Research Paper

Control of *Listeria monocytogenes* growth in soft cheeses by bacteriophage P100

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Abstract

The purpose of this study was to determine the effect of bacteriophage P100 on strains of *Listeria monocytogenes* in artificially inoculated soft cheeses. A mix of *L. monocytogenes* 1/2a and Scott A was inoculated in Minas Frescal and Coalho cheeses (approximately 10^5 cfu/g) with the bacteriophage added thereafter (8.3×10^7 PFU/g). Samples were analyzed immediately, and then stored at 10 °C for seven days. At time zero, 30 min post-infection, the bacteriophage P100 reduced *L. monocytogenes* counts by 2.3 log units in Minas Frescal cheese and by 2.1 log units in Coalho cheese, compared to controls without bacteriophage. However, in samples stored under refrigeration for seven days, the bacteriophage P100 was only weakly antilisterial, with the lowest decimal reduction (DR) for the cheeses: 1.0 log unit for Minas Frescal and 0.8 log units for Coalho cheese. The treatment produced a statistically significant decrease in the counts of viable cells ($p < 0.05$) and in all assays performed, we observed an increase of approximately one log cycle in the number of viable cells of *L. monocytogenes* in the samples under refrigeration for seven days. Moreover, a smaller effect of phages was observed. These results, along with other published data, indicate that the effectiveness of the phage treatment depends on the initial concentration of *L. monocytogenes,* and that a high concentration of phages per unit area is required to ensure sustained inactivation of target pathogens on food surfaces.

Key words: food safety, *Listeria,* biocontrol, bacteriophage, cheese.

Introduction

Minas Frescal and Coalho are very popular traditional cheeses in Brazil. They are classified as soft cheeses and produced by enzymatic coagulation of cow's milk with rennet and/or other appropriate enzymes, and are either complemented or not by the action of specific lactic bacteria.

Reports have indicated that these food products are susceptible to recontamination with *L. monocytogenes* after heat processing (Cox, 1989; Rocourt and Cossart, 1997). Because *L. monocytogenes* is capable of growing at refrigeration temperatures, antimicrobial strategies to overcome the tolerance of this microorganism for low temperatures are essential, and the industry has sought more effective methods to combat this pathogen.

Despite recent advancements in technologies to control pathogen in foods, consumers have been searching for "natural" foods; *i.e.*, foods submitted to less aggressive treatments and free of chemical preservatives (Pranoto *et al.*, 2005). Among these substances, we found the bacteriophages, which are viruses that infect only bacteria and do not cause changes in the food flavor, color or other physical properties (Pao *et al.*, 2004).

Bacteriophages are naturally occurring viruses that are suitable candidates for the environmentally friendly biocontrol of pathogens (Guenther *et al.*, 2009). They do not have their own metabolism, but rely on host bacteria to multiply and exhibit narrow host ranges, usually targeting specific strains (Guenther *et al.*, 2009).

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Phages are omnipresent and accidentally, yet regularly, consumed through ingestion of water and food. For this reason, they are presumed to be safe, since undesirable effects have not been reported. Their benign nature and specificity make them excellent tools for food safety applications (Mahony *et al.*, 2010).

Recently the USFDA announced that it had approved the use of a bacteriophage preparation made from six individually purified bacteriophages to be used on ready-to-eat meat and poultry products as an antimicrobial agent against *L. monocytogenes* (EBI Food Safety, 2007). The commercial product named LISTEX_ P100 was approved as a food biopreservative and granted GRAS (Generally Recognized as Safe) status (Food and Drug Administration, 2006).

The recent approval for the use of phages on *L. monocytogenes* for food safety purposes has increased the impetus for phage research to uncover phage-mediated applications with activity against other food pathogens (Mahony *et al.*, 2010).

Considering that the contamination of dairy products (especially soft cheeses) by *Listeria* spp. is most often caused by faulty or insufficiently sanitized equipment (Taylor *et al.*, 1981), factory-specific flora could be found. Although many foods can serve as vehicles for this pathogen, *Listeria* is often isolated from ready-to-eat (RTE) foods, such as milk and cheeses; cold-cut meats; smoked fish; seafood; and vegetables (Ryser and Marth, 2006). Of particular concern is the fact that these foods are consumed directly, without a final bactericidal processing step.

The present study was undertaken to evaluate the inhibitory effect of the bacteriophage P100 on strains of *L. monocytogenes* in artificially inoculated Minas Frescal and Coalho cheeses at time zero, 30 min post-infection, and after seven days under refrigeration.

