The combined effect of chitosan and high hydrostatic pressure on Listeria monocytogenes and Escherichia coli

Innovative Food Science and Emerging Technologies 94 (2024) 103693

Contents lists available at ScienceDirect

Innovative Food Science and Emerging Technologies

journal homepage: www.elsevier.com/locate/ifset

The combined effect of chitosan and High Hydrostatic Pressure on *Listeria monocytogenes* and *Escherichia coli*

Nikolaos Giannoulis, Kimon Andreas G. Karatzas *

Department of Food & Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6AD, UK

ARTICLE INFO

Keywords:
High-pressure processing (HPP)
Chitosan
Synergistic effect
Hurdle technology
Listeria monocytogenes
Escherichia coli

ABSTRACT

This study explores the combined effect of different High Hydrostatic Pressures (HHP; 200–300 MPa) with various chitosan concentrations (up to 0.2%) on five *Listeria monocytogenes* strains and one of *Escherichia coli*, at temperatures 20 & 35 °C. Cells were resuspended in ACES buffer 1 h prior to HHP. *A synergistic effect of chitosan and HHP was reported for first time in the above bacterial species tested.* Synergistic effect up to 1 log reduction was observed at 300 MPa and chitosan at 20 °C against *L.monocytogenes* LO28 with FBR13 being the most sensitive strain at 250 MPa and 0.1% chitosan. Higher combined effect was found at 35 °C compared to 20 °C at 200 MPa *highlighting for first time the significant role of temperature in the above synergistic action.* Pressure and temperature had a greater impact on inactivation and synergism than chitosan concentrations. Synergistic effect (1 log reduction) was also observed in *E. coli* K12 at 0.1% chitosan and 200 MPa.

Industrial relevance: This study presents the significance of combining HHP with natural antimicrobials to control *L. monocytogenes* and *E. coli*. Even though the technology is used for 3 decades in the food industry, its cost is still relatively high. Therefore, it is important to investigate novel ways to reduce the pressure intensity resulting in reduced costs, lower energy consumption and a broader product portfolio. This research demonstrates for first time the synergistic action of chitosan and HHP on *L. monocytogenes* and *E. coli* and the significant role of temperature that could contribute in the enhancement of the antimicrobial effect and optimization of the processing conditions. This aligns also with the growing demand for more sustainable and natural systems regarding the food production.

1. Introduction

High Hydrostatic Pressure (HHP) is a non-thermal food processing technology which is able to inactivate various spoilage and pathogenic microorganisms ensuring food safety and extending shelf life of food products. It has also the ability to protect the heat-sensitive food constituents (vitamins, minerals, and nutrients) achieving a high nutritional quality food product similar to an untreated one (Aganovic et al., 2021). Fruits and vegetables, meat and fish products, as well as beverages are the main food categories which are treated with HHP today (Huang, Lung, Yang, & Wang, 2014).

Pressures between 100 and 1000 MPa and temperatures ranging from 0 to 120 °C can be used in HHP but pressures ranging from 200 to 800 MPa at temperatures between 20 and 60 °C have been mainly studied and used in the industry (EFSA, 2022; Huang et al., 2014; Huang et al., 2015; Nabi et al., 2021). Vegetative cells of foodborne pathogens and spoilage organisms are inactivated at ambient temperatures with HHP in the range of 200 up to 600 MPa, as usually applied in commercial food applications (Georget et al., 2015; Mota et al., 2013; Rastogi et al., 2007; Wang et al., 2016). Stratakos et al. (2019) found that subjecting raw milk to HHP at 600 MPa for 5 min resulted in a reduction of >5 log units for *L. monocytogenes*, *Salmonella* spp., and *E. coli* cells. The damage caused by HHP on vegetative microbial cells, has been extensively described in many studies. The main mechanisms of cellular inactivation elicited by HHP include changes of cell structural organization and inhibition of metabolic processes, essential for cell maintenance (Aganovic et al., 2021; Torres & Velazquez, 2005).

The economic cost of HHP is relatively high while some food components such as proteins or lipids can be altered during the process (Mujica-Paz et al., 2011). Therefore, it is important to investigate novel ways to reduce the pressure intensity resulting in reduced costs and a broader product portfolio. One way to reduce the pressure intensity increasing the level of inactivation of microorganisms is to combine HHP technology with other hurdles including natural antimicrobial

* Corresponding author at: School of Chemistry, Food & Pharmacy, Department of Food & Nutritional Sciences, University of Reading, Reading RG6 6AD, UK.
E-mail address: k.karatzas@reading.ac.uk (K.A.G. Karatzas).

https://doi.org/10.1016/j.ifset.2024.103693
Received 31 January 2024; Received in revised form 10 May 2024; Accepted 12 May 2024
Available online 14 May 2024

substances such as lysozyme, lactoferrin, bacteriocins (e.g. nisin, pediocin) and chitosan (Li et al., 2016; Malinowska-Panczyk & Kolodziejska, 2009; Raso & Barbosa-Canovas, 2003; Yang et al., 2021).

Among the antimicrobial compounds, chitosan, a linear polysaccharide derived by the deacetylation of chitin, is of great interest. It can be found in the shells of crustaceans, such as crab and shrimps and has been recognized as safe (GRAS) for consumption by the US FDA (GRAS Notices, US FDA, CFSAN, 2001). The antimicrobial activity of chitosan against various groups of microorganisms, such as bacteria and fungi has been broadly investigated (Zheng & Zhu, 2003). *L. monocytogenes* reduced below the detection limit when chitosan applied as an edible film in cold-smoked salmon (Benabbou et al., 2018). Compared to other antimicrobials, chitosan seems to have antibacterial activity against both Gram-positive and Gram-negative bacteria. However, it is challenging to determine whether chitosan is preferably more effective against one of the latter two groups because of major differences in sensitivity (No, Park, Lee, & Meyers, 2002; Tsai et al., 2002).

The inactivation or inhibition of microbial cells by chitosan either in media or food matrices has been investigated by several studies through the years. Due to the protonation of amino groups present in glucosamine residues, chitosan is positively charged when the pH is below 6. The electrostatic interaction between the negatively charged components in the microbial cell membrane and NH_3 groups of chitosan increases the cell permeability releasing several intracellular compounds which finally cause cell death (Liu et al., 2008; Sudarshan et al., 1992). Furthermore, acting as a chelating agent in food the surface of food products, chitosan can selectively attach to metals and vital nutrients, inhibiting the microbial growth (Feng et al., 1997).

