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Phage adsorption to *Lactobacillus plantarum*: Influence of physiological and environmental factors

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ABSTRACT

Bacteriophage infection of lactic acid bacteria (LAB) constitutes one of the major problems in the dairy industry, causing economic losses and a constant risk of low quality and/or unsafe foods. The first step in the phage biology is the adsorption on the host cell surface. In a previous study, a remarkable thermal, chemical and photocatalytic resistance was demonstrated by four phages of *Lactobacillus plantarum* (ATCC 8014-B1, ATCC 8014-B2, FAGK1 and FAGK2). In the present work, these phages were used to characterize the adsorption process on *L. plantarum* ATCC 8014. Clearly, the characterization of this process could increase the possibilities of design useful strategies in order to prevent phage infections. The influence of Ca^{2+}, temperature, pH and physiological cell state on phage adsorption was investigated. Burst sizes of phages ATCC 8014-B1 and ATCC 8014-B2 were 60 and 83 PFU/infective centre, respectively. The four phages exhibited a high infectivity even at pH 4 and pH 11. Calcium or magnesium ions were not indispensable for cell lysis and plaque formation, and more than 99% of phage particles were adsorbed either in the presence or absence of Ca^{2+}, after 15 min at 37 °C. Phage adsorption was only partially affected at 50 °C, while reached its maximum between 30 and 42 °C. The highest adsorption values (99.9%) were observed from pH 5 to 7, after 30 min at 37 °C. Adsorption rates decreased after the thermal inactivation of cells, though, when 20 μg/ml of chloramphenicol was used, adsorption values were similar on treated and untreated cells. All these results showed that the adsorption process was only partially affected by a few conditions: thermally killed host cells, an incubation temperature of 50 °C and pH values of 9 and 10. Nevertheless, and unfortunately, those conditions are not commonly applied during fermented food manufacturing, thus restricting highly the application of strategies currently available to reduce phage infections in industrial environments. This work also contributes to increase the currently knowledge on the biological aspects of *L. plantarum* bacteriophages.

1. Introduction

Lactobacillus plantarum has the coding capacity for the utilization of many different sugars, uptake of peptides and formation of most amino acids (de Vries et al., 2006). Thus, it is involved in the fermentation of vegetables, coffee, meat and silage and it is commonly found in the human gastrointestinal-tract (Chibani-Chennoufi et al., 2004). *L. plantarum* contributes also to cheese quality enhancement when it is used as adjunct culture in manufacturing of Mozzarella (De Angelis et al., 2008), Fiore Sardo (Mangia et al., 2008), Cheddar, Cremoso Argentino (Milesi et al., 2008) and Manchego (Nieto-Arribas et al., 2009) cheeses. On the other hand, probiotic properties have been linked to *L. plantarum* strains (Cebeci and Gürakan, 2003; Golowczyc et al., 2008; Hugo et al., 2008; Maragkoudakis et al., 2006; Mathara et al., 2008; Nguyen et al., 2007; Vizoso Pinto et al., 2006; Wang et al., 2009). Therefore, *L. plantarum* could be used as probiotic starter in manufacturing of several functional foods. However, this metabolic activity can be reduced by phage infection of bacterial cells, which could result in low quality and/or unsafe foods (Yoon et al., 2001). Nowadays, phage infection is considered the main cause of reduction in acid production or complete starter failure, which generates economic losses (Moineau and Lévesque, 2005). Due to the ability of *L. plantarum* to grow in a large range of environmental niches, phages infecting this species were isolated from different sources: meat (Chibani-Chennoufi et al., 2004; Trevors et al., 1983), silage (Caso et al., 1995; Chibani-Chennoufi et al., 2004; Doi et al., 2003), homemade cheese whey (Caso et al., 1995), fermented vegetables (Lu et al., 2003; Yoon et al., 2001), fermented maize and fermented coffee (Chibani-Chennoufi et al., 2004) and kefir grains (De Antoni et al., 2009).

