Meat Science 236 (2026) 110072

Contents lists available at ScienceDirect

Meat Science

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

Nitrite reduction in cooked ham: an organoleptic and food safety concern?

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ARTICLE INFO

Keywords:
Challenge testing
Clean tech
Clostridium sporogenes
Human health
Listeria monocytogenes
Scientific research

ABSTRACT

Cooked meat products, particularly ham, are widely consumed, and reducing nitrite levels has become a priority due to health concerns and regulatory pressure. This study evaluated the microbiological safety, technological performance, physicochemical properties, and sensory attributes of whole cooked ham formulated with reduced nitrite (from 150 to 80 ppm) during shelf life. Microbiological analyses were conducted every 15 days, including total viable counts (TVC), lactic acid bacteria (LAB), *Enterobacteriaceae*, *Staphylococcus aureus* and *Escherichia coli.* TVC and LAB remained below the safety threshold ($<10^4$ CFU/g), while all other parameters were below detection limits. Sulphite reducing *Clostridium* spores, *Salmonella* spp. and *Listeria monocytogenes* were absent from all samples. Challenge testing with *L. monocytogenes* and *Clostridium sporogenes* was performed to assess the product's ability to inhibit pathogen growth under simulated storage conditions (up 35 and 90 days, respectively) and temperature abuse conditions (8 °C). The reduced-nitrite ham formulation effectively inhibited the growth of *C. sporogenes* and delayed the growth of *L. monocytogenes*. Technological assessments included colour measurements, water retention capacity, and texture profile analysis (TPA), with no significant differences observed between the standard and nitrite reduced formulations ($P > 0.05$). Physicochemical parameters such as pH (6.0–6.2), water activity (a_w, 0.9669–0.9482), and residual nitrite content (4 to 1 mg/kg) were evaluated at 0, 45 and 90 days. These findings demonstrate that reducing nitrite levels to 80 ppm can ensure the product safety and quality, as evidenced by stable physicochemical properties and the preservation of sensory characteristics such as appearance, odour, texture, and flavour.

1. Introduction

The rise in pork meat consumption in recent years has been accompanied by increased awareness of food safety and a growing demand for higher quality standards (Nam et al., 2010). Traditional cooked ham, a ready-to-eat (RTE) pork product, is widely consumed due to its convenience, sensory characteristics and high protein content (Jiménez-Colmenero et al., 2010; Melro et al., 2020; Ran et al., 2021).

The production of cooked ham involves several processing steps that significantly alter the product's microbial composition and load. Raw materials, particularly raw meat, harbour a diverse microbiota, including spoilage bacteria such as *Brochothrix* spp., *Pseudomonas* spp., lactic acid bacteria, and members of the *Enterobacteriaceae* family (Blanco–Lizarazo et al., 2022; Pennacchia et al., 2011; Stoops et al., 2015). While the cooking process substantially reduces both spoilage and pathogenic bacteria, the final microbial profile is influenced by factors such as the thermal processing method, storage conditions, and variability among raw material batches. Furthermore, recontamination can occur during post-cooking steps, such as cooling or packaging, stages, particularly in sliced products (Garrett et al., 2008; Kusumaningrum et al., 2003).

When stored at the recommended temperature of 0 °C to 4 °C, the shelf life of whole cooked ham is approximately 70 days, while sliced products typically last around 35 days (Martins & Leal Germano, 2011). Ready-to-eat products are highly susceptible to microbial cross-contamination, including by pathogens such as *Listeria monocytogenes*,

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https://doi.org/10.1016/j.meatsci.2026.110072
Received 10 October 2025; Received in revised form 13 February 2026; Accepted 23 February 2026
Available online 24 February 2026
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during the unmoulding and handling stages (Horita et al., 2018; Nieto et al., 2023). Strict temperature control is therefore essential to maintaining both safety and quality. However, large-scale distribution often exposes RTE products to temperature fluctuations and abuse, during transport and storage period.

Nitrite, an authorised preservative (E-249 and E-250 for potassium and sodium, respectively), has been used for decades in cooked ham formulations. Beyond its role in developing characteristic organoleptic properties, such as cured flavour, pinkish colour, and texture, nitrite also plays a key role in ensuring the microbiological safety, product stability, and shelf life of meat products (Alahakoon et al., 2015; Carvalho et al., 2024; Flores & Toldrá, 2021). However, concerns have been raised regarding the formation of *N*-nitroso compounds (NOCs), including nitrosamines, which are carcinogenic and have been linked to colorectal and gastric cancers (Bouvard et al., 2015; Crowe et al., 2019; IARC, 2015; Lijinsky & Epstein, 1970). In the European Union, the maximum permitted level of nitrite in cooked meat products was established more than 20 years ago at 150 mg/kg of meat (European Commission, Scientific Committee for Food, 1997; EFSA, 2023; European Commission, 2008). Following recent health warnings, including a risk assessment conducted by the French National Agency for Food, Environmental and Occupational Health Safety (French Agency for Food, Environmental and Occupational Health, and Safety, 2022), the European Commission has proposed reducing this limit by nearly 50% to 80 mg/kg of meat (European Commission, 2023). Nevertheless, any reduction in nitrite levels must carefully balance the risks associated with nitrosamine formation with the need to maintain the antimicrobial efficacy of nitrites, particularly against *Clostridium botulinum* (Ávila et al., 2014; Lebrun et al., 2020). To this end, further research is required to evaluate the impact of reduced nitrite levels not only on microbiological safety, including pathogen growth kinetics, but also on physicochemical parameters such as colour stability and lipid oxidation. Monitoring residual nitrite levels throughout the product's shelf life is also important, as degradation over time may compromise preservative efficacy. Studies by Stegeman and Verkleij (2008) and Guéraud et al. (2023b) have demonstrated that residual nitrite levels in finished products typically decrease to 10–25% of the initially added amount.

In this context, this study aimed to evaluate the impact of reduced nitrite concentrations on the microbiological quality of cooked ham, with a particular focus on the growth of *L. monocytogenes* and *C. sporogenes* strain PA3679, a validated surrogate for proteolytic *C. botulinum* (Brown et al., 2012), (Diao et al., 2014; Lorenzo et al., 2018). In addition, key physicochemical properties were analysed, including pH, water activity (a_w), colour, texture profile, and nitrite concentration. Sensory analysis was conducted to evaluate the organoleptic acceptability of formulations with varying nitrite levels.

2. Material and methods

2.1. Sample preparation

Cooked ham was produced on a pilot scale. The formulations used in this study comprised 86% pork leg (8 mm sieve), water, ice, salt, phosphate, antioxidant, thickener and varying concentrations of sodium nitrite.

According to Regulation n° 1333/2008, the maximum limit of added sodium nitrite is 150 ppm, which corresponds to an addition of 150 mg of sodium nitrite per kg of meat, defining the standard product (A). Regulation n° 2108/2023 has updated this limit to 80 ppm of sodium nitrite, corresponding to product (B). A third formulation, product (C), contained a reduced nitrite concentration (ranging from 50 to 20 ppm of sodium nitrite), sufficient to maintain colour properties (Hornsey, 1957). A fourth formulation, product (D), was prepared without nitrite.