The molecular weight (MW) and the degree of acetylation (DA) have been reported as the most significant factors affecting the chitosan antimicrobial activity (Sekiguchi et al., 1992). Also, it is suggested that the influence of the MW on the antimicrobial activity is greater than the influence of the DA. Tsai and Su (1999) reported that temperature, (influencing the chemical reaction rate) and pH value, (changing the protonated numbers of chitosan) also play significant role on the bactericidal activity. However, there are lots of studies discussing chitosan's antimicrobial activity in different conditions, with contradictory results (Zheng & Zhu, 2003).

The concept of hurdle technology in food preservation has been extensively studied combining multiple methods such as pH control, temperature, and packaging techniques to create synergistic barriers against microbial growth, thereby extending the shelf life of food products (Leistner, 1995; Leistner & Gorris, 1995). Currently, the combination of different non thermal technologies is under consideration to ensure the safety while still maintaining the characteristics of fresh food. More specifically, combining preservative factors can significantly improve the quality of foods while producing a clean label safe product with acceptable shelf life. HHP inactivation of microorganisms both alone or combined with antimicrobials (e.g. nisin, essential oils, carvacrol) has been studied in different growth media or food matrices (Gayán et al., 2012; Karatzas et al., 2001; Oner, 2020). However, only few studies have been conducted combining HHP with chitosan. Martillanes et al. (2021) reported that *L. monocytogenes* in sliced dry-cured Iberian ham packaged in a chitosan-based film with added nisin was reduced by 4 log units when subjected to HHP at 600 MPa for 8 min. As such, a systematic study regarding the synergistic or additive effect of HHP treatment in various pressures combined with a range of chitosan concentrations in different process temperatures against *L. monocytogenes* and *E. coli* is lacking. Moreover, to the authors' best knowledge, there are very few studies investigating the behavior of the different piezotolerant and piezosensitive *L. monocytogenes* strains against HHP combined with chitosan treatment.

In this respect, *this study for first time aims to identify the combined and synergistic effect of HHP and chitosan at a range of pressures, concentrations, and process temperatures on Listeria monocytogenes (Gram positive) and Escherichia coli (Gram negative)*. A potential synergism could allow the reduction of equipment manufacture and operation costs resulting in safer and novel products by HHP at an affordable price. *This study also emphasizes the importance of temperature in the reduction of chitosan-treated L. monocytogenes cells during HHP treatment.* Furthermore, the results of this research can be used for validation studies in HHP with chitosan treated food products.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Five strains of *Listeria monocytogenes* (Transit collection) and *E. coli* K12 wild type (Keio collection, Department of Food & Nutritional Sciences, University of Reading) were used throughout this study (Table 1). *L. monocytogenes* strains were selected based on their tolerance to HHP (two piezotolerant, two intermediate, one piezosensitive; Tsagkaropoulou & Karatzas, 2024). *E. coli* K12 wild type was selected as a Gram-negative representative because it is one of the most widely used model strains and additionally, it is a strain with intermediate piezotolerance and as such, it was chosen to better assess the potential synergistic effect (Tsagkaropoulou & Karatzas, 2024). Frozen stock cultures were prepared in 7% *v*/v dimethyl sulfoxide (DMSO; Sigma Aldrich, Dorset, UK) and stored at −80 °C. Stock cultures for storage (slant stock) in the fridge (4–5 °C) were made by streaking from frozen stock on BHI agar plates (BHI agar, Neogen Lancashire, UK) for *L. monocytogenes* and LB agar plates (Neogen Lancashire, UK) for *E. coli* K12. The plates were incubated overnight at 37 °C and then stored in the fridge for one month.

Prior to each experiment, *L. monocytogenes* cells were revived by selecting three separate colonies from slant stock and inoculated in 3 mL of BHI broth in plastic tubes (Neogen Lancashire, UK). The tubes were incubated in 37 °C for 24 h with shaking (120 rpm). Subsequently, stationary phase cells were prepared by inoculating 20 mL BHI broth conical flasks with 1% (*v*/v) of the above primary cultures and incubating them at 37 °C for 16–18 h with shaking (120 rpm). Similarly for *E. coli*, three individual colonies were transferred to LB broth (Neogen Lancashire, UK) for 6–7 h at 37 °C. One percent of these cultures were then inoculated to 10 mL fresh LB broth and incubated for 16–18 h at 37 °C without shaking until stationary phase cells.

2.2. Chitosan preparation

Low molecular weight chitosan (Merck Life Science UK Limited) with deacetylation degree ≥75% was used in this work. Chitosan stock solutions of 10 mg/mL (1% *w/v*) were prepared in 50 mmol/L ACES buffer [N-(2-acetamido)-2-aminoethanesulfonic acid] (ACES Buffer; 0.5 M, pH 7.0; Thermo Fisher Scientific) containing 1% (*v*/v) acetic acid. The pH was adjusted to 6.0 by the addition of 5 N NaOH solution. New chitosan stock solutions were prepared weekly.

Table 1
L. monocytogenes and *E. coli* strains used in this study.

Strain	Origin/Serotype	Reference
Listeria monocytogenes		
LO28	Healthy pregnant carrier, 1/2c	(Aryani et al., 2015)
L6	Milk, 1/2b	(Aryani et al., 2015)
FBR13	Frozen endive a la crème, 1/2a	(Aryani et al., 2015)
NCTC 10357 (DSM20600)	Rabbit, 1a	Murray, Webb, & Swann, 1926)
F2365	Jalisco cheese, 4b	(Aryani et al., 2015)
Escherichia coli		
K12 (BW25113)		(Bulut & Karatzas, 2021)

2.3. Cell suspension preparation and chitosan application

Cells in the stationary phase of growth harvested by centrifugation at 3600 rpm for 10 min at 4 °C and subsequently were resuspended in 50 mM ACES buffer (pH 6.0 ± 0.1) to a final concentration of 10^8–10^9 CFU/mL viable cells. The effect of various chitosan concentrations was firstly investigated against *L. monocytogenes* LO28 and *E. coli* K12 strains. Therefore, different chitosan concentrations (0.01 to 0.2%) were added to the cell suspensions in ACES buffer which had their pH adjusted to 6.0. Moreover, the acetic acid concentration was adjusted for all concentrations to 0.1 or 0.2% (according to the highest) to clearly assess the effect of chitosan and HHP. Bacterial cells without chitosan were used as controls.

