation and DNA synthesis tempering (De Araújo *et al*., 2014). These extracts also contain members of the phenolic family that are known as effective antimicrobials for food preservation (Galindo-Cuspinera *et al*., 2003; Kramer *et al*., 2015; Silva-Beltrán *et al*., 2015).

In large-scale cheese production there are several chemicals additives used to decrease total bacterial counts, such as potassium sorbate (Soni *et al*., 2010), sodium benzoate, and sodium lactate, among others (Kasrazadeh *et al*., 1995). According to the Food and Drug Administration (FDA) potassium sorbate must not exceed the 0.2% of the total weight of the cheese (Food and Drug Administration, 2015). Additionally, the Official Mexican Norm (NOM-212-SSA1-2002) states that dairy products must maintain a proportion of maximum 1000 mg/Kg of potassium sorbate. The potassium sorbate content, in the formulated chesses, was below the FDA and NOM;-1000-SSA1-2002 mandatory regulation (0.00063, 0.00031 and 0.00016%) and was still effective against *L. monocytogenes* growth when combined with *R. discolor* extracts. As previously mentioned, when *L. monocytogenes* was exposed to the relatively low concentrations of extracts and potassium sorbate viability decreased. Needless to say, this positive effect could be interesting for food processors because it could be an alternative to enhance diary food security and shelf life with additional nutraceutical benefits provided by phytochemicals associated to natural extracts. Additionally, it was demonstrated that the amounts evaluated of E1, E2 and potassium sorbate combinations did not significantly affect consumer general acceptance.

In order to determine the aqueous extract efficiency for *L. monocytogenes* growth inhibition, the optimal extract concentrations with serial dilutions of potassium sorbate at 50, 25, 12.5, 6.3, 3.1 and 1.6 mg/kg were combined; tests were performed in triplicates (Figure 1). Statistically, E1 did not show significant inhibition when compared to the sole extract. However, a block ANOVA, that eliminated the day variable, revealed statistical significance in growth inhibition in the following binary combinations: 800 μg/mL+3.1 mg/kg, 800 μg/mL+1.6 mg/kg, 400 μg/mL+6.3 mg/kg and 400 μg/mL+3.1 mg/kg aqueous extract:potassium sorbate, respectively, when applying E2. These same binary combinations were also applied to E1 for further testing and cheese production.

Table 1. Effect of adding different concentrations of two *R. discolor* aqueous extracts (E1 or E2) to determine minimal inhibitory concentration (MIC) (% viability) against *L. monocytogenes*

Concentration %	Extract Concentration (μg/ml)	Viability % (630 nm)	
		E1 MIC	E2 MIC
Control	0	0 ± 0	0 ± 0
100%	3,200	56.5 ± 6.36	88 ± 8.14
50%	1600	42 ± 4.24	47 ± 7.41
25%	800	34 ± 5.66	20 ± 11.21
12.50%	400	35.5 ± 6.36	43.5 ± 2.12
6.25%	200	36 ± 1.41	45.5 ± 9.54
3.13%	100	31.5 ± 3.54	23 ± 12.73
1.56%	50	35 ± 5.66	64.5 ± 4.95
0.78%	25	54 ± 2.83	53 ± 12.21
0.39%	12.5	81 ± 1.41	40.5 ± 6.71
0.20%	6.30	60 ± 2.83	45.5 ± 11.68
0.10%	3.10	76.5 ± 19.09	45.5 ± 12.02
0.05%	1.60	67.5 ± 0.71	42 ± 10.14