Three independent production batches were prepared on separate days. For each batch, a starting quantity of 80 kg of meat was minced and then divided into portions for each of the treatment formulations. From the resulting products, one ham was selected for analysis from each treatment formulation, within each batch, at each designated storage time point. The production process consists of mixing the minced raw pork meat with cold brine in a vacuum mixer operating at constant speed (TECMAQ, Sentmenat, Spain). The mixture was refrigerated at ≤4 °C for 24 h to allow maturation and nitrite binding to myoglobin. The next day, the mixture was filled into polyethylene casings (1–1.2 kg) and cooked under controlled conditions (high humidity; chamber temperature 77 °C; core temperature 72 °C; Verinox, Italy). After cooking, the cooked hams were immediately refrigerated (≤ 4 °C) and chilled overnight. Samples were transported in a controlled refrigerated environment. Some analyses were carried out using fresh samples. Sampling points included the initial time point after chilling (T0) and every 15 days thereafter, up to a 90-day shelf life. At each sampling point, a new ham was opened.

2.2. Microbiological analysis

At each sampling time, three cooked hams per formulation were aseptically opened. For each ham, a single composite sample was prepared by collecting and pooling 25 g of meat from seven different locations of the piece into one sterile stomacher bag (BagLight PolySilk 400 bag, Interscience, Saint-Nom-la-Bretéche, France), and mixed with 225 mL of sterile Buffered Peptone Water (BPW, Biokar Diagnostics, Beauvais, France). Samples were homogenized using a laboratory blender (BagMixer 400 S, Interscience) for 30 s. Afterwards, serial decimal dilutions were prepared in sterile Ringer's (Ringer solution ¼ strength, Biokar Diagnostics) solution for enumeration of total viable colony counts (TVC) on Plate Count Agar (PCA, Biokar Diagnostics – International Organization for Standardization, 2013) at 30 °C for 72 h; lactic acid bacteria (LAB) on de Man Rogosa & Sharp agar (MRS, Biokar Diagnostics – International Organization for Standardization, 1998) at 30 °C for 72 h; *Enterobacteriaceae* on RAPID *Enterobacteriaceae* Agar (Bio-Rad, Antibes, France – International Organization for Standardization, 2017b) at 37 °C for 24 h; *Escherichia coli* on Tryptone Bile X-glucuronate (TBX, Biokar Diagnostics – Instituto Português da Qualidade, 1986b) at 44 °C for 24 h; *Staphylococcus aureus* on Baird-Parker Agar (BPA, Biokar Diagnostics – International Organization for Standardization, 2021) at 37 °C for 24 + 24 h; and to detect sulphite reducing *Clostridium* spores in a recipe medium prepared according to Instituto Português da Qualidade (1986a), at 37 °C for 5 days. Microbiological analyses were conducted in duplicate, and the results were presented as log CFU/g. Detection of *Salmonella* spp. (International Organization for Standardization, 2017c) and *L. monocytogenes* (International Organization for Standardization, 2017a) were also performed.

2.3. Microbiological challenge tests

Microbiological challenge tests were conducted using two pathogens: *C. sporogenes,* inoculated prior to heat treatment, and *L. monocytogenes*, inoculated post-cooking onto sliced ham. All four ham formulations were inoculated with *C. sporogenes* spores and a cocktail of *L. monocytogenes* strains. The challenge tests followed the methodology described by Shao and Ramaswamy (2008) to *C. sporogenes* and by Sullivan, Jackson-Davis, Niebuhr, et al. (2012) for *L. monocytogenes*, with minor modifications.

2.3.1. Preparation of C. sporogenes spores

Clostridium sporogenes ATCC 7955 (ATCC, 2026) was cultured in Brain-Heart Infusion broth (BHI broth, Biokar Diagnostics) at 37 °C for 48 h under anaerobic conditions. A 0.2 mL aliquot was transferred to Campdem Sporulating Agar (CSA), previously prepared as described by Shao and Ramaswamy (2008) and incubated at 37 °C for 5 days under anaerobic conditions to induce sporulation. Spores were harvested by flooding plates with 10 mL of sterile distilled water and scraping the colonies with a sterile plastic loop (VWR, Mylan, Italy). Next, the spore

suspension was heated for 10 min at 80 °C. Hereafter, the spores were washed with distilled water three times, centrifuged at 4.000 rpm for 15 min at 4 °C (Centrifuge 5425R, Eppendorf, Hamburg-Nord, Germany), and suspended in sterile distilled water (around 10^5 spores/mL). The spore suspension was immediately used to inoculate the ham samples.

2.3.2. Preparation of *L. monocytogenes* inocula

Seven *L. monocytogenes* strains, deposited in the culture collection of *Escola Superior de Biotecnologia* (Porto, Portugal) as described by Carvalho et al. (2024), were included: *L. monocytogenes* 2542 (Magalhães et al., 2009), *L. monocytogenes* FSL J1–177 (De Jesús & Whiting, 2003), *L. monocytogenes* FSL J1–031 (De Jesús & Whiting, 2003), *L. monocytogenes* FSL N3–013 (Bille & Rocourt, 1996), *L. monocytogenes* FSL R2–499 (Centers for Disease Control and Prevention, 2000); *L. monocytogenes* FSL N1–227 (Centers for Disease Control and Prevention (CDC), 1998), and *L. monocytogenes* MF4077 (Møretrø et al., 2017).

A loop of 10 μL of each *L. monocytogenes* strain was suspended in 5 mL of BHI broth and incubated at 37 °C overnight (pre-inoculum). A 1% (*v*/v) aliquot was transferred to fresh BHI broth and refrigerated at 4 °C for 7 days to allow the acclimation of the bacteria to low temperatures, according to International Organization for Standardization, 2019. Once the culture reached 8–9 log CFU/mL, cells were centrifuged at 7000 rpm for 5 min at 20 °C and washed twice with Ringer's solution. The seven strains were combined in one sterile Eppendorf (DeltaLab, Rubi, Spain) and diluted until 6 log CFU/mL, to produce the final cocktail for ham inoculation.

2.3.3. Sample preparation and inoculation

For the challenge test with *C. sporogenes*, uncooked ham mass was frozen to preserve batch consistency. Afterwards, a laboratory-scale ham was produced by mimicking the industry conditions. The inoculum at 1% (*v/w*) was aseptically added to portions of 25 g, which were then cased in aseptic conditions, manually homogenized for 30 s, and sealed under anaerobic conditions to enhance the growth of the spores. Therefore, it was cooked in a laboratory stove until the product reached the optimal temperatures (77 °C bath/stove and 72 °C core temperature), monitored with a data logger thermometer (HH374, Omega, Connecticut, USA). Samples were chilled overnight to simulate industrial contamination occurring prior to heat treatment. This process was repeated for each ham formulation, and from each batch. The samples were stored at 4 °C and 8 °C for the 90-day shelf life. Analyses were conducted at each sampling point (every 15 days) as described previously for the detection of *Clostridium* spores, using CSR agar (Instituto Português da Qualidade, 1986a). Enumeration of vegetative cells of *C. sporogenes* was performed on Tryptone Sulphite Cycloserine (TSC) agar (VWR Chemicals, Pennsylvania, USA - International Organization for Standardization, 2023). A storage temperature of 8 °C was selected based on the findings of Jofré et al. (2019), who reported that domestic refrigerator temperatures typically fluctuate between 6 °C and 8 °C, with 8 °C representing a worst-case scenario.

For *L. monocytogenes*, 10–12 g slices were aseptically placed into sterile sealing bags (PA EVOH PA PEBD, 100 μm; Vizelpas, Portugal) and inoculated with 0.1 mL of the strain cocktail (initial inoculum: 4.8 ± 0.6 log CFU/g), evenly distributed over the surface. The bags were flushed with a gas mixture containing 80% nitrogen and 20% carbon dioxide (UN 1956, FL 20CO2 N2, Gasin, Portugal), sealed using a Multivac-Gastrovac system (Multivac Sepp Haggenmüller KG, A300/41/42, Germany), and stored at 4 °C and 8 °C for 35 days.

