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HIGH PRESSURE PROCESSING (HPP) AND *in-situ* NISIN BIOSYNTHESIS BY *Lactococcus lactis*: A HURDLE APPROACH TO IMPROVE *Listeria* spp. INACTIVATION IN BOVINE MILK

COMBINACIÓN DE ALTA PRESIÓN HIDROSTÁTICA (APH) Y NISINA SINTETIZADA *in-situ* POR *Lactococcus lactis*: TECNOLOGÍA DE OBSTÁCULOS PARA LA INACTIVACIÓN DE *Listeria* spp. EN LECHE DE VACA

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Abstract

Cold pasteurization of raw milk may be achieved by combining high pressure processing (HPP) with other (a)biotic factors. In this study, a combined approach involving HPP and biopreservation by *in-situ* nisin synthesis was evaluated. UHT and raw bovine milk were inoculated with *Listeria innocua* and treated with HPP (350-600 MPa), or HPP+nisin synthesized *in-situ* by *Lactococcus lactis* Rif L^+ (LUQ2). HPP treatments of commercial UHT milk yielded two decimal reductions ($SV_{HPP} = 2 \log_{10}$ reductions) of *L. innocua* with 550 MPa/3min, while the HPP+nisin approach yielded <10 cfu ml^{-1} under the same pressure-time conditions. Native *Listeria monocytogenes* was found in raw milk ($\sim 10^2$ cfu ml^{-1}) and the total *Listeria* spp. counts increased to $\sim 10^9$ cfu ml^{-1} with the *L. innocua* inoculum. HPP milk pasteurization ($SV_{HPP} \geq 5 \log_{10}$, aerobic plate count; <1 cfu ml^{-1}, *Listeria* spp.) was viable by using 600 MPa/12 min, whereas HPP+nisin resulted in milk pasteurization at holding times between 6 and 9 min for the same pressure level. Furthermore, LUQ2 synthesized 9.75±0.54 IU ml^{-1} of nisin, which is below the FAO/WHO limit (500 IU g^{-1}) suggesting that the promising HPP+nisin approach could be optimized to pasteurize milk at lower pressure levels and/or shorter pressure holding times.

Keywords: high pressure processing (HPP), nisin, *Listeria*, raw milk; *Lactococcus lactis*.

Resumen

La pasteurización en frío de leche bovina bronca podría lograrse mediante la combinación de alta presión hidrostática (APH) con factores (a)bióticos. En el presente estudio se evaluó la combinación de APH y bioconservación a partir de la biosíntesis *in-situ* de nisina. Muestras de leche comercial UHT y leche bronca fueron inoculadas con *Listeria innocua* y procesadas con APH (350-600 MPa) o APH+nisina sintetizada por *Lactococcus lactis* Rif L^+ (LUQ2). El tratamiento de leche UHT a 550 MPa/3 min redujo dos ciclos logarítmicos ($SV_{HPP} = 2 \log_{10}$) de *L. innocua*, mientras que la combinación APH+nisina resultó en <10 ufc ml^{-1} bajo las mismas condiciones. La presencia de *Listeria monocytogenes* en leche bronca ($\sim 10^2$ ufc ml^{-1}) incrementó la cuenta de *Listeria* spp. a $\sim 10^9$ ufc ml^{-1} después de inocular con *L. innocua*. Tratamientos de 12 min fueron requeridos para pasteurizar leche bronca ($SV_{HPP} \geq 5 \log_{10}$, cuenta total; <1 ufc ml^{-1}, *Listeria* spp.) con APH a 600 MPa, mientras que APH+nisina pasteurizó la leche después de 6-9 min. La concentración de nisina sintetizada por LUQ2 (9.75±0.54 UI ml^{-1}) está por debajo del máximo establecido por la FAO/OMS (500 UI g^{-1}) y el desarrollo pudiese ser optimizado en trabajos futuros.

