

# Characterization of Two Temperate *Lactobacillus paracasei* Bacteriophages: Morphology, Kinetics and Adsorption

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## Key Words

*Lactobacillus* · Bacteriophages · Phage adsorption · Phage kinetics · Lactic acid bacteria

## Abstract

**Background/Aims:** Adsorption and kinetic parameters, latent period, burst size and burst time, are characteristics of phage/host systems and can be affected by several environmental factors. As only few studies have focused on temperate dairy phages, we characterized these parameters on temperate *Lactobacillus paracasei* phages  $\Phi$  iLp84 and  $\Phi$  iLp1308, infective for probiotic strains. **Methods:** Phages were characterized by transmission electron microscopy and genomic DNA restriction. Adsorption under different environmental conditions, phage kinetics and efficiency of plating (EOP) were determined using the double-layer titration method. **Results:** Phages  $\Phi$  iLp84 and  $\Phi$  iLp1308 belong to the *Siphoviridae* family and have genome sizes of 38 and 34 kbp, respectively. Adsorption was affected by calcium concentration, pH, temperature and host viability, and reached a limit at very high multiplicity of infection. Latency, burst time and burst size were of 85 min, 131 min and 46 for  $\Phi$  iLp84, and 51 min, 92 min and 28 for  $\Phi$  iLp1308, respectively, at 37°C. A clear influence of temperature on phage kinetics was observed. Regarding EOP,  $\Phi$  iLp84 produced plaques on only 1 out of 8 strains tested. **Conclusion:** Notice-

able differences in adsorption, kinetics and EOP were found for two morphologically identical temperate *L. paracasei* phages of similar origin.

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## Introduction

Adsorption is the first step in phage infection. It is a strain-specific event, involving host recognition of the bacterial surface (reversible binding) followed by irreversible fixation of another tail protein to a secondary receptor molecule [1]. Both adsorption velocity and efficiency are characteristics of every individual phage/host system, but can be considerably affected by environmental factors, as well as by the host's physiological state [2]. The environmental factors include pH, temperature and presence or absence of divalent ions, whereas the influence of the physiological state may correspond to changes in the levels of some cell surface molecules that are crucial for phage recognition [3]. In the infection process of a lytic phage, the time needed to complete one cycle (burst time) and the number of virions released by the infected cell (burst size) are characteristics of every phage/host system studied [1].

The adsorption was studied in many phages of lactic acid bacteria (LAB), including *Streptococcus thermophi-*

lus [4], *Lactococcus lactis* [5–9] and several species of *Lactobacillus* such as *L. casei* and *L. paracasei* [10–12], *L. fermentum* [13], *L. helveticus* [14], *L. delbrueckii* [15–18] and *L. plantarum* [19]. Most adsorption studies focused on lytic phages, while only a few concerned temperate phages of *L. plantarum*, *L. delbrueckii* and *L. helveticus* [15, 18, 20].

Although LAB phages actually occur as free virions in dairy factories, lysogenic strains represent a good source of new phages. Under favourable conditions, a prophage can be induced from the genome of a given strain and infect related bacteria, or even the same lysogenic strain that produced the phage [10]. In a dairy plant, this might lead to large economic losses [21, 22]. Lysogeny is very widespread in the genus *Lactobacillus* [23–25]. Presently, most probiotic strains used in the dairy industry belong to the *L. casei* group [25]. As any probiotic strain has unique traits, it is hard to replace it by another strain with the same characteristics. Consequently, phage infections have worse consequences for probiotics than for LAB used for acidification only, for which relatively easy industrial anti-phage strategies exist, such as strain rotation [10].

In a previous study, we confirmed the high incidence of lysogeny in the *L. casei* group [25]. In the present work, we phenotypically characterized two phages induced by mitomycin C and able to multiply on related *L. paracasei* strains. We particularly focused on the influence of diverse physicochemical factors on phage adsorption to indicator strains, as well as the efficiency of this process on related bacteria. The effect of lowering temperature on phage multiplication parameters such as burst size, burst time and latent time was also recorded. Our results illustrate the dynamics of temperate phage infection of potentially probiotic bacteria and thus help improving current strategies of phage control in the dairy industry.