The first step in the phage biology is the adsorption on the host cell surface. Phage adsorption process was studied for several species of lactic acid bacteria (Binetti et al., 2002; Capra et al., 2006; Foschino et al., 1995; Monteville et al., 1994; Müller-Merbach et al., 2007; Quiberoni and Reinheimer, 1998; Quiberoni et al., 2004; Sijtsma et al., 1988; Suárez et al., 2008; Valsayevi et al., 1990), although information

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on *L. plantarum* is very scarce and biased (Caso et al., 1995; Lu et al., 2003).

In a previous study, four *L. plantarum* phages demonstrated a remarkable thermal, chemical and photocatalytic resistance (Briggiler Marcó et al., 2009), restricting highly the possibilities to apply the strategies currently available to their inactivation. Thus, the aim of the present work was to characterize the interaction of those four *L. plantarum* phages with their host strain, intending also to establish the basis of new and efficient methodologies to diminish phage infections on *L. plantarum* in industrial environments. The influence of physicochemical parameters on phage infectivity and adsorption to sensitive cells were especially investigated.

2. Materials and methods

2.1. Bacterial strains, phages and culture conditions

Two *L. plantarum* collection phages, ATCC 8014-B1 and ATCC 8014-B2 (from now on B1 and B2, respectively), and two phages (FAGK1 and FAGK2) isolated from kefir grains (De Antoni et al., 2009) were studied in this work. The host strain *L. plantarum* ATCC 8014 was maintained as frozen stock at −80 °C in Man Rogosa Sharpe (MRS) broth in the presence of 15% glycerol, routinely reactivated overnight at 37 °C in MRS broth, and counted on MRS agar (48 h at 37 °C). MRS broth and MRS agar supplemented with 10 mmol/l $CaCl_2$ (MRS-Ca) were used to propagate and count the phages. Phage stocks were prepared as described by Neviani et al. (1992) and stored at 4 °C (MRS) and −80 °C (MRS broth added of 15% v/v glycerol). Phage enumerations (PFU/ml) were determined by the double-layer plaque titration method (Svensson and Christiansson, 1991).

2.2. One-step growth curves

This experiment was carried out for phages B1, B2, FAGK1 and FAGK2 on the sensitive strain *L. plantarum* ATCC 8014. The host strain *L. plantarum* ATCC 8014, in exponential growth (OD_{560nm} = 0.5), was harvested and suspended in 1/5 of initial volume of MRS-Ca broth. Phages were added at a multiplicity of infection (m.o.i.) of 0.5. After adsorption (30 min at 37 °C), cells were harvested by centrifugation (10,000 *g* for 5 min), resuspended in 10 ml of MRS-Ca broth and decimal dilutions of this suspension were carried out and incubated at 37 °C. At regular intervals, 100 µl of each dilution were collected for bacteriophage counts (Capra et al., 2006). Latent periods, burst times and burst sizes were calculated from one-step growth curves.

Growth parameters for phages FAGK1 and FAGK2 on *L. plantarum* ATCC 8014 were previously reported by De Antoni et al. (2009).

2.3. Influence of pH on phage infectivity and strain viability

Phages (10^7 PFU/ml) or strain culture (10^8 CFU/ml) were suspended in MRS broth with a previous pH adjustment (pH values ranging from 2 to 11 for phages and from 4 to 10 for host strain), placed into Eppendorf tubes (1 ml final volume) and used to test the pH influence on phage infectivity or strain viability. After 30 min of incubation at 37 °C, tubes were removed from thermal bath and the surviving phage particles or bacterial cells were immediately plated and counted as described above. The results were expressed as a percentage of the initial phage or cell counts.

2.4. Influence of temperature on phage infectivity and strain viability

Phages (10^7 PFU/ml) or strain culture (10^8 CFU/ml) were suspended in MRS broth, placed into Eppendorf tubes (1 ml final volume) and incubated at 0, 10, 20, 30, 37, 42 and 50 °C for 30 min. The surviving phage particles or bacterial cells were immediately counted and the results were expressed as a percentage of the initial phage or cell counts.

