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# Non-thermal approach to *Listeria monocytogenes* inactivation in milk: The combined effect of high pressure, pediocin PA-1 and bacteriophage P100

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ABSTRACT

Non-thermal food processing and replacement of chemical additives by natural antimicrobials are promising trends in the food industry. The objective of the present work was to evaluate the effect of a process which combines mild high hydrostatic pressure – HHP (200 and 300 MPa, 5 min, 10 °C), phage Listex™ P100 and the bacteriocin pediocin PA-1 as a new non-thermal process for destruction of *Listeria monocytogenes* ($10^4$ CFU $mL^{-1}$ or $10^7$ CFU $mL^{-1}$) in milk. For inoculum levels of $10^4$ CFU $mL^{-1}$, HHP combined with phage P100 eliminated *L. monocytogenes* immediately after pressurization. When L. *monocytogenes* was inoculated at levels of $10^7$ CFU $mL^{-1}$, a synergistic effect between phage P100, pediocin PA-1 and HHP (300 MPa) on the inactivation of *L. monocytogenes* was observed during storage of milk at 4 °C. For non-pressure treated samples inoculated with phage or pediocin or both, *L. monocytogenes* counts decreased immediately after biocontrol application, but regrowth was observed in a few samples during storage. Phage particles were stable during refrigerated storage for seven days while pediocin PA-1 remained stable only during three days. Further studies will have to be performed to validate the findings of this work in specific applications (e.g. production of raw milk cheese).

## 1. Introduction

*Listeria monocytogenes* is a Gram-positive, facultatively anaerobic, nonsporeforming bacillus belonging to the family *Listeriaceae*. It is an intracellular pathogen that causes a rare, although severe, foodborne disease named listeriosis (Lebreton et al., 2015). In recent years, the incidence of listeriosis has increased across Europe, being the leading cause of hospitalization and death, with the highest case fatality rate among the zoonoses monitored in 37 European countries (i.e. 13.8%; EFSA, 2018).

*Listeria monocytogenes* can be ubiquitously found in soil, food processing environments, raw materials as well as in a wide variety of foods; *L. monocytogenes* is commonly associated to dairy products, specially raw and soft cheeses (Almeida et al., 2013; Magalhães et al., 2015; Martinez-Rios and Dalgaard, 2018; Melo et al., 2015).

The multi-hurdle approach or the hurdle technology of Leistner designates a process that deliberately combines hurdles to achieve a microbiologically safe food product, preserving its nutritional and organoleptic properties; more than 60 potential hurdles for food processing have been described, with temperature, water activity ($a_w$), pH, redox potential and chemical food preservatives being the most commonly applied (Leistner, 2000; Leistner and Gorris, 1995). The main concept of the hurdle technology is that a microorganism requires a certain amount of effort to overcome each hurdle; the higher the hurdle, the greater the number of cells needed to overcome it. Application of hurdle technology results in three possible effects: (a) additive, (b) synergistic and (c) antagonistic. Additive effect means that the resulting effect from combining individual hurdles is the sum of the effect of each hurdle; synergism denotates that a combination of hurdles has a significantly greater effect than the simple addition of individual effects. On the order hand, the antagonism definition is applied when the combined hurdles result in a lower effect than the sum of the effects of each hurdle employed individually (Bidlas and Lambert, 2008). A combination of hurdles should maintain food safety due to the microorganism inability to circumvent the combined hurdles.

The hurdle technology has become an attractive strategy to meet the

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increasing demand of consumers for minimally processed, fresh, healthy and "green" labelled food products. In fact, the development of new applications of food biopreservation combining natural antimicrobial agents and high hydrostatic pressure (HHP) not only ensures safety of food products, but also potentiates the implementation/emergence of energy-sustainable and environment-friendly food decontamination technologies (Oliveira et al., 2015). The emergence of novel biocontrol approaches with the use of natural antimicrobials such as bacteriocins, essential oils and bacteriophages introduced new variables for bacteriostatic and bactericidal effects in hurdle technology applications (Hygreeva and Pandey, 2016). The use of bacteriocins (nisin is an exception) and bacteriophages is not allowed in Europe. However, several studies have shown encouraging results in the food safety field and the safety and efficacy of Listex™ P100 for reduction of pathogens on different ready-to-eat food products has been the subject of a scientific opinion of the EFSA Panel on Biological Hazards (2016). Moreover, in other developed countries such as the U.S.A, Canada, Australia and New Zealand, food law policies have already been updated regarding bacteriophage applications (Chibeu et al., 2013). The combined effect of bacteriocins and bacteriophages in food biocontrol systems (Baños et al., 2016; Leverentz et al., 2003; Rodríguez-Rubio et al., 2015; Soni et al., 2014; Wang et al., 2017), as well as synergistic effect of the mild HHP with bacteriocins (de Alba et al., 2013; Hereu et al., 2012; Jofré et al., 2009, 2008; Li et al., 2012; Marcos et al., 2013; Pérez Pulido et al., 2015; Toledo del Árbol et al., 2016) are well documented. With respect to the combination of mild HHP and bacteriophages (Ahmadi et al., 2015; Tabla et al., 2012), scarce information is currently available and further studies are needed. Despite the promising results obtained by these combined hurdles in the inactivation of the target pathogens and, at the same time, in the preservation of endogenous microbiota and product's freshness, a noticeable bacteria regrowth has been observed during refrigerated storage of bio-treated foods, especially in studies targeting *L. monocytogenes*, a bacterium well known for its ability to multiply at refrigeration temperatures (Baños et al., 2016; Leverentz et al., 2003).

Due to the relevance of *L. monocytogenes* in the food industry, the pathogen has been described as a target bacteria for the development of bacteriophage applications towards biocontrol and food decontamination (Strydom and Witthuhn, 2015).

