Journal Pre-proofs

Innovative hurdle system towards *Listeria monocytogenes* inactivation in a fermented meat sausage model - high pressure processing assisted by bacteriophage P100 and bacteriocinogenic *Pediococcus acidilactici*

Norton Komora, Cláudia Maciel, Renata Amaral, Rui Fernandes, Sónia Marília Castro, Jorge A. Saraiva, Paula Teixeira

PII: S0963-9969(21)00527-5
DOI: https://doi.org/10.1016/j.foodres.2021.110628
Reference: FRIN 110628

To appear in: *Food Research International*

Received Date: 30 December 2020
Revised Date: 19 July 2021
Accepted Date: 21 July 2021

Please cite this article as: Komora, N., Maciel, C., Amaral, R., Fernandes, R., Marília Castro, S., Saraiva, J.A., Teixeira, P., Innovative hurdle system towards *Listeria monocytogenes* inactivation in a fermented meat sausage model - high pressure processing assisted by bacteriophage P100 and bacteriocinogenic *Pediococcus acidilactici*, *Food Research International* (2021), doi: https://doi.org/10.1016/j.foodres.2021.110628

Innovative hurdle system towards *Listeria monocytogenes* inactivation in a fermented meat sausage model - high pressure processing assisted by bacteriophage P100 and bacteriocinogenic *Pediococcus acidilactici*

Norton Komora[a†], Cláudia Maciel[a†], Renata Amaral[b], Rui Fernandes[c], Sónia Marília Castro[a,b], Jorge A. Saraiva[b] and Paula Teixeira[a*]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal

[b] LAQV-REQUIMTE – Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal.

[c] Histology and Electron Microscopy (HEMS), Instituto de Investigação e Inovação em Saúde - i3S, Instituto de Biologia Molecular e Celular - IBMC e Universidade do Porto, Porto, 4200-135, Portugal.

*Corresponding author at: CBQF - CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal

† These authors contributed equally to this work

Abstract

The consumers' quest for healthier, locally produced foods, renders the demand for these products increasingly prominent. The purpose of the present work was to evaluate the impact of a non-thermal multi-hurdle approach, which associated mild high hydrostatic pressure (HHP, 300 MPa), the bacteriophage Listex™ P100, and the pediocin PA-1 producing *Pediococcus acidilactici* HA 6111-2, as a novel minimal processing towards *Listeria monocytogenes* eradication in *Alheira* (a traditional fermented meat sausage from Northern Portugal).

The combination of the three hurdles achieved the USDA-FSIS 5 log reduction (in accordance with the standard guidelines for ready-to-eat foods), being the only treatment to elicit the absence of *L. monocytogenes* immediately following processing ($p < 0.05$). The pair association of HHP with Listex™ P100 was unable to eliminate *L. monocytogenes*, whilst in the HHP-pediocin PA-1 producing *P. acidilactici* treated samples the eradication was delayed when compared to the three hurdles combination.

In addition to the listericidal effect of the HHP-phage-lactic acid bacterium treatment, no significant differences ($p > 0.05$) in the pH values were observed, and the semi-quantification of the *in situ* biosynthesized pediocin PA-1 was documented for the first time in a fermented meat sausage model.

Keywords: *L. monocytogenes*; high hydrostatic pressure; biocontrol approaches, bacteriophage; *P. acidilactici*; pediocin PA-1.

1. Introduction

Alheira is a traditional fermented meat sausage, typically produced in the Northern region of Portugal and elected as one of the Portuguese seven gastronomic wonders. It is a not-ready-to-eat (NRTE) product, semi-dried (usually ≥ 50% moisture and $a_w \geq 0.95$), and naturally fermented (Azevedo, Barbosa, Albano, & Teixeira, 2020; Ferreira et al., 2007; Ferreira et al., 2006). The *Alheira* paste entails a mixture of different types of meats (pork, chicken, turkey, veal, along with others) in combination with bread and fat; specific spices (pepper, paprika), salt, aromatics, olive oil, and garlic are also included in the formulation, being the final product obtained by curing the sausages in a smoking room (Marcos, Viegas, de Almeida, & Guerra, 2016).

Listeria monocytogenes is a ubiquitous and psychrotrophic Gram-positive foodborne pathogen, being the etiological agent of listeriosis, a rare, albeit severe illness associated with the highest percentage of hospitalized cases of all zoonosis under European Union (EU) surveillance (European Food Safety Authority & European Centre for Disease Prevention and Control, 2021). Despite the assumption that fermented sausages do not support the growth of *L. monocytogenes*, hence being rarely associated with listeriosis outbreaks (EFSA, 2017), Ferreira et al. (2007) reported over 60% of industrially produced *Alheira* batches as being contaminated with *L. monocytogenes* at levels higher than 100 CFU g^{-1} (Felicio, Hogg, Gibbs, Teixeira, & Wiedmann, 2007; Ferreira et al., 2007, 2011).

The noticeable ability of *L. monocytogenes* to overcome low pH values and high salt concentrations, characteristic features of the Mediterranean-style fermented sausages, has been extensively documented (Meloni, 2015). Moreover, its capability to grow, and

become more resistant to the human gastrointestinal passage, following adaptation to the cold storage of these products, has been reported (Alves et al., 2020).

The United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS), under the directive 7111.1 from 2017, does not require lethality treatment for NRTE products partially cooked, since they will be cooked by the consumer (USDA-FSIS, 2017). Nonetheless, the domestic cooking of *Alheira* is a critical point concerning food safety since the heat distribution is irregular and the time/temperature binomen is not always adequately accomplished (Felício et al., 2011). Moreover, most of the standard guidelines established for lethality and stabilization processes in ready-to-eat (RTE) food products, including meat and poultry products, require at least a 5 log reduction for a proper decontamination validation (Chikthimmah, GUYER, & KNABEL, 2001; Karolenko, Bhusal, Nelson, & Muriana, 2020).

Food safety surveillance and technological improvement of safety and quality attributes of traditional foods are important emerging concerns (Fusco et al., 2015). Research organizations and social media have been engaged in promoting the consumers' knowledge concerning novel food processing technologies as well as the application of natural antimicrobials, in order to increase the confidence towards the consumption of traditional and ethnic food products (Hygreeva & Pandey, 2016). Likewise, this on-going trend for innovative technologies also attends consumers' expectations for greener food processing and sustainability, "clean label", natural, and fresh-like regional food products (Aschemann-Witzel, Varela, & Peschel, 2019; Hernández-Hernández, Moreno-Vilet, & Villanueva-Rodríguez, 2019).

The proper application of the hurdle technology is a promising opportunity to eliminate, or to reduce to extremely low levels, *L. monocytogenes* contamination (Meloni, 2015). Innovative applications for hurdle technology by combining natural antimicrobials

are well documented, including plant extracts (e.g. essentials oils, phenolic compounds), microbial-derived (e.g. bacteriocins, bacteriophages, endolysins), and animal-derived antimicrobials (e.g. lysozyme) (Oliveira, Ramos, Ramos, Piccoli, & Cristianini, 2015; Rodríguez-Rubio et al., 2015).

Bacterio(phages) are viruses that specifically infect bacterial cells, being considered "Generally Recognized As Safe" (GRAS). Nowadays, phages are available and legally authorized for application in a plethora of food products for the biocontrol of *L. monocytogenes* in the US, Canada, Australia, and New Zealand (Chibeu et al., 2013). In Europe, the European Food Safety Agency (EFSA) has issued a positive scientific opinion regarding the bacteriophage P100 application in RTE food products. Moreover, a Court Order of the European Court of Justice authorized the listeriophages' use in those products, albeit a specific regulation, established by the European Commission, is still pending (EFSA Panel on Biological Hazards, 2016). The incorporation of phages in RTE foods appears not to impact the flavor, color and taste of the treated products, including different types of meat products (Perera, Abuladze, Li, Woolston, & Sulakvelidze, 2015). Furthermore, consumers would be willing to pay an extra price for fresh produce which had been treated with phages (Naanwaab, Yeboah, Ofori Kyei, Sulakvelidze, & Goktepe, 2014).

A bacteriocinogenic lactic acid bacterium is defined as a Gram-positive, catalase and oxidase-negative microorganism, which biosynthesizes lactic acid as the main metabolic end product of carbohydrate fermentation and bacteriocins (heat-stable, ribosomally synthesized antimicrobial peptides, with narrow or broad spectrum). Commercial starter cultures comprising bacteriocinogenic LAB are available, as well as bacteriocins solutions (e.g. Nisaplin® and MicroGARD®, nisin and pediocin PA-1 based fermented products, respectively) (Silva, Silva, & Ribeiro, 2018).

High hydrostatic pressure (HHP) is a well-established innovative non-thermal technology that is becoming more conventionally used concerning food processing (Yang et al., 2021). Indeed, its industrial utilization is increasing in the US and EU. On the order hand, only a few studies exploited the potential of the association of conventional biocontrol agents (namely, bacteriocinogenic lactic acid bacteria and phages) with the emerging high-pressure processing. Preliminary results demonstrated that the effectiveness of the process (lethality) was dependent on the biocontrol agent baroresistance/stability and on the components of the food matrix and should be validated for each single food product/process binomial (García-Anaya et al., 2020; Komora et al., 2018; Shannon, Radford, & Balamurugan, 2020).

One of the main drawbacks of the HHP technology is the high cost of the commercial equipment (€500,000-3,500,000) (Zhu et al., 2021), hence outsourcing or tolling may constitute viable alternatives to small and medium-size companies. The HHP processing costs are estimated to be between €0.05 to 0.140 kg^{-1} (Escobedo-Avellaneda, Espiricueta-Candelaria, Calvo-Segura, Welti-Chanes, & Chuck-Hernández, 2021; Tonello-Samson, Queirós, & González-Angulo, 2020; Yamamoto, 2017), according to the operating parameters (holding time and pressure magnitude) and operations scale. In order to overcome this economic limitation, the employment of the hurdle technology may allow to minimize the operating parameters, thus improving the cost-effectiveness of the processing. For instance, bacteriophages (€0.022 to 0.088 kg^{-1}) (Vikram, Woolston, & Sulakvelidze, 2021) or bacteriocinogenic lactic acid bacteria (€0.010 kg^{-1}) (van Wagenberg, van Horne, & van Asseldonk, 2020) could constitute affordable adjuvants to high-pressure processing. Moreover, the HHP-minimally processed *Alheira* may guarantee the compliance with the FDA "zero-tolerance" policy (FDA, 2017) for *L. monocytogenes*, which poses a pivotal challenge to the exportation. Hence, the economic

benefits obtained by surpassing this constraint, may outweigh the mentioned high capital investment.

In this sense, innovative food processing technologies which promote food safety and ensure a minimal impact on the biochemical and organoleptic properties of traditional foods are imperatively needed. The purpose of this work was to evaluate the combined effect of mild high-pressure processing (300 MPa) with phage P100 and *Pediococcus acidilactici* HA6111-2, as a novel decontamination method to inactivate *L. monocytogenes* in fermented meat sausages, immediately following processing.

2. Material and Methods

2.1 Microorganisms and inoculum preparation

The microorganisms utilized in this study are deposited in the *Listeria* Research Center of Escola Superior de Biotecnologia (LRCESB) and were selected based on their origin and on the pressure range resistance previously described (Bruschi et al., 2017; Castro et al., 2015; Komora et al., 2018).

2.1.1 *Listeria monocytogenes* isolates

Two different strains of *L. monocytogenes* belonging to serotype 4b were used to perform independent challenge assays, namely *L. monocytogenes* Scott A (Lm ScottA), a well described strain concerning HHP inactivation (Bover-Cid, Serra-Castelló, Dalgaard, Garriga, & Jofré, 2019) and *L. monocytogenes* 1942 (Lm 1942), isolated from

Alheira and previously characterized regarding its behaviour in HHP processing (Bruschi et al., 2017).

Listeria monocytogenes ATCC 19116 (serotype 4c) was used as phage Listex™ P100 host (Veloso, 2014).

