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Occurrence of *Listeria* spp. in Brazilian fresh sausage and control of *Listeria monocytogenes* using bacteriophage P100

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ABSTRACT

Since the 1980s, an increase in outbreaks of human listeriosis linked to contaminated food has been a concern of health authorities. Intensively manipulated foods, such as Brazilian fresh sausage, are frequently responsible for food-borne diseases. In this work the occurrence of *Listeria monocytogenes* and the efficacy of bacteriophage P100 (LISTEX™) to control the microorganism was evaluated in Brazilian fresh sausage. Eighty samples were analyzed, 40 each of swine and chicken Brazilian fresh sausage. *Listeria* spp. were isolated from 12 samples (15%), of which three (3.75%) were positive for *L. monocytogenes*. *L. monocytogenes* strains isolated belonged to serotype 1/2a. *L. monocytogenes* 1/2a was inoculated in Brazilian fresh sausage (2.1×10^4 cfu/g) with the bacteriophage added thereafter (3.0×10^7 pfu/g). Samples were analysed immediately (day zero) and then stored at 4 °C for 10 days. The bacteriophage P100 reduced *L. monocytogenes* counts by 2.5 log units at both 0 and 10 days compared to controls without bacteriophage. In spite of this, the populations of *L. moncytogenes* increased over the 10 day storage. Our data demonstrate that in one of the samples the use of the bacteriophage dropped the bacteria count below the level of direct detection. This study demonstrates a new alternative for pathogen control in the food industry, especially in the processes used to produce Brazilian fresh sausage.

1. Introduction

Brazilian fresh sausage ("linguiça") is a mixture of minced pork and/or minced chicken, curing salts and spices, in a natural gut casing. This product is very popular in Brazil and is frequently consumed under-cooked.

In Brazil, food-borne listeriosis outbreaks have not yet been documented, but in recent studies, the presence of *Listeria monocytogenes* has been described in several products including Brazilian fresh sausage (Lima, Rossini, & Pompermayer, 2003; Silva et al., 2004; Sousa, Figueiredo, Maia, & Frizzo, 2006). Research conducted in Brazil reported an incidence of 100.0% of the *Listeria* spp. in raw meat used to make the product and the strain of *L. monocytogenes* was isolated in 16.6% of the final product (Silva et al., 2004).

Over the last few years, despite the recent technological advancements in pathogen control in foods, consumers have sought natural foods, i.e., foods submitted to less aggressive treatments without chemical preservatives. Moreover, the extensive use of sanitizers has led to the development of resistant bacteria, rendering these procedures less effective (García, Martínez, Obeso, & Rodríguez, 2008). In recent years, it has become widely recognized that bacteriophages have several potential applications in the food industry. Bacteriophages are naturally occurring viruses that are suitable candidates for the environmentally friendly biocontrol of pathogens (Guenther, Huwyler, Richard, & Loessner, 2009). They have been proposed as an alternative to antibiotics in animal health, as biopreservatives in food, and as tools for detecting pathogenic bacteria throughout the food chain (Atterbury, Connerton, Dodd, Rees, & Connerton, 2003; Leverentz et al., 2001). They are safe to use as they are harmless to mammalian cells, and their high host specificity allows proper starter performance in fermented products and keeps the natural microbiota

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undisturbed (García et al., 2008). The attributes of bacteriophages include the following: (i) They kill bacterial target cells; (ii) They generally do not cross species or genus boundaries, and will therefore not affect (a) the desired bacteria in foods (e.g., starter cultures); (b) commensals in the gastrointestinal tract, or (c) the accompanying bacterial flora in the environment; (iii) Bacteriophages are generally composed entirely of proteins and nucleic acids, therefore their breakdown products consist exclusively of amino acids and nucleic acids (Carlton, Noordman, Biswas, Meester, & Loessner, 2005). Thus, they are not xenobiotics, and, unlike antibiotics and antiseptic agents, their introduction into and distribution within a given environment may be seen as an extension of a natural process (Carlton et al., 2005). 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 approved bacteriophage preparation is reported to be effective against 170 strains of *L. monocytogenes* (Lang, 2006). The commercial product named LISTEX™ P100 was approved as a food biopreservative and granted GRAS (Generally Recognized as Safe) status (FDA, 2006). This study was undertaken to investigate the presence of *Listeria* spp. in Brazilian fresh sausage and to investigate the efficacy of P100 in reducing populations of *L. monocytogenes* on laboratory-inoculated Brazilian fresh sausage.