The objective of the present work was to evaluate the effect of a multi-hurdle process which combines mild HHP, phage Listex™ P100 and pediocin PA-1 as a new non-thermal process for *L. monocytogenes* destruction in dairy matrices, using UHT whole milk as an initial model. As this was a preliminary study UHT whole milk was used to reduce the number of uncontrolled confounding variables that could affect the activity of both phage and bacteriocin (e.g. the microbiota of pasteurized or raw milk).

## 2. Material and methods

### 2.1. Microorganisms and preparation of the inoculum

All bacterial isolates are deposited in the *Listeria* Research Centre of Escola Superior de Biotecnologia (LRCESB).

#### 2.1.1. Listeria spp. isolates

*Listeria monocytogenes* strains used in this study were Lm Scott A (clinical isolate, ATCC 49594, serotype 4b) and Lm 1751 (isolated from dairy product, LRCESB, serotype 4b); Lm Scott A was selected as a representative of a human isolate widely used in stress-related studies including HHP inactivation (Bover-Cid et al., 2019) and Lm 1751 was selected as it was isolated from a dairy matrix (cheese), was quite resistant to HHP at 300 MPa, heat resistant and resistant to tetracycline (Bruschi et al., 2017).

*Listeria monocytogenes* ATCC 19116 (serotype 4c) was used as phage Listex™ P100 host (Veloso, 2014). *Listeria innocua* 2030c (Public Health Laboratory Services (PHLS) Colindale, UK) was used as target bacteria for bacteriocin titration.

*Listeria* spp. isolates were preserved at −20 °C 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 inoculum, one single colony of each *Listeria* isolate was transferred from tryptic soy agar (Pronadisa) supplemented with 0.6% (w/v) of yeast extract (TSAYE) into 10 mL of TSBYE and incubated at 37 °C for 24 h, separately. The cultures were subsequently diluted 1:100 in TSBYE and incubated in the same conditions. Cells were harvested by centrifugation (7000 × *g* for 10 min at 4 °C; 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.* $1 \times 10^9$ colony forming units (CFU $mL^{-1}$).

#### 2.1.2. Bacteriocinogenic lactic acid bacteria

*Pediococcus acidilactici* strain HA-6111-2, a bacteriocin producer (pediocin PA-1) lactic acid bacteria, previously isolated from "Alheira", a Portuguese traditional fermented meat sausage (Albano et al., 2007a), was preserved and cultured under the same conditions described above, except that the growth media used were *de* Man, Rogosa & Sharpe (MRS) broth and agar (Lab M). Antilisterial activity (AU $mL^{-1}$) of pressurized pediocin PA-1 from *P. acidilactici* strain HA-6111-2 was previously studied by Castro et al. (2015).

#### 2.1.3. Bacteriophage

Bacterio(phage) Listex™ P100 stock solution (Micreos Food Safety, The Netherlands) was stored at 4 °C in saline buffer and initial concentration was $10^{11}$ plaque forming units (PFU $mL^{-1}$). Phage P100 is active against multiple serovars of *L. monocytogenes* (1/2 and 4) and also against *L. ivanovii* (Carlton et al., 2005). 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). Pressure stability of phage P100 and the capability to be used under high pressure processing in different food matrices was previously reported by Komora et al. (2018).

### 2.2. Partial purification of pediocin PA-1

The partial purification of pediocin PA-1 from *P. acidilactici* strain HA 6111-2 was performed according to Albano et al. (2007b) with the following modification: after recovering and neutralizing the supernatant from the *P. acidilactici* culture, the ammonium sulphate precipitated extract was dialyzed overnight (4 °C) in ultra-pure water through a 1-kDa MW cut-off dialysis membrane, with two changes of water. The final extract was sterilised by filtration (0.22 μm, Millipore, Belford, MA, USA). The stock solution was stored at 4 °C in 25 mM ammonium acetate buffer (pH 6.5) and antilisterial activity was further evaluated as described by van Reenen et al. (1998).

Pediocin PA-1, the most studied bacteriocin from class IIa, was selected based on its rather narrow spectrum when compared to nisin, a class I bacteriocin recognized by its wide spectrum of action. For the main propose of this work, a minimal processing technology for elimination of *L. monocytogenes* in milk, pediocin PA-1 was a more suitable option to be combined with the narrow spectrum action of phage P100 (Cotter et al., 2005).

### 2.3. Preparation and artificial contamination of the food matrix

Ultra-high temperature (UHT) whole milk (3.6% fat content) was purchased from a local supermarket (Porto, Portugal). One hundred mililitres of UHT whole milk were aseptically transferred from the commercial packages into sterile glass flasks (250 mL), inoculated with two strains of *L. monocytogenes*, Lm Scott A and Lm 1751, separately,

