Efficacy of phage P100 on *L. monocytogenes* in refrigerated vacuum packaged cooked ham

By Frank Devlieghere and Lieve Vermeiren

In this study we investigated the effect of an anti-Listeria phage product on outgrowth of *Listeria monocytogenes* in vacuum packaged cooked ham free from vegetative background flora. Cooked ham was made without organic acids such as lactate or acetate and aseptically sliced and packaged. The inoculation level was low (~17 CFU/g). Phage treatment was performed between slicing and packaging at two levels (1x10^7 and 5x10^7 PFU/cm^2). Phage treatment kept *Listeria monocytogenes* below or at the detection level of 1 CFU/g after 28 (low treatment level) and after 42 days (high treatment level) whereas in the control levels exceeded 1x10^2 CFU/g already after 14 days. In the treated group first outgrowth occurred after 8 weeks with high variation in contamination levels as some samples reached levels of ~10^7 CFU/g after 120 days. The research shows however that phages can be an effective food safety tool for cooked meat products.

KEYWORDS

- Phages
- Cooked ham
- *Listeria monocytogenes*
- Safety
- Storage
- Shelf life

Listeria monocytogenes is an organism ubiquitously distributed in the environment. It is able to grow at temperatures as low as 0 °C and it is able to grow at rather low water activities (a_{wmin} = 0.92). As such persistence and growth in food processing facilities and on refrigerated foods does occur. Also cooked meats are at risk of pre-packaging contamination during slicing and packaging. It is the causative agent of listeriosis in humans and animals, transmitted by contaminated food or feed (Slutzker and Schuchat 1999). Consumption of food contaminated at levels over 100 colony forming units (CFU) is generally believed to cause the majority of listeriosis cases according to a 2004 FAO risk assessment (Anonymous 2004). In Europe, in the period 2008–2016, a statistically significant increasing trend of confirmed listeriosis cases was observed in the EU/EEA ($p < 0.01$), as well as in the last five years (2012–2016) (Anonymous, 2017). The very young, the elderly, pregnant women and their fetuses and immunocompromised individuals are at highest risk of contracting the serious form of the disease (Vazquez-Boland et al. 2001). Alternative approaches are therefore necessary to reduce contamination of food as current processes seem insufficient to prevent contamination and growth of *Listeria*.

Bacteriophages or phages for short, are a relatively new tool for the food industry. Several commercial products are available against *Listeria*, *Salmonella* and *E. coli* 0157. Phages are specific for their host and generally do not cross genus boundaries. This is an advantageous property for their application in foods that are produced with the aid of other bacteria, since phages specific for *Listeria* will in no way interact with fermentation bacteria for example. Nonetheless many food producers use antimicrobials for the sole reason of controlling *Listeria*. The use of phages in foods has been reviewed extensively (Hagens and Loessner 2007, Hagens and Loessner 2010, Kazi and Annapure 2016). Phage P100, the phage used in the experiments carried out in this study has also been used in numerous studies involving a variety of RTE foods. (Carlton et al. 2005, Guenther et al. 2009, Soni et al. 2012, Silva et al. 2014, Oliveira et al. 2014, Iacumin et al. 2017). Briefly, all authors report initial reductions of *Listeria* on different foods varying between 1–3 $\log_{10}$, depending on dose applied. A short period of phage activity of around 24 hours was observed in all cases with re-growth of survivors after this period of time. This appears to be the case for all phages and food stuffs and one author speculates that the phage particles become absorbed to the food matrix which would inhibit interaction with the host (Guenther et al. 2009). The low level of initial contamination found in modern processing facilities is likely to result in safer foods with these reduction levels. A study involving over 30.000 RTE food samples at varying points in their shelf-life found *Listeria* levels at the detection limit of 1 bacteria in 25 g for the majority of positive samples (Gombas et al. 2003). As a consequence a 1 $\log_{10}$ reduction at prior to packaging stage would lead to a significant reduction in the number of positive packages.