2.5. Adsorption studies

2.5.1. Influence of divalent cations

The influence of Ca^{2+} and Mg^{2+} on cell lysis was investigated by incubation (37 °C) of infected (m.o.i = 0.1) *L. plantarum* cultures in MRS broth, with and without $CaCl_2$ or $MgCl_2$ (10 mmol/l). Plaque formation was investigated using the double-layer plaque technique, as previously described, in MRS agar, with and without $CaCl_2$ or $MgCl_2$ (10 mmol/l).

The effect of calcium ions on phage adsorption was investigated by the analysis of the adsorption kinetics in MRS and MRS-Ca as described by Capra et al. (2006). Exponentially growing (OD_{560nm} = 0.5) *L. plantarum* host strain cultures in MRS broth were centrifuged and resuspended (1.10^8–2.10^8 CFU/ml) in MRS and MRS-Ca broths. Phages were added (m.o.i = 0.07) and the mixtures incubated at 37 °C for adsorption after distributing the infected cultures in Eppendorf tubes. At intervals (5, 15, 30 and 45 min), tubes were removed and centrifuged (10,000 *g* for 5 min) to sediment the phage-adsorbed bacteria. Then, the supernatants were assayed for unadsorbed phages (double-layer plate titration), and the counts were compared with the titre of a control without cells. The results were expressed as percentages of the initial phage counts.

2.5.2. Influence of temperature

The adsorption of phages on *L. plantarum* ATCC 8014 was determined at 0, 10, 20, 30, 37, 42 and 50 °C, on the basis of the results obtained in the temperature sensitivity assays, as described by Capra et al. (2006). Results were expressed as percentages of adsorption after 30 min and plotted against temperature values.

2.5.3. Influence of pH

The adsorption of phages on *L. plantarum* cells was determined at pH values of 5, 6, 7, 8, 9 and 10, on the basis of the results obtained in the pH sensitivity assays, according to Capra et al. (2006). Results were expressed as percentages of adsorption after 30 min and plotted against pH values.

2.5.4. Influence of the thermal treatment on cells

Adsorption kinetics were determined both on viable and nonviable cells. Nonviable cells were obtained by keeping a *L. plantarum* cell suspension in boiling water for 2 min (Capra et al., 2006). Nonviability (100% of cell death) of treated cells was checked by plate counts.

2.5.5. Influence of cell protein-synthesis inhibitors

The minimum concentration of chloramphenicol (Elea, Buenos Aires, Argentina) needed to inhibit the protein-synthesis in *L. plantarum* ATCC 8014 was determined as follows. Exponentially growing (OD_{560nm} = 0.5) cell cultures in MRS broth were centrifuged and cells resuspended (1.10^8–2.10^8 CFU/ml) in MRS broth with 20 µg/ml or 100 µg/ml of chloramphenicol and then incubated at 37 °C (during 120 min) after distributing into Eppendorf tubes. A cell culture subjected to a similar treatment but without chloramphenicol was used as control. At regular intervals, OD_{560nm} was measured and cell counts were assayed. The concentration of chloramphenicol selected to perform the adsorption experiment was the minimum one producing stable values in the OD_{560nm} and plate counts during the incubation time. The inhibition of protein-synthesis was achieved after 75 min of incubation.

Additionally, it was also evaluated if the effects of the protein-synthesis inhibitor were maintained after the removal of chloramphenicol. Culture with the same treatment described above was incubated at 37 °C to inhibit the protein-synthesis (75 min). Then,

cells were centrifuged, washed and resuspended in MRS-Ca broth, and finally incubated at 37 °C. At regular intervals, OD_{560nm} was measured.

On the basis of the preceding experiments, phage adsorption assays were performed on host cells treated with chloramphenicol (20 μg/ml) that was removed after the inhibition of protein-synthesis was achieved. Treated cells were infected with each phage (m.o.i = 0.07) and then incubated at 37 °C during 30 min. After centrifugation (10,000 *g* for 5 min), the titres of unadsorbed free phages in the supernatants were assayed as indicated, and the results expressed as percentages of the adsorption. A cell culture subjected to a similar treatment but without chloramphenicol was used as adsorption control.

