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Research Paper

Efficacy of a Natural Antimicrobial System as a Protective Hurdle Against *Listeria monocytogenes* and Lactic Acid Bacteria in Sliced Fully Cooked Coarse-Ground Cured Brazilian Calabrese Pork Sausage

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ABSTRACT

Meat products, such as sliced Calabrese sausage, are widely consumed due to their convenience and affordability. In response to the increasing demand for healthier foods, the meat industry has sought to replace synthetic additives, particularly nitrites, with natural, clean-label alternatives, especially in cured products. Vegetables such as celery, beetroot, and Swiss chard, naturally rich in endogenous nitrates, have shown promising potential in this context. This study evaluated the effect of different antimicrobial compounds on the growth of *Listeria monocytogenes* in cooked, sliced Calabrese sausage stored at 4 °C for 75 days. Two inoculum levels (2.0 and 4.0 log CFU/g) were used to assess pathogen behavior. The alternative treatment, comprising preconverted celery juice extract, acerola powder, and cultured sugar and vinegar blend, consistently inhibited *L. monocytogenes* growth at both inoculation levels. Additionally, this treatment suppressed the growth of lactic acid bacteria (LAB), which were monitored as spoilage indicators. The results suggest that the use of alternative antimicrobial additives, combined with intrinsic product characteristics such as a pH of 5.78 and water activity (a_w) below 0.948, constitutes an effective strategy for controlling *L. monocytogenes* in sliced cooked pork sausage. Consequently, this approach may enhance food safety and extend product shelf life during refrigerated storage.

Brazilian Calabrese sausage is among the most widely consumed cured meat products in Brazil, available in both raw and cooked forms. This traditional sausage is composed of coarsely ground pork and pork backfat, which are stuffed in either natural or artificial casings. Cooked sausages undergo thermal treatment that includes both dry and moist heat cycles, depending on the desired sensory characteristics and commercial application, with smoking being an optional step in the process.

The term “Calabrese” refers to the distinctive spicy flavor profile of the sausage, resulting from the addition of red pepper, known locally as pimenta Calabresa (Brazil, 2000). Although the sausage is generally heated before consumption, it must still comply with cooking standards applicable to ready-to-eat (RTE) products. Sliced Calabrese sausage is particularly popular as a pizza topping in Brazil. However, the thermal process during pizza preparation is not standardized or controlled, potentially compromising the microbiological safety of the final product. The final quality of the product, including its shelf life, is directly influenced by the nature of the raw materials, the use of additives, and the specific manufacturing process.

Due to its high consumer demand, Calabrese sausage is often sold presliced and vacuum-packaged, enhancing convenience and catering to the rapidly growing food service sector in Brazil (ABIA, 2023), which has established itself as a critical component of the national food industry. Although fully cooked and labeled as RTE, the product may still pose a risk to consumers due to potential postprocessing contamination during slicing, packaging, or handling, which can introduce pathogens such as *Listeria monocytogenes* (Casco, 2015). This risk is exacerbated because consumers may consume the product without further cooking, assuming it is completely safe. These considerations highlight the need for stringent hygiene practices, intrinsic

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product properties, sufficient lethality, proper storage, and adherence to regulatory standards throughout the production and supply chain to minimize public health risks associated with RTE meat products.

L. monocytogenes is a significant pathogen in RTE foods, known for its ability to survive and grow under refrigeration and its resistance to adverse environmental conditions such as low pH and elevated salt levels (Bucur et al., 2018; Osek et al., 2022). Furthermore, sliced processed meats are especially vulnerable to cross-contamination during production. The handling, slicing, and repackaging stages provide multiple opportunities for transfer of pathogenic microorganisms, as well as spoilage, from surfaces and the surrounding environment (Lin et al., 2006). The multiplication of foodborne and spoilage microorganisms should be controlled to ensure food safety and quality.

In Brazil, listeriosis cases and outbreaks are not routinely reported, which may be attributed to the absence of mandatory disease notification, challenges in detecting *L. monocytogenes* in clinical samples, and the inherent difficulty in correlating contaminated food products to clinical cases (Brazil, 2024; Rodrigues et al., 2016). Nonetheless, the presence of this pathogen in food has been documented. In 2018, Brazil's National Health Surveillance Agency (ANVISA) prohibited the sale of a domestic chicken breast batch and several frozen vegetable batches imported from Europe due to *L. monocytogenes* contamination (Brazil, 2022a; 2022b). In the same year, a national survey by the Ministry of Agriculture, Livestock, and Food Supply (MAPA) detected the pathogen in 0.96% of 1,035 analyzed samples, which included meat and seafood products (Brazil, 2020). Research conducted from 2011 to 2022 reported an average incidence of 22.21% of *L. monocytogenes* in meat and meat products, encompassing whole cuts (Ristori et al., 2014), sliced products (Forauer et al., 2021), carcasses (Iglesias et al., 2017), and raw meat (Soares et al., 2021).

