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Virulence of *Leuconostoc* phages: Influence of stress conditions associated to dairy processes on their host-phage interactions

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ABSTRACT

In this work, we assessed the impact of technological cell stress conditions, commonly present in industrial dairy processes, on the host strain-phage interactions in *Leuconostoc*. Adsorption and burst size of LDG (*Leuconostoc pseudomesenteroides*) and Ln-9 (*Leuconostoc mesenteroides*) phages were evaluated under the following conditions: i) MRS broth, 30 °C; ii) MRS broth at pH 5.5, 30 °C (acidic stress); iii) MRS broth added of NaCl at 4% w/v, 30 °C (osmotic stress) and iv) MRS broth, 10 °C (cold stress). Experiences were performed with the host strains growing both in MRS broth (30 °C) and under stress conditions. On the other hand, the effect of diverse levels of NaCl, KCl, saccharose and glucose on the adsorption for LDG phage was evaluated. Acidic and cold conditions did not significantly affect the adsorption rates for any phage. However, adsorption rate of phage LDG was highly reduced under osmotic stress (NaCl), except when the host strain previously grew in presence of the salt. LDG phage adsorption was not modified by addition of saccharides, but it drastically decreased in presence of salts. Acidic conditions did not affect the burst size for LDG phage, but Ln-9 phage diminished this parameter (61 phage particles/infected cell). Latency time showed a lengthening of 10 min for both phages, while the burst time remained unaltered for LDG and it was delayed 10 min for Ln-9. LDG phage did not propagate under osmotic conditions, but Ln-9 phage released phage particles with an important increase of its latent period and burst time. No phage particles were released within 90 min after the adsorption step under cold stress. This is the first report about this subject. Under certain conditions of technological stress (osmotic and cold) associated to dairy processes, phage infections on the two systems studied in this work could be delayed/inhibited.

1. Introduction

Leuconostoc is a genus of heterofermentative lactic acid bacteria (LAB) that produce lactic acid, ethanol, acetate and CO_2. Strains belonging to this genus are used as primary starter in butter and cream fermentation because of their capacity to produce diacetyl, acetoin and 2, 3-butanediol. Moreover, *Leuconostoc* is commonly used in combination with *Lactococcus* (mixed starters) in traditional cheeses, contributing to their distinctive flavor (D'Angelo et al., 2017). On the other side, *Leuconostoc* is naturally present in raw milk as non-starter LAB (NSLAB) and gives, together with other NSLAB, special characteristics to cheeses manufactured with raw milk (Montel et al., 2014).

Virulent phages are recognized to adversely affect dairy fermentations by inhibiting the growth of lactic acid bacteria due to the cellular lysis (Mahony and van Sinderen, 2014) and accordingly, dairy phages have been studied for decades (Samson and Moineau, 2013). Phages infecting LAB used as acidifying starters (*Lactococcus lactis, Streptococcus thermophilus* and *Lactobacillus sp.*) have been studied in detail (Mahony et al., 2012; Quiberoni et al., 2010; Villion and Moineau, 2009). *Leuconostoc* strains participate mainly in flavor development rather than in lactic acid production. In view of acidification defects can be detect much easier and earlier than aroma/flavor failures, the study of their phages and interactions with the host strains has been postponed. However, the presence of virulent *Leuconostoc* phages can negatively affect the quality and aroma of the final fermented dairy product (Ali et al., 2013). In this sense, the levels of *Leuconostoc* phages found in dairy products can vary between 10^2 and 10^7 plaque-forming units (pfu) per gram or per ml (Atamer et al., 2011).

On the other hand, LAB used as starter cultures are exposed to many adverse factors (stress factors) during their preparation and storage as well as during the fermented product manufacture. The stress factors are diverse and include pH variation (acidity or alkalinity), temperature (heat and cold), oxidative and osmotic changes, among others (Ferrando et al., 2015; Parente et al., 2010; Van de Guchte et al., 2002; Zotta et al., 2008). All these factors can affect the viability and technological performance of strains used as starters and, in this sense,

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studies on strains belonging to *Leuconostoc* genus have been recently reported (Cicotello et al., 2018; D'Angelo et al., 2017). Nevertheless, influence of these adverse conditions on the interactions of the lytic phages with host strains remains unexplored.