2.6. Statistical analysis

All data were analyzed using the Statgraphics™ Plus software (v 3.0, Statistical Graphics Corp.). Experiments were replicated three times. Means were compared using the one-way ANOVA procedure followed by Duncan's multiple range tests at $P<0.05$.

3. Results

3.1. One-step growth curves

Multiplication phage parameters were calculated from one-step growth curves (Fig. 1). A latent period of 30 min and a burst time of 105 min were exhibited for both phages. Phage B2 showed the highest burst size value (83 PFU per infection centre) while 60 PFU per infection centre was achieved for phage B1. Burst size values for phages FAGK1 and FAGK2 on *L. plantarum* ATCC 8014 were 10.8 and 12 PFU per infected cell, respectively (De Antoni et al., 2009).

3.2. Influence of pH on phage infectivity and strain viability

The highest infectivity (>80%) for all phages was observed in the pH range from 5 to 11 (Fig. 2). The viral suspensions were inactivated completely after 30 min at pH 2. The lowest infectivity at pH 3 was exhibited by phages B1 and B2. Excepting at pH 4, the phage B1 showed lower resistance than phages B2, FAGK1 and FAGK2 in the range of pH assayed.

The viability of *L. plantarum* ATCC 8014 was not influenced by the pH, since cell counts remained constant during 30 min within the range of pH evaluated (data not shown).

3.3. Influence of temperature on phage infectivity and strain viability

The infectivity of phage suspensions was maintained even at 50 °C. More than 95% of initial phage particles remained viable after 30 min of treatment in MRS broth (data not shown). Likewise, the viability of *L. plantarum* ATCC 8014 was not influenced when cell suspensions were subjected to temperatures ranging from 0 to 50 °C during 30 min (data not shown).

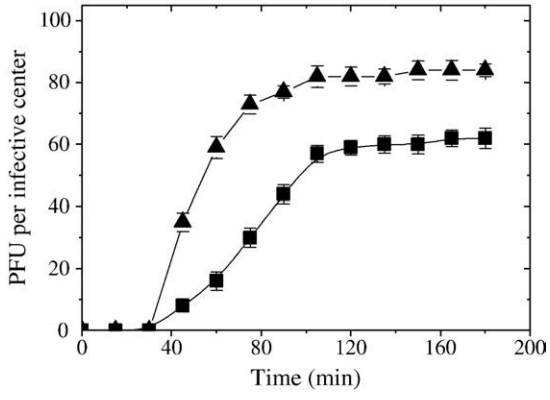

Fig. 1. One-step growth curves of phages B1 (■) and B2 (▲) on the host strain *L. plantarum* ATCC 8014. Values are the mean of three determinations.

3.4. Adsorption studies

3.4.1. Influence of divalent cations

Cell lysis in MRS broth was achieved even without the addition of Ca^{2+} or Mg^{2+} for all phages, but it was faster in the presence of Ca^{2+}. Regarding lysis plaques, they were very clear in the presence of Ca^{2+} whereas in the presence of Mg^{2+} the plaques were diffuse and smaller, and the titres fall between 0.4 and 0.7 $\log_{10}$ orders. In contrast, in the absence of cations, the lysis plaques were extremely diffuse or failed to appear (data not shown).

In general, calcium ions did not have a significant ($P>0.05$) influence on phage adsorption kinetics (Fig. 3). After 15 min of incubation, 99% of phage particles were adsorbed in MRS broth with and without Ca^{2+}. The maximum adsorption rates (>99.6%) were achieved at 45 min of incubation.

3.4.2. Influence of temperature

The effect of temperature on phage adsorption is shown in Fig. 4. More than 82% of phage particles were absorbed after 30 min in all the conditions studied, except at 50 °C, which caused an increase in the titres of unadsorbed phages particles (adsorption values smaller than 39%). The maximum adsorption values (more than 99%) were achieved at temperatures ranging from 30 to 42 °C for all phages studied.

3.4.3. Influence of pH

Fig. 5 showed that the highest adsorption values on *L. plantarum* ATCC 8014 were obtained at pH ranging from 5 to 7 (99.9%) for all phages. On the contrary, at pH 9, the extent of adsorption ranged from 23 (phage B1) to 45% (phage B2) whereas at pH 10, about 20% (phages B2 and FAGK1) and 5% (phages B1 and FAGK2) of phage particles were adsorbed after 30 min of incubation.

