



## Enhancing the safety and shelf life of beef and plant-based burgers by combining High Hydrostatic Pressure (HHP) with nisin or a blueberry-derived product

Nikolaos Giannoulis<sup>a</sup>, Theocharia Tsagkaropoulou<sup>a</sup>, Carolina Bocigas Martin<sup>b</sup>,  
Miriam Ortega-Heras<sup>b</sup>, Mario González-Angulo<sup>c</sup>, Beatriz Melero<sup>b</sup>,  
Kimon Andreas G. Karatzas<sup>a,\*</sup>

<sup>a</sup> Department of Food & Nutritional Sciences, University of Reading, Whiteknights, Reading RG6 6AD, UK

<sup>b</sup> Department of Biotechnology and Food Science, University of Burgos, Plaza Misael Bañuelos, Burgos, Spain

<sup>c</sup> Hiperbaric, S.A., C/Condado de Treviño, 6, 09001 Burgos, Spain

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### ABSTRACT

The growing demand for sustainable and healthy dietary options has led to significant interest in plant-based meat alternatives though traditional meats, such as beef, remain dominant in the protein market. High Hydrostatic Pressure (HHP) stands out as a promising technology improving food safety and extending shelf life, while combining HHP with clean-label additives offers potential for process optimization. This study investigates the synergistic effect of HHP combined with nisin (500 IU/g) or blueberry-derived product (4 %) in beef and plant-based burgers to control *L. monocytogenes* and extend shelf life under slight temperature abuse. In plant-based burgers, HHP (600 MPa, 3 min) combined with additives, effectively delayed *L. monocytogenes* growth for 104 days during storage, outperforming HHP alone. At lower pressures (300–500 MPa), HHP combined with nisin or blueberry product significantly enhanced pathogen reduction in both matrices, achieving a synergistic effect of up to 1.4 log reduction. HHP (600 MPa), with or without the additives, also extended the storage period of non-inoculated plant-based burgers, maintaining the natural microflora below 3 log CFU/g for 83 days. The blueberry product notably influenced the physicochemical properties (e.g. pH, color) of both matrices, while HHP significantly affected the color of beef burgers. This study provides novel insights into the potential applications of HHP combined with natural antimicrobials, highlighting its effectiveness in plant-based meat alternatives and the significant role of the matrix in the synergistic effect. Future research should focus on sensory analysis and consumer acceptance to align these advancements with market demands.

### 1. Introduction

In recent years, there has been a notable increase in interest and development of plant-based meat alternatives, driven by consumer demand for sustainable and health-conscious dietary options (Xiao, Zou, Hu, Zhu, and Wei, 2023; Zhao et al., 2022). Nevertheless, traditional meats, such as beef, pork, and poultry, remain dominant in the protein market due to their high nutritional value offering higher protein levels and better bioavailability of essential nutrients like iron and zinc (Falowo, 2021; Williams, 2007). Additionally, cultural traditions, dietary habits, and the sensory attributes of conventional meat, such as taste and flavor, further contribute to their consumer preference

(Daszkiewicz, Florek, Wodzak, Kubiak and Burczyk, 2023; Lee et al., 2023). Both plant-based and traditional meat products encounter significant food safety challenges. Beef burgers, being animal-derived, are rich in nutrients such as proteins and lipids with high levels of moisture content and water activity, all of which provide an ideal environment for the growth of spoilage microorganisms and pathogens, including *Listeria monocytogenes*. Plant-based meat analogues, typically made from ingredients such as legumes, grains, and oils, create a different set of challenges for food safety and shelf life. Their unique physicochemical characteristics, such as higher pH levels and specific water activity profiles, along with the vegetable nature of the constituents, may influence the presence and behavior of spoilage and pathogenic

\* 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](mailto:k.karatzas@reading.ac.uk) (K.A.G. Karatzas).

microorganisms, which require appropriate control (Liu et al., 2023). Although burger patties are typically cooked prior to consumption, the control of *L. monocytogenes* in raw meat and plant-based products remains important due to the risk of cross-contamination with kitchen utensils or undercooking. Furthermore, the significant extension of shelf life provided by processes such as HHP requires the control of hazards that may grow during prolonged refrigerated storage, (e.g. *L. monocytogenes*) to ensure that it does not reach high levels at the time of consumption.

Due to the growing demand for safe, sustainable, and clean-label food options, there has been considerable research focused on innovative preservation techniques for both animal and plant-based food matrices. High Hydrostatic Pressure (HHP) has emerged as a promising non-thermal food preservation method that effectively enhances food safety and extends shelf life while maintaining the sensory and nutritional qualities of the products (Buzrul, 2015; Wang, Huang, Hsu, and Yang, 2016). By applying pressures within the industrially relevant range of 200–600 MPa, HHP is able to inactivate pathogenic and spoilage microorganisms without the adverse effects associated with traditional thermal processing methods (Aganovic et al., 2021; Sánchez-Basurto et al., 2012; Wang et al., 2016). Common commercial applications of HHP include the preservation of fruit juices, ready-to-eat meals, and meat products, where it helps maintaining the physicochemical characteristics and nutritional value (Bermúdez-Aguirre and Barbosa-Cánovas, 2011; Wang, Dekker, Heising, Zhao, and Fogliano, 2024). However, despite its advantages, the implementation of HHP technology is often limited by high operational costs, including the initial investment in specialized equipment and ongoing energy expenses (Rastogi, Raghavarao, Balasubramaniam, Niranjana and Knorr, 2007; Rendueles et al., 2011). To address these limitations, it is crucial to explore innovative strategies aiming to reduce these costs by extending the shelf life of the HHP-treated products, or even reducing the pressure intensity required for effective treatment. Thus, the efficiency of HHP technology can be improved making it suitable for a wider variety of products, including plant-based meat alternatives which are considered as ultra-processed (Martín-Miguel et al., 2024). Importantly, since HHP is applied to products in their final packaging, this process eliminates the need for conventional antimicrobial additives typically used to delay microbial growth, enabling their replacement with clean-label alternatives. Consequently, combining HHP with additional hurdles, such as natural antimicrobial compounds derived from plants and bacteriocins (e.g., nisin, pediocin), emerges as a promising strategy to develop healthier, minimally processed plant-based products with extended shelf life and enhanced safety.

Nisin, a natural antimicrobial peptide produced by *Lactococcus lactis*, has gained significant interest as an effective antimicrobial agent in food preservation. According to Commission Regulation (EU) No 1129/2011 (EU, 2011) and U.S. Food and Drug Administration (GRAS Notice ID: 65), it is one of the few natural antimicrobials approved for direct use in food products. Additionally, nisin demonstrates strong antimicrobial activity against a wide range of Gram-positive bacteria making it a valuable tool for enhancing the safety and shelf life of various food products (Benkerroum and Sandine, 1988; De Martinis, Alves, and Franco, 2002). Its mechanism of action involves binding to lipid II, a key component of bacterial cell wall synthesis, leading to pore formation and subsequent cell lysis (Simons, Alhanout, and Duval, 2020). Various studies have demonstrated that nisin can effectively reduce *L. monocytogenes* populations in different food matrices, including meat, fish and plant-based products (Gharsallaoui, Oulahal, Joly and Degraeve, 2016; Hara et al., 2009). Mohamed, Elnawawi, and Yousef (2011) and Dai et al. (2022) demonstrated the effectiveness of nisin in reducing *L. monocytogenes* counts, achieving a 1.2 log reduction in young steers and significantly reducing the pathogen in plant-based meat products respectively, highlighting its potential as a natural preservative to enhance food safety.

Natural products obtained from plants have emerged as promising

natural antimicrobial agents, offering an effective alternative to synthetic preservatives in food preservation. These extracts contain a variety of bioactive compounds, such as phenolic compounds (flavonoids, anthocyanins, flavan-3-oles) and terpenoids among others, which have demonstrated antimicrobial properties against a range of foodborne pathogens (Das, Islam, Marcone, Warriner and Diarra, 2017; Manandhar, Luitel and Dahal, 2019; Zhou et al., 2020). For example, cranberry extract at a concentration of 7.5 % w/w has been shown to effectively inactivate *E. coli* O157:H7 in ground beef while maintaining the product's taste and sensory qualities (Wu, Qiu, Bushway and Harper, 2008). Among various plant-derived antimicrobials, extracts from blueberry products (e.g. skin, leaves and pomace), have shown great potential due to their rich composition of anthocyanins and other phenolic compounds that enhance their antimicrobial efficacy (Das et al., 2017; Gonçalves et al., 2023; Salaheen, Nguyen, Hewes, and Biswas, 2014). Lacombe, Tadepalli, Hwang and Wu (2013) indicated that blueberry extracts can effectively reduce *E. coli* O157:H7 by damaging the cell membrane, highlighting their antimicrobial potential in food matrices. Furthermore, a study by Shen et al. (2014), showed that four blueberries' cultivars had significant antibacterial effect against *L. monocytogenes* and *S. enteritidis* in broth media. Beyond their antimicrobial properties, blueberry products are increasingly used in the food industry as natural colorants due to their high anthocyanin content (Duan et al., 2022), as well as sources of fiber and nutrients that help improve the nutritional profile of foods through their content of vitamins, sugars, organic acids, and minerals (Huang et al., 2025). They have also been incorporated into edible packaging and pH-indicator films (Griep et al., 2024; Kurek, Garofulić, Bakić, Šcetar and Uzelac, 2018; Singh, Gu, Castellarin, Kitts and Pratap-Singh, 2020). These findings highlight the growing use of blueberry extracts in food preservation and processing, particularly as part of clean-label strategies that align with consumer demand for natural and minimally processed products.

Nowadays, the concept of hurdle technology (Leistner, 1995) has become a key approach of modern food preservation, taking advantage of the combined and synergistic effect of multiple preservation methods to enhance microbial safety, extend shelf life, and maintain the sensory and nutritional qualities of food products. Promising results have been demonstrated in studies combining HHP with natural antimicrobials in both growth media and food matrices (Gayán, Torres and Paredes-Sabja, 2012; Oner, 2020; Pokhrel et al., 2019). For example, Mizi et al. (2019) reported that combining HHP with sage extract enhanced the antimicrobial and antioxidant properties of beef burgers, significantly reducing *L. monocytogenes* counts and extending shelf life during chilled storage. Additionally, nisin has shown notable efficacy when combined with HHP at 600 MPa, achieving a 4-log reduction of *L. monocytogenes* in sliced dry-cured Iberian ham (Martillanes et al., 2021). Despite these findings, limited research has explored the combination of HHP with powdered products derived from blueberry or nisin, particularly in beef and plant-based matrices. Therefore, this study aims to investigate the combined effect of HHP (600 MPa, 3 min) and nisin or blueberry product in both beef and plant-based burgers. The primary objective is to assess whether these combinations can enhance food safety by controlling *L. monocytogenes*, a pathogen recognized for its high resistance to HHP, while extending shelf life during storage under slight temperature abuse conditions (7 °C). Additionally, this study explores the potential for achieving effective *L. monocytogenes* reduction at lower pressures (300–500 MPa) by combining HHP with these natural antimicrobials.

