Shelf Life Extension of Liquid Whole Eggs by Heat and Bacteriocin Treatment

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Abstract

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During a 15-month period, samples of commercially pasteurised liquid whole egg (LWE) were tested for the presence of spoilage microflora. The total bacterial counts were 2.2 ± 0.6 log CFU/g and total lactic acid bacteria (LAB) counts were 1.9 ± 0.6 log CFU/g. *Enterobacteriaceae* were detected in 2 samples. Out of the tested samples, 45 LAB were isolated and identified, with 30 strains identified as *Enterococcus faecium*, 12 as *Enterococcus faecalis*, and 3 as *Lactobacillus paracasei* subsp. *paracasei.* All strains, except 6 strains of *E. faecium,* possessed lipolytic activity. All the *E. faecalis* strains and one strain of *E. faecium* showed a high proteolytic activity, while moderate proteolytic activity was shown by 3 lactobacilli strains. Minimum inhibitory concentration (MIC) of nisin and Micocin X was measured against groups of isolated strains, and ranged from 10.4 µg/ml to 41.7 µg/ml for nisin and from 0.2 mg/ml to 1.6 mg/ml for Micocin X. The LWEs supplemented with 6.25 mg/l of nisin or with 500 mg/ml of Micocin X were pasteurised at 65°C for 2.5 minutes. The shelf life of LWE with the addition of nisin or Micocin X stored under refrigerator conditions was extended by a minimum of 5 weeks.

Keywords: liquid whole egg; bacteriocin; pasteurisation; extended shelf life; lactic acid bacteria

Hen's eggs are a significant farm commodity worldwide, with the eggs production of 91 × 10^9 in the United States and 126 ×10^9 in EU in 2008 (Anonymous 2008a; Roubalova 2009). In EU, the average human consumption of eggs per capita is 236 while in the Czech Republic the average consumption reached 317 in 2008 (Roubalova 2009). Although shell eggs are important, further processing of eggs has gained the market share. In Canada the production of eggs for further processing accounts for 25% to 30% of all eggs sold, liquid processed eggs being the prevalent egg product (Anonymous 2009). In 2007, the United States produced 7.6 × 10^8 kg of edible liquid whole eggs (Anonymous 2008a).

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Liquid whole egg is made by homogenisation and pasteurisation of broken whole eggs, which extends the shelf life when stored chilled or frozen. The initial microflora of LWE is composed of a mixture of Gram-positive and Gram-negative bacteria similar to the indigenous microflora of shell eggs, as the shell is typically the source. The processing equipment, environment, and eggs contamination also play an important role in determining the microflora of LWE (ICMSF 2005). Low pasteurisation temperature, limited by the low thermostability of egg proteins, and short shelf life of this product suggest that some heat resistant bacteria can survive the heat treatment. Pasteurisation destroys the spoilage bacteria such as *Pseudomonas*, *Acinetobacter*, and *Enterobacter* spp. and can leave bacteria like micrococci, staphylococci, bacilli, and enterococci (ICMSF 2005; Lai 2006). *Salmonella* spp. are the most important pathogen in LWE and a pasteurisation process was designed to eliminate this pathogen. LWEs are a potential source of enterohemorrhagic *Escherichia coli,* but their occurrence in eggs is low and the pasteurisation temperatures are sufficient to inactivate *Enterobacteriaceae* (Lai 2006). *Staphylococcus aureus* does not grow in LWE at the refrigeration temperatures and it must reach 10^5 CFU/ml to produce toxins, which would require severe temperature abuse, inadequate pasteurisation or secondary contamination (ICMSF 2005). The presence of *Listeria monocytogenes* in unpasteurised processed eggs has been reported (Moore & Madden 1993; Ohkochi *et al.* 2009), but to date, pasteurised LWEs have not been associated with the cases of human listeriosis (ICMSF 2005).