3.4.4. Influence of the thermal treatment on cells

When cell suspensions were inactivated by heat, adsorption kinetics of the four phages were significantly different ($P<0.05$) with respect to those obtained on viable cells. At 45 min, adsorption on nonviable cells ranged between 77 and 91%, depending on phage, while adsorption on viable cells reached 99% for all phages after 45 min (Fig. 6).

3.4.5. Influence of cell protein-synthesis inhibitors

The chloramphenicol concentration used in the experience was 20 μg/ml; in that condition, the inhibition of protein-synthesis on cells was maintained after the removal of chloramphenicol. Therefore, phage adsorption process was carried out in the absence of antibiotic. Treatment with chloramphenicol on cells did not influence the adsorption values ($P>0.05$) when they were compared with those attained with untreated cells. After 30 min of incubation, from 96 to 99% of initial phage particles were adsorbed on cells with and without chloramphenicol treatment (data not shown).

4. Discussion

Burst size values of phages B1 and B2 (60 and 83 PFU per infected cell) resulted significantly higher than those reported by De Antoni et al. (2009) for *L. plantarum* phages isolated from kefir grains (FAGK1 and FAGK2) (10.8–12 PFU per infected cell). Nes et al. (1988) reported a lower burst size value (12–14 phages per infection centre) for phage B2, but unlike our study, the experiments were carried out at 30 °C. Similarly, phage ϕJL-1, isolated from cucumber fermentation, showed

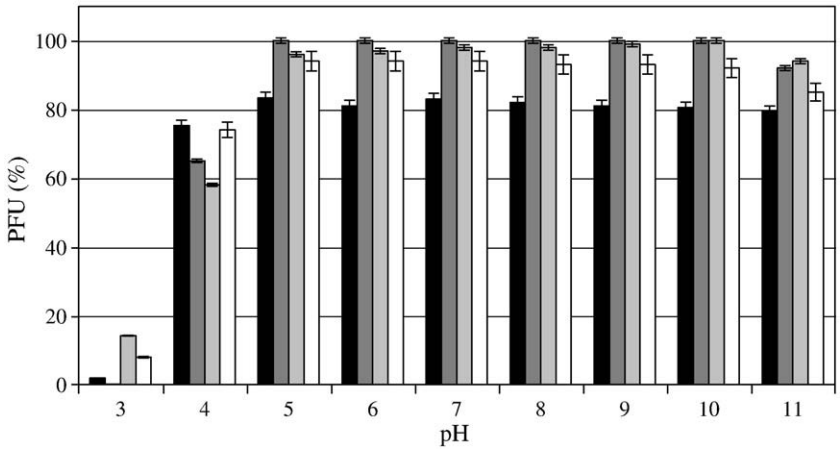

Fig. 2. Effect of pH on infectivity (after 30 min at 37 °C, in MRS broth) of phages B1 (■), B2 (■), FAGK1 (■) and FAGK2 (□). Values are the mean of three determinations.

low burst size (22 PFU per infected cell) at 30 °C (Lu et al., 2003). However, a value unusually high of 200 PFU per infected cell was reported for phage fri (Trevors et al., 1983) also at 30 °C. The influence of the incubation temperature on the propagation of *L. plantarum* phages was previously reported by Caso et al. (1995), being more effective at 30 or 37 °C depending on the phage and host combinations. The high thermal, chemical and photocatalytic resistance reported for phages B1, B2, FAGK1 and FAGK2 (Briggiler Marcó, et al., 2009) restrict the strategies that could be applied to diminish their infections. Therefore, the ability of *L. plantarum* to grow in a wide range of temperatures (even at 10–15 °C) (Corsetti and Gobetti, 2003) might become an advantage when designing new phage control strategies in fermentation processes involving this bacterium.