Listeria isolates were preserved at -20 ºC in tryptic soy broth (Pronadisa, Madrid, Spain) supplemented with 0.6% (w/v) of yeast extract (Lab M, Lancashire, United Kingdom) (TSBYE) containing 30% (v/v) of glycerol (Sigma, Steinheim, Germany). For the preparation of the inocula, one single colony of each *L. monocytogenes* isolate was transferred from tryptic soy agar (Pronadisa) supplemented with 0.6% (w/v) of yeast extract (Lab M) (TSAYE) into 10 mL of TSBYE and incubated at 37 ºC for 24 h. This culture was then subsequently diluted 1:100 in TSBYE and incubated in the same conditions. Cells were harvested by centrifugation (7000 x g for 10 min at 4 ºC; Rotina 35R, Hettich, Germany), re-suspended in 10 mL of sterile ¼-strength Ringer's solution (R/4; Lab M) and homogenized to obtain an initial inoculum concentration of *ca.* 10^9 colony forming units (CFU) mL^{-1}.

2.1.2. Pediocin PA-1 producing lactic acid bacterium

Pediococcus acidilactici strain HA-6111-2, an autochthone bacteriocinogenic LAB isolated from *Alheira* (Albano et al., 2007), was selected as the bioprotective culture; the baroresistance of *P. acidilactici* along with the impact of HHP on the proteinaceous synthesis following pressure exposure was also previously documented (Castro et al., 2015).

Pediococcus acidilactici strain HA-6111-2 was preserved and cultured under the same conditions described above, except that the growth media employed were de Man, Rogosa & Sharpe (MRS) broth and agar (Lab M).

2.1.3. Bacteriophage

Bacterio(phage) Listex™ P100 stock solution (Micreos Food Safety, The Netherlands) was stored at 4° C in the original buffer and the initial concentration was 10^{11} plaque forming units (PFU) mL^{-1}. A working solution of phage (*ca.* 10^{10} PFU mL^{-1}) was freshly prepared on the day of the experiment by diluting the stock solution in phosphate buffered saline (PBS, 0.1 M, pH 7.4, VWR Chemicals, Ohio, USA).

2.2 Preparation and artificial inoculation of the fermented meat sausage model

Alheira was selected based on the previously reported prevalence of *L. monocytogenes* in this type of food matrix (fermented meat sausage) and samples were purchased from a local supermarket (Porto, Portugal).

Fermented meat sausage paste was previously sterilized by autoclaving (121 ºC, 15 min) before being inoculated as described by Castro et al. (2017), in order to avoid interferences and the variability of the initial endogenous microbiota on the phage Listex™ P100 stability. The *Alheira* pastes (*ca.* 100 g) were placed into sterile stomacher bags and inoculated as described in Table 1. Homogenisation was manually performed by gently massaging the sample (*ca*. 3 min). The final phage Listex™ P100 had a multiplicity of infection (MOI; ratio of phage to bacteria cells) of 1000 in the inoculated samples. Afterwards, the *Alheira* pastes were double vacuum sealed in low permeability

polyamide-polyethylene bags (PA/PE-90, Albipack - Packaging Solutions, Portugal). One set of samples was submitted to mild HHP (300 MPa, 5 min, 10 ºC) while the other set was stored at 4 °C under atmospheric pressure (non-pressure treated control samples). All experiments were performed in three independent replicates.

2.3 High hydrostatic pressure treatments

The samples were pressurized at 300 MPa (10 ºC) in a hydrostatic press from Hiperbaric 55 (Burgos, Spain) using water as the pressure-transmitting fluid, and a pressurization rate of *ca.* 100 MPa *per* 7 s. After 5 min, the pressure was released (< 3 s) and the samples were immediately cooled in an ice-water bath and then transferred to refrigerated storage (4 ºC). The pressure magnitude was selected based on what was previously documented concerning the bacteriophage P100 stability upon exposure to HHP, and the maintenance of its infectivity was observed until 300 MPa (Komora et al., 2018). The samples were treated in three independent batches.

2.4 Bacterial enumeration and phage Listex™ P100 titration

Listeria monocytogenes detection (enrichment protocol) and enumeration were performed according to the ISO 11290-1:2017 and ISO 11290-2:2017 standards, respectively (ISO, 2017). The selective agar medium used was PALCAM agar (Merck, Darmstadt, Germany) plus selective supplement (Merck).

Pediococcus acidilactici was serially diluted in sterile ¼ Ringer's solution and plated in duplicate for enumeration by the drop count technique (Miles, Misra, & Irwin, 1938) on MRS agar and incubated at 37 ºC for 48 h, aerobically.

Phage Listex™ P100 titre (PFU mL^{-1}) was determined by the double-layer method (plaque assay) as previously described by (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009) with modifications in the media and diluent. TSAYE was selected as the solid media (underlay) and TSBYE, containing 7g L^{-1} of bacteriological agar (VWR Chemicals), was used as molten soft agar (overlay). The diluent used in the experiments was PBS (0.1 M, pH 7.4, VWR Chemicals) and the detection limit of the enumeration technique was 10 PFU mL^{-1}.

2.5 Pediocin PA-1 biosynthesis during refrigerated storage

Bacteriocin recovery from the food matrices was performed according to Baños et al. (2016) with some modifications. Briefly, 5 g of sample were dissolved (1:10) in 50 mM sodium acetate, 100 mM EDTA (Sigma) and 0.2% Triton X100 (Sigma) at pH 5 and homogenized in BagMixer (Interscience, Saint Nom, France) for 1 min, boiled for 15 min, cooled and centrifuged (5000 x g for 10 min at 4 °C). The bacteriocin in the interphase was carefully transferred to a new glass flask and precipitated with 300 g L^{-1} ammonium sulphate (VWR Chemicals, Leuven, Belgium) for 48 h at 4 °C. The resulting pellet was dissolved in phosphate buffer (50 mM, pH 7.2) and heated at 80 °C for 10 min.

The antilisterial activity of pediocin PA-1 was measured by two-fold dilutions and the spot-on-the-lawn method as described by van Reenen et al. (1998) and expressed in arbitrary units (AU) mL^{-1}. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition of the target bacterium, *L. monocytogenes* Scott A.

2.6 Phage Listex™ P100 stability during refrigerated storage

The Listex™ P100 ability to maintain its stability and viability during shelf-life storage (4 °C) following HHP treatment was assessed. At pre-set time intervals (before treatment, 7, 14, 21, 28, 45 and 60 days), non- and pressure-treated samples were analysed. All experiments were conducted in three independent replicates.

2.7 Determination of pH

The pH of non- and minimally processed *Alheira* model was measured throughout refrigerated storage, using a Meat pH meter (HI 99163, Hanna Instruments, Póvoa de Varzim, Portugal) equipped with a probe for solids inserted directly into the *Alheira* paste. All experiments were conducted in three independent replicates.

2.8 Mechanistic insights on *Listeria monocytogenes* inactivation through the non-thermal multi-hurdle approach

In order to deeply investigate the inactivation mechanism of *L. monocytogenes* 1942 (10^7 CFU mL^{-1}) *post* exposure to HHP (300 MPa, 5 min, 10 °C), pediocin PA-1 (1280 AU mL^{-1}) and phage P100 (10^8 PFU mL^{-1}) or the three hurdles associated, following a two-hour challenge at 10 °C were visualized by transmission electron microscopy (TEM). Briefly, 1 mL aliquots of each hurdle and the combined system prepared in PBS were submitted to HHP (300 MPa, 5 min, 10 °C) and one sample was maintained at atmospheric pressure (0.1 MPa, 4 °C) to serve as a control. The samples were deposited on Formvar/carbon film-coated mesh nickel grids (Electron Microscopy Sciences, Hatfield, PA, USA) and left standing for 2 min, negatively stained with 2% uranyl acetate (pH 4.0) and examined using a JEOL JEM 1400 TEM at 120kV (Tokyo, Japan). Images were digitally recorded using a CCD digital camera Orious 1100W (Tokyo, Japan).

2.9 Statistical analysis

Bacterial and phage titration values were submitted to a logarithmic reduction using the equation: log (N/N0), where N is the bacterial cell density/ phage titre at a particular sampling time and N0 is the initial cell density/ phage titre. The differences were analysed using the one-way analysis of variances (ANOVA) with Tukey's post hoc test (SPSS, Version 23.0, Inc., Chicago, IL) when homogeneity of variance was assumed. The data obtained from the detection procedure was presented as presence or absence of *L. monocytogenes* and significant differences were determined using the chi-square test.

The additive effects and the corresponding confidence intervals at 95% of hurdles applied in *L. monocytogenes* inactivation were determined according to Komora et al. (2020). A synergistic effect was considered when, applied together, the hurdle conditions cooperate for an enhanced effect producing an effect greater than the sum of the individual effects.

3. Results and Discussion

3.1 Inactivation of *L. monocytogenes* in fermented meat sausage model

Results of *L. monocytogenes* inactivation in fermented meat sausage model are presented in Figure 1; Table 1 summarizes the results obtained for the detection of *Listeria.* Both strains evaluated (Lm Scott A and Lm 1942) were inactivated by all the treatments to values below the detection limit of the enumeration technique during the refrigerated storage at 4 ºC, except for the control samples inoculated exclusively with 10^5 CFU g^{-1} of

L. monocytogenes (pressurized and non-pressurized). Concerning those samples in which only the pathogenic bacterium was inoculated, a slight growth of *L. monocytogenes* was observed in non-pressurized samples (1.27 and 1.20 log increase for Lm Scott A and Lm 1942, respectively) over the 60 days of storage. In HHP treated samples, as expected, after a sub-lethal stress owing to mild high-pressure (300 MPa) exposure, a bacteriostatic effect was observed on *L. monocytogenes* throughout the refrigerated storage (0.29 and 0.32 log increase at the end point of storage for Lm Scott A and Lm 1942, respectively). Sub-lethal injuries induced by mild pressures (e.g. 300 MPa) in *L. monocytogenes* have been documented, mainly related to the cytoplasmic membrane (structural damage, increasing the cell permeability and thereby improving the effectiveness of the biocontrol agents). A simple and robust assay for assessing the bacterial cell injury/viability was described, relying on the comparison of the recovery of HHP submitted cells in selective, differential, and non-selective medium; injured cells are unable to grow in the selective medium (Bruschi et al., 2017; Ferreira, Almeida, Delgadillo, Saraiva, & Cunha, 2016). Furthermore, sub-lethal injuries in *L. monocytogenes* strains herein investigated were previously evaluated in TSBYE model, along with the promising pronounced effect of pediocin PA-1 in these injured cells (Bruschi et al., 2017).

Concerning both pressurized (300 MPa, 5 min, 10 ºC) and non-pressurized samples, treatments comprising only phage P100 as the biocontrol agent were unable to eliminate *L. monocytogenes* over the 60 days of storage. Nonetheless, synergism between HHP and phage P100 was observed in samples treated at 300 MPa, in which *L. monocytogenes* counts decreased immediately following HHP treatment to below the detection limit of the enumeration technique (i.e. 2 log), eliciting 3.10 and 3.15 log declines for Lm Scott A and Lm 1942, respectively. In non-pressurized samples (0.1 MPa, 4 ºC), the same result was only attained following 14 days of refrigerated storage. The anti-listerial effect of

phage P100 has been extensively investigated in a multitude of distinct food matrices, namely meat, dairy, fish, vegetables, and fruits (Axelsson et al., 2020; Gutiérrez et al., 2017; Iacumin et al., 2016; Oliveira et al., 2014; Rossi et al., 2011; Silva et al., 2014). While phage P100 loses stability when submitted to treatments in liquid acidic food matrices (Komora et al., 2018; Oliveira et al., 2014), RTE and NRTE meat products appear to guarantee the maintenance of phage's stability required for application in these products. Similarly to the results herein obtained, Rossi et al. (2011), Gutiérrez et al. (2017) and Iacumin et al. (2016) reported that processing fresh sausage and dry-cured ham inoculated with *L. monocytogenes* and treated with phage P100 achieved over 2.5 log decrease, which in some cases resulted in reductions to values below the detection limit of the enumeration technique, or the bacterial absence. The pronounced antimicrobial effect of phage application concomitant with high-pressure processing has also been previously described (Ahmadi, Anany, Walking-Ribeiro, & Griffiths, 2015; Komora et al., 2020; Tabla et al., 2012). Indeed, the synergistic effect between phages and mild high-pressure processing in liquid food matrices as UHT whole milk has been demonstrated as an innovative model system to eliminate *L. monocytogenes* and *Staphyloccocus aureus* (Komora et al., 2020; Tabla et al., 2012). Concerning solid food matrices, Ahmadi et al. (2015) documented no *Shigella flexneri* recovery (> 3.8 log reductions) in ground beef with the combination of HHP (350 MPa, 5 min or 450 MPa, 5 min) and virulent *S. flexneri* phages (10^9 PFU g^{-1}), albeit samples treated exclusively with HHP or phage resulted in the bacterial recovery under optimal conditions. In the same study, the authors also evaluated the *Vibrio cholerae* inactivation in salmon fillets and mussels; the findings suggested that phage application was only effective when performed after mild high-pressure processing (350 MPa, 5 min), eliciting no bacterial regrowth (over 3.8 and 3.9 log reduction in salmon fillets and mussels, respectively),

whereas virulent phage exposure prior to HHP, and HHP *per se*, resulted in bacterial regrowth.

