



Food Microbiology

Antibacterial efficacy of nisin, bacteriophage P100 and sodium lactate against *Listeria monocytogenes* in ready-to-eat sliced pork ham



Ana Cláudia L. Figueiredo^a, Rogeria C.C. Almeida^{b,*}

^a Faculdade de Farmácia, Universidade Federal da Bahia, Rua Barão de Geremoabo, s/n, Ondina, Salvador, BA, Brazil

^b Departamento de Ciência dos Alimentos, Escola de Nutrição, Universidade Federal da Bahia, Salvador, BA, Brazil

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ABSTRACT

The effectiveness of bacteriophage P100, nisin and sodium lactate, individually and in combination, in inhibiting *Listeria monocytogenes* in ready-to-eat pork ham slices was assessed. The antimicrobials were applied to the surfaces of ready-to-eat pork ham slices, which were inoculated with a mixture of *L. monocytogenes*. Among the individual antimicrobial treatments, bacteriophage P100 was the most effective, decreasing *L. monocytogenes* to undetectable levels at zero and 72 h post-infection. Sodium lactate was the least effective treatment. Treatment with nisin at zero h significantly reduced initial cell density ($p < 0.05$). However, this pattern was not observed at 72 h of storage. A significant difference ($p < 0.05$) existed between the results of separate bacteriophage and nisin treatments after refrigerated storage, but not immediately upon inoculation of the bacteria. The results showed that the use of bacteriophage P100 is the method of choice for the control of bacteria.

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Introduction

The first conclusive connection of *Listeria monocytogenes* with a foodborne outbreak occurred in 1981, stimulating researchers to determine the ubiquity of the organism and its mode of transmission. Since then, when the mortality rate did not seem to diminish over years, *L. monocytogenes* has gained recognition as an important foodborne pathogen.¹

Although *L. monocytogenes* is inactivated by thermal treatments in processed food, post-processing cross-contamination from equipment and the environment may

occur due to the pathogen's persistence and ability to form biofilms.^{2,3}

Considering that *L. monocytogenes* is capable of growing at refrigerated temperatures, antimicrobial strategies to overcome this microorganism's tolerance for low temperatures are essential, and the food industry has sought more effective methods to control this pathogen.⁴

Despite advances in hurdle technology, food preservation techniques are still evolving, not only in developing countries but also in the industrialized world. The concept of hurdle technology has been applied in the food industry following the observations that the rate of microorganism survival

* Corresponding author at: Escola de Nutrição, Universidade Federal da Bahia, Salvador, BA, Brazil.

E-mail: rogeriac@ufba.br (R.C. Almeida).

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decreases greatly when they are confronted with multiple antimicrobial factors, or hurdles.⁵

Currently, the application of bacteriocins as part of hurdle technology has gained attention. Bacteriocins are antimicrobial active peptides or proteins synthesized in the ribosomes of bacterial cells and secreted by some bacteria against microorganisms that are closely related to the producer organism.⁶ They are bacteriostatic or bactericidal against Gram-positive bacteria,^{7–10} but bacteriocins synthesized by Gram negative bacteria (e.g. colicins) are active against Gram negative bacteria. These antimicrobial peptides have been traditionally used as bio-preservatives to extend the shelf lives of food products without compromising their nutritional and organoleptic properties^{7,11,12} and they have already been successfully applied in several food systems to control the growth of *L. monocytogenes*.^{13,14}

Several bacteriocins show synergism when used in combination with other antimicrobials, including chemical preservatives, phenolic compounds, and other natural antimicrobial proteins.^{15,16} The effectiveness of bacteriocins is often dependent upon environmental factors such as pH and temperature, interaction with food components, precipitation, inactivation, or uneven distribution of bacteriocin in the food matrix, and food microbiota.¹⁷

Nisin, the bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, has been successfully used as an antibacterial agent in various food products.^{8,16} Currently, nisin is the only bacteriocin widely used as a food preservative, and it has been accepted by the World Health Organization as a food bio-preservative.^{6,15} However, its successful application in meat systems has been limited due its interaction with phospholipids, low solubility and inactivation by endogenous meat enzymes.¹⁵