In addition to the food safety concerns associated with *L. monocytogenes*, RTE meat products are also susceptible to microbiological spoilage, which can lead to significant economic losses within the meat industry. As the predominant microbial population in meat products, LAB play a dual role in foods. Beneficially, they ferment carbohydrates into lactic acid, lowering pH, inhibiting pathogens through bacteriocin, hydrogen peroxide, etc., and contributing to flavor, texture, and shelf life in meat products. Some strains also provide probiotic effects. However, uncontrolled growth of LAB in nonfermented foods can cause spoilage, leading to off-flavors, excessive sourness, gas production, and textural defects. Therefore, although LAB are indispensable in many traditional fermentations, their presence in undesirable contexts can compromise product quality and contribute significantly to spoilage (Korkeala & Björkroth, 1997). LAB are commonly found in soil, vegetation, animal skin, and epithelial tissues, as well as on abiotic surfaces and within food processing facilities. In general, the heat treatment applied during sausage processing is adequate to ensure microbiological quality and safety, as it effectively inactivates the majority of microorganisms. However, several studies have reported the thermotolerant nature of certain LAB strains isolated from cooked meat products (Franz & von Holy, 1996; Houben, 1982; Milbourne, 1983). Consequently, LAB may persist in processed meat either by surviving thermal processing due to their thermoduric properties (Magnus et al., 1986) or through postprocessing contamination, particularly during slicing and packaging operations. Therefore, cross-contamination within slaughterhouses and processing environments can readily occur due to suboptimal manufacturing and handling practices.

To ensure the microbiological safety and quality of RTE cured meat products, the industry relies on technological interventions, including synthetic preservatives such as sodium nitrite. This additive not only inhibits specific pathogens but also enhances oxidative stability and contributes to the characteristic pink coloration of cured meats (Shakil et al., 2022). Additional antimicrobials, such as sodium lactate and sodium diacetate, are commonly employed to control *L. monocytogenes* growth, extend product shelf life, and maintain sensory quality. However, increasing consumer demand for naturally formulated products, free from synthetic additives, has led to growing interest in alternatives aligned with the clean label movement (McDonnell et al., 2013). In response, plant-based sources rich in nitrate, such as celery, beetroot, spinach, and leek extracts, have been investigated as potential natural substitutes for sodium nitrite. These ingredients have demonstrated promising results in maintaining the microbiological safety of cured meat products (Jackson, 2010; McDonnell et al., 2013; Sebranek et al., 2012; Weyker et al., 2016). However, the use of such plant concentrates or extracts as sources of nitrate or nitrite is not yet regulated in Brazil. Their application must undergo evaluation by ANVISA and receive approval from the Federal Inspection Service (SIF) of MAPA for regulatory implementation.

Evaluating the effectiveness of natural antimicrobials in controlling *L. monocytogenes* requires microbiological assessments that simulate real-world storage and retail conditions. The challenge test method is widely employed for this purpose, as it allows researchers to determine whether a particular antimicrobial intervention can inhibit the growth of pathogens throughout the shelf life of refrigerated RTE foods (Health Canada, 2012; NACMCF, 2010).

In light of these considerations, the present study aimed to assess the efficacy of natural antimicrobial agents, specifically, fermented celery juice powder (as a preconverted nitrite source), cultured sugar, and vinegar blend, in inhibiting *L. monocytogenes* and LAB in coarsely ground, sliced, fully cooked, and refrigerated Brazilian Calabrese pork sausage. This treatment was compared to conventionally used synthetic antimicrobials, including a mixture of sodium nitrite, sodium lactate, and sodium acetate.

Materials and methods

Experimental design. The experiment was conducted as a randomized complete block design with two replications. This study evaluated three formulations of fully cooked Calabrese sausage, produced in two independent replications. The treatments included a control batch (C), manufactured without the addition of curing agents or antimicrobials; a traditional batch (T), containing synthetic nitrite, a cure accelerator, and synthetic antimicrobials; and an alternative batch (A), in which nitrite originated from vegetable extracts and natural antimicrobials were incorporated.