In this study, it was demonstrated that neither Ca^{2+} nor Mg^{2+} were indispensable either to adsorption or to complete the lytic cycle of the four phages studied. Unfortunately, the use of phage inhibitory media (PIM) that contain chelating agents (phosphates and citrates) might not be useful to control those bacteriophages since they do not require cations to complete their lytic cycle (Suárez et al., 2002). For other lactobacilli phages, the requirement of calcium either to adsorption or lysis was variable (Binetti et al., 2002; Capra et al., 2006; Quiberoni and Reinheimer 1998; Quiberoni et al., 2004; Suárez et al., 2008).

Regardless of the isolation source (corn silage, anaerobic sewage sludge and kefir grains) (De Antoni et al., 2009; Douglas and Wolin, 1971) the four phages exhibited a high infectivity even at pH 4 and pH 11, similarly to phage fri (Trevors et al., 1983) isolated from a meat starter culture. The four phages adsorbed efficiently on *L. plantarum* ATCC 8014 cells in the pH range from 5 to 8, and even at 0 °C, though the optimal adsorption conditions were pH ranging from 5 to 7 and incubation temperatures from 30 to 42 °C. Watanabe et al. (1993) have demonstrated that the adsorption of phage PL-1 (*Lactobacillus casei*) at 0 °C was almost identical with that at 37 °C. Nevertheless, the number of ghost particles did not increase during the incubation at 0 °C, suggesting that actively functioning bacteria are required for the injection of DNA from this phage. They also showed that, although in this process the initial decrease in the amount of free phages (reversible adsorption) was not affected by lowering the reaction temperature, the formation of irreversibly bound phage-cell complexes was inhibited at lower temperatures.

In this work, the ability of thermally treated cells to adsorb phages was also evaluated. For all the systems studied, the adsorption rates on heat-treated cells were lower, but still considerable in comparison with viable cells. Similar behavior was exhibited by phages specific of *L. casei* (Capra et al., 2006) and *Lactococcus lactis* (Suárez et al., 2008). The diminished phage adsorption observed on thermally treated cells could be linked to a disorganization of phage receptor sites and/or to the physiological cellular state. Taking into account this hypothesis, the phage adsorption on the host strain subjected to the protein-synthesis inhibition was tested. Immediately after the antibiotic treatment, the non-proliferating cells, which are expected to maintain almost the original ATP content (Hahn et al., 1955; Watanabe et al., 1991), were able to adsorb the phages. Although it would not be expected that phage binding is an energy-dependent process, several authors have investigated this issue, by obtaining dissimilar results. When several energy inhibitors were used, it was demonstrated that

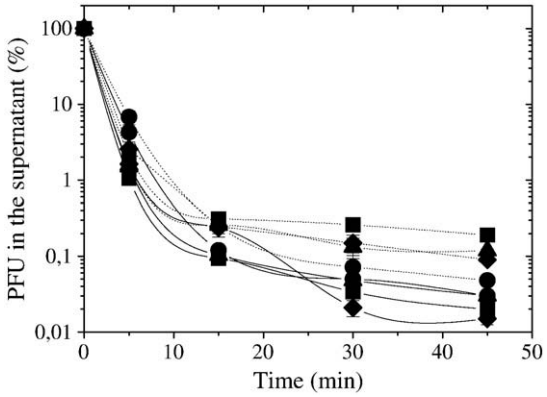

Fig. 3. Influence of calcium ions on the adsorption kinetics (at 37 °C) of phages B1 (■), B2 (▲), FAGK1 (♦) and FAGK2 (●) on the strain *L. plantarum* ATCC 8014, in MRS broth with (—) and without (---) Ca^{2+} 10 mmol l^{-1}. Values are the mean of three determinations.

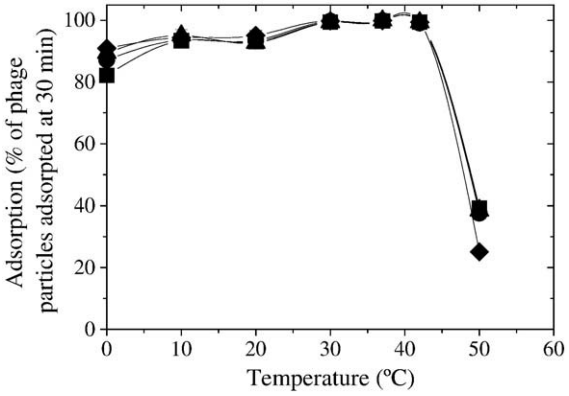

Fig. 4. Influence of temperature on the adsorption (after 30 min, in MRS-Ca broth) of phages B1 (■), B2 (▲), FAGK1 (♦) and FAGK2 (●) on the strain *L. plantarum* ATCC 8014. Values are the mean of three determinations.