Materials and Methods

Cheeses

Minas Frescal is classified as a soft cheese with very high moisture, raw mass and a soft or lightly acidic flavor. Coalho is a curdle cheese, classified as has middle range humidity at half-cooking or cooking mass.

The distinctive characteristics of the elaboration process of the cheeses include approximately 40 min of coagulation, cutting and mixing of the mass, partial removal of the serum, heating of the mass with hot water or indirect steam (Coalho cheese) until attainment of half-cooking (45 °C) or cooking mass (between 45 and 55 °C), addition of salt (sodium chloride) to the mass, pressing, drying, packaging and sale. These products should be stored between 1.0 and 10 °C, and consumed within seven days after opening (Brasil, 1997; Brasil, 2001).

Bacterial strains, media and culture conditions

The bacteria used in this study were as follows: a *Listeria monocytogenes* (serotype 1/2a) isolated from raw milk in a cheese manufacturing plant of Bahia state, Brazil; *Listeria monocytogenes* Scott A - ATCC 15313 (serotype 4b); *Listeria ivanovii* WSLC 3009 (SLCC 4769); and bacteriophage P100 (LISTEX P100), provided by EBI Food Safety (Wageningen, The Netherlands) (Food and Drug Administration, 2006). *L. ivanovii* was used as a helper strain to determine the titer of the P100 bacteriophage (Loessner and Busse, 1990; Carlton *et al.*, 2005).

The cultures of *L. monocytogenes* and *L. ivanovii* were stored in Hogness medium (1.3 mM K_2HPO_4. $3H_2O$ 1.3 mM KH_2PO_4, 2.0 mM citrate-Na.2 H_2O, 1.0 mM $MgSO_4$.7 H_2O, and 4.4% (v/v) glycerol) and frozen at -80 °C. Before use, *L. monocytogenes* cultures were activated in tryptic soy broth (Difco, Detroit, MI, USA) supplemented with 0.5% (w/v) of yeast extract (Difco, Detroit, MI, USA) (TSB-YE) and incubated at 35 °C overnight in a shaker (Cientec, model CT 712, Brazil) at 150 rpm. *L. ivanovii* culture was grown overnight at 30 °C in a half-concentrated brain-heart infusion broth (BHI, 1/2 v/v, Difco, Detroit, MI, USA) with the NaCl concentration adjusted to 5 g/L.

In all experiments, the top layer agar (semi-soft agar or overlay agar) was prepared by adding 0.4% (w/v) of agar to BHI. To improve the visualization of bacteriophage plaques, 0.75% (w/v) of glycine (Sigma Aldrich - Poole, United Kingdom) was added to the top layer of agar (Lillehaug, 1997). Appropriate bacterial dilutions were made in lambda buffer (6 mM Tris buffer, pH 7.2; 10 mM Mg $(SO_4)_2.7H_2O$; and 50 μg/mL gelatin).

Bacterial survival following treatment with the bacteriophage P100 or no treatment was determined by colony-forming units (CFU/g) on lithium chloride-phenylethanol-moxalactam (LPM, Difco, Detroit, MI, USA) agar with 0.1% of esculin and 0.05% of ferric citrate ammoniac.

Titration of P100 bacteriophage

The titer of the P100 was determined according to a protocol suggested by EBI Food Safety (personal communication). For this protocol, serial dilutions of the bacteriophage suspension in a lambda buffer were made, and 100 μL of each dilution were mixed into 3.5 mL of the molten overlay agar cooled to 45 °C, which contained 150 μL of log-phase *L. ivanovii* culture grown overnight at 30 °C in a 1/2 strength BHI. This mixture was poured onto BHI agar (1.2% w/v agar) plates and incubated at 30 °C for 20-24 h. Plaques were counted, and the titer was determined in plaque-forming units (PFU).

To recover the bacteriophage from food without *L. monocytogenes* (phage control), the sample was diluted in lambda buffer, and an aliquot of 100 μL was incorporated into 3.5 mL of the molten overlay agar cooled to 45 °C,

which contained 150 μL of *L. ivanovii* (helper strain). As mentioned above, the mixture was poured onto BHI agar (1.2% w/v agar) plates and incubated at 30 °C for 20-24 h. Plaques were counted, and the titer was determined as plaque forming units (PFU/g).

Preparation of bacterial inoculums

L. monocytogenes 1/2a and Scott A (serotype 4b) were cultured by loop inoculation of 10 mL volumes of tryptic soy broth containing 0.5% (w/v) yeast extract (TSB-YE), followed by incubation at 35 °C for 18-20 h in a shaker at 150 rpm. The cell suspensions were transferred to sterile Eppendorf tubes, and inoculum levels were confirmed by the surface plating of duplicate samples onto LPM agar. The plates were incubated at 35 °C for 24 h before the colony counts were obtained. Cell suspensions were diluted in an appropriate amount of 0.1% (w/v) of peptone water to yield a cell number of 10^5 cfu/mL, and were used immediately for sample inoculation.

