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The protective effect of food matrices on *Listeria* lytic bacteriophage P100 application towards high pressure processing

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ABSTRACT

The application of lytic phages as biocontrol agents is emerging as a promising strategy towards elimination or reduction of foodborne pathogens in a variety of food products. This technology is particularly advantageous for minimally processed and ready-to-eat (RTE) foods. In this study, the potential use of Listex™ P100 combined with high hydrostatic pressure (HPP), to enhance the control of *Listeria monocytogenes* in food was evaluated. For that, the effect of three pressures (200, 300 or 400 MPa; 5 min, 10 °C) on phage P100 stability was tested when inoculated in six different matrices: phosphate buffered saline (PBS, pH 7.4); apple juice (pH 3.41); orange/carrot nectar (pH 3.54); UHT whole milk (pH 6.73); and, two traditional Portuguese fermented products, "Serra da Estrela" cheese (pH 5.66) and "Alheira", a meat sausage (pH 6.07). The results showed that treatment at 400 MPa reduced phage titres to below the detection level in all matrices, whereas at milder pressures the survival of the phage was matrix dependent. "Alheira", "Serra da Estrela" cheese and UHT whole milk were shown to be baroprotective matrices that support phage P100 application in HHP up to 300 MPa; however, an accentuated phage inactivation was observed in apple and orange/carrot nectar, which may be related to the acidic pH values of these matrices. The initial phage load did not affect the inactivation rate during HHP processing (300 MPa, 5 min, 10 °C) in PBS, cheese, sausage or milk matrices, and the phage titres were stable in these matrices during storage at 4 °C for 28 days for milk and 60 days for "Alheira" and "Serra da Estrela" cheese. In addition, a baroprotective effect on phage stability was observed when PBS was supplemented with reducing sugars, dextrin, casein, and tween 80. In conclusion, at mild HHP treatment, phage P100 remained active in specific matrices and seems to present potential to be added in non-thermal inactivation of *L. monocytogenes*.

1. Introduction

Bacteriophages (or phages) are viruses that specifically infect bacterial cells and, in the case of lytic phages, disrupt bacterial metabolism and eventually cause lysis of the host bacterial cell. They are harmless to humans, animals and plants and are the most abundant microorganisms on Earth (*ca.* 10^{31} particles), ubiquitous in nature and spread in soil, water and various foods. Host specificity is generally found at species level or, more rarely, genus level or class level, which makes phages potential candidates for control of target bacteria (Brüssow and Kutter, 2005). Considering the current demand in the food market for minimally processed, healthy and fresh-like foods, as well as the increasing consumer concerns towards chemical food additives, sanitizers and disinfectants, phages could be considered a natural alternative for food decontamination and preservation. The use of phages for biocontrol is considered an environmentally friendly technology, which minimizes the impact on the nutritional and organoleptic food properties and, at the same time, the endogenous and often beneficial microbiota is preserved (García et al., 2010; Sillankorva et al., 2012). Furthermore, lytic phages are a promising alternative to antibiotic/disinfectants in the control of resistant bacteria (Sulakvelidze, 2013).

The successful biocontrol of target bacteria in food systems by phages depends on several factors: (i) external conditions (e.g. pH, temperature, food matrix composition); (ii) host and phage physiological state, such as membrane and capsid integrity; (iii) both phage and host concentration; (iv) homogeneous distribution and sufficient

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diffusion ability of the phage particles (Denes and Wiedmann, 2014; Jończyk et al., 2011; Kazi and Annapure, 2016). These factors mainly affect the adsorption in the infectious process and need to be carefully addressed in a food decontamination process to ensure that phages are viable and active to be used as biocontrol agents. As reviewed by Mahony et al. (2011), if a phage is proposed for use in a particular food matrix, its ability to function in such model food systems should be evaluated.

High hydrostatic pressure (HHP) is a non-thermal emerging technology that employs elevated pressures, transmitted homogeneously and instantaneously by water, resulting in minimal changes in quality attributes (flavour, colour, texture, nutrients) and, at the same time, product safety is also achieved (Balasubramaniam and Farkas, 2008). HHP gives food processing the opportunity for cleaner ingredients and fewer additives in microbial decontamination; however, its efficacy is mostly affected by the food matrix composition (Mújica-Paz et al., 2011; San Martín et al., 2002). Effective inactivation of several pathogenic microorganisms in commercial food products is achieved within the pressure range of 400–600 MPa (San Martín et al., 2002). However, safety of HHP-treated foods can be impaired due to the occurrence of sub-lethal damages or even the induction of baro-resistance in bacterial cells after HHP treatment (Rendueles et al., 2011).

Overall, phages seem to be an interesting additional hurdle technology to be combined with HHP in the food industry, with already some encouraging results to improve biocontrol, as well as the development of more energy-efficient and environmentally friendly processes (Ahmadi et al., 2015; Oliveira et al., 2015; Tabla et al., 2012). Evaluating the synergistic effect of HHP and phages in biocontrol of *Staphylococcus aureus* in UHT whole milk, Tabla et al. (2012) demonstrated the improvement of phage performance when applied concomitantly with HHP (400 MPa, 5 min, 10 °C) compared to both hurdles used separately. Ahmadi et al. (2015) obtained complete inactivation of *Shigella flexneri* in ground beef and *Vibrio cholerae* in salmon and mussels when HHP (350 MPa, 5 min, 20 °C) was combined with specific phages.

To date, the possible combination of HHP and phages to biocontrol *Listeria monocytogenes* is still unexplored. *Listeria monocytogenes* has been recently described as a target for evaluation of the antimicrobial potential of bacteriophage control in food systems, because it is one of the most studied foodborne pathogens and also because the first two commercial phage products approved by the U.S. Food and Drug Administration (FDA) have been developed targeting *L. monocytogenes* in food products (Hagens and Loessner, 2014; Strydom and Witthuhn, 2015). Furthermore, a recent study on the safety and efficacy of Listex™ P100 (commercial antilisterial phage solution) was conducted by the European Food Safety Authority (EFSA) and a partial positive opinion for its application on ready-to-eat (RTE) foods was reported (EFSA, 2016).

The aim of this study was to evaluate the stability of phage P100 after exposure to HHP treatment in different food matrices, exploring the potential of a synergistic combination of both technologies in future applications.

2. Material and methods

2.1. Listeria monocytogenes

Listeria monocytogenes ATCC 19116 (serotype 4c) was used as phage Listex™ P100 host (Veloso, 2014). The host was daily prepared following the same procedure; briefly, stock culture was grown on tryptone soy agar (Pronadisa, Madrid, Spain) supplemented with 6 g L^{-1} of yeast extract (Lab M, Lancashire, United Kingdom) (TSAYE) and then a single colony was transferred into 10 mL of tryptic soy broth (Pronadisa) supplemented with 6 g L^{-1} of yeast extract (TSBYE) and incubated at 37 °C for 24 h. This culture was then subsequently diluted 1:100 in TSBYE and incubated in the same conditions.

