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Phages of dairy *Leuconostoc mesenteroides*: Genomics and factors influencing their adsorption

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ABSTRACT

Phages infecting *Leuconostoc mesenteroides* strains can be overlooked during milk fermentation because they do not slowdown the acidification process. However, they can negatively impact the flavor profile of the final product. Yet, the information about these phages is still scarce. In this work, we investigated diverse factors influencing the adsorption of seven virulent *Ln. mesenteroides* phages, isolated from blue cheese manufacture in Argentina, to their host cells. The addition of calcium ions was generally necessary to observe complete cell lysis and plaque formation for four of the seven phages, but adsorption was very high even in the absence of this cation for all phages. The temperature barely influenced the adsorption process as it was high within the temperature range tested (0 to 50 °C). Moreover, the kinetics of adsorption were similar on viable and non-viable cells, revealing that phage adsorption does not depend on physiological state of the bacterial cells. The adsorption rates were also high at pH values from 4 to 9 for all *Ln. mesenteroides* phages. We also analyzed the complete genome sequences of two of these phages. Complete nucleotide analysis of phages Ln-8 and Ln-9 showed dsDNA genomes with sizes of 28.5 and 28.9 kb, and the presence of 45 and 48 open reading frames (ORFs), respectively. These genomes were highly similar to those of previously characterized Φ1-A4 (USA, sauerkraut, fermentation) and ΦLN25 (England, whey), both virulent *Ln. mesenteroides* phages. A detailed understanding of these phages will lead to better control strategies.

1. Introduction

Leuconostoc strains are Gram-positive bacteria and cells are generally ovoid cocci, often forming chains during exponential growth. They are found on plants, from where they disseminate to various habitats including raw milk (Hemme and Foucaud-Schneumann, 2004). Despite their relative poor growth in milk, *Leuconostoc* play an important role in the dairy industry. When combined with lactic acid-producing lactococci strains, *Leuconostoc* cells often act as flavor producers (Server-Busson et al., 1999). Lactose and citrate metabolism by *Leuconostoc* contributes to the organoleptic properties of buttermilk, sour cream and fresh cheeses (Kot et al., 2014a,b). Moreover, their heterofermentative metabolism produces CO_2, allowing openness and colonization of *Penicillium roqueforti* in some blue-veined cheeses (Hemme and Foucaud-Scheunemann, 2004). *Ln. mesenteroides* subsp. *mesenteroides* and subsp. *cremoris*, *Ln. lactis*, and *Ln. pseudomesenteroides* are mostly used as aroma-producing cultures in dairy fermentations (Farrow et al., 1989).

Virulent phages are recognized to adversely affect dairy fermentations by inhibiting the growth of lactic acid bacteria (Mahony and van Sinderen, 2014) and accordingly, dairy phages have been studied for decades (Samson and Moineau, 2013). While several phages infecting LAB such as *Lactococcus lactis* (Mahony et al., 2012), *Streptococcus thermophilus* (Quiberoni et al., 2010) and *Lactobacillus* sp. (Villion and Moineau, 2009) have been analyzed in great details, virulent phages of dairy *Leuconostoc* have been rather ignored. Because lactic acid production is often the rate-limiting step in various milk fermentation processes, the performance of acid-producing starter cultures is rigorously monitored as well as the virulent phages infecting them. As *Leuconsotoc* cells participate in flavor development rather than lactic acid production and acid defects are easier to detect than faulty aroma, their phages have been mostly overlooked. However, the presence of virulent *Leuconostoc* phages can negatively affect the quality and aroma of the final milk fermented product (Ali et al., 2013).

For a list of all the *Leuconostoc* phages reported to date, the readers are directed to a recent review on this topic (Kot et al., 2014b). The first description of phages affecting *Leuconostoc* and their negative impact on butter aroma was published in 1946 (Mosimann and Ritter, 1946; Kot et al., 2014b). Typically, *Leuconostoc* phage titers in dairy

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products vary between 10^2 and 10^7 plaque-forming units (pfu) per gram or per ml (Atamer et al., 2011). Recently, the morphotypes of 83 *Ln. pseudomesenteroides* and of *Ln. mesenteroides* dairy phages were studied (Ali et al., 2013). All these phages had a small isometric capsid containing a double-stranded DNA genome (dsDNA) and connected to non-contractile 140-nm long tails (Kot et al., 2014a,b). Morphological diversity was observed at the end of their tail, namely the host recognition device or baseplate (Ali et al., 2013). Nonetheless, all virulent *Leuconostoc* phages involved in dairy fermentations have generally been shown to be members of the *Siphoviridae* family of the Caudovirales order (Kot et al., 2014a,b).

Twelve complete dsDNA genomes of phages infecting *Leuconostoc* sp. are currently available in public databases (Jang et al., 2010; Kleppen et al., 2012; Kot et al., 2014a; Lu et al., 2010). They range in size from 25.7 to 38.7 kb, have a GC content from 36.1 to 38.7%, contain from 38 to 50 predicted genes, and exhibit high similarity in regard to genome organization (Kot et al., 2014a). A conserved region within the gene coding for the major tail protein was recently used for the development of a PCR-based detection system for lytic phages of *Ln. mesenteroides* and *Ln. pseudomesenteroides* (Ali et al., 2013). A *Leuconostoc* phage gene coding for host recognization has been experimentally identified (Kot et al., 2013) but the host-encoded receptor for these phages remains unknown (Kot et al., 2014a,b).

Studies on *Ln. mesenteroides* phages have been mainly limited and focused on host range and thermal inactivation (Ali et al., 2013; Atamer et al., 2011; Pujato et al., 2014). Phage adsorption to its host cell is the first step in the lytic cycle. This interaction is highly specific and primarily dependent on the presence of specific binding sites on the bacterial cell surface (Guglielmotti et al., 2012a,b). Both efficiency and adsorption rate may be affected by pH, temperature, presence of calcium ions and physiological cell state (Kutter and Goldman, 2008) but little is known about adsorption of *Leuconostoc* phages (Arendt et al., 1991). Understanding this adsorption step may improve the design of useful strategies in order to prevent phage infections.

The present study was undertaken to investigate factors influencing adsorption on seven *Ln. mesenteroides* phages. The complete genomic sequences of two of these phages were determined and compared to other *Leuconostoc* phages sequences available.