The control batch (C) was included specifically for baseline microbial enumeration, enabling the assessment of the effectiveness of both synthetic and alternative curing methods. All batches were processed under controlled conditions at the Meat Technology Center (CTC) from the Institute of Food Technology (ITAL), located in Campinas, São Paulo, Brazil.

Product manufacture. The formulations used for the production of fully cooked Calabrese sausages are presented in Table 1, with values expressed as percentages. The base formulation consisted of pork shoulder, pork ham, pork fat, chilled water, salt, and a commercial Calabrese sausage seasoning blend (6325, Adeste Produtos Animais e Vegetais Ltda., Santo André, SP, Brazil). The differences between the treatments were related to the type of curing agents and antimicrobial systems applied. The conventional treatment (T) included curing salt with 10% sodium nitrite (Ibrac Aditivos e Condimentos Ltda., Rio Claro, SP, Brazil), sodium erythorbate as a cure accelerator (Doremus Aromas & Ingredientes Ltda., Guarulhos, SP, Brazil), and a blend composed of 50.5–53.5% sodium lactate and 8.9–9.6% sodium acetate (Opti.form Ace S9, Corbion Produtos Renováveis Ltda., Campos dos Goytacazes, RJ, Brazil). In the alternative treatment (A), fermented celery juice powder (Verdad Avanta CS 30, Corbion, Purac America, Lenexa, KS, USA) was used as the nitrite source, containing 30.000 to 35.000 ppm nitrite (i.e., 3.0–3.5%) and 30% salt. Additionally, cherry powder (Liofruit, Liotécnica Tecnologia em Alimentos, Embu das Artes, SP, Brazil) containing 25% ascorbic acid was added as a natural cure accelerator. A natural antimicrobial blend composed of

Table 1
Treatment formulations for cooked Calabrese sausage (values expressed as percentages)

Raw materials / Additives	Treatments (%)		
	T[a]	A[b]	C[c]
Pork shoulder	41.50	40.58	42.56
Pork butt	35.00	35.00	35.00
Pork backfat	12.00	12.00	12.00
Iced water	8.00	8.00	8.00
Salt	1.00	1.01	1.14
Synthetic cure	0.15	0.00	0.00
Sodium erythorbate	0.05	0.00	0.00
Sodium lactate/sodium acetate blend	1.00	0.00	0.00
Fermented celery juice powder	0.00	0.45	0.00
Cherry powder	0.00	0.16	0.00
Sugar cane	0.30	0.30	0.30
Calabrese sausage seasoning	1.00	1.00	1.00
Cultured dextrose-vinegar blend	0.00	1.50	0.00

[a] traditional batch, containing synthetic nitrite, a cure accelerator and synthetic antimicrobials.
[b] alternative batch, in which nitrite originated from vegetable extracts and natural antimicrobials were incorporated.
[c] control batch, manufactured without the addition of curing agents or antimicrobials.

cultured dextrose and vinegar (BioVia CL 600, International Flavors & Fragrances Inc., New York, NY, USA) was also incorporated. The control batch (C) received no curing agents or antimicrobials and was formulated exclusively for microbiological monitoring. Salt content was adjusted to maintain consistency across formulations, compensating for its presence or absence in the ingredients.

Frozen pork shoulder, ham, and back fat were obtained from a local commercial pork slaughterhouse and stored at −18 °C until further processing. Prior to processing, the raw materials were thawed under refrigeration at 2–4 °C until their internal temperature reached 4 °C. Each treatment was prepared individually according to the formulations presented in Table 1, following a standardized production protocol. The processing order was randomized to minimize batch effects. The pork shoulder was ground through a 16−mm plate, the ham through a 12−mm plate, and the frozen fat through an 8−mm plate. The ground ingredients were transferred to a paddle mixer (M60, CAF, Brazil) and mixed for 1 min. Salt, seasoning, and curing agents (as specified for each treatment) were then added and mixed for an additional minute. The fat was subsequently incorporated and mixed for 1 min. Sugar and sodium erythorbate, or in the alternative formulations, cherry powder and natural antimicrobials, were then added, followed by 5 min of homogenization. Each 10 kg batch was stuffed into 42 mm diameter cellulose casings using a vacuum filler (VF 610 Plus, Handtmann Maschinenfabrik GmbH & Co. KG, Riss, BW, Germany) and manually tied into approximately 500 g links.