The observed HHP-phage synergism phenomenon may be the result of one of two hypotheses: (i) due to the sample homogenization resultant from HHP processing, which may allow a better phage particles' dispersion in *Alheira* paste, bridging the problem of phage-host interaction in solid matrices, since phage's mobility (i.e. the ability of phage to diffuse through the food matrix) is a core determinant in the phage treatment efficacy (in non-pressurized samples, bacterial cells were not readily available for phage binding) (Lewis & Hill, 2020; Simmons, Drescher, Nadell, & Bucci, 2018); or (ii) due to the sensibilization elicited by mild-HHP (300MPa) that induced sub-lethal injuries (e.g. swellings and protrusions in cell wall, increased cell volume and disintegration of the cell wall from the cell membrane) in the pathogenic bacteria cells, improving the availability of recognition sites for phage's binding (Ferreira et al., 2016).

All the treatments in which *P. acidilactici* HA-6111-2 was incorporated, demonstrated to elicit the *L. monocytogenes* inactivation to undetectable levels by the enrichment protocol throughout the shelf-life of *Alheira* model stored at 4 °C. The synergistic effect of the combination of HHP (300 MPa, 5 min, 10 ºC) and *P. acidilactici* HA-6111-2 (10^7 CFU g^{-1}) decreased the *L. monocytogenes* initial load to undetectable levels within only 72 h following pressurization (over 5 log reductions for both *Listeria* strains). In non-pressure treated samples solely inoculated with *P. acidilactici* HA-6111-2, *L. monocytogenes* was not detected only after 21 days of refrigerated storage. The same result ($P > 0.05$) was observed for non-pressurized samples treated with both phage P100 and *P. acidilactici* HA-6111-2, being the antilisterial effect mainly attributed to the latter, since phage titre decreased over the storage period due to the acidification promoted by the bacteriocinogenic LAB.

Despite the limitations inherent to the complexity of the meat products (e.g., bacteriocin adsorption to fat globules), the biocontrol system associating bacteriocinogenic LAB and/or their secondary metabolites to high-pressure processing, in cured-cooked meat products has been documented as a promising approach based on the hurdle technology concept (Castro et al., 2017, 2018; Dallagnol et al., 2017; Gálvez, Abriouel, López, & Omar, 2007; Garriga, Aymerich, Costa, Monfort, & Hugas, 2002). Previous studies performed in *Alheira* model with *P. acidilactici* HA-6111-2 targeting *L. innocua* inactivation, through the combination of the mentioned LAB with HHP (300 MPa, 5 min, 10 ºC), demonstrated the potential of the synergistic application. Moreover, the authors documented a higher feasibility of *in situ* bacteriocin biosynthesis (*ca*. 1.9 to 4.7 log reductions) than the application of pediocin produced *ex situ*, which resulted in lower logarithmic reductions (< 2 log) (Castro et al., 2018). Despite the synergism observed in both the present study and the one conducted by Castro et al. (2018), only the approach herein exploited elicited the absence of the target bacteria. The differences between these results could be explained by several possibilities: i) the *Listeria* strains used (according to Bruschi et al. (2017), Lm 1942 was more barosensitive at 300 MPa than *L. inoccua* 2030c); ii) *Alheira* composition; and iii) inoculum level of the target bacteria (10^6 and 10^8 CFU g^{-1} of *L. innocua*).

The synergistic treatment between HHP, phage P100, and *P. acidilactici* HA-6111-2 elicited the absence of *L. monocytogenes* (over 5 log reductions for both strains) in fermented meat sausage model, allowing the USDA-FSIS 5 log reduction (in accordance with the standard guidelines for RTE food products) following pressurization, while neither the individual nor the combined treatments achieved the same logarithmic reduction at the processing day ($P < 0.05$). The hurdle approach comprising bacteriophages and bacteriocins/bacteriocinogenic LAB has been documented in

coleslaw, UHT milk, extended shelf life (ESL) milk, RTE sliced pork ham, and fish (Baños et al., 2016; Figueiredo & Almeida, 2017; Komora et al., 2020; Lewis, Bolocan, Draper, Ross, & Hill, 2019; Rodríguez-Rubio et al., 2015).

Few studies have demonstrated the ability of listeriophages to act synergistically with different bacteriocins, namely coagulin C23 (Rodríguez-Rubio et al., 2015), enterocin AS-48 (Baños et al., 2016), and pediocin PA-1 (Komora et al., 2020), in ESL milk, UHT whole milk and raw salmon or hake, respectively. Moreover, Komora et al. (2020) have proven the synergistic listericidal effect of associating the phage-bacteriocin system with HHP processing (200 or 300 MPa, 5 min, 10 ºC. On the other hand, Lewis et al. (2019) and Figueiredo and Almeida (2017) investigated the combined effect of Nisaplin® (commercial solution based on nisin) and nisin, respectively, with phage P100 to control *L. monocytogenes* in coleslaw and sliced pork ham, and did not find neither synergistic nor additive effect by applying both hurdles in association.

Phage P100 titre in non-pressurized samples of *Alheira* model encompassing *P. acidilactici* HA-6111-2 decreased from 8.03 ± 0.04 log PFU g^{-1} to 5.25 ± 0.10 and 5.12 ± 0.07 log PFU g^{-1} for Lm Scott A and Lm 1942, respectively. In pressurized samples (300 MPa, 5 min, 10 ºC), phage P100 titre was 7.82 ± 0.17 log PFU g^{-1} following two hours of the pressure challenge and 4.95 ± 0.07 and 5.03 ± 0.05 log PFU g^{-1} at the endpoint of refrigerated storage of fermented meat sausage model for Lm Scott A and Lm 1942, respectively.

The decreasing pH effect in fermented food matrices on the phage titre concomitant decrease has been reported by other authors (Bueno, García, Martínez, & Rodríguez, 2012; García-Anaya et al., 2020; García, Madera, Martínez, & Rodríguez, 2007; Lewis & Hill, 2020; Shannon et al., 2020).

García et al. (2007) evaluated the stability of the *S. aureus* phages Φ38 and Φ88 during acidic curdling of pasteurized whole milk with *Lactococcus lactis* subsp. *lactis* IPLA 947 (pH declining from *ca*. 6.8 to 4.5) and observed *ca*. 2 log reduction in the phages' titres over a 12 h incubation period, whereas enzymatic curd (stable pH) did not impact phage's stability. In another study with the same phages (Φ38 and Φ88), Bueno et al. (2012) investigated the viruses' stability during fresh and hard type cheese manufacturing; in fresh type cheese, phages' titres decreased due to low pH (~ 4.24) generated during manufacturing, and the indigenous LAB and starter culture increased over 2.5 log during coagulation. Contrastingly, in hard type cheese, the pH ranged from 6.05 to 5.98 after whey drainage, being both pH and phages' titres stable during ripening over 30 days of storage. Moreover, Komora et al. (2018) demonstrated that phage P100 was stable throughout 60 days of storage at 4 ºC in *Alheira* model without the addition of LAB starter culture.

As a whole, considering the economic variables (e.g., costs associated with the phage's production and purification) and the industrial processing of *Alheira* (for instance, *ca*. 72 h of stabilization process after the smoking stage), the incorporation of *P. acidilactici* HA-6111-2 appears to be the most sustainable approach to mitigate the *L. monocytogenes* risk in this traditional fermented sausage. Nonetheless, the presence of different antimicrobial barriers, as well as the proper application of the hurdle technology, is an asset to address *L. monocytogenes* adaptation in overcoming the antagonistic effect exerted by the biocontrol agents, since environmental stresses from real industrial scenarios could hinder the inactivation obtained at the laboratory scale/optimal conditions. *Listeria monocytogenes* strains resistant to bacteriocins are stable, and resistance frequencies previously determined by Gravesen et al. (2002) were in most cases *ca*. 10^{-6}, ranging from 10^{-7} to 10^{-2} for pediocin PA-1 and nisin, respectively. The

percentage of phage P100 insensitive strains across 486 *L. monocytogenes* strains (isolated from over 59 Austrian dairy processing plants) was estimated to be 2.7%, and the emergence of resistant strains was closely related to the phage treatment in those processing plants (Fister et al., 2016). There is also strong evidence that natural antimicrobials as bacteriocins, when combined, could generate a pronounced effect in comparison to the same concentration of the individual antimicrobials. In fact, the use of different classes of natural antimicrobials is also a relevant point since cross-resistance has been observed in the same class bacteriocins and/or between phages (Kaur, Singh, & Malik, 2013; Trudelle, Bryan, Hudson, & Denes, 2019).

3.2 The impact of the starter culture on the pH of the fermented meat sausage model

The pH evaluation throughout the refrigerated storage of non- and minimally processed fermented meat sausage model is outlined in Figure 2. The pH values determined for the different processed, fermented meat sausages were not significantly different ($P > 0.05$) and were slightly higher than the previous documented concerning the utilization of *P. acidilactici* HA 6111-2 as starter culture in *Alheira* model; the observed differences in the pH value could be explained by the different formulations/recipes used in the *Alheira* manufacturing. Albano et al. (2009) were the first to describe the potential of *P. acidilactici* HA 6111-2 to be used as a biopreservative in traditional Portuguese fermented meat products; in pilot assays, the pH value of *Alheira* model was 4.5 prior to the smoking process, whilst throughout the storage time (54 days, 5 ºC), mean pH values decreased approximately 0.5 units in both spontaneous fermented, or inoculated with *P. acidilactici* HA 6111-2, sausage pastes. Thus, this pH value decline

is in accordance with those observed in non- and HHP-phage-LAB treated samples of the present study. In contrast to these findings, Ben Slima et al. (2018) and Van Ba et al. (2016) documented lower pH values when LAB were utilized in the fermentation processes.

3.3 **Mechanistic insights on *Listeria monocytogenes* inactivation through non-thermal hurdles combined application**

Ultrastructural changes in *L. monocytogenes* cells (10^7 CFU mL^{-1}) induced by HHP (300 MPa, 5 min, 10 °C), pediocin PA-1 (1280 AU mL^{-1}) and phage P100 (10^8 PFU mL^{-1}) or the three hurdles association were assayed in PBS (0.1 M), following a two-hour challenge at 10°C, and visualized by TEM. Logarithmic reductions in PBS were 1.78±0.11, 3.78±0.15 and over 5.00 cycles (below the detection limit of the enumeration technique) for HHP, pediocin PA-1 and phage P100, respectively (data not shown). As expected, the three hurdles combined also elicited the complete inactivation of *L. monocytogenes*. Figure 3 presents TEM imaging concerning ultrastructural changes in *L. monocytogenes* cells upon exposure to the different biocontrol approaches. Control samples showed the characteristic structure and morphology of a Gram-positive bacillus, with a thick and uniform cell wall, displaying a compact adhesion to the cell membrane (Fig. 3-A).