2. Materials and methods

2.1. Investigation of Listeria spp. in Brazilian fresh sausage

2.1.1. Sampling and laboratory procedures

Eighty samples, 40 of swine and 40 of chicken Brazilian fresh sausage, were purchased at a local supermarket in Salvador, BA, Brazil. Portions of 250 g of food were aseptically collected between March and October of 2008 and samples were transported from the place of collection to the laboratory in an insulated cold box filled with ice. To detect bacteria in the samples the standard method of the United States Department of Agriculture (USDA)/Food Safety and Inspection Service (FSIS) (USDA/FSIS, 2005, chap. 8.04) was used. One *L. monocytogenes* positive control (*L. monocytogenes* Scott A, a serotype 4b, ATCC 15313) and one uninoculated media negative control were used for each set of concurrently analyzed samples. After aseptically removing the casing in a class II biosafety cabinet (Labconco model 36210 class BII, Brazil), samples of 25 g were blended with 225 ml of Modified University of Vermont broth (UVM, Difco Code No 022317) in a Stomacher (ITR model 1204, serie126, Brazil, 240 bpm) for two min and incubated for 24 h at 30 °C. Secondary enrichment was performed in Fraser broth (FB, Difco Code No. 211767) at 35 °C for 24–48 h. If any degree of FB darkening was evident (esculin hydrolysis), a volume of 100 μl of the broth was streaked onto Modified Oxford Listeria Selective Agar (MOX, Difco Code No. 222530), supplemented with moxalactam 20 mg/l and colistin sulfate 10 mg/l. Plates were incubated at 35 °C for 24–48 h. After that, at least 20 suspect colonies, if available, were streaked on 5% horse blood agar (blood agar base II, Difco Code No. 0045/17) plates and incubated at 35 °C for 24 h. After incubation, plates were examined for colonies surrounded by a small zone of β-hemolysis. Typical colonies were transferred to tryptic soy broth (TSB, Difco Code No. 211825) supplemented with 0.6% (w/v) yeast extract (Difco Code No. 211929) and confirmatory tests were carried out for Gram stain, catalase activity and motility in semi-solid indol motility medium (SIM, Difco Code No. 211578) at 25 °C for seven days, for the typical umbrella shape. For biochemical confirmatory tests, *Listeria* API (BioMerieux® S.A., Marcy L'Etoile, France) was used. Serological slide agglutination tests were done according to Seeliger and Hohne (1979) on all isolates presumed to be *Listeria*, using commercially prepared antisera (Difco).

2.2. Control of L. monocytogenes inoculated in Brazilian fresh sausage using bacteriophage P100

2.2.1. Bacterial strains, bacteriophage, media and culture conditions

The bacteria and bacteriophage used in this study were: a single strain of *L. monocytogenes* 1/2a isolated from Brazilian fresh sausage; *Listeria ivanovii* WSLC 3009 (SLCC 4769); and bacteriophage P100 (LISTEX™ P100) provided by EBI Food Safety (Wageningen, The Netherlands).

L. ivanovii was used as helper strain for the P100 bacteriophage (Carlton et al., 2005; Loessner & Busse, 1990). The culture strains were stored in a Hogness medium (1.3 mM $K_2HPO_4 \cdot 3H_2O$; 1.3 mM KH_2PO_4; 2.0 mM citrate-Na$\cdot 2H_2O$; 1.0 mM $MgSO_4 \cdot 7H_2O$; 4.4 % (v/v) glycerol) and frozen at −80 °C. Before use, the *L. monocytogenes* culture was activated in tryptic soy broth supplemented with 0.5% (w/v) yeast extract (TSB-YE) at 35 °C overnight in a shaker (Cientec model CT 712, Brazil) at 150 rev/min. The *L. ivanovii* culture was grown overnight at 30 °C in a half-concentrated brain-heart infusion broth (BHI, ½ v/v, Difco Code No. 237500) 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) agar to BHI. To improve the bacteriophage plaques, 0.75% (w/v) glycine (Sigma Aldrich - Poole, United Kingdom) was added to the top layer agar (Lillehaug, 1997). Appropriate bacterial dilutions were made in lambda buffer (6 mmol/l Tris buffer, pH 7.2; 10 mmol/l Mg $(SO_4)_2 \cdot 7H_2O$; 50 μg/ml gelatin).

