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Resistance of two temperate *Lactobacillus paracasei* bacteriophages to high pressure homogenization, thermal treatments and chemical biocides of industrial application

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ABSTRACT

Temperate bacteriophages ϕ i*Lp*84 and ϕ i*Lp*1308, previously isolated from mitomycin C-induction of *Lactobacillus paracasei* strains 84 and CNRZ1308, respectively, were tested for their resistance to several physical and chemical treatments applied in dairy industry. Long-term survival at 4 °C, −20 °C and −80 °C, resistance to either thermal treatments of 63 °C, 72 °C and 90 °C, high pressure homogenization (HPH, 100 MPa) or classic (ethanol, sodium hypochlorite and peracetic acid) and new commercial sanitizers, namely 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), were determined. Phages were almost completely inactivated after eight months of storage at 25 °C, but viability was not affected at 4 °C, −20 °C or −80 °C. Both phages tolerated well HPH treatments. Phage i*Lp*1308 showed higher thermal resistance than ϕ i*Lp*84, but neither resisted 90 °C for 2 min. Best chemical inactivation was accomplished using peracetic acid or biocides A, C and E, whereas biocides B and D were completely ineffective. These results help to improve selection of chemical agents and physical treatments to effectively fight against phage infections in dairy plants.

1. Introduction

Bacteriophages of lactic acid bacteria (LAB) are the main cause of fermentation failure in the dairy industry, leading in some cases to large economic losses (Bruttin et al., 1997; Capra et al., 2009a). Raw milk has been regarded as the main source of bacteriophages in dairy plants, either as free virions or as prophages in naturally occurring lysogenic strains. In this regard, lysogeny was demonstrated to represent an actual threat, as long as prophages are susceptible to be induced from a dairy strain and infect another one, or even the same strain which originated the temperate phage (Capra et al., 2010). On account of their origin, bacteriophages are therefore impossible to eradicate from dairy plants, but strategies such as strain rotation schemes, use of spontaneous bacteriophage resistant mutants and application of thermal and/or chemical treatments help to counteract this problem (Everson, 1991).

Among LAB phages, particular attention must be paid to those infecting lactobacilli of the casei group, which comprises most of the probiotic strains currently added to dairy products (Mercanti et al., 2011). Given their nature, probiotic bacteria possess singular traits that make strain rotation unsuitable as an industrial strategy for phage control (Capra et al., 2010). Bacteriophage insensitive mutants could be a helpful approach, but present however several drawbacks, such as narrow phage specificity and reversion of the phenotype (Moineau, 1999; Capra et al., 2011). In general, application of hygienic measures and correct cleansing of industrial equipments are valuable to keep phage levels under the threshold necessary to cause perceptible damage in a dairy fermentation process. Thermal treatments are applied to raw milk primarily to kill pathogenic bacteria, but at the same time reduce phage numbers. However, its efficiency depends on thermal resistance, which remarkably varies from phage to phage (Atamer et al., 2009, 2011; Capra et al., 2009a).

High pressure homogenization (HPH) is a dynamic (continuous flow) "non-thermal" technology used in pharmaceutical, chemical and food industry, with several advantages over high hydrostatic pressure (batch) systems (Moroni et al., 2002). Formerly, its main purpose was to prepare and stabilize emulsions and suspensions, but its application field has been extended to food preservation (Diels and Michiels, 2006). Treatment of liquid milk by HPH was

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applied to improve either physicochemical properties of milk or fermented milks and cheese quality (Sandra and Dalgleish, 2007; Zamora et al., 2007; Burns et al., 2008). In addition, HPH has been also considered as a convenient alternative for low-temperature inactivation of pathogenic and spoiling microorganisms (Vachon et al., 2002; Fonberg-Broczek et al., 2005; Burns et al., 2008).

Considerable work has been focused on the thermal and chemical resistance of phages infecting the genus *Lactococcus* (Suárez and Reinheimer, 2002; Atamer et al., 2009). Resistance to heat and biocides was studied also on phages of *Lactobacillus delbrueckii* (Quiberoni et al., 2003; Ebrecht et al., 2010) and, at a lesser extent, on phages of *Streptococcus thermophilus* (Binetti and Reinheimer, 2000), *Leuconostoc* (Atamer et al., 2011), *Lactobacillus helveticus* (Quiberoni et al., 1999), and *Lactobacillus plantarum* (Briggiler Marcó et al., 2009). Capra et al. (2004, 2006; 2009a; 2010) focused on this subject on phages of *Lactobacillus casei* and *Lactobacillus paracasei*, but these species have not otherwise received much attention. On the other hand, thermal and chemical resistance was almost exclusively reported for virulent phages, whereas only a few reports for temperate phages of *L. delbrueckii* (Ebrecht et al., 2010) and *L. paracasei* (Capra et al., 2010) were found. Data concerning HPH utilization for virus inactivation are scarce and, to our knowledge, there are only two publications specifically referring to inactivation of bacteriophages of lactic acid and probiotic bacteria (Moroni et al., 2002; Capra et al., 2009b).

