International Journal of Food Microbiology 257 (2017) 128–137

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Leuconostoc mesenteroides and *Leuconostoc pseudomesenteroides* bacteriophages: Genomics and cross-species host ranges

Silvina A. Pujato[a,*], Daniela M. Guglielmotti[a], Manuel Martínez-García[b], Andrea Quiberoni[a], Francisco J.M. Mojica[b]

[a] Instituto de Lactología Industrial (Universidad Nacional del Litoral – Consejo Nacional de Investigaciones Científicas y Técnicas), Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santiago del Estero 2829, 3000 Santa Fe, Argentina
[b] Departamento de Fisiología, Genética y Microbiología, Universidad de Alicante, Campus de San Vicente, E-03080 Alicante, Spain

ARTICLE INFO

Keywords:
Leuconostoc
Phage infection
Host range
Phage genomic

ABSTRACT

Unveiling virus-host interactions are relevant for understanding the biology and evolution of microbes globally, but in particular, it has also a paramount impact on the manufacture of fermented dairy products. In this study, we aim at characterizing phages infecting the commonly used heterofermentative *Leuconostoc* spp. on the basis of host range patterns and genome analysis. Host range of six *Leuconostoc* phages was investigated using three methods (efficiency of plaquing, spot and turbidity tests) against *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains. Complete genome sequencing from four out of the six studied *Leuconostoc* phages were obtained in this work, while the remaining two have been sequenced previously. According to our results, cross-species host specificity was demonstrated, as all phages tested were capable of infecting both *Ln. pseudomesenteroides* and *Ln. mesenteroides* strains, although with different efficiency of plaquing (EOP). Phage adsorption rates and ability of low-EOP host strains to propagate phages by crossing the *Leuconostoc* species' barrier confirm results. At the genome level, phages CHA, CHB, Ln-7, Ln-8 and Ln-9 revealed high similarity with previously characterized phages infecting mostly *Ln. mesenteroides* strains, while phage LDG was highly similar to phages infecting *Ln. pseudomesenteroides*. Additionally, correlation between receptor binding protein (RBP) and host range patterns allowed us to unveil a finer clustering of *Leuconostoc* phages studied into four groups. This is the first report of overlapped phage host ranges between *Leuconostoc* species.

1. Introduction

Strains belonging to the *Leuconostoc* genus are heterofermentative lactic acid bacteria (LAB) frequently used in dairy industry, where they can play various important roles, such as production of flavor compounds, dextran and gas (CO_2) in cheeses when curd openness is needed (Hemme and Foucaud-Scheunemann, 2004). *Leuconostoc* strains are usually used in mesophilic mixed (DL-type) dairy starters together with acid-producing *Lactococcus*; this association helps with aroma generation by the former (Server-Busson et al., 1999). *Leuconostoc* species/subspecies of relevance for dairy industry comprise *Leuconostoc mesenteroides* (subsp. *mesenteroides*, *dextranicum* and *cremoris*), *Leuconostoc pseudomesenteroides* and *Leuconostoc lactis*, mostly used as aroma-producing cultures in dairy fermentations (Farrow et al., 1989; Frantzen et al., 2017).

Dairy fermentative processes are susceptible to bacteriophage infections, causing deficiencies in product quality which can lead to dramatic economic consequences. Despite the relevance of *Leuconostoc* in the dairy industry, phages attacking strains of this genus were overlooked in the past and few incidents were reported, probably because *Leuconostoc* failure affects some flavor and texture characteristics of the product but it does not affect acid production (Ali et al., 2013).

In the last years, *Leuconostoc* phages have been receiving growing attention. A variety of virulent *Leuconostoc* phages has recently been isolated from blue-veined cheese manufactures (Pujato et al., 2014), where the lack of curd openness was the most outstanding failure. These viruses have been characterized regarding their resistance to diverse physicochemical treatments and factors influencing adsorption to their host cells (Pujato et al., 2014, 2015). To date, fourteen complete genome sequences of *Leuconostoc* phages have been deposited in public databases, including two lytic *Leuconostoc mesenteroides* phages sequenced by our group (Pujato et al., 2015). All the analyzed sequences of *Leuconostoc* phages (which includes a temperate phage) present linear, double-stranded DNA genomes, ranging from 26 to

* Corresponding author.
E-mail address: spujato@unl.edu.ar (S.A. Pujato).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.06.009
Received 29 March 2017; Received in revised form 7 June 2017; Accepted 9 June 2017
Available online 12 June 2017
0168-1605/

39 kb in length, with GC content of 36 to 39%, and are composed of between 38 and 50 predicted open reading frames (ORFs) (Jang et al., 2010; Kleppen et al., 2012; Kot et al., 2014; Lu et al., 2010; Pujato et al., 2015).

It is known that for most phages investigated in the laboratory, host species specificity is the rule, since phages attach to very particular receptors placed on the surface of host cells (Chibani-Chennoufi et al., 2004; Legrand et al., 2016). In agreement, most phages attacking lactic acid bacteria have a quite narrow individual host range limited to a variable number of strains within a particular species (Guglielmotti et al., 2009; Rousseau and Moineau, 2009), and phage sensitivity depends on the presence of specific binding sites (called receptors) on the cell wall (Briggiler Marcó et al., 2011). A recent study demonstrated that the baseplate structure of phage Tuc2009 contains, in contrast to other characterized lactococcal phages, two different carbohydrate binding modules that may bind different motifs of the host's surface polysaccharide (Legrand et al., 2016). Unusually, a broad host range was observed for particular phages that were able to infect two strains belonging to different lactobacilli species (Lu et al., 2003). Regarding *Leuconostoc*, phages infecting *Ln. mesenteroides* strains have been reported to be unable to infect *Ln. pseudomesenteroides* strains and vice versa (Ali et al., 2013; Kot et al., 2014). Accordingly, comparison of the complete sequence of nine phages lytic of *Ln. mesenteroides* or *Ln. pseudomesenteroides* allowed grouping them into two classes, which correlated with host species. Moreover, similarity at nucleotide and protein level within each class was very high and significantly different from the other one (Kot et al., 2014).

In the present work, host range of six *Leuconostoc* phages was studied by three methods: spot and turbidity tests and efficiency of plaquing. This allowed us to demonstrate cross-species host specificity, as phages were capable of infecting both *Ln. pseudomesenteroides* and *Ln. mesenteroides* strains. Additionally, complete genome sequences from four out of the six studied *Leuconostoc* phages were obtained in this work, while the other two had been previously sequenced. Genome organization of the studied phages was analyzed, and comparison with previously sequenced genomes was carried out. A direct relationship between the amino acid sequence deduced from the gene coding for the receptor binding protein (RBP) and host range pattern was found. According to our knowledge, this is the first report of overlapped phage host ranges between *Leuconostoc* species.

2. Materials and methods

2.1. Bacterial strains, phages and culture conditions

Six *Siphoviridae* phages named LDG, CHA, CHB, Ln-7, Ln-8 and Ln-9, as well as their respective indicator strains, *Leuconostoc pseudomesenteroides* R707 and *Leuconostoc mesenteroides* C19A, C19B, D4b, D6a and L79-1, were used (Pujato et al., 2014, 2015) (Table 1). These *Leuconostoc* phages were previously isolated during faulty industrial manufactures of blue-veined cheeses (Pujato et al., 2014). Each strain used for the initial isolation of the respective phage was defined as its indicator strain. Ten *Ln. mesenteroides* and three *Ln. pseudomesenteroides* strains (Table 1) were additionally used in the host range analysis. Identification of strains was verified by sequencing of the 16S rRNA gene (1500 bp fragment) according to Edwards et al. (1989). It is worth noting that strain R707 (indicator for phage LDG), previously identified as *Ln. mesenteroides* (Pujato et al., 2014) was reclassified as *Ln. pseudomesenteroides*. Phages were propagated on the corresponding indicator strain and stocks prepared as described by Neviani et al. (1992) in de Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France), supplemented with 10 mM $CaCl_2$ (MRS-Ca). Phage enumerations were performed by the double-layer plaque titration method (Svensson and Christiansson, 1991), using MRS-Ca agar (1.2% w/v) supplemented with 100 mM glycine (Lillehaug, 1997). Working phage stocks were stored at 8 °C. Bacterial strains and phages are maintained at the INLAIN Collection (Argentina) and at the Department of Physiology, Genetics and Microbiology (Alicante, Spain) as frozen (− 80 °C) stocks in MRS broth using 15% (v/v) glycerol as cryoprotectant. Bacterial strains were routinely reactivated overnight (16–18 h) at 32 °C in MRS broth.

2.2. Host range, efficiency of plaquing and adsorption rates

Strain cross-sensitivity was investigated using the spot and turbidity tests as described by Svensson and Christiansson (1991) and efficiency of plaquing (EOP) assay. EOP was defined as the ratio between pfu/mL for a given phage on a sensitive strain and pfu/mL on its indicator strain. The three methods were carried out for the six phages on all the strains listed in Table 1.

