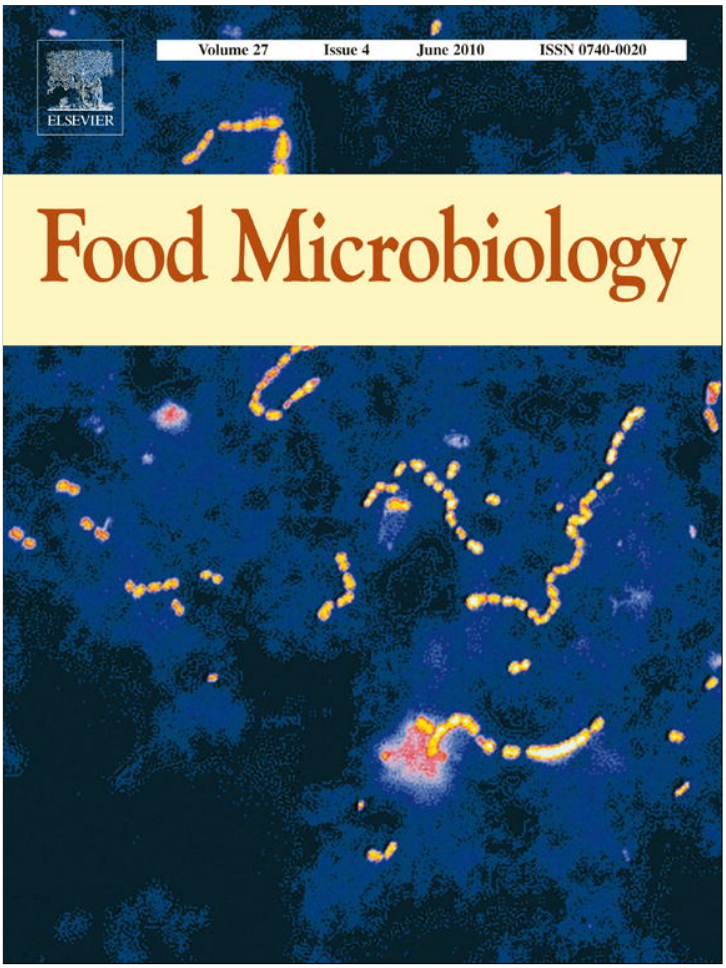

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Temperate and virulent *Lactobacillus delbrueckii* bacteriophages: Comparison of their thermal and chemical resistance

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ABSTRACT

The aim of this work was to study the efficiency of diverse chemical and thermal treatments usually used in dairy industries to control the number of virulent and temperate *Lactobacillus delbrueckii* bacteriophages. Two temperate (Cb1/204 and Cb1/342) and three virulent (BYM, YAB and Ib3) phages were studied. The thermal treatments applied were: 63 °C for 30 min (low temperature – long time, LTLT), 72 °C for 15 s (high temperature – short time, HTST), 82 °C for 5 min (milk destined to yogurt elaboration) and 90 °C for 15 min (FIL-IDF). The chemical agents studied were: sodium hypochlorite, ethanol, isopropanol, peracetic acid, biocides A (quaternary ammonium chloride), B (hydrogen peroxide, peracetic acid and peroctanoic acid), C (alkaline chloride foam), D (p-toluensulfonchloroamide, sodium salt) and E (ethoxylated nonylphenol and phosphoric acid). The kinetics of inactivation were drew and T_{99} (time necessary to eliminate the 99% of phage particles) calculated. Results obtained showed that temperate phages revealed lower resistance than the virulent ones to the treatment temperatures. Biocides A, C, E and peracetic acid showed a notable efficiency to inactivate high concentrations of temperate and virulent *L. delbrueckii* phages. Biocide B evidenced, in general, a good capacity to eliminate the phage particles. Particularly for this biocide virulent phage Ib3 showed the highest resistance in comparison to the rest of temperate and virulent ones. On the contrary, biocide D and isopropanol presented a very low capacity to inactivate all phages studied. The efficiency of ethanol and hypochlorite was variable depending to the phages considered. These results allow a better knowledge and give useful information to outline more effective treatments to reduce the phage infections in dairy plants.