The minimum pasteurisation temperature and holding time for LWE pasteurising is 60°C for 3.5 min in the United States and Canada (Stadelman & Cotteril 1990; Anonymous 2010). The EU does not stipulate pasteurisation conditions, but has implemented microbial standards for the finished products. The product must be free of *Salmonella* (0 CFU in 25 g) and contain no more than 100 CFU/g of *Enterobacteriaceae* (Anonymous 2005).

Pasteurisation is usually done using a high temperature-short time process with a plate or tube heat exchanger (Lai 2006). A newer technology using an ultrapasteurisation process involving heating with a specially designed heat exchanger or with injections of steam separately to yolk and egg white followed by aseptic homogenisation and aseptic packaging allows producing LWE with a shelf life of several months (Swartzel *et al.* 1990; Swartzel & Ball 1991). Several novel methods that do not use heat or combine heat with other techniques have been developed in the last two decades: (*i*) irradiation followed by heat (Alvarez *et al.* 2007) and the combination of heat and nisin (Boziaris *et al.* 1998), (*ii*) pulsed electric field (Amiali *et al.* 2004) and its combination with heat (Bazhal *et al*. 2006; JIN *et al.* 2009) or nisin addition (Calderon-Miranda *et al.* 1999), (*iii*) high hydrostatic pressure (Lee *et al.* 2001), hydrostatic pressure pulsing (Bari *et al.* 2008), and ultra high pressure homogenisation (Velazquez-Estrada *et al.* 2008), (*iv*) combination of high hydrostatic pressure with high ultra sound treatment (Lee *et al.* 2003), hydrogen peroxide treatment (Isiker *et al.* 2003), or nisin treatment (Ponce *et al.* 1998; Lee *et al.* 2003), and (*v*) pH-adjusted pasteurisation (Schuman & Sheldon 2003). All these methods are focused on the elimination of pathogens like *Salmonella* spp., *Listeria monocytogenes*, *E. coli* O157:H7 or *Bacillus cereus*.

Bacteriocins can be used for the reduction of the intensity of the heat treatment in foods without compromising microbial inactivation, which could save the cost of the heat treatment and decrease the impact of heat on the food (Galvez *et al*. 2007). Bacteriocins from LAB are small antimicrobial proteins or peptides that kill or inhibit the growth of closely related bacteria. They inhibit the growth of a wide range of Gram-positive bacteria including *Bacillus, Clostridium, Enterococcus, Listeria,* and *Staphylococcus* (Tagg *et al.* 1976; Martin-Visscher *et al*. 2008). Nisin, produced by *Lactococcus lactis*, belongs to the family of bacteriocins called lantibiotics (Sahl *et al*. 1995). Nisin can be obtained as a commercial product that is regulated as a food additive and is approved for use in most major food-producing countries (Davidson *et al.* 2005). Micocin X is a commercial product that is approved in US, Canada, and a few South American countries as a product to control spoilage (Anonymous 2008b). It contains three bactericocins (piscicolin 126, carnobacteriocin BM1, and recently discovered carnocyclin A) produced by a single strain of *Carnobacterium maltaromaticum.* Piscicolin 126 and carnobacteriocin BM1 are both class IIa bacteriocins and carnocyclin A belongs to a small group of cyclic bacteriocins (Martin-Visscher *et al*. 2008).

The objective of this study was to determine the major spoilage bacteria of pasteurised LWE and

to find an efficient and affordable method for the extending of the shelf life of this egg product.

MATERIAL AND METHODS

Materials. Commercial samples (15) of pasteurised liquid whole eggs were obtained monthly from various Czech producers not using the ultrapasteurisation process followed by aseptic packaging. The refrigerated samples, received 2 to 4 days after packing, were used for the determination of the total and LAB counts and for the identification of microflora. Unpasteurised liquid whole eggs, received on refrigeration for 3 to 5 days after cracking from a Canadian supplier, were used for the pasteurisation experiments.

