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Technological performance of spontaneous phage-resistant derivatives of *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* during milk fermentation

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ABSTRACT

A total of 142 presumptive phage-resistant derivatives were isolated from six commercial phage-sensitive *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* strains, using six phages either individually or in combination (cocktail). Genetic diversity, efficiency of plaquing (EOP), phage-resistance stability, lysogeny, adsorption rates and technological performance were determined for the 89 confirmed phage-resistant variants. Derivatives showed very low EOP values ($<10^{-10}$) and high stability of phage-resistance phenotype. Some mutants showed low adsorption rates (10–42%) thus indicating adsorption interferences. Additional resistance mechanisms (operative at later stages) were suggested for mutants revealing high phage adsorption rates. A good performance during milk fermentation and the subsequent refrigerated storage in the presence of phages was demonstrated for four mutants, selected on the basis of their stability and low or null phage adsorption. These phage-resistant variants could be used in industrial rotation schemes when commercial strains become sensitive to phages present in the environment.

1. Introduction

Bacteria of the genus *Leuconostoc* are incorporated into dairy starter cultures due to their ability to produce important metabolites such as diacetyl and CO_2 (Hemme, 2012; Server-Busson, Foucaud, & Leveau, 1999). Specifically, CO_2 expands the mechanical openings in blue-veined cheeses, where *Penicillium roqueforti* is able to colonise the eyes formed. However, production of these metabolites can be affected by phage infections.

Leuconostoc phages cause failures during the fermentation of several foods, including wine, various types of cheese (blue cheese, Camembert, Cottage, Edam, Cream cheese) and other dairy products (Ali et al., 2013; Wagner et al., 2017). In previous studies (Pujato et al., 2014), a variety of virulent *Leuconostoc* phages were isolated from blue-veined cheese manufacture, where the lack of curd openness was the most outstanding failure. Numerous strategies can be applied to minimise phage dissemination in dairy plants, such as strain rotation programs, use of direct vat inoculation of starters, optimised sanitation and use of phage-resistant starter cultures (Carminati et al., 2016; Moineau, Tremblay, & Labrie, 2002). In particular, the isolation of spontaneous bacteriophage-insensitive mutants (BIMs) is a simple and natural strategy, since there are no regulatory restrictions regarding the use of these improved strains in industrial environments (Emond & Moineau, 2007; Moineau & Lévesque, 2005; Zago et al., 2017). Some authors have reported the successful isolation of BIMs from several lactic acid bacteria (LAB), such as *Lactococcus* (Coffey, Coakley, Mc Garry, Fitzgerald, & Ross, 1998; Limsowtin & Terzaghi, 1976; Weimer, Blake, Hillier, & Davidson, 1993), *Streptococcus thermophilus* (Binetti, Bailo, & Reinheimer, 2007; Viscardi et al., 2003), *Lactobacillus helveticus* (Carminati, Zennaro, Neviani, & Giraffa, 1993; Quiberoni, Reinheimer, & Tailliez, 1998b), *Lactobacillus delbrueckii* (Guglielmotti et al., 2006), *Lactobacillus paracasei* (Capra, Mercanti, Rossetti, Reinheimer, & Quiberoni, 2011) and *Lactobacillus plantarum* (Briggiler Marcó, Mercanti, Reinheimer, & Quiberoni, 2011). Nevertheless, there are no published articles reporting the isolation of BIMs from *Leuconostoc* strains with promising technological capabilities.

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The aim of this work was to isolate spontaneous BIMs, derived from six phage-sensitive *Leuconostoc* strains, with adequate technological characteristics for industrial use.

2. Material and methods

2.1. Bacterial strains, phages and culture conditions

Spontaneous BIMs were obtained from five phage-sensitive *Leuconostoc mesenteroides* strains (identified as CH19A, CH19B, D4d, D6a and L79-1) and one *Leuconostoc pseudomesenteroides* strain (identified as R707), isolated from commercial mesophilic mixed starters used in the manufacture of blue-veined cheeses (Pujato et al., 2014). For this aim, six autochthonous *Leuconostoc* phages (LDG, CHA, CHB, Ln-7, Ln-8 and Ln-9) isolated from Argentinean dairy plants (Pujato et al., 2014; 2015), were employed. These *Leuconostoc* phages are capable of infecting all the strains used in this study, although with diverse efficiency (Pujato, Guglielmotti, Martínez-García, Quiberoni, & Mojica, 2017). The strain used for initial isolation of each phage was defined as its indicator. Phage-sensitive strains and their mutants were grown and routinely reactivated overnight (16–18 h, 30 °C) in de Man, Rogosa and Sharpe (MRS) broth or agar (Biokar, Beauvais, France). For milk fermentation assays, commercial strain *Lactococcus lactis* DX33 (INLAIN collection) was used as starter culture, and routinely reactivated and grown overnight (30 °C) in Elliker broth (Biokar). All the strains were maintained as frozen (−80 °C) stocks in MRS or Elliker broth added of glycerol (15%, v/v). Phage enumeration was carried out by the double-layer plaque titration method (Svensson & Christiansson, 1991), and expressed as plaque-forming units per millilitre (pfu mL^{-1}).