Sample inoculation and treatment application

Minas Frescal and Coalho cheeses were purchased at a local supermarket in Salvador, Bahia, Brazil. The samples were transported to the laboratory in insulated, iced containers for analysis. After aseptically removing the package under a class II biosafety cabinet (Labconco, model 36210 class BII, Kansas City, MO, USA), four samples with 30 g of Minas Frescal cheese and four samples with 30 g of Coalho cheese were transferred to a bag mixer. Two samples of each cheese were designated as time zero samples and two were designated as seven-day samples. All samples were inoculated separately with 1 mL of the mixture of *L. monocytogenes* 1/2a and Scott A (approximately 10^5 cfu/mL). Inoculated samples were homogenized gently by manual shaking to ensure an even distribution of organisms, and were air dried under a class II biosafety cabinet for 30 min at 21 °C to allow the attachment of bacteria into the cheese (Singh *et al.*, 2002).

Two samples of each cheese (one time zero sample and one seven-day sample) were treated with 1 mL volumes of the P100 bacteriophage (2.5 x 10^9 PFU/mL) to yield a final application dose of 8.3 x 10^7 PFU/g, and two of the samples were designated as controls (one for time zero and one for seven days). For the controls, 1 mL of saline solution (0.85% w/v NaCl) was added to the two samples. The samples were kept inside a stomacher bag, sealed and incubated at room temperature (21 °C) for 30 min to allow for infection. One of the samples was immediately subjected to *L. monocytogenes* enumeration on LPM agar, and the other was stored at 10 °C for seven days.

For enumeration of *L. monocytogenes*, each 30 g portion was added to 270 mL of lambda buffer with 0.01% (w/v) of Tween-80 and homogenized in a stomacher (ITR, model 1204, series 126, Esteio, RS, Brazil, 240 bpm) for 2 min. Serial dilutions were made in the same solution without Tween-80, and the solutions were spread onto duplicate LPM plates, which were incubated at 35 °C for 48 h. Bacterial survival following treatment with P100 bacteriophage or no treatment (control) was determined by measuring colony-forming units (CFU).

Investigation of *Listeria* spp. in Minas Frescal and Coalho cheeses

The cheese samples were analyzed for the presence or absence of *Listeria* spp. before inoculation with *L. monocytogenes* strains by streaking Half Fraser enrichment broth (Difco, Detroit, MI, USA) on LPM agar (DIFCO) plates to test for the presence of typical *Listeria* spp. colonies. One *L. monocytogenes* positive control (*L. monocytogenes* Scott A, ATCC 15313) and one uninoculated media negative control were used for each set of concurrently analyzed samples.

Statistical analysis

To investigate the effectiveness of bacteriophage P100 in the elimination of *L. monocytogenes* 1/2a and Scott A, bacterial counts were always determined in duplicate, and all of the experiments described here were independently performed three times. All counts of bacteria (cfu/g) recovered from Minas Frescal and Coalho cheeses were converted to logarithms before computing means and standard deviations. The decimal reduction (DR) of the population of the bacteria was calculated by the difference between the counts obtained in the control and treatment preparations. Log values of zero were assumed for samples in which *L. monocytogenes* was not detected (< 2 log cfu/g). The data were subjected to the Statistical Analysis System of variance and Tukey's multiple range (Software ASSISTAT, version 7.6 beta, 2011) to determine if significant differences ($p < 0.05$) in mean log values existed in the populations of *L. monocytogenes* between the treatment groups.

Results

The microbiological evaluation of Minas Frescal and Coalho cheeses before inoculation with *L. monocytogenes* revealed an absence of *Listeria* spp. in the samples investigated.

The effect of bacteriophage P100 (LISTEX P100) on the survival of *L. monocytogenes* strains was evaluated by measurement of viable cell counts and the innoculum used was 10^5 cfu/mL (5.0 log cfu/mL), approximately. The results obtained for Minas Frescal and Coalho cheeses are presented in Tables 1 and 2, respectively. The treatment decreased the cell numbers of *L. monocytogenes* at time zero, 30 min post-infection, and at seven days at 10 °C, relative to cheeses containing no phages. The results indicate that the P100 bacteriophage may reduce the number of *Listeria* cells in soft cheeses.