1. Introduction

Nowadays, phage attacks are still considered the main cause of delayed lactic acid production during milk fermentation (Moineau, 1999; Moineau and Lévesque, 2005; Emond and Moineau, 2007). Considerable efforts have been aimed to minimizing this problem. Several control strategies, such as sanitizers, thermal and chemical treatments, strain rotation regimes, spontaneous mutant phage-resistant strains and phage inhibitory media (PIM), are used in dairy industries (Suárez et al., 2007). In addition, genes that encode natural resistance mechanisms can be introduced into starter strains (Madera et al., 2004). However, phages represent a real and persistent threat and the authors are aware of recent unpublished cases where phage infection actually limited the fermentation process and/or caused product downgrading (McGrath et al., 2004).

Lactic acid bacteria (LAB) are widely used to ferment milk for production of cheese and other fermented products (Lunde et al., 2005).

In particular, *Lactobacillus delbrueckii* is one of dairy LAB species (together with *S. thermophilus*) used in yogurt production and it is a fundamental constituent of whey starter cultures used for hard cheeses (Curry and Crow, 2003; Giraffa and Rossetti, 2004). Because of the potential negative impact on dairy technology, phages of *L. delbrueckii* need additional studies. Virulent and temperate *L. delbrueckii* bacteriophages have been characterized and classified into four groups (*a* to *d*) on the basis of morphology, immunoblotting tests and DNA–DNA hybridizations (Suárez et al., 2008). The number of active temperate *L. delbrueckii* bacteriophages isolated from dairy products is notably lower than the number of virulent ones. Even if a high percentage of lactobacilli strains are inducible, complete phage particles able to propagate on host strains were less frequently isolated (Carminati et al., 1997). The induction of defective phages or 'killer' particles, unable to propagate on suitable indicators was previously reported (Davidson et al., 1990; Carminati et al., 1997; Suárez et al., 2008). Temperate phages

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were less studied than virulent ones regarding to the effect of temperature and biocides on their inactivation. It is well reported that the majority of lytic phages are resistant to the thermal treatments commonly used in dairy industry (Chopin, 1980; Quiberoni et al., 1999, 2003; Binetti and Reinheimer, 2000; Suárez and Reinheimer, 2002; Capra et al., 2004; Madera et al., 2004). However, there are no reports about the effect of those processes on temperate phages.

The selection of chemical agents to inactivate phages in dairy industries is carried out taking into consideration not only their capacity as biocides but others properties such the ease of their manipulation, the lack of deleterious influence on products, the harmlessness of their residues and the speed of action (Schröder, 1984). For several years, our group has intensely studied the effect of diverse biocides on authochtonal LAB bacteriophages (Quiberoni et al., 1999, 2003; Binetti and Reinheimer, 2000; Suárez and Reinheimer, 2002; Capra et al., 2004; Briggiler Marcó et al., 2009). These biocides included sodium hypochlorite, ethylic and isopropyl alcohols and peracetic acid. The selection of new chemical agents is a dynamic activity. To date, peracetic acid is the most used biocide in dairy industries but new biocides have been introduced in the market. However, their effect on phages have not been tested yet and therefore they were included in this study.

The aim of this study was to determine the effect of thermal and chemical treatments on *L. delbrueckii* temperate phages and to compare them with the virulent ones.

2. Materials and methods

2.1. Strains and bacteriophages

The origin of bacteriophages used in this study as well as their host strains are showed in Table 1. Sensitive strains were maintained as frozen stocks at −80 °C in reconstituted (10% p/v) commercial nonfat dried skim milk (RSM), or MRS broth (Biokar, Beauvais, France) supplemented with 15% (v/v) of glycerol, and routinely reactivated overnight at 42 °C in MRS broth. Phage stocks were prepared as described by Neviani et al. (1992) in MRS broth, adding 10 mM of $CaCl_2$ (MRS-Ca), and then stored at 4 °C and frozen at −80 °C in the presence of 15% of glycerol. Phage enumerations (PFU/ml) were performed by the double-layer plaque titration method (Svensson and Christiansson, 1991), using MRS-Ca agar added with 100 mM of glycine (Lillehaug 1997).

2.2. Thermal treatments

The thermal resistance of all bacteriophages was assayed using traditional pasteurization treatments: low temperature – long time (LTLT, 63 °C for 30 min) and high temperature – short time (HTST, 72 °C for 15 s) and the thermal treatment applied to the milk destined to yogurt elaboration (82 °C for 5 min). The conditions (90 °C for 15 min) recommended for FIL – IDF to assured the destruction of phages were used as control. The tests were performed in MRS broth and RSM by triplicate. The inactivation kinetics were drew for all phages and the T_{99} values (time necessary to eliminate the 99% of phage particles) were calculated from them.