2.2. Phage P100

2.2.1. Stock and work solutions

The phage Listex™ P100 (Micreos Food Safety, The Netherlands), recognized as Generally Recognized as Safe (GRAS), by the U.S FDA (U.S. FDA/CFSAN, 2007) and characterized by its wide spectrum of activity against *L. monocytogenes* strains, was used in this study. Commercial stock phage suspensions were stored at 4 °C. Daily working suspensions of phage were freshly prepared from stock solution by dilution in phosphate buffered saline (PBS; 0.1 M, pH 7.4) to achieve a final concentration of *ca.* 10^{10} plaque-forming units (PFU) mL^{-1}.

2.2.2. Determination of phage titre

After each treatment or incubation period, the samples were serially diluted in PBS (0.1 M, pH 7.4) and the phage titre (PFU mL^{-1}) determined by the double-layer plaque assay as previously described by Kropinski et al. (2009) with modifications of media and diluent. TSAYE was selected as the solid media (underlay) and TSBYE, containing 7 g L^{-1} of bacteriological agar (Pronadisa), was used as molten soft agar containing *ca.* 10^6 *Listeria* cells (overlay). For this methodology, the detection limit was 10 PFU mL^{-1}. Plaques formed by phage infection of *L. monocytogenes* were counted and the effect of pressure on plaque morphology and size was monitored for all treatments. Photographs of plaques formed by phage were taken using a Nikon Digital Camera (Nikon Photo Film Co. Ltd., Tokyo, Japan).

2.3. Inoculation of different matrices with phage P100

Six matrices were selected: PBS; “Alheira” a traditional Portuguese fermented sausage (pH 6.07); “Serra da Estrela” cheese (a semi-soft manufactured with raw ewe's milk, pH 5.66); UHT whole milk (pH 6.73), apple juice (pH 3.41); and orange/carrot nectar (pH 3.54). The abovementioned food matrices were purchased from a local supermarket (Porto, Portugal). The “Alheira” sample was previously sterilized by autoclaving (121 °C, 15 min) before being inoculated with *L. monocytogenes*, to avoid interferences and the variability of endogenous microbiota on phage P100 activity. The “Serra da Estrela” cheese sample was initially confirmed for the absence of *L. monocytogenes*. While the solid samples (“Alheira” and “Serra da Estrela” cheese) were placed in sterile stomacher bags; the liquid samples (UHT whole milk, apple juice, orange/carrot nectar, and PBS) were transferred to sterile 250 mL glass flasks. Subsequently, the selected matrices were inoculated to a final phage concentration of *ca.* 10^8 PFU mL^{-1} or g^{-1}. Briefly, 3 mL aliquots of the working suspension of phage P100 were added to liquid (97 mL) and solid (97 g) samples, followed by homogenous distribution of the inoculum through agitation using a magnetic stir bar or by hand (gently mixing for 3 min), respectively.

Before inoculation, the pH value of each sample was measured directly with a Crison MicropH 2002 pH-meter (Crison, Barcelona, Spain) equipped with an InLab 427 puncture electrode (Mettler Toledo, Colombus, USA).

2.4. High hydrostatic pressure treatments

The liquid samples prepared as described in 2.3 were transferred to HHP resistant polyethylene bottles (36-mL), placed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack - Packaging Solutions, Portugal) and double-vacuum-sealed. Solid samples were placed in low permeability PA/PE-90 bags and double-vacuum-sealed. Pressure stability of phage P100 in different food matrices was investigated within the range of 200–700 MPa (5 min, 10 °C), in Hiperbaric 55 high pressure processing equipment (Burgos, Spain). The inactivation kinetic studies were performed at 200, 300 and 400 MPa (10 °C) and samples were collected after 0.1, 5, 15, 30 and 60 min of HHP cycles. Non-pressure treated samples in both PBS (0.1 M, pH 7.4) and food matrices were maintained at atmospheric pressure (0.1 MPa,

4 °C). Phage titers were determined for pressure treated and non-pressure treated samples by resuspending 1 mL (liquid matrix) aliquots in 9 mL of sterile PBS or 5 g (solid matrix) aliquots in 45 mL of sterile PBS, subsequent homogenization, and appropriated ten-fold serial dilutions plated by the double-layer plaque assay as previously detailed (2.2). Three independent experiments were performed.

2.5. Pressure phage stability during refrigerated storage at 4 °C

Stability and activity of phage P100 during shelf-life storage (4 °C) after HHP treatment (300 MPa, 5 min, 10 °C) were evaluated in three selected food matrices (UHT whole milk, "Alheira" and "Serra da Estrela" cheese). At pre-set time intervals (0, 7, 14, 21, 28 days), non- and pressure-treated samples were taken and phage titers determined as previously described (2.2). Two additional time intervals, 45 and 60 days, were considered for "Alheira", "Serra da Estrela" cheese, and for PBS. Three independent experiments were performed.

2.6. Effect of initial phage load

To determine the influence of the initial concentration of phage P100 on the behaviour of phage during the pressure treatment, three initial phage loads (10^6, 10^7 and 10^8 PFU mL^{-1}) were studied, in the three selected food matrices (UHT whole milk, "Alheira" and "Serra da Estrela" cheese), and in PBS (0.1 M, pH 7.4). Samples were inoculated as described in 2.3, with modifications in the working solutions to obtain the initial phage loads, and further submitted to 300 MPa (10 °C, 5 min). Non-pressure treated samples were maintained at atmospheric pressure (0.1 MPa, 4 °C). Three independent experiments were performed.

2.7. Impact of pH and different food components on the phage P100 pressure stability

To evaluate the impact of pH and several food components on the pressure stability of the phage P100, modified PBS solutions were prepared. PBS was adjusted to final pH values of 4.0, 5.0 and 6.0 with lactic acid (DL-lactic acid, Fluka, Neu-Ulm, Germany) or with hydrochloric acid (HCl, Pronalab, Lisbon, Portugal). The effect of different sugars (D(+) sucrose, D(+) raffinose, D(+) glucose, D(+) lactose and D(+) fructose) was assessed by the addition of 5% (w v^{-1}) in PBS. All sugars were purchased from José M. Vaz Pereira (Lisbon, Portugal), with the exception of D (+) raffinose (Fluka). To evaluate the effect of other food components (salt, proteins, emulsifiers, and other sugars), the following solutions were prepared in PBS: (i) 2% (w v^{-1}) NaCl (Panreac, Barcelona, Spain); (ii) 5% (w v^{-1}) beef extract powder (Sigma, Steinheim, Germany) (iii) 3% (w v^{-1}) casein sodium salt (Sigma); (iv) 5% (w v^{-1}) tween 80 (Sigma); and (v) 5% (w v^{-1}) dextrin (Sigma). All solutions were inoculated as described for liquid samples in 2.3 and submitted to 300 MPa (10 °C, 5 min); controls for each sample were maintained at atmospheric pressure (0.1 MPa, 4 °C). Three independent experiments were performed.

