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RESEARCH ARTICLE

Control of *Listeria monocytogenes* in milk by using phage cocktail

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Abstract

Biocontrol applications such as using phages against the contamination of *Listeria monocytogenes* are promising trends in terms of reducing the use of chemical additives in food industry. The aim of this study was to determine the effectiveness of Listex P100 phage (phage P100) on different *Listeria monocytogenes* strains (PL2, PL3, PL9 and PL10) in pasteurized milk and broth. Survival data of *L. monocytogenes* were successfully described by Weibull model. Time parameter of the Weibull model was used to evaluate the phage-resistances of *L. monocytogenes* strains. The reduction of *L. monocytogenes* was greater in broth than in milk regardless of the temperature level and it was significantly higher at 30 °C than at 4 °C in both media. The reductions of *L. monocytogenes* strains by the phage treatment were between 2.7 to 3.4 $\log_{10}$ units at 30 °C and 1.4 to 2.1 $\log_{10}$ units at 4 °C after 4 days of incubation in broth whereas 1.9 to 2.9 $\log_{10}$ units and 1.0 to1.6 $\log_{10}$ units were observed after 4 days of incubation in milk at 30 °C and 4 °C, respectively. It was found that *L. monocytogenes* PL2 is the most phage-resistant strain in broth at 30 °C and at 4 °C, and in milk at 30 °C, while *L. monocytogenes* PL9 is the most phage-resistant *L. monocytogenes* strain in milk at 4 °C. This study demonstrated P100 phage could be used to control *L. monocytogenes* counts in milk.

Keywords: Biocontrol; foodborne pathogens; Listex P100; predictive microbiology; Weibull model.

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1. Introduction

Listeria is a Gram–positive, rod–shaped, non–sporulating, facultative intracellular exquisitely adaptable environmental bacterium belonging to the family *Listeriaceae* (Klump & Loessner, 2013; Korsak & Szuplewska, 2016; Komora et al., 2020). Currently, the genus *Listeria* contains 21 different species (NCBI, 2021). In food industry, one of the most important pathogens in this genus is *Listeria monocytogenes* that can cause listeriosis in humans and animals which is a health concern throughout the world (Korsak et al., 2012; Leylak & Buzrul, 2020). However, very rarely, *L. ivanovii*, *L. innocua*, and *L. seeligeri* have also been associated with disease in humans (Jadhav et al., 2012; Korsak & Szuplewska, 2016). Listeriosis is a sporadic disease, which is often associated with consumption of contaminated foods. Pregnant woman, neonates, adults with underlying disease (cancer, AIDS, diabetes, chronic hepatic disorder, transplant recipients), the elderly (≥ 65 years), and other immunocompromised individuals are at the highest risk (Rahimi et al., 2010).

L. monocytogenes is a serious threat to the food industry since it can survive the most common stress levels present in the food processing environment such as high salinity, acidity, osmotic pressure, refrigeration temperatures and low water activity (Jadhav et al., 2012). *L. monocytogenes* has been detected in many types of environments including rotting vegetables, untreated or treated sewage water, sludge, agricultural produce and in certain types of food items (Safraz et al., 2017). Many raw and processed foods such as unpasteurized milk, soft cheeses, dairy products, red meats, poultry, sea foods, vegetables, fruits, sausages, smoked fish, salads, deli meat, and refrigerated ready–to–eat (RTE) foods have been identified for their risk of listeriosis (Valimaa et al., 2015). Of these foods, milk and other dairy products especially raw and soft cheeses have been implicated in about half of all the listeriosis outbreaks (Sarker & Ahmed, 2015; Komora et al., 2020).

