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Characterization of spontaneous phage-resistant derivatives of *Lactobacillus delbrueckii* commercial strains

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Abstract

A total of 44 spontaneous phage-resistant mutants were isolated from three commercial *Lactobacillus delbrueckii* strains by secondary culture and agar plate methods. Phenotypic characteristics related to their phage-resistance capacities, i.e. plaquing efficiency, phage-resistance stability, lysogeny and adsorption rates were determined. The morphological, biochemical (sugar fermentation patterns) and technological (acidifying and proteolytic activities and acidification kinetics) properties of mutants were also studied. Amplification and restriction analysis of the 16S rRNA gene (PCR-ARDRA) was applied to confirm strain identity at the subspecies level. Random amplification of polymorphic DNA (RAPD-PCR) was used to determine genetic diversity among the isolates and their respective parent strains. The secondary culture method was the most useful for obtaining phage-resistant mutants. Phage resistance stability was a variable property among the isolates, but a high level of resistance was exhibited as quantified by the efficiency of plaquing. Furthermore, a total absence of spontaneous lysogeny was demonstrated. Adsorption rates were heterogeneously distributed among the three groups of mutants. All mutants isolated from two sensitive strains were similar to them with respect to technological properties. Two groups of mutants with distinctive technological properties were isolated from the other sensitive strain. PCR-ARDRA revealed that two out of three sensitive strains identified commercially as *Lb. delbrueckii* subsp. *bulgaricus* were actually *Lb. delbrueckii* subsp. *lactis*. Some of the phage-resistant mutants that were obtained might be used in culture rotation programs without regulatory restrictions when commercial strains become sensitive to phages present in industrial environments.

Keywords: *Lactobacillus delbrueckii*; Phage resistance; Phage-resistant mutants; Dairy fermentations

1. Introduction

Phage infections represent a serious problem in dairy fermentative processes, causing a partial or total delay in the acidifying activity of lactic acid starters. *Lactobacillus delbrueckii* is a lactic acid bacteria species of great technological importance. *Lb. delbrueckii* subsp. *bulgaricus* is part of commercial yogurt cultures. Also, this species and *Lb. delbrueckii* subsp. *lactis* are present in whey cultures used for the production of Argentinean (Reinheimer et al., 1995, 1996) and Italian (Neviani and Carini, 1994; Giraffa et al., 1997, 2004) hard cheeses. Information on phages that attack these two subspecies is scarce, and the first *Lb. delbrueckii* phages isolated in Argentina were characterized only recently (Quiberoni et al., 2003, 2004). To minimize phage dissemination in dairy plants, various strategies are applied; such as strain rotation programs, direct vat-inoculation of starters, optimized sanitation and use and improvement of phage-resistant starter cultures (Moineau et al., 2002). The isolation of spontaneous phage-resistant mutants and the development of transconjugants were the first approaches used to obtain phage-resistant derivatives from phage sensitive strains. These techniques have the convenience of simplicity and they are considered "natural" strategies to improve strains, since there are no regulatory restrictions on use of the improved strains in industrial

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environments (Sing and Klaenhammer, 1993; Weimer et al., 1993; Moineau and Lévesque, 2005).

Some workers have described the isolation of spontaneous phage-resistant variants from sensitive strains of lactococci (Limsowtin and Terzaghi, 1976; Weimer et al., 1993), *Streptococcus thermophilus* (Viscardi et al., 2003), and *Lactobacillus helveticus* (Neviani et al., 1992; Carminati et al., 1993; Reinheimer et al., 1993; Quiberoni et al., 1998a,b), but there are no reports about this type of mutants isolated from *Lb. delbrueckii* strains. When these phage-resistant mutants are isolated, their relationship to the parent strain must be confirmed, principally to verify that the isolates are not the result of contamination. For this purpose, molecular methods are more accurate than biochemical tests. Some previous studies have focused on the application of molecular techniques for reliable identification of many organisms at the species and subspecies level and determining the diversity among strains belonging to a species. In this regard, restriction analysis of the amplified rRNA (PCR-ARDRA) has been successfully used to differentiate *Lb. delbrueckii* subsp. *bulgaricus* from *Lb. delbrueckii* subsp. *lactis* (Miteva et al., 2001; Roy et al., 2001), identify at the subspecies level *Lb. delbrueckii* strains isolated from dairy products (Giraffa, 1998) and differentiate this species from *Lb. helveticus* and *Lb. acidophilus* (Giraffa et al., 1998). Therefore, this method might be used to determine the species/subspecies of phage-resistant variants in order to avoid mistaking contaminating strains for real variants. On the other hand, randomly amplified polymorphic DNA (RAPD-PCR) can be used to evaluate intraspecific genetic diversity (Quiberoni et al., 1998b; Tailliez et al., 1998; Torriani et al., 1999; Giraffa et al., 2004).

The technological performance of *Lb. delbrueckii* phage-resistant derivatives must be confirmed, because some desirable properties can be absent in mutants. For commercial *Lactococcus* strains, the mutants obtained usually grew slowly, and exhibited diminished acidifying power, low proteolytic activity, limited phage resistance specificity and, in many cases, suffered reversion of the phage resistance phenotype (Klaenhammer, 1984; Weimer et al., 1993; Coffey et al., 1998; Moineau, 1999; Sturino and Klaenhammer, 2004). However, mutants isolated from *Lb. helveticus* showed technological properties that were identical to or better than those of their parent strains, and some of the mutants were successfully used for commercial cheese manufacture (Quiberoni et al., 1998a).

The aim of this work was to investigate the possibility of isolating spontaneous phage-resistant mutants with adequate technological characteristics for industrial uses from three commercial strains of *Lb. delbrueckii*.

