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A fast PCR-based method for the characterization of prophage profiles in strains of the *Lactobacillus casei* group

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ABSTRACT

Lysogeny is widespread among *Lactobacillus* strains of the *casei* group (*L. casei*, *L. paracasei* and *L. rhamnosus*), and prophages account for most strain-specific DNA. Numerous PCR based methods have been developed to detect free phages of lactic acid bacteria, but they do not take in consideration prophages. In this study, a new PCR method for the detection of lysogeny was developed using genome sequences of *L. casei* group strains (including BL23) and bacteriophages. Nine pairs of primers were designed to selectively amplify the highly conserved prophage iA2 (pairs #1–#3) and fragments of two groups phages of temperate origin: C_L1/C_L2/i*Lp*1308/i*Lp*84 (pairs #4 and #5) and Lrm1/J-1/PL-1/A2/AT3/Lc-Nu (pairs #6 to #9). Forty-nine strains of the *casei* group were subjected to PCR. Strains containing remnants of lytic phages outnumbered those containing iA2-related prophages. The combination of pair #2, annealing on the terminase large subunit (TLS), and pair #3, annealing on the helicase (forward) and a non-coding region (reverse), showed the best diagnostic performance for *iA2-like* prophages. For the assessment of remnants of phages C_L1/C_L2/i*Lp*1308/i*Lp*84, pair #4 (annealing on the TLS) was preferred over pair #5 (portal protein). Detection of phages Lrm1/J-1/PL-1/A2/AT3/Lc-Nu was optimal with primers of pair #6, designed on non-coding regions of phage genomes; pair #6 also evidenced a high conservation of certain prophage remnants. Overall, our PCR-based method successfully detected and discriminated groups of prophages or remnants in *L. casei* group strains.

1. Introduction

Bacteriophages (phages) of lactic acid bacteria (LAB) are ubiquitous in dairies (Capra et al., 2009; Guglielmotti et al., 2012). Phages have been extensively studied and numerous strategies have been developed in order to control their infections (Everson, 1991), including strain rotation schemes, use of physical and chemical treatments, and development or isolation of bacteriophage insensitive mutants (BIMs) (Briggiler Marcó et al., 2011; Mercanti et al., 2012; Moineau and Levesque, 2005; Samson and Moineau, 2013). However, phages cannot be completely eradicated and represent a major risk on the manufacture of dairy products, especially in large scale plants with intensive fermentation processes, causing economic losses not only due to fermentation arrest, but also to a negative impact on product taste and texture (Capra et al., 2009; Mahony et al., 2014; Samson and Moineau, 2013).

Most probiotic strains commercially exploited today belong to the *casei* group of *Lactobacillus* (*L. casei*, *L. paracasei* and *L. rhamnosus*) (Desai et al., 2006; Mercanti et al., 2011; Mercanti et al., 2016), in which a high percentage of strains are lysogenic or even polylysogenic (Canchaya et al., 2003; Mercanti et al., 2011). Prophages and prophage remnants are prone to recombine with DNA from the host or from another invading phage, greatly contributing to horizontal gene transfer (HGT) and leading to phage and host diversity. Although at low rate, HGT might allow phages to cross the species barrier (Baugher et al., 2014; Canchaya et al., 2003; Mercanti et al., 2011; Mercanti et al., 2016). Several published PCR methods have resulted in successful detection and discrimination of LAB phages (Binetti et al., 2008; Binetti et al., 2005; del Rio et al., 2007; del Rio et al., 2008; Dupont et al., 2005; Labrie and Moineau, 2000; Zago et al., 2006; Zago et al., 2008). In addition, lysogenic *Lactococcus lactis* strains have been identified using a PCR strategy (Martín et al., 2006), but the presence of prophages has not been assessed by similar approaches in strains of the *casei* group.

L. casei BL23, a broadly used laboratory strain (Maze et al., 2010), contains a prophage highly similar (nucleotide identity > 99.9%) to iA2 present in the commercial strain *L. paracasei* A (Mercanti et al., 2016), and to other prophages found in the related *L. casei* strains BD-II (Ai et al., 2011), LC2W (Chen et al., 2011) and W56 (Hochwind et al.,

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Table 1
PCR assay for all the strains and primers tested.