**Table 1**
Formulations of combined hurdles used in UHT whole milk (mL/100 mL).

| Treatments | Final concentration | *L. monocytogenes* (6 log CFUmL$^{-1}$) | *L. monocytogenes* (9 log CFUmL$^{-1}$) | phage P100 (10 log PFUmL$^{-1}$) | pediocin PA-1 (128000AU mL$^{-1}$) |
|---|---|---|---|---|---|
| L1 | *L. monocytogenes* ($10^4$ CFUmL$^{-1}$) | 1.0 mL | 0 | 0 | 0 |
| LP1 | *L. monocytogenes* ($10^4$ CFUmL$^{-1}$), + Phage ($10^8$ PFUmL$^{-1}$) | 1.0 mL | 0 | 3.0 mL | 0 |
| LB1 | *L. monocytogenes* ($10^4$ CFUmL$^{-1}$) + pediocin PA-1 (1280 AUmL$^{-1}$) | 1.0 mL | 0 | 0 | 1.0 mL |
| LPB1 | *L. monocytogenes* ($10^4$ CFUmL$^{-1}$) + Phage ($10^8$ PFU mL$^{-1}$) + pediocin PA-1 (1280 AUmL$^{-1}$) | 1.0 mL | 0 | 3.0 mL | 1.0 mL |
| L2 | *L. monocytogenes* ($10^7$ CFUmL$^{-1}$) | 0 | 1.0 mL | 0 | 0 |
| LP2 | *L. monocytogenes* ($10^7$ CFUmL$^{-1}$) + Phage ($10^8$ PFU mL$^{-1}$) | 0 | 1.0 mL | 3.0 mL | 0 |
| LB2 | *L. monocytogenes* ($10^7$ CFUmL$^{-1}$) + pediocin PA-1 (1280 AUmL$^{-1}$) | 0 | 1.0 mL | 0 | 1.0 mL |
| LPB2 | *L. monocytogenes* ($10^7$ CFUmL$^{-1}$) + Phage ($10^8$ PFU mL$^{-1}$) + pediocin PA-1 (1280 AU mL$^{-1}$) | 0 | 1.0 mL | 3.0 mL | 1.0 mL |

L - *L. monocytogenes* untreated control; LP - *L. monocytogenes* + phage P100; LB - *L. monocytogenes* + pediocin PA-1; LPB - *L. monocytogenes* + phage P100 + pediocin PA-1; 1 - Inoculum of L. *monocytogenes* ($10^6$ CFUmL$^{-1}$); 2 - Inoculum of L. *monocytogenes* ($10^9$ CFUmL$^{-1}$).

and submitted to different biocontrol treatments as described in Table 1. Homogenisation was performed by magnetic agitation to ensure a uniform distribution of the inoculum. Final phage P100 concentration had a multiplicity of infection (MOI) of 10000 or 10, to $10^4$ or $10^7$ log CFU mL$^{-1}$ of *L. monocytogenes*, respectively, in the inoculated samples. The antilisterial activity of pediocin PA-1 in samples inoculated with the semi-purified extract from *P. acidilactici* HA 6111-2 was 1280 AU mL$^{-1}$. This value was established based in a previous report about the stability of this peptide throughout simulated gastrointestinal digestion (Kheadr et al., 2010), and in biocontrol experiments employing whole milk (Hartmann et al., 2011).

One set of samples was subjected to mild HHP (200 or 300 MPa, 5 min, 10 °C) and the other set was stored at atmospheric pressure (0.1 MPa, i.e. non-pressure treated) under refrigeration (4 °C), to serve as control. Additionally, a third set was inoculated with *L. monocytogenes* cultures and submitted to a conventional heat treatment (high temperature short time pasteurization, HTST) as described in section 2.5, in order to have a standard during refrigerated storage at 4 °C for further comparisons.

### 2.4. High hydrostatic pressure treatments

The samples, prepared as described in section 2.3 and pre-cooled at 4 °C, were transferred to HHP resistant polyethylene bottles (36 mL), previously sterilized, and double vacuum sealed in low permeability polyamide-polyethylene bags (PA/PE-90, Albipack - Packaging Solutions, Portugal). Samples were pressurized in hydrostatic press from Hiperbaric 55 (Burgos, Spain) and the pressure-transmitting fluid was water. The pressure treatment was set at 200 and 300 MPa (10 °C), with 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 °C). HHP parameters (pressure range and holding time of pressurization) were selected based on previous results of phage P100 stability at high pressure processing, which was capable to maintain its infectivity until 300 MPa, and was more stable in short holding times of pressurization, similar to holding times applied in commercial high pressure processing (i.e. 3–20 min) (Komora et al., 2018; Tomasula et al., 2014). During each HHP batch, the temperature of water inside the press was automatically monitored and did not exceed 13 °C. Three independent replicates of all experiments were performed.

### 2.5. High temperature short time pasteurization simulation on milk

Milk samples inoculated with *L. monocytogenes* cultures were submitted to high temperature short time pasteurization (HTST) to have a comparative standard during refrigerated storage of *L. monocytogenes* artificially contaminated samples. Briefly, UHT whole milk was pre-warmed at 72 °C for 60 min in a shaking water bath (50 rpm), to allow temperature equilibration and then cells were inoculated at the same level as described in 2.3 and exposed to this temperature for 25 s. Temperature was monitored using a calibrated glass thermometer in a control flask containing non-inoculated milk and submitted to the same experimental conditions. Samples were cooled with water at room temperature before storage at 4 °C. Three independent replicates were performed for each isolate.

### 2.6. Bacterial enumeration and phage P100 and bacteriocin titration

*Listeria monocytogenes* enumeration and detection procedure (enrichment protocol) were performed according to the methods recommended by the International Organization for Standardization (ISO, 2017a; 2017b).

Phage Listex™ P100 titre (PFU mL$^{-1}$) was determined by the double-layer method (plaque assay) as previously described by Kropinski et al. (2009). TSAYE was selected as solid media (underlay)

and TSBYE, containing 7 g $L^{-1}$ of bacteriological agar (Pronadisa, Madrid, Spain), was used as molten soft agar (overlay). The diluent chosen was PBS (0.1 M, pH 7.4) and the detection limit of the enumeration technique was 10 PFU $mL^{-1}$.

Antilisterial activity of pediocin PA-1 was measured by two-fold dilutions and the spot-on-the-lawn method 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 target bacteria. *Listeria monocytogenes* Scott A and *L. innocua* 2030c were selected as target bacteria.

### 2.7. Bacteriocin PA-1 stability in milk during refrigerated storage at 4 °C

The antilisterial activity of pediocin PA-1 produced by *P. acidilactici* strain HA-6111-2 was evaluated in the UHT whole milk and in ammonium acetate buffer (pH 6.5) as a control during the 7 days of refrigerated storage at 4 °C. At pre-set time intervals (0, 1, 3 and 7 days) samples were subjected to titration protocol as described in 2.6. All experiments were conducted in three independent replicates.