2. Materials and methods

2.1. Bacterial strains, bacteriophages and culture conditions

Three commercial strains (identified as YSD V, Ab_1 and Ib_3) of *Lb. delbrueckii* subsp. *bulgaricus* (identification provided by the respective suppliers) were used. They were isolated from commercial starters used in Argentinean milk fermentation processes that were sensitive to phages BYM, YAB and Ib_3, respectively. These phages were isolated from yogurt samples that showed slow acidification.

Phage sensitive strains and their mutants were grown and routinely reactivated overnight (42 °C) in de Man, Rogosa and Sharpe (MRS) broth or agar (Biokar, Beauvais, France). They were maintained as frozen (−80 °C) stock cultures in sterile, reconstituted (10%, w/v), commercial, nonfat, dry skim milk (RSM; Merck, Darmstadt, Germany). Phage stocks were prepared as described by Neviani et al. (1992) and stored at 4 °C and −80 °C with 15% (v/v) of glycerol. Phage enumerations were performed by the double-layer plaque titration method (Svensson and Christiansson, 1991), and numbers were expressed as plaque-forming units (PFU) per milliliter.

2.2. Isolation of phage-resistant mutants

Two methodologies were used; the agar plate method (AP), as previously described (Reinheimer et al., 1995), and the secondary culture method (SC) (Callegari, 1992; Carminati et al., 1993) modified as follows. An overnight culture of each strain in MRS-Ca broth (MRS broth supplemented with 10 mmol l^{-1} $CaCl_2$) was infected, at different infection ratios (multiplicity of infection, m.o.i. of 1, 0.1 and 0.01), with suspensions of the corresponding lytic phage. After incubation at 42 °C for 6 to 8 h, cultures exhibiting complete lysis and secondary growth after further incubation for up to 48 h at 42 °C were selected and streaked on MRS agar plates. After incubation for 48 h at 42 °C, isolated colonies from each host strain–lytic phage system were selected and cultured in MRS broth. These isolates were purified by three consecutive streakings on MRS agar. Phage resistance was confirmed by challenging each isolate cultivated in MRS-Ca broth with the corresponding lytic phage. Three subcultures were performed, and isolates that were able to grow normally under these conditions were retained as true phage-resistant mutants (Reinheimer et al., 1993) and stored (−80 °C). The efficiency of each isolation methodology was calculated as follows: (number of confirmed phage-resistant mutants/number of presumptive phage-resistant mutants) × 100.

2.3. Characterization of phage resistance phenotype

Efficiency of plaquing (EOP) values for phages YAB, BYM and Ib_3 on their respective resistant mutants were determined using the mutants as sensitive strains in double layer plaque titrations. The ratio PFU ml^{-1} obtained with each mutant/PFU ml^{-1} obtained with the corresponding sensitive strain was defined as the EOP (Carminati et al., 1993).

Phage resistance stability was assayed by seven sequential subcultures (2%) of the mutants in MRS-Ca broth with independent infection with phages at each subculture (Carminati et al., 1993). The loss of phage resistance was determined by the culture lysis in comparison with a control (mutant subculture without phage addition). The subculture at which lysis occurred was recorded.

To examine mutants for lysogeny, an overnight MRS broth culture of each phage-resistant mutant was centrifuged at 12,000 ×*g* for 4 min and the supernatant was mixed with the corresponding sensitive strain in MRS soft agar and then over-

laid on a plate of MRS-Ca agar (Reinheimer et al., 1993), to reveal phage particles released spontaneously by mutants that were able to infect sensitive strains.

To determine adsorption rates for parent and mutant strains, exponentially growing cultures in MRS-Ca broth were mixed with each phage (m.o.i: 0.02), and then incubated for 30 min at 42 °C for adsorption. The phage-bacterial cell mixtures were centrifuged at 12,000 ×*g* for 4 min, and the presence of phage particles in the supernatants was determined by the double layer method. To evaluate the degree of phage adsorption the results were compared with the titer of a control without cells (Neviani et al., 1992).

2.4. Genetic analysis

2.4.1. Extraction of total DNA

Total DNA from washed cell pellets of each sensitive strain and their mutants, was extracted by a chelex-based procedure according to the method for Gram positive (and acid fast) bacteria described in the MicroSeq™ protocol (Perkin Elmer Europe B. V., Monza, Italy) which uses DNA extraction reagents included in the kit (Giraffa et al., 2000).

2.4.2. Amplification and restriction analysis of the 16S rRNA gene (PCR-ARDRA)

PCR-ARDRA was applied to sensitive strains and their respective phage-resistant mutants in order to confirm their identities. Two type strains were included as positive controls, *Lb. delbrueckii* subsp. *lactis* ATCC 12315^T and *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842^T.

PCR-ARDRA was performed according to Giraffa et al. (1998). DNA fragments of approximately 1.5 kbp corresponding to the 16S rRNA genes were amplified from extracted DNA using the primers described by Rodtong and Tannock (1993), 5′-CCGAGCTCAACAGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTCGACCGTTAATACGACTCACTATAGGGATACCTTGTTACGACTT-3′. DNA amplifications were performed in a thermal cycler model 9700 (Perkin Elmer). Restriction was carried out using enzyme *Eco*RI (Life Technologies), and restriction patterns were visualized by electrophoresis in 1.5% agarose gels.