## Materials and Methods

### Bacterial Strains, Phages and Culture Conditions

Bacteriophages  $\Phi$  iLp84 and  $\Phi$  iLp1308 were induced by mitomycin C (Sigma-Aldrich Chemical Co., St. Louis, Mo., USA) from the strains *L. paracasei* 84 (isolated from dairy products) and *L. paracasei* CNRZ1308 (CNRZ collection) [25]. Both phages were propagated on their respective indicator strains:  $\Phi$  iLp84 on *L. paracasei* INL3 (INLAIN collection strain) and  $\Phi$  iLp1308 on *L. paracasei* A14 (commercial strain). Phages were stocked at  $-80^{\circ}\text{C}$  in De Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France) with 15% (v/v) glycerol (Cicarelli, San Lorenzo, Argentina), but a single high-titred 100-ml suspension of each one, prepared as described by Neviani et al. [26], was stored at  $4^{\circ}\text{C}$  and

used for all the assays. Phages were in all cases counted by the double-layer plate titration method, as described by Svensson and Christiansson [27], and using glycine for a better visualization of lysis plaques [28]. Incubation was carried out at  $34^{\circ}\text{C}$  for 18 h under microaerophilic conditions; phage titres (plaque forming units; pfu  $\text{ml}^{-1}$ ) were determined by counting lysis plaques.

In addition to the two above-mentioned indicator strains, seven additional *L. paracasei* strains were used for some tests, namely A, Dn, Hn, Yk, A13 (commercial strains), ATCC 27092 and ATCC 27139 (ATCC collection strains). These seven strains were selected because they showed a diverse degree of sensitivity to bacteriophages  $\Phi$  iLp84 and  $\Phi$  iLp1308 in a previous work [25]. Strains were stocked at  $-80^{\circ}\text{C}$  in MRS broth (Biokar) supplemented with 15% (v/v) glycerol (Cicarelli) and periodically cultured overnight at  $37^{\circ}\text{C}$  in MRS broth.

### Transmission Electron Microscopy

Several micrographs of each phage were obtained using exactly the same method and equipment as described by Capra et al. [12].

### Phage DNA Extraction and Restriction Analysis

Phage DNA extraction started from 50 ml of high-titred lysates in MRS with 10 mM  $\text{CaCl}_2$  (MRS-Ca) broth. Lysates were centrifuged (5,000 g, 10 min,  $4^{\circ}\text{C}$ ), filtered with Millipore (Sao Paulo, Brazil) membranes (pore diameter 0.45  $\mu\text{m}$ ), and phages were precipitated by addition of 0.5 M NaCl (Cicarelli) and 10% (w/v) polyethylene glycol 8000 (USB, Cleveland, Ohio, USA), followed by incubation at  $4^{\circ}\text{C}$  for 18 h. After a new centrifuging step (10,000 g, 1 h,  $4^{\circ}\text{C}$ ), concentrated phages were resuspended in 1 ml SM buffer [29] and DNA was extracted as described by Binetti et al. [30]. Restriction analysis was carried out with *Bam*HI, *Bgl*II and *Eco*RV enzymes according to the manufacturer's recommendations (Genbiotech, Buenos Aires, Argentina), and restriction fragments were resolved by 0.8% (w/v) agarose gel electrophoresis in  $1\times$  TAE buffer, using a 1 kbp DNA ladder (GE Healthcare Life Sciences, Bucks, UK) as molecular weight marker and GelRed™ (Biotium, Inc., Hayward, Calif., USA) as nucleic acid binding dye.

### Factors Influencing Adsorption

Influence of calcium on adsorption of  $\Phi$  iLp1308 and  $\Phi$  iLp84 was evaluated in both MRS and MRS-Ca as described by Capra et al. [12] and starting from a multiplicity of infection (MOI) between  $5\times 10^{-3}$  and  $1\times 10^{-2}$ . Sampling was carried out at 2, 5, 15, 30, 45 and 60 min of incubation.