2.8. Transmission electron microscopy

In order to better understand the inactivation mechanism of phage P100 during HHP, phage particles were visualized by transmission electron microscopy (TEM). Briefly, 1 mL aliquots of the P100 phage commercial stock solution were submitted to HPP (200, 300 and 400 MPa, 5 min, 10 °C), one non-pressure sample was kept as a control at atmospheric pressure (0.1 MPa, 4 °C). The samples were deposited on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min, negatively stained with 2% uranyl acetate (pH 4.0) and examined using a JEOL JEM 1400 TEM at 120 kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100 W (Tokyo, Japan).

2.9. SDS-polyacrylamide gel electrophoresis

The protein profile of non- and pressure-treated (200, 300 and 400 MPa) phage P100 was assessed by Tricine-SDS-PAGE as described by Schägger (2006). The resolution of proteins was performed in a 4–16% gradient polyacrylamide gel with the Tris/Tricine/SDS buffer system. Phage proteins were visualized by Coomassie staining and analysed by comparing relative mobilities to those of the known molecular weight standard, within the range of 6.5–270 kDa (GRISP, Porto, Portugal), run under the same electrophoretic conditions.

2.10. Data fitting and analysis

2.10.1. Weibull model

Weibull model has been applied to the nonlinear viruses inactivation pattern after HHP processing (Avsaroglu et al., 2006; Kingsley et al., 2007; Zhang et al., 2015). Data from phage inactivation pattern after HHP were fitted with the Weibull model utilizing Eq. (1):

$$\log\left(\frac{N}{N_0}\right) = 1 - e^{\left(\frac{\tau}{\alpha}\right)^{\beta}} \quad (1)$$

where N is the phage titre at a particular sampling time and N_0 is the initial phage titre; parameter α is the scale factor; β is the shape factor. The β value gives an idea of the form of the curve, if $\beta > 1$, the curve is convex (it forms shoulders), if $\beta < 1$, the curve is concave (it forms tails), and if $\beta = 1$, the curve is a straight line and can be described by a linear model. τ is the treatment time (min). The Weibull model was analysed by nonlinear regression applying Eq. (1) using software SPPS (Version 23.0, Inc., Chicago, IL, USA).

2.10.2. Statistical analysis

Phage P100 titres were transformed to logarithmic reduction using the equation: log (N/N_0), where N is the phage titre at a particular sampling time and N_0 is the initial phage titre. Statistically significant differences between phage survival through the tested conditions (200, 300 and 400 MPa), and food matrix were evaluated using the one-way analysis of variances (ANOVA) with Tukey pos hoc test (SPSS, Version 23.0) when homogeneity of variance was assumed.

3. Results and discussion

3.1. The impact of food matrix on bacteriophage P100 application towards HHP

The pressure inactivation of phage P100 in PBS (0.1 M, pH 7.4), "Alheira", "Serra da Estrela" cheese, UHT whole milk, apple juice and orange/carrot nectar, at different pressures (200–400 MPa, 5 min, 10 °C), is presented in Fig. 1. At 400 MPa, phage P100 was inactivated to below the detection limit in all the matrices, while at 200 MPa a significant reduction ($P < 0.05$) in phage numbers was only observed in apple juice and carrot/orange nectar (*ca.* 3 $\log_{10}$ cycles). At 300 MPa, the phage demonstrated ability to survive HHP when inoculated in PBS, fermented sausage, cheese or milk, with a reduction of phage titres ranging from 0.79 to 2.60 $\log_{10}$ cycles.

A high variability in the pressure magnitudes (200–800 MPa) required to achieve inactivation of foodborne viruses by HHP has been reported (Avsaroglu et al., 2009; Grove et al., 2008; Kingsley et al., 2007; Müller-Merbach et al., 2005; Tabla et al., 2012; Zhang et al., 2015). Among the factors that result in a high variability in phage inactivation by HHP, the physical state of food seems to play an important role.

Although previous studies have demonstrated that virus and phages are less sensitive to hydrostatic pressure on food than in liquid suspension (Sharma et al., 2008; Smiddy et al., 2006), in this study the susceptibility of P100 to HHP treatment in apple juice and orange/

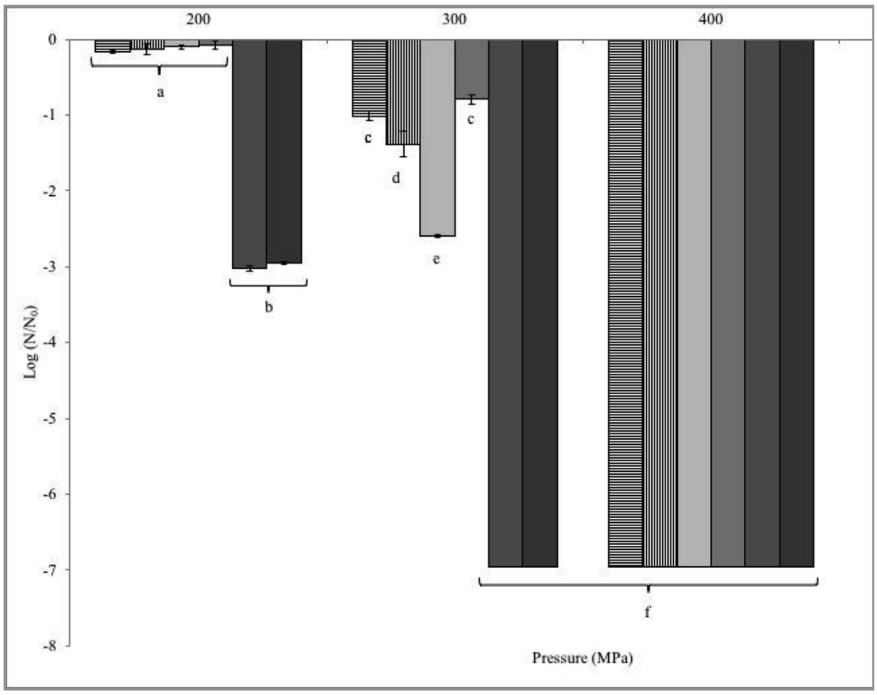

Fig. 1. Inactivation of phage P100 in different food matrices at 200, 300 and 400 MPa (10 °C, 5 min). "Alheira" fermented sausage - pH 6.07 ± 0.02 (▤); "Serra da Estrela" cheese - pH 5.66 ± 0.01 (▥); PBS - pH 7.42 ± 0.01 (▩); UHT whole milk - pH 6.73 ± 0.03 (■); apple juice - pH 3.41 ± 0.04 (■) and orange/carrot nectar - pH 3.54 ± 0.01 (■). Data reported are mean values of three independent experiments ± standard deviation. Means with the same letter are not statistically different from each other ($P > 0.05$).

carrot nectar matrices is likely to be related to the low pH of these samples (< 4.0), as in PBS and UHT milk (> 6.5) the reduction at 200 MPa was 0.07 and 0.08 $\log_{10}$ cycles. Oliveira et al. (2014) also reported a 7 $\log_{10}$ cycles reduction of phage P100 in apple juice after 8 days of storage at 10 °C, attributed to the sensitivity of phage to the acidic environment (pH 3.70).