Briefly, to carry out the spot test, log-phase bacterial cultures were mixed with MRS soft agar (0.6% w/v) and plated as thin top layer on MRS-Ca agar plates. Aliquots of 10 µL of high titer stock phages (between 5×10^9 and 5×10^{10} pfu/mL) were spotted onto the plates. After incubation at 32 °C for 18 h, the presence or absence of lysis zones was observed and recorded (Svensson and Christiansson, 1991). Turbidity test was performed by inoculation of tubes containing 5 mL of MRS-Ca broth with 0.2 mL of an overnight culture of indicator strains and 0.2 mL of stock phages (between 5×10^9 and 5×10^{10} pfu/mL). Tubes containing only the bacterial cultures were used as controls. A total of five subcultures were made (32 °C), and a decrease in turbidity in test tubes compared with that of the control, was considered as positive (Svensson and Christiansson, 1991). The EOP was calculated for the six phages on all the strains listed in Table 1. The three methods were performed by triplicate in independent trials.

Adsorption rates were determined for all phages on all the indicator *Leuconostoc* strains listed in Table 2, as previously described (Pujato et al., 2015). Briefly, each strain was grown in MRS broth until OD_{560} reached 0.5 (early exponential growth phase), centrifuged (5000 × *g*, 5 min, 4 °C) and resuspended in MRS broth (final cell concentration between 3×10^8 and 5×10^8 cfu/mL). Phages were added to bacterial suspensions and incubated 20 min at 32 °C for adsorption to take place. After adsorption, aliquots of mixtures were centrifuged (10,000 × *g*, 5 min, 4 °C) to sediment phage-adsorbed bacteria. Then, the phage titer was determined in the supernatant (non-adsorbed phages); the number of adsorbed phages was calculated as the percentage of the difference respect to the initial phage count. The assays were performed by triplicate in independent trials.

In the case of low EOP values (Table 1), phages from these plaques were verified for their ability to propagate on the low-EOP hosts. Briefly, after the EOP was obtained (first assay), one or two lysis plaques obtained from the titration on the low-EOP host strain, were picked up and suspended in 5 mL of MRS-Ca broth. Phage suspensions were kept 16 h at 4 °C and then inoculated with an overnight culture of the same strain (low-EOP host) and incubated at 32 °C until lysis. The lysate obtained was filtered and titrated on the same low-EOP host strain. All assays were performed in triplicate.

2.3. Viral DNA purification

DNA to be sequenced was purified from phages LDG, CHA, CHB and Ln-7. Aliquots of 15 mL of phage stocks were centrifuged (10,000 × *g*, 10 min, 4 °C) and the supernatants filtered (0.2 µm filters, GV Durapore, Millipore). Viruses were then ultracentrifuged at 186,000 × *g* for 4 h at 20 °C (Optima MAX-XP Ultracentrifuge, TLA-S5 rotor Beckman Coulter) and finally re-suspended in 0.5 mL of ultrapure water. Virus concentrates were mixed with equal volumes of 1.6% (w/v) low-melting-point agarose (Pronadisa), dispensed into 100 µL moulds, and allowed to solidify at 4 °C. Agarose plugs were then incubated for 90 min with 5 µL of Turbo DNA-free kit (Ambion) to digest the dissolved DNA (according to the manufacturer's protocol, 2–3 µL of Turbo DNase digest up to 500 µg/mL of DNA in 30 min). The plugs

Table 1
Host range of *Leuconostoc* phages of dairy origin.

Strain	Phage																	
	CHA			CHB			Ln–7			Ln–8			Ln–9			LDG		
	TT	ST	EOP	TT	ST	EOP	TT	ST	EOP	TT	ST	EOP	TT	ST	EOP	TT	ST	EOP
C19A[a, A]	+	+	1	+	+	1	+	–	6.0×10^{-9}	+	–	5.0×10^{-9}	+	–	9.0×10^{-9}	+	+	2.0×10^{-7}
C19B[a, A]	+	+	0.2	+	+	1	+	–	2.0×10^{-8}	+	–	9.0×10^{-9}	+	–	9.0×10^{-9}	+	+	3.0×10^{-7}
D4b[a, A]	+	+	2.0×10^{-8}	+	–	9.0×10^{-6}	+	+	1	+	+	1	+	+	3.0×10^{-7}	+	+	1.0×10^{-7}
D6a[a, A]	+	+	4.0×10^{-10}	+	+	4.0×10^{-6}	+	+	1	+	+	1	+	+	2.0×10^{-7}	+	+	8.0×10^{-7}
L79–1[a, A]	+	–	6.6×10^{-10}	+	–	4.0×10^{-7}	+	–	1.0×10^{-8}	+	–	1.0×10^{-8}	+	+	1	+	+	1.0×10^{-7}
L72[a, B]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	+	+	1	–	–	$<3.6 \times 10^{-10}$
L74[a, C]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	+	+	1	–	–	3.6×10^{-9}
DG5A[a, B]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	+	+	1	–	–	4.7×10^{-9}
DM5B[a, C]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	+	+	1	–	–	3.6×10^{-9}
DG6B[a]	+	–	2.0×10^{-9}	+	–	7.1×10^{-9}	+	–	2.5×10^{-7}	+	–	1.9×10^{-9}	+	–	6.7×10^{-9}	+	–	3.6×10^{-9}
L1612[a, B]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	+	+	1	+	+	1	+	–	7.0×10^{-8}	–	–	7.1×10^{-9}
LR3[a, B]	+	+	1	+	+	1	+	–	9.1×10^{-9}	+	–	3.6×10^{-9}	+	+	3.1×10^{-7}	–	–	4.7×10^{-9}
LC4[a, C]	+	+	1	+	+	1	+	–	4.5×10^{-7}	+	–	3.5×10^{-7}	+	+	2.0×10^{-6}	–	–	1.2×10^{-8}
LN3[a, C]	+	+	1	+	+	1	+	–	1.1×10^{-7}	+	–	2.6×10^{-9}	–	–	$<1.0 \times 10^{-9}$	–	–	$<3.6 \times 10^{-10}$
LMG 6893[T, a]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	–	–	$<1.0 \times 10^{-9}$	–	–	$<3.6 \times 10^{-10}$
R707[b,A]	+	–	1.0×10^{-8}	+	–	2.0×10^{-5}	+	–	3.0×10^{-8}	+	–	2.0×10^{-7}	–	–	3.0×10^{-10}	+	+	1
LD–6[b, A]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	–	–	$<1.0 \times 10^{-9}$	–	–	$<3.6 \times 10^{-10}$
LM3[b, B]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	–	–	$<1.0 \times 10^{-9}$	–	–	$<3.6 \times 10^{-10}$
LMG 11482[T, b]	–	–	$<2 \times 10^{-10}$	–	–	$<7.1 \times 10^{-10}$	–	–	$<9.1 \times 10^{-11}$	–	–	$<7.1 \times 10^{-11}$	–	–	$<1.0 \times 10^{-9}$	–	–	$<3.6 \times 10^{-10}$

TT: turbidity test; ST: spot test; EOP: efficiency of plaquing. Grey shading corresponds to indicator strain for each phage. [A, B, C] Strain from commercial mesophilic mixed starters used in plant A, B or C, respectively. [T] Type strains from the LMG Bacteria Collection (Belgian Coordinated Collections of Microorganisms). [a] *Ln. mesenteroides*. [b] *Ln. pseudomesenteroides*.

were then incubated overnight at 50 °C in ESP (0.5 M EDTA, pH 9.0; 1% *N*-lauroylsarcosine; 1 mg/mL proteinase K) for digestion of DNase and viral capsids. For DNA extraction, plugs were washed with TE-Pefabloc (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 3 mM Pefabloc, Roche) to inactivate the proteinase K and incubated at 65 °C for 15 min. The mixture of viral DNA and melted agarose was treated with β-agarase (New England BioLabs) for 1.5 h at 42 °C (1 enzyme unit per 0.1 g of melted mixture) and DNA was finally purified using Microcon YM-100 centrifugal filter devices (Millipore). DNA quality was checked by electrophoresis and its concentration determined by Nanodrop (Thermo Fisher Scientific Inc.) (Martínez-García et al., 2014).

2.4. Phage DNA sequencing

Phage DNAs were sequenced at the Fundación para el Fomento de la Investigación Sanitaria y Biomédica de la Comunidad Valenciana (FISABIO), Valencia, Spain. The sequencing libraries were prepared with the Nextera XT DNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. The library was sequenced using a MiSeq Reagent Kit v2 (Illumina – 500 cycles) on a MiSeq system. De novo assembly was performed with Ray assembler versions 2.1.1-devel and 2.2.0-devel using a kmer size of 31 (Džunková et al., 2014). Complete genome sequences were obtained after assembling of multiple individual reads into one single contig. Correct genome assembly was confirmed by PCR. Genome extremities were amplified using converging primers, and nucleotide sequences of purified amplicons were determined at the DNA Sequencing Service of Macrogen (Seoul, Korea). DNA sequences were then assembled with Staden software (Staden, 1996).