Biocontrol assays have received renewed attention in recent years as a possible antibiotic alternative to eliminate or control foodborne pathogens infections. Many studies have investigated the use of various phages to control of foodborne pathogens, including *L. monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, *Campylobacter jejuni*, *Bacillus cereus* and *Escherichia coli* (Goodridge & Bisha, 2011). Biological agents such as

bacteriophage (also called phage) or bacteriocin are also used to reduce the viable counts of bacteria on foods (Criscuolo et al., 2017; Komora et al., 2020). Phages are often used in high concentrations to inactivate foodborne pathogens, such as *E. coli* O157:H7, *Salmonella*, *L. monocytogenes* and *Camplobacter* spp. in different foods (Akhtar et al., 2014). The use of phages in foods is a promising tool for food safety at pre–harvest and post–harvest stages of food production, and storage. There are some advantages of phage application in foods, such as safe, effective, and specific in reducing pathogens in several types of foods, without affecting either normal flora or the organoleptic properties of the food. Bacterial reductions from 0.9 to 6.8 $\log_{10}$ Colony Forming Units (CFU) or even total elimination was reported in many phage applications studies on food (Galarce et al., 2016). *Listeria* specific phages have been investigated in detail as biocontrol agents on foods so far (Leverentz et al., 2003; Soni et al., 2010; Rossi et al., 2011; Oliveria et al., 2014; Lee et al., 2017; Komora et al., 2020). To date, more than 500 listerial phages isolated from several food products such as meat, dairy, vegetables, fish etc. have been characterized. Most of them have been used in biocontrol assays (Soni & Nannapaneni, 2010; Klump & Loessner, 2013). Some phage preparations have been approved as safe status by the Food and Drug Administration (FDA) and recognized by the United States Department of Agriculture (USDA) as an antimicrobial processing aid to combat *L. monocytogenes* in foods. ListShield (LMP–102) and Listex P100 are two listerial phage preparations commercialized and marketed so far. ListShield LMP102 was the first phage–based products developed by Intralytic Inc., USA for use as an antimicrobial agent against *L. monocytogenes* contamination of RTE foods. ListShield LMP102 containing a cocktail of six phages is also used for surfaces in food production facilities. In 2011, Listex P100 containing a single phage was another anti–*Listeria* phage preparation developed by Micreos Food Safety, The Netherlands, formerly EBI Food Safety (Oliveira et al., 2014; Teng–Hern et al., 2014). Of these two phages, Listex P100 is approved for food preservation on RTE meats and deli products at levels not to exceed 10^9 plaque forming units (PFU) g^{-1} (Soni & Nannapaneni, 2010). This phage preparation has also been used in several studies showing its efficacy in removing *L. monocytogenes* contaminations (Soni & Nannapaneni, 2010; Soni et al., 2010; Rossi et al., 2011; Soni et al., 2012; Silva et al., 2014; Oliveira et al., 2014; Gutierrez et al., 2017; Komora et al. 2020). Many listerial phage preparations have also been tested in the dairy products to reduce the occurrence of *L. monocytogenes* strains (Carlton et al., 2005; Guenther & Loessner, 2011; Silva et al., 2014; Perera et al., 2015; Lee et al., 2017).

On the other hand, there is little information about the phage application on *L. monocytogenes* in milk. If there is a flaw during the pasteurization of milk, there may be a risk of *L. monocytogenes* existence. Moreover, there may also be a possibility of contamination of milk by *L. monocytogenes* after the heat treatment.

Therefore, the objectives of this study were (i) to determine the effectiveness of Listex P100 in reducing *L. monocytogenes* in pasteurized milk and broth as a function of storage temperature and storage duration (ii) to describe the reduction of *L. monocytogenes* counts in broth and milk by use of a suitable mathematical model.

2. Materials and methods

Pasteurized milk samples

Pasteurized (72 °C for 15 s) cow's milk used in this study was purchased commercially on the day of the assay in 1000 mL tetra packs or glass bottle from local supermarket in Ankara, Turkey. Shelf life of the milk was between 10 - 14 days if stored in refrigerator without opening the package. Milk samples close to the expiry date were not selected. Prior to the experimental studies, covers of pasteurized milk samples were opened in a sterile cabin at room temperature.