2.2. Isolation of BIMs from sensitive strains

The secondary culture technique (Carminati et al., 1993), modified as stated later, was applied to obtain BIMs from the six *Leuc. mesenteroides* and *Leuc. pseudomesenteroides* strains. Overnight cultures of each indicator strain in MRS-Ca broth (MRS broth supplemented with 10 mM $CaCl_2$) were infected with suspensions of the corresponding lytic phage at a multiplicity of infection (MOI) of 0.1 (Table 1). In addition, *Leuc. mesenteroides* L79-1 was infected with a cocktail of phages LDG, CHA, CHB, Ln-7, Ln-8 and Ln-9. Cultures exhibiting complete lysis after incubation for 6–8 h at 30 °C and subsequent growth after 2–10 days at 30 °C were selected and streaked on MRS agar plates (48 h at 30 °C). Single colonies were selected and cultured in MRS broth (16 h at 30 °C). These isolates were purified by three consecutive streakings on MRS agar and registered as presumptive BIMs. Phage resistance was confirmed by infecting presumptive BIMs with the corresponding phage or cocktail; strains able to grow normally after three subcultures in MRS-Ca broth (turbidity test) were considered true BIMs and stored at −80 °C in MRS broth supplemented with 15% (v/v) glycerol. The efficiency of recovery of phage-resistant variants was expressed as follows: (number of confirmed phage-resistant variants/number of presumptive phage-resistant variants) × 100.

On the other hand, all the isolated BIMs were tested for stability and level (i.e., efficiency of plaquing, EOP) of phage-resistance phenotype and host spectrum according to Capra et al. (2011). In the case of mutants isolated with the cocktail of six phages, the stability was assayed using this cocktail.

2.3. Phage-resistance mechanisms

Lysogeny and adsorption rates were determined for a total of 21 BIMs, derived from *Leuc. pseudomesenteroides* R707 using phage LDG (six strains), *Leuc. mesenteroides* D6a using phage Ln-8 (six strains) and *Leuc. mesenteroides* L79-1 using phage Ln-9 (six strains) or the phage cocktail (three strains). Adsorption assays were carried out at 30 °C for 20 min (Pujato et al., 2015), employing the same phage used to isolate the corresponding BIM; for those BIMs isolated with the phage cocktail, adsorption of each phage of the cocktail was independently tested. To evaluate the spontaneous release of phage particles, an overnight culture of each BIM was centrifuged (10,000 × *g*, 5 min) and aliquots of the supernatant were titred, separately, on the respective phage-sensitive *Leuc. pseudomesenteroides* or *Leuc. mesenteroides* strain and on the BIM itself, using the double layer titration method, as described by Guglielmotti et al. (2006). Incubations were carried out at 30 °C for 16 h. The presence of visible lysis plaques was considered evidence of phage particles spontaneously released from the BIMs.

2.4. Genotypic characterisation of BIMs

Bacterial DNA was obtained using the GenElute™ Bacterial Genomic DNA kit (Sigma, St. Louis, MO, USA), according to the manufacturer's instructions, and quantified by electrophoresis on 1% (w/v) agarose gels in 1 × TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0), after staining with GelRed™ (Biotium, Inc., Hayward, CA, U.S.A.) as nucleic acid binding dye (Pujato et al., 2014).

Random amplification of polymorphic DNA (RAPD-PCR) was applied to phage-sensitive strains and their BIMs to determine genetic diversity. Two primers namely 1254 (5′-CCGCAGCCAA-3′; Akopyanz, Bukanov, Westblom, Kresovich, & Berg, 1992) and M13 (5′-GAGGGTGGCGGTTCT-3′; Huey & Hall, 1989; Stendid, Karlsson, & Hogberg, 1994) were assayed in separate PCR reactions. Amplification conditions were performed according to Pujato et al. (2014). PCR products were analysed by electrophoresis in 1.2% (w/v) agarose gels stained with GelRed™.

2.5. Characterisation of phage-resistant variants

Bacterial cells of all the indicator strains and their BIMs were observed and compared using a Jenamed 2 Carl Zeiss (Jena, Germany) phase-contrast microscope. Colony morphology was observed from streaking in MRS agar.