2.4. HHP treatment

A fixed volume of 1 mL of each culture/chitosan solution was transferred into sterile plastic stomacher bags (Seward, London, UK) with dimensions of 3.5 cm × 4 cm. The bags were double sealed before HPP exposure to prevent cross contamination. Subsequently, the bags were kept at room temperature for 1 h and then submerged in the pressure transmitting medium (20% v/v castor oil in ethanol) of the HHP equipment (Stansted Fluid Power Products Ltd., Harlow, UK) in a vessel with 1.8 cm internal diameter and 29 mL working volume. Pressures of 200 and 300 MPa for 10 min at 20 ± 2 °C were used to investigate the combined effect of HHP and chitosan against *L. monocytogenes* LO28 and *E. coli* K12 which served as reference strains for each species. Furthermore, experiments combining HHP and chitosan against *L. monocytogenes* LO28 were carried out in higher process temperature (35 ± 3 °C). In these experiments, after the chitosan application, the bags were kept at 37 °C for 1 h before being subjected to a pressure of 200 MPa for 10 min. The temperature of the pressure fluid was adjusted at 35 °C to achieve a process temperature at 35 ± 3 °C. The pressure come-up time was 1–2 min depending on the pressure while decompression was instant (2–3 s). The temperature of the chamber in the HHP machine was monitored during the treatment by a digital thermometer (PCE-T 390, PCE Instruments, Southampton, UK) connected to a K-type thermocouple which was in contact with the pressurization liquid.

2.5. L. monocytogenes strain variability in HHP and chitosan combination

Two piezotolerant (L6, F2365), two intermediate (LO28, FBR13) and one piezosensitive (NCTC 10357) *L. monocytogenes* strains were selected (Table 1) to investigate the behavior of different strains and their tolerance profile to the HHP – chitosan combination. One HHP condition (250 MPa), one chitosan concentration (0.1%) and one process temperature (20 °C) were selected as the most appropriate conditions to study the combined/synergistic effect and assess the potential variability between the strains. Bacterial cells without chitosan were always used as control.

2.6. Enumeration of viable cells

Enumeration of viable cells was carried out before and after the HHP treatment to accurately assess the synergistic effect. More specifically, the HHP-treated, the chitosan-treated, and the untreated samples were aseptically opened and serially diluted in Maximum Recovery Diluent (MRD; FisherScientific, UK) with spot or spread plating of appropriate dilutions onto BHI agar for *L. monocytogenes* and LB agar for *E. coli*. Moreover, to quantify the initial population, the untreated cultures were serially diluted and spot/spread plated as previously described. The lower detection limit for this method of enumeration was 2.5 log CFU/mL. The plates were incubated at 37 °C for 48 h. All experiments were carried out in three independent biological replicates with one technical replicate for each treatment.

2.7. Synergistic effect calculation

The synergistic effect between HHP and chitosan was defined as the effect without considering the reduction due to chitosan and due to pressure alone (Eq. 1).

$$\text{Synergistic effect} = log\,(N_{combined}) - [log\,(N_{HHP}) + log\,(N_{chitosan})] \quad (1)$$

where:

- log ($N_{combined}$) = Reduction due to HHP and chitosan combination.
- log (N_{HHP}) = Reduction due to HHP alone without the chitosan effect.
- log ($N_{chitosan}$) = Reduction due to the effect of chitosan alone.

2.8. Statistical analysis

Statistical comparisons among different chitosan concentrations were conducted using Tukey's Honestly Significant Difference (HSD) test as a post-hoc analysis following the analysis of variance (ANOVA). Unpaired two-tailed *t*-tests were performed to compare the combined effect with the individual effects of chitosan or HHP at each concentration. A probability value of <0.05 (*P-value* < 0.05) was considered statistically significant for comparisons between variables. Furthermore, to confirm the existence of the synergistic effect, unpaired *t*-tests with 0 (zero) were conducted with a significance level of 0.05 (*P-value* < 0.05). All statistical analyses were performed using GraphPad Prism (version 10.2.2) software.

3. Results

3.1. The effect of HHP and chitosan on L. monocytogenes LO28

The combined effect of HHP (200 MPa) and chitosan (0.02 to 0.2%) immediately after application ranged between 0.88 and 1.1 log reduction at process temperature of 20 °C without any statistically significant difference between the concentrations ($p < 0.05$; Fig. 1a). Pressure up to 200 MPa for 10 min had no effect (0.03 log reduction) on LO28 cells without chitosan (Fig. 1a; pattern filled bar). >80% of the entire reduction in any concentration was due to chitosan indicating that it was more effective than HHP at pressure of 200 MPa. No statistically significant synergism was observed at 200 MPa and room temperature (20 °C) for all chitosan concentrations (*t*-test; $p < 0.05$; Fig. 2; **Suppl. Table ST2**).

The combined effect of HHP (200 MPa) and chitosan in higher process temperature (35 °C) was also investigated (Fig. 1b). Pressure at 200 MPa / 35 °C had no effect (0.19 ± 0.11 log reduction) to LO28 cells without chitosan similarly to experiments in room temperature (**Suppl. Table ST1**). Temperature (35 ± 3 °C) alone did not affect the bacterial cells (**Suppl. Table ST7**) indicating that any potential reduction was due to HHP, chitosan or the combination. Chitosan reduced the number of viable *L. monocytogenes* cells by about 0.7 and 1.1 log units depending on the concentration (0.02–0.2%), during the incubation of samples for 1 h at 37 °C (Fig. 1b). However, combined HHP and chitosan resulted in 1.2 to 1.6 log reduction while the highest was noticed at 0.1%, 0.15% and 0.2% chitosan (Fig. 1b). This reduction at 35 °C was significantly higher ($p < 0.05$) compared to that of the same experiment carried out at ambient temperature (**Suppl. Table ST1**). Finally, the reduction due to the combined effect was statistically significantly higher than the reduction due to chitosan alone in all concentrations ($p < 0.05$) indicating that the combined treatment was much more effective (Fig. 1b). Synergistic effect (Eq. 1) was observed (*t*-test; $p < 0.05$) in all chitosan concentrations combining HHP (200 MPa/10 min) and chitosan at process temperature of 35 °C while the highest (0.71 log reduction) was