Bacteria, phage and growth condition

The bacteria used in this study were as follows: *L. ivanovii* ATCC19119, *L. monocytogenes* ATCC7644, *L. monocytogenes* PL2, *L. monocytogenes* PL3, *L. monocytogenes* PL9, and *L. monocytogenes* PL10. *L. monocytogenes* PL2, PL3, PL9, and PL10 strains (serotype 1/2a) were isolated from RTE foods in Ankara/Turkey and molecular characterized previously. *L. ivanovii* ATCC19119 was used as a helper strain to determine the titer of the Listex P100 (phage P100 or P100 phage) as described by Silva et al. (2014). All strains used in this study were obtained from the culture collection of Food Microbiology Laboratory, Department of Food Engineering, Ankara University, Ankara, Turkey. Strains were grown individually on Tryptic Soy Broth supplemented with 0.6% of yeast extract (TSBYE) (Sigma, Germany) at 35 °C for 20 – 24 h. The *Listeria* spp. strains were maintained at –20 °C with 30% (v/v) glycerol (Merck, Germany).

Phage P100 characterized by its broad spectrum toward *L. monocytogenes* strains, was used in this study. The phage concentration was approximately 10^{11} PFU mL^{-1} in physiological saline buffer (PBS) (0.85% NaCl). Phage P100 was stored at refrigerated conditions (4 °C) during the study. Phage P100 stock concentration was also approximately 10^{11} PFU mL^{-1} by plaque formation assay. Stock solution of phage P100 was diluted in PBS for preparing the desired concentrations for phage application.

Preparation of bacteria for inoculum

L. monocytogenes strains were grown individually on Tryptic Soy Agar supplemented with 0.6% of yeast extract (TSAYE) (Sigma, Germany) at 35 °C for 20 – 24 h. An individual colony of each strain was transferred into a flask with 10 mL of TSBYE at 35 °C for 20–24 h. Overnight broth cultures of *L. monocytogenes* strains were centrifuged at 6000×g for 5 min at 4 °C and resuspended in PBS. Suspensions were diluted with PBS to approximately 10^6 CFU mL^{-1}. (Oliveira et al., 2014). The concentration of *L. monocytogenes* strains was confirmed by MacFarland Densitometers (Den–1B).

Determination of host range of phage

To determine phage lytic spectrum against four different *L. monocytogenes* strains used in this study, double agar overlay plaque assay was used first to screen the strains as

previously described by Akhtar et al. (2017) with a few minor modifications. Briefly, overnight cultures were used to prepare an exponential phase culture. Phage suspensions (100 µL phage solution with a titer of 10^8 PFU mL^{-1} and 10 mM $CaCl_2$) were prepared in a sterile test tube, and then 150 µL of the exponential phase *L. monocytogenes* strains with a concentration of approximately 10^6 CFU mL^{-1} was added, separately. After 30 min of incubation at 35 °C, 5 mL of soft TSAYE (7.5% agar) was poured on the phage–bacterium mixture. The resulting mixture was gently vortexed and spotted on the pre–solidified TSAYE plate containing 10 mM $CaCl_2$. After solidification of agar for 30 min at room temperature, the plates were incubated at 35 °C for 20 – 24 h to determine plaque formation. The lytic activity was checked for the formation of clear areas. Control plates were untreated with phage.

Preparation of pasteurized milk samples

Pasteurized milk samples were screened for the presence or absence of *Listeria* spp. Pasteurized milks used in this study were diluted in PBS, followed by spread plating on Palcam agar (Merk, Germany) to test for the presence of typical *Listeria* spp. colonies as described by Oliveira et al. (2014). *L. monocytogenes* ATCC7644 positive control and one uninoculated media negative control were used for each set of concurrently analyzed samples. After incubated at 35 °C for 20 – 24 h, samples found to be negative were used in biocontrol studies.

