MICREOS

Challenge studies on the effect of LISTEX™ P100 bacteriophages against *Listeria monocytogenes* on beef franks, roast beef and pastrami

1 Background

The experiments were performed to evaluate the effect of anti-listerial phages, LISTEX™ P100, on *Listeria monocytogenes* inoculated beef franks, roast beef and pastrami. The challenge testing was performed at Micreos, Nieuwe Kanaal 7P, Wageningen, the Netherlands and shall provide producers of the tested meat products with data that validates a 1.5 to 2 log reduction of *Listeria.* All of the reduction levels reported in this document meet the criteria as laid down by USDA-FSIS's final rule in 2003 for "The Control of Listeria in Ready-To-Eat Products". The use of LISTEX™ qualifies for alternative 2 on tested products by achieving a greater than one log reduction of *Listeria monocytogenes*. Furthermore, the use of LISTEX™ is approved under the USDA FSIS Directive 7120.1 "Safe and Suitable Ingredients Used in the Production of Meat, Poultry, and Egg Products" and does not require labeling.

2 Materials and methods

2.1 Materials

Samples and chemical parameters characterizing the samples

Table 1: Chemical parameters characterizing the samples

Sample	pH*	a_w	NaCl (g/100g)	Protein (%)
Beef franks	4.1	0.963	1.8	13.3
Roast beef	5.6	0.972	1.9	20.4
Pastrami	6.1	0.965	2.3	23

*measured at 19ºC

Bacteria/bacteriophage

- Food isolates *L.monocytogenes* 10403S: SV 1/2a ; *L.monocyotgenes* EDGe: SV 1/2 a; *L.monocytogenes* WLSC1042: SV 4b and outbreak strain *L.monocytoges* ScottA: SV4b
- Bacteriophage LISTEX™ P100

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Media
1/2 strength BHI (Brain Heart Infusion) broth
1/2 strength BHI agar plates (non-selective)
Selective agar plates
1 x PBS buffer (Phosphate buffered saline preparation)
0.1% peptone water (+ 5g sodium chloride/l)

Other materials
Vacuum sealer + shrink bags
37ºC, 30ºC + 30ºC (shaking) incubators
Flow cabinet
Water bath (92ºC)
Laboratory stomacher

2.2 Methods

BEEF FRANKS

Bacterial overnight cultures
One colony of each *L. monocytogenes* strain was inoculated in 4ml broth and incubated overnight at 30ºC shaking.
A four-strain mixture was prepared by mixing an equal volume of cell suspensions of all four strains.

Preparation of samples
The beef franks were pricked on wooden sticks and placed in a rack to allow artificial *Listeria* contamination and phage treatment without bringing the samples into contact with hands or other contact surfaces.

Artificial contamination
An appropriate dilution of the *Listeria* four-strain overnight culture was prepared in PBS buffer to allow the contamination of the franks with a final concentration of approximately $1x10^4$ cfu/cm². To control the concentration of the dilution used to contaminate the samples, the titer was determined by plating an appropriate dilution on selective agar plates (in duplicate).
In the laminar flow hood 2µl/cm² of the dilution was transferred to each beef frank and rubbed in evenly with the pipette tip. After leaving the franks in the flow hood to dry they were placed in shrink bags.

Treatment with LISTEX™ P100
To allow the treatment of the samples with a final phage concentration of $4x10^6$ pfu/cm², a dilution of LISTEX™ was prepared in PBS buffer. In the fume hood 5µl/cm² was transferred onto the beef franks placed in the shrink bags. The liquid was distributed over the franks manually prior to sealing of the bags.
Control samples (not phage-treated) were treated the same way by transferring 5µl/cm² PBS buffer not containing phages onto the samples.

The bags were sealed and dipped into a 92ºC water bath for 2 seconds. Samples were stored for 24 or 48 hours at 4ºC, respectively (in duplicate each).
To test for *Listeria* cell concentration on the samples at t=0 and check for changes in cells counts during storage, one duplicate was tested immediately after contamination and packaging (procedure see 'Retrieval of *L. monocytogenes*')

Retrieval of *L. monocytogenes*
After refrigerated incubation, *Listeria* cells were retrieved from the samples to evaluate the efficacy of LISTEX™. The packages containing the samples were placed in stomacher bags and opened with a sterile scalpel. To allow a high retrieval rate of cells, peptone water was added to the bags and samples were homogenized in a stomacher for 180 seconds inclusive the shrink bag.
25µl of the untreated controls and 100µl and 400µl of the treated samples were plated in duplicate on selective agar plates.
Plates were incubated for 24 to 48 hours at 37ºC until typical *Listeria* colonies could be enumerated.
After incubation cell concentrations per cm² sample were calculated.

ROAST BEEF & PASTRAMI

For the challenge testing on roast beef and pastrami the same procedure as described for beef franks was used, besides applying some minor modifications:

1) Instead of using the whole sample, pieces with a surface size (original surface/non-cut surface) of 10cm² were prepared.
2) Only the non-cut surface (10cm²) was contaminated with *Listeria*, while the total surface (34cm²) was treated with LISTEX™.
3) The contaminated and treated samples were incubated for 24 hours only (beef franks: 24 and 48 hours).