In a recent study, a widespread occurrence of lysogeny among strains of *L. casei* and *L. paracasei* was confirmed, as well as the ability of two temperate phages to attack probiotic strains currently incorporated into dairy products (Mercanti et al., 2011). The present work was intended to evaluate the survival of those bacteriophages at different storage temperatures, their resistance to heat treatments and biocides of common application in dairy plants, and their ability to survive HPH treatments, an emerging technology with potential to control phage infections in the dairy industry.

2. Materials and methods

2.1. Strains and bacteriophages

Temperate bacteriophages used in this study were isolated in a previous work during mitomycin C-induction assays on a set of *L. paracasei* strains (Mercanti et al., 2011). Specifically, ϕ i*Lp*84 was induced from *L. paracasei* 84, a strain isolated from dairy products, and propagated on the INLAIN collection strain *L. paracasei* INL3, whilst ϕ i*Lp*1308 was induced from the collection strain *L. paracasei* CNRZ1308 and propagated on the commercial strain *L. paracasei* A14. All the assays were carried out starting from a unique 100-ml stock of each phage, prepared as described by Neviani et al. (1992) and stored at 4 °C in de Man, Rogosa, and Sharpe (MRS) broth (Biokar, Beauvais, France). Sensitive strains were maintained as frozen stocks (−80 °C) in MRS broth with 15% (v/v) glycerol and routinely reactivated overnight at 37 °C in MRS broth.

2.2. Long-term storage

Aliquots of each phage were maintained at 25 °C, 4 °C (MRS broth), −20 °C and −80 °C (MRS broth with 15% v/v glycerol). Phage enumerations (pfu/ml) were carried out periodically for up to ten months of storage by the double-layer plate titration method (Svensson and Christiansson, 1991); mixes of 0.1 ml of decimal dilutions of phages, 0.1 ml of a log-phase culture of the sensitive strain and 1 ml of warm MRS soft agar (0.6% w/v) were poured onto a bottom layer of MRS agar (1.2% w/v) supplemented with 10 mM $CaCl_2$ and 100 mM (MRS-Ca-Gly) (Lillehaug, 1997). Incubation was carried out at 34 °C for 18 h under microaerophilic conditions, and phage titers determined by enumeration of lysis plaques. Assays were carried out in triplicate.

2.3. Influence of pH on phage viability

Phages were diluted to a final concentration of 10^6–10^7 pfu/ml in MRS broth with the pH adjusted to values ranging from 2 to 11. MRS broth without adjustment (pH 6.4) was used as control. After 30 min of incubation at 25 °C or 37 °C, phages were enumerated by the double-layer plate titration method as previously described (Svensson and Christiansson, 1991). Assays were carried out in triplicate.

2.4. Thermal treatments

Dilutions of phages containing between 10^6 and 10^7 pfu/ml were prepared in three different suspension media and then subjected to thermal treatments. Media assayed were MRS broth, reconstituted (10% p/v) commercial skim milk (RSM) and Tris-magnesium gelatine (TMG) buffer: 10 mM Tris–Cl, 10 mM $MgSO_4$ and 0.1% (w/v) gelatine. Temperatures of 63 °C and 72 °C, corresponding to traditional low- and high-temperature pasteurization, respectively, and 90 °C, the temperature recommended for FIL-IDF to guarantee a total phage destruction after 15 min, were tested. Phage suspensions were immediately cooled after 2, 5, 15, 30 and 45 min of treatment and enumerated by the double-layer plate titration method. Assays were carried out in triplicate. Time required to eliminate 99% of phage particles (T_{99}) was calculated from inactivation curves according to Quiberoni et al. (2003).