Acidification kinetics were evaluated by strain growth in milk. With this purpose, strains were inoculated (2%, v/v) in reconstituted commercial skim milk (RSM; 10%, w/v) and incubated for 24 h at 30 °C. Evolution of pH was measured (pH meter model SA 720, Orion, Beverly, Massachusetts, USA) and plotted against time; the acidity developed was determined by titration with NaOH (0.1 M) to pH 8.4, and expressed as percentage (%) of lactic acid.

2.6. Technological performance of BIMs

Technological performance of BIMs was studied in a milk fermentation model. Aliquots of RSM were inoculated with overnight cultures of *L. lactis* DX33 (starter culture; 0.1%, v/v; about 10^7 cfu mL^{-1}) and either phage-sensitive strains (R707, D6a or L79-1; controls) or their BIMs (adjunct cultures; 0.01%, v/v; about 10^6 cfu mL^{-1}). Strain performance in the presence of phages was studied by infecting both phage-sensitive and BIM strains with the corresponding phage or phage cocktail (about 10^4 pfu mL^{-1}; MOI of 10^{-2}), following incubation for 8 h at 30 °C and refrigerated storage at 15 °C (between 8 and 48 h). The acidification proceeded at 30 °C in a thermostatic bath until the non-infected cultures reached a pH value of 4.8. During milk fermentation, evolution of pH was assessed with a pH meter (Orion). Bacterial cell counts (MRS agar

supplemented with vancomycin 30 μg mL^{-1} for *Leuconostoc* and Elliker agar for *Lactococcus*; 48 h at 30 °C) and phage enumeration (if applicable) (Svensson & Christiansson, 1991) were also carried out during milk fermentation.

3. Results and discussion

3.1. Isolation of BIMs from sensitive strains

After complete lysis of the infected cultures, secondary growth was evident for five out of the six *Leuc. mesenteroides* and *Leuc. pseudomesenteroides* strains examined. However, only three of the strains with developed secondary growth rendered true BIMs. Specifically, the 56 presumptive BIMs isolated from *Leuc. mesenteroides* L79-1 and most (87%) variants derived from *Leuc. pseudomesenteroides* R707 were confirmed in their phage-resistance, while this phenotype was validated in only 40% of the presumptive BIMs obtained from *Leuc. mesenteroides* D6a (Table 1). Regarding the 56 BIMs derived from *Leuc. mesenteroides* L79-1, 31 were isolated using phage Ln-9 and 25 using the phage cocktail.

These results show two different situations. On one hand, three (*Leuc. mesenteroides* D6a and L79-1 and *Leuc. pseudomesenteroides* R707) out of the six commercial strains evaluated allowed the isolation of BIMs. All these BIMs exhibited high stability, since they maintained the phage-resistance phenotype after seven sequential subcultures with a phage dose added at each subculture (Table 1). Furthermore, all true BIMs derived from *Leuc. mesenteroides* L79-1, *Leuc. mesenteroides* D6a and *Leuc. pseudomesenteroides* R707 were resistant to phages LDG, CHA, CHB, Ln-7, Ln-8 and Ln-9 during turbidity tests in MRS-Ca broth (data not shown). Also, when phages at high titres (~10^9 pfu mL^{-1}) were used to infect BIMs (MRS-Ca-Gly agar), no plaque forming ability was demonstrated (EOP values <10^{-10}). As acknowledged, BIMs with a high level of resistance to phages (EOP values between 10^{-9} and 10^{-7}) are the basis for the production of defined phage-resistant starter cultures, while those with low resistance (EOP values between 10^{-3} and 10^{-1}) may not be safe during fermentation processes (Moineau & Lévesque, 2005). The second situation revealed no recovery of true BIMs from *Leuc. mesenteroides* C19A, C19B and D4b even after 10 days of post-lysis incubation. This could be attributed to either a likely very low proportion of spontaneous phage-resistant variants naturally present in these bacterial populations (Briggiler Marcó et al., 2011), or the possible absence or the lack of functionality of systems known to be responsible of BIMs generation under phage pressure, such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats).

Table 1
Bacteriophage-insensitive mutants isolated from *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* strains using secondary culture.[a]

Indicator strain	Phage	Number of BIMs isolated		Isolation efficiency of confirmed BIMs (%)	Phage-resistance stability (%)
		Presumptive	Confirmed		
R707	LDG	24	21	87	100
C19A	CHA	0	0	–	–
C19B	CHB	12	0	0	–
D4b	Ln-7	20	0	0	–
D6a	Ln-8	30	12	40	100
L79-1	Ln-9	31	31	100	100
	Phage cocktail	25	25	100	100

[a] R707 is *Leuconostoc pseudomesenteroides*; all other strains are *Leuconostoc mesenteroides*. Phage cocktail comprises LDG, CHA, CHB, Ln-7, Ln-8 and Ln-9. Phage-resistance stability is the percentage of variants able to overcome seven subcultures under phage pressure.