Phage treatment in fresh broth medium and pasteurized milk

Phage application assays were carried out at 4 and 30 °C in parallel batches in 30 mL fresh TSBYE and pasteurized milk added of 10 mM $CaCl_2$. All the batches were incubated at several incubation times (on days 1, 2, 3, 4 and 5) in this study. Three experimental sets were formed in this study: (i) fresh TSBYE and pasteurized milk untreated with phage and bacteria (negative control), (ii) fresh TSBYE and pasteurized milk contaminated with only bacteria (positive control), and (iii) fresh TSBYE and pasteurized milk inoculated with bacteria and after phage (biocontrol experiments).

All the batches of 30 mL were contaminated with 100 µL of bacterial suspensions with a concentration of approximately 10^6 CFU mL^{-1}. Then, one aliquot of each *L. monocytogenes* infected batch was inoculated with 100 µL of the phage suspension at approximately 10^8 PFU mL^{-1}. Finally, 10 mM $CaCl_2$ was added. All samples were mixed thoroughly to ensure homogenous distribution of the pathogen and phage, as reported by McLean et al. (2013) with some minor modifications. All samples were then incubated at 4 and at 30 °C for a total of 5 days.

Determination of bacterial counts and phage titer

Bacterial viable counts (CFU mL^{-1}) and phage concentrations (PFU mL^{-1}) from samples were performed immediately, after addiction of bacteria and phage, and after 1, 2, 3, 4 and 5 days of incubation of storage at 4 and 30 °C. To determine viable bacterial counts, aliquots were removed from each sample at defined intervals and serially diluted in PBS. Then, 100 µL of dilutions were spread plated on TSAYE and incubated at 35 °C for 24 – 48 h until typical *Listeria* colonies could be enumerated (Guenther et al., 2009). The data were plotted as $\log_{10}$ CFU mL^{-1}.

To determine the phage titration from broth medium and pasteurized milk samples of phage treatments, the samples were centrifuged at 6000×g for 10 min. The supernatants of TSBYE medium samples were then filtered through a 0.45 µm por size filter (Sartorius, Germany), but supernatants of pasteurized milk samples were treated with chloroform (Merck, Germany) at 1% (v/v) after centrifugation to destroy bacterial cells and other potential contaminant bacteria (Arachchi et al., 2013). In this assay, the bacteria free samples of TSBYE medium and pasteurized milk were first serially diluted in PBS later. Phage titers were determined using double agar overlay plaque assay using a TSAYE as described previously (Soni et al., 2010). The plates were incubated at 35 °C for 24 – 48 h until plagues could be enumerated. At the end of the incubation period, the visible plaques were counted, and the data were expressed as $\log_{10}$ PFU mL^{-1}.

Model

Weibull model proposed by Mafart et al. (2002) was used to describe the reduction of *L. monocytogenes* by the phage Listex P100 in this study:

$$\log_{10} S(t) = -\left(\frac{t}{\delta}\right)^n \quad (1)$$

where S(t) is the survival ratio [$S(t) = N_0/N(t)$ with N_0 and N(t) are the initial (t = 0) and momentary (at time t) numbers of *L. monocytogenes*, respectively], δ is the time parameter (in day) which represents time necessary to obtain the first decimal reduction i.e., it is the time required reduce N_0 to $N_0/10$, n is the unitless shape parameter which determines the shape of the survival curve. If $n < 1$ then the shape is concave, if $n = 1$ then a straight line is observed and if $n > 1$ the shape is convex.

Note that different versions of the Weibull model were proposed by different researchers (see Peleg, 1999 and van Boekel, 2002 for example); however, they all produce the same fit since their shape parameters are common (Buzrul, 2007). The above equation was used because time parameter (δ) could be used to understand the phage resistance of *L. monocytogenes* – see below.

The non-linear regression was performed by using SigmaPlot (Version 12, Chicago, IL, USA). The goodness-of-fit of the Weibull model was evaluated by using adjusted determination coefficient (R^2_{adj}) and root mean square error (RMSE) value. Although R^2 is generally used to understand how good the model fits the data, it is not recommended (Granato et al., 2014; Leylak et al., 2020). R^2_{adj} may be preferable since it also considers the number of parameters in the model (Granato et al., 2014). Nevertheless, RMSE is the most informative indices of all (Ratkowsky, 2004; Öksüz & Buzrul, 2020). Smaller RMSE values indicate better the fit.