2. Materials and methods

2.1. Strains, phages and culture conditions

Seven *Siphoviridae* phages of *Leuconostoc* isolated during faulty industrial manufactures of blue-veined cheeses and identified as LDG, CHA, CHB, CyC1, Ln-7, Ln-8 and Ln-9, as well as their respective *Ln. mesenteroides* subsp. *mesenteroides* sensitive strains R707, C19A, C19B, CyC, D4b, D6a and L79-1, were used. These phages were partially characterized by our group in a previous study (Pujato et al., 2014). The bacterial strains were routinely reactivated overnight (16–18 h) at 32 °C in de Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France). Phage stocks were prepared as described by Neviani et al. (1992) in MRS broth, adding 10 mmol/l $CaCl_2$ (MRS-Ca). Phage enumerations were performed by the double-layer plaque titration method (Svensson and Christiansson, 1991), using MRS-Ca agar added with 100 mmol/l glycine (Lillehaug, 1997). Working phage lysates were maintained at 8 °C. Bacterial strains and phages are maintained at the INLAIN Collection (Argentina) and at the Félix d'Hérelle Reference Center for Bacterial Viruses (Canada, http://www.phage.ulaval.ca) as frozen stocks in MRS broth using a 15% (*v*/*v*) glycerol cryoprotectant.

2.2. Adsorption studies

The influence of pH, temperature, calcium, cell viability and multiplicity of infection (MOI, calculated as the ratio between initial number of phages and bacterial cells) on phage adsorption was investigated according to Capra et al. (2006). Bacterial strains were 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 of 3×10^8–5×10^8 cfu/ml). MRS broth was previously adjusted to the conditions required for each experiment. Phages were added to bacterial suspensions and incubated at 32 °C for adsorption to take place. At certain time intervals, aliquots of 1 ml 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 effect of pH was studied by suspending *Ln. mesenteroides* cells in MRS-Ca broth adjusted to pH values between 4 and 9. Mixtures of phages and bacterial cells (MOI ~ 0.02) were incubated for 20 min at 32 °C. Concurrently, phage stability was monitored in MRS-Ca with the adjusted pH to ensure that reductions in phage counts were due to adsorption and not to phage sensitivity to pH.

The influence of temperature was determined by incubating phages/bacterial cell mixtures (MOI ~ 0.02) at 0, 10, 20, 32, 42 and 50 °C for 20 min. The selected time-temperature parameters do not affect the stability of these phages in MRS-Ca broth, as previously demonstrated (Pujato et al., 2014).

To investigate the influence of Ca^{2+} on phage adsorption, *Leuconostoc* cells were recovered by centrifugation and resuspended in MRS or MRS-Ca broths. After 0, 2, 10 and 20 min of incubation at 32 °C, aliquots of phage/cell mixtures (MOI ~ 0.02) were centrifuged (10,000 ×*g*, 5 min, 4 °C) and phages in the supernatants were enumerated as previously described. The effect of calcium ions on cell lysis in MRS broth was also investigated by incubating infected *Ln. mesenteroides* cultures (MOI ~ 0.02) in MRS broth at 32 °C, with and without $CaCl_2$ (10 mmol/l). Plaque formation was determined by the double-layer plaque technique in MRS and MRS-Ca agar.

Phage adsorption was also measured on bacterial cells in three different states: (i) exponential growth phase, (ii) nonviable cells obtained by keeping *Ln. mesenteroides* cell suspension in boiling water for 2 min, and (iii) non-proliferating cells by using chloramphenicol, a protein-synthesis inhibitor. For (ii), 100% of cell death after heat-treatment was confirmed by plate counts. Non-proliferating cells on (iii) were obtained according to Briggiler Marcó et al. (2010) with some modifications: 250 μg/ml was the minimum concentration required to completely stop cell growth of *Ln. mesenteroides* strains (followed by OD_{560} measurement), after 75 min of incubation at 32 °C in MRS-Ca broth. Chloramphenicol was not removed after blocking bacterial cell growth. Phage stability in MRS broth with and without chloramphenicol was monitored throughout the assay. Phage addition and enumeration were determined as described previously.

The influence of the MOI on phage adsorption was also determined. The number of phages adsorbed per cell was plotted against MOIc. MOIc is defined as the ratio between MOI and the average number of cells per cfu (assuming that every chain in MRS broth generates one colony in agar plates), which was in turn determined by counting (1000×, optical microscope Jenamed 2 Carl Zeiss, Jena, Germany) the number of cocci in at least 50 chains for each *Leuconostoc* strain. Cell numbers were determined by plate counts in MRS agar prior to phage addition divided by the average number of cells per cfu.

2.3. One-step growth curves

The one-step growth curve was determined for each of the seven phages on their respective *Ln. mesenteroides* host strain. Each host strain was harvested (10,000 ×*g*, 5 min, 4 °C) in exponential growth phase (OD_{560} ~ 0.5), suspended in 1/5 of initial volume of MRS-Ca broth, and phages were added at a MOI of 0.01. After adsorption (20 min at 32 °C), phage-adsorbed bacteria were harvested by centrifugation (10,000 ×*g*, 5 min, 4 °C), resuspended in MRS-Ca broth, and decimal

dilutions of this suspension were incubated at 32 °C. At regular time intervals, 100 μl from each dilution were collected for phage counts (Capra et al., 2006). Latent period, burst time and burst size were calculated from the curves by using DMFit web edition (http://modelling.combase.cc/DMFit.aspx). The complete model of Baranyi and Roberts (1994) was used for data fit.