Another strategy to ensure food safety is the use of bacteriophages, which are viruses that infect and kill bacteria. Phages are components of the natural microflora that are present throughout food production, from the farm to the retail outlet.¹⁷ They are stable and recovered from soil, sewage, water, farm and processing plant effluents, feces, and retail foods.^{17,18} The USFDA has approved a bacteriophage preparation made from six individually purified bacteriophages for use as an antimicrobial agent against *L. monocytogenes* on ready-to-eat meat and poultry products.¹⁹ The commercial product named LISTEX P100 was approved as a food bio-preservative and granted GRAS (Generally Recognized as Safe) status.²⁰

Organic acids with short chains and/or their salts are frequently used as chemical decontaminants and have also been granted GRAS status.^{21,22} Organic acids cross the cell membrane in their undissociated form and dissociate in the cytoplasm, causing a decrease in intracellular pH, which significantly impacts cell metabolism, resulting in reduced growth.^{23,24} They are extensively used in the meat and poultry industries to enhance antimicrobial benefits.^{25–27}

The antimicrobial effects of the salts of organic acids, either alone or in combination with other food additives, on the survival and growth of *L. monocytogenes* has been examined and reported^{4,27–30}; however, little is known about their effects in combination with other antimicrobial substances. The growth

of *L. monocytogenes* in solutions containing sodium lactate depends mainly on a product's water content, storage temperature and, to a lesser extent, the amount of nitrite that it contains.²⁹

The present study was undertaken to evaluate the inhibitory effect of nisin, alone and in combination with sodium lactate and bacteriophage P100, on a mixture of two strains of *L. monocytogenes* in artificially inoculated ready-to-eat sliced pork ham both immediately and after three days under refrigerated temperature.

Materials and methods

Pork ham product: Approximately 500 g of ready-to-eat pork ham slices were collected at a local supermarket of Salvador, BA, Brazil, and transported in iced containers to the laboratory for analysis. The product was sliced, weighed and packaged immediately before purchased (three days of shelf life).

Nisin: Nisin from *L. lactis* was purchased from Sigma–Aldrich (Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil, ref. n° 5764). A stock solution was prepared with 0.1 g of nisin and 10 mL of 0.02 M HCl filter-sterilized through a 0.22- μ m membrane (Millipore). Before each experiment, nisin was diluted with 10 mM phosphate buffer (pH 6.4) to 50 μ g/L.¹⁰

Sodium lactate: Commercial sodium DL-lactate (50% purity, U.S.P.) was obtained from Synth (Synth Brasil Ltda, São Paulo, SP, Brazil). A stock solution at 2% (v/w) was prepared and sterilized at 121 °C for 15 min.

Bacteriophage P100: LISTEX™ was purchased from EBI Food Safety (Nieuwe Kanaal 7P, 6709 PA Wageningen, The Netherlands). Before use, the titer of phage P100 was determined according to a protocol suggested by EBI Food Safety (personal communication).

Bacteria, phage and growth conditions

L. monocytogenes, serotype 1/2a (B7, AL48/15, institutional strain collection) isolated from ready-to-eat turkey breast slices in a previous study in our laboratory and *L. monocytogenes* strain Scott A – ATCC 15313 (serotype 4b) were used to inoculate samples of ready-to-eat pork ham slices. *L. ivanovii* WSLC 3009 (SLCC 4769, institutional strain collection) was used as a helper strain to determine the titer of the P100 bacteriophage (LISTEX™ P100), provided by EBI Food Safety.

The cultures of *L. monocytogenes* and *L. ivanovii* were stored in Hogness medium (1.3 mM K₂HPO₄·3H₂O, 1.3 mM KH₂PO₄, 2.0 mM citrate-Na·2H₂O, 1.0 mM MgSO₄·7H₂O, and 4.4% (v/v) glycerol) and frozen at –80 °C.³²