Bacterial group[a]	Strain[b]	Origin	Phage group 1 *iA2-like* prophages			Phage group 2 C_L1, C_L2, *iLp*84, *iLp*1308		Phage group 3 A2, Lrm1, PL-1, J-1, AT3			
			Pair 1 55 °C[d]	Pair 2 60 °C[d]	Pair 3 55 °C[d]	Pair 4[c] 55 °C[d]	Pair 5[c] 50 °C[d]	Pair 6 55 °C[d]	Pair 7[c] 55 °C[d]	Pair 8 55 °C[d]	Pair 9[c] 60 °C[d]
A	*Lactobacillus casei* BL23[(1)]	Commercial	+	+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei A[(1)]	Commercial	+	+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei Dn[(1)]	Commercial	+	+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei Hn[(1)]	Commercial	+	+	+			+	+(2)	+	+(2)
	Lactobacillus paracasei A13[(1)]	Commercial		+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei A14[(2)]	Commercial	+	+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei ATCC 27092[(2)]	ATCC Collection	+	+	+	+		+	+(2)	+	+(2)
	Lactobacillus paracasei Bio[(1)]	Commercial				+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei L26	Commercial	+	+		+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei SA	Commercial				+(#)	+	+	+(2,#)		
	Lactobacillus rhamnosus CNRZ 1224	CNRZ Collection									
	Lactobacillus paracasei CNRZ 1308	CNRZ Collection						+	+	+	+
	Lactobacillus paracasei CNRZ 318	CNRZ Collection				+	+	+	+(2)	+	+(2)
	Lactobacillus rhamnosus CNRZ 1976	CNRZ Collection				+		+		+	+(2)
	Lactobacillus paracasei Jp-1	INLAIN Collection				+	+	+	+(2)	+	+(2)
	Lactobacillus rhamnosus PR	Commercial					+(#)	+	+(#)	+	+(#)
	Lactobacillus paracasei ATCC 27139[(2)]	ATCC Collection	+	+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus paracasei INL3[(2)]	INLAIN Collection		+	+	+	+	+	+(2)	+	+(2)
	Lactobacillus casei CNRZ 1874	CNRZ Collection						+			+
	Lactobacillus paracasei 72	INLAIN Collection						+		+	+
	Lactobacillus paracasei 81	INLAIN Collection				+	+	+		+	+
	Lactobacillus paracasei 84	INLAIN Collection				+	+	+	+(2,#)	+	+(#)
	Lactobacillus paracasei 85	INLAIN Collection				+	+	+		+	+(2)
	Lactobacillus paracasei 86	INLAIN Collection				+	+	+		+	+(2)
	Lactobacillus paracasei 88	INLAIN Collection				+	+	+		+	+(2)
	Lactobacillus rhamnosus 90	INLAIN Collection					+(#)				
	Lactobacillus rhamnosus INL1	INLAIN Collection					+(#)	+	+	+	+
	Lactobacillus rhamnosus INL2	INLAIN Collection						+		+	+
	Lactobacillus casei SA	Commercial						+		+	+
B	*Lactobacillus casei* INL 20	INLAIN Collection	+	+				+	+	+	+
	Lactobacillus casei INL 23	INLAIN Collection	+	+				+		+	+(#)
	Lactobacillus casei INL 43	INLAIN Collection				+		+	+(2)	+	+(2)
	Lactobacillus casei INL 46	INLAIN Collection						+	+	+	+
	Lactobacillus casei INL 47	INLAIN Collection						+	+(2)	+	+(2)
	Lactobacillus casei INL 136	INLAIN Collection				+		+	+(2)	+	+(2)
	Lactobacillus casei INL 241	INLAIN Collection		+		+	+	+	+	+	+
	Lactobacillus casei INL 264	INLAIN Collection				+		+	+(2)	+	
	Lactobacillus casei INL 274	INLAIN Collection	+	+		+	+	+	+	+	+
	Lactobacillus casei INL 276	INLAIN Collection				+	+	+	+(2)	+	+(2)
	Lactobacillus casei INL 17	INLAIN Collection				+	+	+	+(2)	+	+(2)
	Lactobacillus casei INL 279	INLAIN Collection				+	+	+	+(2)	+	+(2)
	Lactobacillus casei YOL-G	Commercial						+	+(2)	+	+(3)
	Lactobacillus casei YOL-CH	Commercial						+	+(2)	+	+(3)
	Lactobacillus paracasei ATCC 25302	ATCC Collection						+		+	+(3)
	Lactobacillus rhamnosus ATCC 7469	ATCC Collection									
	Lactobacillus rhamnosus GG	ATCC Collection									
	Lactobacillus paracasei 906	INLAIN Collection	+	+				+	+(#)	+	+(#)
	Lactobacillus casei 17051	Commercial									
	Lactobacillus casei 17052	Commercial	+	+	+	+	+	+	+(2,#)	+	+(2,#)
Negative controls	*Lactobacillus plantarum* ATCC 8014	ATCC Collection									
	Leuconostoc mesenteroides R707	Commercial									
	Lactococcus lactis Mo9	Commercial									
	Lactobacillus delbrueckii Ab1	INLAIN Collection									
	Escherichia coli Dh5α	Commercial									
	Leuconostoc mesenteroides MB1	INLAIN Collection									
	Leuconostoc mesenteroides D11	INLAIN Collection									

[a] Strains of group A were previously tested for the presence of MMC inducible prophages (Capra et al., 2010; Mercanti et al., 2011), and strains of group B were not. Strains on the group C do not belong to the casei group and were used as negative controls in PCR assays.

[b] Strains of group A with MMC-inducible prophages sharing restriction profiles are indicated:[(1)]*iA2-like*;[(2)]non *iA2-like*.

[c] Brackets indicate the amplification of the expected fragment plus one (2) or two (3) fragments of different size, or the amplification of only one (#) or two (2,#) fragments of different than expected size.

[d] Annealing temperature used in the PCR assay.

2012). With lower but still high nucleotide identity to iA2, prophages have been also found in *L. casei* LOCK919 (Koryszewska-Baginska et al., 2013), *L. paracasei* N1115 (Wang et al., 2014) and *L. paracasei* 8700:2 (unpublished). On the other hand, related temperate phages C_L1, C_L2, i*Lp*84 and i*Lp*1308 have been recently sequenced (Mercanti et al., 2016). As it was observed for other reported phages of the *casei* group (Alemayehu et al., 2009; Dieterle et al., 2014; Durmaz et al., 2008; Garcia et al., 2003; Lo et al., 2005; Proux et al., 2002; Tuohimaa et al., 2006; Ventura et al., 2006), large fragments of the genomes of these phages have been found in bacterial strains.

Taking advantage of the currently growing number of genome sequences publicly available, the aim of this study was to design a simple and economic PCR-based assay to classify strains of the *casei* group according to prophage profiles, using diverse sets of primers to selective identify prophages grouped by homology.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study are detailed in Table 1. Strains of *Lactobacillus* of the *casei* group were divided into groups A and B. Strains of group A had been assessed in a previous study (Mercanti et al., 2011) for the presence of mitomycin C (MMC)-inducible prophages in their genomes. The genome sequence of strain *L. casei* BL23 was primarily used for primer design and, consequently, as positive control for PCR tests. Group B includes 20 lactobacilli strains of the *casei* group not previously tested for the presence of MMC-inducible prophages. Diverse strains that do not belong to the *casei* group were used as negative control in PCR assays (Table 1). Strains were maintained as frozen stocks at −80 °C in de Man, Rogosa, and Sharpe (MRS; Biokar, Beauvais, France) (*Lactobacillus* and *Leuconostoc*), M17 (Biokar, Beauvais, France) (*Lactococcus*) or Hershey (Difco, Detroit, MI, USA) (*Escherichia coli*) broths supplemented with 15% (v/v) glycerol (Cicarelli, San Lorenzo, Argentina). The strains were reactivated and routinely cultured in their corresponding broth without glycerol.

2.2. Design of primers for PCR

Bacterial and phage genome sequences used to design PCR primers are listed in Table 2; they were either obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) or extracted from bacterial genome sequences using PHAST (Phage Search Tool; http://phast.wishartlab.com/). Multiple sequence alignments were carried out with the program Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) to determine conserved sequences present in most phages. Primers were designed on zones of those conserved phage sequences which were also present in bacterial genomes, using the tools Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and GEMI (Sobhy and Colson, 2012). Perfect matches between selected primers and the genome of the strain *L. casei* BL23 were confirmed using the program BioEdit v7.2.5 (Hall, 1999). Oligonucleotide primer pairs were checked for the potential generation of secondary structures and homo or heterodimers.