The influence of pH and temperature were also assessed according to Capra et al. [12]. In the first case, MRS-Ca broth was adjusted to pH 4, 5, 6, 7, 8 and 9. The influence of temperature was determined in aliquots of infected cells incubated in MRS-Ca at 0, 10, 20, 30, 37 (control) or  $45^{\circ}\text{C}$ . In both experiments, samples were taken after 30 min (for  $\Phi$  iLp1308) or 45 min (for  $\Phi$  iLp84) of incubation, i.e. the minimum time required to reach a plateau in the number of phages adsorbed in MRS-Ca broth at pH 7 (see Results).

To evaluate the influence of cell viability, adsorption kinetics of phages  $\Phi$  iLp1308 and  $\Phi$  iLp84 at  $37^{\circ}\text{C}$  in MRS-Ca broth were determined both on cells in the mid-exponential growth phase (control) and on one aliquot of the same cells heated at  $100^{\circ}\text{C}$  for 2 min in a boiling water bath [19]. The thermal inactivation of the cells was verified by plate counts in MRS agar. The effect of chloramphenicol (Sigma, St. Louis, Mo., USA), a protein synthesis inhibitor, on phage adsorption was also recorded. Among four concen-

trations tested (10, 20, 50 and 100 µg/ml), 50 µg/ml was the minimum required to completely stop cell growth of both bacterial strains (as followed by OD<sub>560</sub> measurement) after 75 min of incubation at 37°C in MRS-Ca broth (data not shown). As the bacteriostatic effect after chloramphenicol removal lasted for at least the duration of the assays, cells were centrifuged (10,000 g for 5 min) and suspended in MRS-Ca broth free of chloramphenicol to conduct adsorption studies.

Finally, the influence of MOI on adsorption of Φ iLp1308 and Φ iLp84 was determined in cultures infected at different initial MOI, at 37°C in MRS-Ca broth.

For all the factors studied, adsorption rates were always calculated as the ratio of initial phages, measured in the corresponding dilution of the original phage suspension, and non-adsorbed phages, measured in the supernatants after centrifugation [12]. Reduction in the number of phages due to adsorption was plotted against time (for the assessment of the influence of calcium and cell viability), pH, temperature or MOI.

#### One-Step Growth Curve

Both Φ iLp84 on *L. paracasei* INL3 and Φ iLp1308 on *L. paracasei* A14 were subjected to one-step growth assays, which were carried out as described by Briggiler et al. [19], but using a MOI of about  $4 \times 10^{-4}$ . An aliquot (1 ml) of the culture was centrifuged (10,000 g, 5 min, 4°C), suspended in 9 ml of MRS-Ca broth (first decimal dilution), and subsequent decimal dilutions were made and incubated at 30 or 37°C. At defined time points, 100 µl of each dilution were collected for phage enumeration [12].

#### Efficiency of Plating

The efficiency of plating (EOP), defined as the ratio between pfu ml<sup>-1</sup> for a given phage on a sensitive strain and pfu ml<sup>-1</sup> on its indicator strain, was investigated for all strains that were sensitive to phages Φ iLp84 or Φ iLp1308 in MRS-Ca broth.

#### Statistical Analysis

Experiments were carried out in triplicate and data analysed using the Statgraphics™ Plus software (v 3.0, Statistical Graphics Corp.). For adsorption studies, one-way ANOVA and Duncan's multiple range tests at  $p < 0.05$  were applied. For the determination of latent period, burst time and burst size on the one-step growth curves, DMFit web edition was used (available online at: <http://modelling.combase.cc/DMFit.aspx>); data were fitted using the complete model of Baranyi and Roberts [31].

## Results and Discussion

#### Phage Morphology and Restriction Analysis

Both Φ iLp84 and Φ iLp1308 have identical dimensions as measured on 10 phage particles each: icosahedral heads of  $61 \pm 3$  nm in diameter and long non-contractile tails of  $184 \pm 4$  nm  $\times$   $8 \pm 1$  nm. Hence, they belong to the morphotype B1 of the *Siphoviridae* family (online suppl. fig. S1; for all online suppl. material, see [www.karger.com/doi/10.1159/000369207](http://www.karger.com/doi/10.1159/000369207)) [32]. They also show cross-striations with a periodicity of approximately 4 nm along

the tail and a tiny basal plate (about  $13 \times 10$  nm). As expected, *Bgl*III profiles of both phages matched those previously obtained from the supernatants of induction during their isolation (before propagation on sensitive strains) (data not shown) [25]. Genome sizes, roughly estimated from restriction fragments (online suppl. fig. S2) in  $38 \pm 2$  kbp for Φ iLp84 and  $34 \pm 4$  kbp for Φ iLp1308, were similar to those of most LAB phages.