Listeria cells treated with pediocin PA-1 presented a remarkable transparent area between the cytoplasm membrane and cell wall, implying a possible distention of these layers (Fig. 3-B). These observations were in accordance with the previously documented by Dallagnol et al. (2017), namely the enlargement of an electron transparent area between these layers after exposure to lactocin AL705 (105 AU mL^{-1}). In accordance with the results herein visualized, class IIa bacteriocins are well-known to induce

permeabilization of the cell membrane of the target bacteria by forming ion-selective pores, leading to disruption of the cell membrane of *L. monocytogenes* and, subsequently, lysis (Kumariya et al., 2019). TEM visualization of *L. monocytogenes* treated at 300 MPa unraveled slight changes in ultrastructural morphology. Albeit a scarce roughness could be observed throughout the cell wall, the major damages were membrane rupture with leakage of the intracellular substrates and nuclear content condensation (which, in this case, may be reversible) (Fig. 3-C). The enlargement of fibrillar regions of DNA was previously described as an effect from high-pressure treatment in *L. monocytogenes* cells, along with invaginations provoked by constraining the cell membrane within the rigid cell wall, supporting membrane rupture (Mackey, Forestière, Isaacs, Stenning, & Brooker, 1994). Phage P100 application resulted in *L. monocytogenes* cells completely surrounded by attached phages particles, many of which leading to injection of the genetic material into the host cell and, following the lytic cycle, cell lysis eliciting the cytoplasmatic material leakage (Fig. 3-D). When the three hurdles were combined, no intact cell was observed, with numerous plasmatic material residues dispersed throughout the visualized preparation, some of which were still attached to the phage (Fig. 3-E).

Conclusion

The present study demonstrated that the non-thermal treatment combining mild high hydrostatic pressure, phage P100, and bacteriocinogenic *P. acidilactici* was able to eradicate *L. monocytogenes* in *Alheira* model immediately following processing, with no regrowth being observed during the 60-days of storage. Moreover, this innovative multi-hurdle technology elicited the USDA-FSIS 5 log reduction through a synergistic effect generated by the combined hurdles. Mild high hydrostatic pressure in combination with natural antimicrobials of narrow spectrum may represent a feasible option of minimal processing concerning microbial decontamination of cured and fermented meat

products, while preserving the authenticity of the organoleptic features of traditional food products. To the best of our knowledge, this is the first comprehensive study evaluating the impact of the association of bacteriophage P100, pediocin PA-1 producing LAB, and HHP towards *L. monocytogenes* inactivation in a fermented meat sausage model.

Acknowledgments

This work was supported by the project "Safe meat products" GO77 PDR2020-1.0.1-FEADER-031359. The authors would like to thank the scientific collaboration under the FCT (Fundação para a Ciência e a Tecnologia) project UIDB/50016/2020 and to University of Aveiro and FCT/MCT for the financial support for LAQV-QUIMTE Research Unit (FCT UIDB/50006/2020) through national funds and, where applicable, co-financed by the FEDER, within the PT2020 Partnership Agreement. Financial support for authors Sónia Marília Castro and Cláudia Maciel was provided by FCT through fellowships SFRH/BPD/71723/2010 and SFRH/BD/104016/2014, respectively.

Credit author statement

Norton Komora: Conceptualization, Investigation, Methodology, Data curation, Writing- Original draft. **Cláudia Maciel**: Conceptualization, Investigation, Methodology, Data curation, Writing- Original draft, Writing- Review and Editing. **Renata Amaral**: Investigation. **Rui Fernandes**: Investigation, Data curation. **Sónia Marília Castro**: Conceptualization, Methodology, Supervision. **Jorge A. Saraiva**: Writing- Review and

Editing, Funding acquisition. **Paula Teixeira**: Conceptualization, Supervision, Validation, Funding acquisition and Project administration.

References

Ahmadi, H., Anany, H., Walking-Ribeiro, M., & Griffiths, M. W. (2015). Biocontrol of *Shigella flexneri* in ground beef and *Vibrio cholerae* in seafood with Bacteriophage-Assisted High Hydrostatic Pressure (HHP) treatment. *Food and Bioprocess Technology*, *8*, 1160–1167. https://doi.org/10.1007/s11947-015-1471-6

Albano, H., Pinho, C., Leite, D., Barbosa, J., Silva, J., Carneiro, L., … Teixeira, P. (2009). Evaluation of a bacteriocin-producing strain of *Pediococcus acidilactici* as a biopreservative for "Alheira", a fermented meat sausage. *Food Control*, *20*(8), 764–770. https://doi.org/10.1016/j.foodcont.2008.09.021

Albano, H., Todorov, S. D., van Reenen, C. A., Hogg, T., Dicks, L. M. T., & Teixeira, P. (2007). Characterization of two bacteriocins produced by *Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology*, *116*(2), 239–247. https://doi.org/10.1016/j.ijfoodmicro.2007.01.011

Alves, Â., Magalhães, R., Brandão, T. R. S., Pimentel, L., Rodríguez-Alcalá, L. M., Teixeira, P., & Ferreira, V. (2020). Impact of exposure to cold and cold-osmotic stresses on virulence-associated characteristics of *Listeria monocytogenes* strains. *Food Microbiology*, *87*, 103351. https://doi.org/10.1016/j.fm.2019.103351

Aschemann-Witzel, J., Varela, P., & Peschel, A. O. (2019). Consumers' categorization of food ingredients: Do consumers perceive them as 'clean label' producers

expect? An exploration with projective mapping. *Food Quality and Preference*, *71*(January 2018), 117–128. https://doi.org/10.1016/j.foodqual.2018.06.003

Axelsson, L., Bjerke, G. A., McLeod, A., Berget, I., & Holck, A. L. (2020). Growth behavior of *Listeria monocytogenes* in a traditional norwegian fermented fish product (Rakfisk), and its inhibition through bacteriophage addition. *Foods*, *9*(2). https://doi.org/10.3390/foods9020119

Azevedo, I., Barbosa, J., Albano, H., & Teixeira, P. (2020). Non meat-based alheiras– a safer novel trend? *Food Control*, *113*(October 2019), 107177. https://doi.org/10.1016/j.foodcont.2020.107177

Baños, A., García-López, J. D., Núñez, C., Martínez-Bueno, M., Maqueda, M., & Valdivia, E. (2016). Biocontrol of *Listeria monocytogenes* in fish by enterocin AS-48 and *Listeria* lytic bacteriophage P100. *LWT - Food Science and Technology*, *66*, 672–677. https://doi.org/10.1016/j.lwt.2015.11.025

Ben Slima, S., Ktari, N., Triki, M., Trabelsi, I., Abdeslam, A., Moussa, H., … Ben Salah, R. (2018). Effects of probiotic strains, *Lactobacillus plantarum* TN8 and *Pediococcus acidilactici*, on microbiological and physico-chemical characteristics of beef sausages. *LWT*, *92*(February), 195–203. https://doi.org/10.1016/j.lwt.2018.02.038

Bover-Cid, S., Serra-Castelló, C., Dalgaard, P., Garriga, M., & Jofré, A. (2019). New insights on *Listeria monocytogenes* growth in pressurised cooked ham: A piezo-stimulation effect enhanced by organic acids during storage. *International Journal of Food Microbiology*, *290*, 150–158. https://doi.org/10.1016/j.ijfoodmicro.2018.10.008

Bruschi, C., Komora, N., Castro, S. M., Saraiva, J., Ferreira, V. B., & Teixeira, P. (2017). High hydrostatic pressure effects on *Listeria monocytogenes* and *L.*

innocua: Evidence for variability in inactivation behaviour and in resistance to pediocin bacHA-6111-2. *Food Microbiology*, *64*, 226–231. https://doi.org/10.1016/j.fm.2017.01.011

Bueno, E., García, P., Martínez, B., & Rodríguez, A. (2012). Phage inactivation of *Staphylococcus aureus* in fresh and hard-type cheeses. *International Journal of Food Microbiology*, *158*(1), 23–27. https://doi.org/10.1016/j.ijfoodmicro.2012.06.012

Castro, S. M., Kolomeytseva, M., Casquete, R., Silva, J., Queirós, R., Saraiva, J. A., & Teixeira, P. (2017). Biopreservation strategies in combination with mild high pressure treatments in traditional Portuguese ready-to-eat meat sausage. *Food Bioscience*, *19*, 65–72. https://doi.org/10.1016/j.fbio.2017.05.008

Castro, S. M., Kolomeytseva, M., Casquete, R., Silva, J., Saraiva, J. A., & Teixeira, P. (2015). Effect of high pressure on growth and bacteriocin production of *Pediococcus acidilactici* HA-6111-2. *High Pressure Research*, *35*(4), 405–418. https://doi.org/10.1080/08957959.2015.1101095

Castro, S. M., Silva, J., Casquete, R., Queirós, R., Saraiva, J. A., & Teixeira, P. (2018). Combined effect of pediocin bacHA-6111-2 and high hydrostatic pressure to control *Listeria innocua* in fermented meat sausage. *International Food Research Journal*, *25*(2), 553–560.

Chibeu, 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 monocytogenes* in cooked turkey and roast beef. *International Journal of Food Microbiology*, *167*(2), 208–214. https://doi.org/10.1016/j.ijfoodmicro.2013.08.018

Chikthimmah, N., GUYER, R. B., & KNABEL, S. J. (2001). Validation of a 5-Log10

Reduction of *Listeria monocytogenes* following simulated commercial processing of Lebanon Bologna in a model system. *Journal of Food Protection*, *64*(6), 873–876. https://doi.org/10.4315/0362-028X-64.6.873

Dallagnol, A. M., Barrio, Y., Cap, M., Szerman, N., Castellano, P., Vaudagna, S. R., & Vignolo, G. (2017). *Listeria Inactivation* by the combination of High Hydrostatic Pressure and Lactocin AL705 on cured-cooked pork loin slices. https://doi.org/10.1007/s11947-017-1956-6

EFSA. (2016). Evaluation of the safety and efficacy of Listex™ P100 for reduction of pathogens on different ready-to-eat (RTE) food products. *EFSA Journal*, *14*(8), e04565. https://doi.org/10.2903/j.efsa.2016.4565

EFSA. (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal*, *15*(12). https://doi.org/10.2903/j.efsa.2017.5077

Escobedo-Avellaneda, Z., Espiricueta-Candelaria, R. S., Calvo-Segura, S., Welti-Chanes, J., & Chuck-Hernández, C. (2021). Changes induced by high hydrostatic pressure in acidified and non-acidified milk during Oaxaca cheese production. *International Journal of Food Science and Technology*, 1–11. https://doi.org/10.1111/ijfs.15134

European Food Safety Authority, & European Centre for Disease Prevention and Control. (2021). The European Union One Health 2019 Zoonoses Report. *EFSA Journal. European Food Safety Authority*, *19*(2), e06406. https://doi.org/10.2903/j.efsa.2021.6406

FDA. (2017). Control of *Listeria monocytogenes* in ready-to-eat foods: guidance for industry. *International Journal of Pharmaceutical & Biological Archives*, *3*(Draft Guidance), 1–49. Retrieved from

https://www.fda.gov/downloads/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/UCM535981.pdf%0Ahttps://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/ucm535981.pdf

Felicio, M. T. S., Hogg, T., Gibbs, P., Teixeira, P., & Wiedmann, M. (2007). Recurrent and sporadic *Listeria monocytogenes* contamination in Alheiras represents considerable diversity, including virulence-attenuated isolates. *Applied and Environmental Microbiology*, *73*(12), 3887–3895. https://doi.org/10.1128/AEM.02912-06

Felício, M. T. S., Ramalheira, R., Ferreira, V., Brandão, T., Silva, J., Hogg, T., & Teixeira, P. (2011). Thermal inactivation of *Listeria monocytogenes* from alheiras, traditional Portuguese sausage during cooking. *Food Control*, *22*(12), 1960–1964. https://doi.org/10.1016/j.foodcont.2011.05.011

Ferreira, M., Almeida, A., Delgadillo, I., Saraiva, J., & Cunha, Â. (2016). Susceptibility of *Listeria monocytogenes* to high pressure processing: A review. *Food Reviews International*, *32*(4), 377–399. https://doi.org/10.1080/87559129.2015.1094816