Bacterial survival following treatment with the P100 phage was determined by measuring colony-forming units (cfu/g) on Modified Oxford Listeria Selective Agar (MOX) supplemented with moxalactam 20 mg/l and colistin sulfate 10 mg/l.

2.2.2. Titration of P100 bacteriophage

The titer of the P100 was determined according to a protocol suggested by EBI Food Safety (personal communication). This consisted of serial dilutions of the bacteriophage suspension in a lambda buffer, followed by the incorporation of 100 μl into 3.5 ml of the molten overlay agar cooled to 45 °C, which contained 150 μl of *L. ivanovii* culture grown overnight at 30 °C in a ½ strength BHI. This 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/ml).

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).

2.2.3. Preparation of bacteria inoculum

L. monocytogenes 1/2a was subcultured at least twice by loop inoculation of 10-ml volumes of tryptic soy broth containing 0.5% (w/v) yeast extract (TSB-YE), which was then incubated at 35 °C for 18–20 h in a shaker at 150 rev/min. The cell suspensions were transferred to sterile eppendorf tubes and inoculum levels were confirmed by surface plating duplicate samples on MOX. The plates were incubated at 37 °C for 24 h before colony counts were obtained. This experiment was repeated three times in duplicate (Valadares, 2000). Cell suspensions were diluted in an appropriate amount of 0.1% (w/v) peptone water to give a cell number of 10^5 cfu/ml and were used immediately for sample inoculation.

2.2.4. Sample inoculation and treatment application

Frozen Brazilian sausage made with pork meat (weight approx. 250 g), lard, nitrate, flavouring and spices was purchased at a local supermarket in Salvador, Bahia, Brazil. Samples were transported from the place of collection to the laboratory in an insulated cold box filled with ice. They were defrosted under refrigeration at 4 °C for 24 h. After aseptically removing the casing, the pieces were distributed in six Petri dishes (65 cm^2, ca. 40 g) and samples of thirty grams each were transferred to a bag mixer: four were inoculated separately with a 1-ml volume of the suspensions of *L. monocytogenes* 1/2a (ca. 10^5 cfu/ml) and two samples were inoculated separately with 1-ml volume of bacteriophage P100 (ca. 10^8 pfu/ml) without the addition of *L. monocytogenes* to measure the survival of the bacteriophage in the sausage samples at zero and ten days.

The samples with *L. monocytogenes* were air-dried under a class II biosafety cabinet for 1 h at 21 °C to allow the attachment of bacteria to the meat before undergoing treatment with bacteriophage (Singh, Singh, Bhunia, & Stroshine, 2002). Inoculated samples were homogenized gently by hand to ensure an even distribution of organisms. After that, two samples were treated with 1-ml volumes of the P100 bacteriophage (ca. 10^8 pfu/ml) to yield a final application dose of 10^7 pfu/g. For the no phage control, 1 ml saline solution (0.85% w/v NaCl) was added to two samples. The samples (control and treatment) were kept inside a stomacher bag and sealed for incubation at room temperature (21 °C) for 15–20 min to allow infection. One of them was immediately subjected to *L. monocytogenes* enumeration on MOX agar and the other stored at 4 °C for 10 days. The bacteriophage titers of the two samples that were inoculated with bacteriophage without the addition of *L. monocytogenes* were determined as described above (Section 2.2) at days 0 and 10.

For enumerating *L. monocytogenes*, each portion of 30 g was added to 270 ml of 0.1% (w/v) peptone water with 0.01% (w/v) tween-80 and homogenized in a stomacher for 2 min. Serial dilutions were made in the same solution without tween-80 and spread onto MOX plates in duplicate and incubated at 37 °C for 48 h. Bacterial survival following treatment with the P100 phage or not (control) was determined by measuring colony-forming units (cfu).

2.3. Statistical analysis

To investigate the effectiveness of bacteriophage P100 in the elimination of *L. monocytogenes* 1/2a, bacterial and bacteriophage counts were always determined by duplicate plating and all the experiments described here were independently performed five times. All counts of bacteria (cfu/g) recovered from Brazilian fresh sausage were transformed to logarithms before computing means and standard deviations. The decimal reduction (DR) in the population of the bacteria was calculated by the difference between the counts obtained in the control and each treatment. Log values of zero were assumed for samples in which *L. monocytogenes* was not detected (<1 log cfu/g). Data were subjected to the Statistical Analysis System for analysis of variance and Tukey's multiple range test (SPSS 2.3 for the Windows pocket program) to determine if significant differences ($P < 0.05$) in populations of *L. monocytogenes* existed among mean log values.