The sausage links were hung on metal rods and randomly arranged on a cooking cart, then thermally processed in a single-chamber smokehouse (HR1, Schröter Technology, Borgholzhausen, NW, Germany). The thermal process (Fig. 1, Table 2) included the following stages: preheating at 40 °C for 10 min; drying at 55 °C for 20 min (with open damper); drying at 55 °C for 15 min (with closed damper); drying at 60 °C for 30 min (with closed damper); two consecutive drying stages at 65 °C and 70 °C (with open damper), each for 40 min; and cooking with direct steam until an internal temperature of 73 °C was reached, followed by a drying phase at 80 °C for 20 min (with open damper). After thermal processing, the products were cooled to 20 °C inside the smokehouse and subsequently refrigerated at 4 °C.

Product analysis. Cooking weight loss (CWL) was determined approximately 3 h after cooling, in accordance with the methodology described by Honikel (1998). The initial (IW) and final (FW) weights of the sausages were recorded, and CWL was calculated. Cooking yield was subsequently calculated as 100 – CWL.

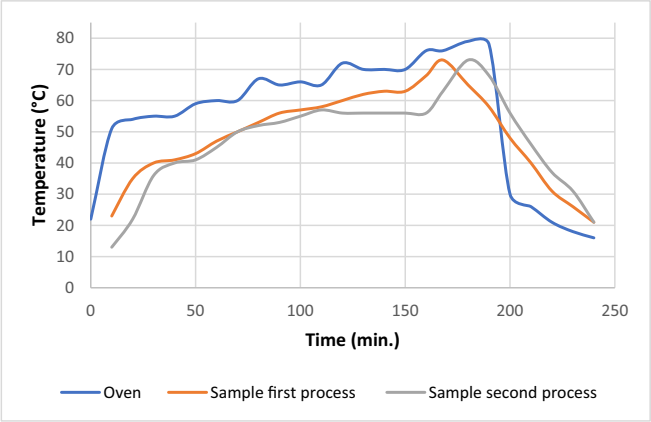

Figure 1. Temperature profile recorded during the cooking of Calabrese sausage.

Samples from the various sausage treatments were collected 24 h after thermal processing for physicochemical and microbiological analyses, aimed at verifying compliance with Brazilian regulatory standards and confirming product suitability for the subsequent challenge test. Separate packages were intended for physicochemical and microbiological analysis. All analyses were conducted in triplicate for the three treatments, and mean values were calculated for each storage time point. The control samples, whose centesimal composition and formulation (raw material, salt, and sugar content) did not differ from those of the other treatments, were included solely to monitor microbiological results.

Physicochemical analyses included determinations of centesimal composition, pH, a_w, and residual nitrite and nitrate levels. Moisture content was measured by drying 10 g of the sample at 102 °C for 24 h (AOAC 950.46, 2012a). Total lipids were determined using Soxhlet extraction with petroleum ether (AOAC 960.39, 2012b) and quantified by sample weight loss. Protein content was analyzed by the Kjeldahl method (AOAC 981.10, 2012c), with nitrogen values multiplied by 6.25 to obtain protein percentage. Ash content was determined by incinerating 5 g of sample in a muffle furnace at 550 ± 25 °C for 24 h (ISO 936:1998a). Nitrite content was determined by extraction at 70 °C for 15 min, followed by clarification, diazotization, and coupling with sulphanilic acid and α-naphthylethylenedia mine dihydrochloride to form a stable pink complex, which was measured spectrophotometrically at 538 nm (ISO 2918:1975, 1975a). Nitrate content was determined from the same extract after reduction to nitrite by passage through a cadmium column, followed by the same colorimetric reaction (ISO 3091:1975, 1975b). Nitrate and nitrite were reported in mg/kg. To determine pH, 50 g of sample were homogenized with 20 mL of deionized water to prepare slurries, and three measurements of each sample were taken using a DM-21 pH meter fitted with a DME-CF1 electrode (Digimed, São Paulo, SP, Brazil). Water activity (a_w) was measured at 25 ± 0.3 °C using an Aqualab 4TE instrument (Decagon Devices, USA).

