International Journal of Food Microbiology 177 (2014) 81–88

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Leuconostoc bacteriophages from blue cheese manufacture: long-term survival, resistance to thermal treatments, high pressure homogenization and chemical biocides of industrial application

Silvina A. Pujato [a], Daniela M. Guglielmotti [a,*], Hans-W. Ackermann [b], Francesca Patrignani [c], Rosalba Lanciotti [c], Jorge A. Reinheimer [a], Andrea Quiberoni [a]

[a] Instituto de Lactología Industrial, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829, 3000 Santa Fe, Argentina
[b] Département de Microbiologie, Infectiologie et Immunologie, Faculté de Médecine, Université Laval, Québec G1K 7P4, Canada
[c] Dipartimento di Scienze e Tecnologie Agro-Alimentari, University of Bologna, Piazza Goidanich 60, 47023 Cesena, Italy

ARTICLE INFO

Article history:
Received 3 December 2013
Received in revised form 12 February 2014
Accepted 15 February 2014
Available online 21 February 2014

Keywords:
Leuconostoc
Bacteriophages
Inactivation
Heat
Biocides
HPH

ABSTRACT

Nine *Leuconostoc mesenteroides* phages were isolated during blue cheese manufacture yielding faulty products with reduced eye formation. Their morphologies, restriction profiles, host ranges and long-term survival rates (25 °C, 8 °C, −20 °C and −80 °C) were analysed. Based on restriction analysis, six of them were further examined regarding resistance to physical (heat and high pressure homogenization, HPH) and chemical treatments (ethanol, sodium hypochlorite, peracetic acid, biocides A, C, E and F). According to their morphology, *L. mesenteroides* phages studied in the present work belonged to the *Caudovirales* order and *Siphoviridae* family. Six distinct restriction patterns were obtained with EcoRV, HindIII, ClaI and XhoI enzymes, revealing interesting phage diversity in the dairy environment. No significant reductions in phage counts were observed after ten months of storage at –20 °C and −80 °C, while slightly and moderate decrease in phage numbers were noticed at 8 °C and 25 °C, respectively. The phages subjected to heat treatments generally showed high resistance at 63 °C and moderate resistance at 72 °C. However, 80 °C for 30 min and 90 °C for 2 min led to complete inactivation of viral particles. In general, the best ethanol concentration tested was 75%, as complete inactivation for most *Leuconostoc* phages within 30 min of incubation was achieved. Peracetic acid, and biocides A, C, E and F were highly effective when used at the same or at a moderately lower concentration as recommended by the producer. Usually, moderate or high concentrations (600–1600 ppm) of sodium hypochlorite were necessary to completely inactivate phage particles. *Leuconostoc* phages were partially inactivated by HPH treatments as remaining viral particles were found even after 8 passes at 100 MPa. This is the first report of *L. mesenteroides* phages isolated from an Argentinean dairy cheese plant. The results of this work could be useful for establishing the most effective physical and chemical treatments for inactivating phages in industrial plants and laboratory environments.

1. Introduction

Leuconostocs are heterofermentative lactic acid bacteria (LAB) frequently used in dairy industry, being *Leuconostoc mesenteroides* subsp. *cremoris* the predominant flavour-producing starter or adjunct culture in various fermented milk products. The metabolism of lactose and citrate by *Leuconostoc* provides flavour and texture compounds, allowing also for the eye formation in some cheeses. Specifically, *Leuconostoc* plays a role expanding the mechanical openings in blue-veined cheeses such as Roquefort, where *Penicillium roqueforti* is able to colonize the eyes formed by CO_2.

As acknowledged, bacteriophage infection is a problem confronted by all dairy industries manufacturing cheeses and fermented milks (Moineau, 1999; Neve, 1996). Nevertheless and despite the significant role of *Leuconostoc* in dairies, only a few phage infection incidents are documented in the literature. The slow growth of *Leuconostoc* in milk might explain the low frequency of phage infections of this genus. Alternatively, attacks by bacteriophages could have been overlooked, since failure of *Leuconostoc* culture would affect some flavour features but not acid production. Phages infecting dairy *Leuconostoc* were isolated from cheeses, butter cream (Sozzi et al., 1978), "Viili" (a fermented milk product typical of Finland and some parts of Sweden) (Saxelin et al., 1986) and other dairy products (Ali et al., 2013; Boizet et al., 1992; Neve et al., 1988) including whey samples (Davey et al., 1995; Johansen and Kibenich, 1992).

Microbiologists and technologists largely agree on the unfeasibility of phage eradication from dairy plant environments (Briggiler Marcó et al., 2012). In consequence, several approaches are routinely employed to minimize phage spreading in dairies. Heat treatments

* Corresponding author. Tel.: +54 342 4530302; fax: +54 342 4571162.
E-mail address: dgugliel@fiq.unl.edu.ar (D.M. Guglielmotti).

http://dx.doi.org/10.1016/j.ijfoodmicro.2014.02.012
0168-1605/

applied to reduce pathogens and spoilage levels from raw milk, indirectly reduce viral titers. However, a remarkably high thermal resistance has been reported for some phages infecting *Lactococcus lactis* (Suárez and Reinheimer, 2002), *Streptococcus thermophilus* (Binetti and Reinheimer, 2000), *Leuconostoc* (Atamer et al., 2011), *Lactobacillus casei* and *Lactobacillus paracasei*, even up to 5 min at 95 °C (Capra et al., 2013). High pressure homogenization (HPH) is a "non-thermal" technology considered as an alternative for the inactivation of pathogenic and spoilage microorganisms (Fonberg-Broczek et al., 2005; Vachon et al., 2002; Patrignani et al., 2013a,b). However, data concerning HPH efficiency for bacteriophage inactivation are scarce and only three articles related to phages of lactic acid- and probiotic bacteria could be cited (Capra et al., 2009b; Mercanti et al., 2012; Moroni et al., 2002). Harsh cleaning and disinfection systems are mandatory for keeping phages under the risky level. Substantial studies have been focused on the chemical resistance of phages infecting *Lactococcus* (Suárez and Reinheimer, 2002), *Lactobacillus delbrueckii* (Ebrecht et al., 2010; Quiberoni et al., 2003) and, at a lesser extent, *S. thermophilus* (Binetti and Reinheimer, 2000), *Lactobacillus helveticus* (Quiberoni et al., 1999), *Lactobacillus plantarum* (Briggiler Marcó et al., 2009) and *Lb. casei* group (Capra et al., 2004; Mercanti et al., 2012). Knowledge regarding thermal (Atamer et al., 2011) and chemical resistance of phages infecting dairy *Leuconostoc* is comparatively very scarce.

During the last years, several dairy *L. mesenteroides* phages were isolated from blue cheese manufactures with reduced eye formation. Their morphology, long-term survival, genetic diversity and host range, as well as their thermal, HPH and chemical resistance were investigated.

2. Materials and methods

2.1. Bacteria

2.1.1. Strains and culture conditions

L. mesenteroides strains were isolated from commercial mesophilic mixed starters used in one Argentinean cheese plant manufacturing blue-veined cheeses. Strains were grown and reactivated overnight at 32 °C in the Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvois, France). They were kept as frozen (−80 °C) stocks in MRS broth supplemented with 15% (v/v) glycerol at the INLAIN collection.