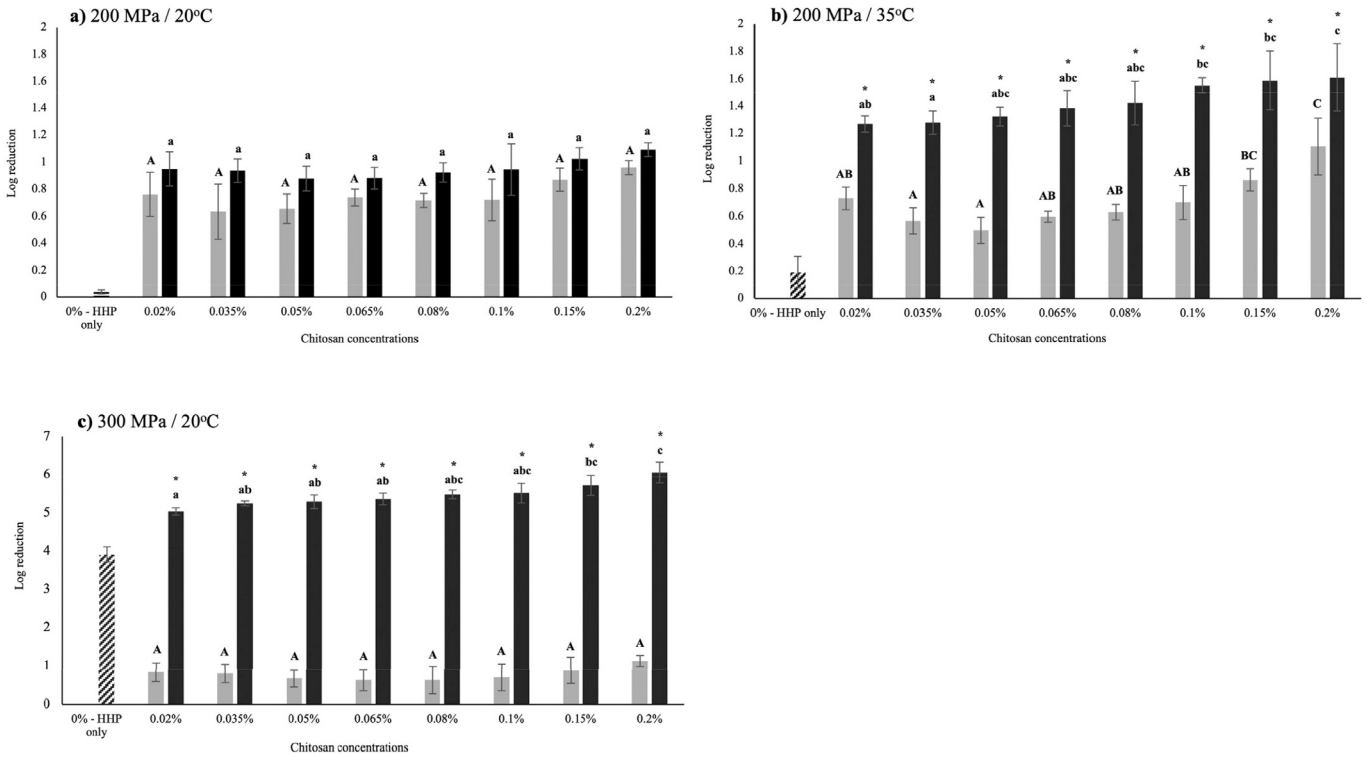

Fig. 1. Effect of chitosan (0.02–0.2%) alone (**grey bars**), HHP alone (**pattern fill**; 0% - HHP only) and combination of chitosan with HHP (**black bars**) against overnight stationary phase *L. monocytogenes* cells (LO28) at three processing conditions **a)** 200 MPa / 20 °C / 10 min **b)** 200 MPa / 35 °C / 10 min **c)** 300 MPa / 20 °C / 10 min. The cultures were centrifuged and resuspended in ACES buffer. All concentrations had similar pH = 6 and acetic acid concentration (0.2%). (a-c) and (A-C) indicate statistically significant differences between the chitosan concentrations regarding the effect of HHP and chitosan combined (black bars) and the effect of chitosan alone (grey bars) respectively. Bars that do not share a letter are significantly different (Tukey HSD test; $p < 0.05$). Asterisk (*) indicates if the combined treatment HHP + chitosan (black bar) was significantly higher than the chitosan effect alone (grey bar) at each concentration (*t*-test; $p < 0.05$). Values are means of three independent biological replicates while error bars indicate the standard deviation ($n = 3$).

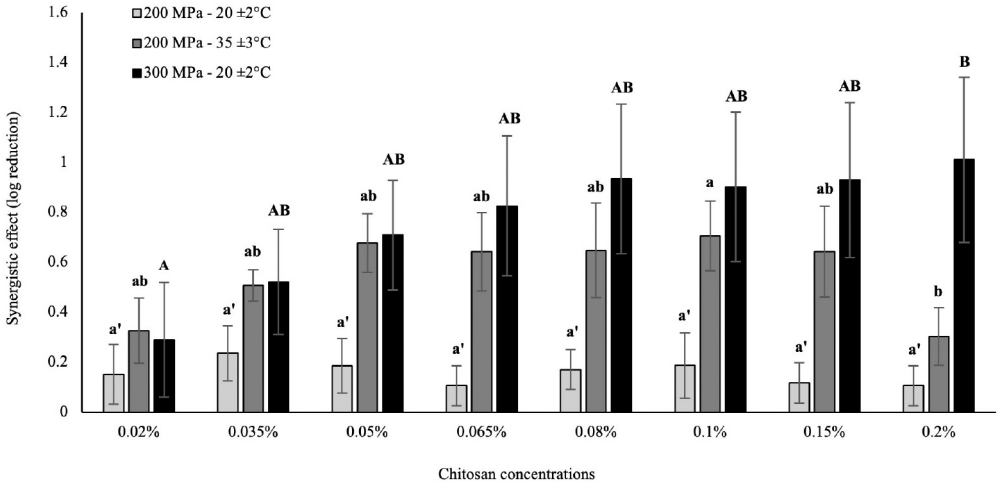

Fig. 2. Synergistic effect of HHP and chitosan (0.02–0.2%) at 200 MPa / 20 °C / 10 min (**light grey bars**), 200 MPa / 35 °C / 10 min (**grey bars**) and 300 MPa / 20 °C / 10 min (**black bars**). (a'-b'), (a-b) and (A-B) indicate statistically significant differences in the synergistic effect between the chitosan concentrations at 200 MPa / 20 °C (light grey bars), 200 MPa / 35 °C (grey bars), 300 MPa / 20 °C (black bars) respectively. Bars that do not share a letter are statistically different (Tukey HSD test; $p < 0.05$). Values are means of three independent biological replicates while error bars represent standard deviation ($n = 3$).

noticed at 0.1% chitosan (Fig. 2).