Since the process of slicing and packaging is not aseptic, bacteria will always be naturally present. However most of the studies undertaken used retail-bought products at some stage in their shelf life. At that time-point these bacteria may have increased compared to initial numbers. Also, many of these products may have contained growth-inhibiting antimicrobials such as lactate. While neither is likely to affect the initial reduction caused by bacteriophage application, both could potentially influence the outgrowth of *Listeria*. This can be observed in an experiment by Guenther et al (2009) on hotdogs at elevated temperatures. Growth in the untreated control slows down markedly after 3 days when *Listeria* numbers reach ~10^8 CFU/g. At six days the final count is ~2x10^9 CFU/g. While one would expect growth to slow down, it is interesting to note that growth in the treated samples slowed exactly after day three with the initial 3 $\log_{10}$ reduction staying constant, i.e. growth slowed down at the same extent as the control. This suggests that other bacteria may be influencing the outgrowth of *Listeria*. In the current study we investigated the efficacy of P100 on cooked ham without a lactic acid bacteria background flora and without the presence of inhibiting antimicrobials such as lactate and acetate to exclude such polar effects.

Materials and methods

Product

The test product was a model cooked ham product, made in a pilot plant at the Research Group for Technology and quality of animal products of KU Leuven – Technology Campus (Gent, Belgium). A batch of 20 kg of product was prepared. The recipe of the model cooked ham (per kg) was: 1 kg of lean pork meat + 200 g of brine. The composition of the brine was 100 mL of water, 12.4 g of nitrite salt, 3.5 g/L glucose syrup, 0.7 g Na-ascorbate and 2 g/L phosphate. The ham was cooked after being canned to a core temperature of 71 °C and a surrounding temperature of 74 °C. The product was transferred to the laboratory of Ghent University under refrigerated conditions and immediately used for the experiment.

Received: 10 May 2019 | reviewed: 15 May 2019 | revised: 8 July 2019 | accepted: 12 September 2019

The product was manually sliced in a laminar flow cabinet. Obtained slices were circular with a diameter of ± 7 cm, a thickness of 3 to 3.5 mm and a weight of ± 15 g (surface area/slice= 38.5 cm^2). Packages of 150 g of product (10 slices) were made. The total treated surface area (per package of 150 g) = 385 cm^2.

Bacterial strains

The product was inoculated with a cocktail of five *Listeria monocytogenes* strains (Tab. 1). The cocktail was prepared based on mixing full grown cultures at equivalent portions, leading to a solution of 1,02x10^9 CFU/ml.

Artificial contamination

Ham was inoculated with a mixture of the cocktail of the five *Listeria monocytogenes* strains at a level of circa 5 CFU/cm^2, corresponding to circa 15 CFU/g (in triplicate). From an appropriate dilution of the mixture of the five grown *L. monocytogenes* strains, 220 µL was divided over and spread on the surface of ± 150 g of product to reach the desired inoculation level.

Phage treatment

Phage P100 with a concentration of 2x10^{11} PFU/mL was obtained from Micreoas Food Safety (Wageningen, The Netherlands) as a commercial phage preparation (Listex™).

Phage treatment occurred within a minute of contamination which mimics contamination and treatment pre-packaging. From an appropriate dilution of the phage solution, 200 µl was divided over and spread on the surface of ± 150 g of product to reach the desired inoculation levels (Exp. 1: ± 1x10^7 PFU/cm^2, Exp. 2: 5x10^7 PFU/cm^2).

Storage of ham

After inoculation, the 150 g portions of product were vacuum packaged and stored at 7 ± 1 °C in a ventilated refrigerator until analysis.