Nisin, obtained from MP Biomedicals, LLC (Montreal, Canada), contained 2.5% (w/w) of pure nisin. Other bacteriocins (piscicolin 126, carnobacteriocin BM1 and carnocyclin A) were used in the form of Micocin^{TM} X (Griffith Laboratories, Alsip, USA) with total antimicrobial activity of 16 000 AU/g against *Listeria monocytogenes*.

Bacterial strains, media, growth conditions. The 45 strains of LAB used in this study were isolated from commercial pasteurised LWE samples as the typical colonies from the highest dilution MRS plates. All isolated strains and *Enterococcus faecium* DMF7050, *Enterococcus faecalis* DMF7051, and *Lactobacillus paracasei* subs. *paracasei* LMG13552 were grown in MRS (Becton, Dickinson and Company, Sparks, USA) broth at 30°C for 16 h prior to use. *Carnobacterium maltaromaticum* UAL9 was grown in APT broth (Becton, Dickinson and Company) at 25°C for 16 h prior to use.

Counts. The samples were serially diluted with 0.85% (w/v) NaCl containing 0.1% (w/v) Tryptone (Becton, Dickinson and Company) and spread- or pour-plated using PCA (Becton, Dickinson and Company) plates for total counts, MRS agar (Becton, Dickinson and Company) plates for LAB counts, and VRBA (Becton, Dickinson and Company) containing 1% (w/v) glucose for *Enterobacteriaceae* counts. The VRBA plates were overlayered with VRBA agar and incubated at 37°C for 24 h, while MRS and PCA plates were incubated at 30°C and 25°C, respectively, for 3 days. The detection limit for spread- and pour-plating was 100 CFU/ml and 10 CFU/ml, respectively.

Strain identification. The isolates were Gram-stained and tested for catalase production. The cell morphology, growth at different temperatures (10–45°C), growth at different pH values (4.5 to 9.6), growth at 6.5% (w/v) of NaCl, bile esculin hydrolysis, gas production from glucose, heat treatment at 60°C for 30 min, and growth on Slanetz-Bartley agar (Oxoid CZ s.r.o., Brno, Czech Republic) were observed (Salminen *et al.*2004; Ammor *et al.* 2005; Niemi & Ahtiainen 1995). API CHL50 strips (bioMérieux S.A., Marcy l'Etoile, France) and ENCOCCUStest strips (PLIVA-Lachema, Brno, Czech Republic) were used for the identification at the species levels. The genomic DNA of each strain isolated was purified using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, Prague, Czech Republic). To amplify the genus- and species-specific amplicons, the primers listed in Table 1 were used. *Enterococcus faecium* DMF7050, *Enterococcus faecalis* DMF7051, and *Lactobacillus paracasei* subs. *paracasei* LMG13552 were used as positive controls.

The reaction mixture (25 µl) contained: 1 µl of the template DNA, 2.5 µl of 10 × PCR buffer, 1 µl of 50mM $MgCl_2$, 0.5 µl of 10mM dNTP and 0.2 µl of 5 U/µl Taq DNA polymerase (Roche s.r.o.,

Table 1. List of primers used in genus- and species-specific PCR

Primer	Sequence (5'→ 3')	Annealing temperature (°C)	Reference
Ent1	TAC TGA CAA ACC ATT CAT GAT G	55	Cupaková *et al.* (2005)
Ent2	AAC TTC GTC ACC AAC GCG AAC		
FL1	ACT TAT GTG ACT AAC TTA ACC	55	Jackson *et al.* (2004)
FL2	TAA TGG TGA ATC TTG GTT TGG		
FM1	GAA AAA ACA ATA GAA GAA TTA T	55	Jackson *et al.* (2004)
FM2	TGC TTT TTT GAA TTC TTC TTT A		
Y2	CCC ACT GCT GCC TCC CGT AGG AGT	45	Ward and Timmins (1999)
Para	CAC CGA GAT TCA ACA TGG		