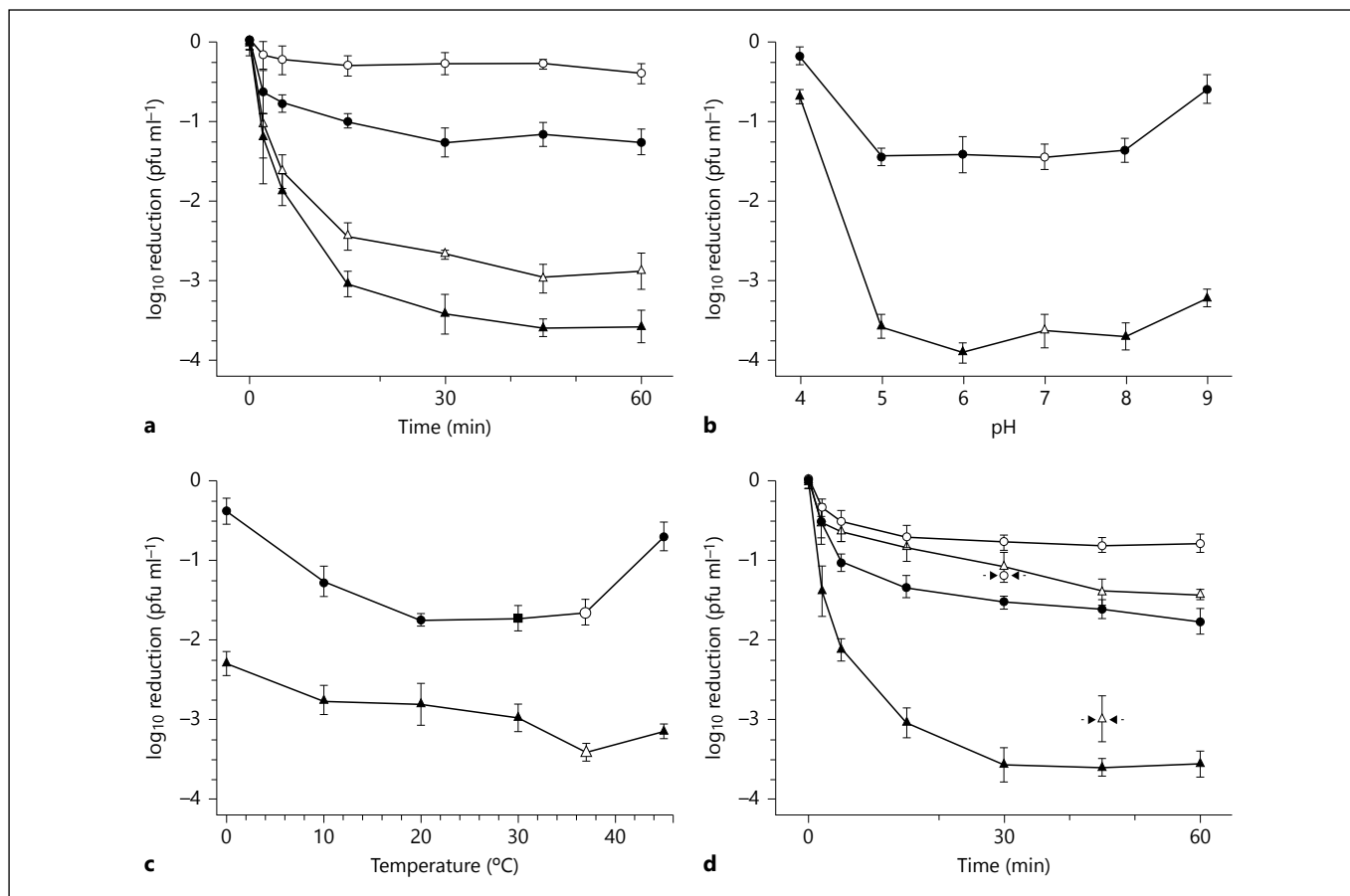
#### Study of Factors Influencing Adsorption