### 2.8. Phage Listex™ P100 stability in milk during refrigerated storage at 4 °C

The ability of phage Listex™ P100 to maintain its stability and viability during shelf-life storage (4 °C) of UHT whole milk after HHP treatment was assessed. At pre-set time intervals (0, 1, 3 and 7 days), non- and pressure-treated samples were analysed. All experiments were conducted in three independent replicates.

### 2.9. Statistical analysis

Microbial and phage titration values were expressed as logarithmic reductions using the equation: log ($N_0$/N), where N is the microbial cell density/phage titre at a particular sampling time and $N_0$ 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 using the detection procedure was expressed as presence or absence of *L. monocytogenes* and significant differences were determined using the chi-square test (SPSS, Version 23.0). The possibility of synergistic effect between the hurdles applied in combination in this study was analysed. The formula applied and the data of logarithmic reduction for theoretical additive effect and for the observed effects are available in Supplementary Table 1.

## 3. Results

### 3.1. Inactivation of L. monocytogenes in UHT whole milk

The survival of *L. monocytogenes*, inoculated at $10^4$ CFU $mL^{-1}$ and submitted to different combined treatments in UHT whole milk, during storage at 4 °C for 7 days is presented in Fig. 1 and in the Supplementary Fig. 1; Table 2 summarizes results obtained for *Listeria* detection method. For both strains and also for non- and pressure-treated samples, the combined effect between phage P100 and pediocin PA-1 resulted in levels of *L. monocytogenes* below the detection limit of the enumeration technique (100 CFU $mL^{-1}$) immediately after treatment; *L. monocytogenes* was absent in samples pressurized at 200 and 300 MPa for 5 min whereas presence was still observed by the enrichment protocol in non-pressure treated samples (0.1 MPa) up to seven days of refrigerated storage. *Listeria monocytogenes* was not detected in pressurized samples (200 and 300 MPa) inoculated with phage P100; a better approach in terms of practical application on the inactivation of *L. monocytogenes* at levels of $10^4$ CFU $mL^{-1}$ compared to the combined application of phage and pediocin PA-1; synergism between HHP (200 and 300 MPa) and phage P100 was observed during all shelf-life for both Lm Scott A and Lm 1751.

Pediocin PA-1 demonstrated to be useful to control Lm Scott A in UHT whole milk, whereas strain Lm 1751 was more resistant to the bacteriocin; in non-pressure treated samples, pediocin PA-1 reduced Lm Scott A to below the detection limit of the enumeration technique and no recovery was observed; for Lm 1751 strain similar results concerning the logarithmic reduction were only achieved in combination with HHP (300 MPa, 5min). Synergism between phage P100 and pediocin PA-1 was observed for Lm 1751 in non-pressurized samples at three and seven days of storage (Fig. 1, G-H); for Lm Scott A, synergism in non-pressurized samples was only observed immediately after treatment (i.e. 2 h after inoculation). The regrowth of *L. monocytogenes* was observed in non-pressurized samples treated with phage P100 for both strains.

Results obtained from samples inoculated with $10^7$ CFU $mL^{-1}$ of *L. monocytogenes* are presented in Fig. 2. In pressurized samples, an initial reduction of *L. monocytogenes* in UHT milk samples immediately after treatment (ranging from 0.01 to 2.86 and from 0.07 to 2.31 log cycles reduction for Lm Scott A and Lm 1751, respectively) was observed. For 200 MPa or 300 MPa treated samples inoculated with both phage P100 and pediocin PA-1, following the initial reduction no growth was detected up to the seventh day of storage at 4 °C. Non-pressurized samples treated with phage P100 or pediocin PA-1 or both registered a decrease on the first day, followed by a regrowth of Lm 1751 during refrigerated storage. *L. monocytogenes* Scott A inoculated with pediocin PA-1 decreased until the end of storage. For Lm Scott A, the most effective treatment was the combination of HHP and pediocin PA-1, which resulted in the higher inactivation rate from day one to the end of storage. Synergism between HHP (200 and 300 MPa) and pediocin PA-1 was observed (Fig. 2 A-D) during all storage; synergism between HHP (200 and 300 MPa) and phage P100 was only observed immediately after treatment.

For samples inoculated with Lm 1751, the most effective treatment was the combination of HHP (300 MPa), phage P100 and pediocin PA-1 ($P < 0.05$); synergism between HHP (300 MPa), phage P100 and pediocin PA-1 was observed during storage. For samples pressurized at 200 MPa synergism between HHP, phage P100 and pediocin was only observed at the end of storage (Fig. 2, H). Synergism between HHP (200 MPa) and P100 was observed for Lm 1751 inoculated samples (Fig. 2 E-F), although a synergistic effect for the combination of HHP (300 MPa) and phage P100 was only observed in the beginning of the storage period (day one) with subsequent simple additive effect detected. Synergism between HHP and pediocin PA-1 was not observed for samples inoculated with Lm 1751 at contamination level of $10^7$ CFU $mL^{-1}$.

### 3.2. Comparison of non-thermal approach to the HTST pasteurization

For samples inoculated with $10^4$ CFU $mL^{-1}$, *L. monocytogenes* was eliminated by both HTST pasteurization and HHP (300 MPa, 5 min, 10 °C) in combination with phage P100 (Table 2). For samples inoculated with $10^7$ CFU $mL^{-1}$, HTST pasteurization resulted in a higher inactivation compared with all non-thermal combinations, which were unable to eliminate *L. monocytogenes*.