Cell injuries and morphological changes in *L. monocytogenes* at mild pressures are well documented in the literature; the main effect which HHP promotes at mild pressures is the destabilization of the cell membrane by disturbance of functional proteins responsible for transport of ions and amino acids (reviewed by Ferreira et al., 2016). Cellular injury may therefore result in more effective phage-host interaction and infection.

The pressure inactivation kinetics of phage P100 in the different matrices at 200 and 300 MPa, up to 60 min, are shown in Fig. 2. Overall, as pressure magnitudes and/or processing times increased, inactivation of phage P100 also increased; after treatment at 400 MPa, phage titres were below the detection limit for all matrices (data not shown). Kinetic parameters for HHP induced inactivation for 60 min at 200 and 300 MPa are detailed in Table 1. The α values ranged from 0.06 ± 0.02 to 220.95 ± 40.63 at 200 MPa and from 3.57 ± 0.39 to 41.25 ± 1.85 at 300 MPa. The increase in the α parameter is associated with a baroprotective effect of the food matrix as the α parameter is inversely proportional to logarithmic reduction. No significant differences were observed for α values between "Alheira", "Serra da Estrela" cheese and PBS at 200 MPa ($P > 0.05$); inactivation of phage P100 HHP in UHT whole milk was slightly accentuated in comparison with "Alheira" and "Serra da Estrela" cheese ($P < 0.05$) while did not differ from PBS ($P > 0.05$). Moreover, phage P100 was more pressure sensitive in apple juice and carrot/orange nectar (Fig. 2E–F), both of which presented a significantly lower α value than the other matrices ($P < 0.05$; Table 1). At 300 MPa, P100 was completely inactivated in apple juice and carrot/orange nectar, while different phage inactivation rates were observed in all the remaining food matrices ($P < 0.05$, Fig. 2A–D).

The sample environment, namely the effect of food/media composition is well known to influence the stability of bacteriophages during HHP (Capra et al., 2009; Guan et al., 2007; Moroni et al., 2002; Sharma et al., 2008; Smiddy et al., 2006). At 300 MPa, in agreement with the preliminary HHP study, the kinetics assay confirmed that UHT whole milk was the most baroprotective matrix, followed by "Alheira" fermented sausage, "Serra da Estrela" cheese and PBS. Moroni et al. (2002), evaluating lactococcal phages inactivation in PBS (0.1 M, pH 7.4), whey permeate powder reconstituted at 6%, and partially (2%) skimmed milk, during treatment by dynamic high pressure at 100 and 200 MPa, verified that those dairy based samples were more protective than PBS. Differences in behaviour of phage P100 in UHT whole milk and "Serra da Estrela" cheese observed in the present study may be explained, at least partially, by the differences in acidity and salt content of these products. In agreement with this result, Modi et al. (2001) investigated the inactivation of phage SJ2, active against *Salmonella* Enteritidis, during cheese production. In comparison with the initial phage titre in milk, a 2 log cycles reduction was observed during the curd drain (pH 5.3–5.4) and in the cheese. "Alheira", a complex food matrix with a high content of fat (*ca.* 16%) demonstrated a baroprotective character for phage P100 during HHP treatment when compared to the PBS control. In agreement with these results, Sharma et al. (2008) evaluating the inactivation of foodborne viruses by HHP, reported a low susceptibility of coliphages (T_4; phiX174; MS2) to pressure treatment at 500 MPa in the sausage matrix.

All β values were concave upward (β < 1) and ranged from 0.07 ± 0.01 (apple juice) to 0.67 ± 0.07 (UHT whole milk). Different values of the shape parameter (β) can have marked effects on the behaviour of the phage population distribution; it means that proximate β values had closely related behaviour towards HHP processing of phage P100. As described by van Boekel (2002) and Avsaroglu et al. (2006), downward concavity (β > 1) indicates that remaining population becomes increasingly damaged, whereas upward concavity (β < 1) indicates a population with different capacities of survival to the stress condition applied. Results from the present study on pressurizations for up to 60 min indicated the presence of a non-homogenous population of phages. Similarly, Avsaroglu et al. (2006) studying the use of Weibull model to describe lactococcal phages inactivation by HHP, showed that the sensitive members of the population were destroyed at a relatively faster rate leaving behind survivors of higher resistance. Moreover, Kingsley et al. (2007) evaluating the inactivation of murine norovirus by HHP (325 MPa, 5 °C and 375 MPa, 20 °C) described non-linear curves characterized by a rapid initial drop in viral counts followed by