1 L. monocytogenes population growth inhibition caused by extract exposure.

2 E1 = Water extract from dry leaves non-boiled. E2 = Water extract from dry leaves boiled.

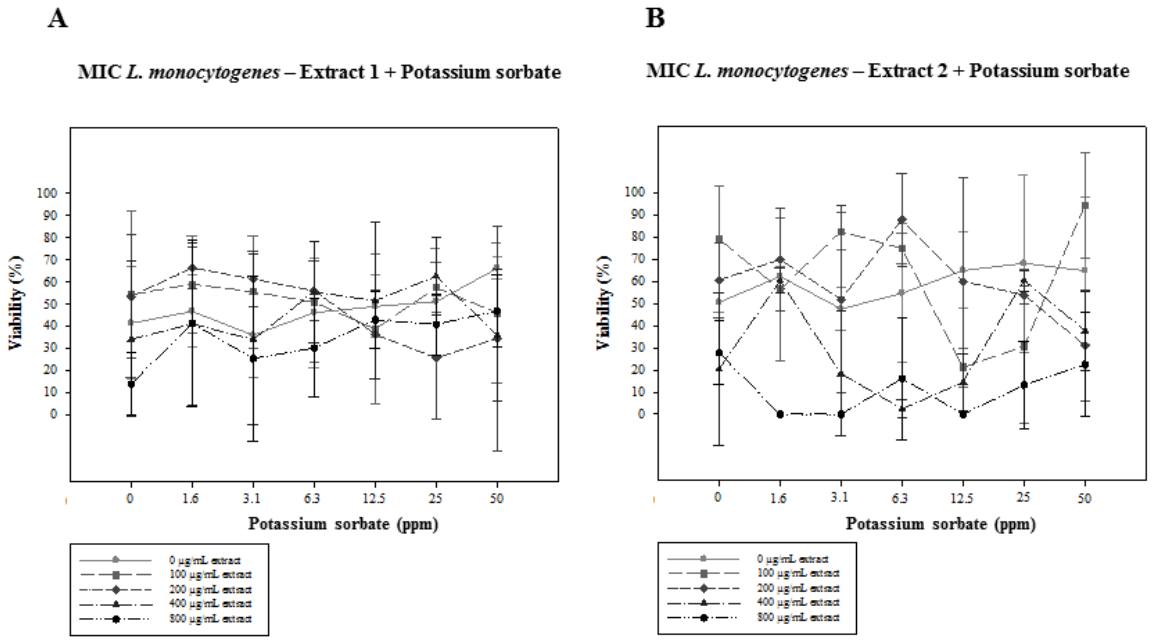

Fig. 1. Block ANOVA statistical analysis interaction plot of non-boiled dry leaves (E1) Extract:potassium sorbate (A) and boiled dry leaves (E2) Extract:potassium sorbate (B), to determine optimal combination with the lowest possible amounts of potassium sorbate, for *L. monocytogenes* challenge preliminary screening (concentrations marked in red rectangles). Optimal interactions are marked inside the rectangles.

The determined amounts of potassium sorbate added to the cheeses were below the FDA regulation. Therefore, it is anticipated that the small quantities of potassium sorbate are not the sole reason for the total microorganism inhibition. The interaction between both the aqueous extracts and the potassium sorbate could have influenced these results. However, we accomplished an important population inhibition with reduced concentrations of potassium sorbate and extracts. It has been demonstrated that different amounts of potassium sorbate are able to reduce bacterial growth (Larson *et al*., 1999); nevertheless, the concentrations applied to the experimental cheeses were so low that without the extracts they were unable to produce a decrease in *L. monocytogenes* growth.

3.2 Microbial challenge after inoculation of the cheese with L. monocytogenes and R. discolor extracts

The cheeses for this stage were formulated using the optimal extract:potassium sorbate combination obtained in the preliminary screening. Two mL of a *L. monocytogenes* culture at 10^8 CFU/mL were added to the cheese formulation along with the optimal concentrations. Microbial challenge was performed in all 9 cheeses for 28 days of evaluation. Both E1:potassium sorbate (Figure 2A) and E2:potassium sorbate (Figure 2B) were able to decrease *L. monocytogenes* microbial population by over 2 log cycles. Significant differences can be observed in all concentrations when compared to the control cheeses. After day 4 in E1 and day 2 in E2, no significant difference between the growth inhibition capacity of *L. monocytogenes* population, caused by the different extract: potassium sorbate concentrations, was observed. All concentrations were able to maintain bacterial populations in a static manner. Thus the use of natural preservatives may reduce the use of regulated antimicrobial compounds, which are usually added to commercial dairy products and therefore lead to more natural cheese (Soni *et al*., 2010).