Sampling was performed every 7 days. Enumeration of *L. monocytogenes* was carried out on Polymyxin Acriflavin Lithium chloride Ceftazidime Aesculin Mannitol (PALCAM) agar (Biokar Diagnostics) at 37 °C for 48 h. Analyses were performed in duplicate, with three independent replicates per formulation. Results were expressed as log CFU/g.

2.3.4. Measurement of package gas composition

The gas composition in each bag containing *L. monocytogenes*-inoculated sliced ham was measured using a Dansensor Checkmate 3 gas analyser (AMETEK MOCON, Ringsted, Denmark). To analyse the headspace gas, a syringe was inserted through the surface of selected packages from each ham formulation, allowing the analyser to measure oxygen and carbon dioxide concentrations (Raines & Hunt, 2010).

2.4. Physical-chemical analysis

All analyses were performed at three time points, during the product's shelf life: the beginning (T0), the midpoint (T45), and the end of the shelf life (T90).

2.4.1. pH and water activity measurements

Three pH measurements for each sample were conducted using a pH-meter (Sension+ PH3 Benchtop pH & ORP meter, Hach, Colorado, USA). The pH meter was calibrated before each use according to the manufacturer's instructions, using commercial standard buffers (pH 4.0, 7.0, and 10.0) that were brought to room temperature (approximately 20–22 °C). To ensure consistency and avoid temperature-related error, ham samples were also equilibrated to the same room temperature before measurement. All calibrations and sample readings were performed at this constant temperature. Water activity was measured using a LabMaster-a_w Neo water activity meter (Novasina AG, Lachen, Switzerland) at a constant temperature, in duplicate for each sample.

2.4.2. Colour analysis

Colour analysis was performed according to Carvalho et al. (2024), with D65 illuminant and 8-mm aperture. The determined ratios were chroma, the hue angle, and delta *E*, which characterizes the colour change over a period between samples ($\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5}$) (King et al., 2022; MacDougall, 1982). Measurements were taken at five randomly selected points on the surface. To ensure the colour stability and for an accurate measurement, the blooming time applied was between 20 and 30 min, according to Škrlep and Čandek-Potokar (2007).

2.4.3. Texture profile analysis

TPA analysis was conducted using a Texture analyser (TA.HDplusC, Stable Micro Systems, Surrey, UK) equipped with a 250 kg load cell and a 100 mm probe as described by Shin and Choi (2021) with minor modifications. Before the analysis, the samples were kept at room temperature, and an aluminium cylinder with 3 × 3 cm (diameter x heigh) was used to cut the samples. TPA test conditions involved a compression speed of 2 mm/s with a maximum strain of 75% and a 5-s wait interval between each compression. Each sample was measured 10 times, and the textural parameters analysed were hardness (N), fracturability (N), adhesiveness (N x s), chewiness (N x s), cohesiveness, springiness (mm), gumminess (N) and resilience.

2.4.4. Nitrite determination

Nitrite was determined by spectrophotometry according to Ribas et al. (2024). The determination was conducted with a sequential injection system equipped with a spectrophotometer to allow a quicker analysis (Fig. S1). For the spectrophotometric detection, an Ocean Optics (USA) USB 2000 charged coupled device detector (CCD), equipped with an illuminated cuvette as an incorporated light source. A Hellma (Germany) 178.711-QS flow cell with a 10 mm light path and 30 μL inner volume was used. Data acquisition was performed at 543 nm using the OceanOptics - Spectrasuite software, running in an HP CZC4401BQX computer.

For nitrite determination (Table S1), Griess reagent was aspired into the holding coil (Table S1, step A), followed by sample aspiration (Table S1, step B) and propelled to the spectrophotometer at wavelength 543 nm (Table S1, step C). Samples were run in triplicate, and the final

concentration was expressed as mg of NO_2 per kg of ham.

2.4.4.1. Reagents and solutions. The Griess reagent was prepared to a final concentration of 20 g/L of sulfanilamide and 2 g/L of N-(1-naphthyl)-ethylenediamine dihydrochloride (N1NED) in 0.5 mol/L of *ortho*-phosphoric acid (Ribas et al., 2024). The 100 mmol/L nitrite stock solution was prepared by dissolving the solid (Merck) in deionized water, diluted to a final concentration of 20 μmol/L, which was posteriorly used to prepare standard solutions in a range of 0.50–7.20 μmol/L. The solutions were prepared monthly by dissolving the corresponding quantity of the solids in deionized water.

The following solutions were prepared according to International Organization for Standardization, 1975 for extraction of meat products. Sodium tetraborate decahydrate (Merck) solution was prepared by dissolving 50 g in 1 L of heated solution water. The potassium hexacyanoferrate trihydrate solution (Reagent I) consisted of dissolving 106 g of the solid (Fluka) in 1 L of deionized water. The zinc acetate dihydrate solution (Reagent II) was obtained by dissolving 220 g of the solid (Sigma) in 1 L of deionized water.

2.4.4.2. Sample preparation. Sample preparation for nitrite extraction consisted of digesting the ham by grinding and homogenising 300 g, using a total of 10 g collected from five random places, and transferred into a 250 mL flask. Add 5 mL of borax, then 100 mL of water previously heated, and let the mixture heat for 15 min. After cooling, 2 mL of reagent I and 2 mL of reagent II were added, and the flask filled until it reached 200 mL. The mixture was left to rest for 30 min to separate the sediment and filter the supernatant with a polyester filter with 0.45 μm of diameter (CHROMAFIL® Pet −45/25, Macherey-Nagel, Germany).

2.5. Sensory analysis

The sensory impact of the reduction of nitrite concentration in cooked ham formulation was evaluated by carrying out a Difference from Control (DFC – Meilgaard, Civille and Carr, 2016) sensory test with 12 panellists from *Escola Superior de Biotecnologia – Universidade Católica Portuguesa.* Only one batch was used to assess the sensory impact, and only one session was held. The sensory evaluation was conducted in a sensory analysis laboratory equipped with individual booths and under white light (6500 K). Each participant received a sample of the traditional ham (A) marked as 'control' plus a sample of the same product marked with a three-digit code (blind control), plus samples of the other two ham formulations (B and C), also coded with a random three-digit code. Coded samples were presented to tasters in a pre-defined randomised order. Panellists were asked to evaluate the control and then the coded samples, and rate the perceived magnitude of difference in appearance, odour (orthonasal), texture and flavour from the "control" sample, using a continuous anchored scale of 10 cm (0 = similar to control, 10 = very different from the control). Water was supplied to clean the palate between tastings. The classifications obtained for each new ham formulation were determined and statistically compared to the "blind" control, using Kruskall-Wallis method. The ham without nitrite (D) was excluded from the sensory analysis due to its uncharacteristic grey colour.

A technological evaluation was also conducted, consisting of assessing the visual properties of the product, relative to water binding capacity (syneresis) and the capacity of slicing, related to the elasticity of the product and its capacity of not tearing apart.

The sensory panel was employed solely as an analytical tool for obtaining objective assessments. No personal data, subjective opinions, or identifiable information were collected, and the procedure posed no risks or discomfort to the assessors.

2.6. Statistical analysis

All data were presented as the mean of three batches, with the corresponding standard error of the mean $\left(\sigma/\sqrt{n}\right)$. Statistical analysis was conducted using IBM® SPSS-IBM Statistic Analytics 28.0 (IBM, Chicago, USA). The normality of the data for each condition was evaluated using the Shapiro-Wilk's test. A Two-Way ANOVA (univariate general linear model) with fixed terms (storage time and ham formulation) and random term (batch) was carried out to assess if the interactions between independent variables (including between storage time vs ham formulation) were significantly different (95% confidence level). The sample averages that followed a normal distribution were also compared using a 95% confidence interval. The Tukey's test was applied for multiple comparison purposes. Regarding samples with a non-normal distribution, Kruskal-Wallis' tests were conducted with 95% confidence to assess the interactions between variables. Regarding sensory analysis, non-parametric tests were used, Wilcoxon (to compare two independent samples) and Friedman (to test for differences between groups). A significance level of 5% was assumed in all tests performed.