3.2. Phage-resistance mechanisms

According to our results, diverse native phage-resistance mechanisms could be present in the isolated BIMs. Free phage particles were evidenced in the supernatant of only three out of 56 BIMs derived from *Leuc. mesenteroides* L79-1 (L79-3, COC 2 and COC 3) (Table 2). These free phages were able to infect the parent (sensitive) strain and other two phage-sensitive strains (*Leuc. mesenteroides* D6a and *Leuc. pseudomesenteroides* R707), while the BIMs releasing these free phages remained resistant. This result indicates that the phage resistance phenotype of these three BIMs (L79-3, COC 2 and COC 3) could have been acquired by superinfection immunity. As it is known, lysogenic strains constitute a hazardous source of phages during fermentative processes and their use as starters might not be safe, because of the possibility of spontaneous phage release (Garneau & Moineau, 2011). Spontaneous viral particles release was detected for BIMs isolated from *S. thermophilus* 149 (Binetti et al., 2007), *Oenococcus oeni* LOF025 and LOF026 (Poblet-Icart, Bordons, & Lonvaud-Funel, 1998) and from other LAB, but it was not previously reported for *Leuconostoc* strains.

Moreover, the ability of *Leuconostoc* phages to adsorb on BIMs isolated in this study was analysed. With this aim, adsorption assays were performed on the parent strain and the corresponding BIMs isolated (Table 2). After 20 min incubation, over 99% of phage particles from LDG, Ln-8 or Ln-9 were adsorbed to *Leuc. pseudomesenteroides* R707, *Leuc. mesenteroides* D6a and *Leuc. mesenteroides* L79-1, respectively, whereas highly variable adsorption rates were observed on BIMs (Table 2). Particularly, BIMs isolated from *Leuc. pseudomesenteroides* R707 showed moderate levels of adsorption (52–79%). Similarly, BIMs of *Leuc. mesenteroides* D6a revealed adsorption rates between 57% and 61%. A broader range of adsorption rates (10–62%) was observed for BIMs isolated from *Leuc. mesenteroides* L79-1 under pressure of phage Ln-9. Furthermore, phages CHA, CHB and Ln-9 efficiently adsorbed (>99%) on BIMs of *Leuc. mesenteroides* L79-1 obtained using the phage cocktail. However, phages Ln-7 and Ln-8 adsorbed only moderately (35–67%) on these BIMs, and adsorption levels of phage LDG were even lower (27–39%). Phage-sensitive strain *Leuc. mesenteroides* L79-1 showed high adsorption rates (>99%) with all the phages tested.

As previously reported (Boucher & Moineau, 2001), resistance to phage infection gained by BIMs is usually attributed to mutations in phage receptors, which lead to reduced adsorption rates. Zago et al. (2017) showed a comparison of the receptor protein sequence between a phage-sensitive *Lb. helveticus* strain and a BIM thereof, revealing the presence of amino acid deletions, which modified its protein folding and decreased the level of adsorption. Nevertheless, *Leuconostoc* BIMs isolated in this work showed moderate or high adsorption (>42%) of phage particles, except for some BIMs of *Leuc. mesenteroides* L79-1 (among them, L79-2, L79-3 and L79-13), for which phage adsorption rates were lower (<42%). Consequently, further studies are required to clarify and identify phage-resistance mechanisms present in BIMs isolated in this study, such as interference with phage DNA injection, restriction-modification, abortive infection or CRISPR systems.

Mechanisms involved in phage resistance of *Leuconostoc* strains have not been elucidated yet. At present, only one presumptive CRISPR system has been identified in *Leuconostoc*, specifically in the genome of *Leuc. mesenteroides* subsp. *mesenteroides* ATCC 8293 (Makarova et al., 2006). However, the number of identified CRISPR/cas loci considerably increases as more sequencing projects are being undertaken, and this could led to the discovery of new CRISPR system in *Leuconostoc* genomes as well.