2.1.2. Bacterial identification

Total DNA of the isolated commercial *Leuconostoc* strains were obtained by using the GeneElute™ Bacterial Genomic DNA kit (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions. The species of the isolated commercial strains was confirmed by sequencing a 1500 bp fragment within their 16S rRNA gene (Edwards et al., 1989). PCR reactions were performed in a GeneAmp PCR System (Applied Biosystems, Foster City, CA, USA) following conditions previously published (Zacarías et al., 2011). Nucleotide sequences of purified amplicons were determined at the DNA Sequencing Service of Macrogen (Seoul, Korea). The identity of isolates was established by nucleotide-nucleotide BLAST of the NCBI database (www.ncbi.nlm.nhi.gov/blast).

2.1.3. Random amplification of polymorphic DNA

Random amplification of polymorphic DNA (RAPD-PCR) was applied to the commercial *L. mesenteroides* strains in order to investigate their genetic diversity. Oligonucleotide primers M13 (5′-GAGGGTGGCG GTTCT-3′ (Huey and Hall, 1989; Stenlid et al., 1994) and 1254 (5′-CCGC AGCCAA-3′ (Akopyanz et al., 1992) were used in separate amplification assays. Amplification conditions were carried out according to Guglielmotti et al. (2006). Amplified DNA fragments were visualized on 0.8% (w/v) agarose gels with GelRed™ (Biotium, Hayward, CA, USA) as nucleic acid binding dye, in TBE buffer (Sambrook and Russell, 2001).

2.2. Bacteriophages

2.2.1. Phage isolation

Whey samples were collected from vats and moulds during industrial manufactures of blue-veined cheeses. In all cases, whey samples were accompanied by an aliquot of the commercial starter lot used in the corresponding blue cheese manufacture process. Collected whey samples were centrifuged (5 min at 5000 ×*g*) and filtered (Millipore membranes, Sao Paulo, Brazil; pore diameter of 0.45 μm). Filtrates with and without a thermal treatment (90 °C, 15 min) were used to investigate the presence of phages according to Svensson and Christiansson (1991). To isolate, purify and enumerate phages, the double-layer plate titration method was performed (Svensson and Christiansson, 1991). The isolated and purified phages were maintained at the INLAIN collection at −80 °C as high-titer stocks (Neviani et al., 1992) with 15% (v/v) of glycerol.

2.2.2. Host range

The host range of the isolated phages was determined by means of the turbidity test (Svensson and Christiansson, 1991). Briefly, overnight cultures of the host strains were inoculated at 2% (v/v) in 5 ml of MRS-Ca broth, added of 0.2 ml of each high-titer (~10^8 pfu/ml) stock of phages and incubated at 32 °C. Cell lysis was observed and recorded throughout three consecutive subcultures.

2.2.3. Electron microscopy

Phages were sedimented in a Beckman J2-21 ultracentrifuge (Beckman, Palo Alto, CA) using a JA-18.1 swinging bucket rotor (60 min at 25,000 ×*g*), and washed twice under the same conditions in 0.1 M neutral ammonium acetate. Phages were deposited on carbon coated copper grids, contrasted with 2% (w/v) potassium phosphotungstate (pH 7.2), or 2% (w/v) uranyl acetate (pH 4.5), and examined in a Philips EM 300 electron microscope (Philips, Eindhoven, The Netherlands) operated at 60 kV. Magnification was monitored using T4 phage tails (113 nm in length) (Ackermann, 2009). Ten particles were measured per phage and stain.

2.2.4. Phage DNA manipulation and restriction analysis

Leuconostoc phages were propagated in 100 ml MRS-Ca broth, treated for 30 min with DNase I (Sigma-Aldrich Corporation, St. Louis, MO, USA) (1 mg/ml) and RNase (USB Corporation, Cleveland, Ohio, USA) (1 mg/ml), centrifuged (10 min at 5000 ×*g*) and filtered (Millipore membranes, pore diameter of 0.45 μm). Phage DNAs were isolated according to Binetti et al. (2008). Briefly, phage particles were concentrated overnight at 4 °C with PEG 8000 (10% w/v) and 0.5 M NaCl (Yamamoto et al., 1970), centrifuged (10 min at 10,000 ×*g*) and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8). Phage DNAs were purified by three phenol–chloroform–isoamyl alcohol extractions and precipitated by addition of absolute ethanol. DNA pellets were suspended in double-distilled and nuclease-free water (Gibco™, Invitrogen, Grand Island, NY, USA). Purified phage DNAs were digested with EcoRV, HindIII, ClaI and XhoI endonuclease enzymes according to the recommendations of the manufacturers (Amersham Biosciences Corporation, Piscataway, New Jersey, USA). Gel electrophoresis in agarose (0.8% w/v) was performed (Sambrook and Russell, 2001) to resolve DNA fragments. Restriction patterns were captured using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Celbio, Milan, Italy) and then analysed with the software package BioNumerics™ (version 5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium). Calculation of restriction profile similarities was based on the Jaccard (band based) correlation coefficient and *Leuconostoc* phages were grouped by using the unweighted pair group method using arithmetic averages (UPGMA) (Vauterin and Vauterin, 1992). Sizes of the phage genomes were estimated by summing up the sizes of the HindIII- and ClaI-digested DNA fragments. Based on

restriction profiles, six out of nine *Leuconostoc* phages were selected for further analysis.

2.2.5. Long-term survival

Aliquots of each phage were maintained at 25 °C, 8 °C (MRS broth), −20 °C and −80 °C (MRS broth with 15% v/v glycerol). Phage enumerations (pfu/ml) were carried out periodically for up to ten months of storage by the double-layer plate titration method (Svensson and Christiansson, 1991).

2.2.6. Thermal treatments

Thermal resistance of phage suspensions (10^6–10^7 pfu/ml) was tested as reported earlier (Quiberoni et al., 2003) at 63, 72 and 90 °C in three suspension media: Tris Magnesium Gelatin (TMG) buffer (10 mM Tris–HCl, 10 mM $MgSO_4$, 0.1% w/v gelatin), reconstituted commercial skim milk (10% w/v) and MRS broth. In order to narrow the range of temperature resistance, treatments at 80 °C were applied afterward but only to those phages that demonstrated high resistance at 72 °C. Assays were performed in triplicate. Time (min) to achieve 99% of inactivation (T_{99}) was calculated from survival curves.

2.2.7. Chemical treatments

Biocides and concentrations used were: ethanol (Cicarelli, Buenos Aires, Argentina) (50, 75, 100% v/v); sodium hypochlorite (Clorox Argentina S. A., Buenos Aires, Argentina) (200–1600 ppm residual-free chlorine); peracetic acid (Proxitane 1512, Química General, Santa Fe, Argentina) (0.15% v/v), and commercial sanitizers A (quaternary ammonium chloride) (0.25 and 0.50% v/v), C (alkaline chloride foam) (2.5% v/v), E (ethoxylated nonylphenol and phosphoric acid) (0.8% v/v) and F [N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine] (2% v/v). All sanitizers were diluted in sterile distilled water, with the exception of sodium hypochlorite, which was diluted in potassium phosphate buffer (0.1 M, pH 7). Each phage (10^6–10^7 pfu/ml approximately) was mixed with the biocide solution and incubated at 25 °C after they were distributed into 1.5-ml microcentrifuge tubes. To test the effect of pH on phage viability, viral particles were suspended in sterilized distilled water adjusted to pH values of each tested biocide (pH control). All assays were performed in triplicate. Surviving phages and T_{99} values were determined according to Quiberoni et al. (2003).