2.5. Bioinformatics analysis

Complete genomes were edited and analyzed with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). Open reading frames (ORFs) were identically predicted using either GeneMark (http://opal.biology.gatech.edu/GeneMark/) (Lukashin and Borodovsky, 1998) or ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). ORFs that encoded 25 or more amino acids (aa) and possessed both a conserved Shine-Dalgarno (SD) sequence (5′-TAGGAGGT-3′) and an AUG, UUG or GUG start codon, were considered functional. In those cases where a potential SD sequence was not identified, the initiation codon could be located near to the putative stop codon of the preceding gene, allowing a potential translational coupling (Brøndsted et al., 2001). Putative functions were assigned to those selected ORFs using Blastp (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Conserved domains in protein sequences were identified with the NCBI CD-search interface to search the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Physicochemical parameters of predicted proteins were estimated using the ProtParam Tool at ExPASy proteomics server (http://web.expasy.org/protparam/). The presence of tRNA genes was investigated employing tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) and ARAGORN (Laslett and Canback, 2004).

The phylogenetic analysis was performed on 28 LAB phages using Gegenees (version 2.2.1), which employs a fragmented 'all against - all comparison' of the genomes and builds a distance matrix file suitable to construct a phylogenetic tree. The phylogenetic tree was built by NJ (Neighbor-joining) method using SplitsTree software (version 4.14.4). The elements of the matrix correspond to ANI (Average Nucleotide Identity) values.

Table 2
Phage adsorption rates on *Leuconostoc* strains.

Strain	Adsorption rate (%)[c]					
	Phage					
	CHA	CHB	Ln–7	Ln–8	Ln–9	LDG
C19A[a]	99.5 ± 1.8	99.1 ± 2.0	87.8 ± 1.3	52.1 ± 1.3	80.8 ± 1.6	50.1 ± 1.9
C19B[a]	99.7 ± 1.6	99.2 ± 1.7	79.3 ± 1.6	50.0 ± 1.9	82.9 ± 1.7	52.2 ± 2.1
D4b[a]	95.5 ± 1.7	80.1 ± 1.4	99.5 ± 2.0	98.2 ± 1.4	74.4 ± 2.3	41.1 ± 1.3
D6a[a]	90.0 ± 1.2	80.1 ± 1.5	99.6 ± 2.1	99.3 ± 2.3	70.2 ± 2.1	46.3 ± 1.4
L79–1[a]	81.0 ± 2.0	90.0 ± 2.0	95.1 ± 1.4	90.2 ± 1.9	99.9 ± 1.8	70.0 ± 2.1
R707[b]	87.7 ± 2.1	79.2 ± 2.0	76.1 ± 1.7	42.8 ± 2.0	63.8 ± 1.6	99.6 ± 1.5

Grey shading corresponds to indicator strain for each phage. [a] *Ln. mesenteroides*; [b] *Ln. pseudomesenteroides*; [c] Values are the mean and standard deviation of three determinations.

2.6. Nucleotide sequence accession numbers

The complete genome sequences of phages LDG, CHA, CHB and Ln-7 were deposited in GenBank under accession numbers KX555527, KX578044, KX578043 and KX578042, respectively.

3. Results and discussion

3.1. Host range, efficiency of plaquing and adsorption rates

Host range of *Leuconostoc* phages LDG, CHA, CHB, Ln-7, Ln-8 and Ln-9 was investigated by applying three methods: spot and turbidity tests, and efficiency of plaquing (EOP). With this aim, fifteen strains of *Ln. mesenteroides* and four of *Ln. pseudomesenteroides* were used (Table 1).

Analysis of EOP values for the studied phages evidenced four different host range patterns, as defined by the list of strains infected by a particular phage with either high or low efficiency. Specifically, similar patterns were revealed for phages CHA and CHB and for Ln-7 and Ln-8; while patterns were distinctive for phages Ln-9 and LDG (Table 1).

According to turbidity and spot tests, phage LDG was able to infect both *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains. Regarding EOP, low values (EOP $< 8 \times 10^{-7}$) were obtained when this phage infected *Ln. mesenteroides* strains. Likewise, phages CHA, CHB, Ln-7, Ln-8 and Ln-9 were also able to infect both *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains, even if different efficiency of plaquing were obtained (Table 1). These results showed an unexpected ability of infecting both *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains by all the phages assayed. This interspecies cross-infection by *Leuconostoc* phages has not been described in the literature before. However, data concerning the virulence of *Leuconostoc* phages are scarce and only a few studies are documented (Atamer et al., 2011; Kot et al., 2014). In general, most phages attacking lactic acid bacteria have a quite narrow host range (Chibani-Chennoufi et al., 2004). The literature only documented a few cases of cross-species phage sensitivity, as two phages isolated from sauerkraut fermentations that were able to infect equally two ecologically related *Lactobacillus* species, namely *Lb. brevis* and *Lb. plantarum* (Lu et al., 2003).

Most studies about host range are usually carried out by applying only spot test. However, the low sensitivity of the method may lead to some false negative results. In the present study, when infection occurred (lysis plaques observed on EOP assays), turbidity test was mostly positive (90% of assays), while a high number of negative results was evidenced in spot test (61% of assays) (Table 1). The use of three methods (spot and turbidity tests, and EOP) undoubtedly increased the accuracy of results. In consequence, it should be recommended the use of at least one more assay, besides spot test, in order to avoid mistaken results.

Regarding adsorption assays, all phages were able to adsorb on all the strains tested, both *Ln. mesenteroides* and *Ln. pseudomesenteroides*, though with different adsorption rates (Table 2). As expected, each phage was able to adsorb at high rate (> 98.2%) both on its indicator strain or on those highly related (Pujato et al., 2014). Also, moderate or high adsorption rates were obtained when phages were assayed on the low-EOP host strains, i.e. 81.0–95.5% (phage CHA), 79.2–90.0% (phage CHB), 76.1–95.1% (phage Ln-7), 42.8–90.2% (phage Ln-8), 63.8–82.9% (phage Ln-9) and 41.1–70.0% (phage LDG) (Table 2). Therefore, no clear correlation between low EOP values and adsorption rates could be established. Besides, in order to verify the ability of low-EOP host strains to propagate phages by crossing the *Leuconostoc* species' barrier, some host/phage systems were selected and investigated (Table 3). According to the results obtained, all phages assayed were able to propagate, achieving high titers ($> 1.2 \times 10^9$ pfu/mL), on the selected low-EOP host strains (Table 3). These results confirm, once more, the ability of the assayed phages to infect and cross the barrier of the two *Leuconostoc* species assayed, namely *Ln. mesenteroides* and *Ln. pseudomesenteroides*.

Table 3
Propagation of *Leuconostoc* phages on selected low-EOP host strains.

Strain	Phage	Assay stage	Titer (pfu/mL)
C19B[a]	CHB	1st	1.4×10^9
R707[b]			4.0×10^3
R707[b]		2nd	1.9×10^9
D4b[a]	Ln–7	1st	1.1×10^{10}
R707[b]			2.7×10^1
R707[b]		2nd	1.8×10^9
D6a[a]	Ln–8	1st	1.4×10^{10}
R707[b]			3.0×10^2
R707[b]		2nd	1.9×10^9
R707[b]	LDG	1st	2.8×10^9
C19A[a]			5.0×10^2
C19A[a]		2nd	1.2×10^9
R707[b]	LDG	1st	2.8×10^9
D4b[a]			5.3×10^1
D4b[a]		2nd	1.3×10^9

Grey shading corresponds to indicator strain for each phage. [a] *Ln. mesenteroides*; [b] *Ln. pseudomesenteroides.*

3.2. Comparative genomics of Leuconostoc phages

Phages LDG, CHA, CHB and Ln-7 all revealed dsDNA, with genome sizes ranging from 26.5 to 28.9 kb, in accordance to those previously reported for other *Leuconostoc* phages (Kot et al., 2014; Pujato et al., 2015). The genomic G + C content ranged from 35.9% (phage CHB) to 36.3% (phage LDG), which is also similar to that reported for *Ln. mesenteroides* subsp. *mesenteroides* strains (37%) and the *Leuconostoc* phages in general (Kot et al., 2014; Makarova et al., 2006; Pujato et al., 2015). Regarding the genome extremities, all phages studied are cos-type. The presence of a cos-site suggests the phage DNA circularization upon entry into the host. The cos sequence of phages CHA, CHB and Ln-7 (5′-GGTTAATAGTAGTCTTTTTGAA-3′) was identical to the 22 nucleotide (nt) cos-site previously reported for other phages infecting *Leuconostoc* strains, namely Ln-8 (Pujato et al., 2015) and 1-A4 (Lu et al., 2010). Additionally, this cos sequence was very similar, though longer than the 12 nt cos-site (5′-CGGTTAGTAGTA-3′) reported for phages ΦLN25, ΦLN34, ΦLNTR2 and ΦLNTR3 (Kot et al., 2014), all specific to *Ln. mesenteroides* strains. Besides, the 23 nt cos-site of phage LDG (5′-TCGTGCAATAGTAGGCGTTTTAA-3′) was also conserved and identical to the 23 nt of phage Lmd1 (Kleppen et al., 2012), specific to *Ln. mesenteroides* subsp. *dextranicum* A1.

Bioinformatic analysis of LDG, CHA, CHB and Ln-7 phage genomes revealed 40, 43, 45 and 47 possible ORFs, respectively. Each ORF was preceded by a region sharing similarities with the Shine–Dalgarno sequence, complementary to the 3′ end of the 16S rRNA of *Ln. mesenteroides* (5′-TAGGAGGT-3′).