Statistical analysis

All experiments were repeated three times and all treatments were replicated at least three times in each experiment in this study. The bacterial and phage data were transformed to $\log_{10}$ units. All statistical analyses were carried out using SPSS (Version 16, Chicago, IL, USA). The analysis of one–way variance (ANOVA) followed by

Tukey's test was applied to determine the differences between broth model system and pasteurized milk treated with phage and control. Significant differences between incubation times were also analyzed. A significant difference was defined at $p \leq 0.05$.

3. Results and discussion

The ability of phage P100 against four different *L. monocytogenes* strains in pasteurized milk and fresh TSBYE samples stored at 4 and 30 °C was tested on days 1, 2, 3, 4 and 5 in this study. The sensitivities of *L. monocytogenes* strains to P100 phage were confirmed by soft agar overlay assay. P100 phage produced large plagues of approximately between 1.7 – 2.0 mm in diameter against four different *L. monocytogenes* strains – results not shown. Although *L. monocytogenes* PL2 and PL9 displayed 1.7 mm and 1.9 mm plaque size, *L. monocytogenes* PL3 and PL10 had both 2.0 mm plaque size. No *Listeria* spp. were found in the samples which was confirmed by the microbiological evaluation of pasteurized milk before inoculation with phage P100.

Fig. 1, Fig. 2, Fig. 3 and Fig. 4 show the impact of Listex P100 on *L. monocytogenes* strains PL2, PL3, PL9 and PL10, respectively. The data of the remaining phage titer in both samples after phage treatment at both temperature levels during storage were not determined in this study. More reductions were observed in broth medium than in pasteurized milk regardless of the temperature level where the strain PL2 was the exception at 4 °C. Almost identical reductions were obtained for *L. monocytogenes* PL2 at 4 °C in broth medium and pasteurized milk (Fig. 2b and 2d). Moreover, reductions were significantly ($p \leq 0.05$) higher at 30 °C than at 4 °C for all the strains showing the effectiveness of phage at higher temperature levels. The reductions of *L. monocytogenes* strains by the phage treatment were between 2.7 to 3.4 $\log_{10}$ units at 30 °C and 1.4 to 2.1 $\log_{10}$ units at 4 °C after 4 days of incubation in broth medium whereas 1.9 to 2.9 $\log_{10}$ units and 1.0 to 1.6 $\log_{10}$ units were observed after 4 days of incubation in milk at 30 and 4 °C, respectively. It should be noted that lower reduction effects of phage P100 were found in this study in comparison to previous works (Soni & Nannapani, 2010; Oliveira et al., 2014; Migueis et al., 2017). *L. monocytogenes* counts in both media could not be eliminated by the phage P100 at both temperature levels and this may be explained by several ways. Firstly, it was possible that some compounds such as proteins, carbohydrates, calcium, and phosphorus in pasteurized milk (Tolba et al., 2014) may inhibit the attachment of phages to cells. This could also be the reason of observing lower reductions in milk than in broth. Secondly, applied phage dosage, which is a critical factor, may be insufficient. High dose treatment of phage (> 10^8 PFU mL^{-1}) can be used to eliminate more *L. monocytogenes* viable cells in pasteurized milk. Another reason to have less phage effectiveness may be the development of phage resistant mutants since phage cocktails were not used in this study. Isolation of natural origin listerial phages may be showed high specify against *L. monocytogenes* isolated in Turkey.