2.2.8. High pressure homogenization

A continuous high-pressure homogenizer PANDA (Niro Soavi, Parma, Italy) supplied with a homogenizing PS type valve with a flow rate of 10 l/h was used in all the homogenization treatments (Capra et al., 2009b). *Leuconostoc* phages were inoculated (10^6 pfu/ml) in 500 ml of sterilized reconstituted skim milk or MRS broth, and subjected to high-pressure homogenization (HPH) for up to 8 successive passes at 100 MPa. The temperature increase during the treatments (about 2.5 °C/10 MPa) was avoided by using a water-cooling system (Niro Soavi, Parma, Italy). Therefore, temperature of phage suspensions after HPH treatments did not exceed 25 °C. Assays were carried out in triplicate. Phage enumerations were performed by the double-layer plate titration method before (control) and after 1, 3, 5 and 8 passes through the homogenizer.

3. Results and discussion

3.1. Identification and diversity of phage-sensitive Leuconostoc strains

Mesophilic, mixed starters used in the manufacture of blue-veined cheeses typically contain Lactococcus *lactis* subsp. *lactis* and subsp. *cremoris*, and limited amounts of citrate-positive strains of *Leuconostoc* species, which produce CO_2 and open up the structure that facilitate development of *P. roqueforti*. In our study, presumptive *Leuconostoc* strains were recovered from all the analysed starters. The eight isolates (R707, C19A, C19B, CyC, D4, D4b, D6a and L79-1) were identified as *L. mesenteroides* subsp. *mesenteroides* based on the nucleotide sequence of a 1500 bp fragment within their 16S rRNA gene (data not shown). RAPD analysis with primers M13 and 1254 allowed us to evaluate the genetic diversity among isolates (Fig. 1). The RAPD-PCR reproducibility for the two primers assayed was of 90%, determined by running duplicate samples of *Leuconostoc* isolate R707. Therefore, similarity coefficients lower than 90% indicated differences between strains. According to this, RAPD analysis revealed three clusters and two further distinct strains (similarity <90%). High similarity between *L. mesenteroides* strains D4b and D6a (>98%), C19A and C19B, as well as D4 and L79-1 (>90%) was shown, while R707 and CyC appear as dissimilar isolates. The low diversity encountered amongst the isolates is probably consequence of the use of a narrow set of *Leuconostoc* strains, which would allow reproducible performance and uniform quality in the final fermented products. In fact, a limited number of commercial *L. mesenteroides* subsp. *mesenteroides* strains possesses the appropriate industrial phenotypes for blue-veined cheeses manufacture, for example, growing during the early cheesemaking stages and producing the optimal concentration of CO_2 (Hemme, 2012). In our work, strains D4b and D6a were isolated from different lots of the same starter, C19A and C19B from the same lot and starter, while D4 and L79-1 belonged to different starters, as well as strains R707 and CyC (Table 1).

3.2. Bacteriophages

3.2.1. Phage isolation and host range

In the present study, a total of nine *L. mesenteroides* subsp. *mesenteroides* phage isolates were obtained from whey samples (both from cheese vats and from cheese moulds) derived from an Argentinean blue-veined cheese plant. A great frequency of phage isolation was encountered during our monitoring though remarkable variations in phage numbers were observed, ranging from 10 to 2.6×10^5 pfu/ml (Table 1). However, very high titers (~10^9–10^{10} pfu/ml) were achieved when these phages were amplified in the laboratory, indicating that they can be highly virulent under the appropriate environmental conditions. A clear dissemination of phages specific for *Leuconostoc* flavour-producing strains was previously documented by Atamer et al. (2011) when analyzing whey samples from ten German dairies. These data confirmed that the factory environment is an important reservoir of phages and it is mainly responsible for phage permanence in dairy plants. Phages attacking acid-producing bacteria are usually the predominant ones (Atamer et al., 2009; Quiberoni et al., 2010; Villion and Moineau, 2013). In this sense, phages attacking *Leuconostoc* starter strains might be unperceived in dairies due both to the low titres in the environments and the secondary role performed by their hosts, though the failure of the *Leuconostoc* culture will impact on some essential flavour and texture aspects of the final products.

Leuconostoc phages showed wide host ranges, having LDG, CHB, Ln7 and Ln8 the broadest ones, since they infect all the eight tested commercial strains, followed by CyC1, CyC2, Ln6 and Ln9 (seven strains), and CHA (six strains) (Table 1). Data concerning the virulence of *Leuconostoc* phages are very scarce and only a few studies are documented. In this sense, Atamer et al. (2011) and Kot et al. (2013) found remarkably conserved host patterns when investigating the virulence of phages lytic for *L. pseudomesenteroides* and *L. mesenteroides* strains. The species-specificity of these phages was outstanding, since *L. pseudomesenteroides* phages did not infect *L. mesenteroides* strains and vice versa. In view of the high and widespread virulence demonstrated by the *Leuconostoc* phages, a meticulous selection of strains must be performed when designing the starters. In addition, this feature highlights and supports the hypothesis of a permanent presence of *Leuconostoc* phages on dairies, due to their ability to proliferate on a broad range of different sensitive hosts.

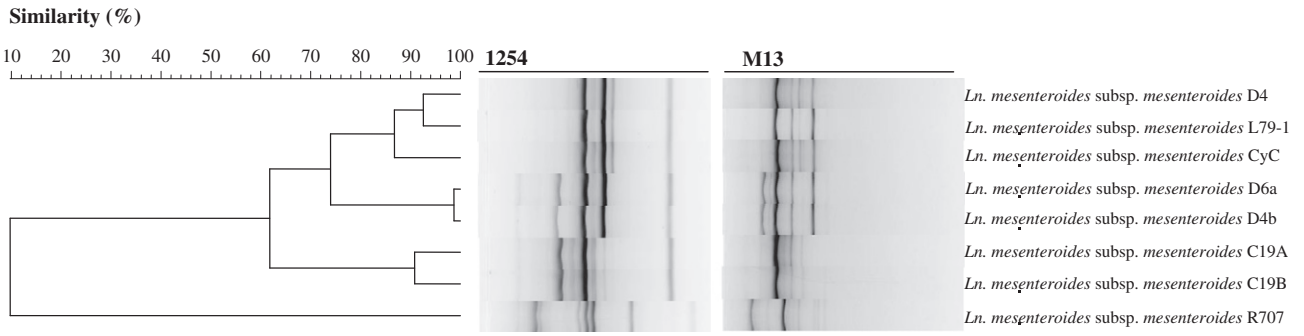

Fig. 1. RAPD-PCR profiles obtained with primers 1254 and M13 of commercial *Leuconostoc mesenteroides* isolates, and corresponding dendrogram obtained from the unweighted pair group average linkage of Pearson correlation coefficients (expressed as a percentage value).

3.2.2. Phage morphology

All phages showed isometric capsids of 53 nm in diameter between opposite apices. Tails were noncontractile, more or less rigid, measured 143 × 8 nm, and showed cross-striations with a periodicity of 4 nm and a base plate of 17 × 10 nm provided with 15 nm-long fibres, as shown in Fig. 2 for a representative phage (i.e., phage CyC1). These features placed the phages into the *Siphoviridae* family in the order *Caudovirales* (Ackermann and Prangishvili, 2012). Unlike that reported by Ali et al. (2013), *L. mesenteroides* phages of our work did not reveal any baseplate appendages, demonstrating a high degree of morphological diversity.