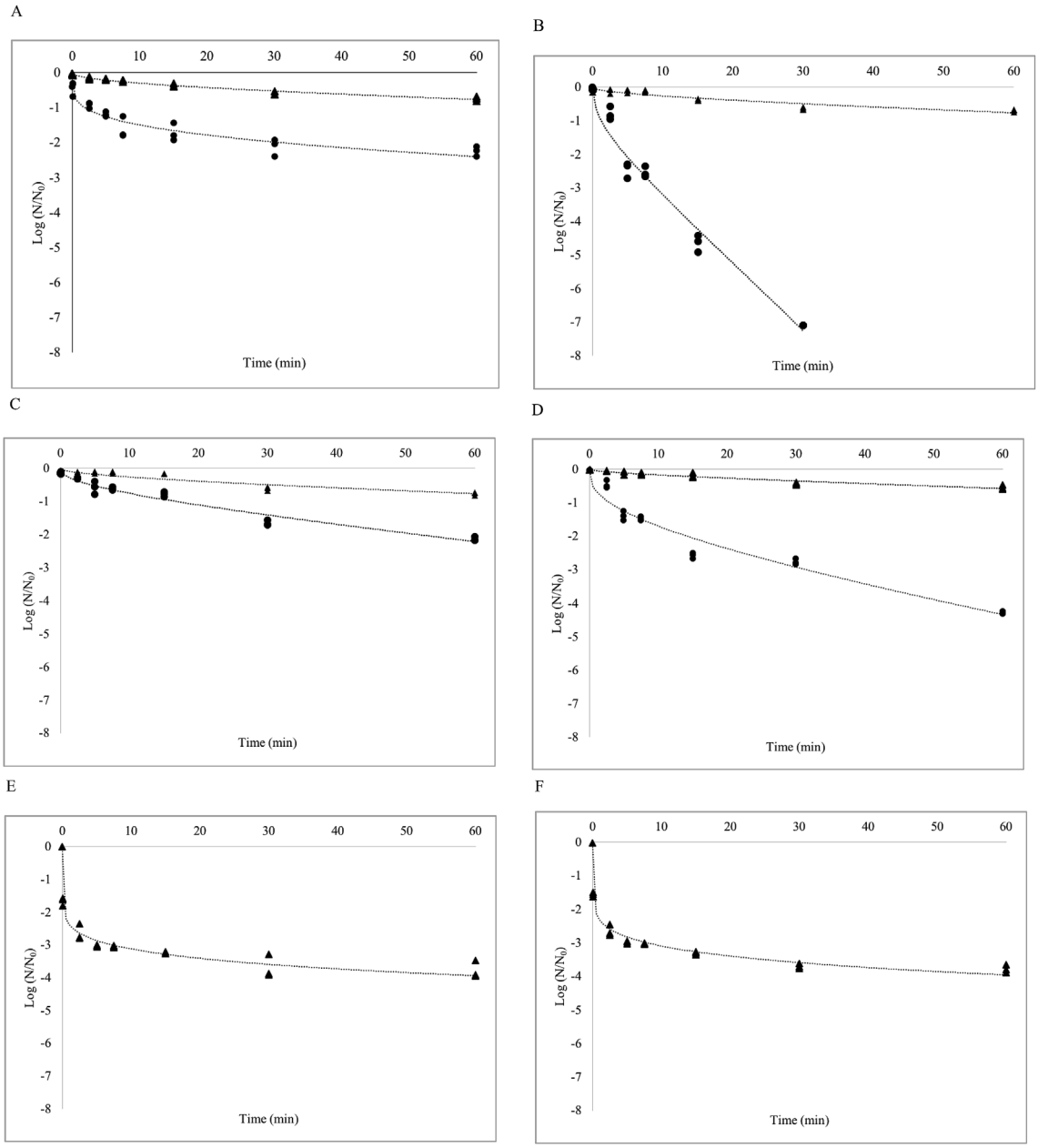

Fig. 2. Kinetics inactivation of phage P100 at 200 MPa (▲) and 300 MPa (●) in different matrices: (A) "Alheira" fermented sausage; (B) PBS; (C) UHT whole milk; (D) "Serra da Estrela" cheese; (E) apple juice; (F) carrot and orange nectar. Inactivation kinetics of phage P100 inoculated in apple juice and orange/carrot juice is not represented at 300 MPa because the phage was completely inactivated. Three independent experiments were performed.

Table 1
Formal kinetic parameters for HHP induced inactivation (200 and 300 MPa) of phage P100 in different food matrices. The values are parameter estimate ± standard deviation. For each parameter, values with the same letter are not statistically different ($P > 0.05$).

Pressure	Parameters	"Alheira" fermented sausage	"Serra da Estrela" cheese	PBS	UHT whole milk	Apple juice	Orange and carrot nectar
200 MPa	α (min)	219.85 ± 27.04^{a}	220.95 ± 40.63^{a}	185.55 ± 38.25^{ab}	133.17 ± 18.03^{b}	0.06 ± 0.02^{c}	0.08 ± 0.03^{c}
	β	0.44 ± 0.02^{c}	0.61 ± 0.06^{ab}	0.51 ± 0.06^{bc}	0.67 ± 0.07^{a}	0.07 ± 0.01^{d}	0.07 ± 0.01^{d}
	R^2	0.96	0.97	0.88	0.90	0.98	0.99
300 MPa	α (min)	17.55 ± 2.17^{b}	10.34 ± 1.08^{c}	3.57 ± 0.39^{d}	41.25 ± 1.85^{a}	n/a	n/a
	β	0.16 ± 0.02^{a}	0.29 ± 0.02^{b}	0.35 ± 0.02^{ab}	0.41 ± 0.03^{a}	n/a	n/a
	R^2	0.91	0.96	0.98	0.96	n/a	n/a

n/a: not applicable.

Table 2
Phage titre of non – and pressure treated samples (UHT whole milk, “Alheira” fermented sausage, “Serra da Estrela” cheese and PBS) during storage at 4 °C for 60 days. Data reported are mean values of three independent experiments ± standard deviation.

Pressure	Matrix	Phage titre (log PFU/g or mL)						
		Time (days)						
		1	7	14	21	28	45	60
0.1 MPa	UHT whole milk	7.95 ± 0.05	7.98 ± 0.03	8.03 ± 0.04	8.07 ± 0.06	8.05 ± 0.09	n/a	n/a
	"Alheira" fermented sausage	8.07 ± 0.03	8.03 ± 0.14	8.03 ± 0.07	7.98 ± 0.10	8.01 ± 0.14	7.96 ± 0.11	7.89 ± 0.09
	"Serra da Estrela" cheese	7.93 ± 0.07	7.89 ± 0.11	7.85 ± 0.12	7.90 ± 0.09	7.82 ± 0.13	7.79 ± 0.07	7.78 ± 0.11
	PBS	8.02 ± 0.04	8.16 ± 0.07	8.06 ± 0.03	8.11 ± 0.05	8.08 ± 0.04	8.08 ± 0.02	8.04 ± 0.07
300 MPa	UHT whole milk	7.23 ± 0.04	7.19 ± 0.07	7.17 ± 0.10	7.15 ± 0.08	7.18 ± 0.11	n/a	n/a
	"Alheira" fermented sausage	7.01 ± 0.13	6.98 ± 0.12	6.90 ± 0.15	6.92 ± 0.08	6.87 ± 0.17	6.85 ± 0.09	6.91 ± 0.13
	"Serra da Estrela" cheese	6.57 ± 0.11	6.48 ± 0.16	6.60 ± 0.09	6.55 ± 0.12	6.47 ± 0.08	6.51 ± 0.10	6.53 ± 0.07
	PBS	5.42 ± 0.04	5.49 ± 0.05	5.45 ± 0.09	5.51 ± 0.07	5.44 ± 0.04	5.49 ± 0.06	5.46 ± 0.03

n/a: not applicable.

tailing caused by diminishing inactivation rate. Curves of lactococcal phages pressurized at 300–600 MPa (25 °C) in Ca-M17 broth also presented tailing indicating a non-homogenous population of these phages (Müller-Merbach et al., 2005).

No correlations between pH and α or β factors were found (data not shown), indicating a complex protective effect from the different components of food matrices resulting in the high variability in the survival of phage P100 exposed to HHP. Likewise, no correlation between α or β factors and pressure range were obtained due to the limited pressure range of survival of phage P100 (0.1–400 MPa). Overall, it is difficult to establish a general nature of scale factor (α) and shape factor (β), indicating that a food process including bacteriophage as a biocontrol agent needs to be carefully studied and evaluated for the specific food matrix.