Microbial analysis

A 15 g sample was taken aseptically and a decimal dilution series in PPS (Peptone Physiologic Salt) solution prepared to plate the appropriate dilutions on the appropriate agar media. Total aerobic psychrotrophic count and number of lactic acid bacteria were determined using NEN-ISO 8552:2004 and NEN-ISO 15214:1998 respectively according to the pour plate technique on Plate Count Agar (PCA) (aerobic incubation at 22 °C for 3–5 days) and de Man Rogosa Sharpe (MRS) agar (aerobic incubation at 30 °C for 3–5 days), respectively.

Quantitative enumeration of *L. monocytogenes* was done following NEN-ISO 11290-2:1998 by spread-plating onto ALOA-plates (aerobic incubation for 2 days at 37 °C) supplemented with ALOA Enrichment selective supplement. For samples below the detection limit of 10 CFU/g, the presence of *L. monocytogenes* in 1, 10 and 25 g samples was determined following NEN-ISO 11290-1: 1997 in three steps: (1) a primary enrichment (24 h, 30 °C) of a 10-fold diluted homogenized sample in demi-Fraser broth, (2) subsequently, 0.1 mL of incubated demi-Fraser broth was transferred to 10 mL Fraser broth for secondary enrichment (24 h, 37 °C) and (3) confirmation by investigating ALOA plates for typical colonies of *L. monocytogenes*. *Listeria* analysis was performed on day 0, 14, 21, 28, 42, 59, 92 and 120. Psychrotrophic counts and lactic acid bacteria numbers were determined on day 0, 28, 42, 59, 92 and 120.

Phage titer determination

For phage number enumeration of phage-treated products, a soft agar overlay technique was used. As a positive control, the Listex™ P100 stock solution was included in the test. The indicator strain used for the phage titer determination was *Listeria innocua* B1488, obtained from Micreos Food Safety (Wageningen, The Netherlands).

Samples (10 g) from phage-treated products were first diluted tenfold in sterile 0.1 M phosphate buffer of pH 7.4 and then filter- sterilized through a membrane filter (0.45 µm) to avoid bacterial contamination of the soft agar double layer plates in the subsequent plaque assay. The phage titers of the filtrates of the phage-treated products and of the ListexTM P100 stock solution were determined by counting plaques from their serial 10-fold dilutions. Volumes of 20 µL of each phage dilution were mixed with 200 µL (10^8 CFU) of cells of the indicator strain *L. innocua*. After incubation for 30 min at 30 °C, the cell-phage mixture was mixed with 3 ml of preheated 4YT (yeast trypton) semi-soft agar (32 g/l trypton, 20 g/l yeast extract, 5 g/l NaCl (VWR) and 7.5 g/L agar) and this final mixture was quickly poured onto pre-heated 4YT-agar plates (32 g/L tryptone, 20 g/L yeast extract, 5 g/L NaCl and 15 g/L agar). Following incubation for 24h at 30°C, plaques were counted. Phage numbers were determined on days 0, 14, 21, 28, 42, 59, 92 and 120.

Results and discussion

Results of direct *Listeria* enumeration are shown in Table 2. For the first 28 days the counts were below or at the detection threshold for the group treated with 1x10^7 PFU/cm^2. In the 5x10^7 PFU/g treatment group this was extended to 42 days. While little variation was seen in the control samples at the various time points growth of *Listeria* differed greatly between the triplicate samples from day 59 on. While some of the phage-treated samples reached equal *Listeria* numbers to those in the control group others did not. At day 92 one sample treated with 1x10^7 PFU/cm^2 had a count of 7.0x10^2 CFU/g compared to an average of 2.4x10^7 CFU/g for the untreated control and at 120 days one sample had a count of 7.8x10^3 CFU/g. In the 5x10^7 PFU/g treatment group one sample had a count of 4.3x10^3 CFU/g compared to an average of 4.6x10^7 CFU/g in the untreated control.

All samples in which a direct count of *Listeria monocytogenes* was not possible were subjected to parallel 1 g, 10 g and 25 g enrichments. The presence of *Listeria monocytogenes* was confirmed for all treated samples on day 0. On day 14 the presence in 25 g could not be confirmed for one of the samples in the 1x10^7 treatment group and for 2 of the samples from the 5x10^7 treatment group. Presence of *Listeria* was confirmed for all later samples that were tested except for 1 sample in the 5x10^7 treatment group at 42 days.