### 3.3. Stability of biocontrol agents during HHP and refrigerated storage at 4 °C

To evaluate the stability of the antimicrobial agents during storage, non- and pressure-treated samples of UHT whole milk were stored at 4 °C for 7 days, and phage and bacteriocin titres were evaluated at specific time periods (Tables 3 and 4 and Supplementary Table 2). In non-pressurized samples, no significant differences were observed for all phage titres ($P > 0.05$). In samples submitted to HHP a slight reduction in P100 titres was registered immediately after treatment, but afterwards phage titres remained stable throughout the 7 days of refrigerated storage ($P > 0.05$). Furthermore, when phage P100 was

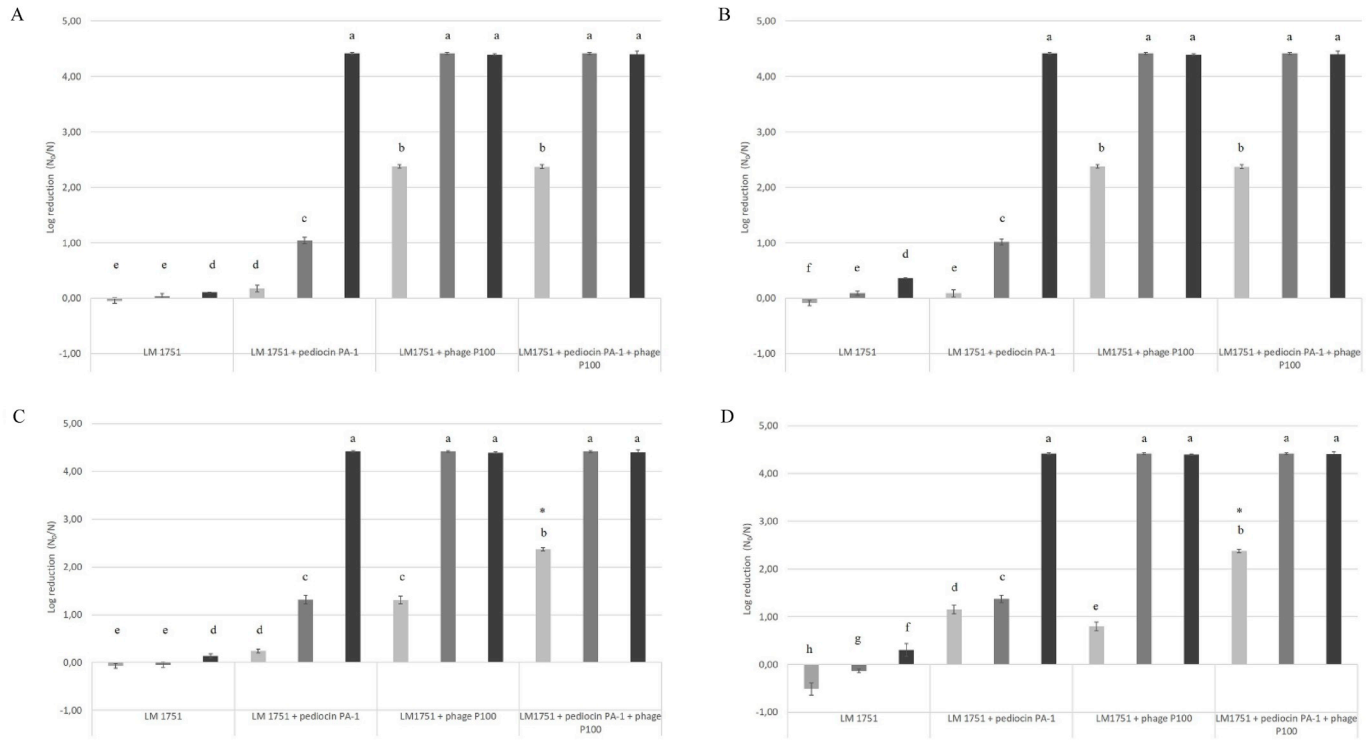


**Fig. 1.** Effect of different biocontrol strategies on the survival of *L. monocytogenes* strains at an initial level of $10^4$ CFU mL$^{-1}$ on UHT milk throughout 7 days of storage at 4 °C: A) Lm 1751 after treatment (2 h). B) Lm 1751 after one day. C) Lm 1751 after three days. D) Lm 1751 after seven days. Values are the average ± standard deviation (error bars) of three independent experiments. Means with the same letter are not statistically different from each other ($P > 0.05$). Pressure: 0.1 MPa (□); 200 MPa (■); 300 MPa (■). * indicates samples where the synergism between phage P100 and pediocin PA-1 was observed.

applied in combination with pediocin PA-1 (1280 AUmL$^{-1}$), the presence of the antimicrobial peptide did not affect the phage titres during the whole storage period ($P > 0.05$). Pediocin PA-1 concentration in milk samples remained stable until the third day of storage, becoming undetectable from this day onwards, until the last sampling. Control samples of semi-purified pediocin PA-1 stored in ammonium acetate buffer at the same conditions showed no significant differences in concentration during the entire storage period ($P > 0.05$).

## 4. Discussion

The combined effect between the applied hurdles has proven to be an efficient system to inactivate *L. monocytogenes*.