Palabras clave: alta presión hidrostática (APH), nisina, *Listeria*, leche bronca, *Lactococcus lactis*.

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1 Introduction

1.1 High pressure processing (HPP) in the dairy industry

Food experts working in the academia, industry and government agencies foresee high pressure processing (HPP) will become the most important nonthermal food processing technology in terms of product commercialization (Jermann *et al*., 2015). Food preservation by nonthermal methods has been used for enzyme inactivation to keep the natural characteristics of foods (Serment-Moreno *et al*., 2012; Castorena-García *et al*., 2013). Despite the current commercial success, no food regulatory agency had previously authorized milk pasteurization using high pressure until the Australian government approved HPP milk in 2016 (New South Wales (NSW) Food Authority, 2016). Subsequently, "Made By Cow^{TM}" (http://www.madebycow.com.au/) became the first company to legally commercialize HPP-treated milk.

Raw milk contains a wide variety of nutrients that support microbial growth, including spoilage microorganisms and pathogenic bacteria that represent a threat to consumers (Black *et al*., 2005; Walter *et al*., 2016). Pressure treatments at 500-600 MPa may inactivate foodborne pathogens commonly found in milk such as *Salmonella* Typhimurium, *Staphylococcus aureus* and *Listeria monocytogenes* (Dogan & Erkmen, 2004; Guan *et al*., 2005; US FDA, 2014). However, ions dissolved in milk and fats may protect microorganisms against the lethal effects of pressure (Huppertz *et al*., 2006; Ferreira *et al*., 2016). The inactivation of bacteria by HPP is a multi-target approach, causing changes in cell membranes (phase transitions of the membrane phospholipids), cell wall, ribosomes, proteins, lipids and enzyme-mediated cellular functions (Campus, 2010), and is also dependent on the strain and growth phase (Baptista *et al*., 2016). Divalent cations like calcium and magnesium stabilize the cellular membrane of microorganisms, whereas dissociated anions such as phosphates and citrates act as buffers that dampen the pH drop occurring under high pressure (Huppertz *et al*., 2006). Other challenges involving microbial food safety include the high variability of pressure resistance even among strains of same species, and the resistance of bacterial spores to pressure levels above 600 MPa which is the limit for current commercial applications (Tay *et al*., 2003; Huppertz *et al*., 2006).

1.2 High pressure processing and biopreservation

HPP alone may be insufficient to ensure milk safety, thus a hurdle approach combining pressure with other preservation technologies like the use of natural antimicrobials is needed. In biopreservation, food grade microorganisms such as lactic acid bacteria (LAB) are grown to synthesize metabolites (i. e. organic acids, peptides) that may retard or inhibit the growth of pathogens and spoilage microorganisms (Gálvez *et al*., 2007; Raybaudi-Massilia *et al*., 2009). Bacteriocins are peptides with antimicrobial properties metabolized by microorganisms, but the use of bacteriocins in the food industry is restricted by regulatory agencies. Nisin is a bacteriocin synthesized by *Lactoccocus lactis* spp. that shows a dual mode of action by blocking the cell wall biosynthesis, and additionally inducing pore formation in the cell membranes of Gram positive bacteria leading to leakage of intracellular compounds, and disruption of the proton motive force. In addition, it also restricts spore germination (Zacharof & Lovitt, 2012; Khan & Oh, 2016). There are few publications showing the combined effect of HPP and a protective nisin producing culture in raw milk, able to inactivate *Listeria* spp., and *Staphylococcus aureus*, in addition to achieving high microbial load reduction. This hurdle approach may represent an alternative way to achieve milk pasteurization without heating.

In the United States, nisin is the only bacteriocin labeled as "GRAS" (Generally Recognized As Safe) by the Food and Drug Administration (FDA), which allows food producers to use nisin as a food additive (US FDA, 2001). Several studies have reported that nisin enhances microbial inactivation of HPP-treated dairy products (Alpas & Bozoglu, 2000; Black *et al*., 2005; Rodriguez *et al*., 2005; Black *et al*., 2008) and meats (Hereu *et al*., 2012; de Alba *et al*., 2013; Marcos *et al*., 2013).