3. Results and discussion

3.1. Microbiological analysis

Microbiological quality and safety were evaluated throughout the 90-day storage period. *Escherichia coli*, *S. aureus* and *Enterobacteriaceae* were below the detection limit of the enumeration techniques in all samples, while *Salmonella* spp., *L. monocytogenes* and *Clostridium* spores were not detected in any formulation. Interactions between fixed terms (storage time vs ham formulation) were significant ($P < 0.05$) whereas batch effects (random term) were not significant ($P > 0.05$).

According to previous studies, cooked ham that remains unopened (i.e., without post-processing handling) typically harbors only total viable counts (TVC) and lactic acid bacteria (LAB) (Doulgeraki et al., 2012; Ercolini et al., 2006), with counts generally not exceeding 4 log CFU/g (European Commission, 2005; FAO and WHO, 2023). Moreover, several studies have reported that spoilage LAB are the dominant microorganisms in unsliced cooked ham (Blanco–Lizarazo et al., 2022; Mustedanagic et al., 2023).

The evolution of TVC and LAB is presented in Figs. 1 and 2. In Fig. 1, a significant increase log values was observed over time ($P < 0.05$), with statistical differences detected at 15, 45, and 75 days. TVC and LAB generally increased up to day 75 and then decreased by day 90. These fluctuations may result from heterogeneous microbial distribution, nutrient depletion, accumulation of metabolic waste products (Pan & Ren, 2022; Wang et al., 2012), resource competition (Garrett et al., 2008), and low-oxygen conditions (Chmiel et al., 2025). Sodium nitrite concentrations (150 to 0 ppm) did not significantly affect bacterial levels ($P > 0.05$), likely due to the low initial microbial load (1–2 log). Despite refrigerated storage and low-oxygen packaging, spoilage bacteria such as LAB can still emerge and drive changes in ham quality (Cooksey, 2014; Doulgeraki et al., 2010). Their presence is attributed to introduction during processing and the survival of stress-tolerant strains through cooking temperatures. LAB are particularly adaptable, capable of forming biofilms on equipment surfaces and resisting stress with low substrate specificity, heightening cross-contamination risks (Luke et al., 2024; Samelis et al., 2006; Zagdoun et al., 2020). As the ham was vacuum-packed without post-cooking handling, the persistence of LAB and TVC over the 90-day shelf life likely reflects the survival and growth of stress-resistant strains that were present before packaging. Sodium nitrite concentration, ranging from 150 to 0 ppm, did not significantly affect their levels ($P > 0.05$). Overall, storage time had a statistically significant effect ($P < 0.05$) on all microbiological indicators analysed, leading to increased microbial counts for TVC and LAB. TVC generally increases over storage time, indicating a clear time effect ($P < 0.001$) on

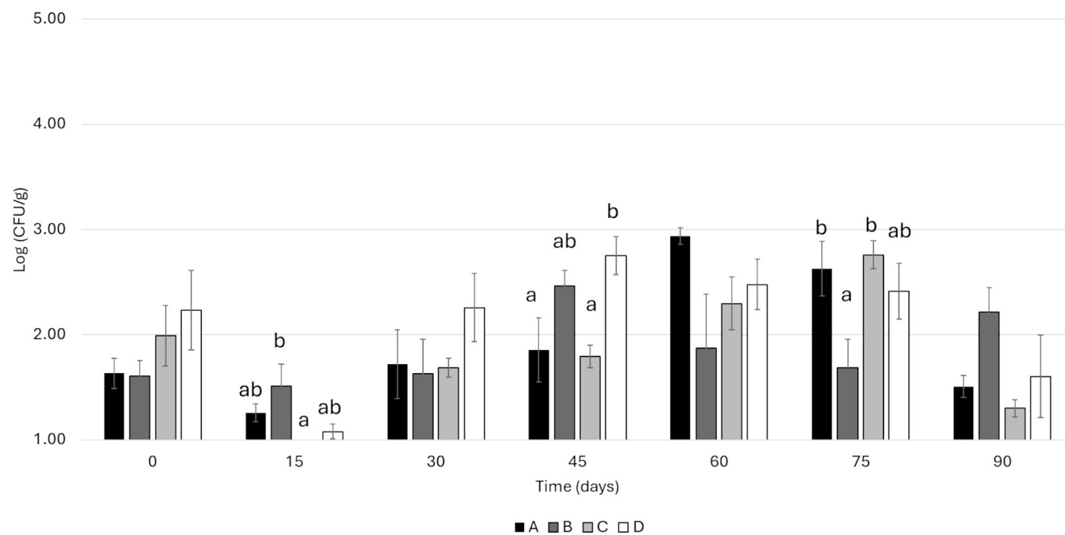

Fig. 1. Total Viable Counts (log CFU/g; mean of three batches ± SE) in ham formulations A (150 ppm added sodium nitrite) to D (no nitrite added). For a given time point, equivalent lowercase letters mean no significant differences between formulations ($P > 0.05$). Time points without any label do not present significant differences ($P > 0.05$).

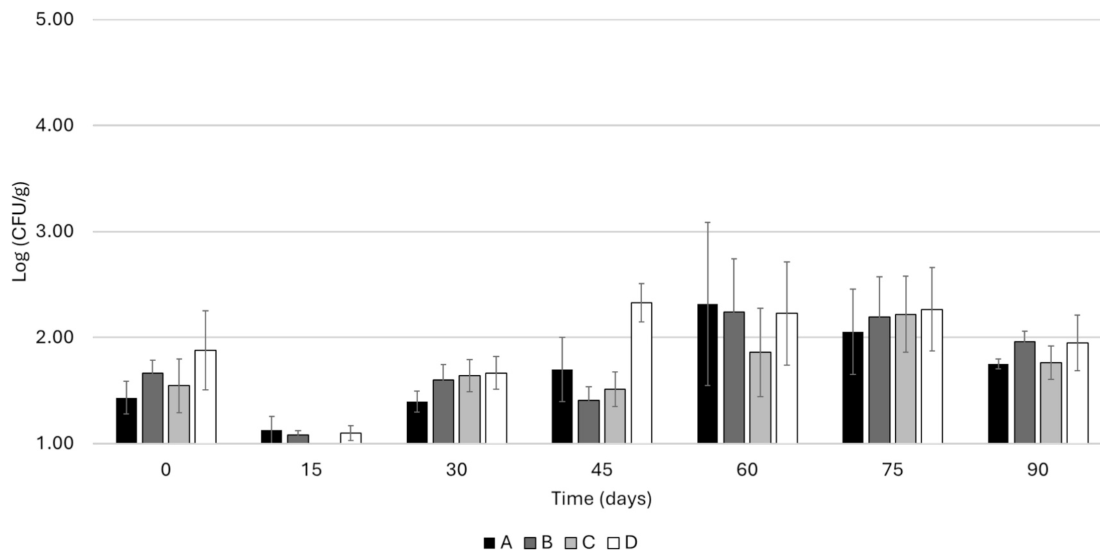

Fig. 2. Lactic acid bacteria (log CFU/g; mean of three batches ± SE) in ham formulations A (150 ppm added sodium nitrite) to D (no nitrite added). Time points without any label do not present significant differences ($P > 0.05$). Time points without any label do not present significant differences ($P > 0.05$).

microbial counts. The changing pattern across days is consistent with a time x sample interaction, meaning no single sample is uniformly best or worst for limiting TVC throughout storage. For LAB, there was no interactive effect ($P = 0.989$).