##### Influence of Calcium

Phage Φ iLp84 adsorbed slightly faster than Φ iLp1308 during the first 2 min at 37°C in MRS-Ca broth. Differences in adsorption were more evident after 5 min, and large after 30 min (fig. 1a). The amount of Φ iLp1308 virions adsorbed was approximately constant after 30 min, but Φ iLp84 particles continued to adsorb for up to 45 min; these times were therefore selected to assess the effect of the rest of the parameters. Adsorption was verified (though at a lesser extent) in MRS broth with no calcium added. Adsorption rates reported for *L. paracasei* phages C<sub>L</sub>1 and C<sub>L</sub>2 were intermediate between those of Φ iLp84 and Φ iLp1308 in MRS-Ca broth but similar to Φ iLp1308 in the absence of calcium [10]. Lysis was not detected without calcium (data not shown), suggesting that this ion is essential at some point after adsorption for both phages to complete the infective cycle. A similar influence of calcium on adsorption and lysis in MRS broth was reported for phages MLC-A [12], C<sub>L</sub>1 and C<sub>L</sub>2 [10], but, on the contrary, both processes showed to be independent of calcium for other *L. casei* and *L. paracasei* phages [11, 33].

##### Influence of pH

Throughout the entire pH range tested, adsorption remained higher for Φ iLp84 than for Φ iLp1308 (fig. 1b). Most reports indicate that adsorption of LAB phages is stable between pH 5 and 7 and decreases more or less markedly at more extreme pHs; in the present work, adsorption was stable between pH 5 and 8. At pH 9, adsorption is in general clearly affected, as is the case of *L. paracasei* phage MLC-A [12]. Phages in our study showed a moderate decrease of adsorption at pH 9 (slightly higher for Φ iLp1308), but phages PL-1 and J-1 adsorbed efficiently even at pH 10 [11]. On the other hand, adsorption of phages Φ iLp84 and Φ iLp1308 was strongly inhibited at pH 4, more than for most LAB phages. It is important to note that both phages retain full viability at pH 4 and 9 (MRS-Ca broth, 37°C, 30 min) [34], so, the observed titre decreases were only due to a decrease in adsorption.



**Fig. 1.** Influence of different parameters on the adsorption kinetics of phages  $\Phi$  iLp84 on *L. paracasei* INL3 ( $\blacktriangle$ ,  $\triangle$ ) and  $\Phi$  iLp1308 on *L. paracasei* A14 ( $\bullet$ ,  $\circ$ ). **a** Influence of calcium at 37°C in MRS (empty symbols) or MRS-Ca (with CaCl<sub>2</sub> 10 mM; filled symbols) broth. **b** Influence of pH at 37°C; empty symbols indicate control samples at neutral pH. **c** Influence of temperature; empty symbols

indicate control samples at 37°C. **d** Adsorption on control host cells (filled symbols), heat-killed cells (2 min at 100°C; empty symbols) or chloramphenicol-treated cells (50 µg/ml, 75 min at 37°C; symbols indicated by arrows) in MRS-Ca broth at 37°C. **b**, **c** Incubation times were 45 min for  $\Phi$  iLp84 and 30 min for  $\Phi$  iLp1308 in MRS-Ca. Experiments were always carried out in triplicate.