Results from previous studies have demonstrated a bacterial growth inhibition of *L. monocytogenes* in several food matrixes, such as cheese and meat when adding extracts rich in natural preservatives from cinnamon, clove, oregano, pomegranate peel, and grape seed. Chemical analysis of these extracts revealed a high content of phenolic compounds to which this effect was attributed (Shan *et al*., 2009, 2011).

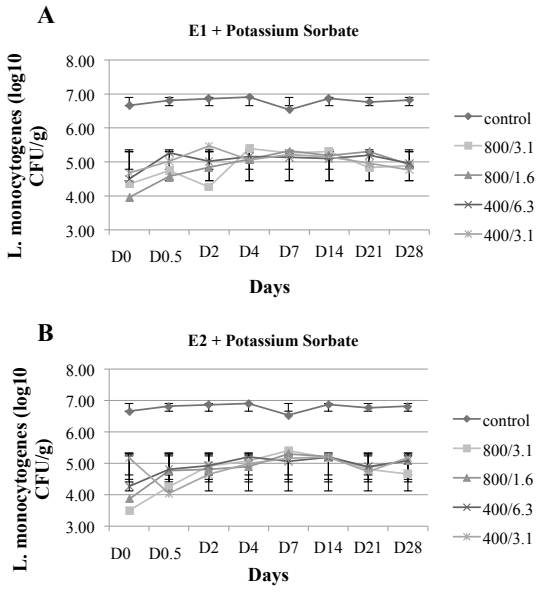

Fig. 2. Effect of two aqueous *Rhoeo discolor* extracts combined with potassium sorbate on growth of *L. monocytogenes* inoculated into *Panela* cheese. Concentration μg/mL-ppm. (A) E1 = water extract from dry leaves non-boiled. (B) E2 = Water extract from dry leaves boiled.

The results obtained in this research supports the premise that natural products, with high phenolic compound content, could be used by the food industries as natural preservatives and nutraceuticals (Tajkarimi *et al*., 2010).

3.3 Sensory quality of Panela cheese supplemented with aqueous extracts

The non-trained panel was presented with 9 different types of cheeses: a control cheese with no extracts or potassium sorbate, 4 cheeses with the optimal E1: potassium sorbate combinations, and 4 additional cheeses with de optimal E2 potassium sorbate combinations. Each member of the panel filled the information sheet for each cheese and statistical analyses were performed to evaluate the general acceptance. The panel determined that the formulated *Panela* cheese with E1 and potassium sorbate had a positive general acceptance (Figure 3A). Compared to the control cheeses, the first two combination samples received a higher acceptance value. However, variation in the evaluated properties resulted non-significant. It can be stated that the addition of these combinations did not affect consumer acceptance. Likewise, when the panel evaluated the E2 and potassium sorbate combination, the general acceptance had no significant difference compared to the control cheese (Figure 3B); however, some cheese samples had better scores than the control counterpart.

Conclusions

Low concentrations of both *R. discolor* extracts (E1 and E2) acting synergistically with very low concentrations of potassium sorbate inhibited *L. monocytogenes* growth when in *Panela* cheese; an effect that was not observed by the sole use of potassium sorbate. These combinations did not compromise the overall quality of the different cheese samples.

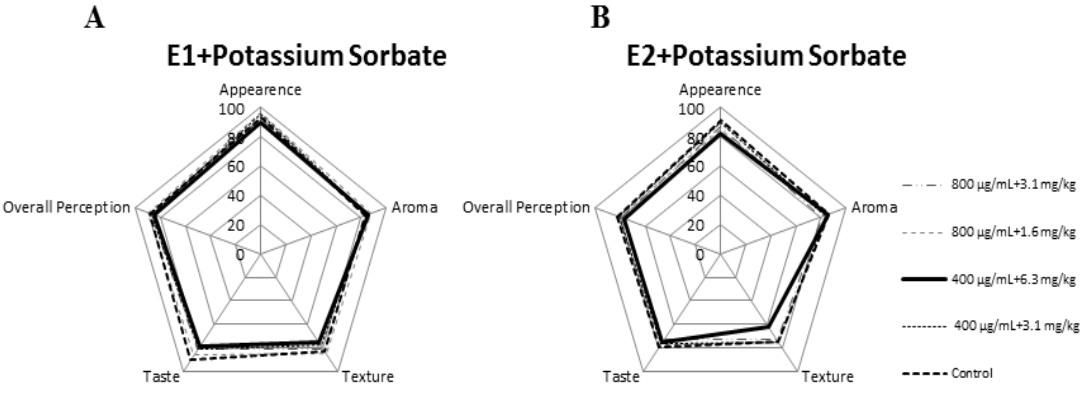

Fig. 3. Hedonic acceptance charts of *Panela* cheeses. E1: potassium sorbate combinations (A). E2: potassium sorbate combinations (B). To the right side of the chart the organoleptic characteristics evaluated are displayed. Acceptance was measured in percentage.