3.2. Microbiological challenge testing

Cooked ham inoculated with *C. sporogenes* was packed in plastic casings to simulate whole-piece conditions with minimal oxygen (Couvert, Divanac'h, Lochardet, Thuault and Huchet, 2019), while sliced ham inoculated with *L. monocytogenes* was packed under a modified atmosphere typical of *charcuterie* industry practices (Iacumin et al., 2020). Challenge tests should assess the growth potential of relevant pathogens, such as *L. monocytogenes*, a psychotropic pathogen with high relevance in refrigerated RTE meats, and *C. botulinum* (Lorenzo et al., 2018). Due to biosafety concerns, *C. sporogenes*, particularly strain PA3679 (ATCC, 2026), is widely used as a validated surrogate in food process validation, given its genetic and physiological similarity to proteolytic *C. botulinum*, especially in thermal resistance studies (Diao et al., 2014). These tests should be conducted under realistic storage conditions, including both recommended (4 °C) and temperature-abuse scenarios (8 °C). Interactions between fixed terms (storage time vs ham formulation) were significant ($P < 0.05$) and the influence of random term (batch) was not significant ($P > 0.05$). For *C. sporogenes*, the effect was observed as significant inactivation. The interaction effects are presented and discussed below.

The results of the challenge tests for *C. sporogenes* are depicted in Fig. 3 (and Table S3). At 4 °C, a reduction in vegetative cells was observed in all formulations containing nitrite (A, B and C) by day 30, with no further changes observed until the end of the storage period. A similar trend was observed at 8 °C for formulations A and B, whereas formulation C only showed a reduction by day 45. As expected, according to the antimicrobial activity of nitrite against *C. sporogenes* (Yetim et al., 2006), the observed reduction only occurred in the formulations with added nitrite (A, B and C), explaining the statistically significant differences ($P < 0.05$) between these and the nitrite-free

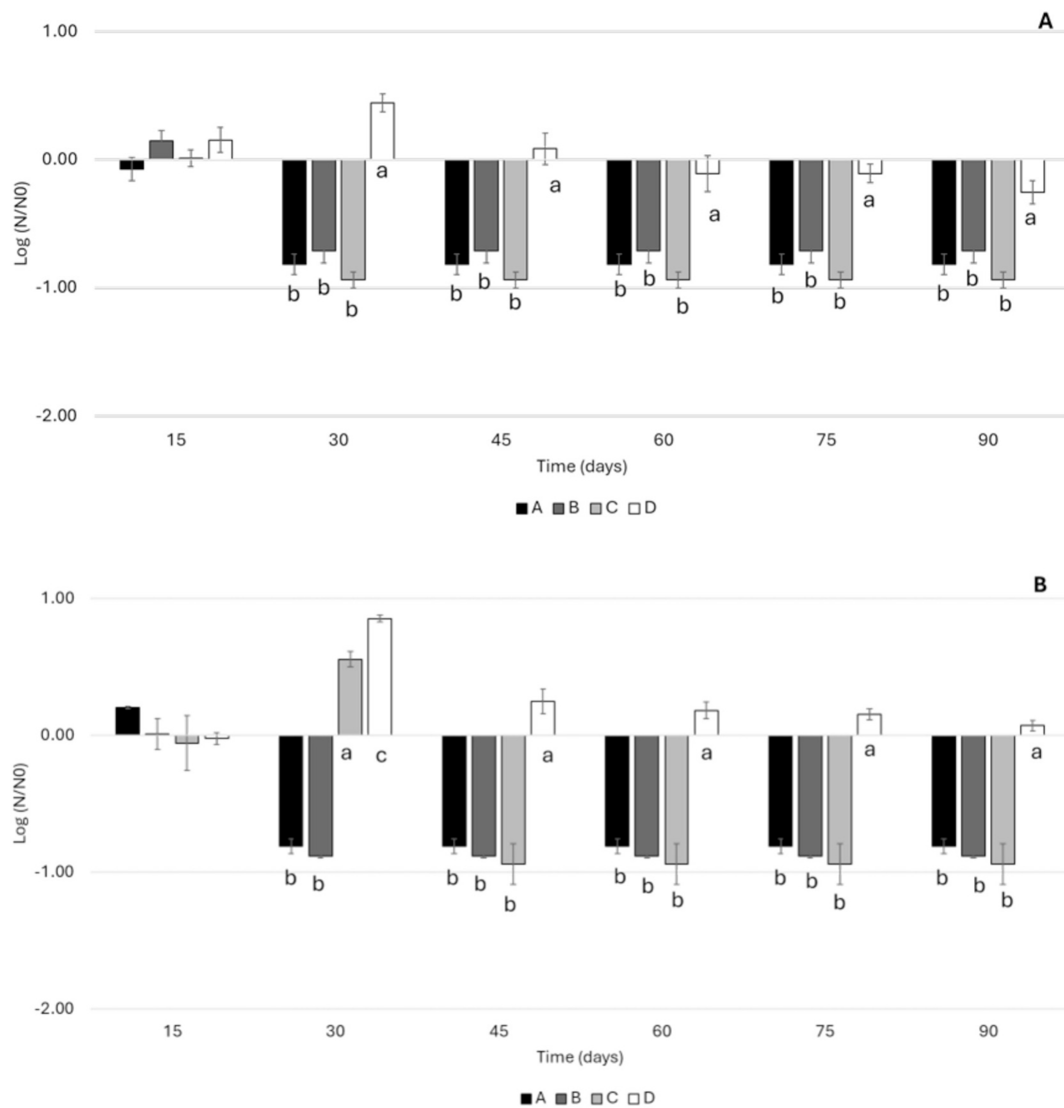

Fig. 3. Microbial challenge test (log N/N0; mean of three batches ± SE) of ham formulations A (150 ppm added sodium nitrite) to D (no nitrite added) for *C. sporogenes* vegetative cells at a refrigeration temperature of 4 °C (A) and abuse temperature of 8 °C (B). For a given time point, equivalent lowercase letters mean no significant differences between formulations ($P > 0.05$). Time points without any label do not present significant differences ($P > 0.05$).

formulation (D). Inhibition of the pathogen followed the patterns described by De Jong, Rombouts and Beumer (2004) and modelled by Hong et al. (2016), who demonstrated that *C. sporogenes* spores may germinate during heat treatment, even when followed by rapid cooling, and that vegetative cells can survive and persist at low temperatures. Although vegetative cell counts fell below the detection limit from day 30 onwards in formulations A, B and C (Fig. 3), spores were detected in 0.1 g of all samples during the 90-day shelf life, absent for 0.01 g of sample (Table S4) and remained at similar levels at both 4 °C and 8 °C. These findings are consistent with the work of Bhusal et al. (2021), who reported that although spores may persist, nitrite effectively inhibits their germination at refrigeration temperatures, eliminating all the vegetative cells.

Inoculation before the cooking process was intended to be a realistic simulation of ham mass contamination either from raw materials or from the environment, where nitrite levels are sufficient to act effectively against *C. sporogenes*. Since cooked ham is a refrigerated product, and storage temperatures should not exceed 8 °C (even in abuse conditions), suggesting that *C. sporogenes* does not germinate under these conditions after 30 days. In fact, vegetative cells were inhibited as early as day 15 at both 4 °C and 8 °C for formulations (A) and (B). However, in formulation (C), a decrease was only observed by day 30, suggesting a potential food safety risk during the early stages of storage. Nevertheless, storage at 4 °C clearly provides better control of *C. sporogenes* than 8 °C across all formulations. For *C. sporogenes*, at both temperatures, there were interactive effects ($P < 0.001$). For untreated samples at 4 °C, *C. sporogenes* shifted from survival to inactivation after 45 days, after which inactivation stabilized at approximately 0.2 log cycle. At 8 °C, no inactivation occurred. Additionally, for 30-day storage, lower nitrite concentrations also allowed the pathogen survival.