The aim of this study was mainly to increase the knowledge about virulence of *Leuconostoc* phages under stress conditions in order to know if they could affect phage propagation during technological processes.

2. Materials and methods

2.1. Bacterial host strains and bacteriophages

Phages LDG (GenBank:KX555527) and Ln-9 (GenBank:KM262192) and their host strains *Leuconostoc pseudomesenteroides* R707 (*Ln* R707) and *Leuconostoc mesenteroides* 79-1 (*Ln* 79-1), respectively, were used in this work (Pujato et al., 2015, 2017). The strains were maintained in MRS broth at 4 °C for the routine work and periodically cultured in fresh MRS broth (Biokar, Beauvais, France) to keep their viability. Phage stocks and phage enumerations (PFU/ml) were performed according to Pujato et al. (2015).

2.2. Stress conditions

The stressors studied in this work were osmotic (MRS broth added of NaCl at 4% *w/v*, 30 °C), acidic (MRS broth at pH 5.5, 30 °C) and cold (MRS broth, 10 °C) factors. MRS broth (30 °C) was used as control condition (Cicotello et al., 2018). These conditions were used for growth of strains as well as for carry out both the adsorption tests and one-step growth curves.

2.3. Adsorption assays

Adsorption assays were performed under stress conditions (Cicotello et al., 2018) with the host strains growing under control and stress conditions. Host strains inoculated (at 2% for control and cold stress and at 4% for acidic and osmotic stress stress) were grown (at 30 or 10 °C) until an optical density ($OD_{560\ nm}$) between 0.5 and 0.6. Then, they were centrifuged (10,000 rpm – 5 min), and pellets suspended in half the initial volume in each studied medium. Phage adsorption kinetics were performed for 20 min at 10 °C or 30 °C, using the phages with m.o.i. (multiplicity of infection) of approximately 1 and taking samples at intervals of 5 min, according to Séchaud et al. (1989).

On the other hand, because of the decrease observed in the adsorption rate of the phage LDG on the strain *Ln* R707 in presence of NaCl (4%, w/v), additional experiments were carried out. Thus, influence of osmotic pressure on phage-strain interaction was investigated. In this sense, adsorption rates in presence of salts (NaCl and KCl) or saccharides (glucose and saccharose) when strain grew in control conditions were performed. The concentrations selected were those required to achieve the same osmotic pressure as concentrations of 1, 2, 3 and 4% (w/v) of NaCl, as follows: i) 0.17 M, 0.34 M, 0.51 M and 0.68 M for salts, and ii) 0.34 M, 0.68 M, 1.02 M and 1.36 M for saccharides. Adsorption rates (at 30 °C) were determined at 20 min of the experience.

2.4. One-step growth curves

To test the influence of stress conditions on phage propagation, the host strains were grown in MRS broth (30 °C, control) and subjected to adsorption test for 10 min (MRS broth, 30 °C). Then cells were suspended in each stress condition and incubated at the respective temperature (30 or 10 °C) during 90 min. Phage particles were counted at intervals of 10 min. Latent period, burst time and burst size were calculated from one-step growth curves (Chow et al., 1988).

To deepen the knowledge about influence of the salt in the one-step growth curve of the LDG phage, additional studies were performed. Thus, the host strain *Ln* R707 was grown in MRS broth added of NaCl (4% w/v) and subjected to adsorption in MRS broth or MRS broth added of NaCl (4% w/v), for 20 min at 30 °C. After adsorption, the one-step growth curves were performed in MRS without and with addition of NaCl (4% w/v).