2.3. Chemical treatments

Assays with sodium hypochlorite (100–300 ppm residual-free chlorine), ethanol (Cicarelli, Buenos Aires, Argentina) (10%, 50%, 75% and 100% v/v), isopropanol (Cicarelli) (10%, 50% and 100% v/v) and peracetic acid (Proxitane1512, Química General, Santa Fe, Argentina) (0.15% v/v) were made according to Quiberoni et al. (2003).

The new commercial biocides used in this study were named: A (quaternary ammonium chloride), B (hydrogen peroxide, peracetic acid and peroctanoic acid), C (alkaline chloride foam), D (p-toluensulfonchloroamide, sodium salt) and E (ethoxylated nonylphenol and phosphoric acid). All products were used according to manufacturer's recommendations. The assays conditions were: A (0.5, 1.0, 1.5, 2.0, 2.5, 3.0%, v/v) until 20 min, B (0.13, 0.26%, v/v) until 20 min, C (2.5%, v/v) until 45 min, D pure until 45 min and E (0.8%, v/v) (40 °C) until 20 min. All dilutions were made in sterilized distilled water. The assays were performed by triplicate and T_{99} values were calculated.

3. Results

3.1. Thermal treatments

Fig. 1 shows the viability loss when high titers suspensions of the phages were heated at 63 °C. The heat-resistance values were similar for both temperate phages (Cb1/204 and Cb1/342). T_{99} values were <2 min when the assays were performed in MRS broth (Table 2) and total phage particles were inactivated at 10 min and 20 min, for Cb1/204 and Cb1/342, respectively. Even though T_{99} values were low when RSM was used as suspension media (≤2.4 min), the remaining phage particles survived perfectly after 30 min of treatment, showing some protective effect of this medium.

Incubation at 72 °C showed a faster inactivation of both temperate phages. The most resistant phage was Cb1/204 since was completely inactivated after only 20 min of treatment in RSM (Fig. 1). In all cases, T_{99} values were lower than 2.4 min (Table 2).

Additional treatments (82 °C and 90 °C) eliminated the totality of phage particles in less than 2 min.

3.2. Chemical treatments

The T_{99} values obtained for commercial biocides assayed are showed in Table 3. Biocides A, C and E were very effective when used at the recommended concentrations against all phages tested. The totality of phage particles was destroyed before 2 min of treatment. The pH values obtained for A (3.0%, v/v), C (2.5%, v/v) and E (0.8%, v/v) and solutions were 10.5, 12.4 and 2.0, respectively. Phages were inactivated before 2 min of exposure at pH 12.4 and 2.0, but were not destroyed completely at pH 10.5 (data not shown).

Table 1
Origin, type and host strains of bacteriophages used in this study.

Phage	Isolated as:	Origin	Host strain
Cb1/204	temperate	*L. delbrueckii* subsp. *lactis* Cb1 (commercial strain)	*L. delbrueckii* subsp. *lactis* 204 (wild strain)
Cb1/342	temperate	*L. delbrueckii* subsp. *lactis* Cb1 (commercial strain)	*L. delbrueckii* subsp. *bulgaricus* 342 (wild strain)
Ib3	virulent	Failed yogurt elaboration	*L. delbrueckii* subsp. *lactis* Ib3 (commercial strain)
YAB	virulent	Failed yogurt elaboration	*L. delbrueckii* subsp. *lactis* Ab1 (commercial strain)
BYM	virulent	Failed yogurt elaboration	*L. delbrueckii* subsp. *bulgaricus* YSD V (commercial strain)