The results of the *L. monocytogenes* challenge testing at both 4 °C and 8 °C are presented in Fig. 4. Growth of the pathogen was observed throughout the 35-day storage period for all formulations. At 4 °C, *L. monocytogenes* growth was higher in formulations C and D, with significant differences ($P < 0.05$) from formulations A and B (with higher nitrite concentrations) observed on days 21, 28 and 35. However, during the first 21 days no significant differences ($P > 0.05$) in pathogen levels were observed between formulations. From days 21 to 28 growth began to diverge, and by day 35, significant increases ($P < 0.05$) were noted. In a general overview, a total growth of 2–2.5 log (N/N0) was observed from day 7 to day 35.

When submitted to the abuse temperature (8 °C), *L. monocytogenes*

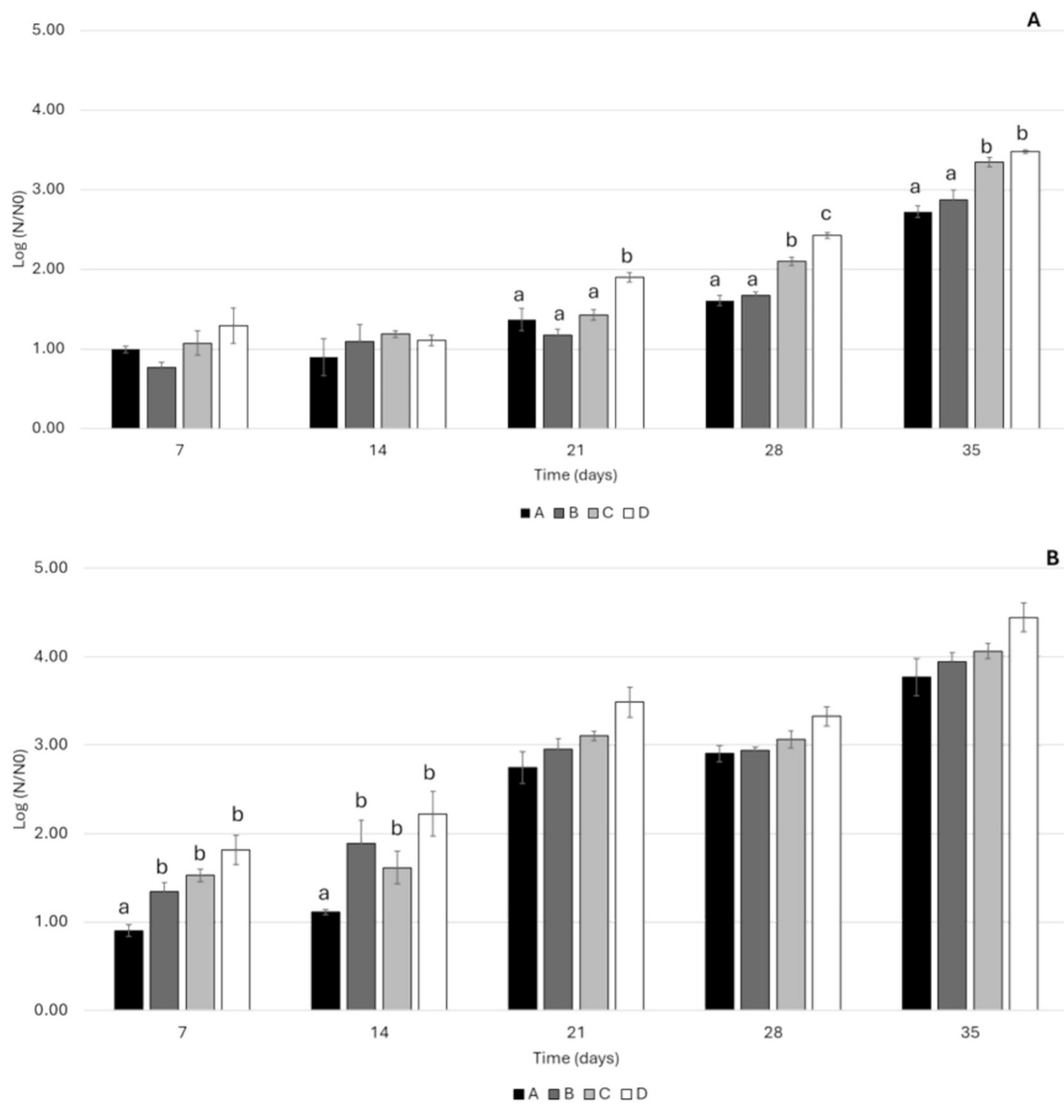

Fig. 4. Microbial challenge test (log N/N0, mean of three batches ± SE) of ham formulations A (150 ppm added sodium nitrite) to D (no nitrite added) for *L. monocytogenes* at a refrigeration temperature of 4 °C (A) and abuse temperature of 8 °C (B). For a given time point, equivalent lowercase letters mean no significant differences between formulations ($P > 0.05$). Time points without any label do not present significant differences ($P > 0.05$).

growth began earlier, between days 14 and 21 ($P < 0.05$) for all formulations, with a second significant increase from days 28 to 35 ($P < 0.05$), resulting in a total growth from day 7 to day 35 of 3–3.5 log (N/N0). Significant differences ($P < 0.05$) were observed for formulations D and A on days 7 and 14, respectively, which may be attributed to sample heterogeneity or a lower initial inoculum (Duffy et al., 1994). Modified atmosphere packaging was used to preserve the product, decelerating its spoilage (Devlieghere et al., 2001; Nychas & Skandamis, 2005; Skandamis & Nychas, 2002), and to guarantee similar conditions to the packed product produced in the industry (80% N_2/20%CO_2). MAP data is show in Table S5, showing that levels are stable during the 35-day of the study ($P > 0.05$). Overall, storage time had a statistically significant effect ($P < 0.05$), leading to increased *Listeria* survival at both temperatures. No interaction effects were observed ($P = 0.527$ for 4 °C and $P = 0.762$ for 8 °C).

Since nitrite concentrations decrease after cooking (Bonifacie et al., 2024), its effectiveness against *L. monocytogenes* may be limited. Studies by Sullivan et al. (2012)Sullivan et al. (2012) and McClure et al. (1991) have shown that nitrite can reduce the growth rate of *L. monocytogenes* in cooked meats but is not sufficient to inhibit it entirely. Cooked ham provides favourable conditions for *L. monocytogenes* growth, including suitable temperature, pH and a_w (Hospital et al., 2014). The *L. monocytogenes* populations in the inoculated products showed a lag phase during the first 14 days at 4 °C, which means that it took more time to reach a given density level (Saraiva et al., 2018). Considering that *L. monocytogenes* grows at low temperatures, studies from Rosso et al. (1996) emphasize the importance of minimizing the storage temperature (≤ 4 °C), to reduce the risk of the prevalence of the pathogen in RTE product (Uyttendaele et al., 2009). Following Regulation n° 2073/2005 and its changes (Regulation n° 2895/2024 - European Commission, 2024) RTE foods should have a *L. monocytogenes* load <100 CFU/g during the product shelf life, which is why the cold chain is one important step to a safer product (Gowda et al., 2024).