2.5. Statistical analysis

All assays were carried out in triplicate, in independent trials. Data were analyzed using the one-way ANOVA procedure of SPSS (SPSS Inc., Chicago, IL), assuming a confidence level of 95%. Tukey's and Dunnett's tests were used to identify significant differences in media values among groups.

3. Results

3.1. Adsorption assays

Phages LDG and Ln-9 showed different behavior in the adsorption experiences performed under diverse stress conditions. The LDG phage evidenced similar adsorption rates for assays carried out under control conditions when bacterial cells were grown either in MRS (control) or under the three stress conditions (acidic, cold, osmotic) (Fig. 1A) ($p > 0.05$ at 20 min of experience). Similarly, adsorption kinetics of this phage were not significantly affected when the adsorption assays were studied under acidic or cold stress conditions (Fig. 1, B and C). In this sense, the adsorption values obtained for all these conditions were higher than 98% at 5 min, even when the host strain was grown in MRS (control) or under stress conditions studied. However, when the adsorption was performed in presence of NaCl (4% w/v), the adsorption values at 20 min were 0%, 23% and 30% for the host strain grown under control, acidic and cold conditions, respectively (Fig. 1 D). These results did not show significant differences among them when one-way

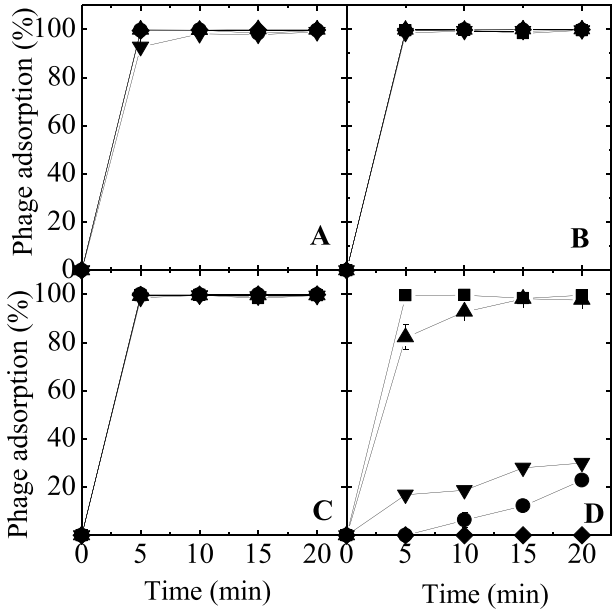

Fig. 1. Adsorption of phage LDG on *Leuconostoc pseudomesenteroides* R707 under control and stress conditions. Adsorption assays were performed for 20 min in: A) MRS broth (30 °C, control); B) acidic stress (MRS broth at pH 5.5, 30 °C); C) cold stress (MRS broth, 10 °C) and D) osmotic stress (MRS broth added of NaCl at 4% w/v, 30 °C). Bacterial cells were previously grown in MRS broth (30 °C, ●), acidic stress (30 °C, ♦), osmotic stress (30 °C, ▲) and cold stress (10 °C, ▼). Control curve corresponds to adsorption kinetic in MRS broth (30 °C) with the bacterial strain previously grown in MRS broth (30 °C) (■).