The microbiological profile was assessed for LAB, coagulase-positive staphylococci, *E. coli, C. perfringens, L. monocytogenes*, and *Salmonella* spp. Samples were diluted 1:10 in 0.1% peptone water and plated on selective media: de Man, Rogosa, and Sharpe (MRS) agar (Merck KGaA, Darmstadt, German y) for LAB (30 °C, 72 h, anaerobic conditions; ISO 15214:1998b); Baird Parker (BP) agar (Neogen Culture Media, Lansing, USA) with egg yolk tellurite enrichment for coagulase-positive staphylococci (34–38 °C, 24–48 h; ISO 6888-1:2021); Triptone Bile X-Glucuronide (TBX) agar (Neogen Culture Media, Lansing, USA) for *E. coli* (44 °C, 24–48 h; ISO 16649-2:2001); Tryptose Sulfite Cycloserine (TSC) agar (Neogen Culture Media, Lansing, USA) for *C. perfringens* (37 °C, 20–24 h, anaerobic conditions;

Table 2
Thermal processing schedule for coarse-ground cured Brazilian Calabrese pork sausage

Step	Step time (min)	Dry bulb (°C)	Wet bulb (°C)	Relative humidity (%)	Exhaust Damper
Preheating	10	40	–[a]	–	Closed
Drying	20	55	0	0	Open
Drying	15	55	0	0	Closed
Drying	30	60	0	0	Closed
Drying	40	65	45	25	Open
Drying	40	70	70	0	Open
Cooking	10	75	75	100	Closed
Drying	20	80	0	0	Auto
Cold Shower	20	–	–	–	Auto

[a] not measured.

Labbe, 2015); and Listeria Selective Agar Base, Ottaviani & Agosti (ALOA) (Merck KGaA, Darmstadt, Germany) for *L. monocytogenes* detection (ISO 11290-2:2017a) after enrichment in Half Fraser broth (Merck KGaA, Darmstadt, Germany; 30 °C, 24 h; then 37 °C, 24–48 h). Colonies were confirmed by standard tests (catalase, Gram staining, motility, and hemolysis). Results (CFU/g) were means of triplicates; detection limits were 1.0 log CFU/g, except 2.0 log CFU/g for staphylococci. *Salmonella* spp. detection (ISO 6579:2020) was performed by preenrichment of 25 g of sample in 225 ml of buffered peptone water (BPW; Neogen Culture Media, Lansing, USA) at 34–38 °C for 18 ± 2 h, followed by selective enrichment in Rappaport-Vassiliadis Soya (RVS) broth (Neogen Culture Media, Lansing, USA) at 41.5 ± 1°C for 24 ± 3 h and Müller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth (Merck KGaA, Darmstadt, Germany) at 34–38 °C for 24 ± 3 h. Cultures were plated on Xylose Lysine Deoxycholate (XLD) agar (Neogen Culture Media, Lansing, USA) and Hektoen Enteric (HE) agar (Merck KGaA, Darmstadt, Germany) and incubated at 34–38 °C for 24 ± 3 h. Presumptive colonies were confirmed by biochemical tests using Triple Sugar Iron (TSI) agar (Neogen Culture Media, Lansing, USA), Lysine Iron (LIA) agar (Neogen Culture Media, Lansing, USA), and Urease agar (Merck KGaA, Darmstadt, Germany), as well as serological testing of somatic and flagellar antigens (Probac do Brasil, São Paulo, Brazil). Results were expressed as presence/absence in 25 g of sample.

Bacterial cultures and inoculum preparation. Six strains of *L. monocytogenes* were employed for the inoculation of cooked Calabrese sausages. These included four type strains from the American Type Culture Collection (ATCC 7644, ATCC 19117, ATCC 15313, and ATCC 19112) and two wild-type strains (AM12-12 and AM14-14) isolated from commercial samples of cooked ham and hamburger, respectively. All strains were obtained from the bacterial culture collection of the Microbiology Laboratory, Meat Technology Center (CTC), ITAL (Campinas, SP, Brazil).

Stock cultures were stored at –80 °C in Tryptic soy broth (BD, Becton Dickinson, USA) with yeast extract (TSB-YE), supplemented with 10% (w/v) glycerol. Prior to use, cultures were activated in TSB-YE by incubation at 37 °C for 18 h. Purity of strains was verified by streaking on Tryptic Soy Agar (TSA, BD, Becton Dickinson, USA) and ALOA agar, with incubation at 35 °C for 48 h. Each strain concentration was adjusted to 8.0 log CFU/mL using the MacFarland scale and a Densimat® photometer (bioMérieux, Marcy l'Etoile, France). Equal volumes of each standardized culture were combined and homogenized to prepare the bacterial cocktail.