Furthermore, the combination of HHP (300 MPa/10 min) with a range of chitosan concentrations from 0.02 to 0.2% was studied at ambient process temperature to assess if the higher pressure increases the synergistic effect (Fig. 1c & 2). In this case, pressure at 300 MPa for 10 min had high effect (3.9 log reduction) to LO28 cells without chitosan (Fig. 1c; pattern fill). By combining HHP and chitosan, 5 to 6 log reduction was achieved depending on the concentration (Fig. 1c). The higher pressure (300 MPa) was considered as the most dominant parameter influencing the inactivation (>70% of the entire reduction was due to the pressure effect) compared to the lower pressure (200 MPa) where chitosan had the greater impact. No statistically significant

differences were observed for the combined treatment (HHP + chitosan) between the chitosan concentrations apart from that of 0.2% which elicited a significantly higher log reduction than those of 0.02%, 0.035%, 0.05% and 0.065% ($p < 0.05$). The combined effect (HHP + chitosan) was always significantly higher than the effect of pressure alone (HHP) regardless the concentration (*t*-test; $p < 0.05$; Fig. 1c).

Regarding the potential synergism between HHP at 300 MPa and chitosan (Fig. 2), namely the effect without considering the reduction due to chitosan and due to pressure alone (Eq. 1), statistically significant (*t*-test; $p < 0.05$) synergistic action was observed in all concentrations except for 0.02%. While no statistically significant differences were found among the other concentrations ($p < 0.05$), the highest level of synergism was observed at 0.2% (1.01 ± 0.33 log reduction). Comparing the synergistic effect expressed in log reduction (Fig. 2; **Suppl. Table ST5**), it was clearly observed that the treatment at 200 MPa and 20 °C of process temperature was the least effective against *L. monocytogenes* LO28 as there was no synergism at any chitosan concentration (t-test; $p < 0.05$). No statistically significant differences were observed between the other two treatments (200 MPa / 35 °C and 300 MPa / 20 °C) at any chitosan concentration except for 0.2% which was significantly higher at HHP (300 MPa) and ambient process temperature (20 °C). Moreover, from Fig. 2, it was observed that increasing the pressure at the same process temperature (20 ± 2 °C), the synergistic effect increased. Additionally, increasing the temperature at 200 MPa, the synergism also increased. In contrast, the synergism was not significantly increased increasing the chitosan concentration in each treatment.

3.2. Combined effect of HHP and chitosan against five L. monocytogenes strains

Since a synergistic effect was predominantly observed in *L. monocytogenes* LO28, further investigation was undertaken to determine its correlation with HHP tolerance. Consequently, the effect of combined HHP and chitosan was assessed on five *L. monocytogenes* strains, including two piezotolerant (L6, F2365), one piezosensitive (NCTC 10357), and two intermediate (FBR13, LO28) strains (Fig. 3). Based on preliminary experiments (data not shown), pressure at 250 MPa/10 min/20 °C and 0.1% chitosan were selected as the appropriate conditions to assess the potential variability between strains as at 200 MPa the more piezotolerant strains were not affected, while at 300 MPa the more sensitive strains were completely inactivated. Variation in the reduction between strains was observed due to the combined effect with most resistant being the L6 and F2365 (0.77 and 0.9 log reduction respectively) while NCTC 10357 the most sensitive (3.7 log reduction; Fig. 3). There were no statistically significant differences between the strains regarding the reduction elicited by 0.1% chitosan ($p < 0.05$). Moreover, the combined effect was significantly higher than the effect of chitosan and HHP alone only in FBR13, LO28 and NCTC 10357 ($p < 0.05$). Finally, regarding the observed synergism (Fig. 4), the highest synergistic effect was observed on FBR13 (1.22 log reduction) while no statistically significant differences ($p < 0.05$) were noted between the other strains.

3.3. The effect of HHP and chitosan on Escherichia coli K12 wild type

The combined effect of HHP (200 MPa/10 min/20 °C) and chitosan

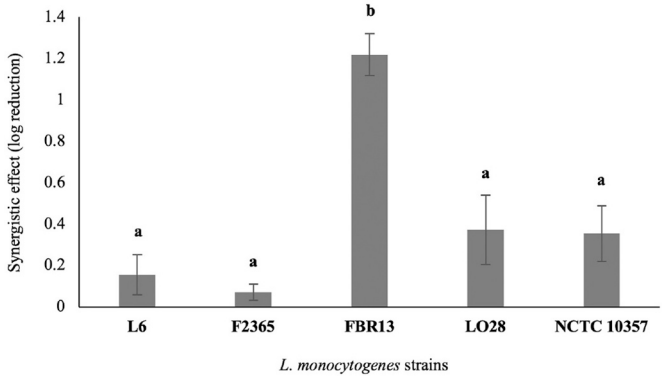

Fig. 4. Synergistic effect of combined HHP (250 MPa/10 min/20 °C) and chitosan (0.1%) against five *L. monocytogenes* strains. Lowercase letters (a-b) indicate statistically significant differences between the strains (Tukey HSD test; $p < 0.05$). Bars that do not share a letter are significantly different. Values are means of three independent biological replicates while error bars represent the standard deviation (n = 3).