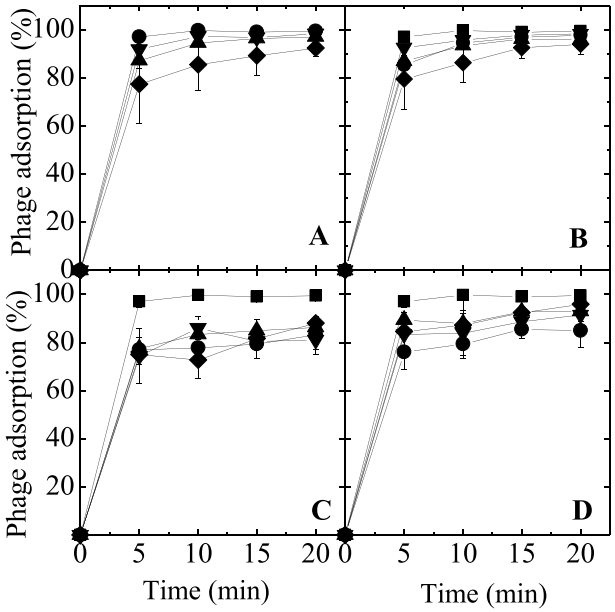

Fig. 2. Adsorption of phage Ln-9 on *Leuconostoc mesenteroides* 79-1 under control and stress conditions. Adsorption assays were performed for 20 min in: A) MRS broth (30 °C, control); B) acidic stress (MRS broth at pH 5.5, 30 °C); C) cold stress (MRS broth, 10 °C) and D) osmotic stress (MRS broth added of NaCl 4% w/v, 30 °C). Bacterial cells were previously grown in MRS broth (30 °C, ●), acidic stress (30 °C, ♦), osmotic stress (30 °C, ▲) and cold stress (10 °C, ▼). Control curve corresponds to adsorption kinetic in MRS broth (30 °C) with the bacterial strain previously grown in MRS broth (30 °C) (■).

ANOVA (Tukey's test, $\alpha = 0.05$) was applied ($p = 0.158$). Moreover, when the host strain was grown in MRS broth added of the salt, similar adsorption rates to the control ones were obtained, reaching values of 98% at 15 min in both cases (Fig. 1D).

The results obtained for the Ln-9 phage showed, in general, a decrease of the adsorption values for all stress conditions in comparison to the control ones, but they varied in a narrow range depending on the condition studied (Fig. 2). Statistical analysis for each adsorption treatment and for the host strains grown under control and stress conditions at 20 min was performed using one-way ANOVA ($\alpha = 0.05$), applying the Dunnet's test *post hoc*. The values obtained for adsorption and cell growth performed in MRS broth were used as control. Dunnett's test showed significant differences between the control and some cell growth and/or adsorption conditions as follows: i) adsorption under control conditions/host strain growing in acidic stress condition ($p = 0.043$), ii) adsorption in osmotic stress condition/host strain growing under control conditions ($p = 0.021$), iii) adsorption in cold stress condition/host strain growing under control conditions ($p = 0.049$) and iv) adsorption in cold stress condition/host strain growing in cold stress condition ($p = 0.032$). Nevertheless, high values of adsorption were obtained for all stress conditions for phage Ln-9, which ranged between 81% and 98% at 20 min of experience.

Results about the osmotic pressure effect on adsorption rates in presence of salts and saccharides are shown in Fig. 3. For the lowest concentration of salts and saccharides (0.17 M and 0.34 M, respectively), no decrease in the adsorption rates related to the control was observed (data not shown). However, a significant decrease of adsorption values was found for both salts with the increase of the osmotic pressure, being the values at 20 min of experience 98.9%, 70.2%, 30.7% and 20.4%, for ascending molar concentrations of the salts. No significant differences (one-way ANOVA test, Tukey's *post hoc*, $\alpha = 0.05$) were found for adsorption rates between the presence of NaCl or KCl for each level of osmotic pressure. On the contrary, the adsorption rates were not affected with the increase of the osmotic pressure produced by the saccharides used.