No lactic acid bacteria were detected in any of the samples at any time-point. Psychrotrophic counts closely resembled *Listeria* numbers in all but one sample (data not shown).

Enumeration confirmed intended levels of treatment where reached. Phage numbers did not evolve in a large extend over time as at day 42 samples treated with 5x10^7 still had an average titer above 1x10^7 PFU/cm^2 (Data not shown). The results show that phages can effectively reduce *Listeria monocytogenes* on cooked ham. The similarities between psy-

Strains

Tab. 1: Investigated *Listeria monocytogenes* strains
Tab. 1: Untersuchte *Listeria monocytogenes*- Stämme

LFMFP*-code / Species	Origin/Serotype
LFMFP 235 / *L. monocytogenes*	serotype 4b Isolate LFMFP – Cooked ham
LFMFP 182 / *L. monocytogenes*	serotype 4b Scott A (outbreak strain)
LFMFP 34 / *L. monocytogenes*	serotype 4b LMG 13305
LFMFP 416 / *L. monocytogenes*	serotype 1/2a IHE2000/099 (Food isolate)
LFMFP 417 / *L. monocytogenes*	1/2b IHE2000/098/04 (Food isolate)

* LFMFP = Laboratory of Food Microbiology and Food Preservation

Source: Devlieghere and Vermeiren FLEISCHWIRTSCHAFT 11_2019

chrotrophic counts and *Listeria* numbers show that no background flora was developing during storage. The control group can be considered absolutely unsafe after 2 weeks whereas the low counts of *Listeria* over the first weeks after treatment resulted in a product that would have been safe to consume for 4 and 6 weeks, respectively for the two treatment groups. After this time-point a lot of variation between samples in the treated group was obvious. While the low *Listeria* numbers for the first few weeks show that treatment was effective on all samples the re-growth of *Listeria* varies after this point in time. The fact that enrichment failed for 1 of the three samples treated with $5x10^7$ at day 42 may indicate why this is so. While higher than the average natural contamination, the number of *Listeria* spread on the ham is low enough to ensure uneven distribution. With an organism able to grow at refrigeration temperatures there is a difference between total local eradication and leaving some cells unaffected. Based on the growth rate in the control and re-growth in the treated samples we estimate a 1–2 log_{10} reduction for the low dose treatment and a 2–3 log_{10} initial reduction for the high dose treatment. Although no information is available at low contamination levels such as in our study, there are some reports available at higher contamination levels on the effectiveness of phages against *Listeria monocytogenes* on cooked meat products. Holck and Berg (2009) reported results with higher levels of inoculated *Listeria monocytogenes* (circa 10^4 CFU/g) and observed a 1 log reduction on cooked ham when $5x10^7$ PFU/cm^2 levels of phages were used at 4 and 10 °C. Also Chibeu et al. (2013) observed a log reduction between 1.5 and 2.1 of *Listeria monocytogenes* on cooked turkey and roast beef slices at 4 and 10 °C when 10^7 PFU/cm^2 LISTEX P100 phages were applied (at inoculation levels of *Listeria monocytogenes* between 103 and 104 cfu/cm^2). While such reduction rates will decimate the contamination it is not high enough to kill all the *Listeria* in the samples. Survivors will remain albeit unevenly spread in these samples. While the phages remained viable they apparently could no longer affect their hosts after the initial reduction. Indeed, although high phage titers were still found after 42 days, they were not able to prevent outgrowth of *Listeria* at this point in time. While this experiment is in one way unrealistic it offers to speculate on real-life situation. With concomitant flora not being affected by *Listeria* phages and with receiving a head start of 4–6 weeks they might slow the growth of *Listeria monocytogenes* significantly and as such an application of phages in factory settings may be even more effective in assuring food safety.