3.2. Phage stability in different matrices after HPP treatment during refrigerated storage

To evaluate the stability of phage P100 particles after HHP treatment during the shelf-life of pressure treated food products, non- and pressure-treated samples of UHT whole milk, “Alheira” fermented sausage, and “Serra da Estrela” cheese inoculated with phage were stored at 4 °C for 60 days, and phage titres evaluated at specific time periods; results obtained are presented in Table 2. Non-pressurized food matrices presented phage titres constant over the 60 days of refrigerated storage ($P > 0.05$); for pressurized samples (300 MPa), after the initial phage inactivation induced by HHP, stable phage titres were observed for all matrices during refrigerated storage ($P > 0.05$). Bacteriophages are well known to survive for long periods in solution and or in food matrices (Modi et al., 2001; Soni et al., 2012; Wang et al., 2016). Modi et al. (2001) observed a stable titre of phage SJ2 at greater than 10^7 (PFU/g) after 90 days of storage at 8 °C in Cheddar cheese; no significant loss in the phage fmb-p1 infectivity over 21 days in fresh chilled pork at 4 °C was observed (Wang et al., 2016). As observed in the present study, phage P 100 was also reported to be stable during 28 days of storage in *queso fresco* (Soni et al., 2012).

3.3. Impact of the initial phage load on the inactivation of phage P100 through HHP treatment

The influence of the initial phage load on inactivation of phage P100 by HHP in PBS, “Alheira” fermented sausage, UHT whole milk, and “Serra da Estrela” cheese, at 300 MPa is presented in Fig. 3. No significant differences were observed between the logarithmic reductions observed for each food matrix tested, with initial phage loads ranging from 10^6 to 10^8 PFU mL^{-1} ($P > 0.05$). In other studies exploring the behaviour of initial phage load towards pressure exposure, differences were observed in the behaviour of phage particles; in a dynamic high pressure treatment, Moroni et al. (2002) showed that greater initial loads (10^8 - 10^9 PFU mL^{-1}) resulted in lower inactivation of lactococcal bacteriophages in PBS at 200 MPa. In contrast, a study evaluating the effect of high pressure homogenization (60 and 100 MPa) on lactic acid bacteria phage in reconstituted skim milk demonstrated that a higher inactivation rate of phage MLC-A was achieved for the higher initial load tested (10^5 - 10^6 PFU mL^{-1}) compared to lower concentrations (10^2 - 10^4 PFU mL^{-1}) (Capra et al., 2009).

3.4. Influence of pH and food components on pressure stability of phage P100

To better explore the inactivation patterns obtained in PBS, the intermediary pressure (300 MPa) was selected to study the influence of the principal food components and pH values on inactivation of phage P100 by HHP. The experiments were performed by testing one variable at a time in order to evaluate the single effect of each factor on phage stability. The results are presented in Fig. 4.

The addition of the several food components did not influence the viability of phage P100 by their incorporation in PBS, as verified in the non HPP-treated control samples (0.1 MPa, data not shown). The addition of 5% (w v^{-1}) of reducing sugars, namely D(+) glucose, D(+) fructose and D(+) lactose, proved to have a protective effect on inactivation of pressurized phage at 300 MPa (10 °C, 5 min) when compared to the control, i.e. PBS ($P < 0.05$; Fig. 4A). In contrast, the addition of D(+) sucrose or D(+) raffinose did not affect phage P100 survival ($P > 0.05$). In agreement with these results, Guan et al. (2007) also reported a baroprotective effect of glucose addition (5% w v^{-1}) in PBS and UHT whole milk (*ca.* 4.5% lactose content) on the pressure stability (600 MPa, 21 °C, 5 min) of Qβ and SP coliphages. Incorporation of sodium casein salt (3% w v^{-1}), tween 80 (5% w v^{-1}) and dextrin (5% w v^{-1}) in PBS demonstrated to have a baroprotective effect ($P < 0.05$), whereas the addition of sodium chloride (2% w v^{-1}) and beef extract powder (5% w v^{-1}) resulted in no significant differences in the phage titres compared to the control ($P > 0.05$; Fig. 4B). Contrarily to the pressure response of modified PBS with tween 80 (5% w v^{-1}) observed in the present work, a study evaluating the pressure response of coliphages with other surfactants (sucrose laurate and monolaurin) and EDTA reported an increased pressure sensitivity of coliphages Qβ and SP in the presence of these compounds (Guan et al., 2007). In addition, Sharma et al. (2008) reported a slight increase in recovered virus titres from sausages inoculated with feline calcivirus and hepatitis A when treated with chelating agents (EDTA and lactoferrin). The addition of sodium chloride to PBS had no effects on phage P100 stability during HHP, whereas a study evaluating different salt concentrations in PBS associated an increased salt concentration to a reduced pressure resistance for Qβ and SP coliphages (600 MPa, 10 °C, 5 min) (Guan