The hydrophobic nature of nisin and its purification costs may limit the HPP+nisin approach in commercial applications (Gálvez *et al*., 2007). This issue may be overcome if nisin is synthesized by a native microorganism that is well adapted to grow in dairy products. Furthermore, the use of native microorganisms for biopreservation may help to retain the original organoleptic properties of foods (Gálvez *et al*., 2007; García-Parra *et al*., 2010). *Lactococcus lactics* UQ2 is a native strain isolated from Mexican-style fresh cheese that synthesizes nisin. *L. lactics* UQ2 has shown antimicrobial activity

against innoculated skim milk (García-Parra *et al*., 2011) and biofilms (García-Almendárez *et al*., 2008) of pathogen *Listeria monoyctogenes*.

Furthermore, the UQ2 strain has been genetically modified via conjugation to incorporate protease-lactose plasmid pLP712 that enhances lactose and protein hydrolysis, allowing *L. lactis* UQ2 to grow more efficiently in milk (García-Parra *et al*., 2010). The resultant transconjugant strain *L. lactis* UQ2 Rif L^+ increased nisin production from 75 to ~200 IU ml^{-1} 12 h after being inoculated in milk, which makes it a promising microbial culture to enhance milk pasteurization or as protective culture for fresh cheese production (García-Parra *et al*., 2010; García-Parra *et al*., 2011).

This study evaluated *L. innocua* survival in UHT commercial milk using a hurdle approach that combines HPP treatments (350-550 MPa) and *in-situ* nisin biosynthesis by *L. lactis* UQ2 Rif L^+ (LUQ2). Furthermore, the combined effect of HPP and nisin on native microbiota and *L. innocua* inoculated in raw whole milk, was evaluated at 550-600 MPa.

2 Materials and methods

2.1 Milk samples

2.1.1 Commercial pasteurized bovine milk

Whole pasteurized UHT milk (LALA, Torreón, Coahuila, México) with 3.2% (w/v) fat content and pH 6.7 was purchased at a local supermarket and stored at 4 °C until used.

2.1.2 Fresh raw bovine milk

Raw bovine milk was collected from a local farm (Agua Fría, Apodaca, Nuevo León, Mexico). Raw milk was immediately kept in ice and transported to Tecnológico de Monterrey facilities. Milk samples (500 ml) were packed in sterile polyethylene bags, vacuum-sealed and stored at 4 °C before use. Fat content and pH were measured as indicated by AOAC (2000).

2.2 Microbial characterization

Quantitative microbiological analysis included the aerobic plate count (APC), *Listeria* spp, and coliforms. Qualitative microbiological analysis consisted on the detection (>1 cfu ml^{-1}) of *Listeria monocytogenes*, *S. aureus* and *Salmonella* spp. All microbiological analysis were performed in accordance to the Bacteriological Analytical Manual (US FDA, 1998). All measurements were performed in triplicate.

2.3 Inoculation of milk samples

Raw and commercial UHT milk samples were inoculated with *L. innocua* to determine HPP inactivation. Alternatively,milk samples were inoculated with *L. innocua* and *L. lactis* UQ2 rif L^+ (LUQ2) to evaluate the combined HPP and biopreservation effect on microbial inactivation. The inoculation procedures are described in the next subsections.

2.3.1 Listeria innocua

L. innocua ATCC 51742 (Científica Senna, Ciudad de México, México) was incubated at 37 °C, 185 rpm (Innova 4000 shaker, New Brunswick Scientific, CT, US) in tryptic soy broth (TSB; Bioxon, Becton Dickinson, NJ, USA). After 15 h, 1 ml was transferred to 120 ml of milk to reach ~10^7 cfu ml^{-1} and samples were packed in 20x15 cm vacuum-sealed polyethylene bags (Filmpack, Guadalupe, Nuevo León, México).