2.4. Complete sequencing of phages Ln-8 and Ln-9

The isolation of phage genomic DNA was conducted using a Maxi lambda DNA purification kit (Qiagen) and following the protocol modified by Deveau et al. (2002). To confirm that each phage was different at the genomic level, the phage DNAs were BglII-restricted according to the manufacturer recommendations (Roche Diagnostics). Restriction fragments were separated by electrophoresis in a 0.8% agarose gel in 40 mM Tris-acetate–1 mM EDTA (TAE) buffer, stained with EZ-Vision ® Three (Amresco), and visualized under UV light. Sample aliquots of each digested DNA were previously heated at 75 °C for 10 min. The presence of an extra band in the non-heated treated sample suggested the presence of cohesive (*cos*-type) genome extremities. 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 (Boisvert et al., 2010). Genomic DNA sequences were obtained after assembling of multiple individual reads into one single contig, with a mean coverage depth of 3690 for phage Ln-8 and 9182 for phage Ln-9. Correct genome assembly was confirmed by PCR. DNA sequences were then analyzed with Staden software (Staden, 1996). For phage Ln-9, genomic DNA extremities were amplified using converging primers, and the obtained PCR product sequenced by the Sanger method with an ABI 3730 xl apparatus at the sequencing and genotyping platform of the Centre Hospitalier of the Université Laval (Quebec, Canada).

2.5. Bioinformatics analysis

Complete phage sequences were analyzed with BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). Open reading frames (ORFs) were predicted using GeneMark (http://opal.biology.gatech.edu/GeneMark/) (Lukashin and Borodovsky, 1998), and confirmed with 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 sequence (5′-TAGGAGGT-3′) and an AUG, UUG or GUG starting codon, were considered functional. Probable functions were assigned to selected ORFs using BLASTp (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi). Functional domains contained on predicted protein sequences were detected with the help of the NCBI 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).

2.6. Analyses of phage structural proteins

Phages were propagated in 1 l of 0.5× MRS-Ca broth, concentrated with polyethylene glycol (PEG) 8000 (Laboratoire Mat), and purified by ultracentrifugation steps on a CsCl gradient (Sambrook and Russell, 2001). First, a discontinuous CsCl gradient was carried out at 35,000 rpm using a Beckman SW41 rotor (210,053 ×*g*) at 20 °C for 3 h. This step was followed by a continuous CsCl gradient at 60,000 rpm on a Beckman NVT65 rotor (342,317 ×*g*), at 20 °C for 18 h. After ultracentrifugation, bands containing concentrated phage particles were recovered and dialyzed against phage buffer (0.05 M Tris–HCl pH 7.5, 0.1 M NaCl, 8 mM $MgSO_4$). Titers of concentrated phage samples reached 10^{11}–10^{12} pfu/ml.

The identification of structural proteins was accomplished from concentrated phage samples, as described by Samson and Moineau (2010). Structural proteins of phage Ln-8 were identified by sending the purified phages directly to the Centre Protéomique de l'Est du Québec (University Laval, Quebec, Canada) for liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis.

2.7. Nucleotide sequence accession numbers

The complete genome sequences of phages Ln-8 and Ln-9 have been deposited in GenBank under accession numbers KM262191 and KM262192, respectively.

3. Results and discussion

3.1. Adsorption studies

The effect of pH on phage adsorption was studied. Phage adsorption and infectivity were not affected by pH values from 4 to 9 for the seven *Ln. mesenteroides* phages tested (Fig. 1A). Adsorption levels were high (>97%) for all phages in the pH range tested, while the maxima (>99%) were between pH 5 and 7. Interestingly, these data indicate that *Leuconostoc* phages can adsorb to their host at the normal pH of milk (≈6.5) as well as in acidified/fermented dairy products. Similar behavior was exhibited by phages infecting other LAB, namely *L. lactis* (Suárez et al., 2008) and *S. thermophilus* (Binetti et al., 2002).

The adsorption of phages LDG, CHA, CyC and Ln-9 to their hosts was also very high (>94%) at all temperatures tested (Fig. 1B). The adsorption of *Lactobacillus* phages is also stable in a wide range of temperatures (0 to 37 °C) (Watanabe et al., 1993). However, for *Leuconostoc* phages Ln-7 and Ln-8, adsorption was high between 0 °C and 42 °C (>94%), but decreased at 50 °C (<79%). The latter temperature is rarely (if at all) used in dairy fermentations. For phage CHB, its adsorption started to slightly reduce (<77%) around 40 °C. Taken altogether, maximum phage adsorption was always achieved at the optimum growth temperature of *Ln. mesenteroides* strains (28 °C–32 °C).

No significant influence of calcium ions was observed on adsorption kinetics for the seven *Ln. mesenteroides* phages studied (data not shown). *Ln. mesenteroides* phages revealed a fast adsorption, higher than 90% after 5 min of incubation, while the maximum adsorption (99%) was reached at 20 min. Arendt et al. (1991) reported slightly lower adsorption rates (between 85% and 87%) for phage P58I (*Leuconostoc oenos*, currently *Oenococcus oeni*). Still, calcium was essential for cell lysis in MRS broth for phages CyC, Ln-7, Ln-8 and Ln-9. On the contrary, phages LDG, CHA and CHB did not need calcium to effectively lyse their host strains in MRS broth, though lysis plaques in MRS plates were smaller in its absence. Influence of calcium on lytic cycle was also reported to be variable for phages of different LAB species and even within a given species. *Lc. lactis* phages QF9 and CHD (Suárez et al., 2008), *Lb. delbrueckii* phages YAB and Ib_3 (Quiberoni et al., 2004) and six *S. thermophilus* phages (Binetti et al., 2002) efficiently completed their lytic cycles (as shown by total cell lysis) only in the presence of calcium ions. For these phages, calcium is likely essential for penetration of phage DNA inside the bacterial cell (Watanabe et al., 1991). In contrast, this cation was not essential for *Lb. delbrueckii* phages BYM and LL-H (Quiberoni et al., 2004) and for *Lb. casei*/*paracasei* phages (Capra et al., 2006), as cell lysis was fast in MRS broth without addition of calcium, even though lysis plaques were smaller in its absence. Many lactococcal phages of the widely dispersed 936 group require calcium to release new virions (Geller et al., 2005). In the presence of calcium ions, the baseplate of the lactococcal phage p2 (936) significantly rotates to open up a channel at the bottom of the baseplate for DNA passage as well as redirects the receptor-binding proteins towards the host cell