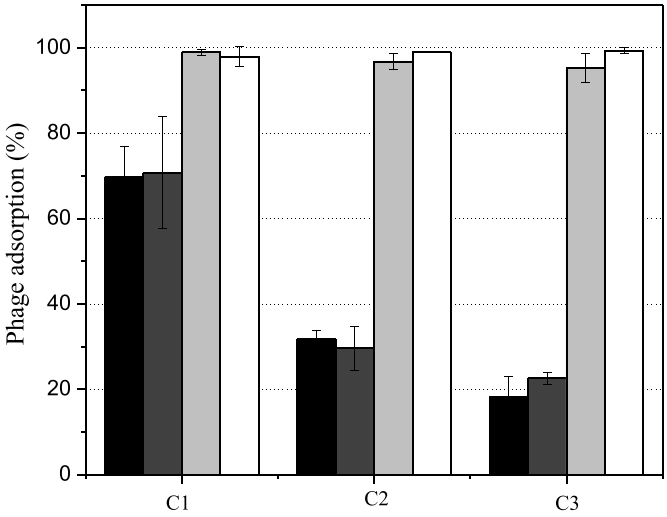

Fig. 3. Influence of the osmotic pressure on the adsorption rates (20 min) of phage LDG on *Leuconostoc pseudomesenteroides* R707. C1, C2 and C3 correspond to concentrations of KCl (■), NaCl (■), saccharose (■) and glucose (□) equivalent to an osmotic pressure of 2, 3 and 4% w/v of NaCl.

3.2. One-step growth curves

One-step growth curves for both phages performed in MRS broth (control) and under stress conditions are shown in Fig. 4. For control, the LDG phage showed higher burst size than Ln-9, with values of 149 ± 15 and 102 ± 11, respectively. The latent periods and burst times were of 30 min and 60 min for the LDG phage and, 20 min and 40 min for the Ln-9 phage, respectively (Fig. 4A and B). The behavior of both phages was variable when their one-step growth curves were studied under stress conditions, except for cold stress factor. For this condition, none of the phages were able to release phage particles within 90 min after the adsorption step (Fig. 4A and B). Similarly, the LDG phage did not propagate under osmotic conditions (Fig. 4A). On the contrary, the Ln-9 phage released phage particles under osmotic stress conditions with a substantial increase of its latent period (70 min) and burst time (90 min), but maintained its burst size (102 ± 2 phage particles/infected cell) (Fig. 4B). Regarding the acidic stress conditions, the behavior of both phages was quite different. The one-step growth curve for the LDG phage was similar to that obtained for control, with a slight lengthening (10 min) in the latency time (Fig. 4A). However, the Ln-9 phage showed a diminished burst size value (61 ± 3 phage particles/infected cell), 10 min of lengthening of the latency time and burst time of 50 min (Fig. 4B).

The results obtained for the LDG phage in presence of NaCl were interesting. When the host strain *Ln* R707 was grown under osmotic stress, the presence of NaCl during the burst size assay also inhibited the release of phage progeny (Fig. 5) independently of the medium in which adsorption assays were performed (MRS broth in absence or presence of NaCl) (data not shown). However, phage propagation in MRS broth (bacterial cells previously grown in presence of NaCl) was observed and the burst size, latent period and burst time were coincident with the corresponding parameters obtained for control (Fig. 5).

4. Discussion

The rate of adsorption is a characteristic of each phage-host system and depends not only on the nature of the receptor, but also on its location, accessibility, spatial arrangement, quantity and density at various sites of the cell wall. In addition to these conditions, which are specifically related to the phage receptor involved, the adsorption rate