Ferreira, V., Barbosa, J., Stasiewicz, M., Vongkamjan, K., Moreno Switt, A., Hogg, T., … Wiedmann, M. (2011). Diverse geno- and phenotypes of persistent *Listeria monocytogenes* isolates from fermented meat sausage production facilities in Portugal. *Applied and Environmental Microbiology*, *77*(8), 2701–2715. https://doi.org/10.1128/AEM.02553-10

Ferreira, Vânia, Barbosa, J., Silva, J., Felício, M. T., Mena, C., Hogg, T., … Teixeira, P. (2007). Characterisation of alheiras, traditional sausages produced in the North of Portugal, with respect to their microbiological safety. *Food Control*, *18*(5), 436–440. https://doi.org/10.1016/j.foodcont.2005.11.011

Ferreira, Vânia, Barbosa, J., Vendeiro, S., Mota, A., Silva, F., Monteiro, M. J., …

Teixeira, P. (2006). Chemical and microbiological characterization of alheira: A typical Portuguese fermented sausage with particular reference to factors relating to food safety. *Meat Science*, *73*(4), 570–575. https://doi.org/10.1016/j.meatsci.2006.02.011

Figueiredo, A. C. L., & Almeida, R. C. C. (2017). Antibacterial efficacy of nisin, bacteriophage P100 and sodium lactate against *Listeria monocytogenes* in ready-to-eat sliced pork ham. *Brazilian Journal of Microbiology*, *48*(4), 724–729. https://doi.org/10.1016/j.bjm.2017.02.010

Fister, S., Fuchs, S., Stessl, B., Schoder, D., Wagner, M., & Rossmanith, P. (2016). Screening and characterisation of bacteriophage P100 insensitive *Listeria monocytogenes* isolates in Austrian dairy plants. *Food Control*, *59*, 108–117. https://doi.org/10.1016/j.foodcont.2015.05.026

Fusco, V., Besten, H. M. den, Logrieco, A. F., Rodriguez, F. P., Skandamis, P. N., Stessl, B., & Teixeira, P. (2015). Food safety aspects on ethnic foods: toxicological and microbial risks. *Current Opinion in Food Science*, *6*(December), 24–32. https://doi.org/10.1016/j.cofs.2015.11.010

Gálvez, A., Abriouel, H., López, R. L., & Omar, N. Ben. (2007). Bacteriocin-based strategies for food biopreservation. *International Journal of Food Microbiology*, *120*(1–2), 51–70. https://doi.org/10.1016/j.ijfoodmicro.2007.06.001

García-Anaya, M. C., Sepúlveda, D. R., Rios-Velasco, C., Zamudio-Flores, P. B., Sáenz-Mendoza, A. I., & Acosta-Muñiz, C. H. (2020). The role of food compounds and emerging technologies on phage stability. *Innovative Food Science & Emerging Technologies*, *64*, 102436. https://doi.org/10.1016/j.ifset.2020.102436

García, P., Madera, C., Martínez, B., & Rodríguez, A. (2007). Biocontrol of *Staphylococcus aureus* in curd manufacturing processes using bacteriophages.

International Dairy Journal, *17*(10), 1232–1239. https://doi.org/10.1016/j.idairyj.2007.03.014

Garriga, M., Aymerich, M. T., Costa, S., Monfort, J. M., & Hugas, M. (2002). Bactericidal synergism through bacteriocins and high pressure in a meat model system during storage. *Food Microbiology*, *19*(5), 509–518. https://doi.org/10.1006/fmic.2002.0498

Gravesen, A., Jydegaard Axelsen, A.-M., Mendes da Silva, J., Hansen, T. B., & Knøchel, S. (2002). Frequency of bacteriocin resistance development and associated fitness costs in *Listeria monocytogenes*. *Applied and Environmental Microbiology*, *68*(2), 756–764. https://doi.org/10.1128/AEM.68.2.756-764.2002

Gutiérrez, D., Rodríguez-Rubio, L., Fernández, L., Martínez, B., Rodríguez, A., & García, P. (2017). Applicability of commercial phage-based products against *Listeria monocytogenes* for improvement of food safety in Spanish dry-cured ham and food contact surfaces. *Food Control*, *73*, 1474–1482. https://doi.org/10.1016/j.foodcont.2016.11.007

Hernández-Hernández, H. M., Moreno-Vilet, L., & Villanueva-Rodríguez, S. J. (2019). Current status of emerging food processing technologies in Latin America: Novel non-thermal processing. *Innovative Food Science & Emerging Technologies*, *58*, 102233. https://doi.org/10.1016/j.ifset.2019.102233

Hygreeva, D., & Pandey, M. C. (2016). Novel approaches in improving the quality and safety aspects of processed meat products through high pressure processing technology - A review. *Trends in Food Science & Technology*, *54*, 175–185. https://doi.org/10.1016/j.tifs.2016.06.002

Iacumin, L., Manzano, M., & Comi, G. (2016). Phage inactivation of *Listeria monocytogenes* on San Daniele dry-cured ham and elimination of biofilms from

equipment and working environments. *Microorganisms*, *4*(1), 4. https://doi.org/10.3390/microorganisms4010004

ISO, (International Organization for Standardization). (2017). *Microbiology of food and animal feeding stuffs—horizontal method for the detection and enumeration of Listeria monocytogenes. Part 1:Detection method. Part 2: Enumeration method. ISO standard 11290-1:1998 and Amd.1:2004.*

Karolenko, C. E., Bhusal, A., Nelson, J. L., & Muriana, P. M. (2020). Processing of Biltong (dried beef) to achieve USDA-FSIS 5-log reduction of *Salmonella* without a heat lethality step. *Microorganisms*, *8*(5), 791. https://doi.org/10.3390/microorganisms8050791

Kaur, G., Singh, T. P., & Malik, R. K. (2013). Antibacterial efficacy of Nisin, Pediocin 34 and Enterocin FH99 against *Listeria monocytogenes* and cross resistance of its bacteriocin resistant variants to common food preservatives. *Brazilian Journal of Microbiology*, *44*(1), 63–71. https://doi.org/10.1590/S1517-83822013005000025

Komora, N., Bruschi, C., Ferreira, V., Maciel, C., Brandão, T. R. S., Fernandes, R., … Teixeira, P. (2018). The protective effect of food matrices on *Listeria* lytic bacteriophage P100 application towards high pressure processing. *Food Microbiology*, *76*, 416–425. https://doi.org/10.1016/j.fm.2018.07.002

Komora, N., Bruschi, C., Magalhães, R., Ferreira, V., & Teixeira, P. (2017). Survival of *Listeria monocytogenes* with different antibiotic resistance patterns to food-associated stresses. *International Journal of Food Microbiology*, *245*, 79–87. https://doi.org/10.1016/j.ijfoodmicro.2017.01.013

Komora, N., Maciel, C., Pinto, C. A., Ferreira, V., Brandão, T. R. S., Saraiva, J. M. A., … Teixeira, P. (2020). Non-thermal approach to *Listeria monocytogenes* inactivation in milk: The combined effect of high pressure, pediocin PA-1 and

bacteriophage P100. *Food Microbiology*, *86*, 103315. https://doi.org/10.1016/j.fm.2019.103315

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., & Johnson, R. P. (2009). Enumeration of bacteriophages by double agar overlay plaque assay. *Methods in Molecular Biology (Clifton, N.J.)*, *501*, 69—76. https://doi.org/10.1007/978-1-60327-164-6_7

Kumariya, R., Garsa, A. K., Rajput, Y. S., Sood, S. K., Akhtar, N., & Patel, S. (2019). Bacteriocins: Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microbial Pathogenesis*, *128*(January), 171–177. https://doi.org/10.1016/j.micpath.2019.01.002

Lewis, Bolocan, Draper, Ross, & Hill. (2019). The effect of a commercially available bacteriophage and bacteriocin on *Listeria monocytogenes* in coleslaw. *Viruses*, *11*(11), 977. https://doi.org/10.3390/v11110977

Lewis, R., & Hill, C. (2020). Overcoming barriers to phage application in food and feed. *Current Opinion in Biotechnology*, *61*, 38–44. https://doi.org/10.1016/j.copbio.2019.09.018

Mackey, B. M., Forestière, K., Isaacs, N. S., Stenning, R., & Brooker, B. (1994). The effect of high hydrostatic pressure on *Salmonella* Thompson and *Listeria monocytogenes* examined by electron microscopy. *Letters in Applied Microbiology*, *19*(6), 429–432. https://doi.org/10.1111/j.1472-765X.1994.tb00973.x

Marcos, C., Viegas, C., de Almeida, A. M., & Guerra, M. M. (2016). Portuguese traditional sausages: different types, nutritional composition, and novel trends. *Journal of Ethnic Foods*, *3*(1), 51–60. https://doi.org/10.1016/j.jef.2016.01.004

Meloni, D. (2015). Presence of *Listeria monocytogenes* in Mediterranean-Style dry

fermented sausages. *Foods*, *4*(4), 34–50. https://doi.org/10.3390/foods4010034

Miles, A. A., Misra, S. S., & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *The Journal of Hygiene*, *38*(6), 732–749. https://doi.org/10.1017/s002217240001158x

Naanwaab, C., Yeboah, O.-A., Ofori Kyei, F., Sulakvelidze, A., & Goktepe, I. (2014). Evaluation of consumers' perception and willingness to pay for bacteriophage treated fresh produce. *Bacteriophage*, *4*(4), e979662. https://doi.org/10.4161/21597081.2014.979662

Oliveira, T. L. C. de, Ramos, A. L. S., Ramos, E. M., Piccoli, R. H., & Cristianini, M. (2015). Natural antimicrobials as additional hurdles to preservation of foods by high pressure processing. *Trends in Food Science & Technology*, *45*(1), 60–85. https://doi.org/10.1016/j.tifs.2015.05.007

Oliveira, M., Viñas, I., Colàs, P., Anguera, M., Usall, J., & Abadias, M. (2014). Effectiveness of a bacteriophage in reducing *Listeria monocytogenes* on fresh-cut fruits and fruit juices. *Food Microbiology*, *38*, 137–142. https://doi.org/10.1016/j.fm.2013.08.018

Perera, M. N., Abuladze, T., Li, M., Woolston, J., & Sulakvelidze, A. (2015). Bacteriophage cocktail significantly reduces or eliminates *Listeria monocytogenes* contamination on lettuce, apples, cheese, smoked salmon and frozen foods. *Food Microbiology*, *52*, 42–48. https://doi.org/10.1016/j.fm.2015.06.006

Rodríguez-Rubio, L., García, P., Rodríguez, A., Billington, C., Hudson, J. A., & Martínez, B. (2015). Listeriaphages and coagulin C23 act synergistically to kill *Listeria monocytogenes* in milk under refrigeration conditions. *International Journal of Food Microbiology*, *205*, 68–72. https://doi.org/10.1016/j.ijfoodmicro.2015.04.007

Rossi, L. P. R., Almeida, R. C. C., Lopes, L. S., Figueiredo, A. C. L., Ramos, M. P. P., & Almeida, P. F. (2011). Occurrence of *Listeria* spp. in Brazilian fresh sausage and control of *Listeria monocytogenes* using bacteriophage P100. *Food Control*, *22*(6), 954–958. https://doi.org/10.1016/j.foodcont.2010.12.001

Shannon, R., Radford, D. R., & Balamurugan, S. (2020). Impacts of food matrix on bacteriophage and endolysin antimicrobial efficacy and performance. *Critical Reviews in Food Science and Nutrition*, *60*(10), 1631–1640. https://doi.org/10.1080/10408398.2019.1584874

Silva, C. C. G., Silva, S. P. M., & Ribeiro, S. C. (2018). Application of bacteriocins and protective cultures in dairy food preservation. *Frontiers in Microbiology*, *9*(APR). https://doi.org/10.3389/fmicb.2018.00594

Silva, E. N. G., Figueiredo, A. C. L., Miranda, F. A., & Almeida, R. C. de C. (2014). Control of *Listeria monocytogenes* growth in soft cheeses by bacteriophage P100. *Brazilian Journal of Microbiology*, *45*(1), 11–16. https://doi.org/10.1590/S1517-83822014000100003

Simmons, M., Drescher, K., Nadell, C. D., & Bucci, V. (2018). Phage mobility is a core determinant of phage–bacteria coexistence in biofilms. *The ISME Journal*, *12*(2), 531–543. https://doi.org/10.1038/ismej.2017.190

Tabla, R., Martínez, B., Rebollo, J. E., González, J., Ramírez, M. R., Roa, I., … García, P. (2012). Bacteriophage performance against *Staphylococcus aureus* in milk is improved by high hydrostatic pressure treatments. *International Journal of Food Microbiology*, *156*(3), 209–213. https://doi.org/10.1016/j.ijfoodmicro.2012.03.023

Tonello-Samson, C., Queirós, R. P., & González-Angulo, M. (2020). Chapter 13 - Advances in high-pressure processing in-pack and in-bulk commercial equipment. In F. J. Barba, C. Tonello-Samson, E. Puértolas, & M. B. T.-P. and F. of H. P. P.