2.4.3. RAPD-PCR amplification

Oligonucleotide primers M13 (Huey and Hall, 1989; Stendid et al., 1994) and 1254 (Akopyanz et al., 1992), 5′-GAGGGTGGCGGTTCT-3′ and 5′-CCGCAGCCAA-3′, respectively, were used in separate amplification assays. PCR was performed in a volume of 25 μl containing 2.5 μl of 10X PCR buffer (Perkin Elmer), 200 μM of each dNTP, 0.6 IU of *Taq* DNA polymerase (Perkin Elmer), 3.0 mM $MgCl_2$, 2.0 μM primer M13 or 0.8 μM primer 1254, and 1.5 μl of template DNA. The PCR program used for primer M13 was carried out according to Giraffa et al. (2000). A first denaturing step of 94 °C for 2 min was followed by 40 cycles of 94 °C for 1 min (denaturation), 45 °C for 20 s (annealing) and 72 °C for 2 min (extension), and final extension at 72 °C for 10 min. For primer 1254 the following program was used. Four initial cycles of 94 °C for 5 min, 36 °C for 5 min and 72 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 10 min. The amplification products were visualized by electrophoresis in 1.5% agarose gels and staining with ethidium bromide. The DNA molecular weight marker 1 kbp Plus DNA Ladder (Invitrogen, Milan, Italy) was used as a standard. The amplification pattern images of the gels were captured using the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Celbio, Milan, Italy) equipped with the EDAS 290 imaging cabinet. Images were saved as TIFF files and analyzed with the pattern analysis software package BioNumerics™ (Version 3.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium). Calculation of similarity of band profiles was based on the Pearson correlation coefficient *r*, and strains were grouped using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) (Vauterin and Vauterin, 1992).

2.5. Characterization of phage-resistant mutants

Cells were examined microscopically, under phase contrast illumination (1000×, Microscope Jenamed 2 CARL ZEISS); and colony morphologies on MRS agar were determined. API 50 CHL test strips (API System, Montalieu-Vercieu, France) were used, according to the manufacturer's instructions, to evaluate sugar fermentation patterns. Milk acidification kinetics, as well as proteolytic (*o*-phthaldialdehyde spectrophotometric assay, OPA Test, Church et al., 1983) and acidifying activities were determined by inoculation (2%, v/v) of the strains in sterile, reconstituted (10%, w/v), commercial, dry skim milk (RSM) and incubation for 24 h at 42 °C. pH values were measured with a model SA 720 pH meter (Orion, Beverly, Massachusetts, USA) and plotted against time. Proteolytic activity values were expressed as the difference in absorbance at 340 nm (A_{340}) between strain cultures and a control of uninoculated milk. The acidity developed, measured by titration with 0.1 N NaOH to pH 8.4, was expressed as % lactic acid. The fast/slow character of the variants was determined by inoculation (2%, v/v) of mutant cultures in milk, milk supplemented with 1% glucose or with 0.25% casein hydrolysate, and milk supplemented with both 1% glucose and 0.25% casein hydrolysate (Mc Kay and Baldwin, 1974; Efstathio and Mc Kay, 1976) and measurement of pH values after 24 h at 42 °C (Reinheimer et al., 1995).

3. Results

3.1. Isolation of phage-resistant mutants

In general, the secondary culture method was more effective than the agar plate method for obtaining phage-resistant variants from all strains studied (Table 1).

A total of 44 phage-resistant variants were obtained from the three phage-sensitive strains.

3.2. Characterization of phage resistance phenotype

When phages YAB, Ib_3 and BYM at high titers were assayed on the mutants previously isolated, no plaque-forming ability was demonstrated (EOP $< 5.10^{-8}$).

Table 1
Phage-resistant mutants isolated from *Lb. delbrueckii* strains using secondary culture (SC) and agar plate (AP) methods

Strain	Phage	Number of presumptive phage-resistant mutants		Number of true phage-resistant mutants		Isolation efficiency of true phage-resistant mutants (%)	
		SC	AP	SC	AP	SC	AP
YSD V	BYM	20	5	12	0	60	0
Ab_1	YAB	23	6	19	1	83	17
Ib_3	Ib_3	33	7	10	2	30	29

Mutants isolated from strain YSD V all maintained the phenotype up to the seventh subculture. All mutants isolated from strain Ab_1 maintained resistance up to the sixth subculture and only one was not resistant at the seventh subculture. Phage resistance stability was lower for mutants from strain Ib_3 because only 41.7% of them were resistant up to the seventh subculture.

Free phages were not detected in the supernatants of broth cultures of phage-resistant mutants.

The mutants isolated from *Lb. delbrueckii* YSD V exhibited very high adsorption rates, while those obtained from *Lb. delbrueckii* Ab_1 showed very low or null adsorption rates (Fig. 1). The phage-resistant mutants obtained from *Lb. delbrueckii* Ib_3 were heterogeneous with respect to adsorption, with rates ranging from 0 to more than 99%. All sensitive strains showed adsorption rates higher than 99%.

3.3. Genetic analysis

3.3.1. ARDRA profiles

PCR-ARDRA profiles of phage-resistant mutants were identical to those of their respective parent strains (Fig. 2). Before restriction, DNA fragments of approximately 1.5 kbp were obtained from all three sensitive strains and phage-resistant mutants used in this work, as well as from the two type strains. After *Eco*RI digestion, a clear difference between *lactis* and *bulgaricus* subspecies patterns was apparent. *Lb. delbrueckii* Ab_1, *Lb. delbrueckii* Ib_3, their respective mutants and *Lb. delbrueckii* subsp. *lactis* ATCC 12315^T showed, after restriction, a DNA band of approximately 1.4 kbp. Consequently, strains Ab_1 and Ib_3 were classified as *Lb. delbrueckii* subsp. *lactis* strains. *Lb. delbrueckii* YSD V, its mutants and *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842^T yielded two fragments of approximately 0.6 and 0.8 kbp, so strain YSD V was classified as *Lb. delbrueckii* subsp. *bulgaricus*.