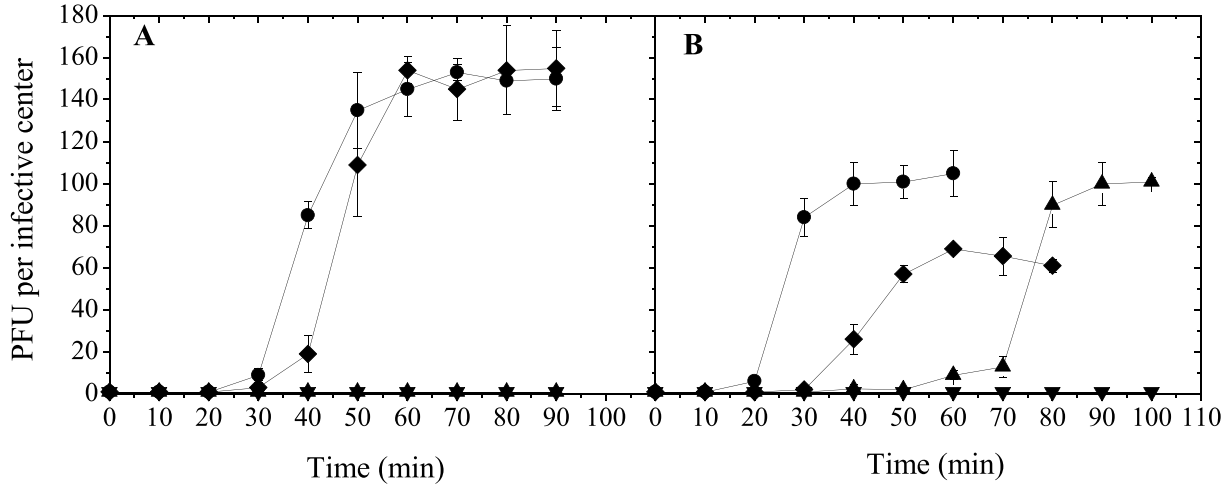

Fig. 4. One-step growth curves for phages LDG (A) and Ln-9 (B), performed under control (MRS broth, 30 °C, ●) and conditions of acidic (MRS broth at pH 5.5, 30 °C, ♦), osmotic (MRS broth added of NaCl at 4% w/v, 30 °C, ▲) and cold (MRS broth, 10 °C, ▼) stress.

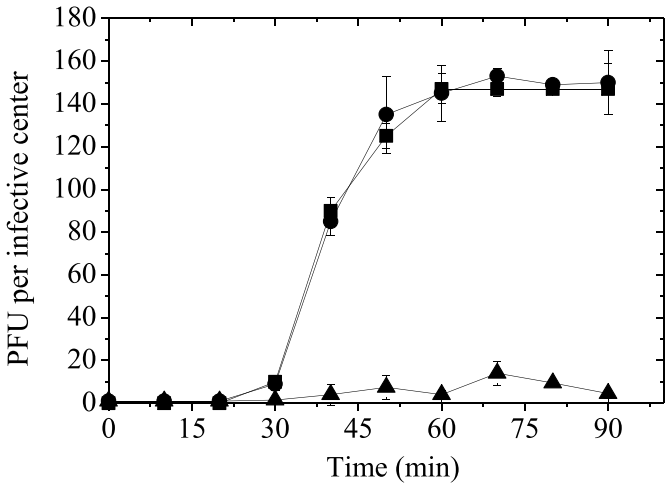

Fig. 5. One-step growth curves for the phage LDG performed under control (MRS broth, 30 °C, ●, ■) and osmotic stress (MRS broth added of NaCl at 4% w/v, 30 °C, ▲) conditions. The host strain *Leuconostoc pseudomesenteroides* R707 was grown in MRS broth (●) and under osmotic stress (■,▲). The adsorption step was performed in MRS broth.

is also controlled by diverse physicochemical factors, such as pH, temperature, ion presence (divalent cations as Ca^{2+} and/or Mg^{2+}) and physiological host state (Rakhuba et al., 2010). The results obtained in this study agree with those reported for phages infective to lactic acid bacteria in assays performed on cells grown in MRS or Elliker broth. None or slight effect of an acidic environment (pH 5.5) in the adsorption was reported for diverse LABs, such as *Lactobacillus casei/paracasei* (Capra et al., 2006a, 2006b), *Lactobacillus plantarum* (Briggiler Marcó et al., 2010; Trevors et al., 1983), *Lactobacillus delbrueckii* (Trucco et al., 2011), *Lactococcus lactis* (Suárez et al., 2008), *Streptococcus thermophilus* (Binetti et al., 2002) and *Leuconostoc* spp.(Pujato et al., 2015). On the other hand, previous studies at 10 °C reported a decrease of adsorption values until 30% (respect to the control) depending on the genera/species considered (Binetti et al., 2002; Briggiler Marcó et al., 2010; Capra et al., 2006a, 2006b; Pujato et al., 2015; Quiberoni et al., 2004; Quiberoni and Reinheimer, 1998; Suárez et al., 2008; Trucco et al., 2011; Watanabe et al., 1993). The temperature can modify the formation capacity of the carbohydrate - TSP (tail spike proteins) complexes that lead the adsorption step, depending on the nature of both TSPs and the cell wall receptors. In carbohydrate binding sites, energetic contributions for the complex formation are provided by hydrogen bond formation and desolvation of the partners, and from van der Waals interactions (Broeker et al., 2013). It is expected that temperatures lower than the optimal one could produce a decrease in the adsorption rate, since low temperatures cause higher protein solvation, which do not facilitate free interaction between receptor - anti-receptor. On the other hand, and even when the interaction energies involved are low, a decrease in temperature could also be unfavorable to the formation and stability of the bonds. In this context, also the minor Brownian movement of phages and host bacteria could difficult the adsorption because of this energetic barrier (Zemb et al., 2013).