Prague, Czech Republic) and 1 µl of each of 50mM primers (Generi Biotech s.r.o., Hradec Králové, Czech Republic). The reaction was carried out under the following conditions: initial denaturation at 94°C for 5 min, amplification by 30 cycles of denaturation at 94°C for 40 s, annealing at 55°C (primers Ent1/Ent2, FL1/FL2, FM1/FM2) or 45°C (primers Y2/Para) for 45 s elongation at 72°C for 1 min and final extension at 72°C for 5 minutes. The samples were run on an agarose (2% w/v) electrophoresis in 0.5 × TBE buffer, stained with 0.5 µg/ml ethidium bromide, and visualised with a microDOC gel documentation system (Cleaver Scientific Ltd., Warwickshire, UK).

Proteolytic and lipolytic activity assay. To test lipolytic activity, 50 µl of the overnight culture of the tested strain were spotted into a well in tributyrin agar base supplemented with 1% (v/v) of tributyrin (Merck spol. s r.o., Prague, Czech Republic). Lipase activity was measured by observing the zone of clearing around the well after 5 days of incubation at 30°C.

To test proteolytic activity, each of the isolates was grown at 30°C for 16 h in skim milk and subcultured 3 times prior to use. A 2.5 ml volume of the culture was mixed with 1 ml of distilled water, to which 5 ml of 0.75 mol/l trichloracetic acid was added. After 10 min the mixture was filtered through a standard filter 390 (Filtrak, Wiesbaden, Germany). The filtrate (50 µl) was mixed with 1 ml of OPA (0.04 g *O*-phtaldialdehyde (OPA) (Sigma-Aldrich) in 1 ml of methanol), 25 ml of sodium tetraborate (0.1 mol/l, pH 9.5) (Penta, Czech Republic), 2.5 ml of 20% (w/v) SDS (Sigma-Aldrich), 0.05 g of *N*-acetylcysteine (Sigma-Aldrich) and made up to 50 ml with distilled water. The absorbance at 340 nm was measured, and glutamic acid was used as the standard for the calibration curve. Proteolytic activity was expressed in mmol of glutamic acid per liter of milk (mmol/l L-Glu).

Minimum inhibitory activity assay (MIC). A series of 2-fold dilutions of the Nisin and Micocin X in MRS broth was prepared in a 96-well microtiter plate. The overnight cultures of LAB strains were mixed into cocktails containing equal amounts of 5 strains listed in Table 2. The medium was inoculated with 1% (v/v) of the cocktail and added to the wells. The microtiter plates were incubated overnight at 30°C and the MICs were calculated as the lowest concentration causing the inhibition of the cocktails growth.

Pasteurisation of liquid whole eggs. Unpasteurised LWE were heated in a UHT/HTST System (model FT74T, Armfield Inc., Whitmire, USA) equipped with the standard tubular heat exchanger (20-MkIII). The holding tube was extended to 150 s with a tube of the same internal diameter as the original holding tube at the flowrate of 12 l/h (pump frequency 7.3 Hz). The whole system was heated at 80°C for 30 min prior to its using to inactivate all viable cells, and was equilibrated with cold water to the required conditions. Thermal treatment of the unpasteurised LWE was performed at 65°C for 2.5 min in 4 l batches. Bacteriocin-treated batches were supplemented with either 6.25 mg/l of nisin or 500 mg/l of Micocin X. The pasteurised LWE was cooled in the system with cold water, collected in sterile 1 l screw cap flasks, and aseptically dispensed into sterile 50 ml screwcap tubes. All samples were stored at 4°C for the duration of the experiment. After each batch, the system was cleaned with 4% NaOH at 60°C for 30 min to remove organic compounds, flushed with cold water and cleaned with 5% citric acid (60°C, 30 min) to remove inorganic compounds. Total, LAB, and *Enterobacteriaceae* counts of each sample were determined weekly and all experiments were done in triplicate.