2.5. Chemical treatments

Phage suspensions containing between 10^6 and 10^7 pfu/ml were incubated with biocide solutions in 1.5 ml micro centrifuge tubes and enumerated by the double-layer plate titration method. Specific assay conditions for each biocide tested are detailed in Table 1. All the biocides were diluted using sterile distilled water, with the exception of sodium hypochlorite, which was diluted in phosphate buffer (pH 7). Incubations were accomplished at 25 °C, except for commercial sanitizer E, tested at 40 °C, and peracetic acid, tested at both 25 °C and 40 °C (Schröder, 1984). Assays were

Table 1
Assay conditions for the different biocides tested.

	Concentrations assayed	Temperature of incubation (°C)	Times of incubation (min)
Ethanol	10–50–75–100% (v/v)	25	2–5–15–30–45
Sodium hypochlorite	100–400–800 ppm	25	2–5–15–30–45
Peracetic acid	0.15% (v/v)	25 and 40	2–5
Biocide A (quaternary ammonium chloride)	0.25–0.50–1.00–2.00% (v/v)	25	5–10–15–20
Biocide B (hydrogen peroxide, peracetic acid and peroctanoic acid)	0.13–0.26% (v/v)	25	2–5–10–20
Biocide C (alkaline chloride foam)	2.5% (v/v)	25	2–5–15
Biocide D (p-toluensulfonchloroamide, sodium salt)	Pure	25	5–20–45–120
Biocide E (ethoxylated nonylphenol and phosphoric acid)	0.8% (v/v)	40	2–5–15

carried out in triplicate. T_{99} was calculated from inactivation curves (Quiberoni et al., 2003).

2.6. High pressure homogenization

Phages were inoculated at a concentration of 10^7 pfu/ml in RSM and subjected to high pressure homogenization (HPH) for up to 8 passes in a continuous high-pressure homogenizer PANDA (Niro Soavi, Parma, Italy) supplied with a homogenizing PS type valve with a flow rate of 10 l h^{-1}. This valve incorporates a ball-type ceramic impact head, a stainless steel impact ring and a tungsten carbide passage head. Pressure applied was of 100 MPa. Temperature of phage suspensions was constant (about 25 °C) all through their passage, on account of a refrigeration system with running water built onto the homogenizer. Assays were carried out in triplicate. Phages were enumerated by the double-layer plate titration method before their passage through the homogenizer (control) and immediately after 1, 3, 5 and 8 passes.

3. Results and discussion

3.1. Long-term storage

No significant reduction in phage counts was observed after 10 months of storage at 4 °C, −20 °C or −80 °C, for either ϕ i*Lp*84 or ϕ i*Lp*1308 (Fig. 1). Similarly, *L. paracasei* ϕ MLC-A, a virulent phage that shares host spectrum with ϕ i*Lp*84 and ϕ i*Lp*1308 (Capra et al., 2006; Mercanti et al., 2011), presented a titer reduction of less than 1 $\log_{10}$ order after 15 months at 4 °C (Capra et al., 2006). In contrast, ϕ i*Lp*84 and ϕ i*Lp*1308 suffered a much more significant loss of viability (higher than 6 $\log_{10}$ orders) after eight months of storage at 25 °C. We have not found previous reports testing phage survival at 25 °C, but it will be interesting to include this temperature in future studies, given the remarkably lower phage survival observed with respect to the storage at refrigeration temperature.

3.2. Phage survival at different pH

Phages ϕ i*Lp*84 and ϕ i*Lp*1308 showed similar resistance within the range of pH values analyzed. None of them was affected by pH between 3 and 8 (Fig. 2). However, phage particles were undetectable when they were incubated at pH 11 (25 °C and 37 °C), and at pH 2 (37 °C). At pH 2 and 25 °C, a reduction of only 1 $\log_{10}$ order was observed. Only at pH 9 (37 °C) and 10 (25 °C and 37 °C) a slightly higher resistance of ϕ i*Lp*1308 with respect to ϕ i*Lp*84 was perceived.