It is important to point out that there is a real advantage in the use of natural bioactive aqueous extracts. The benefit is not only in economic value of obtaining the extracts, since water is relatively inexpensive, and easy to eliminate and discard. Our findings confirm that the traditional extraction process of *R. discolor* leaves was the most efficient technique to obtain aqueous extracts with antimicrobial activity against *L. monocytogenes*. Applying *R. discolor* extracts, to cheese formulated with pasteurized cow milk, could signify a decrease in the addition of artificial food preservatives reducing chemical exposure for consumers and a decrease in product cost. However, further research is required to determine better combinations of *R. discolor* aqueous extracts with other possible natural or synthetic food preservatives. Also the possibility of exploring other dairy products where the application of these extracts could aid in extending shelf life and avoid foodborne illnesses.

Acknowledgements

We thank our colleagues for their assistance in this study and preparation of the manuscript: Dr. Javier and Patricia Kutz for providing the necessary plant leaves for this research; Dr. Edgardo Escalante for his contribution in statistical analysis and Dr. Cesar I. Beristain for providing specific botanical information regarding *R. discolor*. We thank Dr. Bertha A. Barba for her input. We thank CONACYT for Scholarship number 267172 awarded to R.G.V. and for providing support.

References

Akagawa, M., Shigemitsu, T. and Suyama, K. (2003). Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Bioscience, Biotechnology, and Biochemistry 67*, 2632-2640.

Al-Zeyara, S. A., Jarvis, B. andMackey, B. M. (2011). The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths. *International Journal of Food Microbiology 145*, 98-105.

Alvarado, C., García-Almendárez, B. E., Martin, S. E. and Regalado, C. (2005). Anti-*Listeria monocytogenes* bacteriocin-like inhibitory substances from enterococcus faecium uq31 isolated from artisan mexican-style cheese. *Current Microbiology 51*, 110-15.

Arriaga-Alba, M., Blasco, J.L., Ruiz-Pérez, N.J., Sanchez-Navarrete, J., Rivera-Sánchez, R. and Gonzalez-Avila, M. (2011). Antimutagenicity mechanisms of the *Rhoeo discolor* ethanolic extract. *Experimental and Toxicologic Pathology: Official Journal of the Gesellschaft Für Toxikologische Pathologie 63*, 243-248.

Bankar, A., Joshi, B., Kumar, A.R. and Zinjarde, S. (2010). Banana peel extract mediated synthesis of gold nanoparticles. *Colloids and Surfaces B: Biointerfaces 80*, 45-50.

Bouton, Y., Buchin, S., Duboz, G., Pochet, S., and Beuvier, E. (2009). Effect of mesophilic lactobacilli and enterococci adjunct cultures on the final characteristics of a microfiltered milk swiss-type cheese. *Food Microbiology 26*, 183-191.

Cardenas-Sandoval, B.A., Lopez-Laredo, A.R., Martínez-Bonfil, B.P., Bermudez-Torres, K. and Trejo-Tapia, G. (2012). Advances in the phytochemistry of *Cuphea aequipetala, C. aequipetala* var. hispida and *C. lanceolata*: extraction and quantification of phenolic compounds and antioxidant activity. *Revista Mexicana de Ingeniería Química 11*, 401-13.