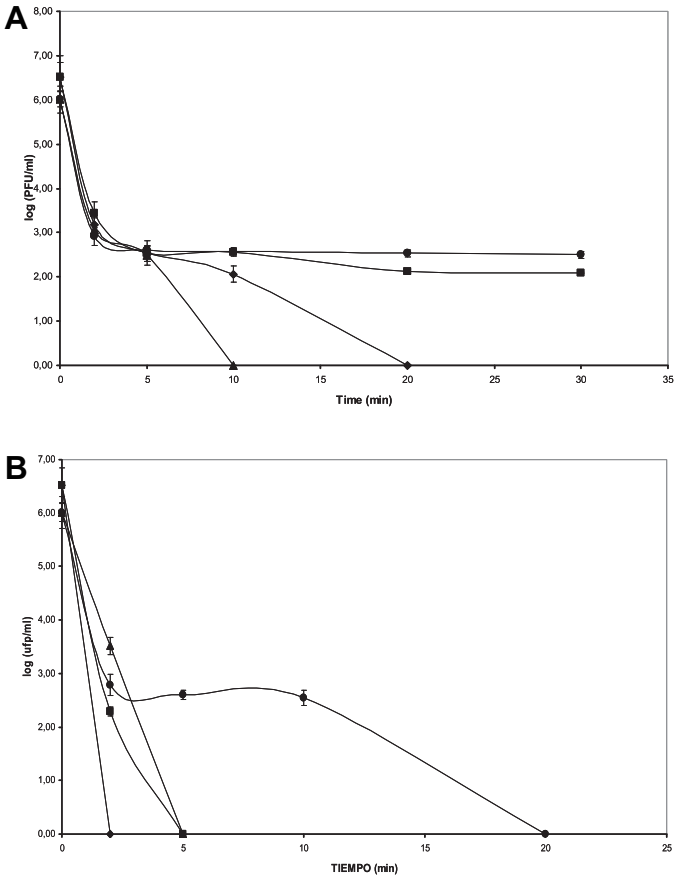

Fig. 1. Thermal inactivation kinetics at 63 °C (A) and 72 °C (B) of temperate *L. delbrueckii* phages Cb1/204 and Cb1/342 in MRS broth (▲ and ◆) and RSM (● and ■).

Table 4 shows T_{99} for different concentrations of the biocide A. A concentration of 0.5% (v/v) was not effective to inactivate phages BYM, YAM and Ib3, and their counts decreased slightly after an exposure of 20 min. On the contrary, this concentration was enough to inactivate all suspensions of temperate phages after 20 min (Fig. 2).

Biocide B (peracetic acid in a hydrogen peroxide base) was efficient to rapidly destroy temperate and virulent phages, except for phage Ib3. Fig. 3 shows inactivation kinetics of phage Ib3 when 0.13% and 0.26% (v/v) of biocide B were used. These solutions showed pH values of 3.45. However, when the tested phages were incubated at this low pH (without biocides) for 2 min, none of them was inactivated. On the other hand, phage Ib3 was incubated at this pH for 10 min and 20 min, because it showed to be very resistant to biocide B (0.26% and 0.13%, respectively). These phage particles were not affected by the solution pH value (data not shown).

Table 2
T_{99} values for temperate Cb1/204 and Cb1/342 *L. delbrueckii* phages treated at different temperatures using two suspension media.

Phage	T_{99}[a] (min)							
	MRS broth				RSM[b] (10% w/v)			
	63 °C	72 °C	82 °C	90 °C	63 °C	72 °C	82 °C	90 °C
Cb1/342	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Cb1/204	<2.0	<2.0	<2.0	<2.0	2.1	2.4	<2.0	<2.0

[a] Time to inactivate the 99% of phage particles.
[b] Reconstituted skim milk.

Table 3
T_{99} values for temperate (Cb1/342 and Cb1/204) and virulent (BYM, YAB and Ib3) *L. delbrueckii* phages treated with commercial biocides.

Biocide (concentration)/pH value	T_{99}[a] (min)				
	Cb1/342	Cb1/204	BYM	YAB	Ib3
B (0.13 % v/v)/3.4	<2.0	<2.0	<2.0	<2.0	8.4
B (0.26 % v/v)/3.4	<2.0	<2.0	<2.0	<2.0	<2.0
C (2.5 % v/v)/12.4	<2.0	<2.0	<2.0	<2.0	<2.0
D (pure)/6.1	>45	17.3	>45	>45	>45
E (0.8 % v/v)/2.0	<2.0	<2.0	<2.0	<2.0	<2.0

[a] Time to inactivate the 99% of phage particles.

Peracetic acid (0.15%, pH 2) produced a fast and complete inactivation of temperate phage particles since no detectable counts were obtained after 5 min of treatment (data not shown). Ethanol 100% produced the fastest inactivation of the temperate viral particles (Fig. 4). Phage Cb1/342 was more resistant than phage Cb1/204 to all ethanol concentrations assayed. For phage Cb1/204, after 45 min of treatment it was possible to inactivate the complete phage population when ethanol 100% was used. Concentrations of 10, 50 and 75% were able to reduce only one logarithmic order from the initial phage counts. Phage sensitivity was slightly influenced by isopropanol (Fig. 5). Only isopropanol 100% was able to reduce, for both temperate phages, two logarithmic orders considering initial phage particles counts.