It was demonstrated that at a contamination level of $10^4$ CFU mL$^{-1}$ different synergisms were able to reduce *L. monocytogenes* below the detection limit of the enumeration technique. The combination of HHP and phage resulted in the complete inactivation of *Listeria* ($10^4$ CFU mL$^{-1}$) in artificially contaminated milk samples, for the two strains tested; globally (for both strains and both inoculum levels

**Table 2**
Recovery of L. *monocytogenes* after enrichment protocol in UHT whole milk during refrigerated storage (4 °C).

| Process condition | Antimicrobial agent | Recovery of *Listeria monocytogenes* after enrichment protocol ISO 11290-2:2017 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Sampling time | | | | | | | |
| | | AT | | 1 day | | 3 days | | 7 days | |
| | | Lm Scott A | Lm 1751 | Lm Scott A | Lm 1751 | Lm Scott A | Lm 1751 | Lm Scott A | Lm 1751 |
| 0.1 MPa (control) | Control* | + | + | + | + | + | + | + | + |
| | Phage P100* | + | + | + | + | + | + | + | + |
| | Pediocin PA-1* | - | + | - | + | - | + | - | + |
| | Phage P100 + pediocin PA-1* | + | + | + | + | + | + | + | + |
| 200 MPa HHP | Control* | + | + | + | + | + | + | + | + |
| | Phage P100* | - | - | - | - | - | - | - | - |
| | Pediocin PA-1* | - | + | - | + | - | + | - | + |
| | Phage P100 + pediocin PA-1* | - | - | - | - | - | - | - | - |
| 300 MPa HHP | Control* | + | + | + | + | + | + | + | + |
| | Phage P100* | - | - | - | - | - | - | - | - |
| | Pediocin PA-1* | - | – | - | – | - | – | - | – |
| | Phage P100 + pediocin PA-1* | - | - | - | - | - | - | - | - |
| HTST pasteurization | Control* | - | - | - | - | - | - | - | - |
| | Control** | - | - | - | - | - | - | - | - |

Lm – *L. monocytogenes*; AT – 2 h after treatment; (+) presence of *L. monocytogenes*; (−) absence of *L. monocytogenes*; (*) $10^4$ CFU mL$^{-1}$ of *L. monocytogenes*; (**) $10^7$ CFU mL$^{-1}$ of *L. monocytogenes.*

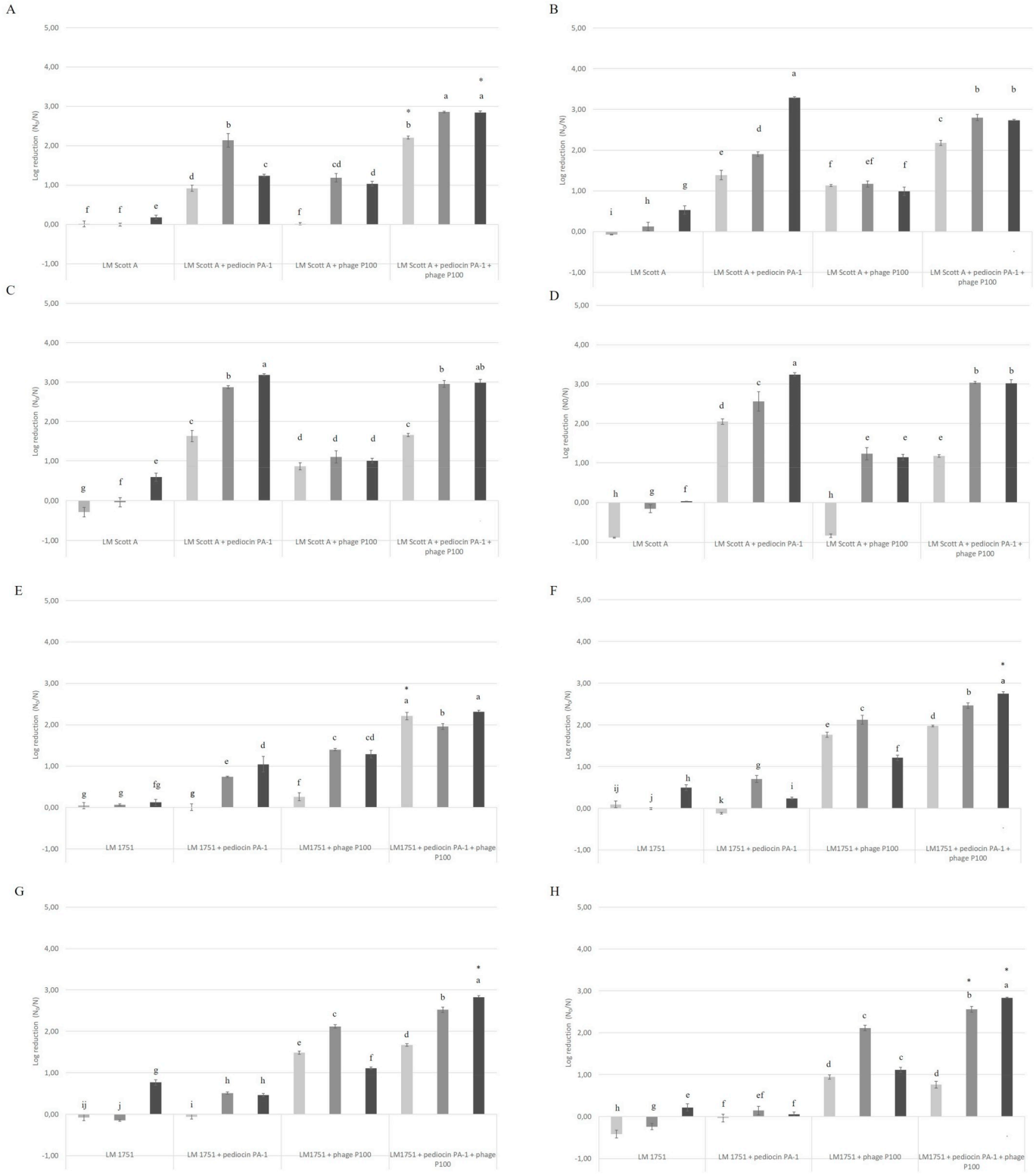


**Fig. 2.** Effect of different biocontrol strategies on the survival of *L. monocytogenes* strains at an initial level of $10^7$ CFU $mL^{-1}$ on UHT milk throughout 7 days of storage at 4 °C: A) Lm Scott A after treatment (2 h). B) Lm Scott A after 1 day C) Lm Scott A after three days. D) Lm Scott A after seven days. E) Lm 1751 after treatment (2 h). F) Lm 1751 after one day. G) Lm 1751 after three days. H) Lm 1751 after seven days. Values are the average ± standard deviation (error bars) of three independent experiments. Means with the same letter are not statistically different from each other ($P > 0.05$). Pressure: 0.1 MPa (□); 200 MPa (■); 300 MPa (■).* indicates samples where the synergism between phage P100 and pediocin PA-1 was observed.