2.3. Nucleic acids extraction

Bacterial DNA was extracted by differential elution using the commercial kit GenElute™ Bacterial Genomic DNA (Sigma Aldrich, Argentina), following manufacturer instructions for Gram-positive bacteria. DNA was quantified by electrophoresis on 0.8% (w/v) agarose gels in 1X Tris-acetate EDTA (1X TAE: 40 mM Tris acetate, 1 mM EDTA, pH 8.0) buffer, using GelRed™ (Biotium, Inc., California, USA) as nucleic acid binding dye. DNA was diluted in sterile distilled water (GIBCO™, Invitrogen, USA) and stored at −18 °C.

Prophage DNA extraction was carried out starting from 50 ml of filtered supernatants of cultures induced with mitomycin C (MMC), following a previously described procedure (Mercanti et al., 2011). DNA pellets were dissolved in 50 µl of sterile distilled water (GIBCO™, Invitrogen, USA) and stored at −18 °C. DNA was quantified by agarose gel electrophoresis likewise bacterial DNA.

2.4. Prophage analysis

The presence of inducible prophages on the strains of group B was tested by MMC treatment, following a previous protocol (Mercanti et al., 2011). DNA from induced prophages was extracted as described and digested with BglII restriction enzyme according to manufacturer's recommendations (GE Healthcare Life Sciences, Bucks, UK). Restriction patterns were visualized after electrophoresis on 0.8% (w/v) agarose gels in 1X TAE buffer, using GelRed™ (Biotium, Inc., California, USA) as nucleic acid binding dye. Images were processed with the analysis software package BioNumerics™ (version 5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium).

2.5. PCR conditions

PCR reactions were carried out in a Veriti® 96-Wells thermal cycler (Thermo Fisher Scientific), in a final reaction volume of 20 µl containing 2 µl of 10x Taq reaction Buffer (Sigma Aldrich, Argentina), 0.5 µl of a mix of dATP, dCTP, dGTP and dTTP (200 mM each), direct and reverse primers (0.5 mM each), 0.5 U of Taq DNA Polimerase (Sigma Aldrich, Argentina) and 1 µl of extracted bacterial DNA. The cycling program consisted of an initial heating at 94 °C for 3 min (denaturation), followed by 35 cycles of 1 min at 94 °C, 2 min at 50, 55 or 60 °C, and 2 min at 72 °C, and a final elongation step at 72 °C for 7 min. The temperature of annealing was variable according to the pair of primers utilized and the obtained results. PCR products were resolved by electrophoresis on 1.8% (w/v) agarose gels in 1x TAE buffer, using GelRed™ (Biotium, Inc., California, USA) as nucleic acid binding dye.

3. Results

3.1. Selection of phages

A set of phage genomes classified into three groups (1–3) was used for the design of the assay, in order to maximize the number and diversity of phages to be detected. Primers were separately designed to amplify groups 1, 2 and 3 (Table 2). Prophage iA2 and other related prophages (group 1, referred here as "*iA2-like*") were found to be highly conserved in several strains (Mercanti et al., 2016). In the same study, phages C_L1, C_L2, i*Lp*1308 and i*Lp*84 (group 2) were classified in a new cluster based on amino acid identity for predicted proteins and terminase phylogeny. Prophages PLE1 and PLE2 of *L. casei* BL23 (Dieterle et al., 2016) are the same previously reported as *L. casei* BL23–prophages 1 and 2 (respectively) (Mercanti et al., 2016). Dieterle et al. (2016) reported also a third complete prophage (PLE3), highly similar to temperate phage i*Lp*84. The rest of *L. casei* phages with genome sequences available in databases, namely Lrm1, J-1, PL-1, A2, AT3 and Lc-Nu (group 3), were used to design the third group of primers.

3.2. PCR assay for the detection of iA2-like prophages

Group 1 contains prophages that possess many ORFs with very high amino acid identity (> 99%) (Mercanti et al., 2016) and they are present almost intact on bacterial genomes. Moreover, several related strains contain two prophages (identified as subgroups I and II), which could be discriminated with primers specific for either one or another subgroup. Consequently, three pairs of primers were designed (Table 3). Primers of pair #1 were designed on the ORFs encoding HNH endonuclease (forward) and terminase large subunit (TLS) (reverse). TLS was selected because it is one of the most conserved genes in this group, and it had been used for phylogenetic classification (Casjens et al., 2005; Mercanti et al., 2016). The HNH endonuclease is involved in phage DNA replication, and the region of this ORF selected for primer design was, likewise for TLS, common to all the prophages of group 1. Pair #2 amplifies internal fragments of TLS; it was specifically designed to amplify all *iA2-like* prophages except subgroup II.

Primers of pair #3 anneal on a helicase (forward) and a non-coding DNA region (reverse); contrary to pair #2, pair #3 amplifies only subgroup II of *iA2-like* prophages. A BLAST search (blastn, adjusted for a short sequence) of the eight primers designed for the group 1 of

Table 2
DNA sequences used for the design of PCR primers (Douillard et al., 2013; Ai et al., 2011; Maze et al, 2010; Chen et al., 2011; Zhang et al., 2010b; Hochwind et al., 2012; Koryszewska-Baginska et al., 2013; Wang et al., 2014; Aleksandrzak-Piekarczyk et al., 2013; Kankainen et al., 2009; Mercanti et al., 2016; Durmaz et al., 2008; Dieterle et al., 2014; Lo et al., 2005; Garcia et al., 2003; Tuohimaa et al., 2006).

Strains	Accession number	Reference
Lactobacillus casei LcA	CM001861	Douillard et al. (2013)
Lactobacillus casei BD-II	NC_017474.1	Ai et al. (2011)
Lactobacillus casei BL23	NC_010999.1	Maze et al. (2010)
Lactobacillus casei LC2W	NC_017473.1	Chen et al. (2011)
Lactobacillus casei LcY	NZ_CM001848.2	Douillard et al. (2013)
Lactobacillus casei LOCK919	NC_021721.1	Koryszewska-Baginska et al. (2013)
Lactobacillus casei Zhang	NC_014334.2	Zhang et al. (2010b)
Lactobacillus casei W56	NC_018641.1	Hochwind et al. (2012)
Lactobacillus paracasei 8700:2	NC_022112.1	Unpublished
Lactobacillus paracasei N1115	CP007122	Wang et al. (2014)
Lactobacillus rhamnosus LOCK900	NC_021723.1	Aleksandrzak-Piekarczyk et al.(2013)
Lactobacillus rhamnosus GG (ATCC 53103)	NC_013198.1	Kankainen et al. (2009)