Phage Cb1/342 revealed to be more resistant than phage Cb1/204 when it was treated with sodium hypochlorite (Fig. 6). For Cb1/204, 200 ppm of this biocide were enough to rapidly inactivate all phage particles (<2 min), while 300 ppm were needed to inactivate similar initial population of Cb1/342 in the same period of exposure.

On the other hand, biocide D showed low efficiency to destroy phage particles, being the phage Ib3 the most resistant. Only phage Cb1/204 showed a T_{99} value lower than 45 min, while the others overcame this time (Fig. 7).

4. Discussion

In Argentina, *L. delbrueckii* is used together with *S. thermophilus* as part of commercial starters for yogurt and certain soft cheese processes. The economic losses and the public health consequences incurred when phage infections occur may be very significant and have been well documented (Daly, 1983; Daly and Fitzgerald, 1987). Some specific virulent phages were isolated in our laboratory from yogurt samples coming from batches with acidifying problems (named as Ib3, YAB and BYM). They were previously studied by our group against diverse chemical and thermal treatments (Quiberoni et al., 2003). However, these studies have been carried out only on virulent phages, and there is

Table 4
T_{99} values for temperate (Cb1/342 and Cb1/204) and virulent (BYM, YAB and Ib3) *L. delbrueckii* phages treated with biocide A at diverse concentrations.

Biocide A Concentration (% v/v)/pH value	T_{99}[a] (min)				
	Cb1/342	Cb1/204	BYM	YAB	Ib3
3.0/10.5	<2.0	<2.0	<2.0	<2.0	<2.0
2.5/9.9	<2.0	<2.0	<2.0	<2.0	<2.0
2.0/9.7	<2.0	<2.0	<2.0	<2.0	<2.0
1.5/9.6	<2.0	<2.0	<2.0	<2.0	<2.0
1.0/9.3	<2.0	<2.0	<2.0	<2.0	<2.0
0.5/8.8	>20.0	>20.0	15.0	14.0	>20.0

[a] Time to inactivate the 99% of phage particles.

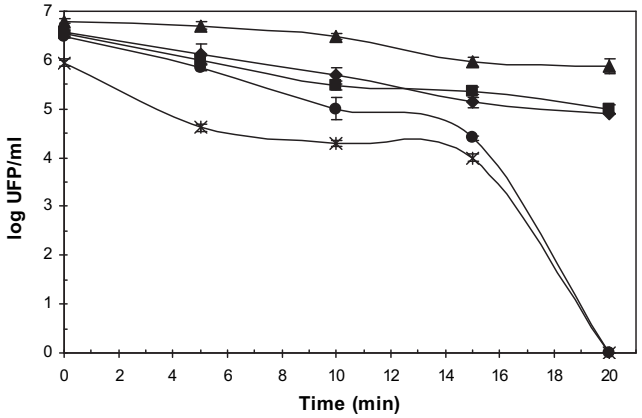

Fig. 2. Chemical inactivation kinetics of temperate Cb1/204 (◆), Cb1/342 (■), and virulent Ib3 (▲), YAB (●) and BYM (*) *L. delbrueckii* phages treated with 0.5% (v/v) of biocide A.

no information available about the effect of same agents on autochthonous temperate phages.

Two temperate phages were isolated in our laboratory from a commercial *L. delbrueckii* subsp. *lactis* strain (*L.l* Cb1) used in dairy industries (Suárez et al., 2008). This finding was the confirmation that lysogenic strains are used in commercial starters. During lactic fermentation processes, the BAL are exposed to various stress conditions, including fluctuations in temperature, pH, osmotic pressure and lack of available nutrients. Many of these conditions are usually overlapped. Like other bacteria, LAB have evolved and generate stress response systems, allowing them to adapt to adverse conditions in order to survive (Lunde et al., 2005). In recent years, the interest of stress response of LAB of industrial importance have increased and some reports include studies on responses to cold shock and heat, acid pH, UV light, salt concentration and oxidation (Guerzoni et al., 2001; Van de Guthche et al., 2002; Streit et al., 2007, 2008; Rivals et al., 2007). However, few studies have been aimed to investigate the effect of environmental factors on the release of BAL prophages (Lunde et al., 2005; Madera et al., 2009). Therefore, it is essential to know the effect of thermal and chemical treatments against these phages released from strains used as starters.