Table 1 - Reduction in the viable counts of *Listeria monocytogenes* in Minas Frescal cheese by bacteriophage P100, in a mix of 1/2a and Scott A serovars, at zero and seven days (10 °C).

Experiment	*Listeria monocytogenes* (Log$_{10}$ cfu/g)					
	Time zero			Seven days		
	Control	Treatment[b]	DR[c]	Control	Treatment[b]	DR[c]
1	5.9	3.6	2.3	6.6	5.6	1.0
2	6.0	3.5	2.5	6.5	5.3	1.2
3	5.1	3.4	1.7	6.5	5.6	0.9
Mean[a]	5.8 ± 0.4^{A*}	3.5 ± 0.08^{B}	2.3	6.5 ± 0.04^{A}	5.5 ± 0.14^{B}	1.0

[a]Mean ± standard deviation. [b]Bacteriophage P100 (title 2.5 x 10^9 PFU/mL). [c]Decimal reduction. *Means followed by different letters are significantly different by Tukey's test (p < 0.05).

Table 2 - Reduction in the viable counts of *Listeria monocytogenes* in Coalho cheese by bacteriophage P100, in a mix of 1/2a and Scott A serovars, at zero and seven days (10 °C).

Experiment	*Listeria monocytogenes* (Log$_{10}$ cfu/g)					
	Time zero			Seven days		
	Control	Treatment[b]	DR[c]	Control	Treatment[b]	DR[c]
1	5.1	3.1	2.1	6.5	5.6	0.8
2	5.6	3.6	2.3	6.6	5.7	1.1
3	5.6	3.3	1.7	6.4	5.8	0.8
Mean[a]	5.5 ± 0.2^{A*}	3.4 ± 0.2^{B}	2.1	6.5 ± 0.08^{A}	5.7 ± 0.08^{B}	0.8

[a]Mean ± standard deviation. [b]Bacteriophage P100 (title 2.5 x 10^9 PFU/mL). [c]Decimal reduction. *Means followed by different letters are significantly different by Tukey's test (p < 0.05).

A mean reduction of greater than 2.0 log cycles in viable counts was observed at time zero: 6.6 x 10^5 to 3.2 x 10^3 cfu/g for Minas Frescal cheese, and 2.9 x 10^5 to 2.3 x 10^3 cfu/g for Coalho cheese. However, in samples stored under refrigeration for seven days, the bacteriophage P100 was only weakly antilisterial, with the lowest decimal reduction (DR) for the cheeses: approximately 1.0 log unit (3.4 x 10^6 to 3.5 x 10^5 cfu/g) for Minas Frescal and 0.8 log units (3.2 x 10^6 to 4.7 x 10^5 cfu/g) for Coalho. In untreated samples, the numbers of *L. monocytogenes* exceeded 6.0 log cfu/g.

For both cheeses, the statistical analysis indicated a significant difference (p < 0.05) between the treated samples and control, at zero and seven days (Tables 1 and 2).

The results obtained from the control samples of bacteriophage alone indicate that the concentration of P100 was lowest after seven days of storage: mean value of 4.4 x 10^7 PFU/g ± 0.12 at zero day and 3.0 x 10^4 PFU/g ± 0.13 at seven days for Minas Frescal cheese, and 4.8 x 10^8 PFU/g ± 0.05 at zero day and 4.2 x 10^5 PFU/g ± 0.13 at seven days for Coalho cheese.

Discussion

Previous studies have demonstrated that cheese is a potential reservoir for *L. monocytogenes* because of the higher availability of nutrients on cut cheese surfaces and the greater potential for contamination due to the increased amount of handling (James *et al.*, 1983; Schlech *et al.*, 1983; Beckers *et al.*, 1987; Farber *et al.*, 1987; Pintado *et al.*, 2004; Makino *et al.*, 2005;). In our country, the incidence of *L. monocytogenes* in three cheese manufacturing plants from the northeastern region of Sao Paulo was evaluated recently, and *L. monocytogenes* was isolated from two of the three plants. *L. monocytogenes* was also isolated from the surface of Prato cheese and brine from one of the evaluated plants. Serotype 4b predominated in the plants studied, and these results indicate the need of control strategies to prevent the dispersion of *L. monocytogenes* in the environment of cheese manufacturing plants (Barancelli *et al.*, 2011).

This paper reports valuable information about the antimicrobial properties of bacteriophage P100 against two serotypes of *L. monocytogenes*. Antimicrobial effects are measured in terms of decimal reduction, which allows for comparisons between different studies.