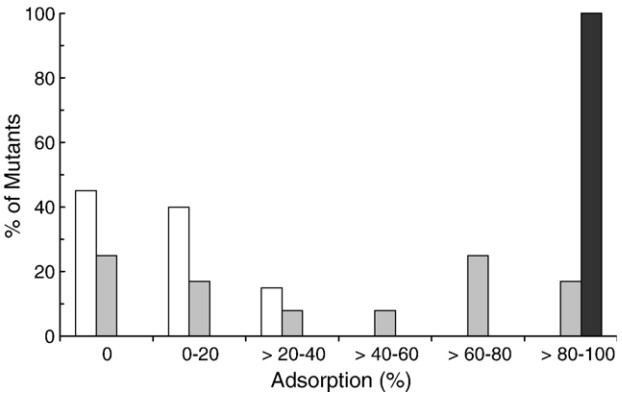

Fig. 1. Distribution of adsorption rates (% of adsorbed phage particles in MRS-Ca broth after 30 min at 42 °C) among spontaneous phage-resistant mutants isolated from *Lb. delbrueckii* YSD V ($n=12$), *Lb. delbrueckii* Ab_1 ($n=20$) and *Lb. delbrueckii* Ib_3 ($n=12$) using phages BYM (■), YAB (□) and Ib_3 (▣), respectively.

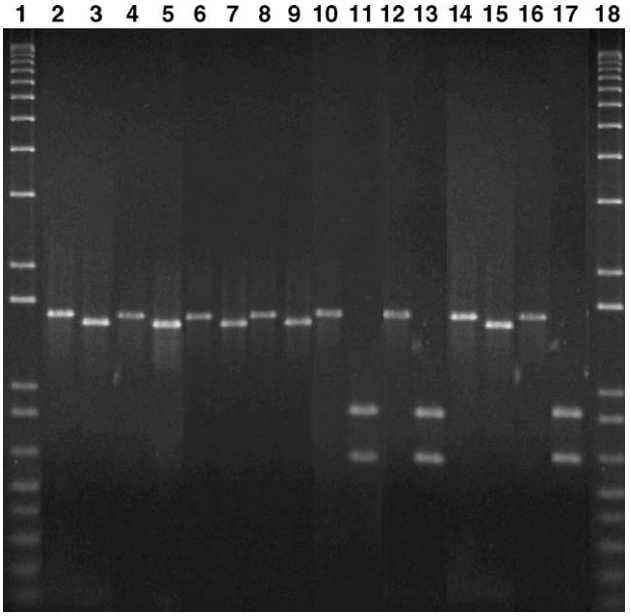

Fig. 2. 16S rRNA ARDRA of *Lb. delbrueckii* subsp. *bulgaricus* YSD V and *Lb. delbrueckii* subsp. *lactis* Ab_1 and Ib_3, and some phage-resistant mutants isolated from them, digested (lanes 3, 5, 7, 9, 11, 13, 15, 17) or undigested (lanes 2, 4, 6, 8, 10, 12, 14, 16) with restriction enzyme *Eco*RI. Lanes 2 and 3: Ab_1, Lanes 4 and 5: Ab_1 mutant, Lanes 6 and 7: Ib_3, Lanes 8 and 9: Ib_3 mutant, Lanes 10 and 11: YSD V, Lanes 12 and 13: YSD V mutant, Lanes 14 and 15: *Lb. delbrueckii* subsp. *lactis* ATCC 12315^T, Lanes 16 and 17: *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842^T, Lanes 1 and 18: molecular weight marker 1 kbp Plus Ladder.

3.3.2. RAPD profiles

With profiles from the primers M13 and 1254, two separate cluster structures were firstly constructed, but good resolution was not observed. Therefore, the profiles were combined, with clear separation of the mutants into three distinct clusters, which included the respective parent strains (Fig. 3). For each cluster, similarity coefficients higher than 94% were obtained, revealing a strong genetic homology between the parent strain and the mutants derived from it.

3.4. Characterization of phage-resistant mutants

All phage-resistant mutants were identical to the corresponding parent strain in cell and colony morphologies and sugar fermentation patterns.

Acidifying and proteolytic characteristics were revealed as phenotypes distributed heterogeneously among the 20 phage-resistant mutants isolated from *Lb. delbrueckii* subsp. *lactis* Ab_1. These mutants were divided into two groups according to the pH