Group	Phages		Accession number	Reference
1	iA2		KR905068.1	Mercanti et al. (2016)
1	*L. casei* BD-II, prophages I and II*	*iA2-like* prophages	NC_017474.1†	Mercanti et al. (2016)
1	*L. casei* BL23, prophages I and II*		NC_010999.1†	Mercanti et al. (2016)
1	*L. casei* LC2W, prophages I and II*		NC_017473.1†	Mercanti et al. (2016)
1	*L. casei* LOCK919 prophage		NC_021721.1†	Mercanti et al. (2016)
1	*L. casei* W56, prophages I and II*		NC_018641.1†	Mercanti et al. (2016)
1	*L. casei* 8700:2 prophage		NC_022112.1†	Mercanti et al. (2016)
1	*L. casei* N1115 prophage		CP007122†	Mercanti et al. (2016)
2	C_L1		KR905066	Mercanti et al. (2016)
2	C_L2		KR905067	Mercanti et al. (2016)
2	i*Lp*84		KR905069	Mercanti et al. (2016)
2	i*Lp*1308		KR905070	Mercanti et al. (2016)
3	Lrm1		EU246945	Durmaz et al. (2008)
3	J-1		KC171646	Dieterle et al. (2014)
3	PL-1		KC171647	Dieterle et al. (2014)
3	A2		AJ251789	Garcia et al. (2003)
3	AT3		AY605066	Lo et al. (2005)
3	Lc-Nu		AY131267	Tuohimaa et al. (2006)

*The strain contains complete prophages of subgroups I and II, detectable with PHAST.
†The accession number corresponds to the genomic sequence of the bacterial strain that contains the prophage(s) extracted with PHAST.

phages (Table 3) rendered exact matches only to phages and bacteria of the *casei* group.

All PCR reactions were carried out with the annealing temperature set to both 60 and 55 °C. Table 1 shows the best results (at optimal temperature for each primer set) obtained for all the strains. Amplification of primers pairs #1 to #3 on the strain *L. casei* BL23 (positive control) is shown in Fig. 1. Pair #1 did not amplify from two strains of the group A (*L. paracasei* strains A13 and INL3) that produced amplifications using pair #2. When the test was applied to group B, six strains amplified when using pair #2, but only one of them (a commercial strain) was positive to pair #3.

Table 3
Primers designed in this study.

Phage group	Pair(s) #	Primers	Amplicon size (kb)	Sequence (5′ → 3′)	Genomic target
1 (*iA2-like* prophages)	1	p1_F	960	TGTCGGTGGATTGTGTGAGC	HNH endonuclease
		p1_R		CAAGCACGTGATTACCACGAC	TLS
	2	p2_F	651	CATGCAGATTGCCGATGGTG	TLS
		p2_R		AAAGGTCAGCCACGACTCAG	TLS
	3	p3_F	668	CGCGAACGACCAACGAATAC	Helicase
		p3_R		TGGTTGCGGCTCTATGTGTT	NCR
2 (C_L1, C_L2, i*Lp*84, i*Lp*1308)	4	p4_F	287	GGCCCGTATCACTGGTTCAA	TLS
		p4_R		GTGGGGTTCTGTGTGCCATA	TLS
	5	p5_F	117	GCAGCTCATACGTTCAAGAACAC	Portal protein
		p5_R		TCATCAGTCGGGAAAATAAACAC	Portal protein
3 (A2, Lrm1, PL-1, J-1, AT3)	6[a]	p6_F	565	AAACAATTGAAAACGCCAAAGAG	Unknown ORF
		p6_R		ATCGAACCCGCCTGACTA	Unknown ORF/NCR[b]
	7[a]	p6_F	764	AAACAATTGAAAACGCCAAAGAG	Unknown ORF
		P9_R		CAAGCTGTGTCCGGTCG	Unknown ORF/NCR[b]
	8[a]	p9_F	545	GAGGTCTTGGGGAACTAC	Unknown ORF
		p6_R		ATCGAACCCGCCTGACTA	Unknown ORF/NCR[b]
	9[a]	p9_F	744	GAGGTCTTGGGGAACTAC	Unknown ORF
		p9_R		CAAGCTGTGTCCGGTCG	Unknown ORF/NCR[b]

TLS: terminase large subunit; NCR: non-coding region.
[a] Note that pairs 6, 7, 8 and 9 result from the combinations of two forward (p6_F, p9_F) with two reverse (p6_R, p9_R) primers; amplicon size varies according to the pairing selected.
[b] The sequence corresponds to an ORF of unknown function in the genome of phage A2, but is found outside predicted ORFs in the genomes of other phages.

3.3. PCR assay for the detection of remnant of phages C_L1, C_L2, iLp1308 and iLp84

Phages of group 2 share the indicator strain (*L. paracasei* A), but their ORFs share very low amino acid identity with prophage iA2, harbored by that strain. Also, these four phages have particular features that distinguished them from the rest of the phages of *L. casei* group reported previously (Mercanti et al., 2016). Particularly, large fragments covering most of their genomes can be found in bacterial strains. For group 2, primer pairs #4 and #5 (Table 3) were designed within the ORFs encoding TLS and portal protein, respectively. Both enzymes, involved in DNA packaging, are encoded by the largest ORFs within the most conserved module of these phages genomes. Several pairs of primers were designed on each zone, but only few fulfilled the requirement of matching both forward and reverse primers the genome of *L. casei* BL23.

For pair #4, PCR reactions were carried out using annealing temperatures of 60 and 55 °C, and best amplifications were achieved at the lower value. For pair #5, a drop to 50 °C in the annealing temperature was necessary to achieve good amplifications. Fig. 1 shows amplification of both primers pairs on the strain *L. casei* BL23. Most strains of group A were positive for both primers pairs (Table 1), with no clear correlation to the presence of MMC-inducible prophages. However, commercial strains with prophages detected using primers of group 1 also amplified when using primers #5 and/or #6, except *L. paracasei* Hn. Pair #4 amplified an additional, larger fragment on *L. paracasei* SA,