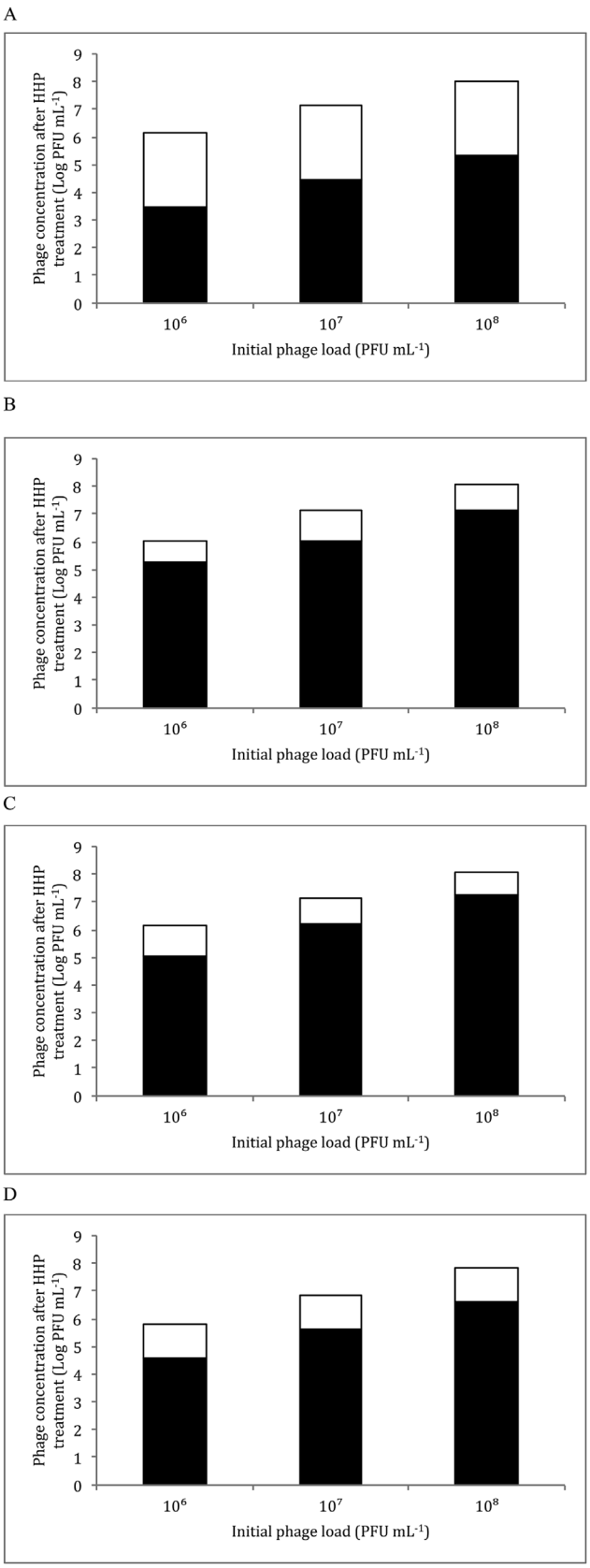

Fig. 3. Effect of the initial phage load in the inactivation of phage P100 treated with HHP (300 MPa, 10 °C, 5 min) in different food matrices. (A) PBS; (B) "Alheira" fermented sausage; (C) UHT whole milk; (D) "Serra da Estrela" cheese. Legend: (□) inactivated phages; (■) viable phages. Three independent experiments were performed.

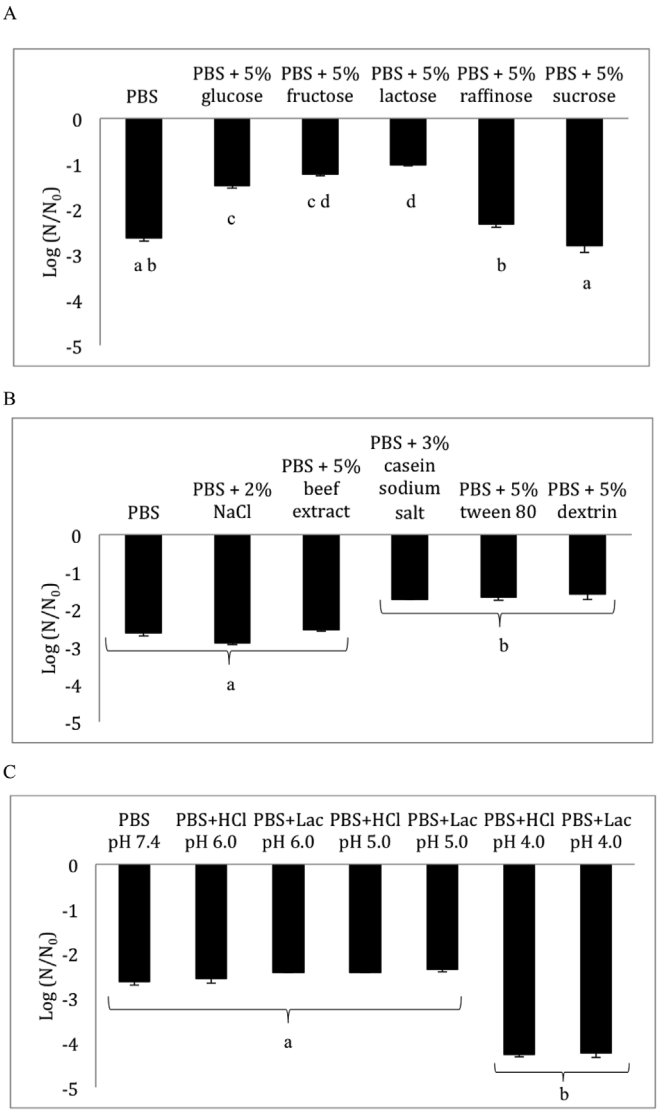

Fig. 4. Inactivation of phage P100 at 300 MPa (10 °C, 5 min) as a function of (A) sugars; (B) food components and (C) acids (HCl and lactic acid) added in PBS. Data reported are mean values of three independent experiments ± standard deviation. Means with the same letter are not statistically different from each other ($P > 0.05$) Legend: Lac – lactic acid.

et al., 2007). In contrast, some studies postulated that the higher the ionic strength of the medium or food, the more HHP resistance is afforded to foodborne viruses (Hirneisen et al., 2010; Kingsley et al., 2005; Murchie et al., 2007); hepatitis A virus also had the pressure sensitivity diminished in seawater (27.4 ppm sodium chloride concentration) when compared to isotonic culture medium (Kingsley et al., 2002).

In the range of PBS pH values of 4.0–7.4 a lower baroresistance of phage P100 was observed for the lowest pH, independently of the acid used (organic and inorganic) when compared to all other pH values tested ($P < 0.05$; Fig. 4C). The inability of bacteriophages to tolerate an acidic environment is documented (Dini et al., 2012; Fister et al., 2016; Leverentz et al., 2003; Oliveira et al., 2014). In a study evaluating the influence of environmental factors on phage-bacteria interaction, Fister et al. (2016) reported an inability of phage P100 to maintain its stability below pH 4 in TSB acidified with HCl, resulting in complete inactivation at pH 2 after 1 h. In the present study, at pH 4, an accentuated phage titre reduction of phage P100 HHP treated was observed, that may be explained by the combination of acidic and pressure