3.3. Physical-chemical analysis

Physical-chemical analyses for all parameters were performed at three distinct sampling times along the product's shelf life, namely day 0 (immediately before product cooling), day 45 (mid-shelf life) and 90 days (on the expiry date).

3.3.1. pH and water activity measurements

The pH and water activity (a_w) values are shown in Table S3. A significant decrease in pH was observed over time ($P < 0.05$), with differences between formulations evident at the end of storage (90 days), particularly for samples C and D ($P < 0.05$). These changes may be associated with microbial activity and strain-specific metabolic traits, as previously reported (Ducic et al., 2014; Pateiro et al., 2019). Formulations A and B remained relatively stable over time, suggesting that product (B) effectively maintains the technological properties equal to the standard product (A). Overall, pH values were consistent with those typically reported for unsliced cooked ham (6.0 and 6.2; Kročko et al., 2014)).

Water activity, a critical parameter affecting microbial stability and texture in cooked ham also decreased significantly during storage, with values ranging from 0.9669 to 0.9482, in line with literature data for cooked ham (Rakotondramavo et al., 2019). Formulation D consistently showed lower a_w values than the other formulations ($P < 0.05$), suggesting a potential effect of nitrite reduction on water-binding capacity (Visy et al., 2020). Significant interactions between storage time and ham formulation were observed for both pH and a_w ($P < 0.05$), while batch effects were not significant ($P > 0.05$). Despite these statistical differences, all values remained within technologically acceptable ranges (Kročko et al., 2014; Rakotondramavo et al., 2019).

3.3.2. Colour analysis

Colour is an important quality attribute of cured cooked meat products, strongly influencing consumer perception and purchasing decisions. Table 1 presents the colour parameters (L^*, a^*, b^*, Chroma, Hue and ΔE) of formulations A to D during storage. Lightness (L^*) did not differ among formulations or over time ($P > 0.05$). In contrast, redness (a^*) was consistently lower in formulation D ($P < 0.05$), which contained no nitrite, while formulations A to C showed comparable values, in agreement with Melios et al. (2024). Yellowness (b^*) differed only for formulation D at the end of storage ($P < 0.05$). Overall colour parameters were also calculated, based on $L^*a^*b^*$ values, and include Chroma, Hue and ΔE. Chroma remained unaffected by formulation or storage time ($P > 0.05$), whereas Hue values were significantly higher in formulation D at all sampling times ($P < 0.05$). ΔE, which translates the colour difference between the standard formulation (A) and all the other formulations, also showed significant differences for formulation D ($P < 0.05$), while formulations B and C remained below the perceptibility threshold to the human eye (<2), consistent with sensory results and previous reports (King et al., 2022). These differences are mainly attributed to nitrite reduction, which directly influences cured meat colour development (Karwowska et al., 2019; Macdougall et al., 1975). Overall, reducing sodium nitrite from 150 ppm to 80 ppm, as imposed by the regulation, or even to 50–20 ppm, did not compromise the typical pink colour of the products, in line with earlier findings (Melios et al., 2024; Shin et al., 2017). No significant individual or combined effects of time were observed for Chroma, Hue, or ΔE ($P > 0.05$).

3.3.3. Texture profile analysis (TPA)

Some authors (Romero et al., 2014; Schreuders et al., 2021) suggested that TPA can be used to perceive whether the product has a similar behaviour when some conditions are changed (e.g. food additives), mimicking a mastication process. TPA results are depicted in supplementary data - Fig. S2. No significant differences ($P > 0.05$) were found in TPA parameters (hardness, fracturability, cohesiveness, springiness, adhesiveness, chewiness, gumminess, and resilience) across formulations or time, indicating that nitrite reduction does not compromise texture.

3.3.4. Nitrite determination

Nitrite has a very particular role in the colour development of cured meat products through its interaction with myoglobin, directly influencing visual quality and consumer perception (Karwowska et al., 2019; Shakil et al., 2022). The analytical determination quantified only free nitrite ions (NO_2^-), not the added sodium nitrite ($NaNO_2$) salt. Consequently, the measured values were inherently lower than the formulation levels ($P < 0.05$); for example, addition levels of 150, 80, and 50–20 mg/kg $NaNO_2$ correspond to approximately 100, 53, and 33–13 mg/kg of nitrite ions, respectively (Fig. 5).

Residual nitrite levels were highest at day 0 and decreased significantly over time ($P < 0.05$), reflecting both chemical reactions during cooking, such as the formation of nitrosylmyoglobin and binding to proteins and lipids, and the natural conversion of nitrite to nitrate under acidic environments (Honikel, 2008; Lebrun et al., 2020; Merino et al., 2016; Pegg & Shahidi, 1997; Skibsted, 2011). Additionally, Honikel (2008), Pegg and Shahidi (1997), and Sebranek and Bacus (2007) describe that 10–30% of nitrite react with myoglobin, 20–40% react

Table 1
Colour measurements throughout storage (mean of combined batches ± SE) for ham formulations with varying nitrite concentrations.

Ham formulation	Storage time (days)	L^*	a^*	b^*	Chroma	Hue	ΔE
	0	58.0 ± 0.2^{a}	14.8 ± 0.1^{a}	1.5 ± 0.2^{a}	14.9 ± 0.1^{a}	5.9 ± 0.6^{a}	–
A	45	58.1 ± 0.8^{a}	15.2 ± 0.2^{a}	1.9 ± 0.1^{a}	15.3 ± 0.2^{a}	7.0 ± 0.3^{a}	–
	90	57.2 ± 0.9^{a}	14.4 ± 0.1^{a}	1.3 ± 0.1^{a*}	14.5 ± 0.1^{a}	5.3 ± 0.4^{a}	–
	0	58.8 ± 0.2^{a}	14.6 ± 0.1^{a}	1.7 ± 0.1^{a}	14.6 ± 0.1^{a}	4.3 ± 0.2^{a}	0.9 ± 0.1^{a}
B	45	58.3 ± 0.6^{a}	14.8 ± 0.2^{a}	1.2 ± 0.1^{a}	14.9 ± 0.2^{a}	4.4 ± 0.4^{a}	0.9 ± 0.1^{a}
	90	57.9 ± 0.9^{a}	14.1 ± 0.1^{a}	1.1 ± 0.1^{a*}	14.1 ± 0.1^{a}	4.6 ± 0.3^{a}	0.9 ± 0.1^{a}
	0	58.5 ± 0.1^{a}	14.5 ± 0.1^{a}	1.3 ± 0.1^{a}	14.6 ± 0.1^{a}	5.3 ± 0.4^{a}	0.9 ± 0.1^{a}
C	45	58.3 ± 0.2^{a}	14.5 ± 0.2^{a}	1.2 ± 0.1^{a}	14.5 ± 0.2^{a}	4.9 ± 0.3^{a}	1.7 ± 0.2^{a}
	90	57.6 ± 0.4^{a}	14.2 ± 0.1^{a}	1.0 ± 0.1^{a*}	14.2 ± 0.1^{a}	4.2 ± 0.2^{a}	0.9 ± 0.4^{a}
	0	58.9 ± 0.3^{a}	11.9 ± 0.2^{a*}	1.9 ± 0.2^{a}	12.1 ± 0.2^{a}	9.2 ± 0.7^{a*}	3.3 ± 0.2^{a*}
D	45	58.9 ± 0.3^{a}	11.1 ± 0.4^{a*}	2.7 ± 0.2^{a}	11.3 ± 0.4^{a}	12.5 ± 0.3^{a*}	4.5 ± 0.6^{a*}
	90	57.8 ± 0.1^{a}	11.2 ± 0.2^{a*}	2.2 ± 0.1^{a}	11.4 ± 0.2^{a}	11.0 ± 0.7^{a*}	4.7 ± 0.7^{a*}

A: 150 ppm added sodium nitrite; B: 80 ppm added sodium nitrite; C: 50–20 ppm added sodium nitrite; D: no nitrite added. Equivalent lower-case letters mean no significant differences between each time point, regardless of formulation ($P > 0.05$). Asterisk means significant differences between each formulation at the same time point ($P < 0.05$).