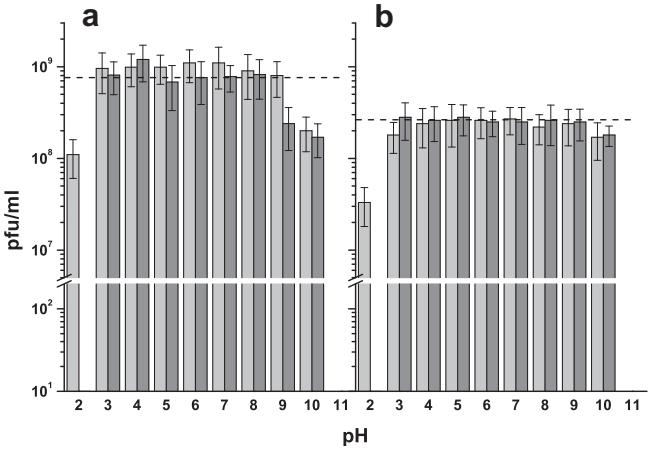

Fig. 2. Survival of phages ϕ i*Lp*84 (a) and ϕ i*Lp*1308 (b) after 30 min at 25 °C (▒) and 37 °C (■) in MRS broth with pH adjusted to values ranging from 2 to 11. Horizontal lines represent the titer for each phage on MRS broth without pH adjustment (control). Values are the mean of three determinations.

3.3. Thermal treatments

Neither ϕ i*Lp*84 nor ϕ i*Lp*1308 resisted a heating of 2 min at 90 °C, which remarkably differs from the extremely high thermal resistance found on eight virulent phages with identical host spectra isolated from industrial samples (Capra et al., 2009a). Seven of them were still active after 2 min at 90 °C, whereas ϕ MLC-A (the remaining one) also did it but in a suspension media not tested here. It was noteworthy that two of those eight phages survived even 45 min at 90 °C in RSM, an extreme heat resistance not frequently found in LAB phages, but some exceptions like *L. delbrueckii* phage Ib3 (Quiberoni et al., 2003), *Leuconostoc* phage P793 (Atamer et al., 2011) and several phages of *Lactococcus lactis* (Atamer et al., 2009) raise the question of the need to revise the current protocol for phage detection in dairy samples (Svensson and Christiansson, 1991). Regarding treatments at 63 °C and at 72 °C, ϕ i*Lp*1308 demonstrated to be slightly more heat resistant than ϕ i*Lp*84, which was undetectable after 30 min at 72 °C (15 min when in buffer TMG) or 45 min at 63 °C (Fig. 3). The lower heat

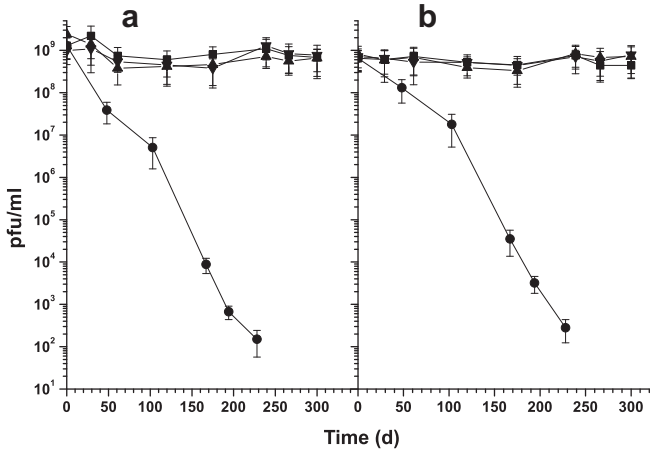

Fig. 1. Reduction of viability of phages ϕ i*Lp*84 (a) and ϕ i*Lp*1308 (b) during storage at 25 °C (●), 4 °C (■) (MRS broth), −20 °C (▲), and −80 °C (▼) (MRS broth with 15% v/v glycerol). Values are the mean of three determinations.

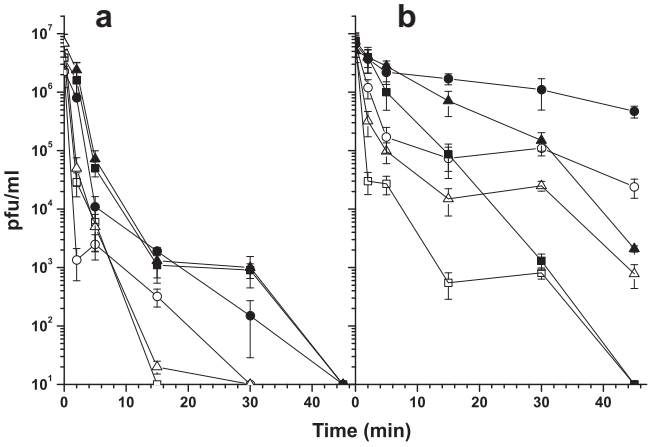

Fig. 3. Thermal resistance of phages ϕ i*Lp*84 (a) and ϕ i*Lp*1308 (b) at 63 °C (filled symbols) and 72 °C (empty symbols) in MRS broth (■), reconstituted (10% p/v) commercial skim milk (RSM; ●) and Tris-magnesium gelatine (TMG) buffer (▲). Values are the mean of three determinations.