Before use, the *L. monocytogenes* cultures were activated separately in tryptic soy broth (Himedia, São Paulo, SP, Brazil) supplemented with 0.6% (w/v) of yeast extract (Himedia) (TSB-YE) at 35 °C overnight in a shaker (Cientec model CT 712, São Paulo, SP, Brazil) at 150 rev/min. *L. ivanovii* culture was grown overnight at 30 °C in a half-concentrated brain-heart infusion broth (BHI 1/2 (v/v), Difco Code No. 237500) with the NaCl concentration adjusted to 5 g/L. Following the incubation, the cultures were centrifuged at 3600 \times g for 5 min in a microcentrifuge (Digital Spectrafuge 24D, Model C2400-24D,

Labnet, São Paulo, SP, Brazil). The resultant pellet was washed twice in PBS (pH 7.0), suspended in the same solution, and adjusted to 0.5 points on the McFarland scale ($\sim 10^8$ CFU/mL) with peptone water.³³

The cultures were then combined and diluted to approximately 10^4 CFU/mL before inoculating onto the food. The presence of viable cells of *L. monocytogenes* was confirmed by plating 100 μ L on *Listeria* agar Ottaviani & Agosti (ALOA™, Laborclin, Pinhais, PR, Brazil) and incubating at 37 °C for 48 h. Bacterial survival in treated and untreated samples was determined by counting colony-forming units (CFU/g) on ALOA agar plates incubated at 37 °C for 24 h.³⁴

Titration of P100 bacteriophage

The titer of the phage P100 was determined according to a protocol suggested by EBI Food Safety (personal communication). For this protocol, serial dilutions of the bacteriophage suspension were made in a lambda buffer (6 mmol/L Tris buffer, pH 7.2; 10 mmol/L Mg (SO₄)₂·7H₂O; 50 μ g/mL gelatin) and 100 μ L of each dilution was mixed into 3.5 mL of molten overlay agar cooled to 45 °C, which contained 150 μ L of a log-phase *L. ivanovii* culture grown overnight at 30 °C in a half-concentrated brain-heart infusion broth (BHI, Himedia) with the NaCl concentration adjusted to 5 g/L. This mixture was poured into BHI agar (1.2%, w/v, agar) plates and incubated at 30 °C for 20–24 h. Following incubation, the titer was determined as plaque-forming units (PFU).

For phage control, the sample was diluted in lambda buffer, and an aliquot of 100 μ L was incorporated into 3.5 mL of molten overlay agar cooled to 45 °C, which contained 150 μ L of *L. ivanovii* (helper strain). As described above, the mixture was poured into BHI agar (1.2%, v/w, 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).¹⁸

Inoculation of the samples

Ready-to-eat pork ham slices were divided into 12 portions, weighed (30 ± 0.5 g) in a laminar flow hood (Labconco Purifier Class IIb, Total Exhaust, model 36210-04, certified ISO 9002, Labconco Corporation, Kansas City, MO, USA). Six of the weighed samples were individually treated with 700 μ L of nisin (0.0012 μ g/g),³¹ 1000 μ L of P100 bacteriophage (5×10^5 UFP/g),³⁵ and 700 μ L of sodium lactate (0.5 μ g/g).³¹ Two samples were individually treated with nisin combined with phage P100, and another two were treated with nisin combined with sodium lactate. The remaining two samples were treated with 700 μ L sterile water to act as a control. The solutions were absorbed on the food surface by using a sterile glass spreader and were air-dried under a class II biosafety cabinet for 15 min, after which the samples were each inoculated on the one-side with 1 mL of the mixture of *L. monocytogenes* 1/2a and Scott A (approximately 10^4 CFU/mL). The inoculated samples were air-dried under a class II biosafety cabinet for 15 min at 21 °C to allow for the attachment of bacteria to the pork ham slices.

Enumeration of *Listeria monocytogenes* in ready-to-eat sliced pork ham

For enumeration of *L. monocytogenes*, each 30 g sample was added to 270 mL of 0.1% peptone water and homogenized in a stomacher (240 bpm; ITR model 1204, series 126, Esteio, RS, Brazil) for 2 min. Aliquots of 100 μ L of the homogenate were spread onto duplicate ALOA plates, which were incubated at 37 °C for 24 h. Bacterial survival in treated and untreated (control) samples was determined by counting colony-forming units (CFU).

Half of the samples were immediately subjected to *L. monocytogenes* enumeration on ALOA agar, and the remaining samples were stored at 6–8 °C for 72 h (pork ham slices shelf life) in aerobic condition before enumeration.