3.3. Characterisation of BIMs

Regarding cell and colony morphologies, all BIMs were identical to their corresponding phage-sensitive parent strain. In addition, high RAPD-PCR similarity coefficients (>87%; Fig. 1) indicated that BIMs would be effectively derived from their parent strains and were not contaminants. Also, phage-sensitive strains (*Leuc. pseudomesenteroides* R707, *Leuc. mesenteroides* D6a, *Leuc. mesenteroides* L79-1) and their respective BIMs were assessed in relation to acidification kinetics (Fig. 2). *Leuc. mesenteroides* L79-1 and its BIMs developed acidity moderately fast (between 0.70 ± 0.02% and 0.80 ± 0.01% acid lactic after 24 h at 30 °C), and pH values in milk cultures after 24 h at 30 °C ranged between 5.40 ± 0.12 and 5.50 ± 0.23 (Fig. 2A). Conversely, all the BIMs isolated from *Leuc. mesenteroides* D6a showed lower acidifying activity (between 0.50 ± 0.03% and 0.60 ± 0.04% acid lactic and pH values 5.20 ± 0.12 after 24 h at 30 °C) than that obtained for the parent strain (0.90 ± 0.02% acid lactic and pH values 4.80 ± 0.16 after 24 h at 30 °C, Fig. 2B). Finally, *Leuc. pseudomesenteroides* R707 and its BIMs were unable to develop acidity in milk (pH > 6.3 after 24 h at 30 °C; 0.20 ± 0.02% acid lactic after 24 h at 30 °C, Fig. 2C). Low acidifying activity observed for these *Leuconostoc* strains is in agreement with heterofermentative metabolism of this genus and it is consistent with results reported previously (Ayad, Nashat, El-Sadek, Metwaly, & El-Soda, 2004; Garabal, Rodriguez-Alonso, & Centeno, 2008; Nieto-Arribas, Sesena, Poveda, Palop, & Cabezas, 2010). For this reason, *Leuconostoc* isolates should be used as starter adjuncts or in combination with acid-producing lactococci during fermentative processes (Hemme, 2012).

3.4. Technological performance of BIMs

Technological performance of BIMs was studied in a milk fermentation model. One BIM of each family was selected on the basis of its phage resistance phenotype (high stability and level of resistance, low or null phage adsorption, absence of spontaneous phage release). Evolution of pH values, bacterial cell counts and phage titres throughout the manufacturing process are shown in Fig. 3.

The growth of *L. lactis* DX33 was similar in all the experiments, as well as the decrease of pH, regardless of the presence of *Leuconostoc* strains (either phage-sensitive or phage-resistant) and phages tested (Fig. 3A). *L. lactis* DX33 reached 9.5 log pfu mL^{-1} after 8 h at 30 °C and maintained at this level all throughout the experiment (15 °C, between 8 h and 48 h). The pH values evolved accordingly, falling to 4.8 and 4.4 after 8 h and 24 h of incubation, respectively (Fig. 3A).

Leuc. pseudomesenteroides R707 and its BIM named R707M1 grew moderately well in milk after 8 h of incubation in the absence of phages, reaching approximately 7.5 log cfu mL^{-1} (Fig. 3B). The growth of R707 in the presence of phage LDG was significantly reduced, reaching 2.5 log cfu mL^{-1} after 8 h of incubation (Fig. 3B). As expected, the titre of phage LDG reached a maximum of 9 log pfu mL^{-1} during the same period (Fig. 3C). In contrast, BIM R707M1 grew normally up to 7.5 log cfu mL^{-1} after 8 h of incubation in the presence of phage LDG, which remained low (3.7 log pfu mL^{-1}) after the 8 h of incubation. Similar behaviour was exhibited by *Leuc. mesenteroides* D6a and its BIM D6a15, since they grew up to 8 log cfu mL^{-1} after 8 h of incubation in milk without phage infection. After 8 h of incubation in the presence of phage, bacterial viable counts of the phage-sensitive strain *Leuc. mesenteroides* D6a was considerably reduced (<2.9 log cfu mL^{-1}) and phage titre increased, reaching 8.6 log pfu mL^{-1}. In the same period and conditions, in contrast, the BIM D6a15 grew adequately (8 log cfu mL^{-1}) while phage titre remained low (<3 log pfu mL^{-1}) (Fig. 3D and E).