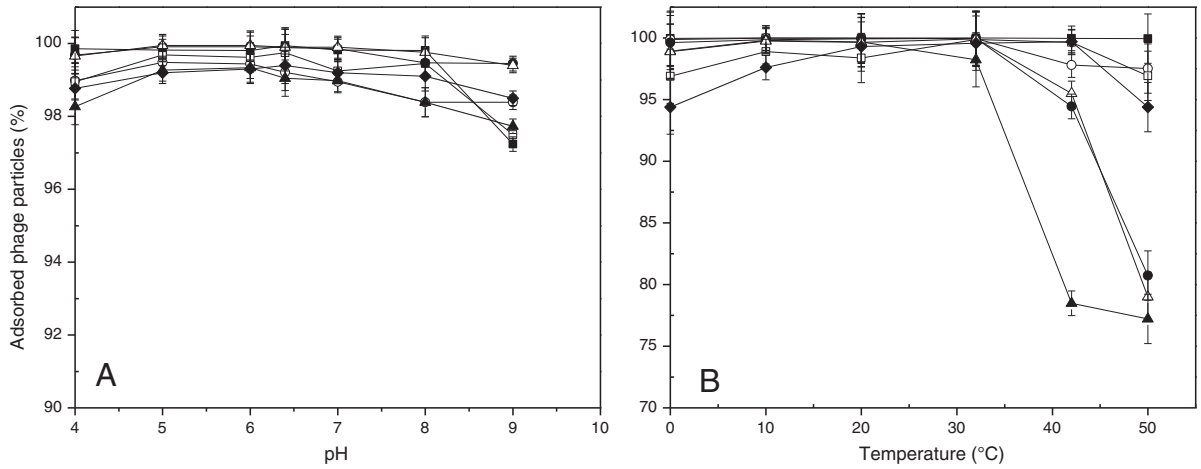

Fig. 1. Influence of pH (A) or temperature (B) on the adsorption (20 min, MRS-Ca broth) of phages LDG (■), CHA (○), CHB (▲), CyC (□), Ln-7 (●), Ln-8 (△) and Ln-9 (♦) on their corresponding host strain. Values are the mean of three determinations ± SD.

surface (Sciara et al., 2010). The adsorption of lactococcal phage 1358 to its host is Ca^{2+} independent, but this cation is mandatory to complete its lytic cycle (Spinelli et al., 2014). Other lactococcal phages do not require calcium (Veesler et al., 2012). Therefore, the need for calcium appears to be phage-dependent as also illustrated with the *Leuconostoc* phages studied here.

The adsorption kinetics were also determined on three types of cells, namely viable, nonviable and non-proliferating. As described above, the nonviable cells were obtained by heat treatment while non-proliferating cells by using chloramphenicol. Adsorption levels of phages CyC and Ln-9 were similar on viable and heat-killed cells (>99%). For phages LDG, CHA, CHB, Ln-7 and Ln-8, adsorption on viable cells was very high (>99%) while it was slightly lower (94–98%) on nonviable cells (data not shown). Adsorption to heat-killed cells was studied for other LAB phages. *Lactococcus* phages (Suárez et al., 2008) showed a slightly higher adsorption rate on viable cells (97.5–99.6%) than on heat-treated ones (82–92%). Contrarily, adsorption of *S. thermophilus* phages was not significantly different when viable and non-viable heated cells were compared (Binetti et al., 2002). Since thermal treatment might affect the structure of phage receptors, non-proliferating cells were obtained by addition of chloramphenicol as a protein-synthesis inhibitor (Briggiler Marcó et al., 2010). Adsorption levels were identical on chloramphenicol-treated and non-treated cells (>99%). Similar behavior was exhibited by *Lb. plantarum* phages (Briggiler Marcó et al., 2010). On the other hand, phage receptors may be more thermoresistant in *Ln. mesenteroides* than in other LAB species, since adsorption on heat-treated cells only slightly decreased in comparison to non-heated cells. Interestingly, the addition of heat-treated cells has been previously proposed as a method of phage decontaminaton in a fermentation medium (Gruss, 1994).

Regarding the influence of MOI, phage adsorption was maximum and constant (≥99%) at values between 1 and 10^{-4} (data not shown) for all *Ln. mesenteroides* phages. The ratio between number of phages adsorbed per cell and MOIc (defined in *Materials and methods* section) is very close to 1 for MOIc ≤ 1, when the number of phages adsorbed is proportional to initial phage number. However, that ratio progressively decreased as initial phage particles started to outnumber phage-sensitive cells (MOIc ≥ 1) (Fig. 2). At higher MOIc, the number of phages particles adsorbed per cell reached a limit of about 3–4. This could be due to the existence of a finite number of available sites for phage attachment on the cell wall, or to some kind of steric impediment.

3.2. One-step growth curves

One-step growth curves allowed calculation of some phage multiplication parameters (Fig. 3). Latent periods were lower than 62 min, burst times ranged from 54 to 96 min, and burst sizes (mean values) from 29 to 111 pfu per infection center. A few studies about multiplication parameters of *Leuconostoc* phages have been conducted. Arendt et al. (1991) reported burst sizes of 16–20 pfu per infection center and latent period of 60 min for phages P58I and P58II (*Ln. oneos*). Sozzi et al. (1978) found a burst size of 170 pfu per infection center and a burst time of 72 min for phage pro2 (*Ln. mesenteroides*).

3.3. Genome analysis of phages Ln-8 and Ln-9

Ln. mesenteroides phages Ln-8 and Ln-9 have dsDNA genomes of 28.5 and 28.9 kb, respectively, sizes that are similar to those reported for other *Ln. mesenteroides* phages (Kot et al., 2014a). The GC content of both genomes (36%) is also similar to that reported for *Ln. mesenteroides* subsp. *mesenteroides* strains (37%) and other *Leuconostoc* phages (Makarova et al., 2006; Kot et al., 2014a). Analysis of the genome extremities indicated that Ln-8 and Ln-9 are *cos*-type phages. The *cos*-sites of phages Ln-8 (5′-GGTTAATAGTAGTCTTTTTGAA-3′) and Ln-9 (5′-GGTTAATAGTAGTCTTTTTTAA-3′) were similar to the 22-nt *cos*-site reported for *Leuconostoc* phage Φ1-A4 (Lu et al., 2010). Bioinformatic analysis revealed the presence of 45 and 48 open reading frames (*orfs*) for Ln-8 (Table 1) and Ln-9 (Table 2), respectively. Predicted *orfs* were numbered consecutively, starting from one of the *cos*-sites on