Product inoculation and sampling procedure. A challenge test was conducted to evaluate the efficacy of the antimicrobial agents against *L. monocytogenes*, following the guidelines of Health Canada (2012). The cellulose casings were removed after 6 days of refrigerated storage, and the Calabrese sausages were aseptically cut into slices approximately 3 mm thick. The slices were arranged individually to avoid overlapping, with five slices per package (approximately 25 g) for each treatment. Half of the packages were then inoculated with the prepared bacterial cocktail. A volume of 100 μL of the diluted inoculum was applied to the surface of all slices within each package, resulting in two inoculation levels: approximately 2.0 and 4.0 log CFU/g. The lower level simulated typical environmental contamination (Amato et al., 2020; Branciari et al., 2020; EURL Lm, 2019; Health Canada, 2012; NACMCF, 2010), whereas the higher level represented a potential abuse scenario (Food Safety Commission of Japan, 2014; Scott et al., 2005; Shen et al., 2024).

Homogeneous distribution of the inoculum was ensured by manually massaging the unsealed packages for 2 min, allowing the inoculum to spread evenly throughout the product. The samples were then left to dry under aseptic conditions for 30 min. Subsequently, the sausage samples were vacuum sealed for 30 s with a 2.5-s sealing time using a Microvac machine (Selovac Ind. Com. Ltda., São Paulo, SP, Brazil) in high-barrier nylon/polyethylene pouches (water vapor transmission rate, 9.3 g/m^2/24 h at 97% RH; oxygen transmission rate, 54.3 cm^3/m^2/24 h at 21 °C and 0% RH; Protervac Ind. Com. Ltda., Jundiaí, SP, Brazil).

Microbiological enumeration was performed in triplicate during refrigerated storage on days 1, 15, 30, 45, 60, and 75 for *L. monocytogenes* (ISO 11290-2:2017b) and LAB (ISO 15214:1998b), using nine packages per treatment (C, T, and A; uninoculated or inoculated with 2.0 or 4.0 log CFU/g), totaling 162 packages. Physicochemical parameters (pH, a_w, nitrite, and nitrate) of sliced samples were assessed from day 15 onward, with analyses performed in triplicate, and three measurements were taken per sample. At each interval, six packages per treatment were analyzed (≈100 g each for T and A). Microbiological and physicochemical measurements were carried out on different samples.

Statistical analysis. The study was conducted in duplicate, with each replicate corresponding to an independent manufacturing day. All raw materials (meat and nonmeat ingredients) originated from a single production lot to minimize variability within blocks. For each replication, experimental treatments were randomly assigned to production batches, and placement of stuffed sausage links in smokehouse trucks followed a random arrangement.

Data were subjected to analysis of variance (ANOVA) using Statistical Analysis Software (version 9.4; Statistica, USA). Treatment, inoculum concentration, and storage time were considered fixed factors, and replication was treated as a random factor. The effect of inoculum concentration was not evaluated for physicochemical traits, which included only treatments T and A. For microbiological counts, all three treatments (C, T, and A) were included, and the inoculum effect was evaluated. The significance of main effects and interactions was determined at $P < 0.05$. When significant differences were detected, Tukey's HSD pairwise comparison test was used to determine differences among means.

Results and discussion

Physical and chemical traits. The significance of the main fixed factors (treatment and storage time) and their interaction effects is presented in Table 3. The proximate composition, residual nitrite and nitrate levels, pH, a_w, and cooking yield of cooked Calabrese sausage 24 h postprocessing are shown in Table 4. The treatment effect was significant ($P < 0.05$) for ash content, a_w, pH, and residual nitrate concentration. Treatment A exhibited a higher ash content (4.17%) than treatment T (3.04%). This result was expected, considering that the fermented celery juice powder contained approximately 30% salt in addition to other minerals and bioactive compounds, and was added at higher levels to achieve an equivalent sodium nitrite concentration (150 ppm) (Awad et al., 2022; Kim et al., 2019; Zhou et al., 2020). Similarly, a_w was significantly ($P < 0.05$) lower in treatment A (0.958) than in treatment T (0.973), likely due to the higher concentrations of salts and sugars present in the fermented celery powder, which contributed to reduced a_w.

Regarding pH, treatment A exhibited significantly ($P < 0.05$) lower values (5.77) than treatment T (6.02). This reduction was likely attributable to the presence of the cultured sugar–vinegar blend, whereas the ascorbic acid in acerola powder, used to replace sodium erythorbate as a curing accelerator, does not significantly influence pH, as previously reported by Sullivan et al. (2012) for ham. No significant differences ($P > 0.05$) were observed between treatments in residual nitrite and nitrate concentrations immediately after processing.