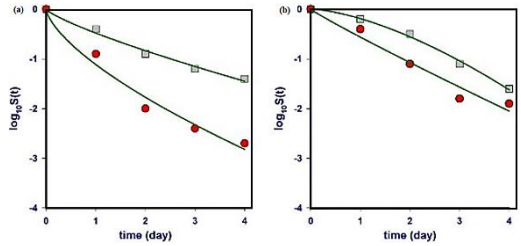

Figure 1. Impact of P100 phage application on *L. monocytogenes* PL2 counts in broth (a) and in pasteurized milk (b). Gray squares and red circles are the experimental data points at 4 and 30 °C, respectively. Solid green lines indicate the fit of the Weibull model [Eq. (1)].

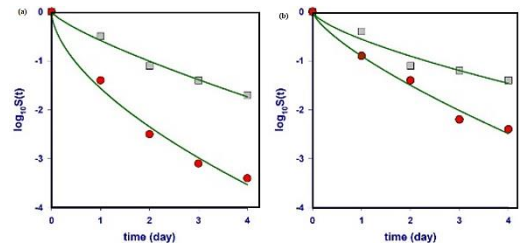

Figure 2. Impact of P100 phage application on *L. monocytogenes* PL3 counts in broth (a) and in pasteurized milk (b). Gray squares and red circles are the experimental data points at 4 and 30 °C, respectively. Solid green lines indicate the fit of the Weibull model [Eq. (1)].

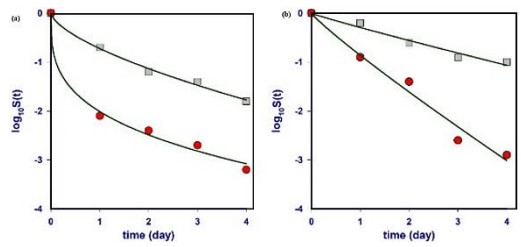

Figure 3. Impact of P100 phage application on *L. monocytogenes* PL9 counts in broth (a) and in pasteurized milk (b). Gray squares and red circles are the experimental data points at 4 and 30 °C, respectively. Solid green lines indicate the fit of the Weibull model [Eq. (1)].

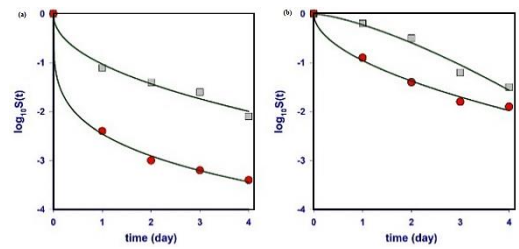

Figure 4. Impact of P100 phage application on *L. monocytogenes* PL10 counts in broth (a) and in pasteurized milk (b). Gray squares and red circles are the experimental data points at 4 and 30 °C, respectively. Solid green lines indicate the fit of the Weibull model [Eq. (1)].

Table 1
Parameters (δ and n) of the Weibull model ± their standard errors and goodness-of-fit indices (R^2_{adj} and RMSE) of each fit

		30 °C				4 °C			
	Strain	δ (day)	n (–)	R^2_{adj}	RMSE	δ (day)	n (–)	R^2_{adj}	RMSE
TSBYE	PL2	0.85 ± 0.20	0.67 ± 0.12	0.969	0.198	2.51 ± 0.12	0.80 ± 0.10	0.985	0.072
	PL3	0.47 ± 0.11	0.59 ± 0.07	0.986	0.164	1.98 ± 0.12	0.78 ± 0.08	0.988	0.076
	PL9	0.10 ± 0.05	0.31 ± 0.05	0.990	0.122	1.65 ± 0.10	0.65 ± 0.08	0.992	0.061
	PL10	0.03 ± 0.01	0.24 ± 0.02	0.998	0.067	0.94 ± 0.18	0.45 ± 0.08	0.980	0.110
Milk	PL2	1.86 ± 0.24	0.93 ± 0.20	0.951	0.186	2.93 ± 0.06	1.55 ± 0.10	0.994	0.049
	PL3	1.15 ± 0.16	0.73 ± 0.10	0.982	0.131	2.31 ± 0.27	0.69 ± 0.18	0.936	0.150
	PL9	1.18 ± 0.20	0.90 ± 0.15	0.969	0.212	3.74 ± 0.26	0.93 ± 0.17	0.961	0.085
	PL10	1.10 ± 0.13	0.52 ± 0.06	0.989	0.081	2.90 ± 0.14	1.40 ± 0.22	0.970	0.111