3. Results and discussion

3.1. Investigation of Listeria spp. in Brazilian fresh sausage

A total of 40 samples of pork and 40 samples of chicken Brazilian fresh sausage, from four different commercial brands, were investigated. *Listeria* spp. were isolated from three different brands of sausage made with chicken meat and from two different brands of sausage made with pork meat. One brand of the product did not show contamination with *Listeria* spp.

Of the 80 samples, *Listeria* spp. was isolated in 12 samples (15%) of which three (3.75%) were positive for *L. monocytogenes.* Among the two species, *L. innocua* was isolated with greater frequency being found in 11 samples (13.75%). The strains of *L. monocytogenes* isolated from the samples belonged to the serotype 1/2a.

In our country, the incidence of *L. monocytogenes* in fresh sausage has been reported in many studies by a number of researchers. Results found in this present work demonstrate lower levels of detection of the *L. monocytogenes* than that reported by Silva (1996), 6.6%, and Lima et al. (2003), 10.4%. An investigation of factories that process Brazilian fresh sausage in Brazil demonstrated that *L. monocytogenes* was isolated from 16.6% of the sausage read to seal (Silva et al., 2004).

In countries other than Brazil, various results were reported on the occurrence of the *L. monocytogenes* in sausages. In Italy, the bacteria was isolated from 13% of fermented sausage samples (Cantoni, Aubert, Valenti, Comi, & Aubert, 1989), in Switzerland, from 15% of the exported fermented sausages (Jemmi, Pak, & Salman, 2002), in Portugal, from 3.7% of samples of Spanish-style sausages (Mena et al., 2004), and in Turkey, the bacteria was detected in 11.6% of the samples of fermented sausage (Colak, Hampikyan, Ulusoy, & Bingol, 2007).

The presence of this pathogenic microorganism in Brazilian fresh sausage, a very popular food in Brazil and frequently consumed under-cooked, demonstrates the necessity of redefining and implementing good manufacturing practices in raw material acquisition, utilization of adequate methods for control, and sanitization of the environment, equipment, and product processing methods, thus avoiding potential risk of listeriosis and contributing to consumer food safety.

3.2. Control of Listeria monocytogenes inoculated in Brazilian fresh sausage using bacteriophage P100

The effect of bacteriophage P100 (Listex™ P100) on the survival of *L. monocytogenes* 1/2a was observed by measurement of viable cell counts. The inoculum used was 6.3×10^5 cfu/ml (5.8 log cfu/ml). All treatments caused a decrease in the number of viable cells. The log reductions or decimal reduction (DR) in viable cells were calculated (Table 1). A mean reduction of more than two log cycles in viable counts was observed, although with one of the samples the treatment with bacteriophage was enough to reduce the cfu/g below the level of direct detection technique used.

In all assays performed, we observed an increase in the number of viable cells in the samples after refrigeration for 10 days. This result demonstrated the psychotropic characteristic of the bacteria.

Table 1
Reduction of viable counts of *Listeria monocytogenes* (serovar 1/2a) by bacteriophage P100 in Brazilian fresh sausage at 0 (zero) and 10 days at 4 °C.

Experiment	*Listeria monocytogenes* 1/2a (Log_{10} cfu/g)[a]					
	0 Day			10 Days		
	Control	Treatment[b]	DR[c]	Control	Treatmen[b]	DR[c]
1	4.0 ± 0.06	1.0 ± 1.41	3.0	4.9 ± 0.18	2.5 ± 0.09	2.4
2	4.4 ± 0.03	2.5 ± 0.34	1.9	5.1 ± 0.04	2.2 ± 0.34	2.9
3	4.1 ± 0.30	0.0 ± 0.00	4.1	5.7 ± 0.11	2.0 ± 0.00	3.7
4	4.0 ± 0.21	3.1 ± 0.05	0.9	5.2 ± 0.17	3.6 ± 0.15	1.6
5	3.6 ± 0.04	1.0 ± 1.41	2.6	4.9 ± 0.04	3.1 ± 0.30	1.8
Mean	4.0 ± 0.17	1.5 ± 1.27	2.5	5.2 ± 0.11	2.7 ± 0.64	2.5

[a] Mean ± standard deviation.
[b] P100 titer: 3.0×10^7 pfu/g (7.5 log pfu/g).
[c] Decimal reduction.