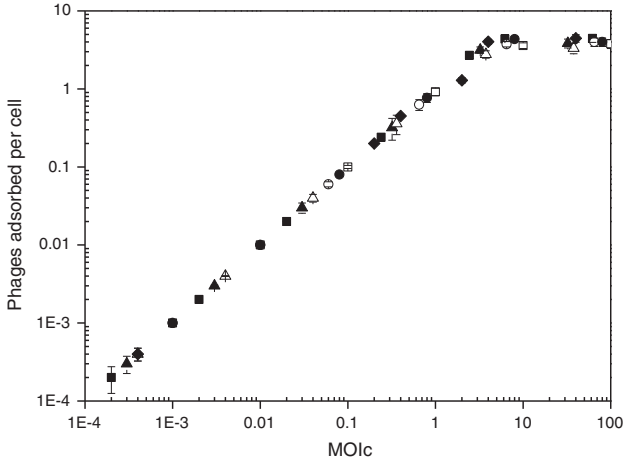

Fig. 2. *Ln. mesenteroides* phages adsorbed per cell. Cell numbers were calculated, for each strain, as cfu × average number of cocci per chain. Symbols indicate phages LDG (■), CHA (○), CHB (▲), CyC (□), Ln-7 (●), Ln-8 (△) and Ln-9 (♦).

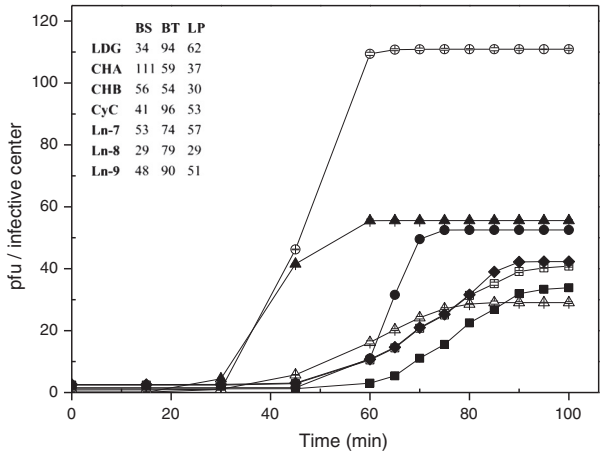

Fig. 3. One-step growth curves of phages LDG (■), CHA (○), CHB (▲), CyC (□), Ln-7 (●), Ln-8 (△) and Ln-9 (⬥). Latent period (LP); burst time (BT) and burst size (BS) were calculated with DMFit web edition using the complete model of Baranyi and Roberts (1994) for data fitting. Values are the mean of three determinations ± SD.

the linear genome. Putative functions of the genes are detailed in Tables 1 and 2 for phages Ln-8 and Ln-9, respectively. Each *orf* is preceded by a region sharing similarities with the Shine–Dalgarno sequence complementary to the 3′ end of the 16S rRNA of *Ln. mesenteroides* (TAGGAGGT; Mahanivong et al., 2001). The genome of phage Ln-8 has 43 ORFs starting with an ATG and 2 ORFs with GTG as initial codons for translation. All the ORFs of phage Ln-9 have an ATG as initial codon for translation. No tRNA was found in both phage genomes.

Comparative analyses revealed that the genomes of Argentinian phages Ln-8 and Ln-9 were highly similar to those of *Ln. mesenteroides* phages ΦLN25 (Kot et al., 2014a) and Φ1-A4 (Lu et al., 2010), with phage Ln-8 more related to ΦLN25 (80%) and Ln-9 to Φ1-A4 (83%) (Fig. 4). Interestingly, phage Φ1-A4 was isolated in the Unites States from sauerkraut fermentation while phage ΦLN25 was isolated from a whey sample from England. This low diversity in *Ln. mesenteroides* phages could be an intrinsic feature, and/or the consequence of a limited number of *Ln. mesenteroides* strains used all over the world.

These phage genomes are organized into the following highly conserved functional modules: replication, packaging, morphogenesis, cell lysis and gene regulation. For phage Ln-9, 26 out of 48 ORFs were assigned with a putative function based on their similarities to proteins with known functions or conserved motifs. In addition, 5 ORFs had no

Table 1
Open reading frames deduced from the genome of *Ln. mesenteroides* phage Ln-8 and their predicted functions.