Although the ability of phage P100 against four different *L. monocytogenes* strains in fresh broth medium and pasteurized milk samples stored at 4 and 30 °C was tested up to 5 days (results not shown), reductions up to 4 days were displayed. This is because slight increases were observed at day 5 (0.2 – 0.6 $\log_{10}$ units at 30 °C and 0.1 – 0.2 $\log_{10}$ units at 4 °C in broth, and 0.2 – 0.4 $\log_{10}$ units at 30 °C and 0.1 – 0.3 $\log_{10}$ units at 4 °C). Hence, for modeling purposes data up to 4 days were used.

The fit of the Weibull model [**Eq. (1)**] are also shown in **Fig. 1, Fig. 2, Fig. 3 and Fig. 4**. It could be said that model produced reasonable fit to the data which can be also judged by the high R^2_{adj} (≥ 0.936) and low RMSE (≤ 0.212) values given in Table 1. Furthermore, survival curves obtained in broth medium were all concave (**Fig. 1a, Fig. 2a, Fig. 3a and Fig. 4a**) indicating that the shape parameters (n) were less than 1 (**Table 1**). On the other hand, both concave (n < 1) and convex (n > 1) survival curves were observed in pasteurized milk. In fact, some of the concave survival curves obtained in milk were close to linear. For example, strains PL2 (**Fig. 1b**), and PL9 at 4 and 30 °C (**Fig. 3b**) were almost linear with n ≥ 0.9 (Note that survival curves are linear if n = 1).

Time parameter (δ) was used to differentiate the phage resistances of the strains. Low *δ* values revealed sensitive strains whereas resistant strains had higher δ values (**Table 1**). It was also possible to evaluate the higher reductions in broth than in milk by comparing the δ values given in **Table 1**. Much lower δ values were estimated by the model fits in broth medium. Moreover, at low temperature higher δ values were observed which indicates reduction at 4 °C was more difficult than 30 °C. Overall, it could be said that PL2 was the most phage-resistant *L. monocytogenes* strain while PL10 was the most sensitive one in broth at both temperature levels (**Table 1**). Maximum reduction in viable counts for the strain PL10 in broth were 3.4 and 2.1 $\log_{10}$ units after 4 days at 30 and 4 °C, respectively. On the other hand, in milk although PL2 was still the most phage-resistant *L. monocytogenes* strain at 30 °C, PL9 was the most-resistant one at 4 °C. Only 1 $\log_{10}$ reduction was observed for PL9 in milk at 4 °C.

Time parameter (δ) of the Weibull model was a good indicator to evaluate the phage-resistances of the strains (**Table 1**); however, it represents only the first decimal reduction (Note that time necessary for 2 $\log_{10}$ reduction is not 2·δ, but $2^{1/n}$ δ. Similarly, time for "m" number of reduction is $m^{1/n}$ δ). It may be a better approach to compare resistances based on the time required for 5 or 6 $\log_{10}$ reduction, but maximum reduction attained in this study was 3.4 $\log_{10}$ (strain PL10 in broth at 30 °C). In some cases, only 1 $\log_{10}$ reduction was observed (strain PL9 in milk at 4 °C). Moreover, the initial inoculum was about 10^6 CFU mL^{-1} for all strains. This is, of course, higher than the expected contamination in pasteurized milk hence δ can be a good option to better understand the phage-resistances of the strains in this study. Comparison of the δ values given in **Table 1** revealed that huge variabilities between strains were possible. Therefore, calculations should be done according to the most phage-resistant strain. In addition, resistant phage in a medium may be sensitive in another medium and this was also observed in this study. PL2 was the most phage-resistant strain among all in broth at 4 °C; however, it was the second after the strain PL9 in milk at the same temperature (**Table 1**).