3 Results
Comparing controls (not phage-treated) at t=0 and after refrigerated storage, *Listeria* cell counts did not change significantly.
Depending on the food sample, a cell retrieval rate of 76% to 92% was seen when comparing *Listeria* cell concentrations of the dilution applied to the samples and after retrieving cells.
Figure 1 shows the *Listeria* cell concentrations (in cfu/cm²) on beef franks, roast beef and pastrami with and without the treatment of phages. With the applied phage concentrations, for all three samples a reduction of ≥1.5 log was seen.

BEEF FRANKS
After 48 hours after phage addition ($4x10^6$ pfu/cm²) bacterial numbers decreased by approximately 1.5 log (Figure 1)

ROAST BEEF

After 24 hours incubation a *Listeria* reduction of approximately 1.5 log could be achieved on roast beef when LISTEX^{TM} was applied in a concentration of 2x10^7 pfu/cm² (Figure 1)

PASTRAMI

On Pastrami cell counts dropped by approximately 1.8 log when treating the samples with 2x10^7 pfu/cm² LISTEX^{TM}.

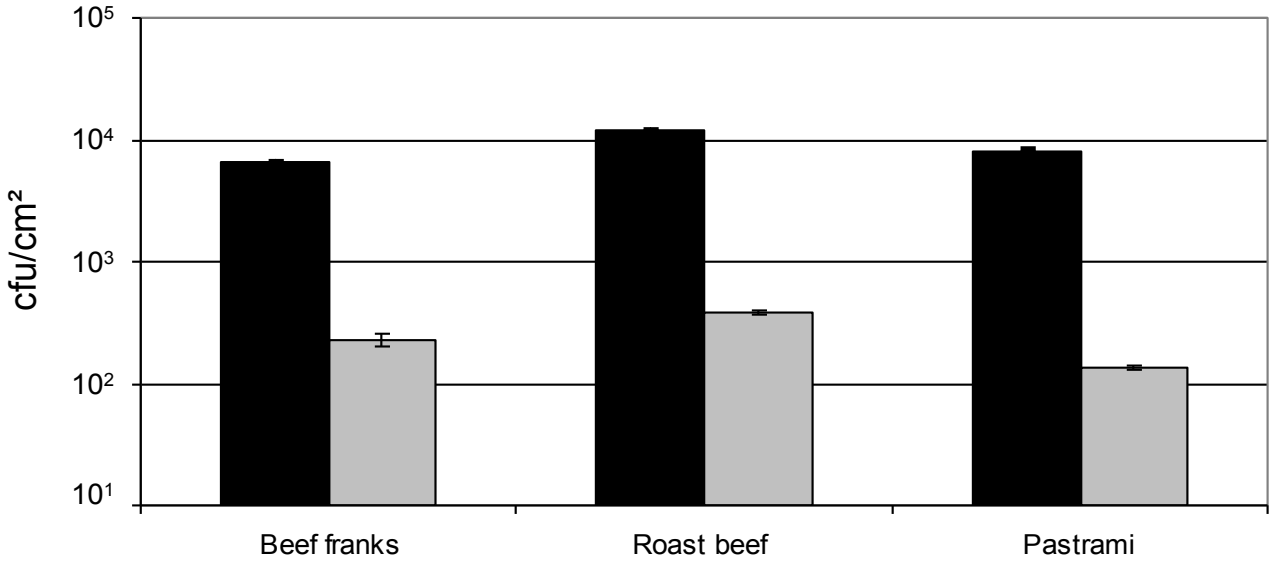

Figure 1: : Phage Listex^{TM} P100 efficacy on *L.* monocytogenes

Artificial contamination of samples with 1x10^4 cfu/cm²; phage treatment with 4x10^6 pfu/cm² (beef franks) or 2x10^7 pfu/cm² (roast beef and pastrami); reaction time: 48 hours (beef franks) and 24 hours (roast beef and pastrami)

- control (no phage treatment)

4 Discussion

Results show that refrigerated storage of the samples does not have a significant influence on *Listeria* cell counts within the applied storage time. A reduction of bacterial cell numbers seen on treated samples can therefore be ascribed to the effect of phages on *Listeria.*

Retrieval rates varied depending on the product, but were higher than 76% and consistent for each product, respectively, making a comparison of treated and non-treated samples reliable.

As can be seen from the results a 1.5 to 1.8 log reduction of *Listeria* can be achieved on the three tested samples when applying a concentration of LISTEX^{TM} of 4x10^6 pfu/cm² for 48 hours on beef franks and 2x10^7 pfu/cm² for 24 hours on roast beef and pastrami.

It has been shown that a uniform distribution of LISTEX™ is of importance to achieve results in this magnitude. The even distributing of liquids is validated is also know as SLIC (Sprayed Lethality In Container). This process has been established by the USDA Agricultural Research Service (ARS) and these systems have a proven track record in effectively controlling pathogens.

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