**Table 3**
Phage titer of non- and pressure-treated samples of UHT whole milk during storage at 4 °C for 7 days.

| Pressure | Applied hurdles | Phage titer (log PFU mL$^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | Time (days) | | | | |
| | | BT | AT | 1 | 3 | 7 |
| 0.1 MPa | phage P100 | 8.08 ± 0.01 | 8.16 ± 0.02 | 8.25 ± 0.01 | 8.24 ± 0.02 | 8.14 ± 0.02 |
| | phage + pediocin PA-1 | 8.24 ± 0.01 | 8.10 ± 0.02 | 8.11 ± 0.03 | 8.11 ± 0.03 | 8.12 ± 0.02 |
| 200 MPa* | phage P100 | 8.17 ± 0.02 | 7.89 ± 0.03 | 7.94 ± 0.02 | 7.88 ± 0.04 | 7.84 ± 0.05 |
| | phage + pediocin PA-1 | 8.14 ± 0.03 | 7.92 ± 0.03 | 7.87 ± 0.04 | 7.89 ± 0.03 | 7.93 ± 0.01 |
| 300 MPa* | phage P100 | 8.13 ± 0.07 | 7.06 ± 0.08 | 7.21 ± 0.09 | 7.07 ± 0.10 | 7.04 ± 0.06 |
| | phage + pediocin PA-1 | 8.20 ± 0.07 | 7.15 ± 0.15 | 7.20 ± 0.04 | 7.15 ± 0.11 | 7.03 ± 0.02 |

(*) Significant differences ($P < 0.05$) observed only between phage titer of non- and HHP-treated samples. Lm inoculum concentration, isolate and combined hurdle did not affect the phage titer during refrigerated storage ($P > 0.05$); complete data can be found in Supplementary Table 2. Results are expressed as means of three independent experiments ± standard deviation.

tested), the synergism between HHP and phage P100 performed better in the lower pressure applied in this study (i.e. 200 MPa), which is in accordance with the previous findings regarding phage P100 stability that is partially lost at 300 MPa, even in a protective matrix as UHT whole milk (Komora et al., 2018). The synergism resulting from the application of HHP (300 MPa) and pediocin PA-1 was able to completely inactivate Lm 1751 at the inoculum of $10^4$ CFU mL$^{-1}$. The effectiveness of each of these natural biocontrol agents *per se* in high pressure processing was shown to be dependent on its stability during HHP as well as on the intrinsic resistance of the different *Listeria* strains to these antimicrobials and the inoculum level.

Overall, besides the promising reductions obtained, the combined application of biocontrol agents (i.e. phage and bacteriocin) in non-pressurized milk samples was unable to result in the elimination of both *L. monocytogenes* strains. Regarding Lm 1751, a synergistic effect between phage P100 and pediocin PA-1 was observed for non-pressurized samples at both contamination levels; at a contamination level of $10^4$ CFU mL$^{-1}$, synergism was only observed in the end points (i.e. three and seven days) of refrigerated storage, whereas at the higher contamination level it was observed immediately after treatment until the day three of shelf-life. Synergism between phage/endolysin and bacteriocins has been described as well as the regrowth of target bacteria or the incomplete bacterial inactivation in this type of synergism, being the effectiveness of the biocontrol directly linked to the complexity of the food matrix and to the behaviour and stability of biocontrol agents (Baños et al., 2016; Ibarra-Sánchez et al., 2018; Leverentz et al., 2003; Rodríguez-Rubio et al., 2015; Wang et al., 2017).

It was demonstrated a synergistic effect of phage P100, pediocin PA-1 and HHP (300 MPa) to eliminate high levels of Lm 1751 (i.e. $10^7$ CFU mL$^{-1}$) in milk. Although promising results from synergistic application of bacteriocins and phages, or HHP and phage, for the non-thermal processing/biocontrol of milk were described (Rodríguez-Rubio et al., 2015; Tabla et al., 2012), to the best of our knowledge, this is the first report combining both phages and bacteriocins as additional hurdles in mild high pressure processing. Rodríguez-Rubio et al. (2015) studied the synergistic effect between coagulin C23 and Listeriophages FWLLm1 and FWLLm3 to eliminate *L. monocytogenes* in milk and reported an effective reduction of *L. monocytogenes* during initial days of storage, followed by a regrowth of *L. monocytogenes* in FWLLm3 treated samples after six days of refrigerated storage (4 °C). Tabla et al. (2012) investigated the improvement of bacteriophages philPLA35 and philPLA88 performance by HHP against *Staphylococcus aureus* in milk and also described an effective synergism and a slight regrowth of *S. aureus* after 48 h of incubation at 25 °C. Besides the regrowth of pathogenic bacteria and the fact that recovery of cells under enrichment conditions was not assessed by both studies, it is important to point out the relevance of sublethal injured cells of *L. monocytogenes* during storage, especially according to the notorious ability of *L. monocytogenes* to recover during refrigerated shelf-life. In addition, the evaluation of the presence or absence of *L. monocytogenes* cells should be addressed for a proposed decontamination process since many countries have adopted the zero-tolerance approach to *L. monocytogenes* (Warriner and Namvar, 2009). Furthermore it would be possible to reduce the pressure required to eliminate *L. monocytogenes* in milk samples, usually 600 MPa for 4–6 min (Buzrul, 2017) using combined mild HHP with biocontrol agents. Operating at lower pressures results in energy saving as well as reduced costs associated with the maintenance of HHP equipment (Oliveira et al., 2015). Another advantage is the minimal processing of milk in terms of biochemical changes, since pressures in the range of 100–300 MPa imply changes in casein micelles sizes, although with minimal and reversible changes in the quaternary, tertiary or secondary structure of milk proteins (Rastogi et al., 2007).