## 2. Materials and methods

### 2.1. Bacterial strains and inoculum preparation

Five strains of *Listeria monocytogenes* available at the Department of Food & Nutritional Sciences (University of Reading) strain collection and kindly provided by the Wageningen University & Research

(Wageningen, the Netherlands) were used in this study (Suppl. Table ST1). These strains were selected based on their tolerance to HHP: L6 and F2365 were classified as piezotolerant, LO28 and FBR13 as intermediate, and NCTC 10357 as piezosensitive, according to Tsagkaropoulou and Karatzas (2024). Frozen stock cultures were prepared from overnight cultures grown in Brain Heart Infusion broth (BHI; NCM0016A, Neogen, Lancashire, UK), supplemented with 20 % glycerol, and stored at  $-80^{\circ}\text{C}$ .

Prior to each experiment, the strains were revived by streaking from frozen stock onto Brain Heart Infusion agar plates (BHI; NCM0080A, Neogen, Lancashire, UK). After overnight incubation at  $37^{\circ}\text{C}$ , a single colony from each strain was selected and inoculated into separate glass tubes containing 10 mL of BHI broth. The cultures were incubated at  $37^{\circ}\text{C}$  for 16–18 h. The overnight cultures were centrifuged at  $6975 \times g$  for 10 min at  $4^{\circ}\text{C}$  in a 5810R Eppendorf centrifuge, and the harvested cells were resuspended in sterile  $\text{H}_2\text{O}$  to achieve a final concentration of  $10^8$  to  $10^9$  CFU/mL. Finally, equal volumes of each individual strain suspensions were mixed to create a five-strain cocktail for inoculation.

## 2.2. Preparation of beef and plant-based matrices

A plant-based meat analogue made from pea protein (17 % w/w) was kindly provided by a local food manufacturer, with organic acids excluded from the original recipe to prevent any potential interference. The formulation also included olive oil (7 %), cocoa butter, rice (5.5 %), methylcellulose as a thickener, and a vegetable concentrate (Suppl. Table ST2). The fat content of the beef was determined by Soxhlet extraction using petroleum ether (VWR, Pennsylvania, USA) as the solvent in a Buchi B-811 extraction system (Buchi, Flawil, Switzerland) and was found to be  $6.62 \pm 0.42$  %.

For each protein source, three batches were prepared: one with nisin, one with blueberry powder, and one control without additives. Each batch was thoroughly mixed after the addition of the respective additive.

### 2.2.1. Nisin application

Nisin, in the form of Nisaplin<sup>®</sup> (containing 2.5 % nisin; distributed by International Flavors & Fragrances, UK), was applied to the first batch. To achieve a nisin concentration of 500 IU/g (0.0125 mg/g), 50 mg of Nisaplin per 100 g of product was added to both the plant-based and beef samples. Sterile water (1 mL per 100 g of product) was used as the dispersive medium to ensure uniform distribution of the additive within the food matrix. This concentration (0.0125 mg/g) corresponds to the maximum permitted level in the European Union under Commission Regulation (EU) No 1129/2011 (EU, 2011) and was identified as the most effective concentration based on preliminary work (data not shown).

### 2.2.2. Blueberry product application

The blueberry product was obtained from blueberry juice processing by-products. These residues were supplied by a company based in Spain specialized in the production of juices, jams, honeys, and liqueurs from various fruits, including wild blueberries (*Vaccinium myrtillus*). The juice was extracted by pressing the berries, and the resulting pomace, composed of skins and seeds, was stored frozen at  $-20^{\circ}\text{C}$  until further processing. For preparation, the thawed pomace was dried in an oven at  $60^{\circ}\text{C}$  for 6 h, ground using a hammer mill (Dietz, Fritsch, Germany) with a 0.5 mm sieve, and then sieved again to obtain a powder with a particle size of less than 0.355 mm. The powder was heat-treated at  $90^{\circ}\text{C}$  for 90 min to ensure microbial stability, and then incorporated into the second batch at a final concentration of 4 %. This concentration was selected based on previous in vitro studies showing bactericidal effect against *L. monocytogenes*.

Characterization of the blueberry product was conducted to provide a comprehensive understanding of its antimicrobial potential (Table 1). More specifically, 1 g of rehydrated powdered product was extracted

**Table 1**  
Bioactive compound characterization of blueberry product.

Parameter	Value	Unit
Total polyphenolic content	$62.2 \pm 9.5$	mg of gallic acid / g of sample
Total anthocyanin content	$79.2 \pm 8.4$	mg of cyanidin-3-O-glucoside/ g of sample
Total antioxidant activity	$0.696 \pm 0.100$	mM of Trolox/ g of sample

Values are expressed as mean  $\pm$  standard deviation ( $n = 6$ ).

using an ultrasonic bath with 10 mL of a methanol:formic acid:distilled water mixture (80:1.5:18.5 v/v) for 10 min. The resulting extract was centrifuged and filtered, and the extraction process was repeated three additional times. The four supernatants were combined and adjusted to a final volume of 50 mL with the same solvent. Total polyphenol content was determined using the Folin–Ciocalteu method (Singleton and Rossi, 1965) and expressed as mg gallic acid/g of sample. Total anthocyanin content was quantified by the pH differential method (Paronetto, 1977) and expressed as mg cyanidin-3-glucoside/g of sample. The antioxidant capacity was evaluated using the ABTS radical cation decolorization assay (Rivero-Pérez, Muniz, and González-Sanjosé, 2007) and expressed as mM Trolox equivalents/g of sample.

## 2.3. Combined treatment of HHP (600 MPa) and blueberry product or nisin in beef and plant-based burgers inoculated with *L. monocytogenes*

### 2.3.1. Inoculation of matrices and HHP treatment

After the application of additives, the three batches of plant-based meat and ground beef matrices were inoculated with the already prepared five-strain cocktail of *L. monocytogenes* (1 mL of inoculum per 100 g of product) in order to achieve final concentration around  $10^7$  CFU/g. After manual mixing,  $10 \pm 0.2$  g patties of the inoculated batches were prepared and vacuum packaged into individual nylon-polyethylene bags. The vacuum packaged samples were then HHP treated at 600 MPa for 3 min in an industrial HHP equipment (Hiperbaric 135, Hiperbaric, Burgos, Spain). The process parameters (pressure, time) were selected based on the most common commercial application for this type of products (Campus, 2010). Water at  $10^{\circ}\text{C}$  was used as pressurizing fluid, while the pressurization rate was 175 MPa/min and instantaneous pressure release. After HHP treatment, the samples were transferred back to the laboratory under refrigeration (within 20 min) and subsequently stored under slight temperature abuse ( $7^{\circ}\text{C}$ ) for up to 132 days, based on the observed microbial growth. Microbial analyses were conducted at regular intervals, as shown in Fig. 1.

### 2.3.2. Microbiological analysis

Microbiological testing for all batches (nisin, blueberry product and no-additive) was carried out immediately after the HHP treatment. Non pressure-treated samples were also analyzed as controls to accurately determine both the initial *L. monocytogenes* concentrations in the matrices prior to HHP treatment and the potential effect of nisin and blueberry alone.

Microbiological analyses were carried out in duplicate for each batch and protein source (plant-based and beef). For *L. monocytogenes* counts and enrichment, two samples from each batch were analyzed on each sampling day. The 10-g patty was aseptically transferred into a sterile Stomacher bag (Scharlau, Spain) using sterile tweezers. A 90 mL aliquot of half-strength Fraser broth (Biokar Diagnostics, France) was added to achieve a 1:10 dilution. Half-strength Fraser was used as diluent for subsequent *L. monocytogenes* enrichment analysis. The samples were then homogenized in a Stomacher 400 (Seward, UK) for 1 min. From each stomacher bag, 1 mL was aseptically withdrawn and serially diluted in Buffered Peptone Water (BPW, Condalab, Spain). Appropriate dilutions were spread-plated on ALOA agar selective medium for *L. monocytogenes* (Microinstant<sup>®</sup> Listeria Selective Agar Base, according

to Ottaviani and Agosti; Scharlau, Spain). For samples with low viable counts, 1 mL aliquots were plated on three ALOA agar plates, enabling a minimum detection limit of 1 log CFU/g. The plates were incubated at 37 °C for 48–72 h.

To detect the presence of *L. monocytogenes*, the Stomacher bags containing the homogenates in half-strength Fraser broth were incubated at 30 °C for 24 h. After incubation, 100 µL was transferred from each bag to sterile glass tubes containing 10 mL of Fraser broth (Biokar Diagnostics) and incubated at 37 °C for 24–48 h. A loopful of the samples from both the half-strength Fraser and Fraser broths was streaked onto ALOA agar and incubated for 48 h at 37 °C. Any colony appearing on the selective medium was indicative of the presence of *L. monocytogenes* in the sample and was recorded as positive (+).

#### 2.4. Combined treatment of HHP (600 MPa) and blueberry product or nisin in non-inoculated beef and plant-based burgers

##### 2.4.1. HHP treatment and storage

For shelf-life assessment after HHP, non-inoculated batches with additives (nisin or blueberry) and without (control) from the two protein sources (beef and plant-based) were prepared as described in Section 2.2. More specifically, 10 ± 0.2 g patties of each non-inoculated batch were prepared and vacuum packaged into individual nylon-polyethylene bags. The samples were then HHP-treated under the same process parameters as described previously. After HHP, the pressurized samples were stored under slight temperature abuse (7 °C) for up to 132 days. Duplicate samples from each batch were analyzed on each sampling day for physicochemical characteristics and microbial evolution.

##### 2.4.2. Physicochemical analysis

The water activity ( $a_w$ ) and pH of the HHP treated samples were monitored during the shelf-life study. The  $a_w$  was determined using an Aqualab 4 TDL (Decagon Devices, USA) equipment and pH was evaluated with a pH-meter Micro pH 2001 (Crison, Barcelona, Spain). The analysis was conducted in duplicate, taking three measurements for samples of each batch on each sampling day.