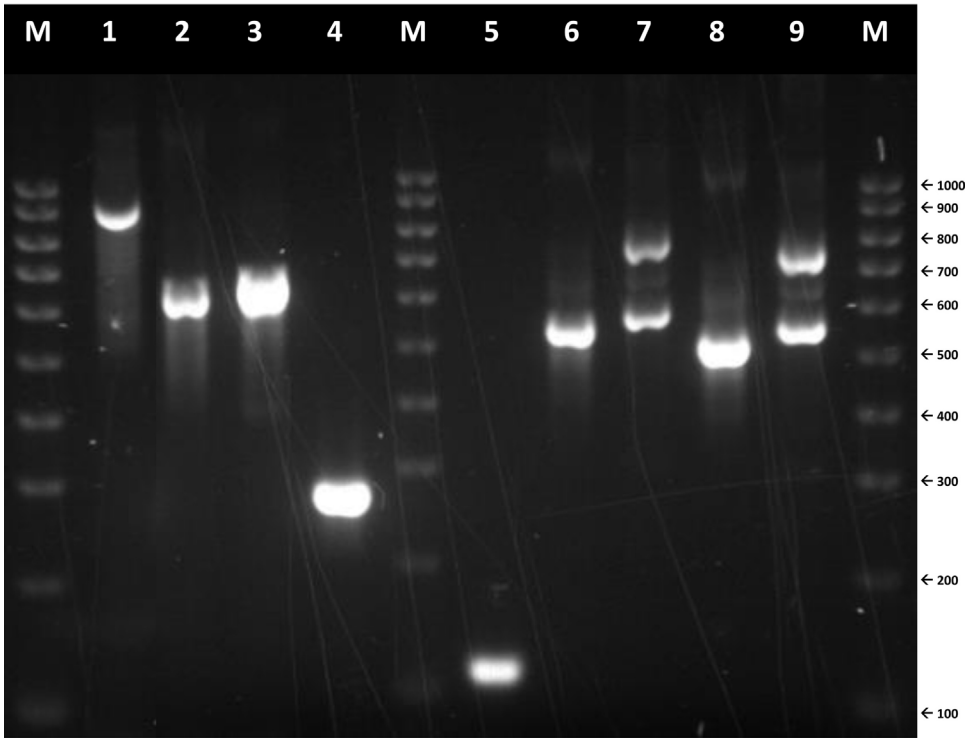

Fig. 1. Agarose gel electrophoresis of PCR products amplified from DNA of *L. casei* BL23 using all the primers designed in the present study. Numbers indicate the pair of primers used; M: molecular weight marker 100 bp DNA ladder (GE Healthcare Life Sciences, Bucks, UK).

while a similar result was observed on three out of six *L. rhamnosus* strains in group A. About half of the strains in group B were positive to the assay.

3.4. PCR assay for the detection of remnant of phages Lrm1, J-1, PL-1, A2, AT3 and Lc-Nu

A multi-alignment of entire phage genomes indicated that there were just few, small regions showing consensus for all the phages in group 3. To avoid making extra phage subgroups, and consequently too many PCR primers, those circumscribed consensus regions were used, even if they corresponded to ORFs of unknown function or non-coding sequences. Besides, phage sequences found in bacterial genomes have in this case a higher degree of fragmentation, thus making difficult to find suitable pairs of primers matching also the genome of *L. casei* BL23. The software GEMI rendered several primers, of which two forward and two reverse were manually selected, checked and eventually combined into pairs #6, #7, #8 and #9 (Table 3). Only pairs #6 and #7 amplified phage Lc-Nu.

Amplification of primers pairs #6 to #9 on the strain *L. casei* BL23 is shown in Fig. 1. Pairs #6 and #8 amplified one fragment of expected size in almost all the strains of the *casei* group tested, either from groups A or B. Some *L. rhamnosus* strains were noticeable exceptions (Table 1). The high percentage of positive cases was somewhat unexpected, considering the process followed for primer design in this case. Pairs #7 and #9 showed a similar pattern of positive cases, but an extra, smaller fragment was observed for most strains. In a few cases, either a unique smaller fragment or two (for pair #9 only) or even three fragments of wrong size were observed (Table 1). Primer p9_R, the only one constituting both pairs #7 and #9 (Table 3) would be responsible of the extra amplifications observed. In view of the high percentage of positive strains within the *casei* group, it is worth remarking that the assay was negative for all other tested bacteria (group C), even for non-*casei* species of *Lactobacillus* (Table 1).

3.5. Correlation between PCR results and presence of inducible prophages

The presence of inducible prophages was determined for strains of group B. Induction with MMC led to either total (13 strains) or partial (5 strains) lysis, but prophage DNA could be extracted only in 10 cases (Table 4), including strains with partial lysis, as it was observed in a previous study (Mercanti et al., 2011). For the remaining eight strains, there was probably a low frequency of induction, phage DNA degradation, or inhibition of bacterial growth without actual prophage induction. At least eight different BglII restriction profiles were evidenced for group B strains (Fig. 2). The commercial strain *L. casei* 17052 was the only one in this group containing a prophage with an *iA2-like* BglII profile (Fig. 2). Similar to *L. casei* BL23, *L. casei* 17052 produced amplifications with all the primers tested in this study (Table 1).

Table 4
Prophage induction of Group B strains.

Strain	Lysis after MMC Induction?	Phage DNA presence in the supernatans of MMC induction
Lactobacillus casei INL 20	Yes, partial	–
Lactobacillus casei INL 23	Yes, complete	+
Lactobacillus casei INL 43	Yes, complete	+
Lactobacillus casei INL 46	Yes, complete	–
Lactobacillus casei INL 47	Yes, complete	+
Lactobacillus casei INL 136	Yes, partial	–
Lactobacillus casei INL 241	Yes, complete	+
Lactobacillus casei INL 264	Yes, partial	–
Lactobacillus casei INL 274	Yes, complete	+
Lactobacillus casei INL 276	Yes, complete	–
Lactobacillus casei INL 17	Yes, partial	+
Lactobacillus casei INL 279	Yes, complete	+
Lactobacillus casei YOL-G	Yes, complete	–
Lactobacillus casei YOL-CH	Yes, complete	–
Lactobacillus paracasei ATCC 25302	Yes, complete	+
Lactobacillus rhamnosus ATCC 7469	Yes, partial	–
Lactobacillus rhamnosus GG	No	–
Lactobacillus paracasei 906	Yes, complete	+
Lactobacillus casei 17051	No	–
Lactobacillus casei 17052	Yes, complete	+

4. Discussion

Regarding LAB, PCR-based methods reported in the past successfully detected phages infecting *Streptococcus thermophilus* (del Rio et al., 2008) and *Lactobacillus* of the *casei* group (Binetti et al., 2008). Other methods allowed also discrimination between dairy lactococcal phage groups (Labrie and Moineau, 2000), or specific detection of phages of *Lc. lactis*, *S. thermophilus* and *L. delbrueckii* using a single multiplex PCR reaction (del Rio et al., 2007). From a different perspective, the present study presents a simple PCR method focused on the classification of strains of the *casei* group based on their prophage content. The assay comprises the selective detection of prophages or remnants unrelated from the viewpoint of DNA homology, with a main division established between complete prophages and fragments of lytic phages.