3.2.3. Phage restriction patterns

Among the nine tested phages, six distinct restriction patterns with EcoRV, HindIII, ClaI and XhoI enzymes were found (Fig. 3). Specifically, CyC1, CyC2, Ln6 and Ln9 could probably be the same phage (>99% similarity), while CHA, CHB, LDG, Ln7 and Ln8 represented different phages, even if similar patterns (>84% similarity) were exhibited by the two last ones. In this sense, phages exhibiting the same restriction patterns exhibited also identical host ranges (phages CyC1, CyC2, Ln6 and Ln9). Data concerning the genetic diversity of phages specific for *Leuconostoc* in dairies is very scarce. Based on preliminary Southern blot analysis, Atamer et al. (2011) reported that all 66 *L. pseudomesenteroides* phages studied were closely related, observing the same for the 11 *L. mesenteroides* phages. Nevertheless, only limited DNA similarity was observed between both groups of *Leuconostoc* phages. In our study, some phages with identical restriction profiles were isolated from samples collected in different periods (e.g., CyC1, CyC2 isolated in 2009, Ln6 and Ln9 in 2010 and 2012, respectively). These results indicated that the resident phage population detected in 2009 would persist in the dairy environment for at least three years. During a monitoring, Guglielmotti et al. (2009) detected an analogous persistence of *S. thermophilus* phages in the dairy environment for at least seven years. In the present study, some phages with different restriction profiles were isolated from the same sample (e.g., CHA and CHB), indicating a remarkable diversity in the phage population. Phage genome sizes ranged from 28 to 35 kb (data not shown), as previously reported for other *L. mesenteroides* bacteriophages (Boizet et al., 1992; Kleppen et al., 2012; Lu et al., 2010).

3.3. Long-term survival

No significant reduction in phage counts was observed after ten months of storage at −20 °C and −80 °C, while a slight decrease in the number of infectious phages was noticed at 8 °C. In contrast, a significant loss of viability (higher than 6 log orders) was suffered by phages after ten months of storage at 25 °C (data not shown). Similar results were previously reported for *Lb. casei* and *paracasei* phages (Capra et al., 2006; Mercanti et al., 2012).

3.4. Thermal treatments

Resistance to heat treatments was phage-dependent, and no clear effect of suspension media was observed. A strong resistance at 63 °C was obtained for most phages studied (LDG, CHA, CHB, Ln7 and Ln8) (T_{99} > 45 min) in the three suspension media used, except for phage CyC1 (Table 2), even though there were still viable phage particles after 45 min of treatment (Fig. 4A). At 72 °C, phages CHA, Ln7 and Ln8 exhibited the highest heat resistance, as this temperature was not sufficient to achieve complete inactivation within 45 min of incubation (Fig. 4B). On the contrary, 5 min was enough for complete inactivation of phage CyC1 in the three suspension media (data not shown). These results demonstrate great resistance of *Leuconostoc* phages to heat treatments, especially to temperatures usually applied in the dairy industry for milk pasteurization. Atamer et al. (2011) evaluated the thermal resistance of 77 *Leuconostoc* phages and demonstrated that approximately 98% of the phages studied survived after 1 min at 75 °C.

Table 1
Origin, isolation date, titers and host range of *Leuconostoc mesenteroides* phages isolated from a blue-veined cheese plant.

Phage	Source	Isolation date (month/year)	Phage titer (pfu/ml)	Host range[a, b]							
				R707 (I)[c]	C19A (II)[c]	C19B (II)[c]	CyC (III)[c]	D4 (IV)[c]	D4b (IV)[c]	D6a (IV)[c]	L79-1 (V)[c]
LDG	Mould whey	5/2009	1.0×10^1	+[d]	+	+	+	+	+	+	+
CHA	Mould whey	5/2009	1.1×10^5	+	+[d]	+	+	+	−	−	+
CHB	Vat whey	5/2009	2.6×10^5	+	+	+[d]	+	+	+	+	+
CyC1	Mould whey	12/2009	1.2×10^1	−	+	+	+[d]	+	+	+	+
CyC2	Mould whey	12/2009	2.3×10^1	−	+	+	+[d]	+	+	+	+
Ln6	Vat whey	4/2010	4.0×10^1	−	+	+	+	+[d]	+	+	+
Ln7	Vat whey	5/2010	3.0×10^1	+	+	+	+	+	+[d]	+	+
Ln8	Vat whey	9/2011	1.5×10^1	+	+	+	+	+	+	+[d]	+
Ln9	Mould whey	2/2012	1.7×10^1	−	+	+	+	+	+	+	+[d]

[a] +, strain lysis; −, no lysis.
[b] Strains identified as *L. mesenteroides subsp. mesenteroides* by sequencing of 1500 bp fragment of the 16S rRNA gene.
[c] Different Roman numerals correspond to different starters.
[d] Sensitive strain used to isolate the phage.

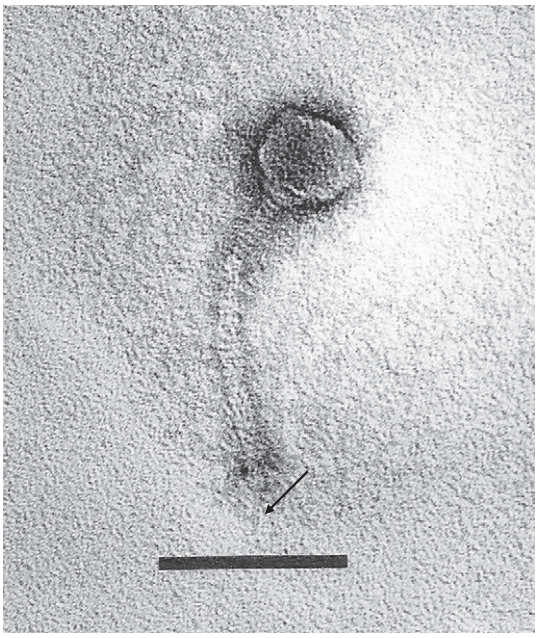

Fig. 2. Electron micrograph of *Leuconostoc mesenteroides* phage CyC1. Uranyl acetate (2% w/v) was used as staining solution. The arrow indicates the 15 nm-long fibres. Bar represents 100 nm.

High diversity on thermo-resistance was found for other phages from LAB (Binetti and Reinheimer, 2000; Briggiler Marcó et al., 2009; Quiberoni et al., 1999, 2003; Suárez and Reinheimer, 2002), showing that thermal resistance depends more on the phage particles than on the infected species (Guglielmotti et al., 2012).

In our study, *Leuconostoc* phages CHA, Ln7 and Ln8, resistant to treatments at 72 °C for 45 min, were subsequently subjected at 80 °C using skim milk as the suspension medium (Fig. 4C and Table 2). Complete inactivation of viral particles was obtained for phages Ln7 and Ln8 after 15 min of treatment (T_{99} = 4.6 and 2.3 min, respectively). Phage CHA showed a marked resistance since total inactivation was only obtained after 30 min of incubation at 80 °C (Fig. 4C). *Leuconostoc* phage P793 (Atamer et al., 2011) was still active after heating at 80 °C for 30 min in skim milk. These results clearly reveal that high-temperature short-time pasteurization (72 °C, 15 s) would not ensure complete inactivation of dairy *Leuconostoc* phages. In our experiments, incubation at 90 °C for 2 min was sufficient to achieve counts <10 pfu/ml in all suspension media for all *Leuconostoc* phages tested (data not shown). There are no other studies on thermal resistance of *Leuconostoc* phages at 90 °C. Atamer et al. (2011) observed that three *Leuconostoc* phages were able to survive at high numbers at 85 °C for 1 min. Although extreme heat resistance was not frequently found in LAB phages, the number of highly-resistant bacteriophages has apparently increased during recent years (Atamer et al., 2009; Capra et al., 2013; Quiberoni et al., 2003).

Table 2
Thermal resistance of *Leuconostoc mesenteroides* phages in different media.