RESULTS AND DISCUSSION

Screening of spoilage microorganisms in pasteurised LWE

Pasteurised LWEs from various Czech producers were sampled monthly during a 15-month period. The average microbial counts and standard deviations were calculated. Total bacterial counts were 2.2 ± 0.6 log CFU/g and total LAB counts were 1.9 ± 0.6 log CFU/g. *Enterobacteriaceae* counts were below the detection limit of the method (10 CFU/g) in all samples except for two that contained 1.2 log CFU/g and 1.6 log CFU/g. All the samples met the EU microbiological criteria (Anonymous 2005) for *Enterobacteriaceae* at the time of sampling but it can be assumed that the two samples with the counts *Enterobacteriaceae* detected would not have met the criteria at the end of the shelf life (4 weeks). These samples also contained some fluorescent colonies on PCA plates, which were found to be Gram-negative and catalase positive rods, and were likely *Pseudomonas* spp., *Enterobacteriaceae* or *Pseudomonas* spp., that do

Table 2. Proteolytic and lipolytic activity and MICs of nisin and Micocin X of strains isolated from pasteurised LWE and indicator strain

Strain	Lipolitic activity[1]	Proteolytic activity[2] (mmol/l L-Glu)	MIC[3]		Strain	Lipolytic acticity[1]	Proteolytic activity[2] (mmol/l L-Glu)	MIC[3]	
			nisin (μg/ml)	Micocin X (mg/ml)				nisin (μg/ml)	Micocin X (mg/ml)
E. faecium 01	+	0.48			*E. faecium* 39	+	0.47		
E. faecium 02	+	0.44			*E. faecalis* 40	+	6.21		
E. faecium 03	+	0.50	10.4	0.8	*E. faecium* 41	+	0.44		
E. faecium 04	+	0.48			*E. faecalis* 42	+	6.39		
E. faecium 05	±	0.46			*E. faecium* 43	+	0.51	41.7	1.6
E. faecium 06	+	0.46			*E. faecium* 44	+	0.45		
E. faecium 07	±	0.53			*E. faecium* 45	+	0.47		
E. faecium 08	+	0.49	5.2	0.2	*E. faecalis* 51	+	6.78		
E. faecium 09	+	0.48			*E. faecium* 52	+	0.50		
E. faecium 10	+	0.46			*E. faecalis* 53	+	6.97	20.8	1.6
E. faecium 11	+	0.48			*E. faecalis* 54	+	6.76		
E. faecium 12	+	0.54			*E. faecium* 55	+	6.81		
L. paracasei 13	+	0.95	41.7	1.6	*E. faecium* 56	+	0.44		
L. paracasei 14	+	1.02			*E. faecalis* 57	+	6.25		
L. paracasei 15	+	1.16			*E. faecium* 58	±	0.49	20.8	1.6
E. faecium 31	±	0.46			*E. faecium* 59	+	0.47		
E. faecium 32	+	0.47			*E. faecalis* 60	+	6.49		
E. faecium 33	±	0.52	10.4	0.8	*E. faecalis* 61	+	5.87		
E. faecium 34	+	0.50			*E. faecalis* 62	+	6.00		
E. faecium 35	+	0.49			*E. faecalis* 63	+	6.41	41.7	1.6
E. faecium 36	±	0.49			*E. faecalis* 64	+	5.70		
E. faecium 37	+	0.47	41.7	1.6	*E. faecalis* 65	+	6.84		
E. faecium 38	+	0.45			*Carnobacterium maltaromaticum* UAL9[4]			10.4	0.8