##### 2.4.3. Color

A Minolta CM-2600d spectrophotometer (Konica Minolta Sensing Inc., Tokyo, Japan; Standard Illuminant D65, 10° observed angle) was used to measure the color characteristics. CIELAB color system was used measuring lightness ( $L^*$ ), green–red coordinate ( $a^*$ ), and blue–yellow coordinate ( $b^*$ ). The measurements were taken at 3 random non-overlapping points on the surface of two independent samples for each batch. Additionally, Chroma ( $C^*$ ) which indicates the intensity or saturation of the color was calculated based on the eq. (1):

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (1)$$

##### 2.4.4. Microbiological analysis

Aerobic Plate Counts (APC), Lactic Acid Bacteria (LAB), *Clostridium* spp., *Enterobacteriaceae*, *Pseudomonas* spp., and *Brochothrix thermosphacta* were monitored throughout the storage period for the non-inoculated samples, using the same time intervals as for the inoculated samples (Figs. 2 and 3). Microbiological analyses were performed in duplicate for each batch and protein source (plant-based and beef). To determine the initial levels of the target microbial groups, samples (with and without additives) were analyzed both prior to HHP treatment and immediately after to evaluate its immediate effect. The 10-g samples from each batch were aseptically transferred into a sterile Stomacher bag and half-strength Fraser broth was added to achieve a 1:10 dilution as described in Section 2.3.2. Following homogenization, 1 mL was serially diluted in BPW, and appropriate dilutions were spread or pour-plated according to the type of microorganism. The lower detection limit

of enumeration for all tested microorganisms was 1 log CFU/g.

For APC and *Enterobacteriaceae* counts, 1 mL of the appropriate dilutions was pour-plated on Plate Count Agar (PCA; Condalab) and Violet Red Bile Glucose Agar (VRBG; Condalab) respectively. Plates were incubated at 30 °C for 48–72 h for APC and 37 °C for 24 h for *Enterobacteriaceae* before colony enumeration. *Clostridium* spp. counts were also evaluated by pour-plating 1 mL of the appropriate decimal dilutions on Tryptone Sulfite Neomycin -agar (TSN agar; Condalab) followed by incubation of the plates in anaerobe jars containing AnaeroGen™ sachets (Thermo Scientific, Basingstoke, UK) for 24–48 h at 45 °C. *Pseudomonas* spp., LAB and *Brochothrix thermosphacta* were determined by spread-plating 0.1 mL on the surface of Pseudomonas Agar (Pseudomonas Agar Base ISO, Condalab), MRS agar (Oxoid, Basingstoke, UK) and Streptomycin Thallous Acetate Actidione agar (STAA; Oxoid) respectively. For samples with low viable counts, 1 mL aliquots were spread-plated onto three agar plates, allowing for a lowest detection limit of 1 log CFU/g. Colony counts were determined following incubation at 30 °C with incubation periods ranging from 24 to 72 h depending on the microorganism. Additionally, the Stomacher bags containing the homogenates in half-strength Fraser broth were used for the detection of *L. monocytogenes* following the same methodology described previously for the inoculated samples (Section 2.3.2).

#### 2.5. Immediate inactivation of *L. monocytogenes* across various pressure levels

To accurately evaluate and quantify the synergistic effect between HHP and the additives (blueberry product and nisin), *L. monocytogenes* inactivation was assessed immediately after HHP at 300, 400 and 500 MPa for 3 min, in samples with and without additives.

##### 2.5.1. Preparation of samples and HHP treatment

Beef and plant-based batches, with the addition of either nisin (500 IU/g) or blueberry product (4 %), and without additives (controls) were prepared as described in Section 2.2. The batches were inoculated with a five-strain cocktail of *L. monocytogenes* and 10-g samples were subsequently prepared, and vacuum-packaged as detailed in Section 2.3.1. The samples were then subjected to HHP at 300, 400 and 500 MPa for 3 min using an industrial HHP equipment (Hiperbaric 55, Hiperbaric, Spain). The pressurizing fluid used was water at 14 ± 1 °C, with a pressurization rate of 150–165 MPa/min and instantaneous pressure release. After HHP, samples were transferred back to the laboratory under refrigeration within 20 min, followed by microbiological analysis. Non-HHP-treated samples were used as controls to evaluate the synergistic effect.

##### 2.5.2. Microbial enumeration

Following HHP, the 10-g samples for each batch (nisin, blueberry, no-additive) and protein source (beef, plant-based) were aseptically transferred into sterile Stomacher bags using sterile tweezers and homogenized with 90 mL of BPW to achieve a 1:10 dilution. Non-pressure-treated samples were also analyzed as controls to determine the initial *L. monocytogenes* concentrations in the matrices prior to HHP treatment. Samples were homogenized in a Stomacher for 1 min, and 1 mL aliquots were serially diluted in BPW. Appropriate dilutions were spread-plated onto ALOA agar and incubated at 37 °C for 48–72 h. For samples with low viable counts, 1 mL aliquots were spread-plated onto three ALOA agar plates, allowing for a lowest detection limit of 1 log CFU/g. For each pressure level (300, 400, and 500 MPa), at least two biological replicates were conducted, with each replicate analyzed in duplicate (two technical replicates).

##### 2.5.3. Synergistic effect calculation

The synergistic effect between HHP and the additives immediately after the HHP treatment of the products was determined based on eq. (2) (Giannoulis and Karatzas, 2024):

$$\text{Synergistic effect} = \log(N_{\text{combined}}) - [\log(N_{\text{HHP}}) + \log(N_{\text{additive}})] \quad (2)$$

where:

- $\log(N_{\text{combined}})$  = Reduction due to the combined effect of HHP and the additive.
- $\log(N_{\text{HHP}})$  = Reduction due to HHP alone.
- $\log(N_{\text{additive}})$  = Reduction due to the additive (nisin or blueberry product) alone before HHP treatment.

The presence or absence of synergistic effect was confirmed based on statistical analysis (*t*-test against 0, *p*-value < 0.05).

### 2.6. Statistical analysis

Statistical comparisons between treatments (HHP alone, HHP + blueberry product, and HHP + nisin) at each time point during the storage period were performed using one-way ANOVA, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test. When only two variables were available, a two-tailed unpaired *t*-test was applied instead. Additionally, unpaired *t*-tests were used to compare the synergistic effect between the two additives (nisin and blueberry product) at each pressure level. Differences in *L. monocytogenes* inactivation immediately after HHP treatment between the two matrices (beef and plant-

based) were also evaluated using two-tailed unpaired *t*-tests. A probability value of <0.05 (*p*-value < 0.05) was considered statistically significant for all comparisons. To confirm the presence or absence of a synergistic effect for each treatment, unpaired *t*-tests were performed against a theoretical value of zero, with significance set at *p* < 0.05. All statistical analyses were carried out using GraphPad Prism (version 10.2.2) and SPSS software.

### 3. Results

#### 3.1. Combined effect of HHP (600 MPa) and blueberry product or nisin in beef and plant-based burgers inoculated with *L. monocytogenes*

The efficacy of HHP (600 MPa, 3 min) combined with blueberry product (4 %) or nisin (500 IU/g) on *L. monocytogenes* in beef and plant-based burgers was assessed over a 132-day storage period under slight temperature abuse conditions (7 °C).

In beef patties, HHP combined with nisin or blueberry product reduced the levels of *L. monocytogenes* below the detection limit (<1 log CFU/g) immediately after treatment, while HHP alone achieved a 5.36 log reduction (Fig. 1a). Throughout the storage, samples without additives (control) showed a steady increase in microbial counts, reaching 7.1 log CFU/g by day 34. A similar growth behavior was observed in samples treated with nisin combined with HHP, which reached 6.2 log

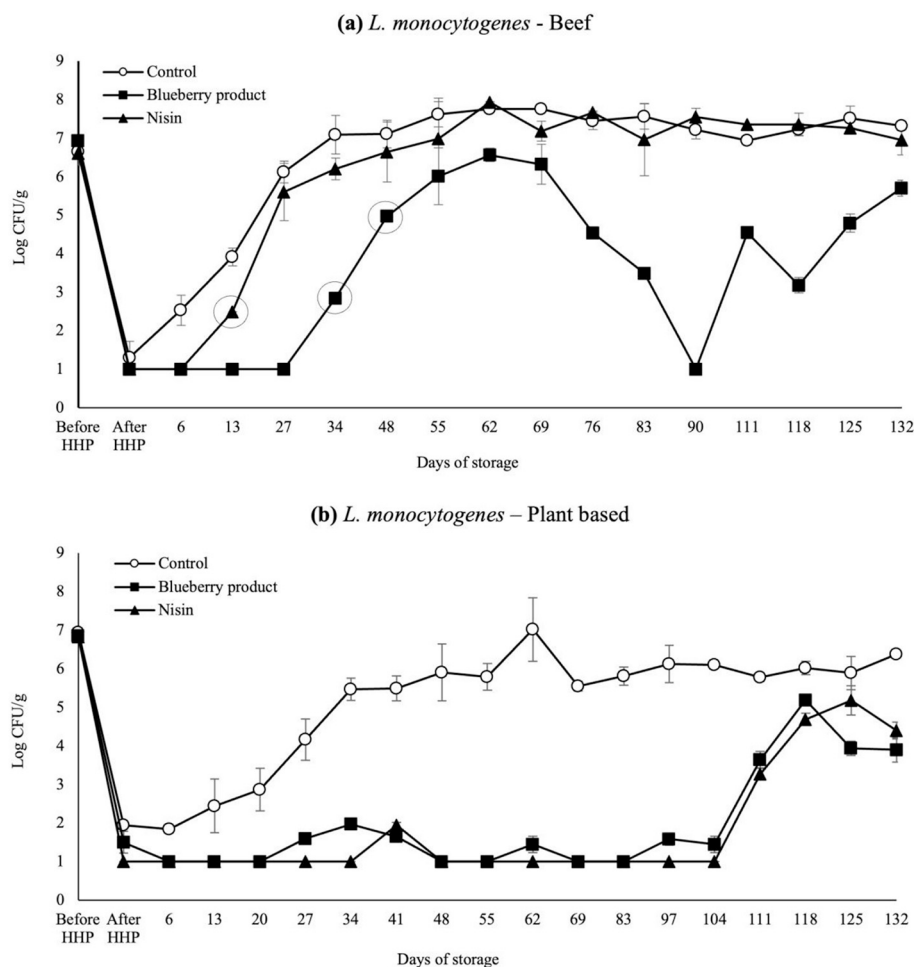


Fig. 1. Concentration of *Listeria monocytogenes* (log CFU/g) through time in artificially inoculated (a) beef and (b) plant-based vacuum-packaged burgers after HHP treatment (600 MPa for 3 min) alone; controls (○) or combined with 4 % blueberry product (■) or nisin 500 IU/g (▲) during 132 days of storage under slight temperature abuse conditions (7 °C). The initial concentration before HHP treatment and the concentration immediately after are also shown. Each point represents the mean of duplicate results (except for circled values, which represent a single replicate), and error bars indicate the standard deviation.