Temperate bacteriophages showed, in general, low resistance at the temperatures assayed ($T_{99} \leq 2.4$). However, the treatment at 63 °C for 30 min in RSM did not guarantee the inactivation of all phage particles. On the contrary, when MRS broth was used as

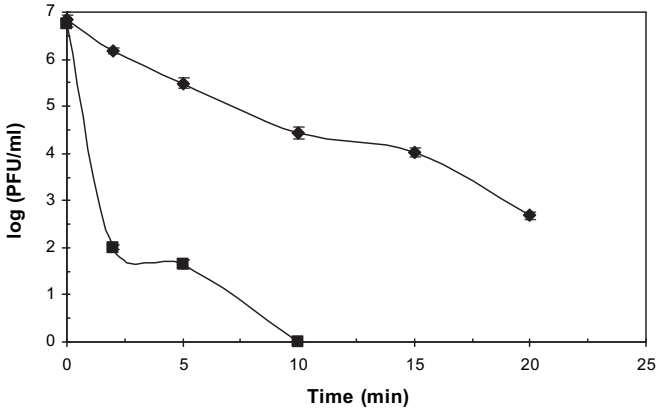

Fig. 3. Chemical inactivation kinetics of virulent phage Ib3 treated with 0.13% (v/v) (◆) and 0.26% (v/v) (■) of biocide B.

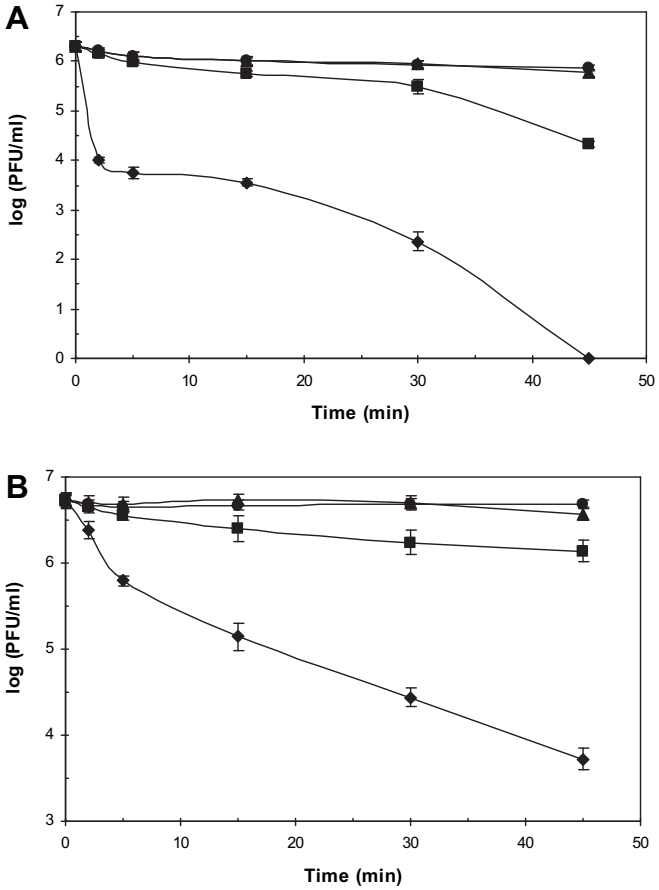

Fig. 4. Chemical inactivation kinetics of temperate phages Cb1/204 (A) and Cb1/342 (B) treated with 10% (●), 50% (▲), 75% (■) and 100% (◆) of ethanol.

suspension media, the two temperate phages were eliminated after 20 min of treatment. Similar behavior was observed at 72 °C. Virulent phages (BYM, YAB and Ib_3) were reported as highly resistant at both temperatures (Quiberoni et al., 2003), specially phage Ib3. According to these authors, treatment at 90 °C for 15 min was necessary to completely inactivate this particularly resistant phage when RSM was used as suspension medium. In general, for other species of LAB, treatments of 63 °C and 72 °C were not sufficient to destroy all population of phage particles, mainly in RSM (Quiberoni et al., 1999; Binetti and Reinheimer, 2000; Suárez and Reinheimer, 2002; Capra et al., 2004; Briggiler Marcó et al., 2009). According to these results, it is possible to affirm that the virulent phages studied (all isometric-headed) were more resistant that temperate ones (prolate-headed) used in this study.