**Table 4**
Stability of pediocin PA-1 produced by *Pediococcus acidilactici* strain HA-6111-2 during storage at 4 °C in non- and pressurized milk and ammonium acetate buffer.

| Pressure | Sample | AU mL$^{-1}$[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Time (days) | | | | | | | | | |
| | | BT | | AT | | 1 | | 3 | | 7 | |
| | | Lm Scott A* | Li 2030c | Lm Scott A* | Li 2030c | Lm Scott A* | Li 2030c | Lm Scott A* | Li 2030c | Lm Scott A* | Li 2030c |
| 0.1 MPa | Milk* | 1280 | 640 | 11280 | 640 | 1280 | 640 | 1280 | 640 | n/d | n/d |
| | Ammonium acetate buffer | 1280 | 640 | 1280 | 640 | 1280 | 640 | 1280 | 640 | 1280 | 640 |
| 200 MPa | Milk* | 1280 | 640 | 1280 | 640 | 1280 | 640 | 1280 | 640 | n/d | n/d |
| | Ammonium acetate buffer | 1280 | 640 | 1280 | 640 | 1280 | 640 | 1280 | 640 | 1280 | 640 |
| 300 MPa | Milk* | 1280 | 640 | 1280 | 640 | 1280 | 640 | 1280 | 640 | n/d | n/d |
| | Ammonium acetate buffer | 1280 | 640 | 640 | 640 | 640 | 640 | 640 | 640 | 640 | 640 |

BT - before treatment; AT- 2 h after treatment; n/d - not detectable; [a] AU - antimicrobial activity expressed as arbitrary units *per* mL. One AU was defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition. *Similar values were observed for Lm 1751

Concerning the MOI value used, a decrease in bactericidal effect of phage synergisms was observed when a high level of contamination was assumed, being the most effective reductions achieved *ca.* 2.86 log cycles. Besides MOI being considered the ratio of adsorbed phage to bacteria, the impact of changes in bacterial density on MOI is not straightforward (Abedon, 2016); Kudva et al. (1999) studied the biocontrol of *Escherichia coli* O157:H7 by O157-specific phages and also described a higher MOI required (i.e. MOI of $10^3$ PFU/CFU) to efficiently killing *E. coli* O157:H7, whereas MOIs ranging from $10^{-3}$ to $10^2$ resulted in minimal decline in bacterial titre. The application of pediocin PA-1 (1280 AU $mL^{-1}$) also had a decrease in its bactericidal activity at high contamination level. Similarly, Carnio et al. (2000), studied the effect of *in-situ* production of micrococcin P1 by food-grade strain *Staphylococcus equorum* WS2733 in soft cheese and also described the phenomenon of contamination level dependence in bactericidal effect of bacteriocin.

The SPO1-like *Listeria* phages are well known for their ability to support refrigerated storage in various food matrices; results from several studies demonstrated that phage P100 and phage A511 can be successfully applied without compromising sensorial characteristics in dairy, meat and fisheries matrices and ready-to-eat products, whereas in fruit and vegetable matrices it is only supported by matrices with high pH (Chibeu et al., 2013; Guenther et al., 2009; Oliveira et al., 2014; Soni et al., 2012). Dairy matrices are specially favourable matrices for application of phages since some phages require divalent ions such as $Ca^{2+}$ for attachment or intracellular growth (Landry and Zsigray, 1980). Under high pressure processing, phage P100 remained stable until 300 MPa according to the complexity of food matrices, being dairy and fermented meat products protective matrices (Komora et al., 2018). Additionally, Tabla et al. (2012) studied the combined effect of HHP (400 MPa, 5 min, 10 °C) with phages philPLA35 and philPLA88 to promote *S. aureus* inactivation in milk and concluded that pressurized phage also maintained its infectivity. Decrease in activity of bacteriocins incorporated in food systems during shelf-life of products is well documented (Ananou et al., 2010; Baños et al., 2016). Baños et al. (2016), evaluating the synergistic effect of phage P100 and enterocin AS-48 to control *L. monocytogenes* in fish fillets, described a gradual decrease in the recovery of enterocin AS-48 from the fillets tissue during storage at 4 °C. Normally, adsorption of bacteriocin is directly related to retention to fat components, as well as to slower diffusion in complex matrices and irregular distribution. In this sense, the lower titration of semi-purified pediocin PA-1 in milk on the final day of storage can be attributed to the retention to fat components, since it maintained the titre when stored in ammonium acetate buffer.

## 5. Conclusion

In this preliminary study, at a contamination level of $10^4$ CFU $mL^{-1}$, the presence of phage P100 in pressurized samples contributed to the non recovery of *L. monocytogenes* during the refrigerated shelf-life of milk, being the efficacy of this treatment comparable to the HTST pasteurization. At a high contamination level ($10^7$ CFU $mL^{-1}$), the synergistic effect of the phage P100, pediocin PA-1 and HHP (300 MPa) against *L. monocytogenes* has been described in a food matrix for the first time. Nevertheless, this treatment was not as effective as the thermal treatment (HTST). Further studies should be carried out in order to better understand the mechanisms responsible for inactivation of *L. monocytogenes* in non-thermal conditions and in the presence of biocontrol agents in different food matrices. It should be highlighted that only two strains of *L. monocytogenes* were investigated, both of serotype 4b. Given the high diversity among the strains belonging to the species *L. monocytogenes*, serotype classification is not definitive for predicting phenotypic behaviour. Nevertheless current findings need to be further validated for other strains.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103315.

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