Concerning their growth in milk, both *Leuc. mesenteroides* L79-1-derived BIMs, L79-2 (isolated using the phage Ln-9) and COC 1

Table 2
Spontaneous phage release and adsorption rates of bacteriophage-insensitive mutants isolated from phage-sensitive *Leuconostoc mesenteroides* and *Leuconostoc pseudomesenteroides* strains.[a]

Strain	Phage release	Adsorption with phage					
		LDG	Ln-7	Ln-8	Ln-9	CHA	CHB
Leuconostoc pseudomesenteroides							
R707	−	99.8 ± 3.2					
R707M1	−	66.6 ± 4.2					
R707M2	−	64.1 ± 3.6					
R707M3	−	72.4 ± 4.5					
R707M4	−	52.1 ± 6.8					
R707M5	−	79.9 ± 3.2					
R707M6	−	64.1 ± 4.1					
Leuconostoc mesenteroides							
D6a	−			99.7 ± 3.2			
D6a10	−			58.9 ± 5.1			
D6a11	−			58.2 ± 1.6			
D6a12	−			56.8 ± 2.2			
D6a13	−			61.4 ± 4.3			
D6a14	−			60.4 ± 3.2			
D6a15	−			61.1 ± 4.2			
L79-1	−				99.6 ± 4.2		
L79-2	−				10.1 ± 5.2		
L79-3	+				33.9 ± 3.2		
L79-13	−				35.1 ± 2.3		
L79-14	−				41.9 ± 4.3		
L79-20	−				62.3 ± 1.2		
L79-21	−				58.2 ± 2.2		
L79-1	−	99.8 ± 4.2	99.7 ± 3.2	99.6 ± 5.3	99.9 ± 2.4	99.2 ± 3.2	99.9 ± 1.4
COC 1	−	31.5 ± 4.5	66.6 ± 2.9	54.8 ± 4.7	99.0 ± 4.1	99.2 ± 3.6	99.1 ± 5.7
COC 2	+	27.6 ± 1.1	58.3 ± 2.4	47.6 ± 1.5	99.0 ± 4.6	99.3 ± 2.2	99.1 ± 1.3
COC 3	+	39.4 ± 3.7	52.7 ± 4.2	34.8 ± 3.2	99.9 ± 4.2	99.0 ± 2.8	99.2 ± 4.9

[a] Phage release is the spontaneous release of free phages detected in the supernatant of the cultures; Adsorption with phage is the percentage of adsorbed phages in MRS-Ca broth after 20 min at 30 °C. All strains were phage-resistant variants except for R707, D6a and L79-1.