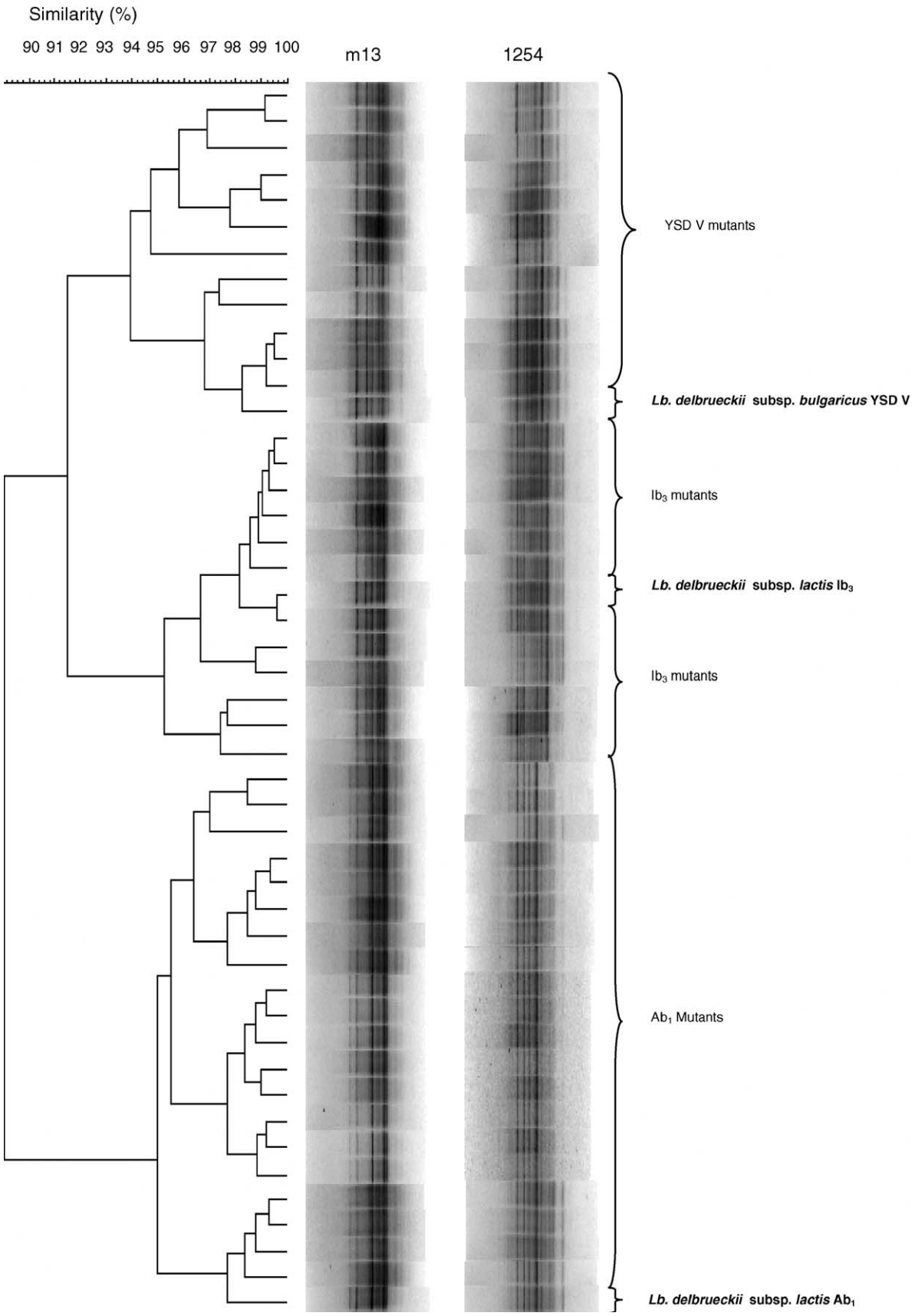

Fig. 3. RAPD profiles obtained from total DNA from *Lb. delbrueckii* subsp. *bulgaricus* YSD V, *Lb. delbrueckii* subsp. *lactis* Ib_3, *Lb. delbrueckii* subsp. *lactis* Ab_1 and their respective phage-resistant mutants using primers M13 and 1254.

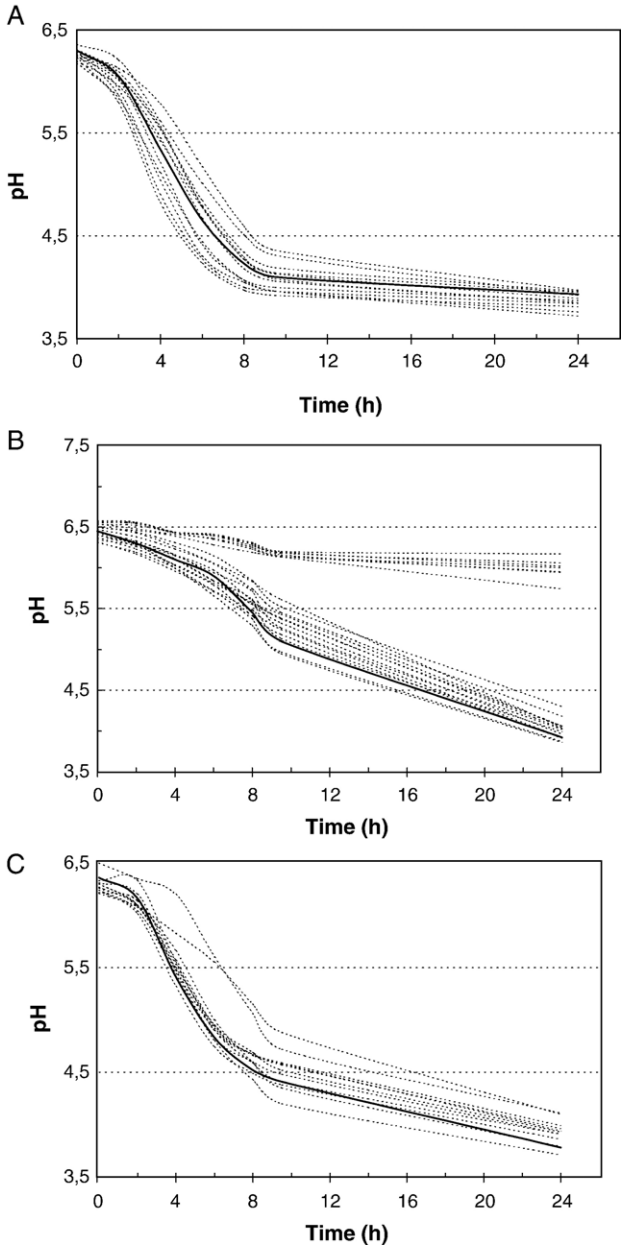

Fig. 4. Milk acidification kinetics of sensitive strains (—) of *Lb. delbrueckii* YSD V (A), *Lb. delbrueckii* Ab_1 (B), *Lb. delbrueckii* Ib_3 (C) and their respective phage-resistant mutants (- - -).

values reached in RSM cultures after 24 h at 42 °C as 'fast' (pH = 3.91 ± 0.06) or 'slow' (pH = 5.76 ± 0.12). 'Fast' mutants exhibited acidifying activity (1.60 ± 0.08% lactic acid) and proteolytic activity (A_{340} 0.58 ± 0.10) similar to those of the parent strain (pH = 3.99 ± 0.12, 1.75 ± 0.06% lactic acid, A_{340} 0.56 ± 0.05). 'Slow' variants showed lower values for acidifying (0.33 ± 0.04% lactic acid) and proteolytic (A_{340} 0.09 ± 0.03) activities.