For the amplification of *iA2-like* prophages, primers pairs #1 and #2 were designed on well conserved ORFs. Although reverse primer of pair #3 was designed on a non-coding DNA region instead of a functional ORF, this region is however well conserved throughout the subgroup (II) of phages to be selectively detected. Given the high degree of conservation throughout genomes, the design was relatively easy to carry out and good results were obtained with three out of four pairs of primers tested. It is important to highlight that all the primers sequences in this study have total or partial identity only to reported bacterial strains of the *casei* group, and no amplification existed for strains of group C, indicating that no false positives would occur when using these PCR assays. Pairs #2 and #3, used separately to detect subgroups I and II, were preferred over pair #1 alone, because the latter did not amplify on some strains. Anyway, most strains harboring *iA2-like* prophages will be positive in case of using only pair #1 for simplicity. The fact that more strains of group B were positive to pair #2 than to pair #3 indicates that prophages of subgroup II would be less widespread than prophages of subgroup I. With one exception (*L. paracasei* Bio), commercial strains known to harbor *iA2-like* prophages (Mercanti et al., 2011) were positive for at least two of the three primers. Four other commercial strains containing a prophage with a restriction profile common but different to iA2 (Mercanti et al., 2011) were also positive. Nevertheless, according to RAPD-PCR fingerprinting both groups of commercial strains are closely related, and likewise could be their prophages (Mercanti et al., 2011). The rest of the strains containing diverse MMC-inducible prophages were negative for this assay, probably indicating that none of those prophages (except that of *L. paracasei* L26) are related to iA2.

In the case of phages C_L1/C_L2/*iLp*1308/*iLp*84 (group 2), only fragments of their genomes were found in lysogenic strains. Hence, DNA sequences available for primer design were circumscribed to certain areas. In spite of this hurdle, two pairs of primers could be designed within conserved genes. According to the high percentage (> 50%) of positive results observed, sequences amplified with these primers have a broader distribution than *iA2-like* prophages. Pairs #4 and #5 displayed a similar pattern of amplification, but three strains were amplified only with pair #4, which was consequently considered better for the assay.

The PCR methodology developed by Binetti et al. (2008) successfully detected phages A2, AT3, Lc-Nu (the only three *casei* group phages fully sequenced at that time), and some other phages recently sequenced (PL-1, J-1), using two pairs of primers. However, the purpose

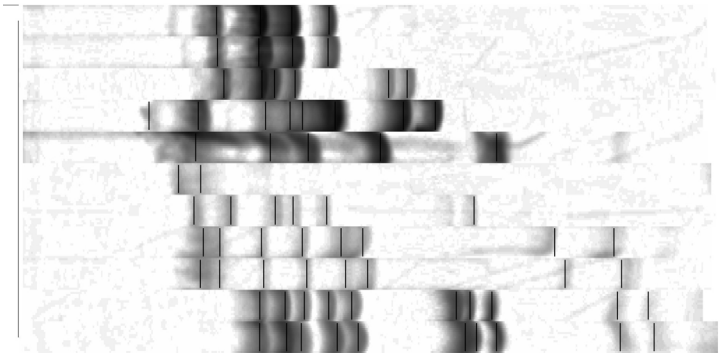

Fig. 2. BglII restriction patterns of prophages for which DNA could be directly isolated from supernatants of MMC induction of group B strains. The names of the corresponding lysogenic strains are indicated at the right. Gel lanes were processed with the software package BioNumerics™ (version 5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium).

of that study was to detect lytic phages instead of lysogenic bacteria. In fact, according to a BLAST search, that method would amplify bacterial DNA of strains *L. casei* ATCC 393 (harboring phage AT3) (Toh et al., 2013), *L. rhamnosus* M1 (harboring prophage Lrm1) (Durmaz et al., 2008), *L. paracasei* KL1 and *L. paracasei* JCM 8130 (Toh et al., 2013), but would not detect unrelated phages also infecting the *casei* group (iA2, C_L1/C_L2/i*Lp*84/i*Lp*1308). In our study, the design of primers for the detection of the same phages studied by Binetti et al. (2008) (group 3) took into account regions of phage genomes usually present in bacterial DNA, and consequently match many additional *casei* group strains: *L. casei* strains W56, BD-II, LC2W, BL23, ATCC 334, LcA, LcY and LOCK919, *L. paracasei* strains N1115, 8700:8, CAUH35 and L9, and *L. rhamnosus* strains BPL5 and Lc 705. The genomes of *L. casei* 12A and *L. rhamnosus* strains LOCK900, LOCK908 and GG (ATCC 53103) contain the sequence of primer p6_R (though not those of forward primers p6_F and p9_F, resulting in no amplification). This spectrum covers most of the *casei* group strains sequenced so far, being exceptions *L. casei* strain Zhang and some *L. rhamnosus* strains. The genome of probiotic *L. casei* Zhang contains less transposases genes than *L. casei* ATCC 334 and BL23. Therefore, genome diversification mediated by prophages and insertion elements would be less common (Zhang et al., 2010b). In fact only one non-functional prophage remnant, Lcazh1, has been identified in the genome of *L. casei* Zhang, sharing similarities with a prophage present in *Lactobacillus plantarum* WCSF1 rather than with other *casei* group strains, probably originated on a genetic exchange between different species of *Lactobacillus* (Zhang et al., 2010a; Zhang et al., 2010b). In concordance, none of the other primers designed in our study (pairs #1 to #5) matched the genome of *L. casei* Zhang. Interestingly, our PCR assay would help detecting the seemingly low percentage of strains lacking prophages.

What is more, it was found that the genomes of all the phages of group 2 (C_L1/C_L2/i*Lp*84/i*Lp*1308) are amplified by pair #6. This pair amplified almost all the tested strains of the *casei* group, including all of those positive to primer pairs #4 and #5 (phages of group 2), and could be consequently regarded as of "broad-spectrum". The fact that many strains share this region raises the question of why it is not eliminated from bacterial genomes. As reviewed by Mills et al. (2013), phages exert a great influence in the composition of human gut microbiota, and lysogeny seems to be the prevailing life cycle within this ecosystem. These authors discussed the relationship between prophage induction in probiotic bacteria and gut microbiota composition. Ideally, probiotics must possess phage resistance mechanisms to avoid phage evolution on the intestinal ecosystem but, on the other hand, phage-resistant derivatives might loss probiotic attributes of the parent strain (Mills et al., 2013). Our results point out once more the extensive occurrence of prophages and their remnants in probiotic bacteria, suggesting that some conserved sequences of phage origin and undetermined function seem to be of high value for the strains. This trend favors phage diversification instead of blocking phage attacks, but certainly justifies more research on this matter.