ORF	Start (bp)	Stop (bp)	Size[a] (aa)	MM (kDa)	pI	Putative RBS and start codon[b]	Predicted function	Best match (% amino acid identity)	Size[c] (aa)
1	286	588	100	11.6	8.3	TAGGAGGTttgaaag**ATG**	Phage HNH endonuclease	ORF1 phage ΦLN25 (100%)	100
2	601	777	58	6.9	9.4	AAGGATAAttaat**ATG**	Hypothetical protein	ORF2 phage ΦLN25 (100%)	58
3	779	1216	145	16.7	6.9	GAAGAGGTatcaaaaa**ATG**	Phage-related protein	ORF3 phage ΦLN25 (99%)	146
4	1213	1359	48	6.0	12.0	AAGGAGCAaaaaagaa**ATG**	Hypothetical protein	ORF4 phage ΦLN25 (100%)	48
5	1356	1730	124	14.9	7.8	CAGGAGGAtaatat**ATG**	Endodeoxyribonuclease	ORF5 phage ΦLN25 (100%)	124
6	2155	3507	450	52.1	5.3	CAGGAGGAaacgcta**ATG**	DNA helicase	ORF1 phage Φ1-A4 (98%)	450
7	3504	4253	249	28.7	5.6	AAAGGGTTgacga**ATG**	DNA primase/polymerase	ORF7 phage ΦLN25 (98%)	251
8	4262	6097	611	69.7	6.7	TAGGAGTAatc**ATG**	DNA polymerase	ORF8 phage ΦLN25 (96%)	611
9	6155	6727	190	21.6	4.9	AAGGAGAAtttatat**ATG**	Hypothetical protein	ORF9 phage ΦLN25 (100%)	190
10	6780	7412	210	24.0	5.6	AGGGAGGAattaaaat**ATG**	Hydrolase	ORF10 phage ΦLN25 (99%)	210
11	7424	7750	108	12.5	5.2	TAGGAGGTaaatat**ATG**	Terminase small subunit	ORF11 phage ΦLN25 (96%)	108
12	7761	7958	65	7.8	9.2	TAGGAGGTtttat**ATG**	Hypothetical protein	ORF12 phage ΦLN25 (100%)	65
13	8023	8181	52	5.9	10.1	AAAATCATtccccagt**ATG**	Terminase small subunit	ORF9 phage Φ1-A4 (88%)	52
14	8165	9811	548	63.1	5.1	AAGGAGGGtaatag**ATG**	Terminase large subunit	ORF13 phage ΦLN25 (100%)	548
15	9824	10,945	373	43.0	4.9	AAGGAGAAaactat**ATG**	Portal protein	ORF14 phage ΦLN25 (99%)	373
16	10,905	11,630	241	26.2	4.3	CAGGAGACactacga**ATG**	Phage prohead protease	ORF14 phage ΦLNTR2 (90%)	239
17	11,682	12,635	317	34.7	5.2	AAGGAGACctataat**ATG**	Major capsid protein	ORF15 phage ΦLN34) (99%)	321
18	12,785	13,057	90	10.4	4.3	TAGGAGGTgacaca**ATG**	Hypothetical protein	ORF17 phage ΦLN25 (94%)	90
19	13,047	13,325	92	10.6	10.1	GAGGAGGCgattagat**ATG**	Phage tail protein	ORF18 phage ΦLN25 (100%)	92
20	13,325	13,642	105	12.3	4.6	GGGGAGGTaatcattta**ATG**	Hypothetical protein	ORF19 phage ΦLN25 (98%)	105
21	13,639	13,968	109	12.4	10.9	AGACAGGTgttaatatt**ATG**	Hypothetical protein	ORF20 phage ΦLN25 (98%)	109
22	14,017	14,598	193	21.3	4.8	AAGGAGAAttaatcaatt**ATG**	Major tail protein	ORF21 phage ΦLN25 (98%)	193
23	14,672	17,239	855	86.7	7.7	AAGGAAATgtattat**ATG**	Phage tail tape measure protein	ORF22 phage ΦLN25 (98%)	889
24	17,303	18,499	398	45.5	4.7	ATGGAGGAaattat**ATG**	Hypothetical protein	ORF22 phage ΦLNTR2 (94%)	398
25	18,502	19,500	332	36.9	4.8	TAGGAGATtaatc**ATG**	Structural Protein	ORF24 phage ΦLN25 (99%)	332
26	19,515	19,655	46	5.4	9.8	AAGGTGGTtctaaatactatagt**GTG**	Hypothetical protein	ORF21 phage ΦLmd1 (53%)	83
27	19,708	20,109	133	15.8	4.8	AAGGAGAAttaaagac**ATG**	Hypothetical protein	ORF25 phage ΦLN25 (98%)	133
28	20,190	20,954	254	27.9	7.8	GAGGAGATctaaaaat**ATG**	Receptor-binding tail protein	ORF26 phage ΦLN25 (99%)	255
29	20,986	21,357	123	14.0	6.4	AAGGAGACcccacatt**ATG**	Holin_I	ORF27 phage ΦLN25 (100%)	123
30	21,424	22,161	245	28.9	4.9	AAGGAGATgggggtt**ATG**	Hypothetical methylotransferase	ORF28 phage ΦLN25 (98%)	242
31	22,158	22,322	54	6.4	8.1	TGGGAGAGaagta**ATG**	Hypothetical protein	ORF29 phage ΦLN25 (100%)	54
32	22,322	22,591	89	10.2	9.0	AAGGAGAAaacaa**ATG**	Putative gag-pol polyprotein	ORF30 phage ΦLN25 (100%)	89
33	22,588	22,827	79	9.3	9.5	ATTGAGGTattata**ATG**	Hypothetical protein	ORF31 phage ΦLN25 (100%)	76
34	22,827	23,063	78	9.1	6.8	AAGGGAAAccatt**ATG**	Hypothetical protein	ORF32 phage ΦLN25 (100%)	78
35	23,053	23,268	71	8.9	7.9	AGAGAGGTtcgcaagta**ATG**	Hypothetical protein	ORF33 phage ΦLN25 (100%)	71
36	23,268	23,519	83	8.7	9.8	AAGGAGAAaacaa**ATG**	Phage-related hydrogenase	ORF34 phage ΦLN25 (100%)	83
37	23,594	23,764	56	6.0	8.6	AAAGAGGAacaaac**GTG**	Hypothetical protein	ORF33 phage Φ1-A4 (95%)	56
38	23,886	24,767	293	31.5	6.2	AAGGAGGAaaagtaat**ATG**	Phage-related amidase	ORF36 phage ΦLN25 (100%)	293
39	24,769	25,116	115	12.6	9.3	ATGGAGGAcacatt**ATG**	Holin_II	ORF34 phage ΦLN34) (91%)	115
40	25,142	27,136	664	72.6	5.5	TAGGAGGAttattat**ATG**	Neck passage structure protein	ORF38 phage ΦLN25 (93%)	664
41	27,246	27,563	105	12.0	5.6	AAGGAGATataaa**ATG**	Hypothetical protein	ORF39 phage ΦLN25 (98%)	105
42	27,565	27,762	65	7.8	4.6	TATGTGGTaaatgtg**ATG**	Hypothetical protein	ORF38 phage ΦLN34) (91%)	69
43	27,768	28,055	95	11.3	6.3	GAGGAAGTgagcaa**ATG**	Hypothetical protein	ORF41 phage Φ1-A4 (57%)	91
44	28,052	28,303	83	9.9	4.2	AGGGAGATaacaa**ATG**	Hypothetical protein	ORF40 phage ΦLN25 (98%)	87
45	28,449	28,619	56	6.4	6.2	AAGGAAATaatatatgac**ATG**	Repressor	ORF41 phage ΦLN25 (96%)	56

Table 2
Open reading frames deduced from the genome of *Leuconostoc mesenteroides* phage Ln-9 and their predicted functions.