resistance found on ϕ i*Lp*84 and ϕ i*Lp*1308 with respect to most of the virulent phages studied by Capra et al. (2009a) could be attributable to their temperate origin. However, ϕ PL-1 and ϕ J-1 showed a very low heat resistance in spite of their virulent character (Capra et al., 2004), whilst the opposite was true for temperate phages C_L1 and C_L2 (Capra et al., 2010), all sharing host spectra with ϕ i*Lp*84 and ϕ i*Lp*1308. Heat inactivation curves for ϕ i*Lp*84 are comparable to those of ϕ PL-1. Even if pasteurization conditions applied to raw milk intended for cheese-making (63 °C for 30 min or 72 °C for 15 s) are not sufficient to inactivate ϕ i*Lp*84 and ϕ i*Lp*1308, thermal treatments used for fermented milks manufacture (85 °C for 30 min or 90–95 °C for 5 min; Tamime, 2002) would be.

Similarly to results observed for phages PL-1 and J-1 (Capra et al., 2004), no clear influence of the suspension media could be distinguished for ϕ i*Lp*84. On the other hand, resistance of ϕ i*Lp*1308 was clearly affected by this factor. RSM and MRS proved to be the most and the least protective suspension media, respectively; this influence was equally exerted on ϕ MLC-A survival (Capra et al., 2006). In effect, treatments of ϕ i*Lp*1308 in RSM for 45 min at 63 °C and 72 °C lead to reductions of about 1 and 2 $\log_{10}$ orders, respectively. This thermal resistance was similar to that of ϕ MLC-A. However, the same conditions produced the total inactivation of ϕ i*Lp*1308 when MRS broth was assayed. Compared to commercial laboratory broths, RSM have also shown a protective effect on several phages of *L. paracasei* related to ϕ MLC-A (Capra et al., 2009a), as well as on phages of *Lactococcus* (Suárez and Reinheimer, 2002; Müller-Merbach et al., 2005; Atamer et al., 2010), *L. delbrueckii* (Quiberoni et al., 2003) and *Leuconostoc* (Atamer et al., 2011). This protection has been ascribed to the buffer capacity of milk, as well as its higher protein, lipid and salt contents (de Fabrizio et al., 1999; Atamer et al., 2010).

3.4. Chemical treatments

Concerning traditionally used sanitizers, peracetic acid (0.15% v/v) was the most effective one, as only 2 min were enough to completely destroy phage particles, either at 25 °C or 40 °C (Table 2). These results are not unusual, since peracetic acid has been unanimously recognized as fast and powerful for the inactivation of a broad spectrum of phages infecting LAB (Quiberoni et al., 1999, 2003; Binetti and Reinheimer, 2000; Suárez and Reinheimer, 2002; Capra et al., 2004, 2006; Briggiler Marcó et al., 2009; Ebrecht et al., 2010). The low pH of peracetic acid solution (1.90) could be partially responsible for the biocide activity, as both phages were significantly (at 25 °C) or completely (at 40 °C) eliminated at pH 2 in MRS broth without biocides (Fig. 2). However, this remains a hypothesis as no phage enumerations prior to 30 min of incubation at diverse pH values were carried out.

Alternatively, sodium hypochlorite was less efficient than peracetic acid. It was able to quickly eliminate ϕ i*Lp*1308 at a concentration of 400 ppm ($T_{99} < 2$ min, no detectable phages after 5 min of treatment) (Fig. 4). Lower concentrations were enough to completely inactivate two temperate *L. delbrueckii* phages (Ebrecht et al., 2010), as well as several phages of *S. thermophilus* (Binetti and Reinheimer, 2000), *L. helveticus* (Quiberoni et al., 1999), and *Lc. lactis* (Suárez and Reinheimer, 2002). However, 800 ppm were necessary to achieve a $T_{99} < 2$ min for ϕ i*Lp*84 (Table 2), and 30 min were required for complete inactivation at this concentration, which was comparable to the resistance calculated for *L. plantarum* phages. This makes chlorine usable only at laboratory scale, since of 800 ppm is not a permitted concentration in the food industry (Briggiler Marcó et al., 2009). In any case, ϕ i*Lp*84 was far from possess the astonishing resistance to chlorine found on *L. delbrueckii* ϕ Ib3, whose complete inactivation needed 45 min at 1200 ppm, and attributed to the long permanence of this phage in the dairy environment (Quiberoni et al., 2003).