Biocides commonly used in dairy industries were assayed against temperate phages Cb1/204 and Cb1/342. Sodium hypochlorite (100 ppm of active chlorine) inactivated these phages after 20 min and 30 min of treatment, respectively. This concentration was reported to be efficient against bacteriophages of *Lactobacillus helveticus* (Quiberoni et al., 1999), *S. thermophilus* (Binetti and Reinheimer, 2000) and *Lc. Lactis* (Suárez and Reinheimer, 2002). Nevertheless, Capra et al. (2004) reported that indigenous phages of *Lactobacillus casei/paracasei* supported concentrations until 800 ppm of active chlorine. Virulent phages of *L. delbrueckii* showed resistance to higher concentrations, since Ib_3 was able to survive until 1200 ppm of active chlorine (Quiberoni et al., 2003).

The results obtained from alcohol (ethanol and isopropanol) treatments showed that *L. delbrueckii* phages were less sensitive

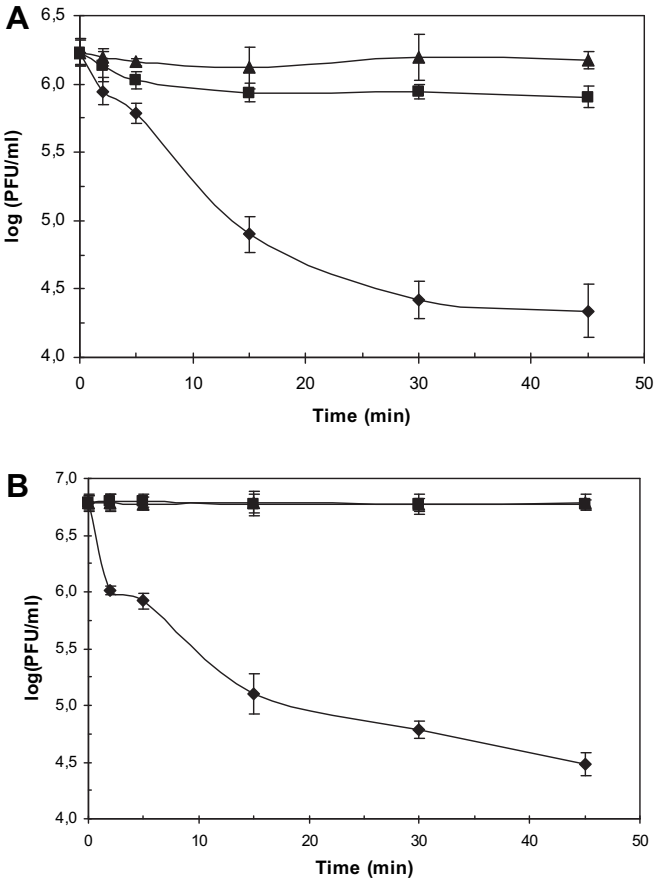

Fig. 5. Chemical inactivation kinetics of temperate phages Cb1/204 (A) and Cb1/342 (B) treated with 10% (▲), 50% (■) and 100% (◆) of isopropanol.