Dantas, A. B., Jesus, V. F., Silva, R., Almada, C. N., Esmerino, E. A., Cappato, L. P. and Sant'Ana, A. S. (2016). Manufacture of probiotic minas frescal cheese with *Lactobacillus* casei zhang. *Journal of Dairy Science 99*, 18-30.

de Araújo, A. A., Soares, L. A. L., Ferreira, M. R. A., de Souza Neto, M. A., da Silva, G. R., de Araújo, R. F. and de Melo, M. C. N. (2014). Quantification of polyphenols and evaluation of antimicrobial, analgesic and anti-inflammatory activities of aqueous and acetone-water extracts of *Libidibia ferrea*, *Parapiptadenia rigida* and *Psidium guajava*. *Journal of Ethnopharmacology 156*, 88-96.

Domínguez-Niño, A.N., Buendía-González, D., Cantú-Lozano, I., Andrade-González and Luna-Solano, G. (2016). Effect of fluidized bed drying on physicochemical and microbiological

properties of mexican fresh cheese. *Revista Mexicana de Ingeniería Química 15*, 869-881.

Felicio, B. A., Pinto, M. S., Oliveira, F. S., Lempk, M. W., Pires, A. C. S., and Lelis, C. A. (2015). Effects of nisin on *Staphylococcus aureus* count and physicochemical properties of minas frescal cheese. *Journal of Dairy Science 98*, 4364-69.

Food and Drug Administration (2015). Food and Drug Administration Department of Health and Human Services. in http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=133.169. Accesed November 20th 2016.

Gadotti, C., Nelson, L. and Diez-Gonzalez, F. (2014). Inhibitory effect of combinations of caprylic acid and nisin on *Listeria monocytogenes* in queso fresco. *Food Microbiology 39*, 1-6.

Galindo-Cuspinera, V., Westhoff, D. C. and Rankin, S. A. (2003). Antimicrobial properties of commercial annatto extracts against selected pathogenic, lactic acid, and spoilage microorganisms. *Journal of Food Protection 66*, 1074-78.

García-Varela, R., García-García, R. M., Barba-Dávila, B. A., Fajardo-Ramírez, O. R., Serna-Saldívar, S. O. and Cardineau, G. A. (2015). Antimicrobial activity of *Rhoeo discolor* phenolic rich extracts determined by flow cytometry. *Molecules 20*, 18685-703.

García-Varela, R., Fajardo Ramírez, O.R., Serna-Saldivar, S.O., Altamirano, J. and Cardineau, G.A. (2016). Cancer cell specific cytotoxic effect of *Rhoeo discolor* extracts and solvent fractions. *Journal of Ethnopharmacology 190*, 46-58.

Gonzalez-Avila, M., Arriaga-Alba, M., De la Garza, M., del Carmen Hernández-Pretelín, M., Domínguez-Ortíz, M. A., Fattel-Fazenda, S. and Villa-Treviño, S. (2003). Antigenotoxic, antimutagenic and ROS scavenging activities of a *Rhoeo discolor* ethanolic crude extract. *Toxicology in Vitro 17*, 77-83.

Gould, G.W. (2012). *New Methods of Food Preservation*. Springer Science & Business Media.

Idaka, E., Ogawa, T., Kondo, T. and Goto, T. (1987). Isolation of highly acylated anthocyanins from commelinaceae plants, *Zebrina pendula*, *Rhoeo spathacea* and *Setcreasea purpurea*. *Agricultural and Biological Chemistry 51*, 2215-20.

Jimenez, M., Castillo, I., Azuara, E. and Beristain, C. I. (2011). Antioxidant and antimicrobial activity of capulin (*Prunus serotina* subsp Capuli) extracts. *Revista Mexicana de Ingeniería Química 10*, 29-37.

Kabara, J. (1991). Phenols and chelators. *Food Preservatives, Blackie, Glasgow* 200-214.

Kasrazadeh, M. and Genigeorgis, C. (1995). Potential growth and control of Escherichia coli O157:H7 in soft hispanic type cheese. *International Journal of Food Microbiology 25*, 289-300.

Kramer, B., Thielmann, J., Hickisch, A., Muranyi, P., Wunderlich, J. and Hauser, C. (2015). Antimicrobial activity of hop extracts against foodborne pathogens for meat applications. *Journal of Applied Microbiology 118*, 648-657.

Larson, A.E., Johnson, E.A. and Nelson, J.H. (1999). Survival of *Listeria monocytogenes* in commercial cheese brines. *Journal of Dairy Science 82*, 1860-1868.