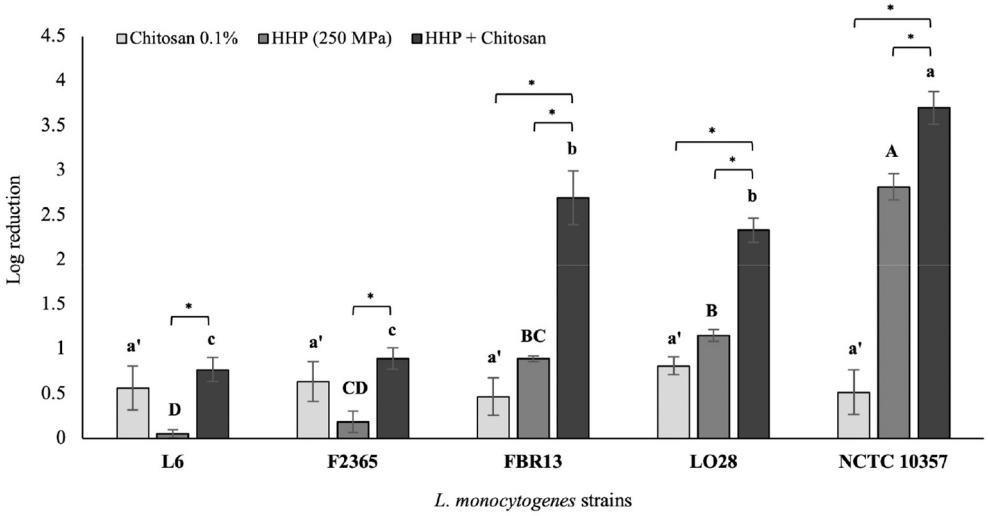

Fig. 3. Effect of 0.1% chitosan alone (**light grey bars**), HHP at 250 MPa/10 min/20 °C alone (**grey bars**) and combination of chitosan (0.1%) and HHP (250 MPa; **black bars**) on the inactivation of 5 WT *L. monocytogenes* strains. The cultures were centrifuged and resuspended in ACES buffer. All concentrations had similar pH = 6 and acetic acid concentration (0.1%). (a-c), (A-C) and (a'-c') indicate statistically significant differences between the strains regarding the effect of HHP and chitosan (black bars), the effect of HHP (250 MPa) alone (grey bars) and the effect of chitosan alone (light grey bars) respectively. Bars that do not share a letter are significantly different (Tukey HSD test; $p < 0.05$). An asterisk (*) indicates if the combined treatment is significantly higher than the chitosan or the HHP effect alone (*t*-test; $p < 0.05$). Values are means of three independent biological replicates while error bars indicate the standard deviation ($n = 3$).

(0.01 to 0.1%) against *E. coli* K12 in ACES buffer was investigated to explore the potential synergism on a Gram-negative representative. More specifically, the combined effect ranged between 2.78 and 4.34 log units depending on the concentration (Fig. 5). Pressure up to 200 MPa for 10 min reduced the number of *E. coli* cells without chitosan by 1.15 log CFU/mL (Fig. 5; pattern filled bar). Chitosan alone reduced the number of viable *E. coli* cells by about 1.8 to 2.1 log CFU/mL (Fig. 5; grey bars) with no statistically significant differences between the concentrations after 1 h application ($p < 0.05$). The log reduction due to the HHP and chitosan combination was significantly higher than the reduction due to chitosan or the pressure alone in all concentrations ($p < 0.05$) indicating that the combination was always more effective (**Suppl. Table ST6**). Furthermore, the combined effect of chitosan and HHP at 300 MPa was assessed to investigate if the higher pressure increases the synergistic effect. However, the reduction was below the detection limit (2.5 log CFU/mL) in all concentrations (**Suppl. Table ST6**) while 4.04 ± 0.25 log reduction was achieved only with HHP treatment. Consequently, it was not possible to accurately determine the synergistic effect between HHP and chitosan at 300 MPa against *E. coli*.

Regarding the synergistic effect (Eq. 1) when combining HHP (200 MPa) and chitosan, no statistically significant (*t*-test; $p < 0.05$) synergism was observed in all concentrations except for 0.1% chitosan (1.01 log reduction; Fig. 6). The level of synergistic action at 0.1% of chitosan was statistically significant different from 0.01%, 0.02% and 0.05% (Tukey HSD test; $p < 0.05$).

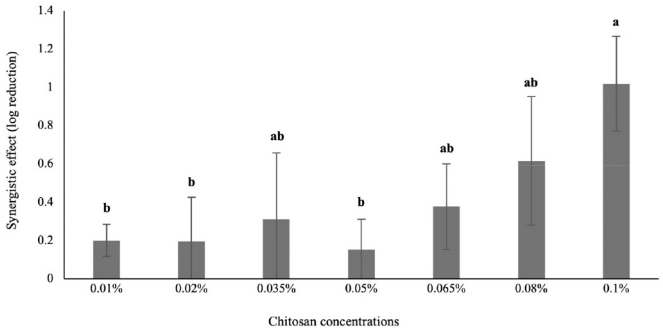

Fig. 6. Synergistic effect (eq. 1) of combined HHP (200 MPa/10 min/20 °C) and chitosan (0.01% - 0.1%) against overnight stationary phase *Escherichia coli* K12 cells. a-c: indicate the statistically significant differences in the synergistic effect between the chitosan concentrations (Tukey HSD test; $p < 0.05$). Bars that do not share a letter are significantly different. Values are means of three independent biological replicates while error bars indicate the standard deviation (n = 3).

4. Discussion

In the present study, the inactivation of the combined HHP (200 MPa) and a range of chitosan concentrations (0.02 to 0.2%) on *L. monocytogenes* LO28 in ACES buffer was initially investigated. Pressure at 200 MPa for 10 min without chitosan had no effect on LO28 cells while 3.9 log reduction was achieved at 300 MPa (Fig. 1). Pressure of 200 MPa for 10 min is considered very low to cause any substantial reduction on the *L. monocytogenes* cell numbers (Karatzas et al., 2001; Karatzas & Bennik, 2002). Pressure higher than 300 MPa was required to achieve a considerable reduction in ACES buffer in this study (>3–4 log CFU/mL). As a consequence, chitosan was more effective than HHP at 200 MPa and vice versa at 300 MPa (Fig. 1). Absence of any synergistic effect was noticed at 200 MPa in all chitosan concentrations at ambient process temperature (Fig. 2; $p < 0.05$). This is also supported by Papineau et al. (1991) where no synergy was found between the activity of chitosan and HHP (238 MPa) against Gram-positive *Staphylococcus aureus*.