ORF	Start (bp)	Stop (bp)	Size (nt)	Size[a] (aa)	MM (kDa)	pI	Putative RBS and start codon[b]	Predicted function	Best match (% amino acid identity)	Size[c] (aa)
1	680	982	303	100	11.6	8.3	AGGAGGTtttaaag**ATG**	Phage HNH endonuclease	ORF1 phage ΦLN25 (99%)	100
2	995	1171	177	58	6.9	9.4	GATAAGGAtaattaat**ATG**	Hypothetical protein	ORF2 phage ΦLN25 (93%)	58
3	1173	1610	438	145	16.7	6.2	AAGGAGCAaagaagaa**ATG**	Phage-related protein	ORF2 phage phiLN34 (94%)	145
4	1607	1981	375	124	14.9	7.8	CAGGAGGAtaatat**ATG**	Endodeoxyribonuclease	ORF5 phage ΦLN25 (100%)	124
5	2408	3760	1353	450	52.1	5.2	CAGGAGGAaaacaatt**ATG**	DNA helicase	ORF1 phage Φ1-A4 (99%)	450
6	3757	4512	756	251	29.1	6.0	AAGGATTGacga**ATG**	DNA primase/polymerase	ORF3 phage Φ1-A4 (96%)	251
7	4588	5145	558	185	21.2	5.4	TAGGAGAAaaaac**ATG**	DNA polymerase	ORF4 phage Φ1-A4 (84%)	182
8	5142	6977	1836	611	69.5	8.0	AATAAGGCaaaaccaatc**ATG**	DNA polymerase	ORF8 phage ΦLN25 (96%)	611
9	7035	7607	573	190	21.6	4.9	AAGGAGAAtttatat**ATG**	Hypothetical protein	ORF9 phage ΦLN25 (99%)	190
10	7660	8292	633	210	24.0	5.6	AGGGAGGAattaaaac**ATG**	Hydrolase	ORF7 phage Φ1-A4 (99%)	210
11	8303	8629	327	108	12.4	5.2	TAGGAGGTaatat**ATG**	Terminase small subunit	ORF11 phage ΦLNTR2 (98%)	108
12	8691	8849	159	52	5.8	10.6	AAAATCATtccccagt**ATG**	Terminase small subunit	ORF9 phage Φ1-A4 (92%)	52
13	8833	10,479	1647	548	62.9	5.0	GAGGAGGGcaatag**ATG**	Terminase large subunit	ORF10 phage Φ1-A4 (99%)	548
14	10,492	11,613	1122	373	43.0	4.9	AAGGAGAAaactat**ATG**	Portal protein	ORF11 phage Φ1-A4 (98%)	373
15	11,573	12,298	726	241	26.2	4.3	CAGGAGACactacga**ATG**	Phage prohead protease	ORF14 phage ΦLNTR2 (90%)	241
16	12,350	13,303	954	317	34.7	5.2	AAGGAGACctataat**ATG**	Major capsid protein	ORF15 phage ΦLN34 (99%)	317
17	13,379	13,651	273	90	10.4	4.2	TAGGAGGTgacaca**ATG**	Hypothetical protein	ORF17 phage ΦLN25 (96%)	90
18	13,641	13,919	279	92	10.6	10.1	GAGGAGGCgatcagat**ATG**	Phage tail protein	ORF15 phage Φ1-A4 (100%)	92
19	13,919	14,236	318	105	12.3	4.5	GGGGAGGTaatcattta**ATG**	Hypothetical protein	ORF19 phage ΦLN25 (98%)	105
20	14,233	14,562	330	109	12.3	10.9	AGGCAGGTgttaatatt**ATG**	Hypothetical protein	ORF17 phage Φ1-A4 (98%)	109
21	14,611	15,186	576	191	21.1	4.8	AAGGAGAAttaatcaatt**ATG**	Major tail protein	ORF18 phage Φ1-A4 (93%)	193
22	15,313	17,982	2670	889	90.9	8.6	AAGGAAATgtattat**ATG**	Phage tail tape measure protein	ORF19 phage Φ1-A4 (95%)	889
23	17,995	18,129	135	44	5.1	9.0	AATAAGGAgacgat**ATG**	–		
24	18,176	19,372	1197	398	45.2	4.7	ATGGAGGAatttac**ATG**	Hypothetical protein	ORF20 phage Φ1-A4 (68%)	398
25	19,375	20,373	999	332	37.3	4.8	TAGGAGATtaaaa**ATG**	Base plate	ORF21 phage Φ1-A4 (71%)	332
26	20,388	20,606	219	72	8.3	9.7	AAAGAGGTaa**TTG**	Hypothetical protein	ORF21 phage ΦLmd1 (56%)	83
27	20,581	20,982	402	133	15.7	4.8	AAGGAGAAttaaagac**ATG**	Hypothetical protein	ORF22 phage Φ1-A4 (96%)	133
28	21,062	21,832	771	256	28.6	5.0	GAGGAGATttaaaat**ATG**	Receptor-binding tail protein	ORF23 phage Φ1-A4 (56%)	255
29	21,868	22,239	372	123	14.0	6.4	AAGGAGACcccacatt**ATG**	Holin_I	ORF27 phage ΦLN25 (97%)	123
30	22,310	22,477	168	55	6.9	10.8	AAGGAGATggattt**ATG**	–		
31	22,474	22,638	165	54	6.4	4.8	TTGGAGAGaagta**ATG**	Hypothetical protein	ORF27 phage ΦLNTR2 (89%)	54
32	22,638	22,868	231	76	9.0	9.3	TTGGAGCGtaatc**ATG**	Hypothetical protein	ORF27 phage Φ1-A4 (96%)	76
33	22,861	23,106	246	81	9.6	9.7	ATTGAGGTtctatg**ATG**	–		
34	23,106	23,342	237	78	9.1	6.0	AAGGAACGagaaa**ATG**	Hypothetical protein	ORF28 phage Φ1-A4 (92%)	78
35	23,332	23,463	132	43	5.4	4.8	AAGGAAAAccgtt**ATG**	–		
36	23,450	23,668	219	72	9.2	7.9	AGAGAGGTtcgcaagta**ATG**	Hypothetical protein	ORF29 phage Φ1-A4 (85%)	71
37	23,668	24,168	501	166	18.8	9.4	AAGGAGAAaacaa**ATG**	Phage-related hydrogenase	ORF31 phage Φ1-A4 (96%)	166
38	24,244	24,414	171	56	6.1	8.4	AAGGAGAAaaa**ATG**	Hypothetical protein	ORF33 phage Φ1-A4 (95%)	56
39	24,528	25,409	882	293	31.6	6.0	AAGGAGGAaaaataac**ATG**	Phage-related amidase	ORF36 phage ΦLN25 (98%)	293
40	25,411	25,758	348	115	12.6	9.3	GATGAGGTaacaa**ATG**	Holin_II	ORF36 phage Φ1-A4 (96%)	94
41	25,751	26,023	273	90	9.8	4.0	TAGGAGATaaatt**ATG**	Hypothetical protein	ORF37 phage Φ1-A4 (90%)	121
42	26,034	26,756	723	240	26.0	7.8	TAGGAGGAattaatttttt**ATG**	Baseplate protein	ORF38 phage Φ1-A4 (37%)	244
43	26,823	27,140	318	105	11.9	9.1	AGGGAGGAgttctaac**ATG**	Hypothetical protein	ORF39 phage Φ1-A4 (94%)	105
44	27,142	27,285	144	47	5.6	4.4	AAGGAGATataat**ATG**	–		
45	27,287	27,484	198	65	7.6	4.7	AGTTTGGTaaatgtg**ATG**	Hypothetical protein	ORF41 phage Φ1-A4 (91%)	69
46	27,490	27,765	276	91	10.7	4.6	AAGGAAGTgagcaa**ATG**	Hypothetical protein	ORF42 phage Φ1-A4 (87%)	91
47	27,762	28,013	252	83	9.8	4.2	AGGGAGAAtaacaa**ATG**	Hypothetical protein	ORF43 phage Φ1-A4 (66%)	83
48	28,162	28,341	180	59	6.8	6.2	ATTGTTGTtta**ATG**	Repressor	ORF45 phage Φ1-A4 (93%)	56