Table 2
Time required for inactivation of 99% phage particles (T_{99}) for ϕ i*Lp*84 and ϕ i*Lp*1308 treated with different biocides.

Biocide	Concentration	T_{99} (min)	
		ϕ i*Lp*84	ϕ i*Lp*1308
Ethanol	10% (v/v)	>45	>45
	50% (v/v)	>45	>45
	75% (v/v)	15.5	9.6
	100% (v/v)	>45	>45
Sodium hypochlorite	100 ppm	>45	>45
	400 ppm	13.1	<2
	800 ppm	<2	<2
Peracetic acid	0.15% (v/v), 25 °C	<2	<2
	0.15% (v/v), 40 °C	<2	<2
Biocide A	0.25% (v/v)	<5	>45
	0.50% (v/v)	<5	<5
	1.00% (v/v)	<5	<5
	2.00% (v/v)	<5	<5
Biocide B	0.13% (v/v)	>20	>20
	0.26% (v/v)	>20	>20
Biocide C	2.5% (v/v)	2.2	<2
Biocide D	pure	>120	>120
Biocide E	0.8% (v/v)	<2	<2

Regarding ethanol, even the best concentration tested (75% v/v) resulted rather useless, as after 45 min of treatment, reductions of only 2- and 3-$\log_{10}$ orders for ϕ i*Lp*84 and ϕ i*Lp*1308, in that order, were obtained (Fig. 5). Ethanol was reported to be equally inefficient for inactivation of *L. plantarum* (Briggiler Marcó et al., 2009) and *L. paracasei* (Capra et al., 2004, 2006) phages. For *L. delbrueckii* phages, variable results were obtained depending on each particular phage (Quiberoni et al., 2003; Ebrecht et al., 2010), whereas *S. thermophilus* (Binetti and Reinheimer, 2000), *L. helveticus* (Quiberoni et al., 1999), and *Lc. lactis* (Suárez and Reinheimer, 2002)

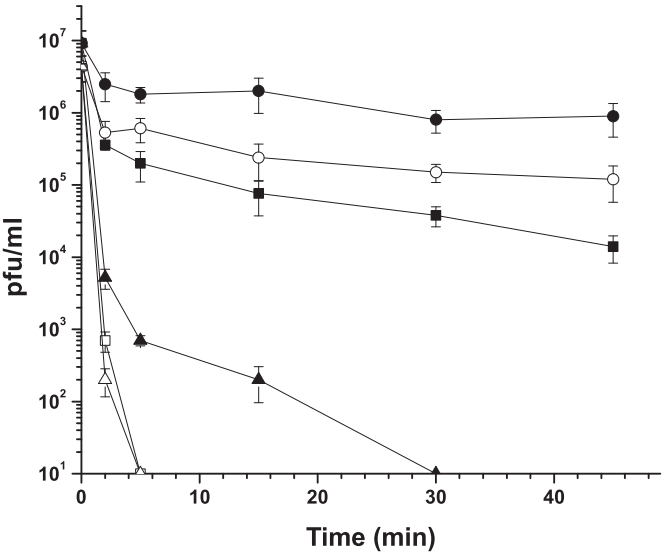

Fig. 4. Viability of phages ϕ i*Lp*84 (filled symbols) and ϕ i*Lp*1308 (empty symbols) after treatment with 100 ppm (●), 400 ppm (■) and 800 ppm (▲) residual-free chlorine (sodium hypochlorite). Values are the mean of three determinations.

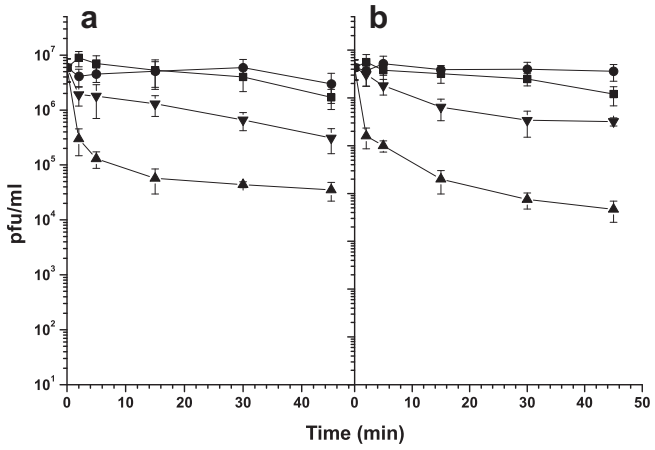

Fig. 5. Viability of phages ϕ i*Lp*84 (a) and ϕ i*Lp*1308 (b) after treatment with 10% (v/v) (●), 50% (v/v) (■), 75% (v/v) (▲) and 100% (v/v) (▼) ethanol. Values are the mean of three determinations.