However, it has been reported that temperature plays a significant role on chitosan antimicrobial activity (Goy et al., 2009; No et al., 2002). More specifically, Tsai and Su (1999) found that within the range of 4 to 37 °C, chitosan's bactericidal activity against *E. coli* increased with increasing temperature. Subsequently, the combined effect of HHP and chitosan at 200 MPa at higher process temperature (35 °C) was investigated (Fig. 1b). In contrast to 20 °C, combined HHP and chitosan resulted in higher reduction while synergistic effect was observed (0.3 to 0.7 log reduction) with all chitosan concentrations (Fig. 1b and 2). According to Tsai and Su (1999), temperature is a significant factor for the antimicrobial activity influencing the structure of cell surface enhancing the damage to the already-affected by HHP cell structural organization (Aganovic et al., 2021). Thus, an enhanced destructive activity against *L. monocytogenes* cells could be considered as synergistic. Moreover, chitosan's chemical reaction rate is increased during the HHP process

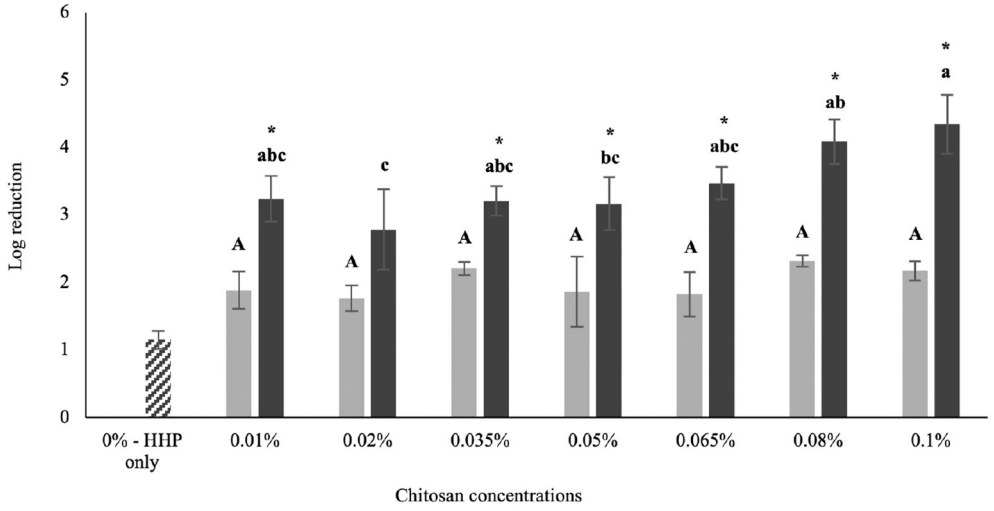

Fig. 5. Effect of chitosan (0.01–0.1%) alone (**grey bars**), HHP alone (**pattern fill**; 0% - HHP only) and combination of chitosan with HHP at 200 MPa/10 min/20 °C (**black bars**) against overnight stationary phase *Escherichia coli* K12 cells. The cultures were centrifuged and resuspended in ACES buffer. All concentrations had similar pH = 6 and acetic acid concentration (0.1%). (a-c) and (A-C) indicate statistically significant differences between the chitosan concentrations regarding the effect of HHP and chitosan combined (black bars) and the effect of chitosan alone (grey bars) respectively. Bars that do not share a letter are significantly different (Tukey HSD test; $p < 0.05$). Asterisk (*) indicates if the combined treatment HHP + chitosan (black bar) is significantly higher than the chitosan effect alone (grey bar) at each concentration (*t*-test; $p < 0.05$). Values are means of three independent biological replicates while error bars indicate the standard deviation ($n = 3$).

due to the higher temperature resulting in higher antimicrobial effect. These are possible explanations for the observed synergistic effect between HHP and chitosan. However, no literature data exist regarding the combined and synergistic effect of HHP and chitosan at a process temperature of 35 °C; this is the first-time reported instance of synergism under these conditions. Furthermore, looking only at the chitosan effect after 1 h incubation (Fig. 1; grey bars; **Suppl. Table ST3**), higher temperature (35 ± 3 °C) did not affect the chitosan antimicrobial activity. Therefore, there was not any statistically significant ($p < 0.05$) additional reduction on LO28 cells in any chitosan concentration compared to the room temperature incubation (**Suppl. Table ST3**). According to Tsai and Su (1999) >2–3 h are required to have an effect of temperature on the antimicrobial activity supporting our results. Also, Malinowska-Panczyk, et al. (2009) reported a minimal reduction of *Staphyloccocus aureus* after 1 h incubation at 37 °C with 0.2% concentration of chitosan.

In addition, by increasing the pressure (300 MPa), the synergistic effect also increased compared to 200 MPa. More specifically, a synergistic action (0.25 to 1 log reduction) was observed when HHP (300 MPa/20 °C) in combination with chitosan (0.02–0.2%) was applied (Fig. 1c and 2). In this case, the main parameter seems to be the higher pressure which enhances the membrane cell permeability, (compared to 200 MPa) increasing the antimicrobial activity of chitosan. As a consequence, a leakage of intracellular constituents occurs which leads to cell inactivation. The increase of chitosan charge (thus enhanced antimicrobial activity) resulting from the reduction in pH of the medium could not be a reason for synergism. The reason for the latter is related with the use of ACES buffer [N-(2-acetamido)-2-aminoethanesulfonic acid] which was selected as resuspension medium of the microbial cells because it has the ability to maintain the pH stable during the HHP processing (Karatzas & Bennik, 2002; Smelt & Hellemons, 1998). However, no literature data are available regarding the HHP and chitosan combination at 300 MPa against *L. monocytogenes*.

At 300 MPa, *L. monocytogenes* LO28 cells were effectively reduced by any chitosan concentration higher than 0.035%. Consequently, changing the chitosan concentration did not have a major impact on the inactivation and the synergistic effect while changing the processing conditions (pressure and temperature) seemed to have a significant impact. Surprisingly, similar synergistic action with 300 MPa could be achieved in lower pressure (200 MPa) increasing the process temperature up to 35 °C indicating for first time that temperature plays a significant role on the synergistic effect, as discussed above.

Furthermore, two piezotolerant (L6, F2365), two intermediate (LO28, FBR13) and one piezosensitive (NCTC 10357) *L. monocytogenes* strains were selected based on previous work by Tsagkaropoulou & Karatzas, 2024, to assess whether the combined effect or synergism are linked to the tolerance to HHP. Subsequently, their response against the combined effect of HHP at 250 MPa and 0.1% chitosan (Fig. 3) was studied. L6 and F2365 showed the highest resistance to the combined effect while NCTC 10357 was the most sensitive. The same resistant profile was also noticed regarding the tolerance to HHP alone at 250 MPa, which is in line with the findings of Tsagkaropoulou & Karatzas, 2024. In addition to this, considering that the majority of chitosan concentrations had overall the same effect in all strains without statistically significant differences, HHP seems to be the main determinant for the variability between the strains against the combined effect.