When 'slow' variants were cultured in RSM supplemented with casein hydrolysate, they were able to coagulate milk and produced a significant ($P < 0.05$) decrease of pH, similar to 'fast' strains. The addition of glucose did not have a significant influence ($P > 0.05$) on the acidifying activities of the mutants (data not shown).

All phage-resistant mutants obtained from *Lb. delbrueckii* subsp. *bulgaricus* YSD V were classified as 'fast' with culture pH values being 4.03 ± 0.08 after 24 h at 42 °C, and they all exhibited high acidifying (1.22 ± 0.12% lactic acid) and proteolytic (A_{340} 0.35 ± 0.05) activities. These values were comparable to those exhibited by the parent strain (pH = 4.07 ± 0.02, 1.14 ± 0.10% lactic acid, A_{340} 0.49 ± 0.05).

A similar behavior was observed among phage-resistant mutants isolated from *Lb. delbrueckii* subsp. *lactis* Ib_3 since all variants were classified as 'fast' with cultures obtaining pH values of 4.01 ± 0.07 and they all exhibited high acidifying (1.39 ± 0.09% lactic acid) and proteolytic (A_{340} 0.49 ± 0.08) activities, like the parent strain (pH = 3.96 ± 0.15, 1.44 ± 0.15% lactic acid, A_{340} 0.55 ± 0.01).

Milk acidification kinetics are shown in Fig. 4. Phage-resistant mutants isolated from *Lb. delbrueckii* subsp. *bulgaricus* YSD V and *Lb. delbrueckii* subsp. *lactis* Ib_3 developed acidity as fast as their respective parent strains, while derivatives from *Lb. delbrueckii* subsp. *lactis* Ab_1 showed the different behaviors of 'fast' and 'slow' mutants.

4. Discussion

Several strategies are proposed to control bacteriophages in dairy environments and one of them includes the use of phage-resistant cultures. Traditionally, these phage-resistant cultures have been obtained by recombinant DNA technology (Hansen, 2002; McGrath et al., 2002) or conjugal transfer of plasmids conferring phage resistance (Pillidge et al., 2000). However, isolation of spontaneous phage-resistant mutants has been revalued recently because it is a simple and 'natural' methodology that involves no genetic manipulation.

In this work the secondary culture method was demonstrated to be more effective than the agar plate method for isolation of phage-resistant mutants. This finding agrees with that of a previous study carried out on *Lb. helveticus* spontaneous phage-resistant mutants (Quiberoni et al., 1998a). Recently, alternative strategies were reported to isolate phage-resistant variants from *S. thermophilus* strains using a combination of immunoselection and flow cytometry (Viscardi et al., 2003).

The mutants isolated in this study from two strains of *Lb. delbrueckii* subsp. *lactis* and one of *Lb. delbrueckii* subsp. *bulgaricus* exhibited a high level of phage resistance and the highest stability was observed for derivatives isolated from *Lb. delbrueckii* subsp. *bulgaricus* YSD V and *Lb. delbrueckii* subsp. *lactis* Ab_1. Spontaneous variants with a high and stable resistance to phages are the basis for the construction of defined phage-resistant starter cultures. Quiberoni et al. (1998a) obtained 66 true phage-resistant mutants using *Lb. helveticus* ATCC 15807 and the lytic phages hv and ATCC 15807-B1. In this case, stability among mutants was variable.

Free phages were not detected in the supernatants of broth cultures of phage-resistant mutants, suggesting the absence of a phage resistance mechanism linked to lysogeny among the variants.

Mutants obtained from *Lb. delbrueckii* subsp. *lactis* Ab_1 were partially or completely unable to adsorb phages, indicating

the presence of adsorption interference. A diminished adsorption was also identified as the phage resistance mechanism for spontaneous mutants isolated from *Lb. helveticus* ATCC 15807 (Quiberoni et al., 1998a) and *Lb. helveticus* CNRZ 892 (Neviani et al., 1992). A relationship between phage resistance and point mutations in the genome was hypothesized. Moreover, the production of a masking substance could reduce the availability of phage receptors, causing a decrease in adsorption efficiency (Moineau and Lévesque, 2005). Phage-resistant mutants isolated from *Lb. delbrueckii* subsp. *bulgaricus* YSD V showed very high adsorption rates suggesting interference with the injection of phage DNA or the presence of some intracellular resistance barrier, or a combination of both types of mechanisms. On the other hand, mutants obtained from *Lb. delbrueckii* subsp. *lactis* Ib_3 showed a heterogeneous distribution of adsorption rates.

Over the last years, amplified ribosomal DNA restriction analysis (ARDRA) has been successfully used for the differentiation of lactobacilli species (Drake et al., 1996; Andrighetto et al., 1998; Giraffa et al., 1998; Roy et al., 2001). In the present study, ARDRA revealed that two out of three strains identified commercially as *Lb. delbrueckii* subsp. *bulgaricus* were actually *Lb. delbrueckii* subsp. *lactis*. Furthermore, ARDRA confirmed that the mutants in each group were the same species as the parent strain. In our study, the restriction analysis of the 16S rRNA was carried out using enzyme *Eco*RI, previously used by Giraffa et al. (1998) for the identification of *Lb. delbrueckii* dairy isolates and to assign them to its three subspecies (*delbrueckii*, *lactis* and *bulgaricus*).