2.3.2 In situ nisin biosynthesis with Lactococcus lactics UQ2 rif L^+ (LUQ2)

Cryopreserved *Lactococcus lactis* UQ2 rif L^+ (LUQ2) strain (García-Parra *et al*., 2010) was activated by adding 1 ml to a mixture consisting of 94 ml of M17 broth (Oxoid, UK) and 5 ml of a sterile lactose solution (10% w/v), and kept at 30 °C for 24 h (Alcántara-Zavala, 2013; Velázquez-Lugo, 2014). An aliquot (1 ml) was resuspended in 100 ml of sterile lactose-enriched M17 broth for 12 h (30 °C). On the final step of LUQ2 activation, 1 ml was added to 100 ml of sterile lactose-enriched M17 broth supplemented with 0.05 g of $MgSO_4$ and 0.01 g of $MnSO_4$ (Desarrollo de Especialidades Químicas, Monterrey, Nuevo León, México), and incubated at 30 °C for 8 h to reach 3×10^8 cfu ml^{-1} in the late log phase.

For HPP and biopreservation treatments, 1 ml of LUQ2 was transferred to 120 ml of milk previously inoculated with ~10^7 cfu ml^{-1} of *L. innocua*, and left 30 min at room temperature (20 °C) to reach ~10^7 cfu ml^{-1} of LUQ2 (Alcántara-Zavala, 2013; Velázquez-Lugo, 2014).

2.4 High pressure processing (HPP) of milk

Milk samples (120 ml) were treated in a 2-liter Welch HPP food processor (Avure Technologies, Middletown, Ohio, United States). For UHT milk, HPP was performed at 350, 450, 550 and 600 MPa, and holding times ranging from come-up time (CUT) to 40 min. In the case of raw milk, HPP treatments were at 550 and 600 MPa, with pressure holding times from CUT to 16 min. For all cycles, pressure remained within ± 3 MPa of the target process level and water vessel averaged temperature 28.6 ± 2.1 °C during pressure holding time. All treatments included a duplicate of milk samples.

2.5 Nisin activity

Nisin activity was determined according to British Standard 4020 (British Standards Institution, 1974).

2.6 Statistical analysis

One-way ANOVA and the Tukey test were used to determine statistically significant differences among treatments considering a significance level of $p = 0.05$. All statistical analyses were performed using Statistica, version 11 (Statsoft®) and Microsoft Excel® 2010.

3 Results

3.1 Inactivation of L. innocua in UHT milk by high pressure processing (HPP)

HPP treatments yielded >6 $\log_{10}$ reductions of *L. innocua* after 40 min at 350 MPa, whereas 3.6-3.7 $\log_{10}$ reductions occurred after 12 and 3 min at 450 and 550 MPa, respectively (Figure 1). The addition of the *L. lactis* UQ2 Rif L^+ (LUQ2) culture significantly improved *L. innocua* inactivation at 550 MPa ($p < 0.05$), yielding microbial counts below 50 cfu ml^{-1} (>5.5 $\log_{10}$ reductions) after 2.5 min (Figure 1). Such processing conditions may be suitable for commercial HPP applications according to the Mexican legislation, which requires absence of pathogen *Listeria monocytogenes* for milk pasteurization (NOM-184-SSA1-2002, 2002). Furthermore, the 2.5 min holding time complies with current commercial practices in which pressure holding times under 10 min are recommended since HPP is a batch processing technology (US FDA, 2014; Serment-Moreno *et al*., 2015). However, it is unlikely that pressurization below 500 MPa will yield non-detectable counts of *L. innocua* within 10 min with the current LUQ2 inoculum level and nisin concentration, since only 1-3 $\log_{10}$ reductions were observed at the end of 350 and 450 MPa treatments (Figure 1).