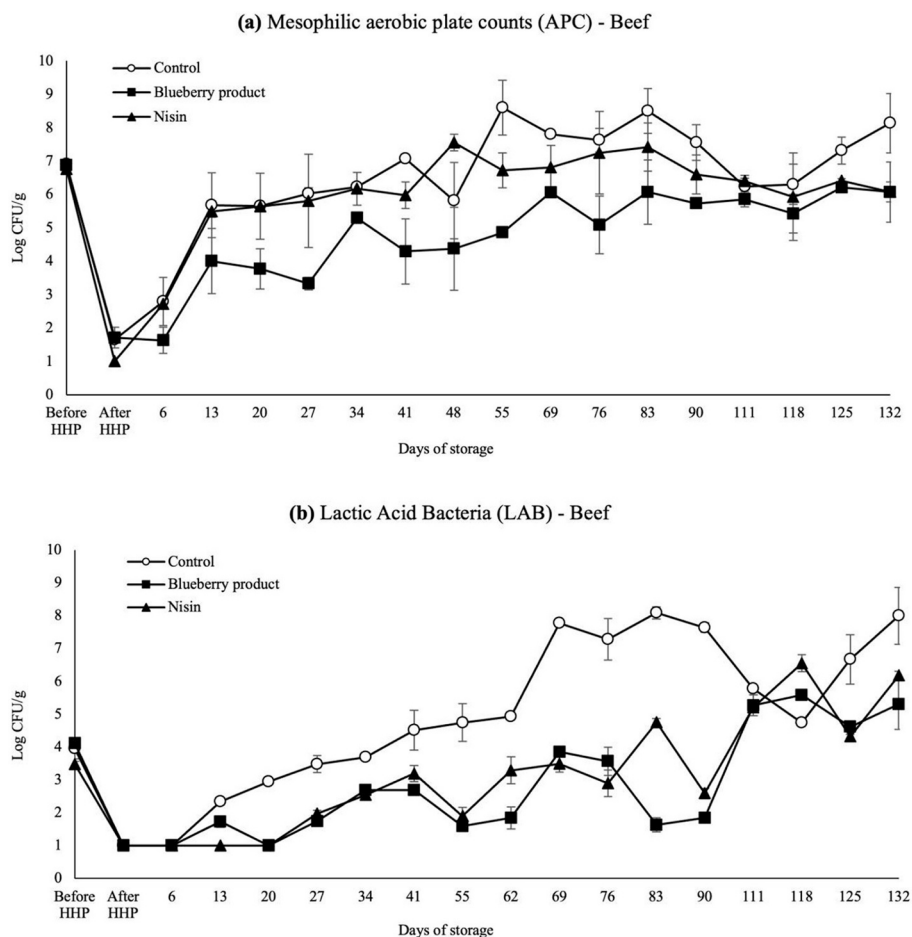
CFU/g by day 34 after a lag phase of 6 days. In contrast, microbial growth in beef burgers with blueberry product was delayed until day 27 (lag phase) after HHP treatment, with the highest counts (6.56 log CFU/g) observed on day 62. *L. monocytogenes* counts in samples treated with blueberry product were significantly lower ( $p < 0.05$ ) than those in the control and nisin-treated samples in most sampling days, indicating its greater effectiveness in beef burgers after HHP treatment. No significant differences ( $p > 0.05$ ) were observed between nisin-treated and control samples at most sampling points (Suppl. Table ST3; Fig. 1a).

In the plant-based matrix, the pathogen was reduced to levels below the detection limit ( $<1$  log CFU/g) immediately after the HHP treatment combined with nisin, corresponding to 5.8 log reduction. A reduction of more than 5 log was achieved with the combination of HHP and blueberry product, while HHP treatment alone reduced the initial *L. monocytogenes* load from 6.95 to 1.95 log CFU/g (Fig. 1b). During storage at 7 °C, pathogen's counts in control samples (without additives) increased rapidly, while both additives effectively delayed growth, maintaining microbial levels below 2 log CFU/g up to day 104, indicating their effectiveness during storage following HHP treatment (Fig. 1b). Presence of *L. monocytogenes* in all samples (beef and plant-based) throughout the storage was observed through the use of the enrichment method mentioned in Materials and Methods section (2.3.2).

### 3.2. Combined effect of HHP (600 MPa) and blueberry product or nisin in non-inoculated beef and plant-based burgers

#### 3.2.1. Microbiological stability

In beef patties, significant reduction ( $>5$  log CFU/g) in mesophilic Aerobic Plate Counts (APC) was observed following treatment with HHP, either alone or combined with the antimicrobials (Fig. 2a). However, microbial survivors grew rapidly after treatment, with APC levels exceeding 5 log CFU/g after 13 days in samples treated with HHP alone or combined with nisin, and after 34 days when combined with blueberry product. No statistically significant differences ( $p > 0.05$ ) were observed between treatments at most time points during storage (Suppl. Table ST4a). Lactic Acid Bacteria (LAB) were reduced to below the detection limit across all treatments immediately after processing. In samples treated with HHP alone, LAB counts reached 7 log CFU/g by day 69, whereas in samples treated with HHP combined with either nisin or blueberry product, LAB counts remained below 5 log CFU/g until day 111 (Fig. 2b). After day 13, LAB counts in control samples were consistently higher than in samples treated with nisin or blueberry product (Suppl. Table ST4a). The combined effect of HHP and the additives was also evident on *B. thermosphacta*, with both nisin and the blueberry product delaying its growth compared to the control (HHP alone) after an initial reduction to below the detection limit (Table 2). For *Enterobacteriaceae* and *Pseudomonas* spp., no synergistic effect between HHP and the additives was observed. However, *Enterobacteriaceae* counts remained below 3 log CFU/g throughout the entire storage



**Fig. 2.** Mesophilic aerobic plate counts (APC) (a) and lactic acid bacteria counts (LAB) (b) through time in vacuum-packaged, non-inoculated beef patties after HHP treatment (600 MPa for 3 min) alone; controls (○) or combined with 4 % blueberry product (■) or nisin 500 IU/g (▲) during 132 days of storage under slight temperature abuse conditions (7 °C). The initial concentration before HHP treatment and the concentration immediately after are also shown. Each point represents the mean of duplicate results and error bars indicate the standard deviation.

**Table 2**

Microbiological counts of *Enterobacteriaceae*, *Pseudomonas* spp., and *Brochothrix thermosphacta* (log CFU/g) in vacuum-packaged, non-inoculated beef burgers during storage (7 °C) after HHP (600 MPa, 3 min) alone (Control) either combined with 4 % blueberry product (Berry) or 500 IU/g nisin.