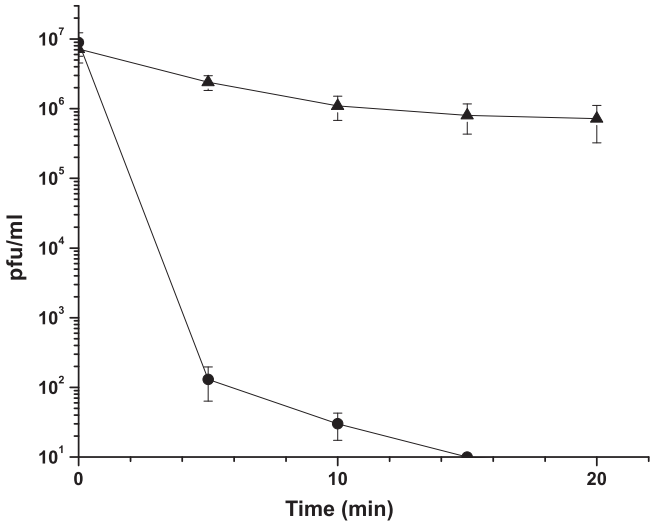

Fig. 6. Viability of phages ϕ i*Lp*84 (●) and ϕ i*Lp*1308 (▲) after treatment with 0.25% (v/v) commercial sanitizer A (quaternary ammonium chloride). Values are the mean of three determinations.

phages seemed to be more sensitive to this alcohol. Anyway, there is no consensus about the optimal ethanol concentration able to inactivate a broad spectrum of LAB phages, as some of them are more sensitive to 75% (v/v) and others to 100% (v/v), regardless their host specificity.

Although peracetic acid is widely used in dairy industry, several new disinfectants are being increasingly applied nowadays. Nonetheless, their effect on phage viability has been barely assessed, with the exception of a few *L. delbrueckii* phages studied by Ebrecht et al. (2010). We tested the same commercial biocides used by these authors and, in general, rather variable levels of efficiency were observed. On one hand, biocides B and D demonstrated not to be apt for inactivating phages. The former could not attain quantifiable phage reductions after 20 min of incubation (Table 2). This was completely unexpected, given the excellent results obtained on *L. delbrueckii* phages (Ebrecht et al., 2010). In that case, though, the mechanism of action was postulated to be other than the low pH. In fact, even if biocide B contains peracetic acid, its final concentration ought to be rather lower than when tested alone (0.15% v/v), thus explaining the higher pH of the dilutions from the commercial product (3.36 and 3.17 for 0.13% v/v and 0.26% v/v dilutions from concentrated commercial biocide B, respectively). These pH values are not low enough to affect ϕ i*Lp*84 and ϕ i*Lp*1308 viability (Fig. 2). Biocide D was completely inefficient as well, because no significant phage reductions were observed even after 2 h of incubation (Table 2). Although *L. delbrueckii* phages were also resistant to this biocide, a slightly inhibition could be observed for some of them, especially temperate ϕ Cb1/204, the only one with a $T_{99} < 45$ min (Ebrecht et al., 2010).

In contrast, biocide A was highly efficient for ϕ i*Lp*84 and ϕ i*Lp*1308 inactivation. Concentrations of 0.50% (v/v) or higher produced a complete phage inactivation after 5 min of treatment (Table 2). Thus, susceptibility of both phages to this biocide was remarkably higher than that reported for *L. delbrueckii* phages (Ebrecht et al., 2010). Even at a lower concentration (0.25% v/v) it caused a quick and effective inactivation of ϕ i*Lp*84 particles ($T_{99} < 5$ min, 15 min required for total destruction), but ϕ i*Lp*1308 counts diminished only 1 $\log_{10}$ order after 45 min of incubation (Fig. 6). Biocide A was the only commercial sanitizer for which both phages showed markedly different levels of resistance. Peculiarly, ϕ i*Lp*84 was more resistant than ϕ i*Lp*1308 when it was incubated with sodium hypochlorite.