[a] Number of amino acids (aa) of the predicted protein.
[b] Underlined, boldfaced indicate nucleotides identical to the consensus RBS; lowercase indicate spacer nucleotides between the RBS and start codon; boldfaced indicate start codon.
[c] Number of amino acids (aa) of the best-matched protein.

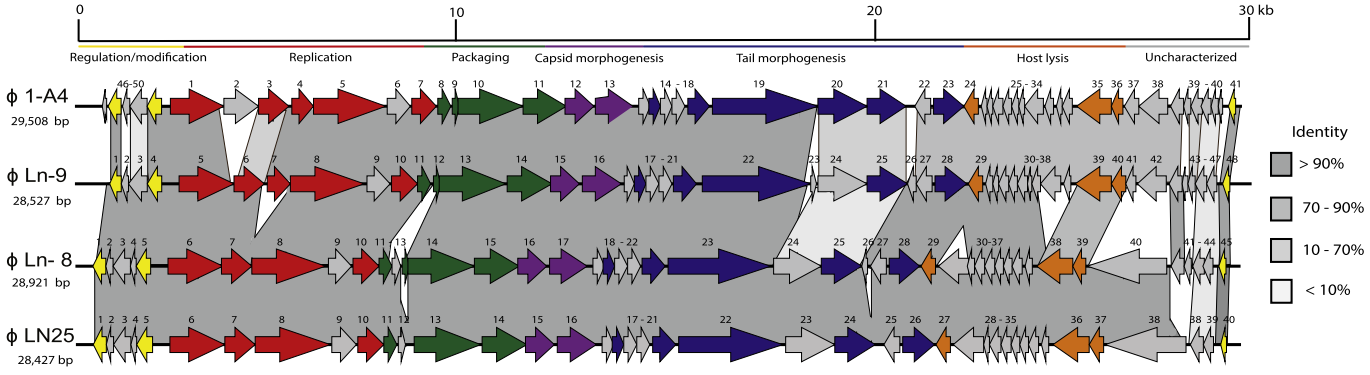

Fig. 4. Schematic overview of four *Leuconostoc mesenteroides* phage genomes. ORFs are indicated by numbered-colored arrows. Arrows represent putative proteins and their color indicates the corresponding module. ORFs connected by a grey box show homologies at the amino acid level; the grey tone indicates percentage of identity. Uncharacterized ORFs are displayed as grey arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

homology to any known sequences and 18 ORFs exhibited similarities to uncharacterized database entries (conserved hypothetical proteins). On the other hand, for phage Ln-8, 24 out of 45 ORF were assigned with a putative function, and 21 exhibited similarities to uncharacterized database entries.

3.4. Protein identification

The structural proteins of phage Ln-8 were analyzed by mass spectrometry and 13 protein were detected, including ORF15 (portal protein), ORF16 (prohead protease), ORF17 (major capsid protein), ORF18 (hypothetical protein), ORF19 (tail protein), ORF20 (hypothetical protein), ORF21 (hypothetical protein), ORF22 (major tail protein), ORF23 (tail tape measure protein), ORF24 (hypothetical protein), ORF25 (baseplate protein), ORF28 (receptor-binding tail protein), and ORF40 (neck passage structure protein). The ORF16 had the lowest coverage and it is unclear at this time if this protein is in the mature phage structure or if it was co-purified with complete or immature virions particles.

4. Conclusions

In this work, we demonstrated that the adsorption of *Ln. mesenteroides* phages to their host strains was a rapid and efficient process. Moreover, we showed that their adsorption was not affected by pH, temperature, the presence of calcium and the physiological state of the cells. A detailed understanding of the first step of phage infection will allow designing strategies to minimize the consequences of such attacks in dairies. For example, the use of chelating agents in the growth medium (i.e., phosphate-containing media) to sequester calcium may offer some protection against *Leuconostoc* phages during the growth of dairy cultures (Moineau and Lévesque, 2005). We also provided the first genomic sequences of *Ln. mesenteroides* phages isolated from Argentina. The overall composition of the modules in both phages was highly similar to previously studied virulent *Ln. mesenteroides* phages. The high identity level between these phages is remarkable considering the distant geographical locations from where they were isolated. While this limited phage diversity is likely due to the few commercial *Ln. mesenteroides* strains used worldwide, it also suggests that the applications of anti-phage control measures, including the use of phage resistance mechanisms, may be relevant for several processes using *Leuconostoc* strains.

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