### Influence of Temperature

For both  $\Phi$  iLp84 and  $\Phi$  iLp1308, the efficiency of adsorption was minimal at 0°C and increased with the temperature up to 37°C. Adsorption in other *L. casei* group phages previously studied was also highest at 37°C [11, 12]. For  $\Phi$  iLp84, adsorption was less dependent on changes of temperature, and even at 0°C it was much more efficient than for  $\Phi$  iLp1308 at 37°C. For  $\Phi$  iLp1308, adsorption was similar to that observed in *L. paracasei* phage MLC-A on *L. paracasei* A [12] and phage J-1 on *L. paracasei* ATCC 27139, although for the latter, adsorption fluctuated when it was tested on other strains and was already affected at 10°C [11] (fig. 1c). In contrast,  $\Phi$  PL-1 was reported to adsorb equally well at 0 and 37°C, either to *L. paracasei* ATCC 27092 [33] or to *L. casei* ATCC 393

[11]. On the other hand, a temperature higher than the optimum (45°C) had little effect on the adsorption of  $\Phi$  iLp84, but it greatly affected that of  $\Phi$  iLp1308 (fig. 1c).

### Influence of Cell Viability

Adsorption was reduced by thermal inactivation of host cells, considerably for  $\Phi$  iLp84 (at least 2 log) and moderately for  $\Phi$  iLp1308 (fig. 1d). Although adsorption rates were still higher for  $\Phi$  iLp84 than for  $\Phi$  iLp1308 on heat-killed cells, differences were not significant up to 15 min and slight thereafter. Phage  $\Phi$  PL-1 (on the strain *L. paracasei* ATCC 27092) [35] adsorbed to heat-inactivated bacteria, indicating that phage receptors are thermostable and that adsorption does not depend on the physiological state of the cells. On the other hand, adsorp-

tion to non-viable cells was moderately or strongly inhibited in phages PL-1 (on *L. paracasei* A), J-1 [11] and MLC-A [12], as well as in phages  $\Phi$  iLp84 and  $\Phi$  iLp1308 in this study. These results suggest that adsorption strongly depends on the physiological state of the culture and/or that phage receptors are thermo-sensitive and suffered conformational changes during heating that made them no longer able to adsorb phage particles. On non-growing chloramphenicol-treated cells, adsorption levels of both phages were somewhat lower than on control cells, but still higher than those obtained on heat-killed cells (fig. 1d). These results indicate that the physiological state actually contributes to the efficiency of phage adsorption, especially for phage  $\Phi$  iLp1308. This factor was, however, less important than changes due to heat treatment of host cells: modifications in the conformation of phage receptors or the dropping in the energy levels (ATP content) of killed cells. To discriminate between the latter, further studies employing drugs which decrease without heating the intracellular concentration of ATP (NaF, NaN<sub>3</sub> or arsenate) would be necessary.

#### Influence of MOI

Adsorption of LAB phages is usually studied at low MOI (<0.02) to avoid variances due to the influence of the relative amounts of phage particles and host cells present. In the present work, adsorption remained more or less constant for both phages at MOI <0.1. At higher MOI values, the percentage of adsorption was sharply reduced and almost blocked (online suppl. fig. S3). Although the intrinsic error of the double-layer plate titration rapidly increases with MOI, an inhibition in the adsorption at MOI of 1 ( $\Phi$  iLp84) and 5 ( $\Phi$  iLp1308) was verified. Capra et al. [11] studied adsorption on *L. paracasei* phages at MOI of up to 1.5, where a decrease in the adsorption is reasonably expected, likely due either to a low number of available sites for phage attachment on the cell wall or to another sort of steric obstruction. Therefore, more attention should be paid to this parameter when evaluating adsorption. To our knowledge, this has not been reported in phages of *Lactobacillus*. In addition, it would be interesting to carry out complementary studies by transmission electron microscopy.