McAuliffe, O., Hill, C. and Ross, R. P. (1999). Inhibition of *Listeria monocytogenes* in cottage cheese manufactured with a lacticin 3147-producing starter culture. *Journal of Applied Microbiology 86*, 251-256.

Min, K. Y., Kim, H. J., Lee, K. A., Kim, K. T. and Paik, H. D. (2014). Antimicrobial activity of acid-hydrolyzed citrus unshiu peel extract in milk. *Journal of Dairy Science 97*, 1955-1960.

Mujeeb, F., Bajpai, P., and Pathak, N. (2014). Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of aegle marmelos. *BioMed Research International 497606*, 11 p.

Oliveira, T. L. C. D., Cardoso, M. D. G., Soares, R. D. A., Ramos, E. M., Piccoli, R. H. and Tebaldi, V. M. R. (2013). Inhibitory activity of *Syzygium aromaticum* and *Cymbopogon citratus* (DC.) stapf. Essential oils against

Listeria monocytogenes inoculated in bovine ground meat. *Brazilian Journal of Microbiology 44*, 357-65.

Patra, J. K., Kim, E. S., Oh, K., Kim, H. J., Kim, Y. and Baek, K. H. (2014). Antibacterial effect of crude extract and metabolites of phytolacca americana on pathogens responsible for periodontal inflammatory diseases and dental caries. *BMC Complementary and Alternative Medicine 14*, 343.

Rosales-Reyes, T., de la Garza, M., Arias-Castro, C., Rodríguez-Mendiola, M., Fattel-Fazenda, S., Arce-Popoca, E. and Villa-Treviño, S. (2007). Aqueous crude extract of *Rhoeo discolor*, a mexican medicinal plant, decreases the formation of liver preneoplastic foci in rats. *Journal of Ethnopharmacology 115*, 381-386.

Shan, B., Cai, Y. Z., Brooks, J. D. and Corke, H. (2011). Potential application of spice and herb extracts as natural preservatives in cheese. *Journal of Medicinal Food 14*, 284-290.

Shan, B., Cai, Y. Z., Brooks, J. D. and Corke, H. (2009). Antibacterial and antioxidant effects of five spice and herb extracts as natural preservatives of raw pork. *Journal of the Science of Food and Agriculture 89*, 1879-85.

Silva-Beltrán, N. P., Ruiz-Cruz, S., Cira-Chávez, L. A., Estrada-Alvarado, M. I., Ornelas-Paz, J. D. J., López-Mata, M. A. and Márquez-Ríos, E. (2015). Total Phenolic, flavonoid, tomatine, and tomatidine contents and antioxidant and antimicrobial activities of extracts of tomato plant. *International Journal of Analytical Chemistry*, 2015.

Smith-Palmer, A., J. Stewart and L. Fyfe. (1998). Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. *Letters in Applied Microbiology 26*, 118-122.

Solomakos, N., Govaris, A., Koidis, P.and Botsoglou, N. (2008). The antimicrobial effect of thyme essential oil, nisin and their combination against Escherichia coli O157:H7 in minced beef during refrigerated storage. *Meat Science 80*, 159-66.

Soni, K. A, Nannapaneni, R., Schilling, M. W. and Jackson, V. (2010). Bactericidal activity of lauric arginate in milk and queso fresco cheese against *Listeria monocytogenes* cold growth. *Journal of Dairy Science 93*, 4518-25.

Su, H., Chen, W., Fu, S., Wu, C., Li, K., Huang, Z. and Li, J. (2014). Antimicrobial effect of bayberry leaf extract for the preservation of large yellow croaker (*Pseudosciaena crocea*). *Journal of the Science of Food and Agriculture 94*, 935-42.

Tajkarimi, M. M., Ibrahim, S. A. and Cliver, D. O. (2010). Antimicrobial herb and spice compounds in food. *Food Control 21*, 1199-1218.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J. and Perez-Álvarez, J. (2008). Antibacterial activity of lemon (*Citrus lemon* L.), mandarin (*Citrus reticulata* L.), grapefruit (*Citrus paradisi* L.) and orange (*Citrus sinensis* L.) essential oils. *Journal of Food Safety 28*, 567-76.