The adsorption data obtained in presence of NaCl were, undoubtedly, influenced by the ionic strength of the medium. For the LDG phage, adsorption rates for the host strain growing under control conditions (MRS broth, 30 °C) as well as under cold and acidic stress were low, while Ln-9 phage was not affected by this factor. To our knowledge, there are no data about influence of ionic strength on the adsorption step for LAB phages and the studies have been mainly focused on phages of pathogenic bacteria. Zemb et al. (2013) found that NaCl concentrations until 100 mM favored the adsorption of phage T2 on *Escherichia coli* XL1. Similar results were informed for adsorption rates of phage P100 on *Listeria monocytogenes* reference strain ATCC BAA-679, which were high even at NaCl concentrations of 2 M (Fister et al., 2016). The interaction between virus and cell surfaces involves electrical forces, and thus changes in environmental ion concentration could modify the binding capacity (Armanious et al., 2016). Preliminary studies (Pujato, 2017) evidenced carbohydrates and possibly polysaccharides acting as phage receptors for both phages used in this study. Hypothesis based on differences in carbohydrates composition and/or their disposition on the cell surface could explain the diverse results observed for the LDG and Ln-9 phages. On the other hand, thermodynamics of oligosaccharide associations show that desolvation is an important driving force for complex formation oligosaccharide - protein and it is directly related to the ionic strength. Broeker et al. (2013) demonstrated that exchange of a single amino acid in the TSP of the bacteriophage HK620 results in thousand-fold increase of its oligosaccharide affinity, because of a hydrophobic binding pocket is shielded from a high-affinity-providing scaffold. Therefore, small differences in the TSP composition of phages could modify their solvation capacity, which could also change with the ionic strength. Moreover, the growth of *Ln* R707 host strain in a medium with NaCl led to a normal adsorption. This change in the behavior of the system could be due to modifications in the receptor disposition on the cell wall because of the need of adaptation of host strain during its growth in adverse conditions.

In this study, osmotic pressure influence on the adsorption rate of phages was also evaluated by using NaCl, KCl, saccharose and glucose. Normal adsorption was observed when osmotic pressure was produced by saccharides instead of ionic compounds. Besides, KCl and NaCl produced similar inhibition when values obtained at the same molar concentration were compared. These results demonstrated that osmotic pressure, at least until the levels evaluated, did not influence the adsorption rates and confirmed that decreased adsorption was due to ionic strength.