Data analysis

When investigating the effectiveness of bacteriophage P100, nisin and sodium lactate in the inhibition of the *L. monocytogenes* 1/2a and Scott A mix, bacterial counts were always determined in duplicate, and all of the experiments described here were independently performed five times. All bacteria counts (CFU/g) recovered from ready-to-eat sliced pork ham were converted to logarithms before computing their means and standard deviations. For each treatment condition, the decimal reduction (DR) of the population of the bacteria was calculated relative to the counts obtained in the control. The data were subjected to Analysis of Variance (ANOVA) and Tukey's multiple range test (Software ASSISTAT, version 7.6 beta, 2011) to determine if significant differences ($p < 0.05$) in the mean log values of the populations of *L. monocytogenes* existed between the treatment groups.

Results

The in situ antilisterial activities of nisin, bacteriophage P100 (LISTEX™ P100) and sodium lactate against two strains of *L. monocytogenes* were studied using pork ham slices as a model substrate.

The average *L. monocytogenes* population recovered from pork ham slices after inoculation was 2.83 ± 0.11 log CFU/g. *L. monocytogenes* was not detected in samples that were not inoculated.

The effect of the treatments containing nisin, bacteriophage P100 and sodium lactate, either individually, or nisin combined with either bacteriophage P100 or sodium lactate, on the survival of the *L. monocytogenes* strains at two time points is shown in Table 1.

Treatment with nisin at zero h significantly reduced initial cell density ($p < 0.05$), and in two assays the bacteriocin led to a more than two-fold inhibition of the growth. However, this pattern was not observed at 72 h of storage at 6–8 °C, and a mean DR value of 1.66 log cycles was found. Indeed, the initial *L. monocytogenes* population (2.83 log CFU/g) increased to a level of 3.23 log CFU/g for the untreated group (inoculated with only *L. monocytogenes*).

Table 1 – Reduction in the viable counts of *L. monocytogenes* in ready-to-eat sliced pork ham by bacteriophage P100 (1.5×10^7 UFP/mL), nisin (50 µg/L) and sodium lactate (2%, v/w) alone or nisin combined with either bacteriophage P100 or sodium lactate, at zero and 72 h (6–8 °C) (mean ± standard deviation, n = 5).

<i>Listeria monocytogenes</i> (Log ₁₀ UFC/g)				
Control	Zero hour		72 h	
	2.83 ± 0.08 a ¹ , x ²		3.27 ± 0.05 a, x	
	Treatment	DR	Treatment	DR
P100	0.0 b, x	2.83 ± 0.08	0.0 b, x	3.27 ± 0.05
Nisin	0.8 ± 0.98 b, x	2.03 ± 1.2	1.57 ± 1.13 a, y	1.67 ± 1.11
Sodium lactate	2.47 ± 0.17 a, x	0.37 ± 0.17	2.10 ± 0.22 a, x	1.13 ± 0.26
Nisin + P100	2.27 ± 0.40 a, x	0.57 ± 0.31	0.33 ± 0.47 b, y	2.9 ± 0.42
Nisin + sodium lactate	2.40 ± 0.24 a, x	0.43 ± 0.26	1.83 ± 0.24 a, x	1.40 ± 0.22

DR, decimal reduction.

Superscript numbers: 1, treatment means with different (a or b) letters are different at the 0.05 significance level (Tukey's test); 2, time means within treatment with different (x or y) letters are different at the 0.05 significance level (Tukey's test).

Among the individual antimicrobial treatments, bacteriophage P100 was the most effective, decreasing *L. monocytogenes* to undetectable levels at zero and 72 h post-infection (6–8 °C).

Sodium lactate was the least effective treatment, reducing *L. monocytogenes* counts by 0.2–0.6 log CFU/g on day 0. Additionally, although a better DR was achieved, no significant reduction in the counts of *L. monocytogenes* was observed for the sliced pork ham stored at 72 h at 6–8 °C (Table 1).