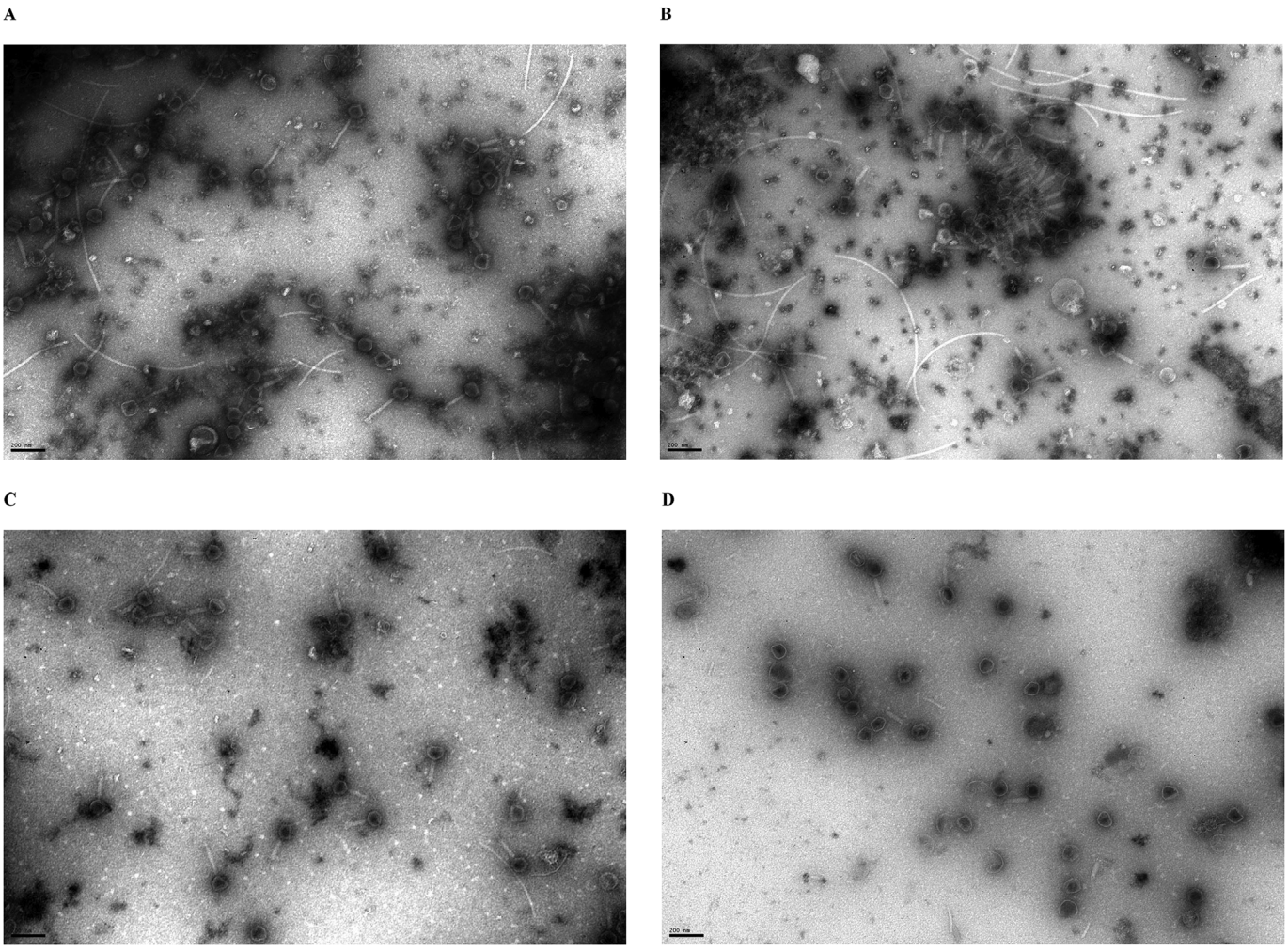

Fig. 5. Electron microscopy of non- and pressure treated phage P100 in saline buffer. (A) 0.1 MPa (control); (B) 200 MPa; (C) 300 MPa; (D) 400 MPa.

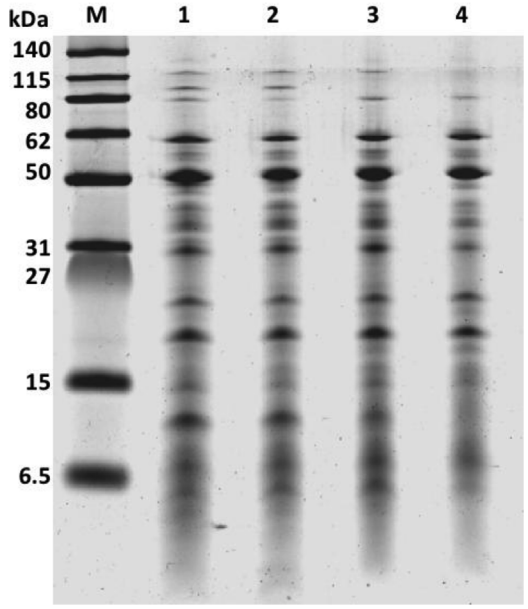

Fig. 6. Tricine SDS-PAGE evaluation of the phage P100 integrity following different pressure treatments. Lane M: Molecular weight ladder (standard band weight indicated in kDa); Lane 1: non-pressure treated phage P100 (control); Lane 2, 3, 4: phage P100 treated at 200, 300 and 400 MPa, respectively.

hurdles. Moreover, these results are in accordance with the previously described inactivation of phage P100 in apple juice and orange/carrot nectar, which demonstrated an effect of low pH in the increased inactivation of phage P100 during HHP.

3.5. The mechanistic analysis of phage P100 inactivation

Results from electron microscopy of phage P100 non- and pressure-treated in saline buffer are shown in Fig. 5. In the non-pressurized sample (0.1 MPa) an intact structure of phage P100 was observed, with an isometric head and non-flexible contractile tail (Fig. 5A). Fig. 5 B–D show the effect of pressure increase on the morphology of phage P100. Phage particles submitted to 200 MPa (5min, 10 °C) appeared to have similar aspects to the non-pressurized sample (Fig. 5B) whereas samples treated at 300 MPa presented some phage particles that had lost their tail or presented just part of it, and some phages appeared with deformed heads (Fig. 5C). At 400 MPa all observed phages were without tails, demonstrating that they became unable to attach to the bacteria; ruptures in phage heads were also observed (Fig. 5D).

These results are in accordance with the previous reports (Moroni et al., 2002; Müller-Merbach et al., 2005). The main assumption is that phage inactivation by HHP may occur via essential phage protein denaturation, namely the structural damages from tail loss and genetic material lost by openings formed in phage heads. Moreover, it may contribute to explain the protective effect of food components described in this study; the viscosity of non-Newtonian food or solutions influence the shear rate during high pressure (Floury et al., 2002, 2000) and it

could also affect phage proteins denaturation, resulting in less or greater damage according to specific matrix viscosity.

As phage A511 and phage P100 are highly similar in morphology and in the whole genome, protein profile from phage A511 was used to compare the protein profile obtained from phage P100 and to analyze the proteins affected by HHP inactivation of phage P100 (Klumpp et al., 2008). As shown in Fig. 6, the 400 MPa treatment resulted in a absence of two bands in the range between 80 and 115 kDa, one in the range of 31 kDa and other between 6.5 and 15 kDa. Inactivation of phage P100 by HHP seems to be linked to the denaturation of functional proteins (estimated between 80 and 115 kDa in phage A511) and putative tail proteins (36 kDa and below 20.1 kDa in phage A511). These findings are in accordance with the results obtained from TEM microscopy, since it showed phage without tails and many capsids preserved.

4. Conclusions

Significant differences in the inactivation behaviour of phage P100, inoculated in food matrices, during HHP were observed and the main factors in food composition that influenced the phage stability were proposed. This study demonstrated that UHT whole milk, “Alheira” fermented sausage and “Serra da Estrela” cheese are baroprotective matrices to support phage P100 application in HHP up to 300 MPa. The presence of reducing sugars, dextrin, casein, and tween 80 were described as baroprotective agents during HHP processing of phage P100 in modified PBS whereas acidic pH values seem to be linked to an accentuated phage inactivation. The initial phage load did not affect the inactivation rate during HHP process and the phage P100 titres in HHP treated (300 MPa, 5 min, 10 °C) samples, were stable during all refrigerated storage at 4 °C.

Furthermore, since *L. monocytogenes* presents cell injuries and damage at mild HHP and phage P100 infectivity is maintained according to the inoculated matrix, the combined effect of these environmentally friendly and minimal processing technologies may represent an efficient synergetic system for *L. monocytogenes* control.

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