5. Conclusions

The PCR method developed in the present study is fast, economic, and able to assess lysogeny in strains of this group, allowing their classification from the viewpoint of prophage content and diversity. In this study, a few primers allowed the detection of diverse, seemingly unrelated prophages or phage remnants. Pairs #2 and #3 (used together), pair # 4 and pair #6 detected the three main groups of temperate phages of *L. casei* group known to date. This assay would also help identifying the seemingly low percentage of strains lacking prophages within this bacterial group.

Conflict of interest

None of the authors have a financial, personal or other conflict of interest with people or organizations that could inappropriately influence or bias the content of the study.

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References

Ai, L., Chen, C., Zhou, F., Wang, L., Zhang, H., Chen, W., Guo, B., 2011. Complete genome sequence of the probiotic strain *Lactobacillus casei* BD-II. J. Bacteriol. 193, 3160–3161.

Aleksandrzak-Piekarczyk, T., Koryszewska-Baginska, A., Bardowski, J., 2013. Genome sequence of the probiotic strain Lactobacillus rhamnosus (formerly *Lactobacillus casei*) LOCK900. Genome Announcements 1.

Alemayehu, D., Ross, R.P., O'Sullivan, O., Coffey, A., Stanton, C., Fitzgerald, G.F., McAuliffe, O., 2009. Genome of a virulent bacteriophage Lb338-1 that lyses the probiotic *Lactobacillus paracasei* cheese strain. Gene 448, 29–39.

Baugher, J.L., Durmaz, E., Klaenhammer, T.R., 2014. Spontaneously induced prophages in *Lactobacillus gasseri* contribute to horizontal gene transfer. Appl. Environ. Microbiol. 80, 3508–3517.

Binetti, A., Del Rio, B., Martin, M., Alvarez, M., 2005. Detection and characterization of *Streptococcus thermophilus* bacteriophages by use of the antireceptor gene sequence. Appl. Environ. Microbiol. 71, 6096–6103.

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

Briggiler Marcó, M., Mercanti, D., Reinheimer, J.A., Quiberoni, A., 2011. Performance of spontaneous phage-resistant derivatives of *Lactobacillus plantarum* in fermented milk manufacture. Int. Dairy J. 21, 857–862.

Canchaya, C., Fournous, G., Chibani-Chennoufi, S., Dillmann, M.L., Brussow, H., 2003. Phage as agents of lateral gene transfer. Curr. Opin. Microbiol. 6, 417–424.

Capra, M.L., Binetti, A.G., Mercanti, D.J., Quiberoni, A., Reinheimer, J.A., 2009. Diversity among *Lactobacillus paracasei* phages isolated from a probiotic dairy product plant. J. Appl. Microbiol. 107, 1350–1357.

Capra, M.L., Mercanti, D.J., Reinheimer, J.A., Quiberoni, A.L., 2010. Characterisation of three temperate phages released from the same *Lactobacillus paracasei* commercial strain. Int. J. Dairy Technol. 63, 396–405.

Casjens, S.R., Gilcrease, E.B., Winn-Stapley, D.A., Schicklmaier, P., Schmieger, H.,