Shape parameters (n) listed in **Table 1** and survival curves shown in **Fig. 1**, **Fig. 2**, **Fig. 3** and **Fig. 4** indicated both concave (n < 1) and convex (n > 1) survival curves. This may be because of temperature and medium (**Peleg & Cole, 2000**). For example, in broth medium only concave survival curves were observed at 30 and 4 °C. On the other hand, in pasteurized milk although concave survival curves were abundant some of them were close to linear (**Fig. 1b** and **3b**). In addition, convex survival curves in milk at 4 °C were also obtained (**Fig. 1b** and **4b**).

Biocontrol assay against contamination by L. *monocytogenes* by using phages is a promising tool of green strategy. The use of phages is also a viable alternative to chemical antimicrobials against foodborne pathogens. Earlier works on using phage to control the growth of *L. monocytogenes* were done in fresh–cut fruits and vegetables (**Leverentz et al., 2003**), honeydew melon (**Leverentz et al., 2004**), fresh–cut apples (**Leverentz et al., 2006**), cooked ham (**Holck & Berg, 2009**), in RTE poultry (**Bigot et al., 2011**), soft ripened white mold and red–smear cheeses (**Guenther & Loessner, 2011**), lettuce, apples, cheese, smoked salmon and frozen foods (**Perera et al., 2015**) and chicken breast (**Yang et al., 2017**).

Phage P100, which was received GRAS status for application in all foods (**Guenther et al., 2009**), has been used effectively against *L. monocytogenes* in soft cheeses (**Carlton et al., 2005**), RTE foods (**Guenther et al., 2009**), catfish fillets (**Soni et al., 2010**), raw salmon fillet tissue (**Soni & Nannapani, 2010**), fresh sausage (**Rossi et al., 2011**), queso fresco cheese (**Soni et al., 2012**), RTE sliced deli meat products, (**Prabhu et al., 2016**), fresh cut fruits and fruit juices (**Oliveira et al., 2014**), soft cheeses (**Silva et al., 2014**), dry–cured ham (**Gutierrez et al., 2017**) and sashimi (**Migueis et al., 2017**) so far. Although *L. monocytogenes* is

effectively controlled by pasteurization, its presence in the finished product is possible because of post–pasteurization contamination from sources in the milk processing plant environment or by the operator error. Even very low numbers of *L. monocytogenes* in processed dairy products can multiply rapidly to reach dangerous levels despite the proper refrigeration (**Xanthiakos et al., 2006**). To the best of our knowledge, little is known about the efficacy of phage P100 to control of *L. monocytogenes* strains in pasteurized milk. A notable example is the work of **Lee et al. (2017)** and **Komora et al. (2020)**. However, to test of the lytic ability of the phage against *E. coli* strains, some researchers have been carried out in milk and milk fermentation so far (**Mclean et al., 2013**; **Tomat et al., 2013**; **Tolba et al., 2014**).

In all assays performed, application of phage P100 in both samples was able to reduce *L. monocytogenes* cells at both temperature levels when the initial number of *Listeria* cells was about 10^6 CFU mL^{-1}. It was interesting that, on day 5, there was no effect on *L. monocytogenes* populations with treatment phage P100 in both samples.

4. Conclusions

Listex P100 was used to reduce *L. monocytogenes* strains in broth and milk in this study. Reductions up to 3.4 $\log_{10}$ in broth and 2.9 $\log_{10}$ in milk were achieved. Survival data was successfully described by the Weibull model and the time parameter of the Weibull model was used to evaluate the phage-resistances of the strains. The results obtained in this study were noteworthy because the use of phages for biocontrol of *L. monocytogenes* in milk has received little attention. Further studies should be carried out at different inoculum levels of both bacterium and phage, and with the use of combined natural chemicals such as nisin.

Conflict of interest

The authors express no conflict of interest associated with this work

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