The evaluation of the physicochemical properties of sliced, cooked Calabrese sausage during 75 days of refrigerated storage (4.0 ± 1.0 °C) is presented in Table 5 (a_w and pH values), Figure 2 (residual nitrite concentration), and Figure 3 (residual nitrate concentration). The treatment effect was significant ($P < 0.05$) for a_w and pH, whereas a significant treatment × storage time interaction ($P < 0.05$) was observed for both residual nitrite and nitrate concentrations.

Throughout storage, the a_w in treatment A (mean 0.948) remained significantly lower ($P < 0.05$) than in treatment T (mean 0.963), reflecting the initial pattern, although values were approximately 0.01 lower than those recorded immediately after processing (Table 5). This slight decrease may be attributed to moisture loss during the six days of refrigerated storage prior to slicing.

Similarly, pH values in treatment A (mean 5.78) remained significantly lower ($P < 0.05$) than those in treatment T (mean 6.00), consistent with the inclusion of the cultured sugar–vinegar blend, whereas the presence of acerola powder had no appreciable effect on pH (Table 5).

Table 3
P values for fixed factors and their interaction effects on the physicochemical traits of cooked Calabrese sausage

Dependent variable Initial (24 h after cooking)	Treatment	Storage time	Treatment X Storage time
Cooking/cooling loss	0.309	nt[a]	Nt
Moisture	ns[b]	nt	Nt
Lipid	ns	nt	Nt
Protein	ns	nt	Nt
Ash	$P < 0.01$	nt	Nt
Nitrite	ns	nt	Nt
Nitrate	ns	nt	Nt
pH	$P < 0.01$	nt	Nt
Water activity	$P < 0.01$	nt	Nt
Slices storage			
Nitrite	$P < 0.01$	$P < 0.01$	$P < 0.05$
Nitrate	$P < 0.01$	$P < 0.01$	3
pH	$P < 0.01$	0.323	0.813
Water activity	$P < 0.01$	0.9794	0.9796

[a] not tested.
[b] not significant.

Regarding residual nitrite concentrations, treatment T exhibited significantly ($P < 0.01$) higher values (45.80 ppm) than treatment A (27.54 ppm) on day 15 of storage (Fig. 2). As previously discussed by Honikel (2008), nitrite depletion is expected during storage due to its reactions with muscle components such as myoglobin and lipids, resulting in nitric oxide formation and nitrosomyoglobin stabilization. Both treatments showed a gradual decline in residual nitrite concentration over time. After day 30, no significant differences ($P > 0.05$) were observed between treatments, indicating similar depletion dynamics for both curing systems.

For residual nitrate concentrations (Fig. 3), the treatment × storage time interaction was significant ($P < 0.05$). Initially, treatment A exhibited higher nitrate levels (41.30 ppm) than treatment T (25.56 ppm), reflecting the naturally higher nitrate content of the fermented celery powder (Sebranek & Bacus, 2007; Sebranek et al., 2012). During storage, nitrate concentrations in treatment A decreased significantly ($P < 0.05$), whereas treatment T maintained relatively stable levels. By day 75, no significant differences ($P > 0.05$) were observed between treatments, suggesting partial nitrate degradation or microbial conversion over time, particularly in the naturally cured product (Sindelar et al., 2007).

On days 15, 30, and 45, residual nitrate concentrations in treatment T were significantly lower ($P < 0.01$) than in treatment A. However, no significant differences ($P > 0.05$) were detected between treatments on days 60 and 75 (Fig. 3). While nitrate levels in treatment A remained relatively stable throughout storage, treatment T exhibited a slight increase after day 45 and a marked rise from day 60, reaching levels comparable to treatment A by day 75.

The oxidation of nitrite to nitrate in the presence of oxygen (Flores & Toldrá, 2021) likely occurred during the six-day storage period prior to slicing, when the sausages were held under refrigeration. The higher and more stable nitrate content observed in treatment A may be attributed to the vegetable-derived origin of the preconverted nitrite and the more stable pH conditions, which could enhance nitrate stability in this formulation.