Deduced ORFs were located on either DNA strand at equivalent proportions. The putative functions of the genes, based on the similarities to already known sequences, are listed in Tables 4 and 5.

A phylogenetic tree was generated comparing whole genomes of a total of 28 phages infecting different lactic acid bacteria (Fig. 1). The bacterial hosts for the 28 phages include *Leuconostoc*, *Lactobacillus*, *Lactococcus* and *Streptococcus*. This analysis revealed that phages infecting *Ln. mesenteroides* and *Ln. pseudomesenteroides* form separate clusters. Within Cluster I, two separate subclusters could be observed. Phage Ln-7 belonged to subcluster IA, and it was similar to *Ln. mesenteroides* phages such as Ln-8 (Pujato et al., 2015) (> 92% ANI) and ΦLN25 (Kot et al., 2014) (> 84% ANI). In addition, phages CHA and CHB, belonging to subcluster IB, were highly similar to phages previously reported as specific to *Ln. mesenteroides*, namely ΦLNTR3,

Table 4
Open reading frames (ORFs) deduced from the genome of *Leuconostoc* phage CHA and homologues ORFs in *Leuconostoc* phages CHB and Ln-7.

Strand	ORF	Start	Stop	Size[a] (aa)	MM (kDa)	pI	SD sequence (5′-TAGGAGGT-3′)[b]	ORF		Predicted function
								Phage CHB	Phage Ln-7	
−	1	683	381	101	11.6	7.7	ACAAGGataattaat**ATG**	1	1	Phage HNH endonuclease
									2	Hypothetical protein
−	2	1122	685	146	16.7	7.0	AAGAGGtactaaaaa**ATG**	2	3	Phage-related protein
−	3	1265	1119	49	6.1	11.3	AGGAGCGaagaagaa**ATG**	3	4	Hypothetical protein
−	4	1636	1262	125	14.8	7.8	AGGAGGgtaacat**ATG**	4	5	Endodeoxyribonuclease
+	5	2018	3331	438	50.5	5.5	AGGAGGaaaacagat**ATG**	5	6	DNA helicase
+	6	3328	4083	252	29.1	6.1	None	6	7	DNA primase/polymerase
+	7	4162	5997	612	69.7	7.1	AGGAGAaaaaagatt**ATG**	7	8	DNA polymerase
+	8	6187	6759	191	21.7	5.6	AGGAGAacatat**ATG**	8	9	Hypothetical protein
								9		HNH endonuclease
+	9	6812	7444	211	24.1	5.9	GGGAGGaattaaagt**ATG**	10	10	Hydrolase
								11		Hydrolase
									11	Terminase small subunit
+	10	7456	7638	61	7.2	5.2	AGGAGGattgact**ATG**	12	12	Hypothetical protein
+	11	7647	7973	109	12.4	5.0	ATGAGGtaatat**ATG**	13		Terminase small subunit
+	12	8035	8193	53	5.9	11.3	None	14	13	Terminase small subunit
+	13	8177	9823	549	62.9	5.1	AGGAGGgtaatag**ATG**	15	14	Terminase large subunit
								16		Terminase large subunit
+	14	9836	10,957	374	43.0	5.0	AGGAGAaaactat**ATG**	17	15	Portal protein
+	15	10,917	11,642	242	26.2	4.5	AGGAGAcactacga**ATG**	18	16	Phage prohead protease
+	16	11,694	12,647	318	34.8	5.2	AGGAGAcctataat**ATG**	19	17	Major capsid protein
+	17	12,777	13,064	96	10.8	4.3	AGGAGGtgacaca**ATG**	20	18	Hypothetical protein
+	18	13,051	13,317	89	10.2	10.0	AGGAGGcgatcagat**ATG**	21	19	Phage tail protein
+	19	13,317	13,634	106	12.3	4.6	GGGAGGtagtcattta**ATG**	22	20	Hypothetical protein
+	20	13,631	13,960	110	12.4	11.1	AGGTGTtaatatt**ATG**	23	21	Hypothetical protein
+	21	14,009	14,590	194	21.2	4.7	AGGAGAattaatcaatt**ATG**	24	22	Major tail protein
+	22	14,657	17,398	914	93.2	9.2	AGAAAGGaaatgtattat**ATG**	25	23	Tail tape measure protein
									24	Tail tape measure protein
+	23	17,462	18,163	234	27.2	4.8	AGAATGGaggaaattat**ATG**	26	25	Hypothetical protein
+	24	18,168	18,785	206	23.0	5.1	None			Hypothetical protein
+	25	18,788	19,786	333	36.7	5.1	AGGAGAttaatc**ATG**	27	26	Structural protein
−	26	20,394	19,993	134	15.6	4.7	AGGAGAattaaagac**ATG**	28	27	Hypothetical protein
									28	Hypothetical protein
+	27	20,474	21,241	256	27.9	5.9	GAGGAGAtttaaaat**ATG**	29	29	Receptor-binding tail protein
−	28	21,274	21,645	124	14.0	6.4	AGGAGAccccgcatt**ATG**	30	30	Holin I
									31	Phage-related methyltransferase
−	29	21,920	21,756	55	6.4	8.1	AGGAGAagta**ATG**	31	32	Hypothetical protein
−	30	22,150	21,920	77	9.0	6.8	TGGAGGTtctata**GTG**	32	33	Hypothetical protein
−	31	22,383	22,150	78	9.1	5.5	AGGAACGAgaaa**ATG**	33	34	Hypothetical protein
−	32	22,504	22,373	44	5.4	5.0	None	34	35	Hypothetical protein
−	33	22,709	22,491	73	9.0	8.0	GAGAGGttcgcaagta**ATG**	35	36	Hypothetical protein
−	34	23,209	22,709	167	18.7	9.5	AGGAGAtttaaa**ATG**	36	37	Phage-related hydrogenase
							AAGAGGaacaaac**GTG**		38	Hypothetical protein
−	35	24,449	23,577	291	31.0	4.9	AGGAGGacaagtaac**ATG**	37	39	Lysin
−	36	24,771	24,424	116	12.8	9.3	AGGAGGaaacaataa**ATG**	38	40	Holin II
−	37	26,299	24,788	504	55.9	5.4	AGGAGGaaattac**ATG**	39	41	Hypothetical protein
−	38	26,507	26,361	49	5.6	5.1	AGGGGTattgta**ATG**	40	42	Hypothetical protein
−	39	26,815	26,507	103	11.6	9.5	AGGAGAtataac**ATG**	41	43	Hypothetical protein
−	40	27,017	26,817	67	7.8	4.9	None	42	44	Hypothetical protein
−	41	27,298	27,020	93	10.7	4.5	TGAGGaagtgagcaa**ATG**	43	45	Hypothetical protein
−	42	27,546	27,295	84	9.8	5.1	ACGAGGagataacaa**ATG**	44	46	Hypothetical protein
−	43	27,862	27,692	57	6.5	6.1	AGGAATaatatatgac**ATG**	45	47	Repressor

[a] Number of amino acids (aa) of the predicted protein.
[b] Uppercase letters indicate nucleotides identical to the consensus SD sequence; boldfaced indicate start codon.

ΦLNTR2 and ΦLN34 (Kot et al., 2014) (> 91% ANI). On the other side, phage LDG was included in Cluster II, and it was highly similar to phages infecting *Ln. pseudomesenteroides* strains, e.g. ΦLN04 (> 72% ANI) and ΦLN12 (> 68% ANI) (Kot et al., 2014).

3.3. Function assignment and genomic organization of Leuconostoc phages

As for many siphophages, the genome of *Leuconostoc* phages is organized into five functional modules (Lu et al., 2010): DNA packaging, morphogenesis (capsid and tail), cell lysis, DNA replication and regulation/modification. Based on their nucleotide sequence, the four analyzed phages can be divided into two groups. The first one (group I) was constituted by phages CHA, CHB and Ln-7, while phage LDG was included into the second group (II) (Fig. 2). Phages included in the first group revealed high similarity with phages infecting mostly *Ln. mesenteroides* strains, while phage LDG (from the second group) was highly similar to a phage infecting *Ln. pseudomesenteroides*.

In general, *Leuconostoc* phages analyzed presented five genes encoding proteins which may be involved in phage DNA replication (Fig. 2). However, the analysis of phage CHB revealed an additional gene (ORF9) predicted to encode for the HNH endonuclease. Furthermore, ORF10 and ORF11 of phage CHB may code for one hydrolase, being strongly similar (99% identity) to the hydrolase encoded by phage LNTR3 (Kot et al., 2014) (Table 4). Experimental data are needed to confirm if these two ORFs produce a functional protein.

The terminase enzymes participate in phage DNA packaging into the procapsids. They are heteromultimers composed of a large subunit and small subunits (Duffy and Feiss, 2002). The genes encoding terminase

Table 5
Open reading frames (ORFs) deduced from the genome of *Leuconostoc* phage LDG and their predicted functions.