The baroresistance of *Listeria monocytogenes* and its surrogate *L. innocua* in milk and dairy products has been extensively reported (Ferreira *et al*., 2016). Serment-Moreno *et al*. (2017), observed that *L. innocua* counts decreased from ~$1x10^6$ cfu ml^{-1} to <25 cfu ml^{-1} in UHT milk with 500 MPa/4 min and 600 MPa/45 s treatments. Conversely, Buzrul *et al*. (2008) needed 20 min at 600 MPa to reduce 9 $\log_{10}$ cycles of *L. innocua* in milk. Chen and Hoover (2004), applied 16 min at 500 MPa to reduce 8 $\log_{10}$ cycles of *L. monocytogenes* Scott A, but Chen and Hoover (2003) shortened the pressure holding time to 5 min by processing milk samples at 50 °C.

3.2 Cold pasteurization of raw bovine milk

3.2.1 Effect of high pressure processing (HPP) on native microbiota

Raw milk samples presented a slightly higher fat content (3.8 ± 0.02% w/v) than the commercial UHT milk used in this study (3.2% w/v). Initially, raw milk samples showed a microbial abuse scenario not suitable for consumption but required to evaluate the performance of HPP and HPP+LUQ2 treatments. Thus, raw milk displayed elevated counts of aerobic mesophiles (7.4 $\log_{10}$ cfu ml^{-1}), and pathogens like *L. monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus* were detected (Table 1). HPP at 550 MPa for 16 min greatly improved the microbial quality of this raw milk, reducing 4.74 $\log_{10}$ cycles of APC and yielded non-detectable levels of coliforms and *S. aureus*, but pathogens *L. monocytogenes* and *Salmonella* spp. were still detected (Table 1).

Processing at 600 MPa enhanced microbial inactivation, and significant differences between non-treated and HPP samples could be observed after 1 min holding time (Table 1). Non-detectable levels were observed for all pathogens by applying 600 MPa/12 min treatments although 2.30 ± 0.02 $\log_{10}$ cfu ml^{-1} of aerobic mesophiles remained in milk samples (Table 1). HPP is a batch process, and is limited to processing volumes of ~50-525 L, thus pressure holding times shorter than 10 min are desired for HPP commercial applications (US FDA, 2014; Serment-Moreno *et al*., 2015).

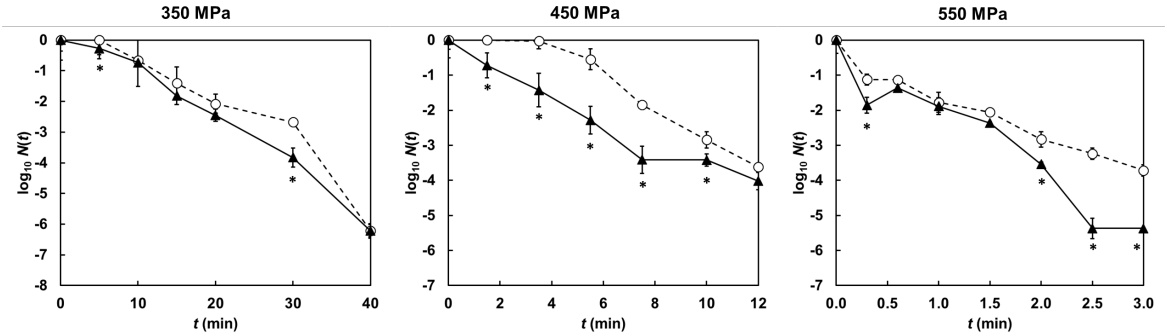

Fig. 1. *Listeria innocua* inactivation in UHT milk: (o) high pressure processing (HPP); (▲) hurdle approach combining HPP and nisin synthesized in situ by *Lactococcus lactis* Rif L^+. Star symbols (*) indicate significant differences ($p < 0.05$) between HPP and HPP+nisin samples.

Table 1. Microbial characterization of untreated and HPP-treated fresh raw bovine milk. Superscript letters indicate significant differences between HPP treatments ($p < 0.05$).