Lavilla (Eds.) (pp. 297–316). Elsevier. https://doi.org/https://doi.org/10.1016/B978-0-12-816405-1.00013-3

Trudelle, D. M., Bryan, D. W., Hudson, L. K., & Denes, T. G. (2019). Cross-resistance to phage infection in *Listeria monocytogenes* serotype 1/2a mutants. *Food Microbiology*, *84*(January), 103239. https://doi.org/10.1016/j.fm.2019.06.003

USDA-FSIS. (2017). *Performance Standards for the Production of Certain Meat and Poultry Products. FSIS Directive 7111.1. Federal Register* (Vol. 66).

Van Ba, H., Seo, H.-W., Kim, J.-H., Cho, S.-H., Kim, Y.-S., Ham, J.-S., … Seong, P.-N. (2016). The effects of starter culture types on the technological quality, lipid oxidation and biogenic amines in fermented sausages. *LWT*, *74*, 191–198. https://doi.org/10.1016/j.lwt.2016.07.019

van Reenen, Dicks, & Chikindas. (1998). Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*, *84*(6), 1131–1137. https://doi.org/10.1046/j.1365-2672.1998.00451.x

van Wagenberg, C. P. A., van Horne, P. L. M., & van Asseldonk, M. A. P. M. (2020). Cost-effectiveness analysis of using probiotics, prebiotics, or synbiotics to control *Campylobacter* in broilers. *Poultry Science*, *99*(8), 4077–4084. https://doi.org/https://doi.org/10.1016/j.psj.2020.05.003

Veloso, P. M. A. (2014). *Improving derived Listeria phage endolysins properties at low temperatures*. Universidade do Minho.

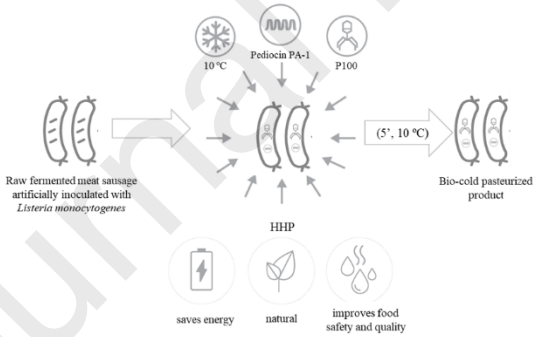
Vikram, A., Woolston, J., & Sulakvelidze, A. (2021). Phage biocontrol applications in food production and processing. *Current Issues in Molecular Biology*, *40*, 267–302. https://doi.org/10.21775/cimb.040.267

Yamamoto, K. (2017). Food processing by high hydrostatic pressure. *Bioscience,*

Biotechnology, and Biochemistry, *81*(4), 672–679. https://doi.org/10.1080/09168451.2017.1281723

Yang, P., Rao, L., Zhao, L., Wu, X., Wang, Y., & Liao, X. (2021). High pressure processing combined with selected hurdles: Enhancement in the inactivation of vegetative microorganisms. *Comprehensive Reviews in Food Science and Food Safety*, *20*(2), 1800–1828. https://doi.org/10.1111/1541-4337.12724

Zhu, J., Li, S., Yan, T., Sun, X., Ma, L., Meng, X., & Lu, X. (2021). Whole transcriptome sequencing analysis of the effect of high hydrostatic pressure on *Escherichia coli* O157:H7. *Journal of Agriculture and Food Research*, *4*, 100147. https://doi.org/https://doi.org/10.1016/j.jafr.2021.100147



Highlights

Feasibility of associating phages to bacteriocinogenic LAB proved in fermented food

L. monocytogenes eliminated in fermented sausage model with HHP-LAB-phage system

Synergistic listericidal effect between hurdles demonstrated in a solid matrix

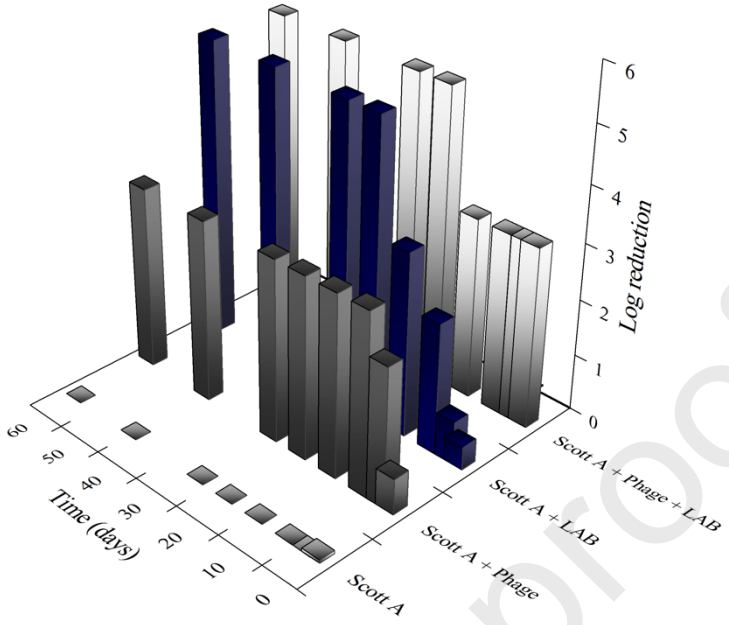
In situ biosynthesis of pediocin PA-1 firstly reported in solid matrix

CRediT author statement

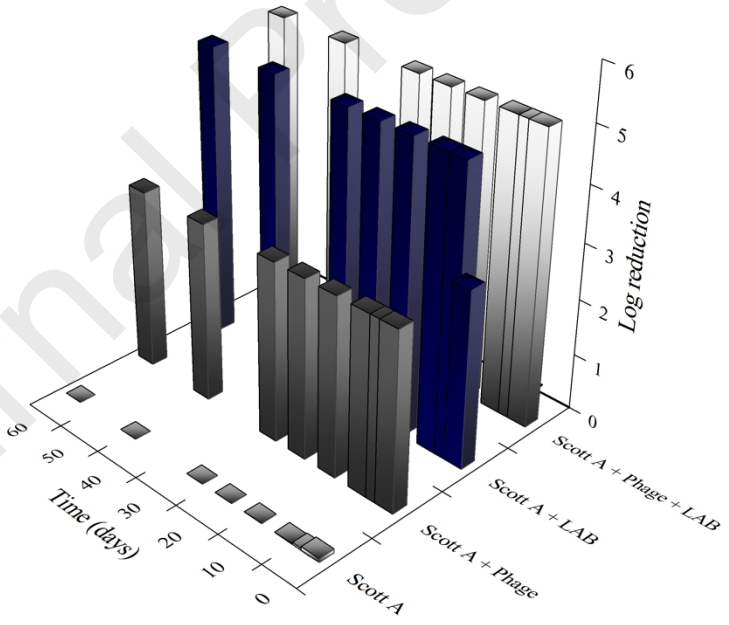
Credit author statement

Norton Komora: Conceptualization, Investigation, Methodology, Data curation, Writing- Original draft. **Cláudia Maciel**: Conceptualization, Investigation, Methodology, Data curation, Writing- Original draft, Writing- Review and Editing. **Renata Amaral**: Investigation. **Rui Fernandes**: Investigation, Data curation. **Sónia Marília Castro**: Conceptualization, Methodology, Supervision. **Jorge M. A. Saraiva**: Writing- Review and Editing, Funding acquisition. **Paula Teixeira**: Conceptualization, Supervision, Validation, Funding acquisition and Project administration.

A



B



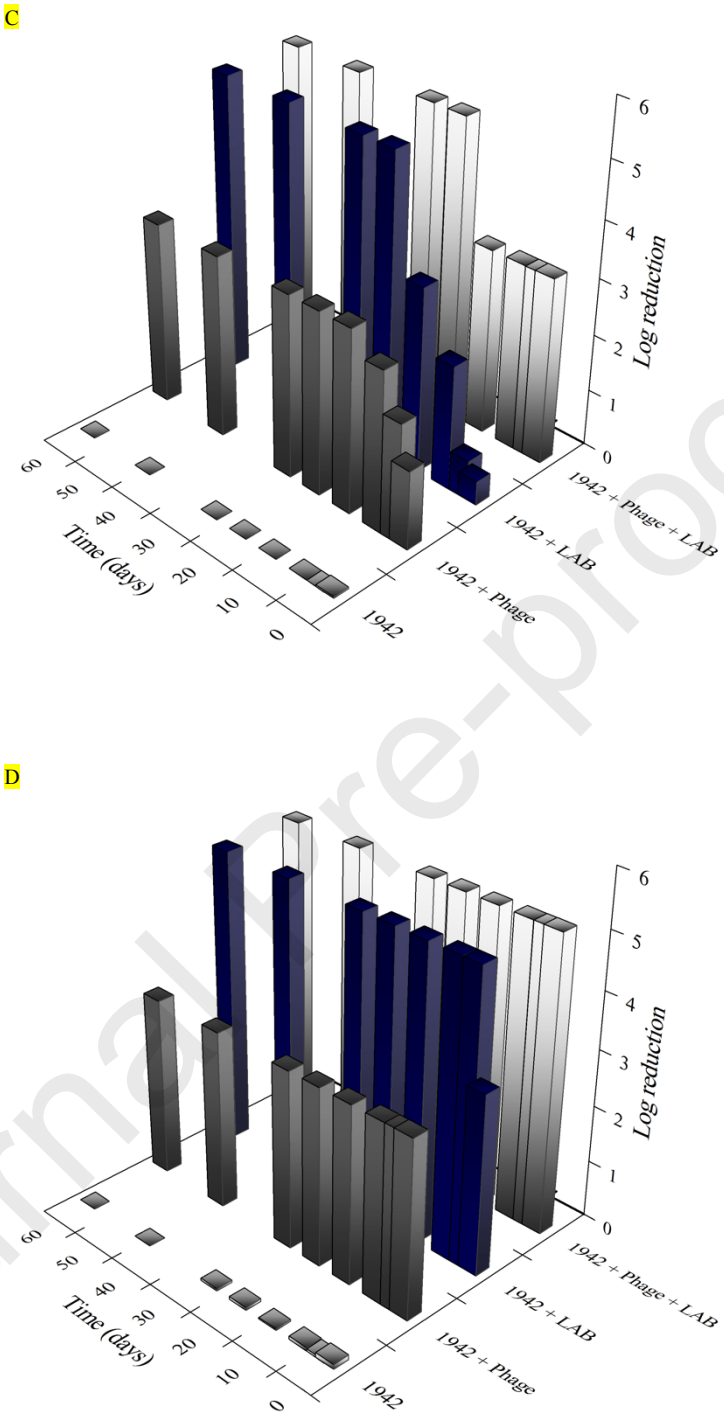


Figure 1. Effect of different biocontrol approaches on the inactivation of *L. monocytogenes* ScottA (A and B) and Lm 1942 (C and D) at an initial level of 10^5 CFU g^{-1} on *Alheira* (fermented meat sausage) throughout 60 days of storage at 4 °C. Pressure: 0.1 MPa (A and C); 300 MPa (B and D).

Values are the average of three independent experiments. Means with the same letter are not statistically different from each other ($P > 0.05$).