Phage	T_{99} (min)[a, b]									
	MRS broth			Skim milk				TMG buffer		
	63 °C	72 °C	90 °C	63 °C	72 °C	80 °C	90 °C	63 °C	72 °C	90 °C
LDG	>45	4.0	<2	>45	22.1	nd	<2	>45	4.0	<2
CHA	>45	>45	<2	>45	>45	5.0	<2	>45	>45	<2
CHB	>45	8.0	<2	>45	20	nd	<2	>45	18	<2
CyC1	32	1.2	<2	29	2.9	nd	<2	18	2.9	<2
Ln7	>45	>45	<2	>45	>45	4.6	<2	>45	>45	<2
Ln8	>45	43	<2	>45	>45	2.3	<2	>45	26	<2

nd: not determined.
[a] Time to achieve 99% inactivation of phage particles.
[b] Treatments at 80 °C were carried out for phages CHA, Ln7 and Ln8 in skim milk.

3.5. Chemical treatments

Among the concentrations assayed, 75% ethanol produced the highest phage inactivation, with the exception of phage CyC1, for which 100% ethanol was more lethal than the former concentration. In general, 75% ethanol produced 99% inactivation in less than 5 min (Table 3), while complete inactivation was achieved within 30 min of incubation (data not shown). According to published data, 75% and 100% ethanol were the most efficient concentrations to inactivate *Lactococcus* phages (Buzrul et al., 2007; Suárez and Reinheimer, 2002).

The effectiveness of sodium hypochlorite was phage-dependent but in general, moderate to high resistance of *Leuconostoc* phages was observed. The most sensitive were phages CHB and CyC1, as 600 ppm was necessary to achieve 99% inactivation of total phage particles (T_{99} < 2 and 3.0, respectively) (Table 3), and 30 min were required for complete inactivation at this concentration (data not shown). Concentrations of 1400 ppm (phage LDG) and 1600 ppm (phages Ln7 and Ln8) of residual-free chlorine were necessary to obtain a total destruction of phage particles within 45 min (data not shown). Fig. 5 illustrates the remarkably high resistance of phage Ln7 against this biocide. However, these chlorine concentrations are much higher than those allowed in the food industry and therefore they could be used only at laboratory scale (Briggiler Marcó et al., 2009). Dairy bacteriophages are generally inactivated by concentrations of 300 ppm or less of sodium hypochlorite (Binetti and Reinheimer, 2000; Ebrecht et al., 2010; Quiberoni et al., 1999; Suárez and Reinheimer, 2002). Studies carried out by Quiberoni et al. (2003) revealed that high concentrations (1200 ppm) of sodium hypochlorite were necessary to inactivate phage *Lb. delbrueckii* Ib_3, although its resistance

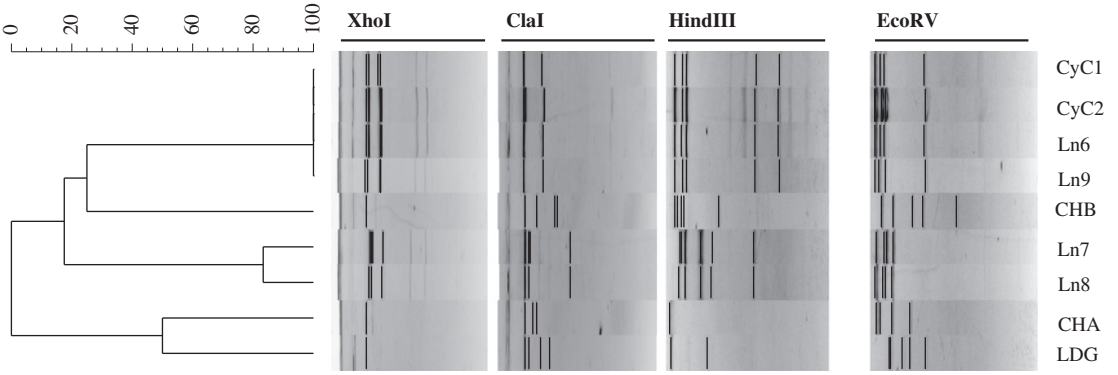

Fig. 3. Restriction enzyme profiles of the *Leuconostoc mesenteroides* phages using XhoI, ClaI, HindIII and EcoRV. Calculation of restriction profile similarities was based on the Jaccard (band based) correlation coefficient and *Leuconostoc* phages were grouped by using the unweighted pair group method using arithmetic averages (UPGMA).

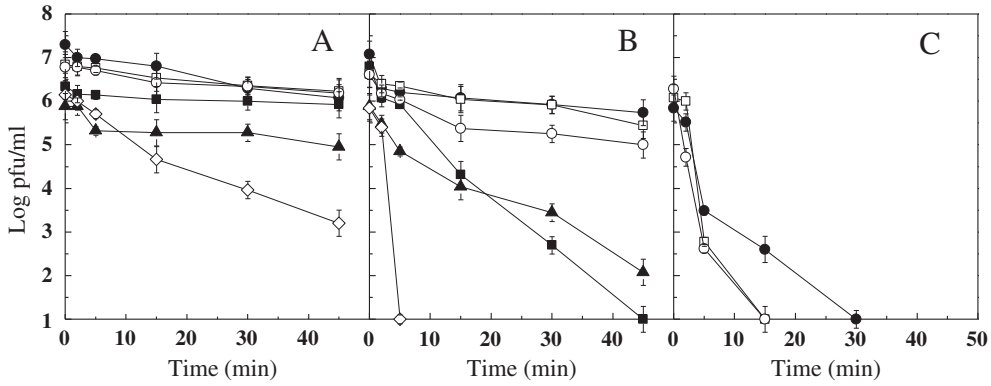

Fig. 4. Thermal resistance of *Leuconostoc mesenteroides* phages LDG (■), CHA (●), CHB (▲), CyC1 (◇), Ln7 (□) and Ln8 (○) at 63 °C (A), 72 °C (B) and 80 °C (C) in sterilized reconstituted commercial skim milk. Values are the mean of three determinations.

was not as remarkable as that shown by *Leuconostoc* phages LDG, Ln7 and Ln8 of our study.

Peracetic acid treatments were highly lethal as it led to the destruction of *Leuconostoc* phage particles before 2 min at 25 °C (Table 3). The high effectiveness of this biocide could be in part due to the extreme acidic pH of its solution (pH 2), as complete inactivation of phage particles incubated in sterilized distilled water adjusted to the pH of the biocide was observed (data not shown). Several authors reported the high effectiveness of peracetic acid against phages infecting LAB (Binetti and Reinheimer, 2000; Briggiler Marcó et al., 2009; Capra et al., 2004, 2006; Ebrecht et al., 2010; Mercanti et al., 2012; Quiberoni et al., 1999, 2003; Suárez and Reinheimer, 2002).

Besides peracetic acid, several chemical agents are used in the dairy industry for the cleaning and sanitation of dairy plants. Nevertheless, their effect on phage viability has been assessed only for phages lytic of *Lb. delbrueckii* (Ebrecht et al., 2010) and *Lb. paracasei* (Mercanti et al., 2012). Based on our results, biocides A, C, E and F were very effective at equal or minor recommended concentrations against all *L. mesenteroides* phages tested. For biocide A (quaternary ammonium chloride), concentrations of 0.25% (v/v) were insufficient to completely inactivate viral particles. On the contrary, 0.50% (v/v) biocide A rapidly inactivated a 99% viral population before 2 min of treatment (Table 3). In contrast to our work, 0.50% biocide A was not capable of inactivating *Lb. delbrueckii* phages during 30 min of treatment (Ebrecht et al., 2010). However, this concentration produced a complete destruction of *Lb. paracasei* phages (Mercanti et al., 2012). Treatments with biocides C (alkaline chloride foam) and E (ethoxylated nonylphenol and phosphoric acid) were highly lethal ($T_{99} < 2$, Table 3) leading to the total destruction of phage particles within 5 min of incubation (data not shown). Comparable effectiveness was found for *Lb. paracasei* and *Lb. delbrueckii* (Ebrecht et al., 2010; Mercanti et al., 2012). Biocide F (2% v/v) also showed high efficiency to inactivate *Leuconostoc* phage particles since 99% inactivation was achieved in less than 7 min of incubation (Table 3).