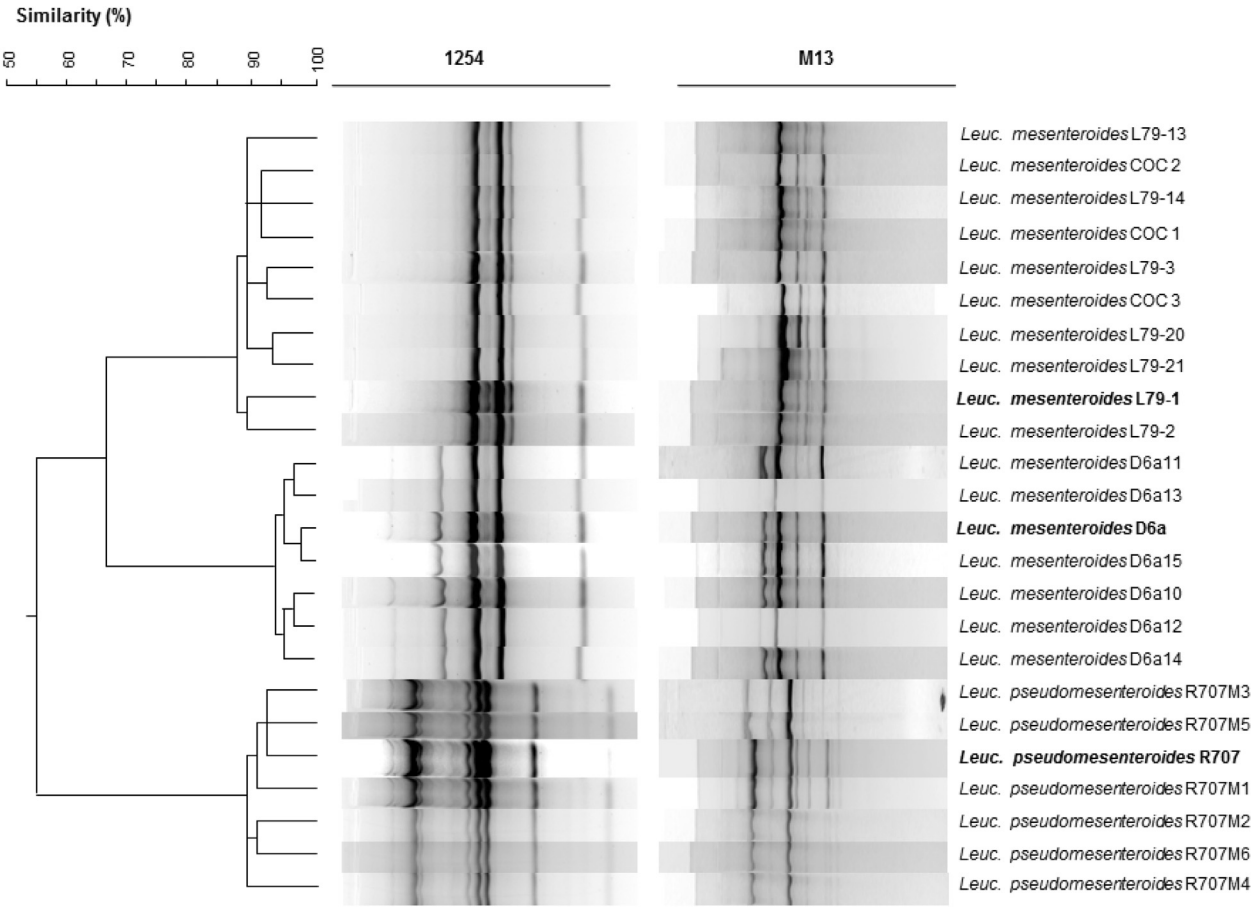

Fig. 1. Dendrogram obtained by comparison (GelCompar, Applied Maths, Saint-Martens-Latem, Belgium) and clustering (UPGMA method, Unweighted Pair Group Method using Arithmetic Averages) of RAPD profiles from *Leuconostoc mesenteroides* L79-1, *Leuconostoc mesenteroides* D6a, *Leuconostoc pseudomesenteroides* R707 and their respective spontaneous BIMs, using primers 1254 and M13. Phage-sensitive strains are indicated in bold letters.

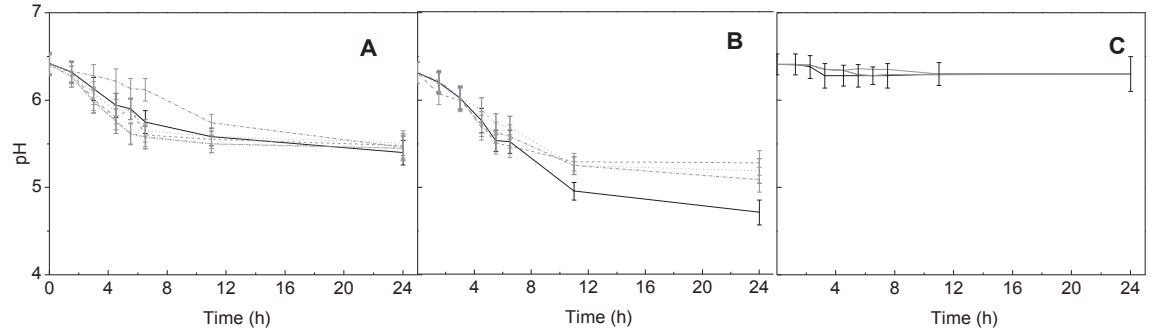

Fig. 2. Milk acidification kinetics of phage-sensitive strains (solid lines) and their bacteriophage-insensitive mutants (dotted lines): A, *Leuconostoc mesenteroides* L79-1; B, *Leuconostoc mesenteroides* D6a; C, *Leuconostoc pseudomesenteroides* R707. Assays were performed in triplicate, and data are reported as means ± standard deviations.

(isolated using the phage cocktail) were similar to their parent strain when grown in the absence of phages (>8 log cfu mL^{-1} after 8 h). When phage Ln-9 or the phage cocktail were added, viable cell counts of the parent strain were notably reduced to 2 log cfu mL^{-1} after 8 h (Fig. 3F), while phage titre reached a maximum of 9 log pfu mL^{-1} within 8 h. In contrast, BIMs named L79-2 and COC 1 grew normally (8.2 log cfu mL^{-1}) in the presence of phage Ln-9 or the phage cocktail, respectively. As shown in Fig. 3G, when phage Ln-9 was assayed on BIM L79-2, phage titre remained constant after 8 h of incubation. However, when phage cocktail was assayed on BIM COC 1, phage titres increased two logarithmic orders. This fact could be attributed to the loss of phage resistance of a small portion of the mutant population, allowing the propagation of phages from the cocktail. During refrigerated storage, all bacterial counts remained constant or decreased slightly from 8 to 48 h. The low pH and the accumulated lactic acid could be responsible of the observed decrease in cell viability.