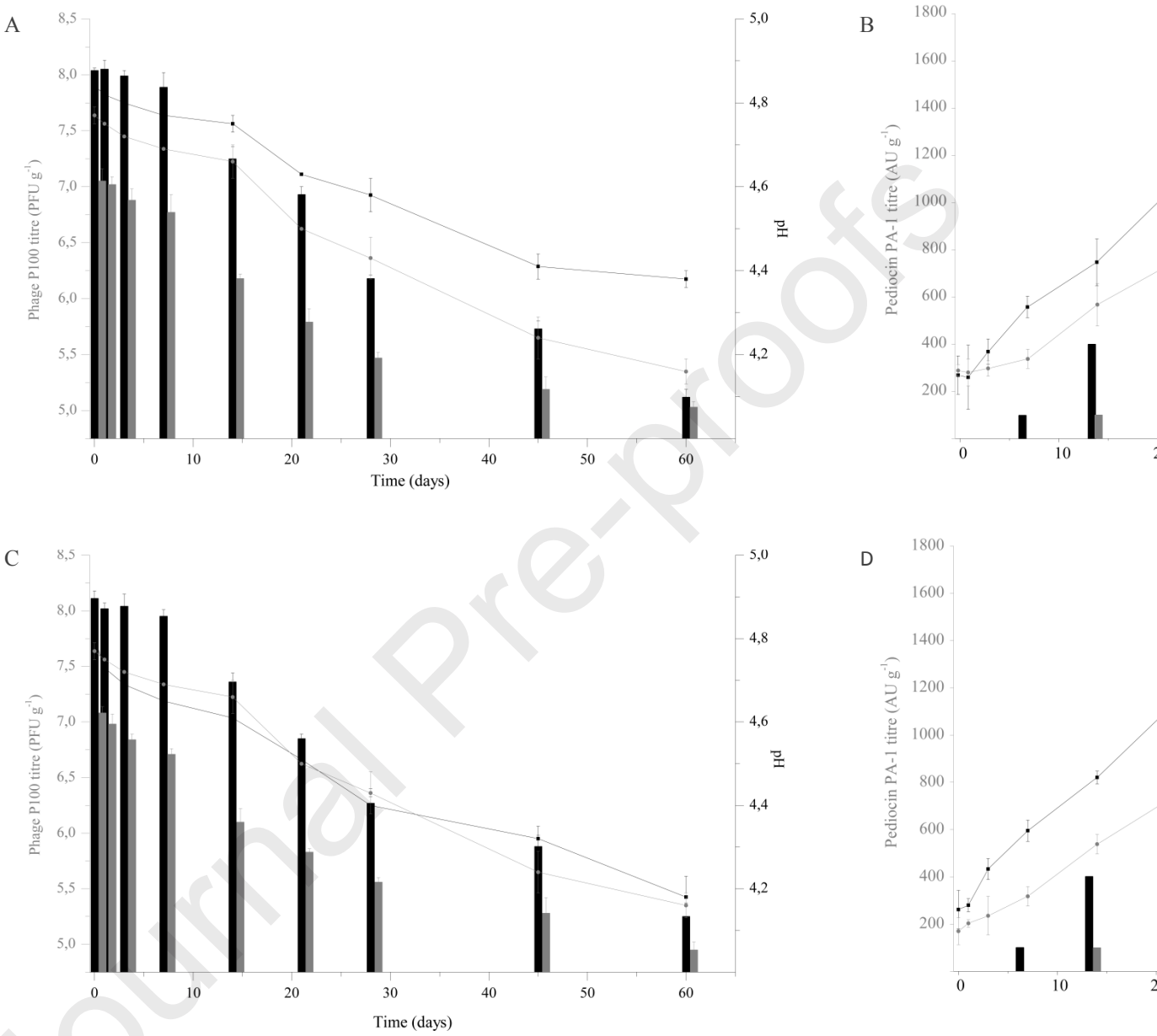
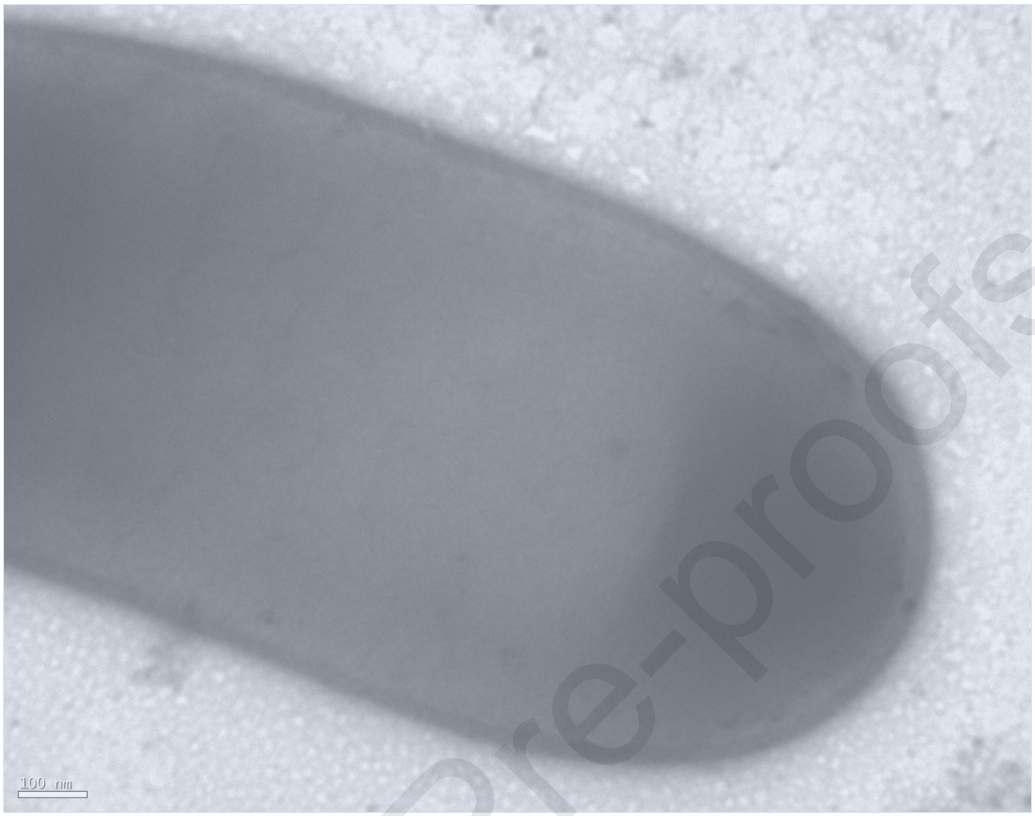
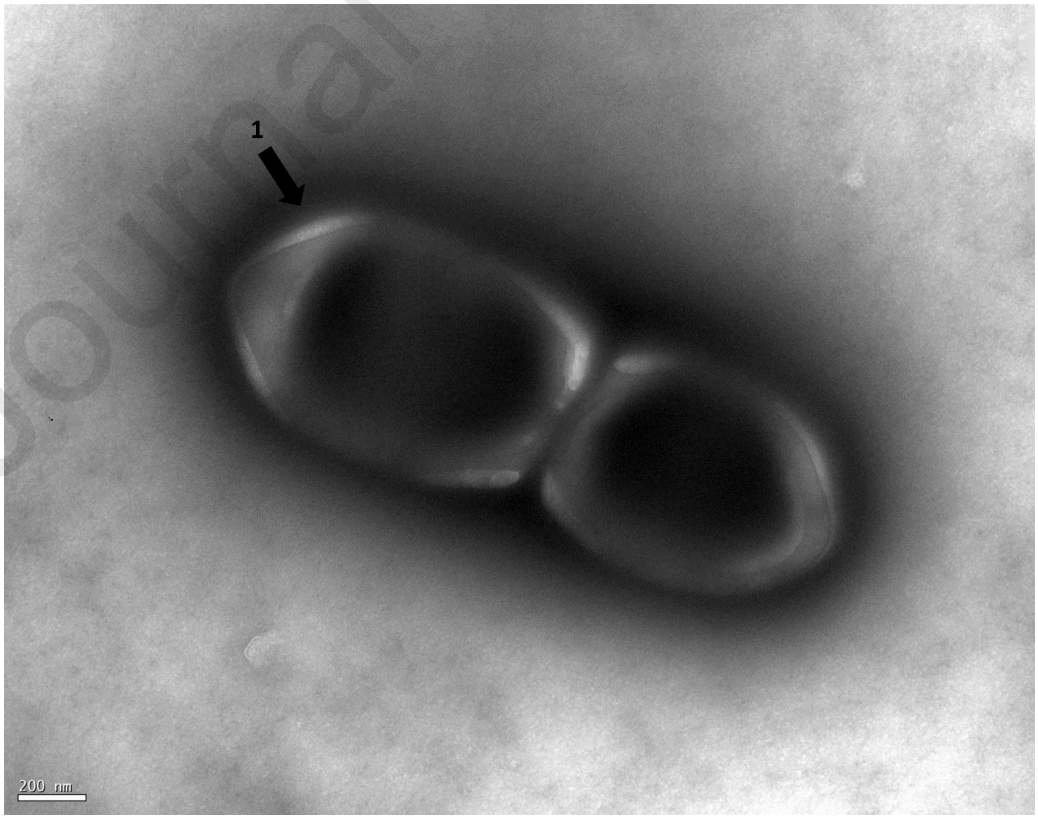


Figure 2. Phage Listex™ P100 titration (column) and pH oscillation (line) (A, C), *P. acidilactici* HA-6112 (LAB) enumeration (line) and pediocin PA-1 biosynthesis (column) (B, D), evaluation throughout the 60 days of refrigerated storage (4 ºC) of the fermented meat sausage artificially inoculated with *L. monocytogenes* 1942 (A, B) or ScottA (C, D), following the application of the HHP-LAB-phage system (grey) and LAB-phage system at 0.1 MPa (black).