Usually, the effectiveness of these commercial biocides is due to their active components. However, the extreme pH value of biocide solutions could be an inactivating factor itself. With this regard, pH values measured for biocide A (0.50% v/v), C (2.5% v/v), E (0.8% v/v) and F (2% v/v) solutions were 9.4, 12.7, 1.7 and 10.25, respectively. *L. mesenteroides* phages were inactivated before 2 min of exposure at pH 12.7 and 1.7 in pH controls, but they were not destroyed completely at pH 9.4 and 10.25 (data not shown) during 45 min of incubation. According to this, the extreme pH of biocide C and E solutions could be partially responsible for the biocide activity, but not for that of biocides A and F. Ebrecht et al. (2010) and Mercanti et al. (2012) reported notable efficiency of biocides C and E to inactivate high titers of *Lb. delbrueckii* and *Lb. paracasei* phages, respectively.

Table 3
Resistance of *Leuconostoc mesenteroides* phages to different biocides.

Biocide	Concentration[a]	T_{99} (min)[b]					
		LDG	CHA	CHB	CyC1	Ln7	Ln8
Ethanol	50	>45	>45	>45	5.7	>45	>45
	75	4.3	12	4.7	2.0	<2	<2
	100	7.7	22	41	<2	>45	33
Sodium hypochlorite	200	>45	>45	33	>45	>45	>45
	300	>45	>45	5.0	28	>45	>45
	400	>45	22	3.9	18	>45	>45
	600	10	4.4	<2	3.0	>45	>45
	800	7.0	6.4	<2	<2	>45	>45
	1000	6.1	2.9	nd	nd	5.6	7.0
	1400	3.9	nd	nd	nd	2.2	5.3
	1600	nd	nd	nd	nd	<2	2.1
Peracetic acid	0.15	<2	<2	<2	<2	<2	<2
Biocide A[c]	0.25	5.1	8.3	7.0	5.3	2.3	2.1
	0.50	<2	<2	<2	<2	<2	<2
Biocide C[c]	2.50	<2	<2	<2	<2	<2	<2
Biocide E[c]	0.80	<2	<2	<2	<2	<2	<2
Biocide F[c]	2.00	6.7	5.0	5.0	2.2	2.4	2.5

nd: not determined.
[a] % (v/v) for ethanol, peracetic acid and biocides A, C, E and F; ppm residual-free chlorine for sodium hypochlorite.
[b] Time to achieve 99% inactivation of phage particles.
[c] Composition for biocides: A (quaternary ammonium chloride), C (alkaline chloride foam), E (ethoxylated nonylphenol and phosphoric acid) and F [N-(3-aminopropyl)-N-dodecylpropane-1,3-diamine].

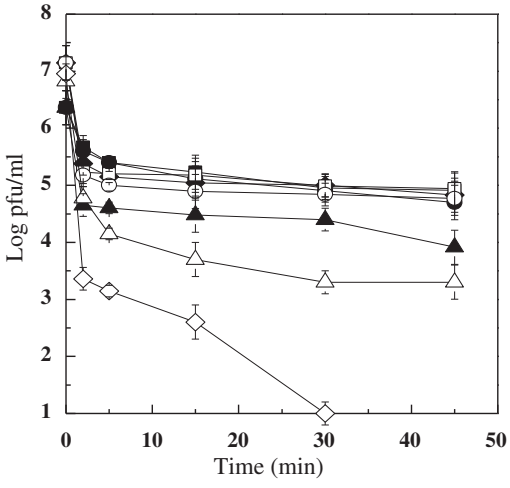

Fig. 5. Viability of *Leuconostoc mesenteroides* phage Ln7 after treatments with 200 ppm (■), 300 ppm (●), 400 ppm (○), 600 ppm (♦), 800 ppm (□), 1000 ppm (▲), 1400 ppm (△) and 1600 ppm (◇) residual-free chlorine (sodium hypochlorite). Values are the mean of three determinations.

3.6. High pressure homogenization

Among the *L. mesenteroides* phages tested, LDG was the most resistant, showing viability decreases of only 1.5 and 2.1 log orders after 8 passes at 100 MPa, in MRS and skim milk as suspension media, respectively (Fig. 6). This result is pointing LDG as the most resistant phage of LAB tested until today (Capra et al., 2009a; Mercanti et al., 2012). The other *L. mesenteroides* phages tested were less resistant to 8 passes at 100 MPa than phage LDG, revealed by falls between 2.9 (phage CHA) and 4.2 (phage Ln7) log orders in MRS broth (Fig. 6A), and between 3.1 (phage CyC1) and 3.6 (phage CHA) log orders in reconstituted skim milk (Fig. 6B). A marked sensitivity was reported for phages of other LABs, i.e., *S. thermophilus* phage 13.2 as well as *L. lactis* phages QP4 and QF12 were completely inactivated (more than 6 log reductions) after only 5 passes at 100 MPa (Capra et al., 2009b). No effect of suspension media on *Leuconostoc* phage survival was observed. Specifically, phage inactivation ranged from 1.5 to 4.2 log orders in MRS broth (phages LDG and Ln7, respectively), and from 2.1 to 3.6 log orders in skim milk (phages LDG and CHA, respectively). Nevertheless, protective effects of whey permeate and milks with different fat contents were suggested by some authors in comparison with buffer solutions (Chen et al., 2004; Diels et al., 2005; Moroni et al, 2002).

Even if a few studies have been conducted on bacteriophages, the current bibliography demonstrates that they resist better against high pressure treatments than bacteria (Capra et al., 2012). Consequently, this technology should not be used as the sole barrier against phages present in the environment of dairies. Some authors suggest combining it with other conventional preservation processes, either thermal or non-thermal, in order to enhance bacteriophage inactivation (Lado and Yousef, 2002; Ross et al., 2003) thus reducing HPH treatment intensities (Capra et al., 2012). A mild heat treatment in combination with HPH applied on milk used for dairy manufactures could allow a more efficient viral inactivation and better retention of physicochemical properties of the product.

4. Conclusion

In this work, phages infecting *L. mesenteroides* commercial strains isolated from blue-veined cheese manufactures of an Argentinean plant were characterized. Treatments at 63 and 72 °C did not guarantee phage inactivation, while 80 °C for 30 min and 90 °C for 5 min should assure the inactivation of *L. mesenteroides* phages. Unfortunately, the latter conditions are too strong to be applied to raw milk used for cheesemaking because it would lead to protein denaturation. HPH treatments were not useful to inactivate *Leuconostoc* phages, which showed the highest resistance in comparison to other LAB phages assayed until now. High resistance to sodium hypochlorite was also observed, since concentrations needed to completely inactivate them (1400–1600 ppm) greatly exceed those allowed in the food industry. Peracetic acid continues to be a very effective option for the sanitation of dairy plants. Other commercial biocides tested resulted effective as well, partially due to their extreme pH values. Thermal and chemical toughness of *Leuconostoc* phages and their important persistence in the environmental (as shown by high viability during long-term storage), might hinder their effective control in dairies. Combination of assorted physical and chemical treatments should be applied in order to optimize phage inactivation.

Acknowledgments

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Project PICT No. 0138; Argentina), the Universidad Nacional del Litoral (Project CAI+D No. 57-275; Argentina) and the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Project PIP No. 112-200801-01206; Argentina).