[1]± – diameter of zone of clearing to 2 mm, + – diameter of zone of clearing to 6 mm; [2]$n = 3$ – coefficient of variance were up to 5% of all samples; [3]MIC – no standard deviations are shown because in all tests identical results were obtained; [4]indicator strain – proteolytic and lipolytic activity was not done

not tolerate high temperatures, cannot generally survive the pasteurisation of LWE. In such a situation where high levels of contamination of raw LWE occur they might be found in the pasteurised LWE. In Canada, all eggs have to be washed before further processing (Anonymous 2010); however, according to EU law (Anonymous 2004) only clean eggs can be used for other processing, from which results that only small part of eggs is washed in EU. To our knowledge, none of the Czech producers washes eggs before processing. Washing dirty eggs before breaking can reduce the aerobic plate counts of liquid egg by several orders of magnitude (ICMSF 2005).

The average LAB counts corresponded to the average total bacteria counts of the pasteurised LWE; furthermore, the LAB counts and total bacterial counts of the individual samples were similar. This observation indicates that the LABs are the prevalent spoilage microflora in pasteurised LWE.

Identification of strains isolated from LWE

A total of 45 strains of bacteria that grew on MRS plates were collected during the 15-month study. Priliminary biochemical testing and confirmatory PCR identified 30 strains as *Enterococcus faecium*, 12 as *Enterococcus faecalis,* and 3 as *Lactobacillus paracasei*. *Lbc. paracasei* isolates were identified by API testing as *Lbc. paracasei* ssp. *paracasei*. Enterococci and *Enterobacteriaceae* that are often selected as indicator organisms (Jay 2000; Mallet *et al.* 2006; Wall *et al.* 2008) are the most common bacteria found on the egg shell (Wall *et al.* 2008). Wall *et al.* (2008) found that enterococci were present on 40% to 60% of the egg shells depending on the age of the hens and the type of breeding.

Enterococci appear to be heat tolerant, with all isolates in this study surviving 60°C for 30 minutes. Kearns (1995) reported that the isolates of enterococci can survive 65°C for at least 10 minutes. The strains of *E. faecium* were more heat tolerant, with some of *E. faecium* surviving 80°C for 3 minutes. Some enterococcal species, in particular *E. faecalis*, can be opportunistic pathogens and, therefore, are undesirable in food. In addition, the propensity of fecal enterococci to be resistant to antibiotics and to transfer such traits by means of mobile genetic elements is also a safety risk (Salminen *et al.* 2004). As *E. faecium* was the most common isolate in the current study on commercially produced pasteurised LWE, these results indicate that such products may be a potential food safety risk. Further work is required to determine if these strains have specific virulence factors associated with pathogenicity of enterococci. *Lbc. paracasei* ssp. *paracasei* is usually isolated from dairy products, sewage, silage, and human and clinical sources, and its presence in LWE is unusual (Wood & Holzapfel 1995).

Proteolytic and lipolytic activities

All isolated strains were tested for proteolytic and lipolytic activities (Table 2). All strains demonstrated lipolytic activity on tributyrin agar plates. The 6 strains of *E. faecium* exhibited a weak lipolytic activity. Sarantinopoulosa *et al.* (2001) showed that 90% of 129 enterococcal isolates hydrolysed all substrates from tributyrin (C4) to tristearin (C18). High proteolytic activities were demonstrated by all of the *E. faecalis* strains and only one strain of *E. faecium*. All isolates of *L. paracasei* showed a moderate proteolytic activity. The highest activity (6.84 mmol/l L-Glu) among all isolates was shown by *E. faecalis* 65. The level of proteolytic activity in enterococci is strain dependent (Veljovic *et al.* 2009) but, in general, enterococci possess a low proteolytic activity with the exception of *E. faecalis* strains (Moreno *et al.*2006).

Most researchers studying proteolytic and lipolytic activities of enterococci focus on dairy isolates. However, eggs contain high amounta of proteins and lipids, and thus lipolytic and proteolytic activities could lead to the formation of undesirable bitter-tasting peptides and fatty acids. These fatty acids might be converted to other aromatic compounds as methyl ketones and lactones, or their oxidation might lead to the formation of various strongly flavoured aldehydes (Moreno *et al.* 2006) that are detrimental to LWE products quality. Thus, the identification of lipolysis and proteolysis in enterococci isolated from LWE is important from the food quality standpoint.