Strand	ORF	Start	Stop	Size[a] (aa)	MM (kDa)	pI	SD sequence (5′-TAGGAGGT-3′)[b]	Predicted function
−	1	746	435	104	12.4	9.0	None	Phage HNH endonuclease
−	2	1140	718	141	15.2	9.4	None	Endodeoxyribonuclease
+	3	1723	3117	465	52.8	5.4	AGGAGGcctacaaacA**TG**	DNA helicase
+	4	3107	3877	257	30.0	5.8	GGGGGTgctttttA**TG**	DNA primase/polymerase
+	5	3937	5754	606	68.5	5.8	AGGAGGacagaaaA**TG**	DNA polymerase
+	6	5812	6369	186	21.1	8.9	AGGAAGTgtaacaA**TG**	Hypothetical protein
+	7	6439	7062	208	24.3	6.5	AAGAGAagataatcA**TG**	Hydrolase
+	8	7081	7431	117	13.5	5.1	TTGAGGtaataaccaatA**TG**	Terminase small subunit
+	9	7434	9074	547	63.1	5.5	CGGAGAattgagtA**TG**	Terminase large subunit
+	10	9065	10,306	414	46.5	5.3	None	Portal protein
+	11	10,257	10,982	242	26.1	4.8	AGGGAGcacggctaA**TG**	Phage prohead protease
+	12	11,040	12,008	323	34.7	6.1	GTGAGGaaaaatattataA**TG**	Major capsid protein
+	13	12,083	12,361	93	11.1	4.6	AGGAAAccgactattA**TG**	Hypothetical protein
+	14	12,358	12,639	94	10.6	10.0	AGGTGGtggcaagaA**TG**	Phage tail protein
+	15	12,639	12,950	104	11.8	5.3	GGGTGGtaatcgctaA**TG**	Hypothetical protein
+	16	12,950	13,306	119	13.6	11.0	ATGTGGtggttctctaA**TG**	Hypothetical protein
+	17	13,357	13,941	195	21.6	5.2	GTGAGGataataaaaaacA**TG**	Major tail protein
+	18	14,091	15,935	615	63.1	9.6	AGGAGCttttaaA**TG**	Tail tape measure protein
+	19	15,913	16,674	254	25.6	11.0	TGGTCAattgA**TG**	Tail tape measure protein
+	20	16,716	17,831	372	41.8	5.0	ATGTGAtataatcgtagtA**TG**	Hypothetical protein
+	21	17,834	18,703	290	32.4	5.4	TGGAGActagagA**TG**	Structural protein
+	22	18,717	19,697	327	34.8	7.9	AGAAAGGtaataacA**TG**	Receptor-binding tail protein
−	23	19,968	19,717	84	9.5	9.5	AGGATAatagcctttctC**ATG**	Hypothetical protein
−	24	20,323	19,943	127	14.7	4.9	AGGACGaaccaacA**TG**	Hypothetical protein
−	25	20,771	20,388	128	14.3	6.6	CGAATGGAaacataataA**TG**	Holin I
−	26	21,040	20,852	63	14.6	4.5	CGGAGAtaaaaatcA**TG**	Hypothetical protein
−	27	21,485	21,033	151	16.3	4.6	TGGTGAaaggataaaacA**TG**	Lysin
−	28	22,433	21,489	315	6.8	5.7	None	Phage-related methyltransferase
−	29	22,708	22,433	92	10.8	9.6	AAAAGGaaaataactaA**TG**	Hypothetical protein
−	30	22,977	22,708	90	10.2	7.8	AGGAGCcagatagA**TG**	Hypothetical protein
−	31	23,192	22,974	73	8.2	5.3	AGGAGAtaacacattG**TG**	Hypothetical protein
−	32	23,493	23,245	83	9.8	4.8	TGGAGTgagtgA**TG**	Hypothetical protein
−	33	23,702	23,493	70	8.3	9.6	AGAGGattttcaaaaaA**TG**	Transcriptional regulator
−	34	23,845	23,699	49	6.2	12.0	AAGGGGtaaattggtA**TG**	Hypothetical protein
−	35	24,111	23,842	90	10.8	10.0	AGGAGCaattataA**TG**	Response regulator
−	36	24,627	24,274	118	13.3	4.6	AGGAGCaattataA**TG**	Hypothetical protein
−	37	24,878	24,627	84	9.9	9.6	AGGAGGtcacaaaagA**TG**	Hypothetical protein
−	38	25,769	24,987	261	29.4	11.0	AGGAGGtaattA**TG**	Lysin
−	39	26,217	25,780	146	15.6	9.1	AAGAGGattaacatA**TG**	Holin II
−	40	26,367	26,221	49	5.7	7.2	AGGAACGataagactA**TG**	Repressor

[a] Number of amino acids (aa) of the predicted protein.
[b] Uppercase letters indicate nucleotides identical to the consensus SD sequence; boldfaced indicate start codon.

small subunits are located upstream the terminase large subunit gene. Phages CHA, CHB and Ln-7 revealed two ORFs upstream the terminase large subunit gene, while products derived from these two ORFs are likely to be the small terminase subunits (Fig. 2 and Table 4). Similar results were previously reported for *Ln. mesenteroides* phage Φ1A-4 (Lu et al., 2010). For phage CHB, merging of ORF15 and ORF16 yields to a sequence similar to the large terminase subunit of *Ln. mesenteroides* phage ΦLNTR2 (Kot et al., 2014). In phage LDG, the deduced proteins of ORF8 and ORF9 share high similarity with the putative small and large terminase subunits from various *Ln. pseudomesenteroides* phages, such as ΦLN6B (Kleppen et al., 2012; Kot et al., 2014) (Table 5).

The gene encoding the tail tape measure protein is located within the morphogenesis module. The name of this protein derives from the length of the corresponding gene, which is proportional to the length of the phage's tail (Katsura and Hendrix, 1984). ORF22 and ORF25 of phages CHA and CHB, respectively, showed several characteristics in common and identical sizes (914 aa) with the tail tape measure protein from phage ΦLNTR3 of *Ln. mesenteroides* (Kot et al., 2014). Moreover, for phage Ln-7, the product derived of merging those of ORF23 (298 aa) and ORF24 (597 aa), shows great similarity (99% identity) to the putative tail tape measure protein encoded by phage Ln-8 (855 aa) (Pujato et al., 2015) (Table 4). For phage LDG, the merged product obtained from ORF18 and ORF19 showed some similarity to the putative tail tape measure protein of *Ln. pseudomesenteroides* phage P793 (Kot et al., 2014) (Table 5). The tail tape measure protein frequently includes a variable number of tandem repeats containing tryptophan and phenylalanine amino acids, which are located at fixed positions. They are used as anchors by small auxiliary proteins to stretch the tape and the actual tail construction. The regular spacing between these anchors seems to be a key structural property of the tail tape measure protein and acts as a marking on the tape (Belcaid et al., 2011). Amino acid sequence of tail tape measure proteins of phages CHA, CHB, Ln-7, Ln-8 and LDG, showed a preserved amount of phenylalanine (44 aa) and tryptophan (5 aa) units, but these proteins did not present tandem repeats. Similar results were observed for other *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages (Kot et al., 2014; Lu et al., 2010; Pujato et al., 2015).

The cell lysis module consists of a putative endolysin and two holins (Daniel et al., 2007). Two different versions of the putative lysin were detected in *Ln. mesenteroides* phages, which showed no significant nucleotide similarities between each other. The putative lysin (ORF39) of phage Ln-7 showed 98% similarity to the amidase from phage Φ1-A4 (Lu et al., 2010). In phages CHA and CHB, lysin (ORF35 and ORF37, respectively) exhibited high similarity to those from phage ΦLNTR3 (Kot et al., 2014). Moreover, putative methyltransferase genes were detected in the genome sequence of the *Leuconostoc* phages. One of them was encoded by ORF31 in phage Ln-7 and the other one by ORF28 of phage LDG (Tables 4 and 5). In prokaryotes, the major role of DNA methylation is to protect host DNA against degradation by restriction enzymes (Cheng, 1995).