Information related to ability of phages to propagate on bacterial cells under stress conditions is truly scarce (Briggiler Marcó et al., 2015; Zaburlin et al., 2017). Some authors have reported that, when the physiological state of the bacterial cell is altered, changes in the host cell's susceptibility to phage infection and/or decrease in the phage particles productivity can be observed (Denes and Wiedmann, 2014; Schrader et al., 1997). Due to the fact that the cell biosynthetic machinery is slowed down at 10 °C, longer time period could be necessary to observe the release of phage particles. Some studies showed the effect of temperature (20–40 °C) on one-step growth curves for infective phages of LAB, including the LDG phage (Müller-Merbach et al., 2007; Murata, 1971; Zaburlin et al., 2017). These authors found that both latent period and burst time increased with temperature decrease (below optimal temperature). The low temperatures could have influenced on injection step of phages. In this sense, DNA injection of phage PL-1 (*Lactobacillus casei*) was inhibited at 0 °C since the presence of particles with empty heads (free of DNA) was not observed by electronic microscopy (Watanabe et al., 1993). In particular, authors suggested that penetration of phage genomes into the cells would require cells containing high energy levels (Watanabe and Takesue, 1973). Also, a presumptive enzymatic reaction using energy would take part in the process of DNA injection (Watanabe et al., 1987). Therefore, if the bacterial cells are not actively growing (as it is the case of cells under stress conditions), this stage of the lytic cycle could be inhibited / delayed, as it was demonstrated for *Lb. casei* phages (Watanabe et al., 1993; Watanabe et al., 1991). However, further researches would be necessary in order to know the metabolic state of our *Leuconostoc* strains when they are subjected to stress conditions.

The release of phage particles for the LDG phage within 90 min of experience for one-step growth curves carried out under osmotic stress was not observed, even when the host cells grew in presence of NaCl. Nevertheless, when the host strain grew under osmotic stress and the one-step growth curve was performed in absence of salt, phage particles were released in a similar way to the control. Evidently, the presence of salt in the medium used to study the parameters of phage multiplication inhibited any step (subsequent to phage adsorption) in the phage lytic cycle, and thus the release of phage particles. In this sense, Evilevitch et al. (2003) reported that DNA injection was inhibited by osmotic pressure (produced by polyethylene glycol) for phage λ (*Escherichia coli*) in *in vitro* studies. In view of phage adsorption and DNA injection would not be independent steps (Rakhuba et al., 2010), penetration of nucleic acid into the cell could also be influenced by alterations in the binding ability between phages and host cell wall, produced by the presence of the salt. As well, Fister et al. (2016) found that the replication of phage P100 (*Listeria monocytogenes*) was influenced by increasing concentrations of NaCl, (0.1–2 M), but the authors related the growth rate of bacterial host to this behavior. On the other hand, phage Ln-9 evidenced a longer latent period but the same burst size value under osmotic stress. Possibly, because of cellular metabolism is slower, additional time is required for production of the new phage particles, as it was mentioned above.

Regarding the ability of phages to propagate under host acidic stress (pH 5.5), the LDG phage showed the same behavior as control with a slight increase in the burst time. However, dissimilar behavior was observed for the Ln-9 phage, which evidenced a longer latent period and lower burst size that those found for control. Similar results were reported for phages infective to lactic acid bacteria under diverse stress conditions (Zaburlin et al., 2017). These authors suggest that the reduced burst size would be linked to a slower cellular metabolism. As it was previously discussed, injection of phage DNA would require high levels of energy (Watanabe and Takesue, 1973). It is likely that, under acidic stress, this stage is not as efficient as under control conditions. Thus, a lower number of phage particles could be produced. In addition, Watanabe and Takesue (1972) reported that the steps of the lytic cycle subsequent to the adsorption might be influenced by pH. In this sense, pH values lower to 6 affected the formation of lysis plaques for phage PL-1 (*Lb. casei*) in spite of the adsorption step not being influenced (Watanabe and Takesue, 1972).

5. Conclusions

The results obtained in this study, although preliminary, could be taken as a starting point for some considerations. It was interesting to observe that some conditions of technological stress, usually present in cheese manufacture where *Leuconostoc* is used as starter, could inhibit or delay the propagation of some phages of this genus. Specifically, it was observed that the salt concentration and the low temperature were able to delay or stop the release of phage progeny for the phages tested, independently of the growth conditions of the host cells. As far as we know, this is the first study on this subject and constitutes an approach to the real behavior of the bacteriophage-host strain systems of the genus *Leuconostoc* under some stress conditions.

Acknowledgements

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