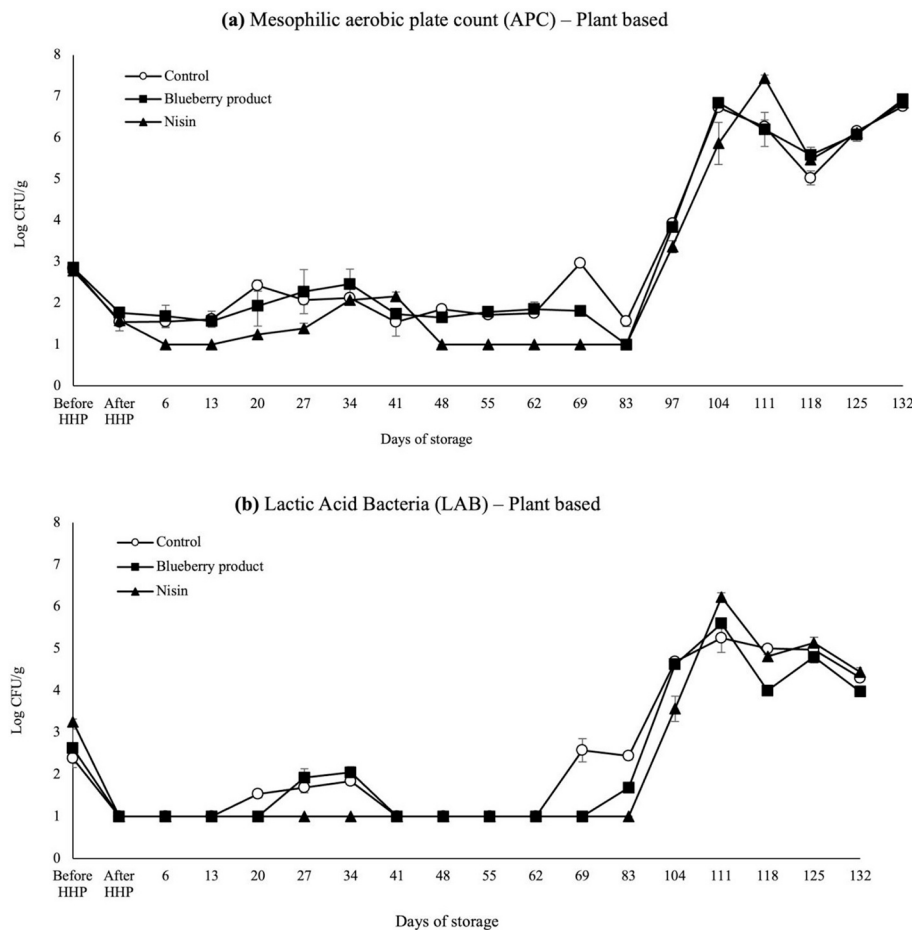
Beef		Before HHP	After HHP	Days of storage																
				6	13	20	27	34	41	48	55	62	69	76	83	90	111	118	125	132
<i>Enterobacteriaceae</i>	Control	<sup>a</sup> 2.54 ± 0.08	nd	nd	nd	1.72 ± 0.17	<sup>a</sup> 2.96 ± 0.02	<sup>a</sup> 2.94 ± 0.06	nd	nd	nd	2.52 ± 0.05	2.56 ± 0.07	<sup>b</sup> 1.93 ± 0.05	2.53 ± 0.21	nd	nd	nd	nd	nd
	Berry	<sup>a</sup> 2.70 ± 0.14	nd	nd	nd	nd	<sup>a</sup> 2.62 ± 0.25	<sup>a</sup> 2.44 ± 0.3	nd	nd	nd	nd	nd	<sup>a</sup> 2.45 ± 0.02	nd	nd	nd	2.02 ± 0.03	nd	nd
	Nisin	<sup>a</sup> 2.96 ± 0.37	nd	nd	2.08 ± 0.13	1.59 ± 0.15	<sup>a</sup> 2.97 ± 0.02	<sup>a</sup> 3.03 ± 0.01	nd	nd	nd	2.15 ± 0.21	2.59 ± 0.01	<sup>c</sup> 1.65 ± 0.06	nd	nd	nd	1.87 ± 0.08	nd	nd
<i>Pseudomonas</i> spp.	Control	<sup>a</sup> 6.88 ± 0.02	nd	<sup>a</sup> 2.58 ± 0.42	<sup>a</sup> 5.77 ± 0.55	<sup>a</sup> 6.13 ± 0.33	<sup>a</sup> 6.21 ± 0.44	<sup>a</sup> 6.15 ± 0.24	<sup>a</sup> 6.09 ± 0.03	<sup>a</sup> 5.92 ± 0.72	<sup>a</sup> 5.91 ± 0.18	<sup>a</sup> 5.85 ± 0.27	<sup>a</sup> 5.97 ± 0.02	<sup>a</sup> 5.50 ± 0.28	<sup>a</sup> 5.59 ± 0.01	<sup>a</sup> 5.14 ± 0.27	<sup>a</sup> 5.93 ± 0.12	<sup>a</sup> 5.53 ± 0.04	<sup>a</sup> 5.89 ± 0.05	<sup>a</sup> 5.57 ± 0.11
	Berry	<sup>a</sup> 6.85 ± 0.08	nd	<sup>a</sup> 2.30 ± 0.18	<sup>a</sup> 5.33 ± 0.001	<sup>a</sup> 5.82 ± 0.03	<sup>a</sup> 5.81 ± 0.05	<sup>a</sup> 5.50 ± 0.06	<sup>a</sup> 5.14 ± 0.04	<sup>a</sup> 6.26 ± 0.06	<sup>a</sup> 5.94 ± 0.20	<sup>a</sup> 6.26 ± 0.14	<sup>a</sup> 5.99 ± 0.01	<sup>a</sup> 5.12 ± 0.55	<sup>a</sup> 5.73 ± 0.72	<sup>a</sup> 5.52 ± 0.09	<sup>a</sup> 5.83 ± 0.22	<sup>a</sup> 5.74 ± 0.24	<sup>a</sup> 5.81 ± 0.13	<sup>a</sup> 5.65 ± 0.20
	Nisin	<sup>a</sup> 6.85 ± 0.02	nd	<sup>a</sup> 2.58 ± 0.19	<sup>a</sup> 5.89 ± 0.84	<sup>a</sup> 5.96 ± 0.11	<sup>a</sup> 6.10 ± 0.02	<sup>a</sup> 6.28 ± 0.36	<sup>a</sup> 6.01 ± 0.56	<sup>a</sup> 5.68 ± 0.67	<sup>a</sup> 6.35 ± 0.33	<sup>a</sup> 5.62 ± 0.11	<sup>a</sup> 5.73 ± 0.14	<sup>a</sup> 5.71 ± 0.31	<sup>a</sup> 5.89 ± 0.31	<sup>a</sup> 5.61 ± 0.11	<sup>a</sup> 5.97 ± 0.01	<sup>a</sup> 6.01 ± 0.14	<sup>a</sup> 5.84 ± 0.02	<sup>a</sup> 5.33 ± 0.01
<i>B. thermosphacta</i>	Control	<sup>a</sup> 4.72 ± 0.01	nd	nd	nd	–	<sup>b</sup> 1.83 ± 0.18	<sup>a</sup> 3.78 ± 0.16	<sup>a</sup> 4.31 ± 0.08	<sup>a</sup> 5.73 ± 0.11	–	<sup>a</sup> 5.67 ± 0.13	<sup>a</sup> 4.50 ± 0.08	4.47 ± 0.04	3.89 ± 0.19	–	<sup>b</sup> 5.22 ± 0.05	<sup>a</sup> 5.18 ± 0.04	<sup>a</sup> 5.89 ± 0.14	<sup>a</sup> 5.83 ± 0.01
	Berry	<sup>a</sup> 4.88 ± 0.04	nd	nd	nd	–	<sup>b</sup> 1.98 ± 0.03	<sup>b</sup> 3.13 ± 0.07	<sup>b</sup> 3.38 ± 0.32	<sup>b</sup> 3.0 ± 0.14	–	<sup>b</sup> 3.10 ± 0.25	<sup>a</sup> 4.90 ± 0.37	5.27	4.78	–	<sup>a</sup> 6.17 ± 0.26	<sup>a</sup> 5.37 ± 0.22	<sup>a</sup> 5.82 ± 0.03	<sup>a</sup> 5.81 ± 0.04
	Nisin	<sup>b</sup> 4.33 ± 0.08	nd	nd	nd	–	<sup>a</sup> 2.48 ± 0.05	<sup>ab</sup> 3.49 ± 0.13	<sup>ab</sup> 3.57 ± 0.11	<sup>b</sup> 3.2 ± 0.09	–	<sup>b</sup> 3.04 ± 0.06	<sup>b</sup> 2.7 ± 0.07	<sup>*</sup> 2.47 ± 0.08	<sup>*</sup> 2.44 ± 0.13	–	<sup>c</sup> 4.00 ± 0.28	<sup>b</sup> 4.18 ± 0.20	<sup>a</sup> 6.03 ± 0.05	<sup>a</sup> 5.80 ± 0.05

Values represent the average of two replicates with ±standard deviations while values without standard deviation represent a single replicate.

nd: Non-detected; Plate counts below the detection limit (1 log CFU/g).

Superscript letters (a–c) indicate statistically significant differences between treatments (HHP alone, HHP + blueberry product, HHP + nisin) at each time point (Tukey's HSD test;  $p < 0.05$ ). This test was applied only when all three treatments were available for comparison. Values that do not share a letter are significantly different.

An asterisk (\*) indicate significant differences between HHP alone and either HHP + blueberry product or HHP + nisin at time points where only two treatments could be statistically compared (unpaired  $t$ -test;  $p < 0.05$ ).



**Fig. 3.** Mesophilic aerobic plate counts (APC) (a) and lactic acid bacteria (LAB) counts (b) through time in vacuum-packaged, non-inoculated plant-based patties after HHP treatment (600 MPa for 3 min) alone; controls (○) or combined with 4 % blueberry product (■) or nisin 500 IU/g (▲) during 132 days of storage under slight temperature abuse conditions (7 °C). The initial concentration before HHP treatment and the concentration immediately after are also shown. Each point represents the mean of duplicate results and error bars indicate the standard deviation.

period across all samples, while *Pseudomonas* spp. counts increased rapidly after treatment (Table 2). *Clostridium* spp. counts were not detected before HHP treatment and remained below the detection limit (<1 log CFU/g) throughout the storage period in all samples (data not shown). In plant-based burgers, the combination of HHP with the antimicrobials did not show any synergistic effect on APC, LAB, *Pseudomonas* spp., or *B. thermosphacta* during storage. The microbial counts of the aforementioned microorganisms remained below 3 log CFU/g in all samples (control; blueberry; nisin) up to day 83 of storage (Fig. 3 & Table 3). *Clostridium* spp. and *Enterobacteriaceae* counts were not detected prior to HHP treatment and remained below the detection limit (1 log CFU/g) throughout the storage period in all samples (data not shown). Finally, it is worth mentioning that neither nisin nor the blueberry product alone had any antimicrobial effect prior to HHP treatment in any of the studied microorganisms across both matrices.

The absence of *L. monocytogenes* in all samples (beef and plant-based) during storage was observed using the enrichment method mentioned in Materials and Methods.

### 3.2.2. Physicochemical properties

The pH values of beef and plant-based burgers before and after HHP treatment, as well as during storage, are presented in Fig. 4. The addition of the blueberry product significantly ( $p < 0.05$ ) reduced the pH in both matrices, from 5.68 to 5.33 in beef and from 6.45 to 5.75 in the plant-based burgers, while the addition of nisin had no significant effect (Suppl. Table ST5). Following HHP treatment, a slight increase in pH was observed in beef patties across all samples, whereas the pH

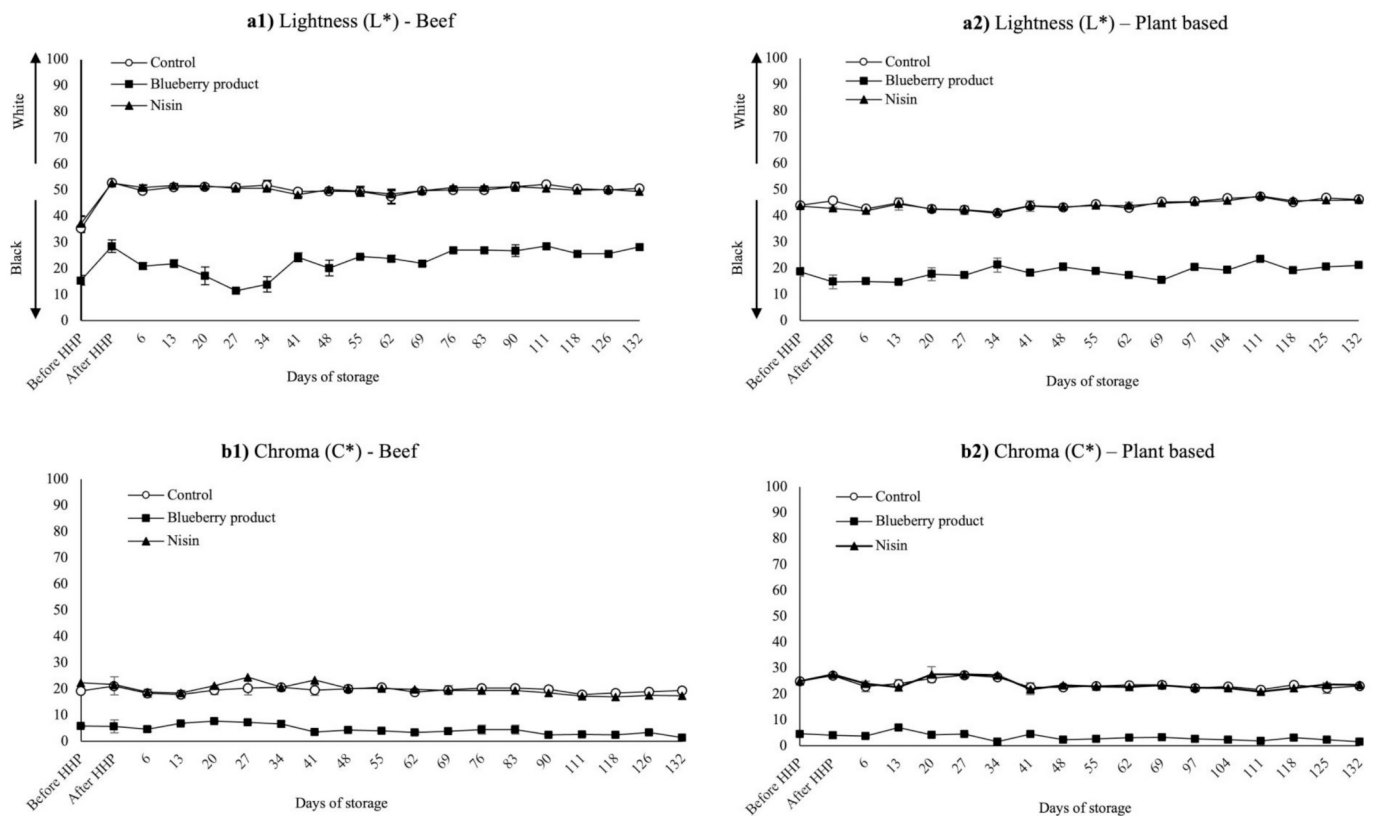
remained stable in the plant-based matrix. During storage, the pH of beef patties remained relatively constant, with lower values in blueberry-treated samples compared to the control and nisin-treated ones. Similarly, the pH of plant-based burgers remained stable throughout storage. The initial pH of beef burgers was significantly lower ( $5.68 \pm 0.03$ ) than that of the plant-based matrix ( $6.45 \pm 0.05$ ).