Minimum inhibitory concentrations

The MICs of nisin and Micocin X were evaluated against cocktails of the strains isolated from the LWE. The MIC of pure nisin ranged from 10.4 μg/ml to 41.7 μg/ml, while the MIC of Micocin X ranged from 0.2 mg/l to 1.6 mg/ml. The cocktail of strains that was most sensitive to Micocin X contained strains *E. faecium* 06, 07, 08, 09, and 10. Six of nine cocktails demonstrated similar results, with the MIC equal to 1.6 mg/ml. Generally, the cocktails that contained only strains of *E. faecium* were more sensitive to nisin and Micocin X. The inhibition of all isolated strains by the bacteriocins used in this research suggested the bacteriocins can be used as a sufficient hurdle in the control of LWE spoilage.

Pasteurisation of liquid whole eggs with addition of bacteriocins

Heat treatment at 64°C for 2.5 min is the lowest heat pasteurisation allowed in the United Kingdom for LWE (Stadelman & Cotteril 1990). The time/temperature parameters used in this study

were chosen to minimise the thermal damage and preserve the functional properties of LWE. The LWE was pasteurised at 65°C for 2.5 min and the pasteurised LWE was stored at 4°C for several weeks. The final concentration of nisin (6.25 mg/l) added to the LWE was 50% of the maximum concentration allowed by EU regulation, which varies from 3 mg/kg to 12.5 mg/kg depending on the foodstuff (Anonymous 1995). Although the use of nisin in egg products is not allowed by this regulation (Anonymous 1995), national legislations may vary, thus nisin was chosen for the use. Micocin X at a concentration of 500 mg/l corresponds to the same level of antibacterial activity against the indicator strain *Carnobacterium maltaromaticum* UAL9 as the 6.25 mg/l of nisin. Micocin X, a product containing 3 bacteriocins of strain *C. maltaromaticum*, is permitted as a food preservative for certain foodstuffs in the United States, Canada, and few South American countries. The addition of bacteriocins was done prior to pasteurisation to simulate fully the manufacturing process.

The initial bacterial counts of LWE before pasteurisation ranged from 3.5 to 4.3 log CFU/ml for *Enterobacteriaceae*, 5.4 to 6.2 log CFU/ml for total microflora, and 4.1 to 4.3 log CFU/ml for LAB, respectively (Figure 1). *Enterobacteriaceae* were not detected in any of the pasteurised samples, with or without the addition of the bacteriocins, during the storage test. In the non-pasteurised samples, *Enterobacteriaceae* reached 7.2 ± 0.2 log CFU/ml by the second week of storage. No bacteria were detected in any of the samples by the end of the first day after pasteurisation. During one replicate of the experiment, the pasteurised samples (with no bacteriocin) reached a total count of 4.2 log CFU/ml after two weeks of storage, but the counts of the samples obtained from the other two replicates were still below the detection limit. All the samples for each replicate were considered to be spoiled when microbial counts of 4.0 ± 0.4 log CFU/ml were reached on day 21 of the storage test. In all cases, the LAB counts were analogous to the total bacterial counts. According to Canadian regulations, LWE should have a total bacterial count of no more than 5×10^4 CFU/g (Anonymous 2010). To our knowledge, this limit is usually adopted by the producers in EU as a limit of the internal quality control. In the current study, the pasteurised LWE without bacteriocins had a storage life of 3 weeks. The addition of bacteriocins (either nisin or Micocin X) delayed the microbial growth throughout the storage study (Figure 1). The counts of all groups of microorganisms were below the detection limit of 100 CFU/ml. No colonies were found on any of the agar plates onto which the samples with nisin or Micocin X were plated. The shelf life of the pasteurised samples with added bacteriocins was extended to minimum of 5 weeks, which was the duration of the study.