Conclusion and practical importance

The results of this study showed that the P100 phages are effective in reducing *Listeria monocytogenes* levels that can reasonably be expected in modern cooked meat processing facilities. However, in cases where the initial contamination load is high total eradication cannot be guaranteed. For manufacturers that are confident that only low-level,

Plate count

Tab. 2: Direct plate count of *Listeria monocytogenes* retrieved from ham untreated and treated with respectively $1x10^7$ PFU/g and $5 x 10^7$ PFU/g P100 phages

Tab. 2: Direkte Keimzahl von *Listeria monocytogenes*, die aus unbehandeltem Schinken und mit jeweils $1x10^7$ PFU/g und $5x10^7$ PFU/g P100-Phagen behandelt gewonnen wurden

Time (days)	Control – untreated ham		
	Sample 1 (CFU/g)	Sample 2 (CFU/g)	Sample 3 (CFU/g)
0	2.0×10^1	1.0×10^1	2.0×10^1
14	1.1×10^2	2.2×10^2	1.0×10^2
21	4.5×10^3	2.8×10^4	2.0×10^4
28	1.4×10^5	1.0×10^5	1.8×10^5
42	2.0×10^6	2.4×10^6	6.8×10^5
59	1.5×10^7	1.5×10^7	2.9×10^7
92	2.1×10^7	1.5×10^7	2.4×10^7
120	2.3×10^7	6.4×10^7	5.2×10^7
		Treatment 1 x 10^7 PFU/g	
1	<1	1.0×10^0	<1
14	1.0×10^0	<1	<1
21	<1	<1	<1
28	<1	<1	<1
42	2.4×10^1	1.0×10^1	7.2×10^1
59	6.6×10^4	3.4×10^5	5.0×10^1
92	1.3×10^6	7.0×10^2	1.2×10^7
120	7.0×10^6	6.6×10^7	7.8×10^3
		Treatment 5 x 10^7 PFU/g	
1	<1	<1	1.0×10^0
14	<1	<1	<1
21	<1	<1	1.0×10^0
28	<1	<1	<1
42	<1	<1	1.0×10^0
59	2.1×10^4	6.8×10^1	<1
92	2.2×10^6	8.1×10^4	7.4×10^6
120	5.2×10^6	1.8×10^8	4.3×10^3

Source: Devlieghere and Vermeiren

sporadic contaminations occur phage treatment provides an ideal, natural alternative to enhance food safety. For those that wish to include an extra level of safety a combination with growth inhibiting ingredients is a viable option.