Pedulla, M.L., Ford, M.E., Houtz, J.M., Hatfull, G.F., Hendrix, R.W., 2005. The generalized transducing *Salmonella* bacteriophage ES18: complete genome sequence and DNA packaging strategy. J. Bacteriol. 187, 1091–1104.
Chen, C., Ai, L., Zhou, F., Wang, L., Zhang, H., Chen, W., Guo, B., 2011. Complete genome sequence of the probiotic bacterium *Lactobacillus casei* LC2W. J. Bacteriol. 193, 3419–3420.
Desai, A.R., Shah, N.P., Powell, I.B., 2006. Discrimination of dairy industry isolates of the *Lactobacillus casei* group. J. Dairy Sci. 89, 3345–3351.
Dieterle, M.E., Jacobs-Sera, D., Russell, D., Hatfull, G., Piuri, M., 2014. Complete genome sequences of *Lactobacillus* phages J-1 and PL-1. Genome Announcements 2.
Dieterle, M.E., Fina Martin, J., Duran, R., Nemirovsky, S.I., Sanchez Rivas, C., Bowman, C., Russell, D., Hatfull, G.F., Cambillau, C., Piuri, M., 2016. Characterization of prophages containing evolved Dit/Tal modules in the genome of *Lactobacillus casei* BL23. Appl. Microbiol. Biotechnol. 100 (21), 9201–9215.
Douillard, F.P., Kant, R., Ritari, J., Paulin, L., Palva, A., de Vos, W.M., 2013. Comparative genome analysis of *Lactobacillus casei* strains isolated from Actimel and Yakult products reveals marked similarities and points to a common origin. Microbiol. Biotechnol. 6, 576–587.
Dupont, K., Vogensen, F., Josephsen, J., 2005. Detection of lactococcal 936-species bacteriophages in whey by magnetic capture hybridization PCR targeting a variable region of receptor-binding protein genes. J. Appl. Microbiol. 98, 1001–1009.
Durmaz, E., Miller, M., Azcarate-Peril, M., Toon, S., Klaenhammer, T., 2008. Genome sequence and characteristics of Lrm1, a prophage from industrial *Lactobacillus rhamnosus* strain M1. Appl. Environ. Microbiol. 74, 4601–4609.
Everson, T.C., 1991. Control of phages in the dairy plant. Bull. Int. Dairy Fed. 263, 4–11.
Garcia, P., Ladero, V., Suarez, J.E., 2003. Analysis of the morphogenetic cluster and genome of the temperate *Lactobacillus casei* bacteriophage A2. Arch. Virol. 148, 1051–1070.
Guglielmotti, D.M., Mercanti, D.J., Reinheimer, J.A., Quiberoni, A., 2012. Review: efficiency of physical and chemical treatments on the inactivation of dairy bacteriophages. Front. Microbiol. 2, 282.
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.
Hochwind, K., Weinmaier, T., Schmid, M., van Hemert, S., Hartmann, A., Rattei, T., Rothballer, M., 2012. Draft genome sequence of *Lactobacillus casei* W56. J. Bacteriol. 194, 6638.
Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P., Lebeer, S., De Keersmaecker, S.C., Vanderleyden, J., Hamalainen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjarvi, T., Auvinen, P., de Vos, W.M., 2009. Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. Proc. Natl. Acad. Sci. U. S. A. 106, 17193–17198.
Koryszewska-Baginska, A., Aleksandrzak-Piekarczyk, T., Bardowski, J., 2013. Complete genome sequence of the probiotic strain *Lactobacillus casei* (formerly *Lactobacillus paracasei*) LOCK919. Genome Announcements 1.
Labrie, S., Moineau, S., 2000. Multiplex PCR for detection and identification of lactococcal bacteriophages. Appl. Environ. Microbiol. 66, 987–994.
Lo, T.C., Shih, T.C., Lin, C.F., Chen, H.W., Lin, T.H., 2005. Complete genomic sequence of the temperate bacteriophage PhiAT3 isolated from *Lactobacillus casei* ATCC 393. Virology 339, 42–55.
Mahony, J., Bottacini, F., van Sinderen, D., Fitzgerald, G.F., 2014. Progress in lactic acid bacterial phage research. Microb. Cell Fact. 13, 1–12.
Martín, M.C., Ladero, V., Alvarez, M.A., 2006. PCR Identification of lysogenic *Lactococcus lactis* strains. J. Verbrauch. Lebensm. 1, 121–124.
Maze, A., Boel, G., Zuniga, M., Bourand, A., Loux, V., Yebra, M.J., Monedero, V., Correia, K., Jacques, N., Beaufils, S., Poncet, S., Joyet, P., Milohanic, E., Casaregola, S., Auffray, Y., Perez-Martinez, G., Gibrat, J.F., Zagorec, M., Francke, C., Hartke, A., Deutscher, J., 2010. Complete genome sequence of the probiotic *Lactobacillus casei* strain BL23. J. Bacteriol. 192, 2647–2648.
Mercanti, D.J., Carminati, D., Reinheimer, J.A., Quiberoni, A., 2011. Widely distributed lysogeny in probiotic lactobacilli represents a potentially high risk for the fermentative dairy industry. Int. J. Food Microbiol. 144, 503–510.
Mercanti, D.J., Guglielmotti, D.M., Patrignani, F., Reinheimer, J.A., Quiberoni, A., 2012. Resistance of two temperate *Lactobacillus paracasei* bacteriophages to high pressure homogenization, thermal treatments and chemical biocides of industrial application. Food Microbiol. 29, 99–104.
Mercanti, D.J., Rousseau, G.M., Capra, M.L., Quiberoni, A., Tremblay, D.M., Labrie, S.J., Moineau, S., 2016. Genomic diversity of phages infecting probiotic strains of *Lactobacillus paracasei*. Appl. Environ. Microbiol. 82, 95–105.
Mills, S., Shanahan, F., Stanton, C., Hill, C., Coffey, A., Ross, R.P., 2013. Movers and shakers: influence of bacteriophages in shaping the mammalian gut microbiota. Gut Microbes 4, 4–16.
Moineau, S., Levesque, C., 2005. The control of bacteriophages in industrial fermentations. Bacteriophages: Biol. Appl. 285–296.
Proux, C., van Sinderen, D., Suarez, J., Garcia, P., Ladero, V., Fitzgerald, G.F., Desiere, F., Brussow, H., 2002. The dilemma of phage taxonomy illustrated by comparative genomics of Sfi21-like *Siphoviridae* in lactic acid bacteria. J. Bacteriol. 184, 6026–6036.
Samson, J.E., Moineau, S., 2013. Bacteriophages in food fermentations: new frontiers in a continuous arms race. Annu. Rev. Food Sci. Technol. 4, 347–368.
Sobhy, H., Colson, P., 2012. Gemi: PCR primers prediction from multiple alignments. Comp. Funct. Genomics 2012, 7831–7838.
Toh, H., Oshima, K., Nakano, A., Takahata, M., Murakami, M., Takaki, T., Nishiyama, H., Igimi, S., Hattori, M., Morita, H., 2013. Genomic adaptation of the *Lactobacillus casei* group. PLoS One 8, e75073.
Tuohimaa, A., Riipinen, K.A., Brandt, K., Alatossava, T., 2006. The genome of the virulent phage Lc-Nu of probiotic *Lactobacillus rhamnosus*, and comparative genomics with *Lactobacillus casei* phages. Arch. Virol. 151, 947–965.
Ventura, M., Canchaya, C., Bernini, V., Altermann, E., Barrangou, R., McGrath, S., Claesson, M.J., Li, Y., Leahy, S., Walker, C.D., Zink, R., Neviani, E., Steele, J., Broadbent, J., Klaenhammer, T.R., Fitzgerald, G.F., O'Toole, P.W., van Sinderen, D., 2006. Comparative genomics and transcriptional analysis of prophages identified in the genomes of *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus casei*. Appl. Environ. Microbiol. 72, 3130–3146.
Wang, S., Zhu, H., He, F., Luo, Y., Kang, Z., Lu, C., Feng, L., Lu, X., Xue, Y., Wang, H., 2014. Whole genome sequence of the probiotic strain Lactobacillus paracasei N1115, isolated from traditional chinese fermented milk. Genome Announcements 2.
Zago, M., De Lorentiis, A., Carminati, D., Comaschi, L., Giraffa, G., 2006. Detection and identification of *Lactobacillus delbrueckii* subsp. *lactis* bacteriophages by PCR. J. Dairy Res. 73, 146–153.
Zago, M., Rossetti, L., Reinheimer, J., Carminati, D., Giraffa, G., 2008. Detection and identification of *Lactobacillus helveticus* bacteriophages by PCR. J. Dairy Res. 75, 196–201.
Zhang, W.-Y., Yu, D.-L., Sun, Z.-H., Chen, W.E.I., Hu, S.-N., Meng, H.E., Zhang, H.-P., 2010a. The comparative analysis of a prophage remnant Lcazh1 in relation to other *Lactobacillus* prophages, particularly Lp3. Int. J. Dairy Technol. 63, 413–417.
Zhang, W., Yu, D., Sun, Z., Wu, R., Chen, X., Chen, W., Meng, H., Hu, S., Zhang, H., 2010b. Complete genome sequence of *Lactobacillus casei* Zhang, a new probiotic strain isolated from traditional homemade koumiss in Inner Mongolia. China. J. Bacteriol. 192, 5268–5269.
del Rio, B., Binetti, A., Martin, M., Fernandez, M., Magadan, A., Alvarez, M., 2007. Multiplex PCR for the detection and identification of dairy bacteriophages in milk. Food Microbiol. 24, 75–81.
del Rio, B., Martin, M., Martinez, N., Magadan, A., Alvarez, M., 2008. Multiplex fast real-time PCR for quantitative detection and identification of *cos*- and *pac*-type *Streptococcus thermophilus* bacteriophages. Appl. Environ. Microbiol. 74, 4779–4781.