On the other hand, biocides C and E displayed an exceptional efficiency against both phages, wiping them out after just 2 min of treatment, with the exception of biocide C on ϕ i*Lp*84, which required 5 min ($T_{99} = 2.2$ min, Table 2). These results are in agreement with those obtained for *L. delbrueckii* phages (Ebrecht et al., 2010). Again, biocide activity could be attributable to extreme pH values (12.69 and 1.68 for biocides C and E solutions, respectively), which are outside the range tolerated by these phages.

In a few words, peracetic acid remains the only good agent of choice for phage inactivation among traditional disinfectants, as long as it is used at a concentration of 0.15% (v/v). However, its efficiency could be significantly reduced when used at lower concentrations and/or mixed with other chemical on commercial products, which displayed rather dissimilar rates of efficiency for phages infecting different LAB species. With respect to other newly developed commercial biocides, those based on alkaline chloride foam or ethoxylated nonylphenol and phosphoric acid seem also adequate for phage inactivation at industrial scale. Alternatively, the optimal concentration of a quaternary ammonium-based biocide appeared to be dependent on each particular phage. Lastly, p-toluensulfonchloroamide demonstrated to be completely useless as an anti-phage option.

3.5. High pressure homogenization

During HPH assays, temperature was kept constant at about 25 °C and, therefore, a partial influence of thermal inactivation on loss of phage viability can be disregarded. Phages ϕ i*Lp*84 and ϕ i*Lp*1308 showed a logarithmic reduction of viability along with the successive passes at 100 MPa in RSM (0.62 and 0.48 $\log_{10}$ orders/pass, respectively, on average; Fig. 7). This reduction was similar to that obtained for ϕ MLC-A and for ϕ B1 of *L. plantarum* under the same assay conditions (Capra et al., 2009a). In contrast, these authors observed a moderate sensitivity to HPH of ϕ 832-B1 (*L. helveticus*), whilst all ϕ MLC-A8 (*L. casei*), ϕ Bym (*L. delbrueckii*), ϕ 13.2 (*S. thermophilus*), ϕ QP4 and ϕ QF12 (*Lc. lactis*) were not detected after only 5 passes through the high-pressure homogenizer. It is worth mentioning that ϕ MLC-A8, although far more sensitive to HPH than either ϕ MLC-A, ϕ i*Lp*84 or ϕ i*Lp*1308, shares host range with these phages.

To sum up, likewise some other LAB phages, both ϕ i*Lp*84 or ϕ i*Lp*1308 proved to be highly resistant to HPH treatments, at least at

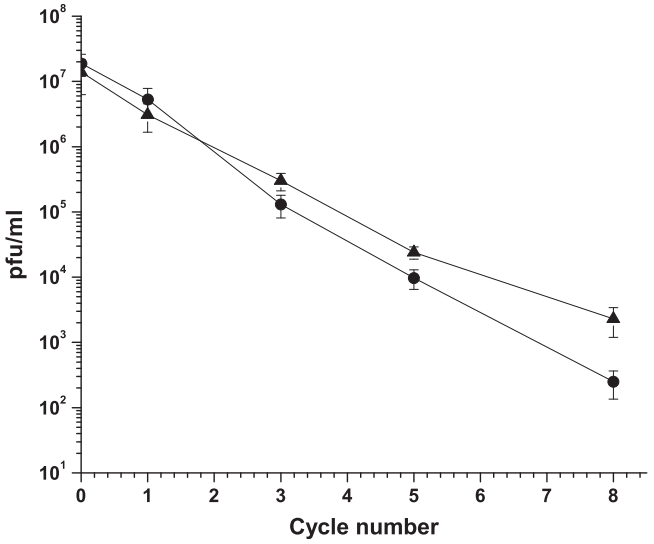

Fig. 7. Viability of phages ϕ i*Lp*84 (●) and ϕ i*Lp*1308 (▲) after successive HPH cycles at 100 MPa (25 °C) in RSM. Values are the mean of three determinations.

the pressure applied (100 MPa). This result emphasizes the already noticed inappropriateness of using HPH alone for phage inactivation in raw milk (Capra et al., 2009a). Instead, it should be regarded as a tool that could be combined with thermal and chemical phage control measures, thus allowing softening the strength of these treatments, bearing always in mind to protect physicochemical and nutritional characteristics, and sensory attributes of milk and dairy products.

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