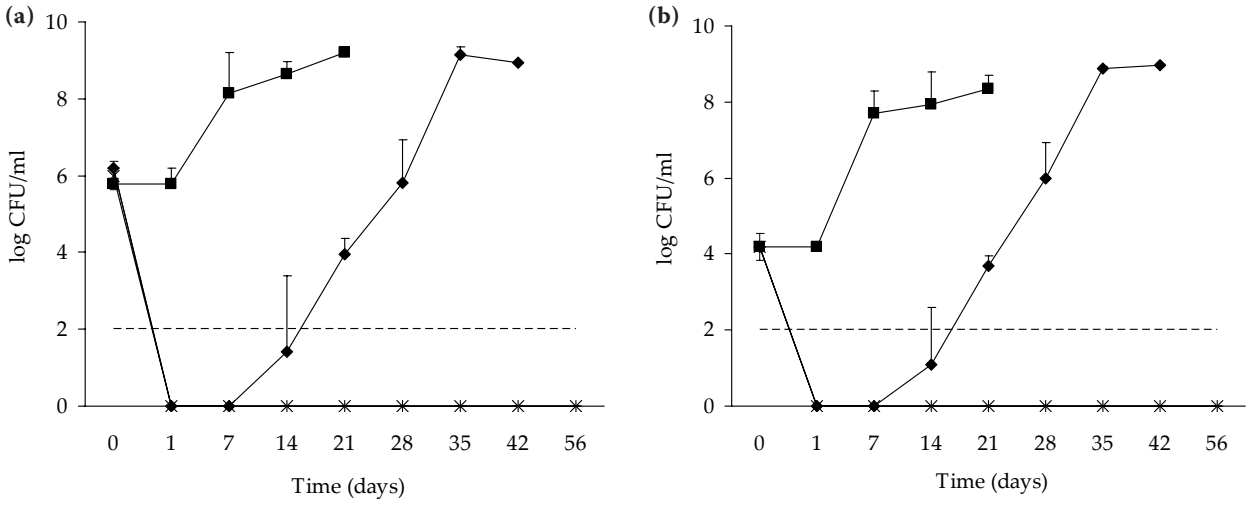

Figure 1. Total bacterial counts (**a**) and total LAB counts (**b**) of non-pasteurised LWE (■), pasteurised at 65ºC for 2.5 min (♦), pasteurised at 65ºC for 2.5 min with addition of 6.25 mg/l of nisin (×), pasteurised at 65ºC for 2.5 min with addition 500 mg/l of Micocin X (+) stored for 8 weeks at 4ºC, ---- detection limit 100 CFU/ml. Counts at 0 days represent the counts prior to pasteurisation. Dashed line represents the detection limit. Error bars indicate the standard deviation of three independent measurements

CONCLUSIONS

While most of researchers have focused on suppressing the growth of pathogenic bacteria such as *Salmonella* spp., in LWE, we aimed at the inhibition of the spoilage microflora of LWE to extend its shelf life. This study demonstrated that the predominant spoilage microorganisms in pasteurised LWE are lactic acid bacteria, most notably enterococci that survive pasteurisation and that have the potential to produce undesirable flavour compounds. The results of this study demonstrated that the addition of bacteriocins to LWE prior to pasteurisation appear to be a promising option for extending the shelf life by at least 5 weeks as compared to pasteurisation alone. Minimal capital cost is required by the producers for a significant gain in the shelf life, especially when compared to the expensive technologies mentioned in the introduction. However, despite the success of bacteriocins in inhibiting spoilage microflora, minimising microbial contamination of raw materials through pre-processing steps is a key to the success of any intervention. Future research should focus on the steps that have the potential to reduce pre-processing microbial contamination.

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