As far as it concerns the synergistic effect, the highest synergism was observed on FBR13 (1.21 log reduction; Fig. 4). Interestingly, FBR13 was not the most sensitive strain to HHP or to chitosan. A possible explanation could be the differences in the cell surface properties such as thickness or surface molecules which under pressure conditions can affect the membrane permeability of chitosan as previously described (Malinowska-Panczyk et al., 2009; Yang et al., 2021). Various studies have reported variability between *L. monocytogenes* strains in the cell surface characteristics which are related to the serotype, genotype or the stress responses (Giovannacci et al., 2000; Meylheuc et al., 2002; Severino et al., 2007). However, to the authors' knowledge, apart from the present study there is no other research demonstrating variations either in the synergistic or the combined effect of chitosan and HHP among strains of *L. monocytogenes*. In further work, the behavior of a larger number of strains will be investigated regarding this synergistic effect and the role of temperature. Furthermore, there is a lack of research studies on the response of different WT *L. monocytogenes* strains against chitosan. Ye, Neetoo, and Chen (2008) reported variability between 12 strains of *L. monocytogenes* when treated with low molecular weight chitosan in the form of coated plastic film for 24 and 48 h. Subsequently, only 1 h exposure to 0.1% chitosan was not sufficient to show any differences between the strains. However, it is well documented that the resistance to various antimicrobial factors, such as nisin, differs among different strains of *L. monocytogenes* (Harris et al., 1991; Prazak et al., 2002; Ukuku & Shelef, 1997).

Finally, the effect of HHP (200 & 300 MPa/10 min/20 °C) and chitosan (0.01 to 0.1%) against WT *Escherichia coli* K12 in ACES buffer was investigated to explore the potential synergistic effect on a Gram-negative representative. As observed in *L. monocytogenes* LO28, no statistically significant differences in the chitosan effect between the different concentrations were noticed (Fig. 5; grey bars; **Suppl. Table ST5**). Moreover, in contrary to other antimicrobials such as nisin (Boziaris & Adams, 1999; de Arauz, et al., 2009; Helander & Mattila-Sandholm, 2000; Mok et al., 2020), low molecular weight chitosan seems to be effective against *E. coli* K12 reducing the cells by 2 ± 0.31 log units, on average, for all concentrations (Fig. 5; grey bars). Statistically significant ($p < 0.05$) synergistic effect was observed only at 0.1% chitosan concentration (Fig. 6; 1.01 ± 0.22 log reduction). Papineau et al. (1991) reported no synergy between chitosan (0.02%) and HHP at 238 MPa against *E. coli* V517. However, lower chitosan concentrations seem to be insufficient to induce synergism between HHP and chitosan (Fig. 6). No literature data exist about higher concentrations combined with HHP against *E. coli*.

Comparing the combined effect of HHP (200 MPa/10 min/20 °C) and chitosan with *L. monocytogenes* LO28, *E. coli* K12 was found to be more sensitive at all chitosan concentrations. This can be explained from the observed higher resistance of LO28 to HHP and chitosan alone. Less than 0.02 log reduction was achieved at 200 MPa against LO28 (Fig. 1a) while *E. coli* cells were reduced by >1 log CFU/mL at the same conditions (Fig. 5). Various studies have reported that Gram-positive bacteria are often more resistant to HHP than the Gram-negative ones (Ariefdjohan et al., 2004; Wuytack, Diels, & Michiels, 2002) due to probably stronger and thicker cell wall or reduced membrane fluidity (Macdonald, 2001). Moreover, the antimicrobial effectiveness of chitosan was greater against *E. coli* than *L. monocytogenes*. Less than 1 log reduction was observed against LO28 due to chitosan effect at all concentrations. In contrast, chitosan reduced the levels of *E. coli* cells from 1.8 to 2.34 log units depending on the concentration (**Suppl. Table ST4**). Higher sensitivity of *E. coli* was also reported by Wang (1992) and Ibañez-Peinado et al. (2020). According to Chung et al. (2004) higher negative charge on the surface of Gram-negative bacteria can enhance the antimicrobial activity of chitosan.

5. Conclusions

This study presents the significance of combining HHP with natural antimicrobials such as chitosan to control *L. monocytogenes* and *E. coli*. Chitosan is a natural antimicrobial that, when combined with HHP, is able to act synergistically with potential applications in enhancing microbial safety and extending the shelf life of various food products. HHP at 300 MPa (10 min/20 °C) combined with any chitosan concentration (0.035 to 0.2%) seems to be the most effective combination reducing *L. monocytogenes* (LO28) by 5–6 log CFU/mL, while the synergistic effect ranged between 0.52 and 1.01 log reduction. Temperature seemed to play a significant role on the reduction of the chitosan treated cells during the HHP treatment. Similar synergistic action with 300 MPa can be achieved at lower pressure (200 MPa) increasing the process

temperature up to 35 °C. Changing the processing conditions (pressure, temperature) had greater impact on the reduction or synergism than increasing the chitosan concentration. Furthermore, it can be concluded that chitosan is effective against *Escherichia coli* K12, a representative of Gram-negative bacteria. This effectiveness is notable when compared to other natural antimicrobials such as lysozyme and nisin, which have been well-studied against Gram-negative bacteria but are known to be inefficient (Helander & Mattila-Sandholm, 2000; Masschalck and Michiels, 2003). Finally, combining HHP with chitosan was more effective in most conditions than applying the hurdles separately. Consequently, chitosan could be used in combination with HHP as a strategy to enhance the inactivation of *L. monocytogenes* and *E. coli* optimizing the processing conditions. However, further studies will be undertaken in actual food products to assess chitosan's organoleptic impact and the influence in the shelf-life. Additionally, in further work, the mode of action and mechanism of the synergistic effect will be identified.

Funding

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement No. 955431.

CRediT authorship contribution statement

Nikolaos Giannoulis: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Kimon Andreas G. Karatzas:** Writing – review & editing, Supervision, Resources, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Acknowledgments

The authors would like to thank Wageningen University & Research (Wageningen, the Netherlands) for providing the L. *monocytogenes* strains.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ifset.2024.103693.

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