References

Ackermann, H.-W., 2009. Basic phage electron microscopy. In: Kropinski, A.M., Clokie, M. (Eds.), Bacteriophages: Methods and Protocols. Methods in Molecular Biology, 501. Humana Press, Totowa, NJ, pp. 113–126.

Ackermann, H.-W., Prangishvili, D., 2012. Prokaryote viruses studied by electron microscopy. Arch. Virol. 157, 1843–1849.

Akopyanz, N., Bukanov, N.O., Westblom, T.U., Kresovich, S., Berg, D.E., 1992. DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. Nucleic Acids Res. 20, 5137–5142.

Ali, Y., Kot, W., Atamer, Z., Hinrichs, J., Vogensen, F.K., Heller, K.J., Neve, H., 2013. Classification of lytic bacteriophages attacking dairy *Leuconostoc* starter strains. Appl. Environ. Microbiol. 79, 3628–3636.

Atamer, Z., Dietrich, J., Müller-Merbach, M., Neve, H., Heller, K.J., Hinrichs, J., 2009. Screening for and characterization of *Lactococcus lactis* bacteriophages with high thermal resistance. Int. Dairy J. 19, 228–235.

Atamer, Z., Ali, Y., Neve, H., Heller, K.J., Hinrichs, J., 2011. Thermal resistance of bacteriophages attacking flavour-producing dairy *Leuconostoc* starter cultures. Int. Dairy J. 21, 327–334.

Binetti, A.G., Reinheimer, J.A., 2000. Thermal and chemical inactivation of indigenous *Streptococcus thermophilus* bacteriophages isolated from Argentinian dairy plants. J. Food Prot. 63, 509–515.

Binetti, A.G., Capra, M.L., Alvarez, M.A., Reinheimer, J.A., 2008. PCR method for detection and identification of *Lactobacillus casei/paracasei* bacteriophages in dairy products. Int. J. Food Microbiol. 124, 147–153.

Boizet, B., Mata, M., Mignot, O., Ritzenthaler, P., Sozzi, T., 1992. Taxonomic characterization of *Leuconostoc mesenteroides* and *Leuconostoc oenos* bacteriophages. FEMS Microbiol. Lett. 90, 211–216.

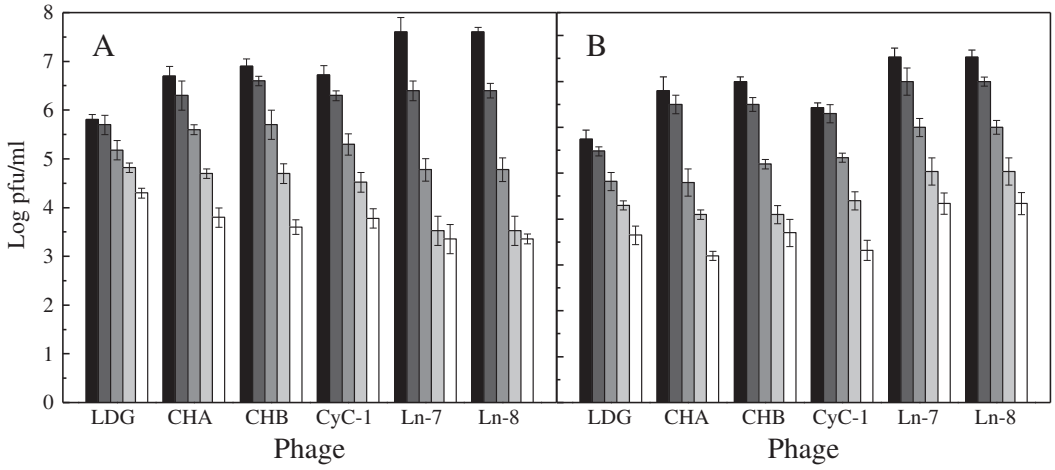

Fig. 6. Effect of multi-pass high pressure homogenization treatments at 100 MPa on the viability of *Leuconostoc mesenteroides* phages suspended in MRS (A) and reconstituted skim milk (B). Values correspond to viable phage particles (as log pfu/ml) of untreated samples (■), and after one (■), three (■), five (■) and eight (□) passes at 100 MPa. Values are the mean of three determinations.