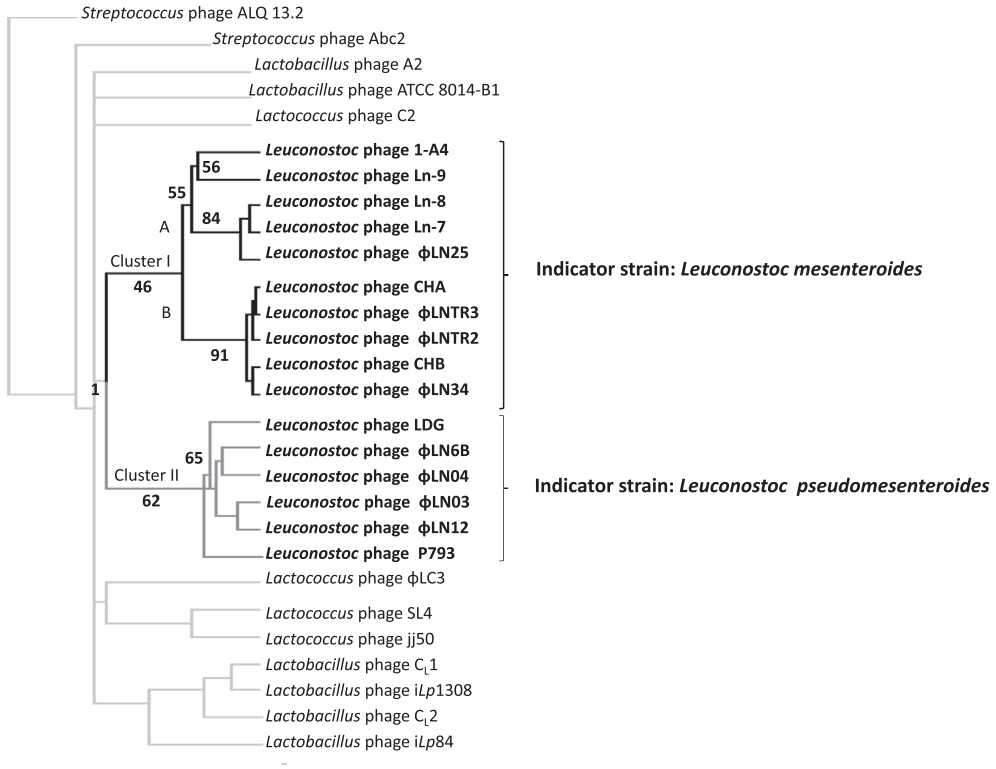

Fig. 1. Phylogenetic tree of 28 LAB infecting phages. The whole genome information was used to build the distance matrix using Gegenees (version 2.2.1). The phylogenetic tree was developed using SplitsTree (version 4.14.4) by Neighbor-Joining method. For *Leuconostoc* phages (in bold-face letters), the species of indicator strains is shown Numbers (%) indicate minimum Average Nucleotide Identity (ANI) values within each cluster or subcluster.

3.4. Correlation between receptor binding protein and host range patterns

The first interaction of a phage particle and a bacterium is mediated through the specific recognition between host receptors distributed over the cell surface and the phage receptor binding protein (RBP), located at the tip of the tail. Regarding receptors, and as Mahony et al. (2014) stated, phages interact with their hosts involving various different host and phage structures, being possible to establish a simplified classification based on the nature of the receptor material: protein or carbohydrate (including lipoteichoic acid). The lactococcal 936 and P335 phages are believed to recognize carbohydrate moieties located at the cell surface (Tremblay et al., 2006; Legrand et al., 2016). For many phages, this binding step is reversible, and phages of the c2 species, for example, require a second irreversible binding step to a predicted membrane-attached protein (PIP). Regarding *Lactobacillus*, the lipoteichoic acids (LTAs) from the cell surface were the responsible of the interaction between phage LL-H and its host *Lactobacillus delbrueckii* ssp. *lactis* ATCC15808 (Raisanen et al., 2004; Munsch-Alatossava and Alatossava, 2013). Even this phage-host interaction has been extensively investigated, scarce knowledge is still available regarding

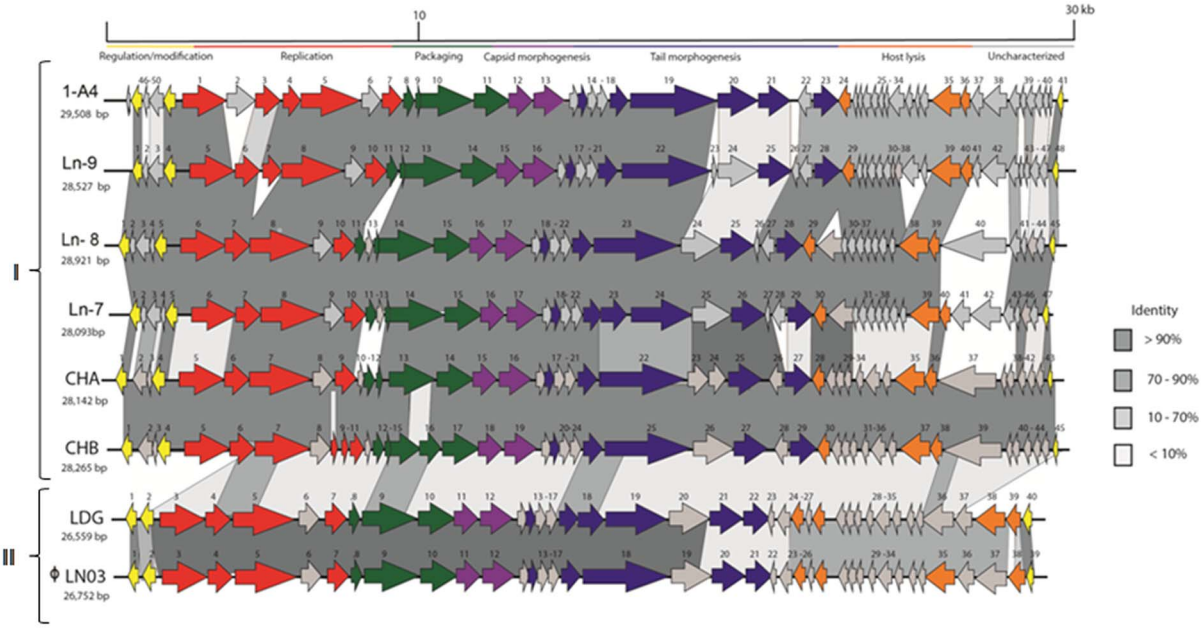

Fig. 2. Schematic overview of eight *Leuconostoc* phage genomes. ORFs are indicated by numbered-colored arrows. Arrows represent putative ORFs and their color indicates the corresponding gene module. ORFs connected by a grey box show homologies at the amino acid level; the grey tone indicates percentage of identity. Uncharacterized ORFs encoding hypothetical proteins are displayed as grey arrows.

specific receptor compounds in *Lactobacillus* strains. Concerning RBPs in phages infecting Gram-positive bacteria, information is sparse compared to that available for phages infecting Gram-negative bacteria, and only a small number of RBPs in phages of lactic acid bacteria has been identified (Kot et al., 2013; Mahony et al., 2013, 2014). The isolation of chimeric phages containing a "swapped" receptor binding domain allowed the identification of the gene responsible for host recognition in *Streptococcus thermophilus* phages DT1 and MD4 (Duplessis and Moineau, 2001). The RBPs of the lactococcal P335 species phages, TP901-1 and Tuc2009 have also been identified (Vegge et al., 2006) as were those of the lactococcal 936 phages, sk1 and bIL170 (Dupont et al., 2004). Furthermore, immunogold labelling electron microscopy allows the identification of the genetic determinants of c2 phages responsible for the interaction with their protein receptor, PIP, i.e. *l10* and *orf31* of phages c2 and bIL67, respectively (Lubbers et al., 1995). More recently, the complete genome sequences of four novel phages capable of infecting the industrial strain *S. thermophilus* ST64987 were reported (McDonnell et al., 2016). These phages were categorized as the novel 987 group, based on their notable differences with those of previously described groups of *S. thermophilus* phages. In this study, the N-terminal end of RBP of phage 9871 shares a high level of amino acid identity (approximately 85%) with the N-terminal portion of the upper baseplate protein (BppU) of TP901-1, Tuc2009, P335, and ORF322 of ul36 and then appears to be extended (relative to BppU) at the C-terminal end. The authors suggest that RBP of phage 9871 has a carbohydrate binding function, hypothesizing that this protein incorporates the receptor binding activities of the upper and lower baseplate proteins (BppU and BppL, respectively) of TP901-1, where BppL is known to be responsible for host interaction and specificity (Vegge et al., 2006).

Previous studies carried out on the RPB genes of two *Ln. pseudomesenteroides* phages, ϕLN04 (ORF23) and P793 (ORF21), which present nonoverlapping host ranges, demonstrated that the deduced putative proteins from these ORFs could be divided into two fragments (Kot et al., 2013). Only the first amino acid portion (N-terminal) shows similarity (75% nucleotide identity), while the C-terminal part did not show any significant similarity between each other. The authors also verified that the construction of chimeric phages with exchanged RPB genes, led to the expected switch in their host ranges (Kot et al., 2013). In the present study, Blastp analysis of ORF22 (phage LDG), ORF27 (CHA), ORF29 (CHB and Ln-7) and ORF28 (Ln-8 and Ln-9) (Pujato et al., 2015) from *Leuconostoc* phages, revealed similarities to the putative RBP of previously sequenced *Leuconostoc* phages. The deduced RBPs from these ORFs were used to perform a comparative analysis, which also included RBP sequences from previously reported *Leuconostoc* phages (Kot et al., 2014; Lu et al., 2010). In coincidence with previous studies (Kot et al., 2013), RBP sequence could be divided into two fragments. The first amino acid portion (N-terminal) is highly conserved within phages isolated on indicator strains belonging to the same *Leuconostoc* species (80–100% aa identity) and moderately conserved between phages isolated on different species of *Leuconostoc* indicator strains (44% aa identity) (Fig. 3A). The second fragment corresponds to the C-terminal part of the protein, which is the most divergent region within the deduced RBP gene. Similarly, phages of *Streptococcus thermophilus* DT1 and MD4 exhibited a variable region at the C-terminal fraction of the protein, which was encoded by ORF18 and could be responsible for host recognition (Duplessis and Moineau, 2001). Further analysis of RBP sequences from phages of lactococcal 936 species revealed that the N-terminal region of the RBPs were very conserved, while the C-terminal portion varied, suggesting also that the latter could play a role in binding and receptor recognition (Dupont et al., 2004). Moreover, Stuer-Lauridsen et al. (2003), demonstrated that ORF35 and ORF115 from phages bIL67 and c2, respectively, were responsible for host range determination. The N-terminal part of the ORF35 protein shows a high level of conservation between the two phages, while the C-terminal end of the deduced amino acid sequence is less conserved. Interestingly, a low level of homology was observed in the middle part of the ORF35 protein. As suggested by the authors, this variable region plays also a relevant role for host range determination. These findings agree with those previously reported by Duplessis and Moineau (2001), who had speculated that the variable middle region of ORF18 from two *S. thermophilus* phages could also be involved in host recognition.