Table 2
Titer of the bacteriophage P100 after inoculation in Brazilian fresh sausage at 0 (zero) and 10 days at 4 °C.

Experiment	Bacteriophage P100 (pfu/g)[a,b]	
	0 Day	10 Days
1	$4.8 \times 10^3 \pm 3.20$	$2.9 \times 10^4 \pm 0.07$
2	$4.3 \times 10^3 \pm 2.80$	$2.2 \times 10^4 \pm 0.30$
3	$3.4 \times 10^3 \pm 1.20$	$3.1 \times 10^3 \pm 2.20$
4	$4.0 \times 10^3 \pm 1.10$	$2.3 \times 10^3 \pm 0.06$
5	$4.5 \times 10^3 \pm 1.00$	$3.0 \times 10^3 \pm 0.02$
Mean	$4.2 \times 10^3 \pm 0.53$	$1.2 \times 10^4 \pm 1.13$

[a] Mean ± standard deviation.
[b] P100 titer: 3.0×10^7 pfu/g (9.0×10^8 pfu/ml).

The results obtained from the control samples of bacteriophage alone show that P100 was viable at low temperatures (mean value of 4.2×10^3 pfu/g ± 0.53 at 0 (zero) day and 1.2×10^4 pfu/g ± 1.13 at 10 days) (Table 2), and that these counts were not statistically different ($P > 0.05$).

The statistical analysis showed a significant difference ($P < 0.05$) between the treated samples and the control, in both at 0 (zero) and at 10 day (Table 1). However, no significant difference ($P > 0.05$) was found between the treated samples at 0 (zero) time and 10 day, 1.5 log cfu/g ± 1.37 and 2.7 log cfu/g ± 0.64, respectively.

The results of this work indicate that this bacteriophage is inhibitory against a specific strain of *L. monocytogenes*, serovar 1/2a, artificially inoculated in Brazilian fresh sausage. Also, this effect was observed in the samples under refrigeration (4 °C) for 10 days (shelf life of the Brazilian fresh sausages), although the counts of the viable cells of the microorganism had increased.

A lower effect of phages was found in this study in comparison to previous works (Carlton et al., 2005; FDA, 2006; Guenther et al., 2009), and this is explained in terms of a lower dose. As a result, the number of surviving cells was much higher at 10 days of storage, although a higher number of cells were killed too, and therefore the risk of *L. monocytogenes* was higher.

Carlton et al. (2005) also used a P100 bacteriophage with a lower titer (1.5×10^8 pfu/ml) and achieved a reduction of 2–3 log cycles in cheese, similar to that observed in the Brazilian fresh sausage in our study.

Other foods have been treated with the broad-host-range bacteriophages A511 and P100 for control of *L. monocytogenes* strains Scott A (serovar 4b) and WSLC 1001 (serovar 1/2a) (Guenther et al., 2009). In liquid foods, such as chocolate milk and mozzarella cheese brine, bacterial counts rapidly dropped below the level of direct detection. In solid foods, according to the authors, the bacteriophages were able to reduce bacterial counts by up to five log units. The authors also considered the application of more bacteriophage particles (3×10^8 pfu/g) to be more effective than lower doses.

The food industry is mostly concerned with the "big four" food pathogens: *L. monocytogenes*, *Salmonella*, *Campylobacter*, and pathogenic *Escherichia coli*. Of these, only *Listeria* regularly colonizes production facilities and is thus able to contaminate food very late in the production process. Therefore, it is logical to introduce this bacteriophage treatment at the stage of the meat processing when this contamination is likely to take place (Hagens & Offerhaus, 2008).

The methodology used in this study for preparation of the Brazilian fresh sausage sample (e.g., elimination of the casing before inoculation of the bacteria and the bacteriophage) was chosen because the casing of the meat product constitutes a barrier for the artificial inoculation of the bacteria and the bacteriophage. Considering the fact that sausage is frequently consumed undercooked we recommend the addition of the bacteriophage in the industrial process just before the casing of the product.

Considering the results obtained, it may be concluded that the application of virulent, broad-host-range P100 bacteriophage could be very effective for the specific biocontrol of *L. monocytogenes* in Brazilian fresh sausage. However, further investigations using a bacteriophage with a higher titer for a complete eradication of the pathogen may be needed. This is due to the capability of *L. monocytogenes* to grow at low temperatures and due to the observation that the target bacteria may be embedded within the rather complex food matrix, thereby shielding them from diffusing phage particles.

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