		Quantification log10 (cfu ml^{-1})			**Detection**	
P (MPa)	**t (min)**	**Aerobic plate counts (APC)**	**Coliforms**	***Staphylococcus aureus***	**Listeria spp.**	**Salmonella spp.**
Untreated	-	7.43±0.07^a	D	D	D	D
550	CUT	6.67±0.01^b	D	D	D	D
	1	6.18±0.06^b	ND	D	D	D
	4	5.19±0.01^c	ND	D	D	D
	8	5.10±0.04^c	ND	D	D	D
	16	2.69±0.06^f	ND	ND	D	D
600	CUT	6.79±0.02^b	D	D	D	ND
	0.5	6.30±0.07^b	D	D	D	ND
	1	5.43±0.06^c	ND	D	D	ND
	3	4.59±0.00^d	ND	D	D	ND
	6	3.77±0.06^e	ND	D	D	ND
	12	2.30±0.02^f	ND	ND	ND	ND

D: detected counts; ND: non-detected counts (<1 cfu in 25 ml)

Despite the significant reduction of aerobic counts with 600 MPa/6 min ($p < 0.05$), microbial analysis still detected pathogens *S. aureus* and *L. monocytogenes* (Table 1). The high baroresistance of *S. aureus* in milk and other food systems is also well documented, where hurdle approaches involving HPP and other food preservation technologies are required to enhance inactivation (Baptista *et al.*, 2016). For instance, 50 °C and pressure levels between 350 and 500 MPa, 5-10 min resulted in 5.5-6.5 $\log_{10}$ reductions of *S. aureus* (Alpas & Bozoglu, 2000; Chen, 2007).

3.2.2 *Effect of HPP and nisin biosynthesis by Lactococcus lactis UQ2 rif L^+ (LUQ2) on Listeria spp. inactivation*

The inoculation of raw milk with ~7 $\log_{10}$ cfu ml^{-1} of *L. innocua* increased the initial counts of *Listeria* spp. to 9.52 $\log_{10}$ cfu ml^{-1}. The LUQ2 inoculum synthesized ~400 μg ml^{-1} of nisin (9.75 ± 0.54 IU ml^{-1}) after 30 min, and *Listeria* spp. decreased by 2.22 $\log_{10}$ cycles. In raw milk, the combination of LUQ2 and HPP treatments at 550/2.5 min reduced 3.0

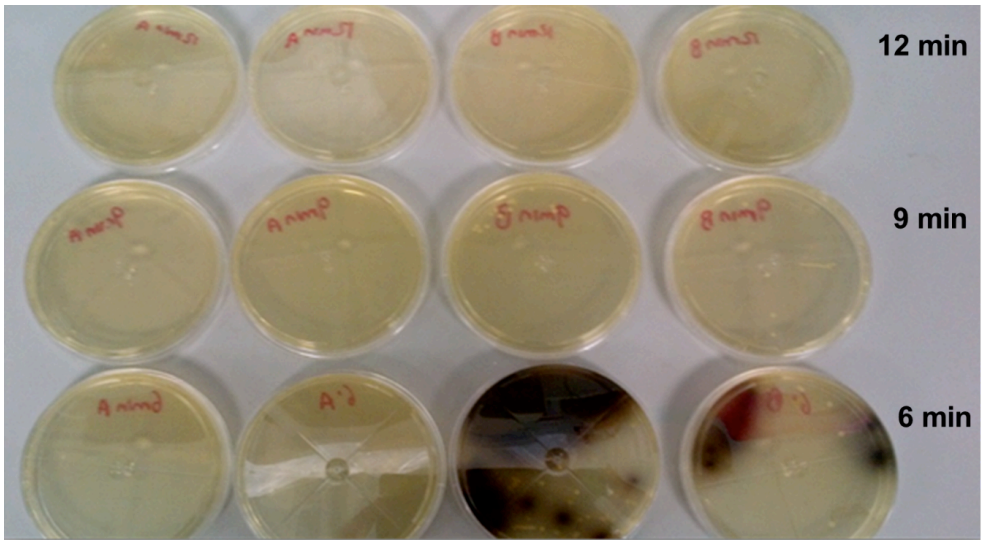

Fig. 2. Qualitative determination of *Listeria* spp. survivors (Oxford agar) in raw bovine milk samples treated at 600 MPa and inoculated with 7 $\log_{10}$ of *Lactococcus lactis* Rif L^+.