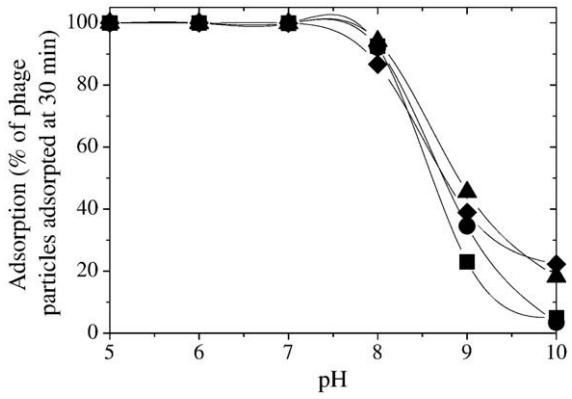

Fig. 5. Influence of pH on the adsorption (after 30 min at 37 °C, in MRS-Ca broth) of phages B1 (■), B2 (▲), FAGK1 (♦) and FAGK2 (●) on the strain *L. plantarum* ATCC 8014. Values are the mean of three determinations.

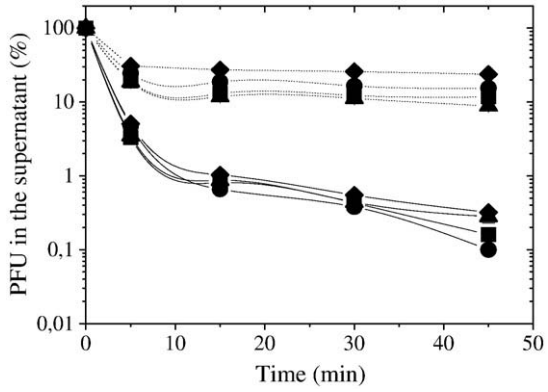

Fig. 6. Influence of cell-thermal treatment on the adsorption kinetics (at 37 °C) of phages B1 (■), B2 (▲), FAGK1 (♦) and FAGK2 (●), in MRS-Ca broth on viable (—) and nonviable (---) cells of the strain *L. plantarum* ATCC 8014. Values are the mean of three determinations.

adsorption of phage fd to *Escherichia coli* male cells was not energy-dependent (Yamamoto et al., 1980). Phage PL-1 was adsorbed to cells of *L. casei* which had been killed by keeping or by exposing to UV light (Watanabe et al., 1993). However, adsorption of phage PRD1 to *Salmonella typhimurium* DS88 strain was drastically reduced when NaN_3, NaF, and arsenate (these inhibitors considerably decreased the intracellular concentration of ATP) were used (Daugelavicius et al., 1997). Taking into account all these data and the results obtained in the present study, further researches would be needed to discern if the decreased adsorption obtained on the thermally treated cells is linked to the disorganization of phage receptor sites or to the complete lack of bacterial cell energy.

5. Conclusion

Knowledge on factors that influence the binding of phages to the sensitive cells allows developing strategies to enhance the performance of the strains in industrial environments. In spite of the relevance of *L. plantarum* in food fermentation processes and the possibility of use as probiotic starter, very limited information is available on the biology of its bacteriophages. Data obtained in this work have demonstrated a scarce influence of environmental and physiological factors on the adsorption of four phages on *L. plantarum* ATCC 8014. This process was only partially affected by a few conditions: thermally killed host cells, an incubation temperature of 50 °C and pH values of 9 and 10. Nevertheless, and unfortunately, those conditions are not commonly applied during fermented food manufacturing, thus limiting greatly the application of strategies currently available to reduce phage infections in industrial environments.

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