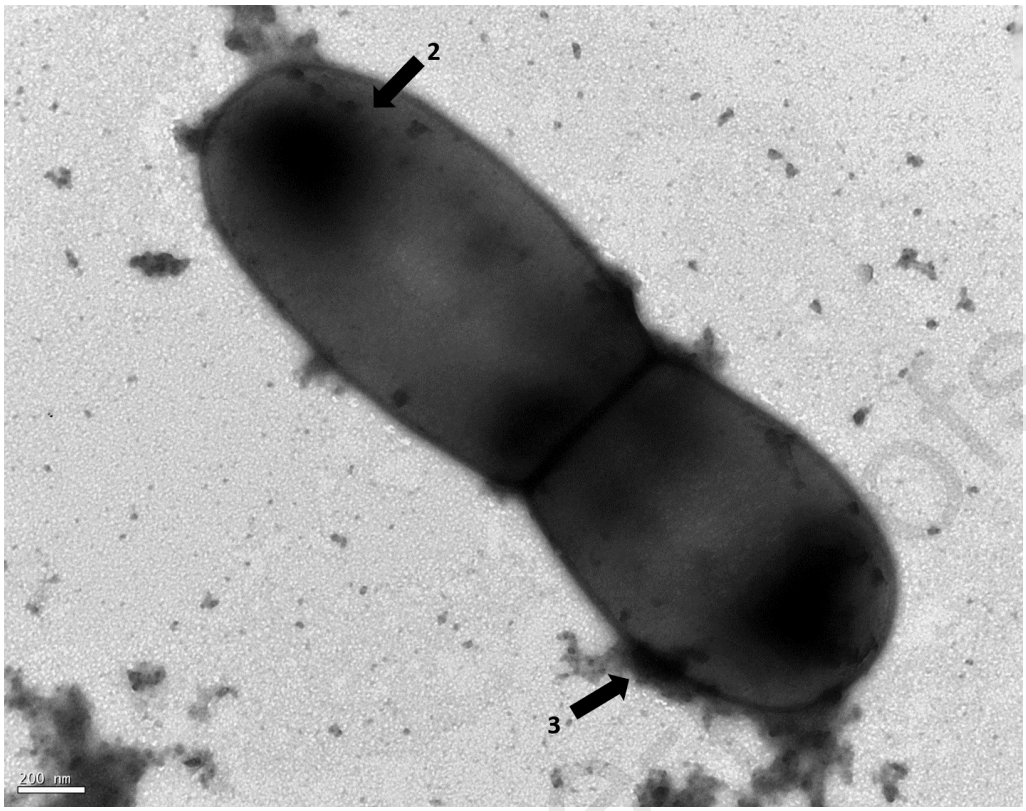
A



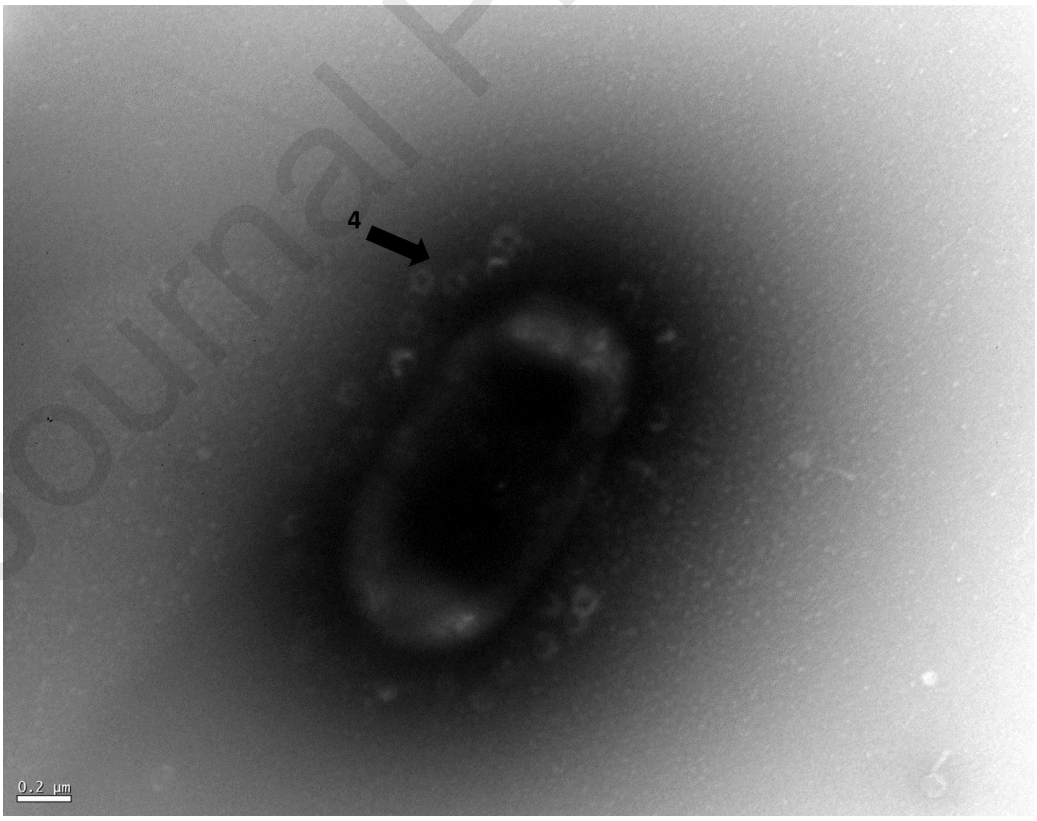
B



C



D



E

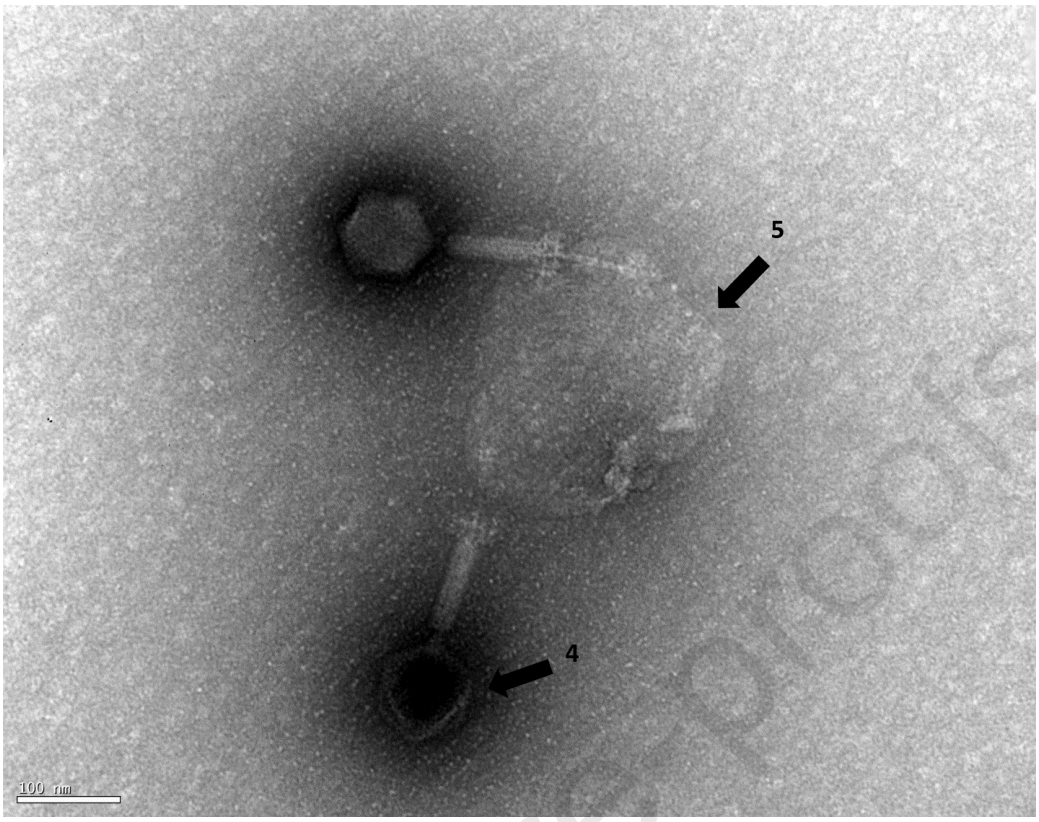


Figure 3. Transmission electron microscopy evaluation of the ultrastructural changes in *L. monocytogenes* cells in PBS (0.1 M) through non-thermal hurdles combined application.
Legend: control (A), pediocin PA-1 (B), HHP 300 MPa, 5 min, 10 °C (C), phage P100 (D) and combined hurdles (E). *Arrows* indicate: 1 – Transparent area between the cytoplasm membrane and cell wall; 2 – Nuclear content condensation; 3 – Membrane rupture; 4 – Attached phage particle; 5- Cytoplasmic material residue from complete cell lysis. Scale bar represents 200 nm in B, C, D micrographs, and 100 nm in A and E.

Table 1. Formulations of the combined hurdles used in *Alheira* fermented sausage (% v/w)

Treatment	Final concentration	*L. monocytogenes* (8 log CFU mL^{-1})	Phage P100 (10 log PFU mL^{-1})	*P. acidilactici* (9 log CFU mL^{-1})
L	*L.monocytogenes* (10^5 CFU g^{-1})	0.1 %	-	-

	Recovery of *Listeria monocytogenes* after enrichment protocol ISO 11290-2:2017														
	Sampling time														
	AT		1 day		3 days		7 days		14 days		21 days		28 days		45 day
bial	Scott A	1942	Scott A	1942	Scott A	1942	Scott A	1942	Scott A	1942	Scott A	1942	Scott A	1942	Scott A
l	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
00	++	++	++	++	++	++	+	++	+	+	+	+	+	+	+
ctici	++	++	++	++	++	++	++	++	+	+	-	-	-	-	-
+ *P.* *ici*	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
l	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
00	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ctici	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
+ *P.* *ici*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

LP	*L.monocytogenes* (10^5 CFU g^{-1}) + Phage (10^8 PFUg^{-1})	0.1 %	1.0 %	-
LL	*L.monocytogenes* (10^5 CFU g^{-1}) + *P. acidilactici* (10^7 CFU g^{-1})	0.1 %	-	1.0 %
LPL	*L.monocytogenes* (10^5 CFU g^{-1}) + Phage (10^8 PFU g^{-1}) + *P. acidilactici* (10^7 CFU g^{-1})	0.1 %	1.0 %	1.0 %

Note: L – *L. monocytogenes* untreated control; LP - *L. monocytogenes* + phage P100; LL - *L. monocytogenes* + *P. acidilactici*; LPL - *L. monocytogenes* + phage P100 + *P. acidilactici*.

Table 2. Recovery of *L. monocytogenes* in fermented meat sausage during refrigerated storage (4 °C).

Legend: Lm – *L. monocytogenes*; AT – 2 hours after treatment; (++) presence and enumeration of *L. monocytogenes*; (+) presence of *L. monocytogenes* at levels below the detection limit of the enumeration technique; (-) absence of *L. monocytogenes*.

Table 3. Theoretical additive, and observed, effects determination

Lm Scott A 10^5 CFU g^{-1}							
Time	Pressure	Theoretical additive effect (Log N/N_0)			Observed effect (Log N/N_0)		
		phage P100	LAB	P100 + LAB	phage P100	LAB	P100 + LAB
AT	0.1 MPa	n/a	n/a	-0.28	n/a	n/a	**-1.66**
	300 MPa	-0.33	-0.07	-0.35	**-3.18**	**-3.15**	**-5.19**
Day 1	0.1 MPa	n/a	n/a	-1.02	n/a	n/a	**-3.21**
	300 MPa	-0.69	-0.45	-1.08	**-3.18**	**-3.15**	**-5.19**
Day 3	0.1 MPa	n/a	n/a	-3.25	n/a	n/a	-3.21
	300 MPa	-2.52	-0.79	-3.28	**-3.18**	**-5.15**	**-5.19**
Day 7	0.1 MPa	n/a	n/a	-5.56	n/a	n/a	-3.21
	300 MPa	2.25	-2.33	-5.57	**-3.18**	**-5.15**	-5.19
Day 14	0.1 MPa	n/a	n/a	-6.50	n/a	n/a	-3.21
	300 MPa	-3.24	-3.26	-6.50	-3.18	**-5.15**	-5.19
Day 21	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.21
	300 MPa	-3.24	-5.26	-8.50	-3.18	-5.15	-5.19
Day 28	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.21
	300 MPa	-3.16	-5.18	-8.42	-3.18	-5.15	-5.19
Day 45	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.21
	300 MPa	-3.09	-5.11	-8.35	**-3.18**	-5.15	-5.19
Day 60	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.21
	300 MPa	-2.93	-4.95	-8.19	**-3.18**	**-5.15**	-5.19

Lm 1942 10^5 CFU g^{-1}							
Time	Pressure	Theoretical additive effect (Log N/N_0)			Observed effect (Log N/N_0)		
		phage P100	LAB	P100 + LAB	phage P100	LAB	P100 + LAB
AT	0.1 MPa	n/a	n/a	-0.20	n/a	n/a	**-1.59**
	300 MPa	-0.20	-0.24	-0.32	**-3.15**	**-3.18**	**-5.20**
Day 1	0.1 MPa	n/a	n/a	-1.80	n/a	n/a	**-3.28**
	300 MPa	-1.47	-0.49	-1.88	**-3.15**	**-3.18**	**-5.20**
Day 3	0.1 MPa	n/a	n/a	-2.81	n/a	n/a	**-3.28**
	300 MPa	-2.16	-0.73	-2.85	**-3.15**	**-5.18**	**-5.20**

Day 7	0.1 MPa	n/a	n/a	-5.03	n/a	n/a	-3.28
	300 MPa	-2.89	-2.24	-5.08	**-3.15**	**-5.18**	**-5.20**
Day 14	0.1 MPa	n/a	n/a	-6.50	n/a	n/a	-3.28
	300 MPa	-3.26	-3.28	-6.52	-3.15	**-5.18**	-5.20
Day 21	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.28
	300 MPa	-3.28	-5.30	-8.54	-3.15	-5.18	-5.20
Day 28	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.28
	300 MPa	-3.27	-5.29	-8.53	-3.15	-5.18	-5.20
Day 45	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.28
	300 MPa	-3.17	-5.19	-8.43	-3.15	-5.18	-5.20
Day 60	0.1 MPa	n/a	n/a	-8.50	n/a	n/a	-5.28
	300 MPa	-3.05	-5.07	-8.31	**-3.15**	**-5.18**	-5.20

No conflict of interest to declare