References

1.Anonymous (2004): Risk assessment of *Listeria monocytogenes* in ready-to-eat foods. WHO, Food and Agriculture organization of the UN. – 2. Anonymous (2017): The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016; EFSA Journal 2017*15* (*12*), 5077. – 3. Carlton RM, W.H., Noordman, B. Biswas, E.D. de Meester and M.J. Loessner (2005): Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. Regul Toxicol Pharmacol *43* (3), 301–312. – 4. Chibee, A., Agius, L. Gao, A. Sabour, P. M. Kropinski, A.M. Balamurugan, S. (2013): Efficacy of bacteriophage LISTEX P100 combined with chemical antimicrobials in reducing Listeria monocyteogenes in cooked turkey and roast beef. Int. J. Food Microbiol. *167*, 208–214. – 5. Gombas D.E., Y. Chen, R.S. Clavero and V.N. Scott (2003): Survey of *Listeria monocytogenes* in ready-to-eat foods. J Food Prot *66* (4), 559–569. – 6. Guenther S., D. Huwyler, S. Richard, M.J. Loessner (2009): Virulent bacteriophage for efficient biocontrol of *Listeria monocytogenes* in ready-to-eat foods. Appl Environ Microbiol *75* (1), 93–100. – 7. Holck, A. and Berg, J. (2009. Inhibition of *Listeria monocytogenes* in cooked ham by virulent bacteriophages and protective cultures. Appl. Enivironm. Microbiol. 75 (21), 6944–9646. – 8. Hagens, S. and M.J. Loessner (2010): Curr Pharm Biotechnol. Bacteriophage for Biocontrol of Foodborne Pathogens: Calculations and Considerations. Jan *11*(1), 58–68. – 9. Hagens S, Loessner MJ. Application of bacteriophages for detection and control of foodborne pathogens. Appl Microbiol Biotechnol. 2007 *76* (3), 513–519. – 10. Iacumin, L., M. Manzano and G. Comi (2016): Phage Inactivation of *Listeria monocytogenes* on San Daniele Dry-Cured Ham and Elimination of Biofilms from Equipment and Working Environments. Microorganisms *4* (1). – 11. Kazi, M. and U.S. Annapure (2016): Bacteriophage biocontrol of foodborne pathogens. J Food Sci Technol. 2016 *53* (3), 1355–1362. –12. Oliveira, M., I. Vinas, P. Colas, M. Anguera, J. Usall and M. Abadias (2014): Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. Food Microbiol *38*, 137–142. – 13. Silva, E. N. Figueredo, A. C. Miranda, F. A. De castro almeida, R. C. (2014): Control of *Listeria monocytogenes* growth in soft cheeses by bacteriophage P100. Braz. J. Microbiol. *45*, 11–16. - 14. Slutzker, L., and A. Schuchat (1999): Listeriosis in humans, 75–95. In E. T. Ryser and E. H. Marth (ed.), *Listeria*, listeriosis and food safety, vol. 2. Marcell Dekker, Inc., New York, NY. – 15. Soni, K.A., M. Desai, A. Oladunjoye, F. Skrobot and R. Nannapaneni (2012): Reduction of *Listeria monocytogenes* in queso fresco cheese by a combination of listericidal and listeriostatic GRAS antimicrobials. Int J Food Microbiol. *155* (1–2), 82–88. – 16. Vazquez-Boland, J. A., Kuhn, M. Bercher, P. Chakraborty, T. Dominguez-Bernal, G. Goebel, W. Gonzalez-Zorn, B. Wehland, J. and Kreft, J. (2001): Listeria pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. *14,* 584–640.

Zusammenfassung

Wirksamkeit von Phagen P100 auf *L. monocytogenes* in gekühlten, vakuumverpackten Kochschinken

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Phagen | Kochschinken | *Listeria monocytogenes* | Sicherheit | Lagerung | Haltbarkeit

In dieser Studie untersuchten die Autoren die Wirkung eines Anti-Listeria-Phagenprodukts auf das Wachstum von *Listeria monocytogenes* in vakuumverpacktem Kochschinken ohne vegetative Hintergrundflora. Gekochter Schinken wurde ohne organische Säuren wie Laktat oder Acetat hergestellt und aseptisch geschnitten und verpackt. Die Impfquote war niedrig (~17 KbE/g). Die Phagenbehandlung wurde zwischen dem Schneiden und dem Verpacken auf zwei Ebenen durchgeführt (1x10^7 und 5x10^7 PFU/cm^2). Die Phagenbehandlung hielt *Listeria monocytogenes* unter oder auf dem Nachweisniveau von 1 KbE/g nach 28 (niedrigem Behandlungsniveau) und nach 42 Tagen (hohem Behandlungsniveau), während in den Kontrollstufen 1x10^2 KbE/g bereits nach 14 Tagen überschritten wurden. In der behandelten Gruppe trat das erste Auswachsen nach 8 Wochen mit hohen Schwankungen der Kontaminationswerte auf, da einige Proben nach 120 Tagen ein Niveau von ~10^7 KbE/g erreichten. Die Forschung zeigt jedoch, dass Phagen ein wirksames Mittel zur Lebensmittelsicherheit für gekochte Fleischprodukte sein können.

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