Our results have demonstrated that the bacteriophage P100 reduced the population of *L. monocytogenes* in Minas Frescal and Coalho cheeses by 2.3 and 2.1 log cycles, respectively, following the addition of the phage. These results are in accordance with Leverentz *et al.* (2003), who reported that lytic *L. monocytogenes*-specific phages reduced *L. monocytogenes* populations on honeydew melons by 2.0 to 4.6 log units relative to the control, and Rossi *et al.*

(2011) who showed that the bacteriophage P100 reduced L. monocytogenes counts by 2.5 log units in Brazilian fresh sausage.

According to Leverentz *et al.* (2003), the effectiveness of the phage treatment depended on the initial concentration of *L. monocytogenes.* Similarly, Bigot *et al.* (2011) reported that a bacteriophage similar to phage A511 prevented the growth of *L. monocytogenes* on the surface of a vacuum-packed, ready-to-eat chicken breast roll. In that study, the bacteria were present at approximately twice its concentration for 7 h, and the authors observed an immediate 2.5 $\log_{10}$ cfu cm^{-2} reduction in pathogen concentration following the addition of phages, and then re-growth.

Carton *et al.* (2005) contaminated cheeses with low concentrations of *L. monocytogenes* at the beginning of the ripening period, and P100 was applied to the surface during the rind washings. Depending on the time points, frequency and dose of phage applications, the authors were able to obtain a significant reduction (at least 3.5 log units) or complete eradication of *Listeria* viable counts.

Soni and Nannapaneni (2010) also investigated the effectiveness of bacteriophage P100 against *L. monocytogenes* serotypes 1/2a and 4b on the surface of raw salmon fillet tissue in a broth model system. According to the authors, phage P100 completely inhibited *L. monocytogenes* growth at 4 °C for 12 days, at 10 °C for 8 days, and at 30 °C for 4 days, and at phage concentrations of 10^4, 10^6, and 10^8 PFU/mL. However, a higher phage concentration of 10^8 PFU/g was required to yield 1.8, 2.5, and 3.5 log cfu/g reductions of *L. monocytogenes* from initial loads of 2.0, 3.0, and 4.5 log cfu/g at 4 or 22 °C, respectively.

One study reported that the reduction in *L. monocytogenes* counts on raw catfish fillet tissue with the P100 phage (2×10^7 PFU/g) was 1.4-2.0 log cfu/g at 4 °C, 1.7-2.1 log cfu/g at 10 °C, and 1.6-2.3 log cfu/g at room temperature (22 °C). The phage contact time of 30 min (the same time used in our study) was adequate to yield a greater than 1.0 log cfu/g reduction in *L. monocytogenes*, whereas a 15 min contact time with phage yielded less than a 1.0 log cfu/g bacterial load reduction (Soni *et al.*, 2010).

In all assays performed, we observed an increase of approximately 1.0 log cycle in the number of viable cells of *L. monocytogenes* in the samples under refrigeration for seven days. Moreover, a lesser effect of phages was observed. This result demonstrated the influence of the storage of the cheeses.

Rossi *et al.* (2011) and Bigot *et al.* (2011) also demonstrated that the populations of *L. monocytogenes* increased over the storage. According to Bigot *et al.* (2011), the re-growth of *L. monocytogenes* in a ready-to-eat chicken breast roll occurred such that the concentration after 24 h of incubation was similar in both control and phage-treated cultures. However, the authors demonstrated that at a temperature in which a chilled food might be stored (5 °C), bacterial regrowth was prevented over a 21-day incubation.

Our results indicate that there are phages remaining in the samples after seven days (shelf life of the cheeses after opening), but the concentration of phage particles was insufficient to infect *Listeria* cells present and to show the same effect demonstrated 30 min post-infection (time zero).

The successful phage infection and subsequent killing of the host cells is strongly dependent on the environmental conditions, *i.e.*, the type of food and its specific matrix (Guenther *et al.*, 2009). According to the authors, the proportion of bacterial cells that can be infected depends on several parameters: First, the binding of phages to their receptors on the bacterial surfaces is influenced by intrinsic factors, such as ionic strength, pH, and substances, which may interfere with this process. These parameters are largely defined by the food itself and may change during the production, ripening, or storage of the items. Second, the concentration of phage at the time of application is crucial for efficacy, *i.e.*, applying more phage generally resulted in greater inactivation.

Conclusions

Our findings demonstrate the efficacy of bacteriophage P100 for rapid reduction of *L. monocytogenes* on soft cheeses followed by a weakly antilisterial effect after storage. These results, along with other published data, indicate that the effectiveness of the phage treatment depends on the initial concentration of *L. monocytogenes* and that a high concentration of phages per unit area is required to ensure sustained inactivation of target pathogens on food surfaces.

Acknowledgments

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