The comparison of RAPD patterns obtained for the phage-resistant mutants and the respective sensitive strains gave, for each group, cluster structures with high similarity coefficients. Therefore, resistant mutants appeared to be very similar to the corresponding parent strain, confirming that each group of mutants studied derived from them, and that contamination did not occur.

All mutants isolated from *Lb. delbrueckii* subsp. *bulgaricus* YSD V and *Lb. delbrueckii* subsp. *lactis* Ib_3 were similar to their parent strains with respect to the technological properties that were assayed. However, two groups of mutants with distinctive technological properties were obtained from *Lb. delbrueckii* subsp. *lactis* Ab_1. Some of these, 'slow' mutants, were unable to coagulate milk. Nevertheless, most of them, identified as 'fast' mutants, revealed milk acidification kinetics, proteolytic activity and acidifying activity values similar to those observed for the parent strain. 'Slow' variants were deficient in proteolytic activity.

The results obtained in this work demonstrated that the secondary culture method is a simple methodology for obtaining spontaneous phage-resistant mutants from *Lb. delbrueckii* strains of high technological performance. These mutants might be used in culture rotation programs without any kind of regulatory restrictions when commercial strains become sensitive to phages present in industrial environments.

Acknowledgments

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References

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

Andrighetto, C., De Dea, P., Lombardi, A., Neviani, E., Rossetti, L., Giraffa, G., 1998. Molecular identification and cluster analysis of homofermentative thermophilic lactobacilli isolated from dairy products. Research in Microbiology 149, 631–643.

Callegari, M.L., 1992. Caratterizzazione dei recettori de pare specifici per l'adesione dei batteri lattici termofili. Thesis, Dottore di Ricerca della Università Católica del Sacro Cuore, Piacenza, Italia.

Carminati, D., Zennaro, R., Neviani, E., Giraffa, G., 1993. Selezione e caratteristiche di mutanti fago-resistenti di *Lactobacillus helveticus*. Scienza e Tecnica Lattiero-Casearia 44 (1), 33–48.

Church, F.C., Swaisgood, H.E., Porter, D.H., Catignain, G.L., 1983. Spectrophometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. Journal of Dairy Science 66, 1219–1227.

Coffey, A., Coakley, M., Mc Garry, A., Fitzgerald, G.F., Ross, R.P., 1998. Increasing phage resistance of cheese starters: a case study using *Lactococcus lactis* DPC4268. Letters in Applied Microbiology 26, 51–55.

Drake, M.A., Small, C.L., Spence, K.D., Swanson, B.G., 1996. Rapid detection and identification of *Lactobacillus* spp. in dairy products by using the polymerase chain reaction. Journal of Food Protection 59, 1031–1036.

Efstathio, J.D., Mc Kay, L.L., 1976. Plasmids in *Streptococcus thermophilus*. Evidence that lactose metabolism and proteinase activity are plasmid linked. Applied and Environmental Microbiology 32, 38–44.

Giraffa, G., 1998. Population dynamics of thermophilic lactobacilli from whey starter to curd: a model study for grana cheese. 12th Forum for Applied Biotechnology, Brugge.

Giraffa, G., De Vecchi, P., Reinheimer, J.A., 1997. Population dynamics of thermophilic lactobacilli in mixed starter whey cultures. Food Research International 30 (2), 137–140.

Giraffa, G., De Vecchi, P., Rossetti, L., 1998. Note: Identification of *Lactobacillus delbrueckii* subspecies *bulgaricus* and subspecies *lactis* dairy isolates by amplified rRNA restriction analysis. Journal of Applied Microbiology 85, 918–924.

Giraffa, G., Rossetti, L., Neviani, E., 2000. An evaluation of chelex-based DNA purification protocols for the typing of lactic acid bacteria. Journal of Microbiological Methods 42, 175–184.

Giraffa, G., Andrighetto, C., Antonello, C., Gatti, M., Lazzi, C., Marcazzan, G., Lombardi, A., Neviani, E., 2004. Genotypic and phenotypic diversity of *Lactobacillus delbrueckii* subsp. *lactis* strains of dairy origin. International Journal of Food Microbiology 91, 129–139.

Hansen, E.B., 2002. Commercial bacterial starter cultures for fermented food of the future. International Journal of Food Microbiology 78, 119–131.

Huey, B., Hall, J., 1989. Hypervariable DNA fingerprinting in *Escherichia coli*. Minisatellite probe from bacteriophage M13. Bacteriology 171, 2528–2532.

Klaenhammer, T.R., 1984. Interactions of bacteriophages with lactic streptococci. Advances in Applied Microbiology 30, 1–29.

Limsowtin, G.K.V., Terzaghi, B.E., 1976. Phage-resistant mutants: their selection and use in cheese factories. New Zealand Journal of Dairy Science and Technology 11, 251–256.

Mc Kay, L.L., Baldwin, K.A., 1974. Simultaneous loss of proteinase and lactose utilizing enzymes activities in *Lactococcus* and reversal of loss by transduction. American Society for Microbiology 20, 342–346.

McGrath, S., van Sinderen, D., Fitzgerald, G.F., 2002. Bacteriophage-derived genetic tools for use in lactic acid bacteria. International Dairy Journal 12, 3–15.

Miteva, V., Boudakov, I., Ivanova-Stoyancheva, G., Marinova, B., Miltev, V., Mengaud, J., 2001. Differentiation of *Lactobacillus delbrueckii* subspecies

by ribotyping and amplified ribosomal DNA restriction analysis (ARDRA). Journal of Applied Microbiology 90, 909–918.