$\log_{10}$ cycles of *Listeria* spp., whereas the same HPP conditions resulted in non-detectable counts for UHT milk. The difference in pressure resistance shown by *Listeria* spp. in raw and UHT milk can be attributed to the higher contents of milk fat in raw milk, which may protect bacteria from the lethal pressure effects (Ferreira, Almeida *et al*., 2016). Moreover, *L. monocytogenes* present in raw milk samples could be better adapted to the media, possibly conferring the microorganism a higher pressure resistance (Baptista *et al*., 2016).

Listeria spp. survivors were detected in half of the samples by combining HPP (600 MPa/6 min) and nisin, whereas no survivors could be observed after extending the pressure holding time to 9 or 12 min (Figure 2). These experimental results suggest that the HPP+nisin combination may achieve milk pasteurization in shorter holding times (6-9 min) at 600 MPa when compared to HPP treatments alone (12 min; Table 1). Furthermore, the nisin activity quantified in this study (9.75±0.54 IU ml^{-1}) is well below the 500 IU g^{-1} nisin activity limit allowed by the FAO/WHO (2010), which suggests that LUQ2 nisin biosynthesis optimization could be explored to shorten holding times or process milk at lower pressure levels (i. e. 500-550 MPa).

Black *et al*. (2005) reported ≥8 $\log_{10}$ reductions of *L. innocua*, *Escherichia coli* and spoilage bacteria by adding 500 IU ml^{-1} of nisin and pressurizing skimmed milk at 500 MPa during 5 min, but ~1.5 $\log_{10}$ cfu ml^{-1} of *L. innocua* were observed when the nisin activity was reduced by half. The nisin activity levels used by Black *et al*. (2005) were ~25-50 times higher than the 9.75±0.54 IU ml^{-1} synthesized by LUQ2 in the present study. Differences on the observed microbial inactivation could be attributed to both intrinsic (strain, milk composition, presence of protective culture) and extrinsic (pressure level, holding time) factors.

Alpas and Bozoglu (2000), analyzed a multi-targeted hurdle approach that included HPP (345 MPa, 5 min), mild heat (50 °C), and a fermentation broth extract with bacteriocins nisin and pediocin (5,000 AU ml^{-1}) that was obtained by simultaneously growing *L. lactis* and *Pediococcus acidilactici*. Over 8 $\log_{10}$ reductions of *E. coli*, *L. monocytogenes*, *S. aureus* and *Salmonella* spp. were reported with the HPP/heat/bacteriocin treatments of orange juice and milk (Alpas & Bozoglu, 2000), although it would be interesting to determine if the nisin-pediocin combination works for HPP at ambient temperature (20-25°C).

Conclusions

The hurdle technology approach combining HPP 600 MPa (6-9 min holding time), and *in-situ* nisin biosynthesis by LUQ2 (9.75±0.54 IU ml^{-1}) produced non-detectable counts of *Listeria* spp. (≥5 $\log_{10}$ reductions), most likely achieving milk pasteurization. It may be feasible to achieve non-detectable *Listeria*

spp. for pressure levels below 600 MPa if growth conditions of LUQ2 are optimized, or by evaluating if alternative native *Lactococcus lactis* strains isolated from dairy products are capable of yielding higher nisin activity levels through *in-situ* biosynthesis. Furthermore, shelf life studies evaluating microbial growth and sensory properties should be evaluated to determine if the HPP+nisin approach is feasible for milk commercialization.

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