Over the last years, only a few articles have reported the isolation of spontaneous BIMs using phage cocktails. In this context,

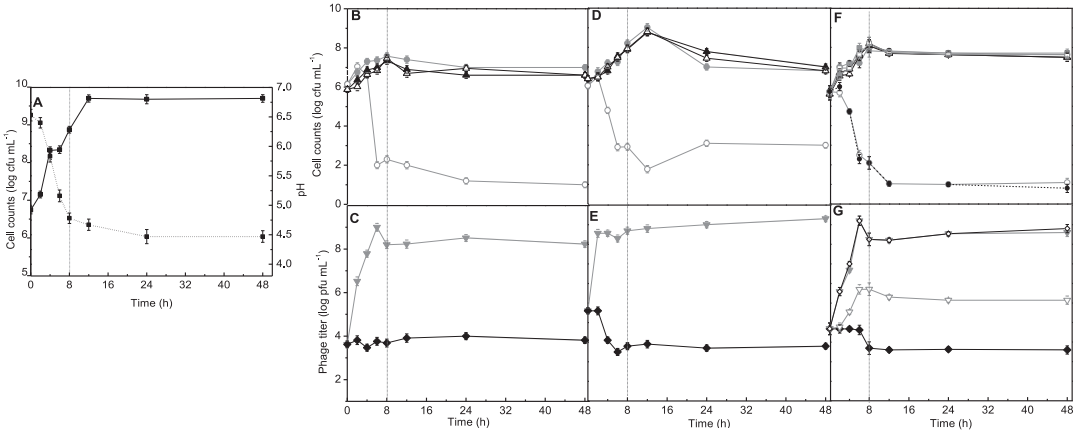

Fig. 3. Panel A: growth kinetics (log cfu mL^{-1}) (solid lines) and pH values (dotted lines) of *Lactococcus lactis* DX33 during milk fermentation (30 °C, 8 h). Panels B and D: growth kinetics (log cfu mL^{-1}) during milk fermentation (30 °C, 8 h) and refrigerated storage (15 °C, between 8 h and 48 h), using sensitive strains (●, ○) of *Leuconostoc pseudomesenteroides* R707 (B) and *Leuconostoc mesenteroides* D6a (D), or their bacteriophage-insensitive mutants (▲, △) R707M1 (B) and D6a15 (D) infected (○, △) or not (●, ▲) with their corresponding phage. Panel F: growth kinetics (log cfu mL^{-1}) during milk fermentation and refrigerated storage, using sensitive strain *Leuc. mesenteroides* L79-1 (●, ○, ●), bacteriophage-insensitive mutant L79-2 (▲, △) or bacteriophage-insensitive mutant COC 1 (■, □) infected with phage Ln-9 (○, △) or phage cocktail (●, □), or not infected (●, ▲, ■). Panels C and E: phage titre during milk fermentation and refrigerated storage using sensitive strain (▼) *Leuc. pseudomesenteroides* R707 (C), *Leuc. mesenteroides* D6a (E), or their bacteriophage-insensitive mutants (◆) R707M1 (C) or D6a15 (E). Panel G: phage titre during milk fermentation and refrigerated storage using sensitive strain *Leuc. mesenteroides* L79-1 (▼) or bacteriophage-insensitive mutant L79-2 (◆) with phage Ln-9; *Leuc. mesenteroides* L79-1 (◇) or bacteriophage-insensitive mutant COC 1 (▽) with phage cocktail. Error bars represent standard deviation of three determinations.

Briggiler Marcó et al. (2011) isolated spontaneous BIMs from *Lb. plantarum* ATCC 8014 with adequate technological properties by using a mixture of two lytic phages.

All the BIMs assayed in this study could be good candidates for their use in the manufacture of fermented products, with the exception of COC 1, which allowed phage propagation, and thus may cause further infections to other sensitive strains present in the starter culture. The performance of BIMs in the presence of phages during a milk fermentation model is a good in vitro evaluation of their potential effectiveness in an industrial scenario (Moineau & Lévesque, 2005). Reports on the use of BIMs as a tool to minimise phage attacks during the manufacture of fermented dairy products showed mixed results (Briggiler Marcó et al., 2011; Quiberoni, Reinheimer, & Suárez, 1998a; Sturino & Klaenhammer, 2004). However, there are no data associated to the use of *Leuconostoc* BIMs in fermented product making.

4. Conclusions

In the present study, spontaneous BIMs isolated from commercial *Leuc. mesenteroides* and *Leuc. pseudomesenteroides* strains, using six phages either individually or in combination (cocktail), showed a strong and stable phage resistance phenotype, while also exhibiting interesting technological abilities. In particular, four selected BIMs were able to resist the presence of lytic phages during a milk fermentation process and the subsequent refrigerated storage of the product. The isolation of spontaneous BIMs is an ongoing venture to appropriately select bacterial starters for long-term industrial use in phage-contaminated environments.

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