Water activity was not affected by HHP treatment or the addition of additives in either matrix. Specifically, in beef burgers,  $a_w$  ranged from 0.970 to 0.994 across all samples throughout the storage period. In plant-based patties,  $a_w$  ranged from 0.970 to 0.987 (Suppl. Table ST7). No statistically significant difference in water activity was observed between the beef and plant-based matrix before HHP treatment (Suppl. Table ST6).

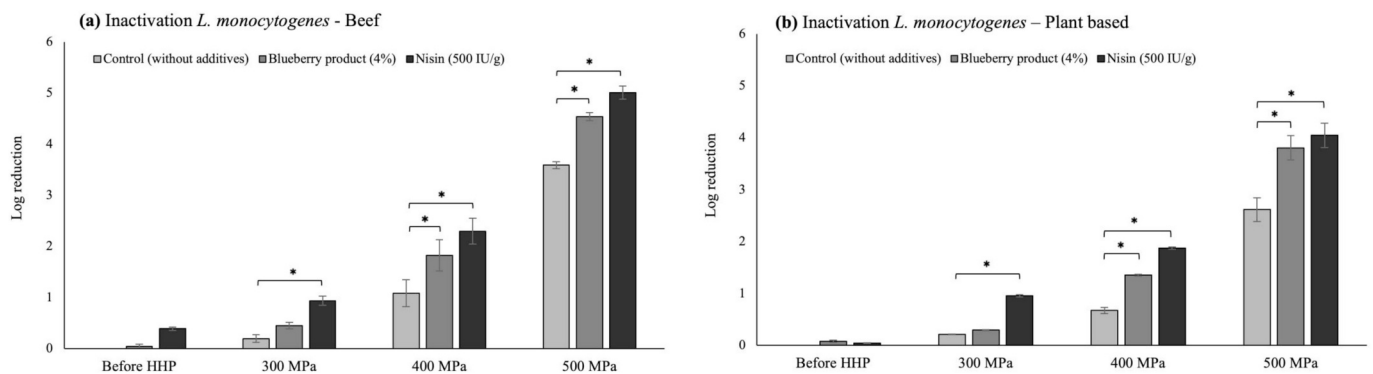
The  $L^*a^*b^*$  color coordinates were measured during the storage of the HHP-treated samples. The chroma ( $C^*$ ), which represents the intensity or saturation of color, was calculated based on the  $a^*$  and  $b^*$  values. As expected, the addition of blueberry product in both matrices significantly affected the lightness ( $L^*$ ) and the chroma ( $C^*$ ) of the samples, making them darker and moving the values closer to 0, which corresponds to total blackness (Fig. 5). The  $a^*$  and  $b^*$  values were also affected and are presented in detail in Supplementary Table 8 (ST8).

In beef patties, lightness ( $L^*$ ) significantly increased ( $p < 0.05$ ) after HHP treatment, indicating that the samples became lighter (Fig. 5a1; Suppl. Table ST9). This trend remained stable throughout the storage period with minimal variation. Chroma ( $C^*$ ) also remained relatively stable across all samples during storage (Fig. 5b1). In blueberry-treated patties, both  $L^*$  and  $C^*$  values were consistently lower than in the





**Fig. 5.** Lightness ( $L^*$ ) (a1, a2) and chroma ( $C^*$ ) (b1, b2) values of vacuum-packaged beef (a1, b1) and plant-based burgers (a2, b2) treated with HHP (600 MPa, 3 min) during 132 days of storage at 7 °C. Samples include control; HHP alone (○), HHP combined with 4 % blueberry product (■), and HHP combined with 500 IU/g nisin (▲). Data are shown as mean ( $n = 6$ )  $\pm$  standard deviation.



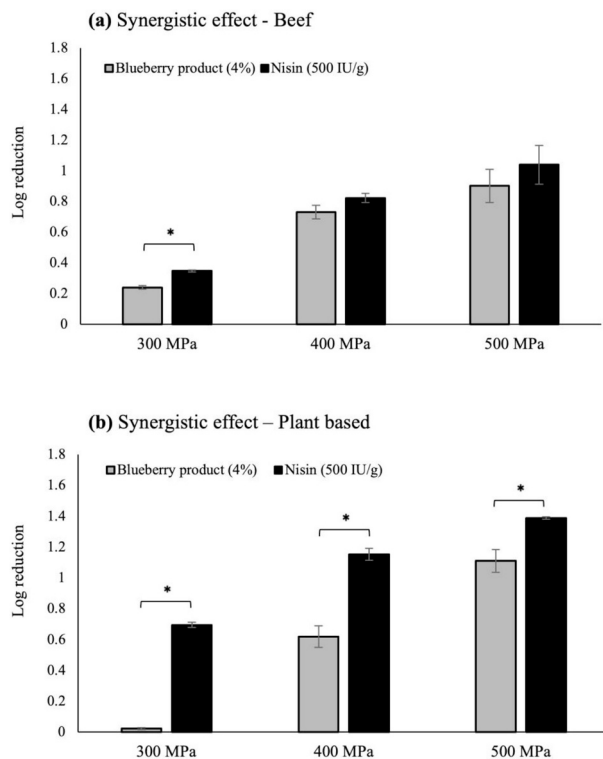
**Fig. 6.** Combined effect of HHP (300, 400 & 500 MPa, 3 min) and nisin (500 IU/g; black bars) or blueberry product (4 %; grey bars) on the inactivation of five-strain cocktail of *L. monocytogenes* in (a) beef and (b) plant-based burgers. The effect of HHP alone (without additives; light grey bars) is represented as control. The inactivation due to the additives alone (before HHP) is also represented. (\*) indicates statistically significant differences within the same pressure level ( $t$ -test;  $p < 0.05$ ). Values are means of two (300 & 400 MPa) or three (500 MPa) independent biological replicates while error bars indicate the standard deviation.

400 and 500 MPa ( $p > 0.05$ ). At 300 MPa, the combination with nisin resulted in a significantly higher synergistic effect ( $p < 0.05$ ) compared to blueberry product while similar synergism was observed at 400 and 500 MPa (Fig. 7a).

In plant-based burgers, combining HHP (500 MPa, 3 min) with nisin or blueberry product resulted in reduction of 3.8 and 4.0 log, respectively, compared to 2.6 log with HHP alone (Fig. 6b). Similar to beef burgers, the additives did not significantly reduce *L. monocytogenes* before HHP treatment. At both 400 and 500 MPa, the combined treatments with additives consistently resulted in significantly higher inactivation than HHP alone. The highest synergism was observed at 500

MPa, with additional reductions of 1.1 and 1.4 log for blueberry product and nisin, respectively (Fig. 7b). Across all pressure levels, combining HHP with nisin resulted in a significantly greater synergistic effect than blueberry product ( $p < 0.05$ ). The synergism between HHP and nisin increased significantly only as the pressure increased from 300 to 400 MPa, while the synergistic effect with blueberry product increased consistently across all pressure levels (Fig. 7b; Suppl. Table ST10).

When comparing the two matrices, the inactivation due to either HHP alone or combined with the additives at 500 MPa was higher in beef than in plant-based matrix ( $t$ -test;  $p < 0.05$ ; Suppl. Figure SF1). However, the synergistic effect with nisin was greater in the plant-based



**Fig. 7.** Synergistic effect of combined HHP (300, 400 & 500 MPa – 3 min) and nisin (black bars) or blueberry product (grey bars) against *L. monocytogenes* in (a) beef & (b) plant-based burgers.

(\*) indicates statistically significant differences between the additives within the same matrix (*t*-test;  $p < 0.05$ ). Values are means of two (300 & 400 MPa) or three (500 MPa) independent biological replicates while error bars indicate the standard deviation.

matrix ( $p < 0.05$ ), while a similar synergism was observed in both matrices at 400 and 500 MPa when HHP was combined with blueberry product (Suppl. Table ST10).

#### 4. Discussion

This study firstly investigated the combined effect of HHP and natural antimicrobials (nisin and blueberry product) in controlling *L. monocytogenes* in beef and plant-based burgers immediately after the treatment and during extended storage under slight temperature abuse (7 °C). In addition to *L. monocytogenes* control, the study also examined the effect of these combinations on the microbiological stability and physicochemical properties of non-inoculated beef and plant-based burgers, providing important insights into the potential applications of combining HHP with natural antimicrobials for enhancing food safety and quality.