A second comparison considering only the variable RBP region (C-terminal) is shown in Fig. 3B. According to this analysis, five groups could be identified; three for phages infecting *Ln. mesenteroides* and two for phages specific to *Ln. pseudomesenteroides*. Phages CHA, CHB, Φ1-A4 (Lu et al., 2010), ΦLNTR2, ΦLNTR3 and ΦLN34 (Kot et al., 2014), were included in Group 1; Group 2 included phages Ln-7, Ln-8 (Pujato et al., 2015) and ΦLN25 (Kot et al., 2014); Group 3 was formed only by phage Ln-9 (Pujato et al., 2015), while Group 4 comprised four phages infecting *Ln. pseudomesenteroides* (Kot et al., 2014). Finally, phages LDG and P793 infecting *Ln. pseudomesenteroides* constituted Group 5. Thus, *Leuconostoc* phages studied by us were included in four different groups according to the variable region of RBPs, this grouping being coincident with that resulting from the four different host range patterns.

This study evidenced wide host ranges for all phages assessed, as they were able to infect both *Ln. pseudomesenteroides* and *Ln. mesenteroides* strains. On the contrary, previous studies reported narrow host ranges when nine *Leuconostoc* phages were analyzed (Kot et al., 2014). Those phages revealed four different host range patterns and four RBP variable regions; two of them were exclusively found in phages specific to *Ln. mesenteroides*, while the other two were exclusive for those specific to *Ln. pseudomesenteroides*. The attachment of phages to their sensitive strains is accomplished by RBP proteins present in baseplate structures of the viral particles. Previous studies on phage morphology have established that the differences in their baseplate structures were perfectly correlated with the diverse and non-overlapping host range profiles of *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains (Ali et al., 2013). However, unlike our results, none of those patterns were overlapping. Moreover, interspecies cross-infection of the phages studied when tested on *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains was not evidenced (Kot et al., 2014). A limited number of hosts and high conservation of host range patterns in *Leuconostoc* phages have been observed before when considering *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains (Atamer et al., 2011).

4. Conclusions

In the present study, six dairy *Leuconostoc* phages were characterized on the basis of host range patterns and genome analysis. Host range studies, carried out by applying three methods, demonstrated cross-species sensitivity, as phages were capable of infecting both *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains, although with diverse efficiency. Phage adsorption rates and ability of low-EOP host strains to propagate phages by crossing the *Leuconostoc* species' barrier, allow us to confirm results from host range studies. The analysis and further comparison of complete genomes of *Leuconostoc* phages, allowed dividing them into *Ln. mesenteroides* and *Ln. pseudomesenteroides* phages, related to the corresponding indicator strains used for their isolation. Comparison of the variable fraction from the deduced amino acid sequence of the receptor binding protein (RBP), let us classify *Leuconostoc* phages studied into four groups, which were coincident with the four different host range patterns. In general, phages containing similar RBP variable region showed equivalent host range patterns. However, some of the studied phages presenting distinct RBP variable regions were able of infecting the same *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains.

Our results show that phages presenting similar RBP region were able of infecting the same *Leuconostoc* strains with high efficiency. However, other phages with dissimilar RBPs were also capable of infecting the same strains but with much lower efficiency. All these data

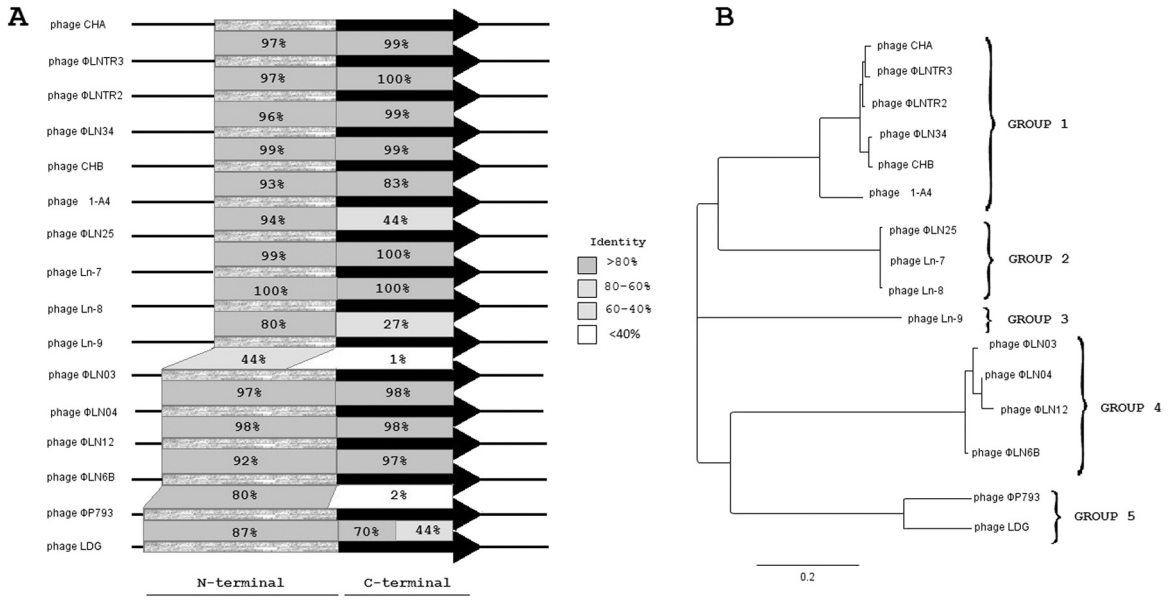

Fig. 3. Genetic overview of the RBP region of *Leuconostoc* phages. A) The figure illustrates the nucleotide similarities of ORF regions from the 16 analyzed phages as calculated by the BLASTN algorithm. Arrows are divided into two fragments; the first one corresponds to the N-terminal portion and the second fragment corresponds to the C-terminal portion of the protein. B) Phylogenetic tree of the RBP variable C-terminal fragment of 16 phages, including the four sequenced phages in this study. The distance matrix and visualization was performed in Geneious 9.0.2 (alignment algorithm with BLOSUM55 substitution matrix) by Neighbor-Joining method.

emphasize principally the overlapped host ranges of *Leuconostoc* phages on both *Ln. mesenteroides* and *Ln. pseudomesenteroides* strains.

Acknowledgments

This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; Project PIP 112-201201-00046; Argentina), the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT; Project PICT 2010-0138; Argentina) and the Universidad Nacional del Litoral (UNL, Project CAI + D PI 501 201101 00039 LI; Argentina). S.A.P. was the recipient of an international scholarship awarded by BEC.AR (Becas de formación en el exterior en Ciencia y Tecnología, Presidencia de la Nación, Argentina). M.M.G. thanks Ministerio de Economía y Competitividad (refs. CGL2013-40564-R and SAF2013-49267-EXP), Generalitat Valenciana (ACOMP/2015/133) and Gordon and Betty Moore Foundation (Grant award ref. 5334). F.J.M.M. is funded by the Spanish Ministerio de Economía y Competitividad (BIO2014-53029P) and the European Commission/Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (291815 Era-Net ANIHWA).

References

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., 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.

Belcaid, M., Bergeron, A., Poisson, G., 2011. The evolution of the tape measure protein: units, duplications and losses. BMC Bioinf. 12 (Suppl. 9), S10.

Briggiler Marcó, M., Reinheimer, J., Quiberoni, A., 2011. Characterization of phage receptors in lactic acid bacteria. In: Medina, D.A., Laine, A.M. (Eds.), Food Quality: Control. Analysis and Consumer Concerns. Nova Science Publishers, Inc., New York, pp. 431–442.

Brøndsted, L., Østergaard, S., Pedersen, M.M., Hammer, K., Vogensen, F.K., 2001. Analysis of the complete DNA sequence of the temperate bacteriophage TP901-1: evolution, structure, and genome organization of lactococcal bacteriophages. Virology 283, 93–109.

Cheng, X., 1995. Structure and function of DNA methyltransferases. Annu. Rev. Biophys. Biomol. Struct. 24, 293–318.

Chibani-Chennoufi, S., Bruttin, A., Dillmann, M.-L., Brüssow, H., 2004. Phage-host interaction: an ecological perspective. J. Bacteriol. 186 (12), 3677–3686.

Daniel, A., Bonnen, P.E., Fischetti, V.A., 2007. First complete genome sequence of two *Staphylococcus epidermidis* bacteriophages. J. Bacteriol. 189, 2086–2100.

Duffy, C., Feiss, M., 2002. The large subunit of bacteriophage lambda's terminase plays a role in DNA translocation and packaging termination. J. Mol. Biol. 316, 547–561.