To evaluate additive and synergistic effects, inoculated pork ham slices was treated with nisin combined with either bacteriophage P100 or sodium lactate. The results showed that the combination of nisin and P100 phage had a small anti-*Listeria* effect at zero h, indicating antagonism between these agents. However, at 72 h, almost 3 log cycles of reduction were observed in the number of viable bacterial cells (Table 1). In contrast, the treatment using sodium lactate in combination with nisin demonstrated no improvement over the efficacy of the bacteriocin alone.

Among the five antimicrobial treatments, at two different time points, ANOVA showed no significant difference between samples treated with sodium lactate and samples treated with sodium lactate and nisin ($p > 0.05$) relative to the control. By contrast, the individual treatments with phage P100 and nisin at zero h showed significant difference from the control ($p < 0.05$). At 72 h, phage P100 and the combination phage P100 and nisin treatments were significantly different ($p < 0.05$) when compared to the control (Table 1).

Discussion

The occurrence of *L. monocytogenes* in RTE foods, especially in meat products, presents a significant health problem.

The emergence of pathogenic bacteria resistant to most available antimicrobials along with the consumer demand for more natural foods without chemical preservatives and with long shelf lives has become a critical issue for the food industry. This reality has opened frontiers for the application of GRAS substances, such as bacteriocins, bacteriophages and salts of organic acids.

We investigated the effects of the antimicrobials nisin, bacteriophage P100, and sodium lactate, individually, and nisin

combined with either bacteriophage P100 or sodium lactate, at two different time points (zero and 72 h, 6–8 °C), on the growth of a mixture of two strains of *L. monocytogenes* on ready-to-eat pork ham slices. Statistical analysis showed that at zero h individual phage P100 and nisin treatments had greater effects on inhibiting the growth of *L. monocytogenes* compared to the control. Although treatments with nisin effectively reduced the initial contamination level of *L. monocytogenes*, the combination of nisin with phage P100 was most effective on inhibiting the growth of *L. monocytogenes* after refrigerated storage.

Similar results were found in catfish fillets,³⁶ fresh sausage,³⁷ and soft cheeses,⁴ showing that bacteriophage P100 reduces the cell viability of *L. monocytogenes*. However, Vermeiren et al.³⁸ observed that treatment with bacteriophage P100 led to a small reduction of only 1.6 log units in *L. monocytogenes* counts on inoculated fish fillets.

One way to lower the necessary concentrations of antimicrobials agents without compromising activity is to use the synergistic effects that are observed between two antimicrobials that feature different mechanisms of action. For example, a study performed by Leverentz et al.³⁹ showed that the use of nisin and the phage mixtures LM-103 and LMP-102, which contained 14 and 6 distinct lytic phages, respectively, reduced *L. monocytogenes* populations by up to 5.7 log units on honeydew melon slices compared to the control.

Recently, a study using two *Listeria* bacteriophages FWLLm1 and FWLLm3, in combination with the bacteriocin coagulin C23 to inhibit *L. monocytogenes* in milk under refrigerated conditions, showed that when used alone the phages and coagulin C23 kept *L. monocytogenes* counts lower than the untreated control throughout storage. When used in combination, *L. monocytogenes* counts were under the detection limits (less than 10 CFU/mL) from day 4 until the end of the experiment.³

Related to sodium lactate, the results in the present study were similar to a study performed by Lung and Johnson⁴⁰ that showed the addition of salt to turkey breast alone or in combination with nisin did not inhibit the growth of *L. monocytogenes* during storage under refrigeration. Also, the addition of organic acid did not increase the bactericidal effect of nisin. However, study have been reported for the combination of nisin and sodium lactate in reducing densities of *L.*

monocytogenes on cold-smoked salmon paté,⁴¹ cold-smoked rainbow trout⁴² and cooked ham.⁴³

Conclusion

Phage P100, when applied as an antimicrobial treatment, was found to be very effective in inactivating and inhibiting the growth of *L. monocytogenes* on sliced pork ham. Overall, we have demonstrated that in sliced pork ham under refrigerated conditions, the combination of bacteriophage P100 and the bacteriocin nisin is more effective as a bio-preservative against *L. monocytogenes* than nisin alone. This combination could be a smart strategy to ensure sliced pork ham safety during storage conditions.

Conflicts of interest

The content of this report solely reflects the opinions of the authors, and we report no conflicts of interest.

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