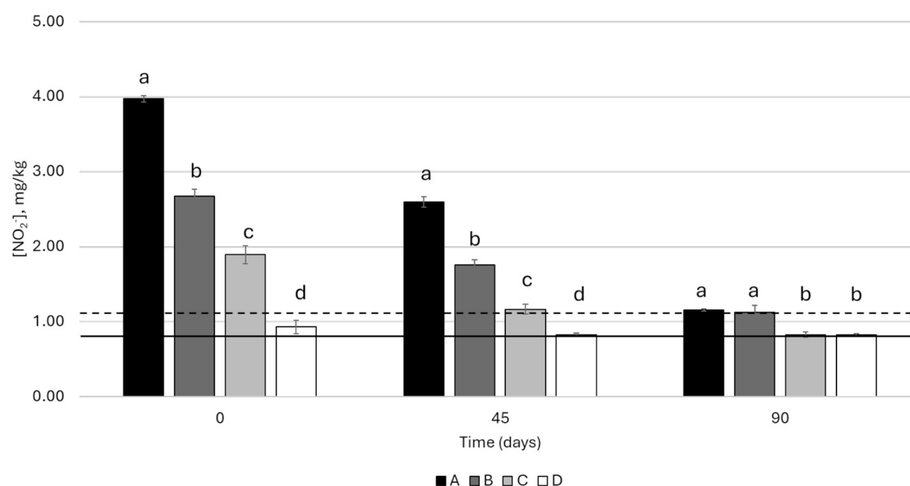

Fig. 5. Nitrite concentration (mg NO_2^-/ kg of ham; mean of three batches ± SE) of ham formulations A (150 ppm added sodium nitrite) to D (no nitrite added) during storage time. For a given time point, equivalent lowercase letters mean no significant differences between formulations ($P > 0.05$). Time points without any letter do not present significant differences ($P > 0.05$). LOQ means the limit of quantification and corresponds to the dashed line (1.16 μmol/L, corresponding to 1.07 mg/kg); LOD means the limit of detection and corresponds to the straight line (0.83 μmol/L, corresponding to 0.76 mg/kg).

with proteins, lipids and amino acids, and 20–40% is oxidized to nitrate, leaving only 5–20% of residual nitrite that can be directly determined by quantification methods. This behaviour is consistent with the known instability of nitrite in cured meat systems and explains the progressive reduction observed throughout storage, in accordance with Surace et al. (2025), Ferreira and Silva (2008) and Armenteros et al. (2012), who reported nitrite concentrations in hams of 3.92 ± 0.05 mg/kg, 0.35–1.25 mg/kg and 2.2 ± 1.4 mg/kg respectively. This kinetic profile correlates with the *C. sporogenes* challenge test results, suggesting that initial nitrite concentrations drive inhibition despite the low levels of 'free' nitrite remaining after heat treatment (Guéraud et al., 2023a).

Formulations B to D showed significantly lower residual nitrite concentrations than the standard formulation (A) ($P < 0.05$) throughout storage, in line with their reduced initial nitrite addition. Although some variability maybe influenced by matrix heterogeneity, all measured concentrations complied with the new Regulation n° 2108/2023, which limits 'the maximum residual amount from all sources for the product ready for marketing throughout the shelf-life of the product shall not exceed 45 mg/kg expressed as NO_2 ion.'. A significant effect of storage time ($P < 0.001$) and a time × formulation interaction ($P < 0.001$) were observed, with differences between formulations diminishing by day 90 as nitrite levels converged towards the regulatory limit.

3.4. Sensory analysis

Table 2 presents the degree of difference from the control (DFC) for formulations B and C relative to the standard formulation (A). Fig. S3 shows the visual difference among formulations, emphasising the distinct colour of formulation D and supporting its exclusion from the sensory analysis.

No significant differences were uncovered between formulations A, B and C for appearance, odour, texture and flavour ($P > 0.05$). This sensory similarity is consistent with the absence of differences previously observed in instrumental colour and texture profile analyses for these formulations, and is in agreement with findings reported by Melios et al. (2024) and King et al. (2022). Additionally, syneresis (water binding capacity) was observed in product without nitrite (D), which goes in accordance with other non-favourable visual characteristics.

Table 2
Mean values ± SE ($n = 12$) of DFC rates of ham formulations B and C relative to ham formulation A. Equivalent lower-case letters indicate no significant differences, regardless of formulation ($P > 0.05$).

Formulation	Appearance	Odour	Texture and Flavour
DFC B	0.06 ± 0.02 [a]	0.20 ± 0.04 [a]	0.07 ± 0.05 [a]
DFC C	0.41 ± 0.16 [a]	0.72 ± 0.26 [a]	0.12 ± 0.07 [a]

4. Conclusion

This study demonstrates that reducing sodium nitrite from 150 ppm to 80 ppm in cooked ham does not compromise microbiological safety or sensory quality. Formulation B (80 ppm) showed inhibitory effects against *C. sporogenes* comparable to the standard formulation and delayed the growth of *L. monocytogenes*, confirming its suitability under both normal and abuse storage conditions. Although formulation C (50–20 ppm) also showed potential, its reduced inhibitory effect against clostridia at elevated temperatures warrants caution. Importantly, the 80 ppm formulation complies with current European regulatory limits while maintaining technologic and sensory characteristics. Further research should focus on nitrosamine formation, lipid oxidation, and microbial community dynamics to fully assess the long-term safety and quality implications of nitrite reduction in cooked meat products.

Consent form

All samples were produced and prepared according to good hygiene and manufacturing practices at the pilot plant of Primor, Charcutaria Prima (Portugal). Participants were informed about the general aim of the study and procedures for handling data and gave informed consent prior to participation.

CRediT authorship contribution statement

Maria J.M. Nunes: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Rui C. Pereira:** Writing – review & editing, Methodology, Investigation. **Lúcia Noronha:** Writing – review & editing, Supervision. **Inês Cruz:** Writing – review & editing, Supervision. **Norton Komora:** Writing – review & editing, Conceptualization. **Joana Bastos Barbosa:** Writing – review & editing, Validation. **Maria João P. Monteiro:** Writing – review & editing, Validation, Investigation, Formal analysis, Conceptualization. **Tânia C.F. Ribas:** Visualization, Validation. **Raquel.B.R. Mesquita:** Writing – review & editing, Visualization, Validation. **António O.S.S. Rangel:** Resources, Project administration, Funding acquisition. **Fátima Carvalho:** Writing – review & editing, Validation. **Teresa R.S.

Brandão: Writing – review & editing, Visualization, Validation, Formal analysis. **Paula Teixeira:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

N.K., I.C. and F.C. are employees of the company Primor Charcutaria Prima – S.A.:, who provided raw materials.

Acknowledgments

M. J. M. N. thanks FCT – Fundação para a Ciência e Tecnologia – for the Grant SFRH/BDANA/02204/2023. This work was supported by agenda VIIAFOOD – Plataforma de Valorização, Industrialização e Inovação Comercial para o setor Agroalimentar (C644929456-00000040), financiado pelo PRR – Plano de Recuperação e Resiliência e pelos Fundos Europeus NextGeneration EU. We would also like to thank the scientific collaboration under the FCT project UIDB/50016/2025.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2026.110072.

Data availability

Data will be made available on request.

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