HHP (600 MPa, 3 min) significantly reduced *L. monocytogenes* immediately after treatment in beef and plant-based patties, consistent with previous studies reporting similar microbial reductions at comparable pressure levels (600 MPa) and treatment durations (1.5–4.5 min; Porto-Fett et al., 2020). However, microbial counts increased rapidly during storage in control at both matrices after HHP treatment (Fig. 1a, b). This phenomenon has been previously documented in ham, with *L. monocytogenes* showing recovery and regrowth following sublethal damage induced by HHP (500 MPa, 3 min) during refrigerated storage (Teixeira, Repková, Gänzle, and McMullen, 2018). Increasing the process intensity by extending the pressure holding time, could improve microbial inactivation and prolong shelf life. Alternatively, as demonstrated in the present study, incorporating antimicrobial agents that act synergistically with HHP can also improve food safety and extend

microbiological shelf life under mild treatment conditions. In beef patties, the combination of HHP with blueberry product showed a better long-term antimicrobial activity compared to HHP with nisin or HHP alone. Following HHP treatment, the blueberry product effectively delayed *L. monocytogenes* growth, maintaining counts consistently lower than both control and nisin-treated samples after day 13 (Fig. 1a). This prolonged antimicrobial effect of blueberry product could be attributed to its total phenolic and anthocyanin content (Table 1), that may inhibit *L. monocytogenes* growth in beef matrices. Additionally, the decrease in pH resulting from the addition of the blueberry product may have further enhanced the antimicrobial activity by inhibiting bacterial growth. Conversely, the reduced effectiveness of nisin in beef patties may be due to interactions with matrix components such as fats and proteins, which could decrease its bioavailability and antimicrobial action (Wang et al., 2024). Moreover, Rose, Sporns, Stiles, and McMullen (1999) reported that the activity of nisin is reduced in raw meat due to an enzymatic reaction with glutathione.

In the plant-based matrix, both nisin and blueberry product were highly effective when combined with HHP (Fig. 1b) against *L. monocytogenes*, highlighting their effectiveness as preservatives in combination with HHP, particularly in plant-based products, which have been found to provide the pathogen with increased resistance to HHP treatment (Porto-Fett et al., 2020). Similar findings have been reported by Teixeira et al. (2018), where the combination of nisin and pressure treatment (500 MPa, 3 min) in ham reduced *L. monocytogenes* by more than 5 log CFU/g, with microbial counts remaining below the detection limit for 4 weeks of storage. Comparable synergistic effect has been observed in milk (Black, Kelly, and Fitzgerald, 2005), cured meats (Hereu, Bover-Cid, Garriga, and Aymerich, 2012) and carrot juice (Pokhrel et al., 2019) during storage after HHP treatment combined with nisin. However, there is currently no available literature on the post-HHP effects of combining nisin or blueberry product with HHP in beef or plant-based patties. This study provides novel insights into the potential of these combinations to enhance food safety and extend product shelf life compared to HHP alone.

Investigating *L. monocytogenes* inactivation immediately after treatment at lower pressure levels (300–500 MPa, 3 min) combined with the additives further confirmed the existence of synergism. In both matrices, a clear synergistic effect was observed when HHP (500 MPa) was combined with nisin in agreement with previous studies reporting similar synergism between HHP and nisin in other food matrices, such as dry-cured hams, cooked hams, avocado dressings, milk and carrot-juice (Black et al., 2005; Jofré, Aymerich, Monfort, and Garriga, 2008; Oner, 2020; Pokhrel et al., 2019). For instance, Hereu et al. (2012) reported the existence of synergism between HHP (600 MPa, 5 min) and nisin (200 AU/cm<sup>2</sup>) in dry-cured hams achieving a higher than 5.5 log reduction of *L. monocytogenes* by the combination, compared to reductions of 3.85 and 0.8 log by HHP and nisin alone, respectively. Combining HHP (500 MPa) with the blueberry product also resulted in a clear synergistic effect in both matrices (Fig. 7a,b). While plant extracts, particularly berry-derived products, have been widely studied for their antimicrobial properties, there is very limited literature available on their combined application with HHP in food products. For instance, Diez-Sánchez, Martínez, Rodrigo, Quiles, and Hernando (2020) found that chokeberry pomace (10 %) combined with HHP (500 MPa, 10 min) enhanced *L. monocytogenes* inactivation in a milkshake product compared to HHP alone. Similarly, the addition of thyme-based natural antimicrobials (NAs) to fresh cheese, combined with HHP (300 MPa), resulted in a synergistic reduction of 1.68 log CFU/g of *L. monocytogenes* compared to HHP alone (Bleoancă et al., 2016). To the best of the authors' knowledge, this is the first study reporting a synergistic effect immediately after the HHP treatment combined with blueberry product or nisin on *L. monocytogenes* in beef and plant-based burgers.

The observed synergistic effect may be attributed to the combined action of HHP and the additives on microbial cells. More specifically, HHP disrupts bacterial membranes and cellular structures, which may

enhance the efficacy of compounds like nisin, known to form pores in the membrane and inhibit peptidoglycan biosynthesis (Simons et al., 2020). The high phenolic and anthocyanin content of the blueberry product (Table 1) may contribute to bacterial cell wall damage, leading to structural alterations and increased membrane permeability (Das et al., 2017; Yang et al., 2021). Additionally, the substantial pH reduction observed upon blueberry product incorporation (Fig. 4) creates an acidic environment that further compromises bacterial membrane integrity and metabolic functions, making cells more susceptible to HHP, since acidic conditions increase microbial susceptibility to HHP (Aganovic et al., 2021). While these mechanisms present a plausible explanation for the observed synergism, further research is required to clearly define the mode of action and identify the underlying molecular mechanisms involved. During storage, the continued presence of these additives prevents regrowth of any surviving *L. monocytogenes* cells, while the structural damage caused by HHP ensures that the cells remain vulnerable. This synergistic interaction ensures the safety of the food product extending the microbiological shelf life by maintaining low levels of spoilage and pathogenic microorganisms. These findings can explain the complementary actions of HHP and nisin or blueberry product and how their combined use can lead to enhanced microbial inactivation and prolonged shelf life of food products.

Matrix appears to have a significant role in the observed differences in inactivation and synergism between beef and plant-based burgers. Inactivation levels at 500 MPa were higher in beef patties (Fig. 6; Suppl. Figure SF1), likely due to their lower pH ( $5.68 \pm 0.03$ ) compared to plant-based patties ( $6.45 \pm 0.05$ ), as microorganisms are generally more pressure-sensitive in acidic environments (Aganovic et al., 2021; Cheftel and Culioli, 1997; EFSA, 2022; Syed, Buffa, Guamis, and Saldo, 2016). Similar results have been reported by Porto-Fett et al. (2020), where STEC and *L. monocytogenes* demonstrated higher sensitivity after HHP treatment of beef burger samples at 600 MPa regardless the processing time. Interestingly, a higher synergistic effect between HHP and nisin was observed in plant-based compared to beef patties (Suppl. Table ST10), consistent with the 132-day storage results, where greater combined effectiveness of both additives was also observed in plant-based burgers following HHP treatment (Fig. 1). The simpler and more homogeneous composition of the plant-based matrix, which likely allows better diffusion and activity of the antimicrobial agents during HHP treatment, may help explain this observation. The composition of a food matrix, including factors such as proteins, lipids, carbohydrates, and fat content significantly influences the efficacy of antimicrobial agents, especially when combined with HHP (Oulahal and Degraeve, 2022). Studies have demonstrated that the presence of fat and certain food additives in meat products can diminish the antimicrobial efficacy of compounds like essential oils, likely due to dilution effects or interactions with lipid content (Wang et al., 2024). A possible enhancement of the synergistic effect could be achieved with higher concentrations of the additives. For nisin, the most recent EFSA opinion (EFSA, 2017) considered the extension of its use to heat-treated meat products with a maximum level of 25 mg/kg, compared to the 12.5 mg/kg applied in this study. This suggests that higher concentrations could be feasible in such matrices and may further strengthen the synergistic effect in beef products. However, additional studies are required to confirm the efficacy and assess potential impacts on product quality and consumer acceptance. Additionally, structural differences between plant-based and meat matrices can impact the stability and bio-accessibility of antimicrobial compounds, further influencing their effectiveness (McClements, Das, Dhar, Nanda, and Chatterjee, 2021).

The effect of HHP, either alone or in combination with nisin and blueberry product, was evaluated in non-inoculated beef and plant-based burgers to investigate the growth behavior of spoilage microorganisms (APC, LAB, *Clostridium* spp., *Enterobacteriaceae*, *Pseudomonas* spp., and *B. thermosphacta*) during a 132-day storage period under slight temperature abuse (7 °C).

Beef is highly perishable due to its rich nutrient composition and

favorable pH and  $a_w$ , which promote microbial growth and subsequent spoilage (Nema et al., 2022; Pellissery, Vinayamohan, Amalaradjou and Venkitanarayanan, 2020). In this study, HHP treatment at 600 MPa for 3 min significantly reduced all the tested microbial groups in beef patties immediately after treatment whereas nisin and blueberry product alone did not result in statistically significant microbial reduction prior to HHP treatment. However, a rapid recovery of APC and *Pseudomonas* spp. was observed during storage (Fig. 2 and Table 2). This regrowth aligns with previous studies reporting similar patterns in HHP-treated meat products, where microbial recovery occurred due to the survival of sub-lethally injured cells. For example, Jung, Ghouh and de Lamballerie-Anton (2003) observed only slight inhibition of aerobic microbial populations in beef meat treated with HHP (520 MPa, 260 s), with microbial recovery occurring 14 days post-treatment during storage (4 °C). Similarly, Argyri, Papadopoulou, Sourri, Chorianopoulos and Tassou (2019) reported an increase in *Pseudomonas* spp. in chicken fillets stored at 4 °C after HHP treatment (500 MPa, 10 min). Despite the observed initial microbial reductions, no synergistic effect between HHP and the tested antimicrobials (nisin and blueberry product) was found for APC and *Pseudomonas* spp. In all samples (control, nisin and blueberry), both microorganisms reached the spoilage threshold of 6-7 log CFU/g, considered the onset of spoilage for meat products (Feiner, 2006), within the first 27 to 34 days of storage. The only exception was the samples treated with the blueberry product, where APC counts remained below 6 log CFU/g for up to 55 days. This lack of combined effect aligns with findings from Malinowska-Pańczyk and Kołodziejska (2009), who demonstrated that the combination of HHP with chitosan did not achieve lower bacterial counts than HHP alone in minced pork during storage (5 °C). The resistance of Gram-negative bacteria to the action of antimicrobial agents, such as *Pseudomonas* spp., due to their protective outer membrane, likely explains this limited effect (Breijyeh, Jubeh and Karaman, 2020). In contrast, the growth of LAB and *B. thermosphacta* was delayed by nisin and blueberry product when combined with HHP. This enhanced efficacy can be attributed to the higher susceptibility of Gram-positive bacteria to antimicrobials like nisin and phenolic compounds contained in blueberry product (Helander and Mattila-Sandholm, 2000). Gram-positive bacteria lack an outer membrane, which makes them more vulnerable to antimicrobial agents. Following the initial injury caused by HHP, nisin and phenolic compounds from the blueberry product appear to further damage the already-affected cells, enhancing microbial control during storage. Campus (2010) and Garriga, Aymerich, Costa, Monfort and Hugas (2002) also highlighted that combining HHP (400 MPa, 10 min) with natural antimicrobials, such as nisin, effectively inhibited the growth of Gram-positive spoilage microorganisms in meat products during storage (4 °C).