Duplessis, M., Moineau, S., 2001. Identification of a genetic determinant responsible for host specificity in *Streptococcus thermophilus* bacteriophages. Mol. Microbiol. 41, 325–336.

Dupont, K., Vogensen, F.K., Neve, H., Bresciani, J., Josephsen, J., 2004. Identification of the receptor-binding protein in 936-species lactococcal bacteriophages. Appl. Environ. Microbiol. 70, 5818–5824.

Džunková, M., Garcia-Garcerà, M., Martínez-Priego, L., D'Auria, G., Calafell, F., Moya, A., 2014. Direct sequencing from the minimal number of DNA molecules needed to fill a 454 picotiterplate. PLoS One 9 (6), e97379.

Edwards, U., Rogall, T., Blocker, H., Emde, M., Bottger, E.C., 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.

Farrow, J.A.E., Facklam, R.R., Collins, M.D., 1989. Nucleic acid homologies of some vancomycin-resistant *Leuconostocs* and description of *Leuconostoc citreum* sp. nov. and *Leuconostoc pseudomesenteroides* sp. nov. Int. J. Syst. Bacteriol. 39, 279–283.

Frantzen, C.A., Kot, W., Pedersen, T.B., Ardö, Y.M., Broadbent, J.R., Neve, H., Hansen, L.H., Dal Bello, F., Østlie, H.M., Kleppen, H.P., Vogensen, F.K., Holo, H., 2017. Genomic characterization of dairy associated *Leuconostoc* species and diversity of leuconostocs in undefined mixed mesophilic starter cultures. Front. Microbiol. 8, 132. http://dx.doi.org/10.3389/fmicb.2017.00132.

Guglielmotti, D.M., Binetti, A.G., Reinheimer, J.A., Quiberoni, A., 2009. *Streptococcus thermophilus* phage monitoring in a cheese factory: Phage characteristics and starter sensitivity. Int. Dairy J. 19, 476–480.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.

Hemme, D., Foucaud-Scheunemann, C., 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int. Dairy J. 14, 467–494.

Jang, S.H., Hwang, M.H., Chang, H.-I., 2010. Complete genome sequence of ΦMH1, a *Leuconostoc* temperate phage. Arch. Virol. 155, 1883–1885.

Katsura, I., Hendrix, R.W., 1984. Length determination in bacteriophage lambda tails. Cell 39, 691–698.

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.

Kot, W., Hansen, L.H., Neve, H., Hammer, K., Jacobsen, S., Pedersen, P.D., Sørensen, S.J., Heller, K.J., Vogensen, F.K., 2014. Sequence and comparative analysis of *Leuconostoc* dairy bacteriophages. Int. J. Food Microbiol. 176, 29–37.

Laslett, D., Canback, B., 2004. ARAGORN, a program for the detection of transfer RNA and transfer-messenger RNA genes in nucleotide sequences. Nucleic Acids Res. 32, 11–16.

Legrand, P., Collins, B., Blangy, S., Murphy, J., Spinelli, S., Gutierrez, C., Richet, N., Kellenberger, C., Desmyter, A., Mahony, J., van Sinderen, D., Cambillaub, C., 2016. The atomic structure of the phage Tuc2009 baseplate tripod suggests that host recognition involves two different carbohydrate binding modules. MBio 7 (1), e01781-15. http://dx.doi.org/10.1128/mBio.01781-15.

Lillehaug, D., 1997. An improved plaque assay for poor plaque-producing temperate

lactococcal bacteriophages. J. Appl. Microbiol. 83, 85–90.
Lu, Z., Breidt, F., Plengvidhya, V., Fleming, H.P., 2003. Bacteriophage ecology in commercial sauerkraut fermentations. Appl. Environ. Microbiol. 69, 3192–3202.
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 (6), 1955–1966.
Lubbers, M.W., Waterfield, N.R., Beresford, T.P., Le Page, R.W., Jarvis, A.W., 1995. Sequencing and analysis of the prolate-headed lactococcal bacteriophage c2 genome and identification of the structural genes. Appl. Environ. Microbiol. 61, 4348–4356.
Lukashin, A.V., Borodovsky, M., 1998. GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res. 26, 1107–1115.
Mahony, J., Kot, W., Murphy, J., Ainsworth, S., Neve, H., Hansen, L.H., Heller, K.J., Sørensen, S.J., Hammer, K., Cambillau, C., Vogensen, F.K., van Sinderen, D., 2013. Investigation of the relationship between lactococcal host cell wall polysaccharide genotype and 936 phage receptor binding protein phylogeny. Appl. Environ. Microbiol. 79, 4385–4392.
Mahony, J., Bottacini, F., van Sinderen, D., Fitzgerald, G.F., 2014. Progress in lactic acid bacterial phage research. Microb. Cell Factories 13 (Suppl. 1), S1.
Makarova, K., Slesarev, A., Wolf, Y., Sorokin, A., Mirkin, B., Koonin, E., Pavlov, A., Pavlova, N., Karamychev, V., Polouchine, N., Shakhova, V., Grigoriev, I., Lou, Y., Rohksar, D., Lucas, S., Huang, K., Goodstein, D.M., Hawkins, T., Plengvidhya, V., Welker, D., Hughes, J., Goh, Y., Benson, A., Baldwin, K., Lee, J.H., Diaz-Muniz, I., Dosti, B., Smeianov, V., Wechter, W., Barabote, R., Lorca, G., Altermann, E., Barrangou, R., Ganesan, B., Xie, Y., Rawsthorne, H., Tamir, D., Parker, C., Breidt, F., Broadbent, J., Hutkins, R., O'Sullivan, D., Steele, J., Unlu, G., Saier, M., Klaenhammer, T., Richardson, P., Kozyavkin, S., Weimer, B., Mills, D., 2006. Comparative genomics of the lactic acid bacteria. Proc. Natl. Acad. Sci. U. S. A. 103, 15611–15616.
Martínez-García, M., Santos, F., Moreno-Paz, M., Parro, V., Antón, J., 2014. Unveiling viral–host interactions within the 'microbial dark matter'. Nat. Commun. http://dx.doi.org/10.1038/ncomms5542.
McDonnell, B., Mahony, J., Neve, H., Hanemaaijer, L., Noben, J.-P., Kouwen, T., van Sinderen, D., 2016. Identification and analysis of a novel group of bacteriophages infecting the lactic acid bacterium *Streptococcus thermophilus*. Appl. Environ. Microbiol. 82, 5153–5165.
Munsch-Alatossava, P., Alatossava, T., 2013. The extracellular phage-host interactions involved in the bacteriophage LL-H infection of *Lactobacillus delbrueckii* ssp. *lactis* ATCC 15808. Front. Microbiol. 4, 408.
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.
Pujato, S.A., Guglielmotti, D.M., Ackermann, H.-W., Patrignani, F., Lanciotti, R., Reinheimer, J.A., Quiberoni, A., 2014. *Leuconostoc* bacteriophages from blue cheese manufacture: long-term survival, resistance to thermal treatments, high pressure homogenization and chemical biocides of industrial application. Int. J. Food Microbiol. 177, 81–88.
Pujato, S.A., Mercanti, D.J., Guglielmotti, D.M., Rousseau, G.M., Moineau, S., Reinheimer, J.A., Quiberoni, A., 2015. Phages of dairy *Leuconostoc mesenteroides*: Genomics and factors influencing their adsorption. Int. J. Food Microbiol. 201, 58–65.
Raisanen, L., Schubert, K., Jaakonsaari, T., Alatossava, T., 2004. Characterization of lipoteichoic acids *as Lactobacillus delbrueckii* phage receptor components. J. Bacteriol. 186, 5529–5532.
Rousseau, G.M., Moineau, S., 2009. Evolution of *Lactococcus lactis* phages within a cheese factory. Appl. Environ. Microbiol. 75, 5336–5344.
Server-Busson, C., Foucaud, C., Leveau, J.Y., 1999. Selection of dairy *Leuconostoc* isolates for important technological properties. J. Dairy Res. 66, 245–256.
Staden, R., 1996. The Staden sequence analysis package. Mol. Biotechnol. 5, 233–241.
Stuer-Lauridsen, B., Janzen, T., Schnabl, J., Johansen, E., 2003. Identification of the host determinant of two prolate-headed phages infecting *Lactococcus lactis*. Virology 309, 10–17.
Svensson, U., Christiansson, A., 1991. Methods for phage monitoring. In: International Dairy Federation, Brussels, Belgium. Bulletin. 263. pp. 29–39.
Tremblay, D.M., Tegoni, M., Spinelli, S., Campanacci, V., Blangy, S., Huyghe, C., Desmyter, A., Labrie, S., Moineau, S., Cambillau, C., 2006. Receptor-binding protein of *Lactococcus lactis* phages: identification and characterization of the saccharide receptor-binding site. J. Bacteriol. 188, 2400–2410.
Vegge, C.S., Vogensen, F.K., Mc Grath, S., Neve, H., van Sinderen, D., Brondsted, L., 2006. Identification of the lower baseplate protein as the antireceptor of the temperate lactococcal bacteriophages TP901-1 and Tuc2009. J. Bacteriol. 188, 55–63.