Briggiler Marcó, M., De Antoni, G.L., Reinheimer, J.A., Quiberoni, A., 2009. Thermal, chemical, and photocatalytic inactivation of *Lactobacillus plantarum* bacteriophages. J. Food Prot. 72, 1012–1019.
Briggiler Marcó, M., Moineau, S., Quiberoni, A., 2012. Bacteriophages and dairy fermentations. Review. Bacteriophage 2 (3), 1–10.
Buzrul, S., Öztürk, P., Alpas, H., Akcelik, M., 2007. Thermal and chemical inactivation of lactococcal bacteriophages. LWT Food Sci. Technol. 40, 1671–1677.
Capra, M.L., Quiberoni, A., Reinheimer, J.A., 2004. Thermal and chemical resistance of *Lactobacillus casei* and *Lactobacillus paracasei* bacteriophages. Lett. Appl. Microbiol. 38, 499–504.
Capra, M.L., Quiberoni, A.L., Ackermann, H.W., Moineau, S., Reinheimer, J.A., 2006. Characterization of a new virulent phage (MLC–A) of *Lactobacillus paracasei*. J. Dairy Sci. 89, 2414–2423.
Capra, M.L., Binetti, A.G., Mercanti, D.J., Quiberoni, A., Reinheimer, J.A., 2009a. Diversity among *Lactobacillus paracasei* phages isolated from a probiotic dairy product plant. J. Appl. Microbiol. 107, 1350–1357.
Capra, M.L., Patrignani, F., Quiberoni, A., Reinheimer, J.A., Lanciotti, R., Guerzoni, M.E., 2009b. Effect of high pressure homogenization on lactic acid bacteria phages and probiotic bacteria phages. Int. Dairy J. 19, 336–341.
Capra, M.L., Patrignani, F., Guerzoni, M.E., Lanciotti, R., 2012. Non thermal technologies: pulsed electric field, high hydrostatic pressure and high pressure homogenization. Application on virus inactivation. In: Quiberoni, A., Reinheimer, J.A. (Eds.), Bacteriophages in Dairy Processing. Nova Science Publishers, Inc., New York, pp. 215–238.
Capra, M.L., Neve, H., Sorati, P.C., Atamer, Z., Hinrichs, J., Heller, K.J., Quiberoni, A., 2013. Extreme thermal resistance of phages isolated from dairy samples: updating traditional phage detection methodologies. Int. Dairy J. 30, 59–63.
Chen, H., Joerger, R.D., Kingsley, D.H., Hoover, D.G., 2004. Pressure inactivation kinetics of phage λ cI 857. J. Food Prot. 67, 505–511.
Davey, G.P., Ward, L.J.H., Brown, J.C.S., 1995. Characterization of four *Leuconostoc* bacteriophages isolated from dairy fermentations. FEMS Microbiol. Lett. 128, 21–26.
Diels, A.M.J., Callewaert, L., Wuytack, E.Y., Masschalck, B., Michiels, C.W., 2005. Inactivation of *Escherichia coli* by high-pressure homogenization is influenced by fluid viscosity but not by water activity and product composition. Int. J. Food Microbiol. 101, 281–291.
Ebrecht, A.C., Guglielmotti, D.M., Tremmel, G., Reinheimer, J.A., Suárez, V.B., 2010. Temperate and virulent *Lactobacillus delbrueckii* bacteriophages: comparison of their thermal and chemical resistance. Food Microbiol. 27, 515–520.
Edwards, U., Rogall, T., Blockerl, H., Emde, M., Bottger, E., 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. 17, 7843–7853.
Fonberg-Broczek, M., Windyga, B., Szczawinski, J., Szezawinska, M., Pietrzak, D., Prestamo, G., 2005. High pressure processing for food safety. Acta Biochim. Pol. 52, 721–724.
Guglielmotti, D.M., Reinheimer, J.A., Binetti, A.G., Giraffa, G., Carminati, D., Quiberoni, A., 2006. Characterization of spontaneous phage-resistance derivatives of *Lactobacillus delbrueckii* commercial strains. Int. J. Food Microbiol. 111, 126–133.
Guglielmotti, D.M., Deveau, H., Binetti, A.G., Reinheimer, J.A., Moineau, S., Quiberoni, A., 2009. Genome analysis of two virulent *Streptococcus thermophilus* phages isolated in Argentina. Int. J. Food Microbiol. 136, 101–109.
Guglielmotti, D.M., Mercanti, D.J., Reinheimer, J.A., Quiberoni, A. del L., 2012. Efficiency of physical and chemical treatments on the inactivation of dairy bacteriophages. Front. Microbiol. 2, 1–11.
Hemme, D., 2012. *Leuconostoc* and its use in dairy technology, In: Hin Hui, Yiu (Ed.), Handbook of Animal-Based Fermented Food and Beverage Technology, Second edition. CRC Press, Boca Raton, Florida, pp. 73–108.
Huey, B., Hall, J., 1989. Hypervariable DNA fingerprinting in *Escherichia coli*: minisatellite probe from bacteriophage M13. J. Bacteriol. 171, 2528–2532.
Johansen, E., Kibenich, A., 1992. Characterization of *Leuconostoc* isolates from commercial mixed strain mesophilic starter cultures. J. Dairy Sci. 75, 1186–1191.
Kleppen, H.P., Nes, I.F., Holo, H., 2012. Characterization of a *Leuconostoc* bacteriophage infecting flavor producers of cheese starter cultures. Appl. Environ. Microbiol. 78, 6769–6772.
Kot, W., Hammer, K., Neve, H., Vogensen, F.K., 2013. Identification of the receptor-binding protein in lytic *Leuconostoc pseudomesenteroides* bacteriophages. Appl. Environ. Microbiol. 79, 3311–3314.
Lado, B.H., Yousef, A.E., 2002. Alternative food-preservation technologies: efficacy and mechanisms. Microbes Infect. 4, 433–440.
Lu, Z., Altermann, E., Breidt, F., Kozyavkin, S., 2010. Sequence analysis of *Leuconostoc mesenteroides* bacteriophage 1-A4 isolated from an industrial vegetable fermentation. Appl. Environ. Microbiol. 76, 1955–1966.
Mercanti, D.J., Guglielmotti, D.M., Patrignani, F., Reinheimer, J.A., Quiberoni, A. del L., 2012. Resistance of two temperate *Lactobacillus paracasei* bacteriophages to high pressure homogenization, thermal treatments and chemical biocides of industrial application. Food Microbiol. 29, 99–104.
Moineau, S., 1999. Application of phage resistance in lactic acid bacteria. Antonie Van Leeuwenhoek 76, 377–382.
Moroni, O., Jean, J., Autret, J., Fliss, I., 2002. Inactivation of lactococcal bacteriophages in liquid media using dynamic high pressure. Int. Dairy J. 12, 907–913.
Neve, H., 1996. Bacteriophage. In: Cogan, T.M., Accolas, J.P. (Eds.), Dairy Starter Cultures. VCH Publishers Inc., New York, pp. 157–189.
Neve, H., Lilischkis, R., Teuber, M., 1988. Characterization of a virulent bacteriophage of *Leuconostoc mesenteroides* subsp. *cremoris*. Kiel. Milchwirtsch. Forschungsber. 40, 205–212.
Neviani, E.N., Carminatti, D., Giraffa, G., 1992. Selection of some bacteriophage- and lysozyme-resistant variants of *Lactobacillus helveticus* CNRZ 892. J. Dairy Sci. 75, 905–913.
Patrignani, F., Tabanelli, G., Siroli, L., Gardini, F., Lanciotti, R., 2013a. Combined effects of high pressure homogenization treatment and citral on microbiological quality of apricot juice. Int. J. Food Microbiol. 160, 273–281.
Patrignani, F., Vannini, L., Sado Kamdem, S.L., Hernando, I., Marco-Molés, R., Guerzoni, M.E., Lanciotti, R., 2013b. High pressure homogenization *vs* heat treatment: safety and functional properties of liquid whole egg. Food Microbiol. 36, 63–69.
Quiberoni, A., Suárez, V.B., Reinheimer, J.A., 1999. Inactivation of *Lactobacillus helveticus* bacteriophages by thermal and chemical treatments. J. Food Prot. 62, 894–898.
Quiberoni, A., Guglielmotti, D.M., Reinheimer, J.A., 2003. Inactivation of *Lactobacillus delbrueckii* bacteriophages by heat and biocides. Int. J. Food Microbiol. 84, 51–62.
Quiberoni, A., Moineau, S., Rousseau, G.M., Reinheimer, J., Ackermann, H.-W., 2010. Review. *Streptococcus thermophilus* bacteriophages. Int. Dairy J. 20, 657–664.
Ross, A.I.V., Griffiths, M.W., Mittal, G.S., Deeth, H.C., 2003. Combining nonthermal technologies to control foodborne microorganisms. Review. Int. J. Food Microbiol. 89, 125–138.
Sambrook, J., Russell, D.W., 2001. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Saxelin, M.-L., Nurmiaho-Lassila, E.-L., Meriläinen, V.T., Forsén, R.I., 1986. Ultrastructure and host specificity of bacteriophages of *Streptococcus cremoris*, *Streptococcus lactis* subsp. *diacetylactis*, and *Leuconostoc cremoris* from Finnish fermented milk "Viili". Appl. Environ. Microbiol. 52, 771–777.
Sozzi, T., Poulin, J.M., Maret, R., Pousaz, R., 1978. Isolation of a bacteriophage of *Leuconostoc mesenteroides* from dairy products. J. Appl. Bacteriol. 44, 159–161.
Stenlid, J., Karlsson, J.-O., Högberg, N., 1994. Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of minisatellite DNA. Mycol. Res. 98, 57–63.
Suárez, V.B., Reinheimer, J.A., 2002. Effectiveness of thermal treatments and biocides in the inactivation of Argentinian *Lactococcus lactis* phages. J. Food Prot. 65, 1756–1759.
Svensson, U., Christiansson, A., 1991. Methods for phage monitoring. Bulletin of the International Dairy Federation, 263. Macmillan Publishers Limited, Brussels, Belgium 29–39.
Vachon, J.F., Kheadr, E.E., Giasson, J., Paquin, P., Fliss, I., 2002. Inactivation of foodborne pathogens in milk using dynamic high pressure. J. Food Prot. 65, 345–352.
Vauterin, L., Vauterin, P., 1992. Computer aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. Eur. Microbiol. 1, 37–41.
Villion, M., Moineau, S., 2013. Phages hijack a host's defence. Nature 494, 433–434.
Yamamoto, K.R., Alberts, B.M., Benzinger, R., Lawhorne, L., Treiber, G., 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology 40, 734–744.
Zacarías, M.F., Binetti, A., Laco, M., Reinheimer, J., Vinderola, G., 2011. Preliminary technological and potential probiotic characterization of bifidobacteria isolated from breast milk for use in dairy products. Int. Dairy J. 21, 548–555.