Moineau, S., 1999. Applications of phage resistance in lactic acid bacteria. In: Konings, W.N., Kuipers, O.P., Huis in 't Veld, J.H.J. (Eds.), Proceedings of the Sixth Symposium on Lactic Acid Bacteria: Genetics, Metabolism and Applications. Kluwer Academic Publisher, Dordrecht, pp. 337–382. Veldhoven, The Netherlands.

Moineau, S., Lévesque, C., 2005. Control of bacteriophages in industrial fermentations. In: Kutter, E., Sulakvelidze, A. (Eds.), Bacteriophages: Biology and Applications. CRC Press, Boca Raton, FL, pp. 285–296. Chap. 10.

Moineau, S., Tremblay, D., Labrie, S., 2002. Phages of lactic acid bacteria: from genomics to industrial applications. American Society for Microbiology News 68, 388–393.

Neviani, E., Carini, S., 1994. Microbiology of Parmesan cheese. Microbiologie, Aliments, Nutrition 12, 1–8.

Neviani, E., Carminati, D., Giraffa, G., 1992. Selection of some bacteriophage- and lysozyme-resistant variants of *Lactobacillus helveticus* CNRZ 892. Journal of Dairy Science 75, 905–913.

Pillidge, C.J., Collins, L.J., Ward, L.J.H., Cantillon, B.M., Shaw, B.D., Timmins, M.J., Heap, H.A., Polzin, K.M., 2000. Efficacy of four lactococcal phage resistance plasmids against phage in commercial *Lactococcus lactis* subsp. *cremoris* cheese starter strains. International Dairy Journal 10, 617–625.

Quiberoni, A., Reinheimer, J.A., Suárez, V.B., 1998a. Performance of *Lactobacillus helveticus* spontaneous phage-resistant mutants in hard cheese production. International Dairy Journal 8, 941–949.

Quiberoni, A., Reinheimer, J.A., Tailliez, P., 1998b. Characterization of *Lactobacillus helveticus* phage-resistant mutants by RAPD fingerprints and phenotypic parameters. Food Research International 31 (8), 537–542.

Quiberoni, A., Guglielmotti, D.M., Reinheimer, J.A., 2003. Inactivation of *Lactobacillus delbrueckii* bacteriophages by heat and biocides. International Journal of Food Microbiology 84, 51–62.

Quiberoni, A., Guglielmotti, D., Binetti, A., Reinheimer, J., 2004. Characterization of three *Lactobacillus delbrueckii* subsp. *bulgaricus* phages and the physicochemical analysis of phage adsorption. Journal of Applied Microbiology 96, 340–351.

Reinheimer, J.A., Morelli, R., Callegari, M.L., Bottazzi, V., 1993. Phage resistance in *Lactobacillus helveticus* CNRZ 328. Microbiologie Aliments Nutrition 11, 235–240.

Reinheimer, J.A., Suárez, V.B., Bailo, N.B., Zalazar, C.A., 1995. Microbiological and technological characteristics of natural whey cultures for Argentinian hard-cheese production. Journal of Food Protection 58 (7), 796–799.

Reinheimer, J.A., Quiberoni, A., Tailliez, P., Binetti, A.G., Suárez, V.B., 1996. The lactic acid microflora of natural whey starters used in Argentina for hard cheese production. International Dairy Journal 6, 869–879.

Rodtong, S., Tannock, G.W., 1993. Differentiation of *Lactobacillus* strains by ribotyping. Applied and Environmental Microbiology 59, 3480–3484.

Roy, D., Sirois, S., Vincent, D., 2001. Molecular discrimination of lactobacilli used as starter and probiotic cultures by amplified ribosomal DNA restriction analysis. Current Microbiology 42, 282–289.

Sing, W.D., Klaenhammer, T.R., 1993. A strategy for rotation of different bacteriophage defenses in a lactococcal single-strain starter culture system. Applied and Environmental Microbiology 59, 365–372.

Stendid, J., Karlsson, J.O., Hogberg, N., 1994. Intraspecific genetic variation in *Heterobasidium annosum* revealed by amplification of minisatellite DNA. Mycological Research 98, 57–63.

Sturino, J.M., Klaenhammer, T.R., 2004. Bacteriophage defense systems and strategies for lactic acid bacteria. Advances in Applied Microbiology 56, 331–378.

Svensson, U., Christiansson, A., 1991. Methods for phage monitoring. Bulletin 263. International Dairy Federation, Brussels, Belgium, pp. 29–39.

Tailliez, P., Tremblay, J., Ehrlich, S.D., Chopin, A., 1998. Molecular diversity and relationship within *Lactococcus lactis*, as revealed by Randomly Amplified Polymorphic DNA (RAPD). Systematic and Applied Microbiology 21, 530–538.

Torriani, S., Zapparoli, G., Dellaglio, F., 1999. Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. Applied and Environmental Microbiology 65, 4351–4356.

Vauterin, L., Vauterin, P., 1992. Computer aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. European Microbiology 1, 37–41.

Viscardi, M., Capparelli, R., Di Matteo, R., Carminati, D., Giraffa, G., Iannelli, D., 2003. Selection of bacteriophage-resistant mutants of *Streptococcus thermophilus*. Journal of Microbiology Methods 55 (1), 109–119.

Weimer, B.C., Blake, M., Hillier, A.J., Davidson, B.E., 1993. Studies on the isolation of phage-resistant derivatives of *Lactococcus lactis* subsp. *cremoris* FG2 with phage sk1. The Australian Journal of Dairy Technology 48, 59–61.