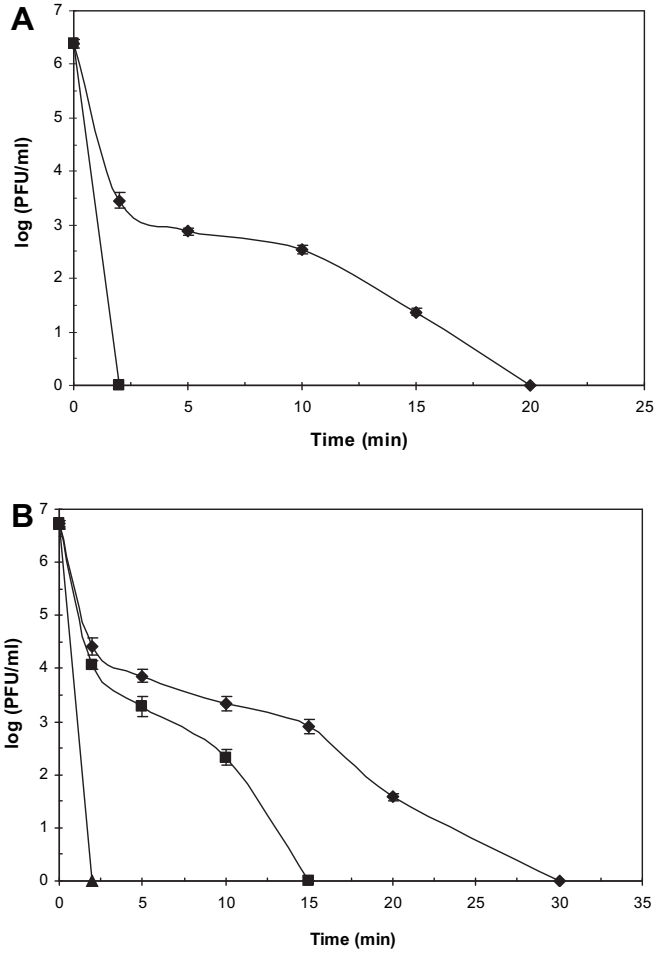

Fig. 6. Chemical inactivation kinetics of temperate phages Cb1/204 (A) and Cb1/342 (B) treated with 100 ppm (◆), 200 ppm (■) and 300 ppm (▲) of sodium hypochlorite.

than specific phages of other LAB. Only ethanol 100% was effective against these phages, while a concentration of 75% was efficient for *S. thermophilus* (Binetti and Reinheimer, 2000), *L. helveticus* (Quiberoni et al., 1999) and *Lc. lactis* (Suárez and Reinheimer, 2002) phages. Isopropanol showed to be extremely harmlessness and was not efficient to inactivate our phages ($T_{99} > 35$ min). Peracetic acid (0.15%, v/v) destroyed all phage particles before 5 min of incubation.

Biocides A, C and E showed a notable efficiency to inactivate high concentrations of *L. delbrueckii* phages. Extreme pH of biocides C and E solutions (controls) (12.4 and 2.0, respectively) produced total destruction of phage particles after 2 min. These results allow us to hypothesize that these extreme pH values could have a great contribution on phage inactivation.

Particularly, biocide A was effective at concentrations lower than those recommended by suppliers. The MIC (minimal inhibitory concentration) was calculated, resulting of 1% (v/v). A solution at this concentration was prepared, which showed a pH of 9.3. Our phages supported this pH for more than 2 min, which is the time required to inhibit the totality of phage particles in all cases.

Biocide B is a sanitizer composed by two active compounds, peroctanoic and peracetic acids. As well as peracetic acid, this biocide is used at room temperature and does not require subsequent rinse. Although pH values of two biocides (B and peracetic acid) were very low (acid solutions), the controls demonstrated that this factor had not influence in phage destruction for *L. delbrueckii* phages. However, on phage Ib3 the treatment with peracetic acid assured its complete elimination, while when biocide B was applied, it was necessary to prepare it at the maximal concentration recommended (0.26%, v/v) and incubated for 10 min to obtain the same result.

In this work was achieved the determination of five *L. delbrueckii* bacteriophages (virulent and temperate ones) resistance against chemical and physical treatments used in the dairy industry. These results allow a better knowledge and give useful information to

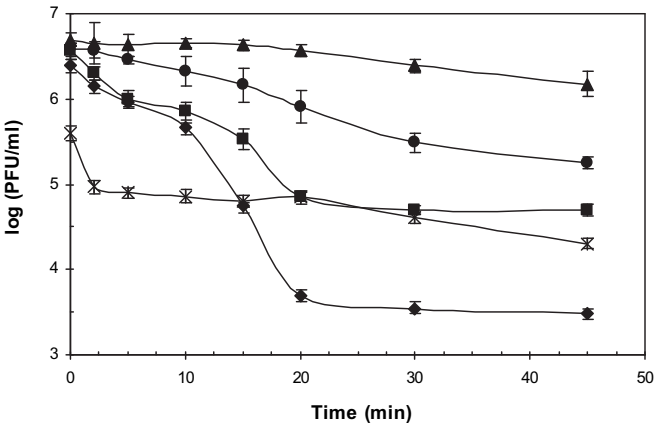

Fig. 7. Chemical inactivation kinetics of temperate Cb1/204 (◆), Cb1/342 (■), and virulent Ib3 (▲), YAB (●) and BYM (*) *L. delbrueckii* phages treated with biocide C (pure).

outline more effective treatments to reduce the phage infections in dairy plants.

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