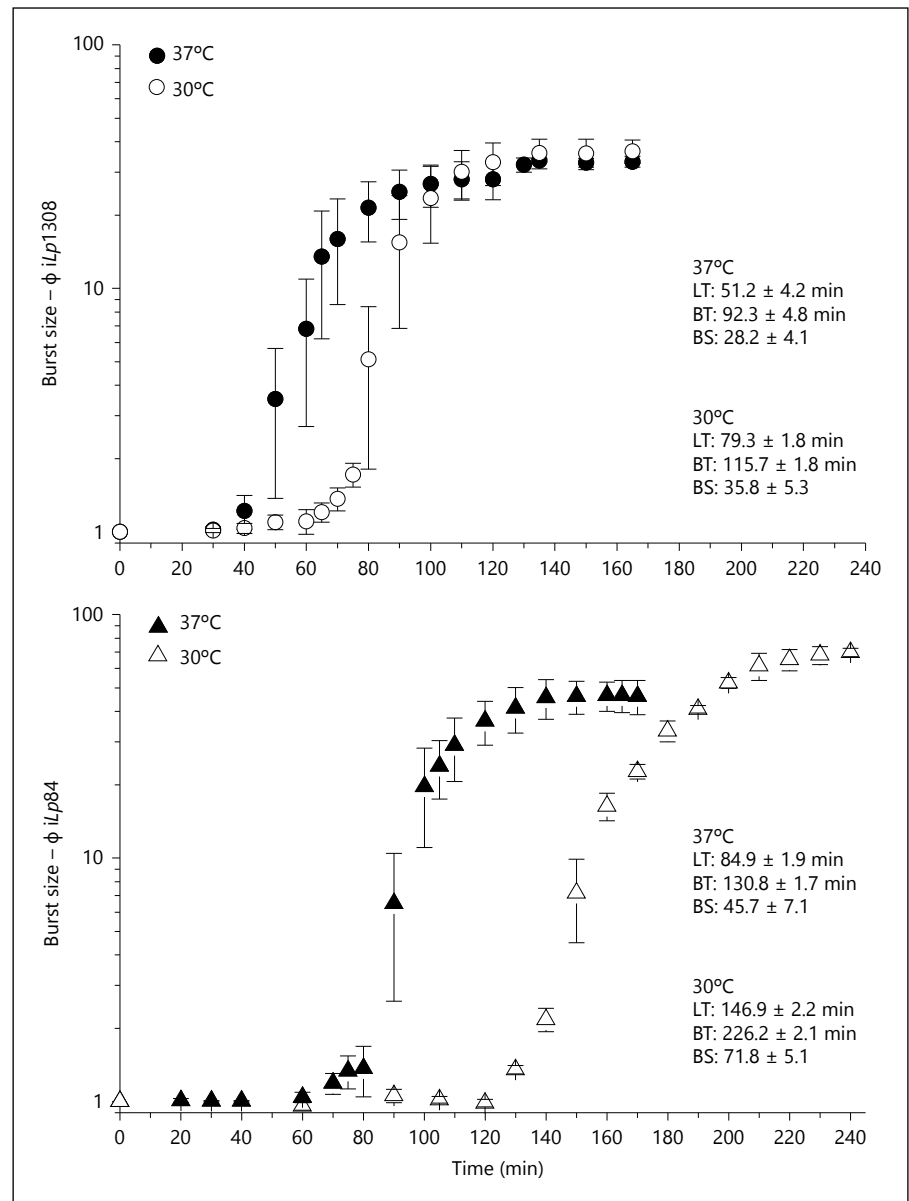
#### One-Step Growth

Figure 2 shows the one-step growth curves and growth parameters estimated for  $\Phi$  iLp84 and  $\Phi$  iLp1308 at 37 and 30°C. At both temperatures, the complete infective cycle of  $\Phi$  iLp1308 was shorter (lower latent and burst time) and the burst size of  $\Phi$  iLp84 was higher. A great

variation in latent time (5–140 min) and burst time (120–240 min) has been reported among phages of *L. casei* and *L. paracasei* [10–12, 36]. At 37°C,  $\Phi$  iLp84 lysed the bacterial host slowly, similar to temperate phage  $\Phi$  393-A2 [36]. It is worth pointing out that growth parameters were in most studies visually deduced from drawn curves, but we have observed that it underestimates latent and burst times in relation to parameters calculated with DMFit (unpublished results), so the latter was used in our work. The influence of external variables on LAB phages multiplication has received little attention, even if changes in temperature and pH are key stress factors in dairy fermentation. Therefore, we decided to carry out one-step growth curves at 30°C. This sub-optimal temperature had the same effect on both phages, but it was more evident for  $\Phi$  iLp84 than for  $\Phi$  iLp1308: infection cycles were slower (burst time 73 and 25% higher, respectively); however, burst sizes were higher (57 and 27%, respectively). Also Müller-Merbach et al. [8] found, for lactococcal phage P008, longer infection cycles at a low temperature but, in contrast, these authors observed larger burst sizes at a higher temperature. Wang et al. [37] observed that the better the physiological condition of host cells, the shorter the latency period; in our study, it corresponds to results obtained at optimal growth temperature. Abedon [38] stated that for two identical phages only differing in lysis time, the one with the longer latent period will have a larger burst size. He postulated eclipse periods of the same length and constant rates of progeny production, so a longer progeny-producing period would produce more phage particles at the time of the burst, in accordance with the results presented here. Future investigations should also consider the influence of factors such as pH and cell density on phage population growth.