The demand for plant-based meat alternatives has grown significantly in recent years, driven by increasing consumer interest in sustainable and health-conscious dietary choices (Zhao et al., 2022). However, Wild et al. (2014) reported that plant-based meats, due to their neutral pH, high protein and moisture content, are just as susceptible to microbial growth as traditional ground beef. In this study, HHP treatment (600 MPa, 3 min), either alone or combined with additives, not only significantly reduced the indigenous microflora in non-inoculated plant-based patties immediately after treatment but also maintained microbial counts at very low levels over an extended storage period. These findings are particularly noteworthy, as the majority of recent studies indicate that plant-based meat analogues stored under refrigerated conditions remain microbiologically stable and unspoiled for only 7–14 days. For instance, Liu et al. (2023) reported that total aerobic bacteria, LAB, coliforms, and yeast/mold in pea-based meat analogues reached the spoilage threshold (6–7 log CFU/g) by day 10 of refrigerated storage. Similarly, plant-based sausage patties stored at 7 °C reached a spoilage threshold of 6 log CFU/g for total aerobic microorganisms within 10 days, while 14 days were required when stored at 4 °C (Cook, Northcutt, and Dawson, 2024). Tóth et al. (2021) observed that aerobic colony counts began increasing within just 5–7 days in

refrigerated vegan meatballs. Additionally, recent studies have highlighted the growth of *Pseudomonas fluorescens* in pea-based meat alternatives, with counts exceeding 6 log CFU/g after 10 days of storage at 4 °C (Liu et al., 2023). In contrast, this study demonstrated that after HHP treatment, *Pseudomonas* spp. counts remained mostly below the detection limit throughout the 132-day storage period at 7 °C (Table 3), indicating the high efficacy of HHP alone or in combination with additives. Although *Clostridium botulinum* has been identified as a concern in vacuum-packaged plant-based meat products (Pernu, Keto-Timonen, Lindström and Korkeala, 2020), *Clostridium* spp. was not detected in all samples before the treatment in our study. Given that both nisin and the blueberry product have demonstrated antimicrobial activity against *Clostridium* spp. in previous research (Das et al., 2017; Garde, Gómez-Torres, Hernández and Ávila, 2014; Gharsallaoui et al., 2016; Udompittkul, Paredes-Sabja and Sarker, 2012), their combination with HHP presents a promising strategy to mitigate this issue in plant-based meat analogues.

It is important to note that no synergism between HHP and the additives (nisin and blueberry product) was observed during storage. This outcome may be explained by the fact that HHP alone was sufficient to keep the samples microbiologically stable and safe for 83 days. Beyond this time point, the antimicrobial activity of nisin and blueberry product likely diminished or was no longer effective. Nisin's stability is influenced by factors such as pH, temperature, and storage conditions (Abee and Delves-Broughton, 2003). For example, Mohammadi and Jodeiri (2014) reported that nisin remains stable at low temperatures but can degrade at higher temperatures or over extended storage periods, leading to reduced antimicrobial efficacy.

When comparing the effectiveness of HHP on the spoilage microorganisms of non-inoculated beef and plant-based burgers, it is evident that HHP maintained the indigenous microflora at low counts for a longer period in plant-based patties. However, it is worth mentioning that the initial microbial loads of APC, LAB, *Enterobacteriaceae*, *Pseudomonas* spp., and *B. thermosphacta* were significantly higher ( $p < 0.05$ ) in beef burgers (Suppl. Table ST11). This could explain the faster growth of these microorganisms in beef compared to plant-based burgers following HHP treatment. The reason for the higher initial microbial load in beef has been extensively described in research and is primarily attributed to potential issues during slaughterhouse operations, handling practices, or chilled storage before reaching the market. In contrast, the lower initial microbial load in plant-based meat products can be attributed to the processing steps they undergo (e.g., extrusion, heating, cooling, drying, and coagulation) before being sold (Kyriakopoulou, Dekkers and van der Goot, 2019; Liu et al., 2023; Zhang, Chen, Kaplan and Wang, 2022).

Finally, the physicochemical properties of the non-inoculated beef and plant-based burgers were evaluated during storage after the HHP treatment. The incorporation of blueberry product significantly reduced the pH and changed the color attributes ( $L^*$  and  $C^*$ ) resulting in a darker appearance in both beef and plant-based burgers, while nisin addition showed no notable effect (Figs. 6, and 7). This pH reduction is due to the presence of organic acids, mainly citric acid, and a diverse range of phenolic compounds, including anthocyanins, phenolic acids, flavonols, and flavan-3-ols, which all together contribute to the acidic nature of the blueberry product (Cortez, Luna-Vital, Margulis and Gonzalez de Mejia, 2017; Khoo, Azlan, Tang and Lim, 2017). The significantly ( $p < 0.05$ ) higher initial pH in plant-based burgers compared to beef patties (Suppl. Table ST6) is consistent with findings in other studies where plant-based analogues generally show a neutral to slightly alkaline pH, which differs from the slightly acidic pH of animal-based meat (Liu et al., 2023; Porto-Fett et al., 2020). This difference in initial pH is a critical factor influencing the HHP efficiency as discussed above. It is worth noting that the commercial formulation of the plant-based matrix used in this study typically includes additives such as antioxidants and acetic acid, which contribute to a lower pH. However, these additives were excluded in the present study to avoid any potential interference as mentioned in

Materials and Methods. Therefore, it can be postulated that their incorporation could enhance microbial inactivation during HHP. Nevertheless, further research is needed to clarify their potential interactions with the antimicrobial agents used in this study, as these interactions may influence the overall efficacy of the treatment.

In terms of color characteristics, HHP treatment significantly increased the lightness ( $L^*$ ) values in beef patties, resulting in a lighter color (Suppl. Table ST9). This change may be attributed to myoglobin denaturation, displacement of heme, and the oxidation of ferrous myoglobin to ferric myoglobin (Rajendran, Mallikarjunan and O'Neill, 2022). Jung et al. (2003) reported that metmyoglobin levels decrease at pressures up to 300 MPa but increase at higher pressures, further influencing color changes. Similarly, Carballo, Fernandez, Carrascosa, Solas and Colmenero (1997) found that higher pressures increased lightness and reduced redness, particularly in high-fat patties, which aligns with the current findings. Such discoloration could pose challenges for marketing pressurized raw meat, as color plays a key role in consumer acceptance. Conversely, the absence of myoglobin in plant-based burgers resulted in no significant changes to their color attributes ( $L^*$  and  $a^*$ ) following HHP treatment (Suppl. Table ST9). These findings are consistent with Porto-Fett et al. (2020), who observed no significant impact of HHP at pressures up to 600 MPa for 12 min on the color of plant-based burgers. The stability of color and other physicochemical properties (pH and  $a_w$ ) during storage after HHP, despite microbial development, is consistent with previous reports on vacuum-packed products. Stable pH values have been observed in beef (Chasco, Alzueta, Beriain and Insausti, 2003; Frank et al., 2020) and in plant-based burgers (Neo, How, Kong, Talib and Pui, 2024) stored under vacuum. Likewise, the color stability aligns with the protective effect of vacuum packaging, where low oxygen levels favor deoxymyoglobin formation and limit oxidative discoloration (Gill, 1992; Zhu et al., 2024). Moreover, in plant-based burgers the pH and color did not significantly change immediately after HHP (in contrast to beef burgers), highlighting the greater potential of HHP for application in plant-based meat analogues compared to beef products. Overall, this enhanced microbial and physicochemical stability could support broader distribution opportunities, facilitating long-distance and international supply chains, while also providing a financial advantage for food business operators (FBOs) by reducing spoilage and minimizing waste. However, factors such as lipid oxidation, color degradation, and sensory quality should also be considered when evaluating overall shelf life and to ensure successful large-scale implementation.

## 5. Conclusions

This study highlights the potential of combining HHP with natural antimicrobials (nisin and blueberry product) as an effective strategy to enhance food safety and quality in beef and plant-based burgers during storage. The matrix composition (beef or plant-based) had a significant role on the synergism between HHP and the antimicrobials with a higher effect observed in plant-based burgers when nisin was combined with HHP. While the combination of nisin or blueberry product with HHP did not further extend the microbiological shelf life of plant-based burgers compared to HHP alone (up to 83 days), it nevertheless provided significant benefits of enhancing food safety by reducing the risks associated with *L. monocytogenes*. In beef burgers, microbial stability was shorter, with regrowth observed within 27–34 days after HHP treatment.

From an industrial perspective, the combination of HHP with nisin and blueberry product emerges as a great potential for developing safe, clean-label, minimally processed plant-based meat analogues with extended shelf life. Moreover, the use of nisin and blueberry product could allow the application of lower pressure levels while maintaining microbial safety, thereby potentially reducing processing costs and energy demands. However, the pigmentation effects of anthocyanins from the addition of blueberry product may pose challenges for consumer

acceptance. Nevertheless, the inclusion of blueberry-derived compounds may provide added health benefits due to their antioxidant properties while also helping to mask the discoloration typically observed in beef after HHP treatment. Although these findings are promising, further research, especially sensory evaluations and consumer studies, is essential before considering large-scale industrial application. This will help ensure that such preservation strategies align with both industry standards and consumer expectations.

#### CRedit authorship contribution statement

**Nikolaos Giannoulis:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Theocharia Tsagkaropoulou:** Writing – review & editing, Visualization, Methodology, Investigation, Conceptualization. **Carolina Bocigas Martin:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Miriam Ortega-Heras:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Mario González-Angulo:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Beatriz Melero:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Kimon Andreas G. Karatzas:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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#### Declaration of competing interest

None.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ifset.2025.104314>.

#### Data availability

Data will be made available on request.

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