#### Efficiency of Plating

Several *L. casei* and *L. paracasei* phages previously characterized in our laboratory and different at the molecular level showed highly conserved host ranges [10–12, 22]. This makes this test less powerful in discriminating between phages of other LAB species. Table 1 shows EOP values for both phages on nine strains shown to be sensitive to one or another phage in MRS-Ca broth [25]. EOP values for  $\Phi$  iLp1308 were of 0.2 for *L. paracasei* INL3 and approximately 1 for the rest of the strains. On the other hand,  $\Phi$  iLp84 did not produce plaques on some strains sensitive to this phage in liquid media [25]. In that case, however, lysis was sometimes partial or achieved after two subcultures, and uneven results were obtained after repeating the test (results not shown).



**Fig. 2.** One-step growth curves in MRS-Ca broth for phages  $\Phi$  iLp84 on *L. paracasei* INL3 and  $\Phi$  iLp1308 on *L. paracasei* A14. Experiments were carried out in triplicate. The three parameters were calculated with DMFit web edition using the complete model of Baranyi and Roberts [31] for data fitting. LT = Latent period; BT = burst time; BS = burst size.

**Table 1.** EOP of phages  $\Phi$  iLp84 and  $\Phi$  iLp1308, defined as the ratio between phage counts on different *L. paracasei* strains and phage counts on the respective indicator strain

Strain	Phage		Strain	Phage	
	$\Phi$ iLp84	$\Phi$ iLp1308		$\Phi$ iLp84	$\Phi$ iLp1308
<i>L. paracasei</i> A	n.d. <sup>a</sup>	1.0	<i>L. paracasei</i> ATCC 27139	n.d. <sup>a</sup>	1.1
<i>L. paracasei</i> Dn	n.d. <sup>a</sup>	0.9	<i>L. paracasei</i> INL3	<sup>b</sup>	0.2
<i>L. paracasei</i> Hn	n.d. <sup>a</sup>	0.8	n.d. = Not detected. <sup>a</sup> EOP in these cases were $<1.0 \times 10^{-8}$ considering both the detection limit ( $10$ pfu $\text{ml}^{-1}$ ) and the titre of $\Phi$ iLp84 on <i>L. paracasei</i> INL3 ( $9.9 \times 10^8$ pfu $\text{ml}^{-1}$ ). <sup>b</sup> Indicator strains for each phage (EOP = 1).		
<i>L. paracasei</i> Yk	n.d. <sup>a</sup>	1.0			
<i>L. paracasei</i> A13	n.d. <sup>a</sup>	1.0			
<i>L. paracasei</i> A14	n.d. <sup>a</sup>	<sup>b</sup>			
<i>L. paracasei</i> ATCC 27092	0.6	1.7			

## Conclusion

Clear differences in adsorption and multiplication kinetics were found for morphologically identical temperate *L. paracasei* phages  $\Phi$  iLp1308 and  $\Phi$  iLp84. While both phages were induced with mitomycin C from *L. paracasei* strains, they showed different EOP. Particularly, the host range of  $\Phi$  iLp84 was narrower than that of most *L. casei* group phages studied until now. Taking this into consideration, and in order to attain a complete phage characterization, it may be interesting to analyse the genomic sequence and organization of both phages while paying special attention to their temperate nature.

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## Disclosure Statement

The authors declare that they have no conflicts of interest.

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