Under meat-curing conditions (pH 5.0–6.0), between 97.5% and 99.8% of nitrous acid (HNO_2) dissociates into nitrite ion (NO_2^-). Nitrite is subsequently reduced to nitric oxide (NO) via two primary pathways: (i) bacterial nitrite reductases and (ii) meat deoxymyoglobin, which exhibits nitrite reductase activity (Honikel, 2008). Additionally, nitrite can be oxidized to peroxynitrite ($ONOO^-$) by hydrogen peroxide. LAB also contribute to nitrite degradation through three mechanisms: enzymatic reduction by nitrite reductase, acidic degradation, and degradation by certain metabolites, with the enzymatic pathway being predominant (Yuan et al., 2024).

These complex dynamics highlight the importance of storage and processing conditions in controlling nitrite and nitrate transformations. The reactivity of these compounds, along with the influence of microbial enzymes, raw materials, curing accelerators, pH, oxygen availability, temperature, and thermal processing duration, contributes to the variability in nitrite and nitrate content (Honikel, 2008; Majou & Christieans, 2018).

The findings of the present study demonstrate that fermented celery juice powder and the cultured sugar–vinegar blend significantly influence the initial physicochemical properties of sliced, cooked Calabrese sausages, including ash content, a_w, pH, and nitrate concentration. Over time, differences in residual nitrite and nitrate concentrations between naturally and conventionally cured products diminished, suggesting comparable stability profiles. These results support the feasibility of using natural curing systems to achieve physicochemical characteristics and storage stability similar to those obtained with conventional curing methods.

***L. monocytogenes* and LAB in sliced cooked calabrese sausage.** Significance was determined at $P < 0.05$. *P* values for the effects of the fixed main factors (treatment and storage time) and their interaction are presented in Table 6 (microbiological counts). Initial microbiolog-

Table 4
Least square means[a] for the main effect of treatment on proximate composition, pH, nitrite, nitrate, a_w, and cooking yield of traditional (T) and alternative cured (A) whole Calabrese sausage 24 h ± 1 h postprocessing

Treatment	Proximate composition				Water activity	pH	Nitrite	Nitrate	Yield
	Moisture	Protein	Lipid	Ash					
	(g/100 g)						(mg/Kg)		(%)
T	60.14^{A}	18.77^{A}	18.38^{A}	3.04^{B}	0.973^{A}	6.02^{A}	49.90^{A}	22.41^{A}	89.5^{A}
A	61.70^{A}	17.96^{A}	18.24^{A}	4.17^{A}	0.958^{B}	5.77^{B}	50.36^{A}	24.80^{A}	89.0^{A}
C	nt[c]	nt	nt	nt	nt	nt	nt	Nt	89.1^{A}
SEM[b]	0.51	0.54	0.73	0.17	0.0015	0.02	9.4	3.6	0.15

[a] means with common letters (A through B) within the same column do not differ significantly ($P < 0.05$).
[b] standard error of mean.
[c] not tested.

Table 5
Least-squares means[a] for the main effect of treatment on a_w and pH values of sliced, cooked Calabrese sausage stored at 4 °C

Treatments	a_w	pH
T	0.963^{A}	6.00^{A}
A	0.948^{B}	5.78^{B}
SEM[b]	0.0014	0.01

[a] means with common letters (A through B) within the same column do not differ significantly ($P < 0.05$).
[b] standard error of mean.

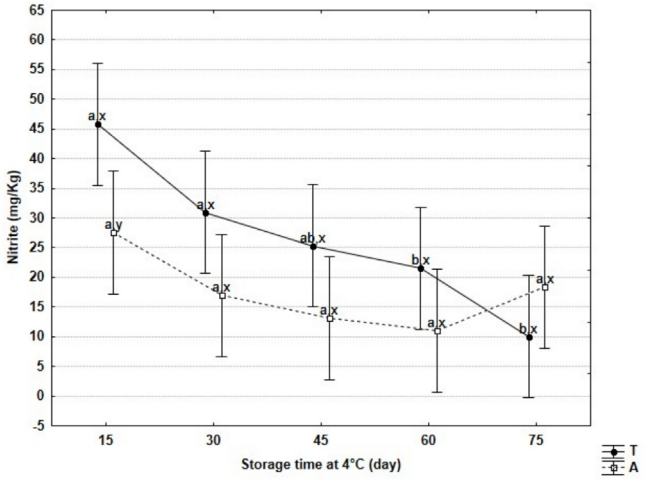

Figure 2. Least-squares means of residual nitrite in sliced cooked Calabrese sausages during storage at 4 °C. Error bars represent ± SEM (=5.136). Significant differences over time within the same treatment are indicated by different letters (a through b), and differences between treatments are indicated by different letters (x through y) ($P < 0.05$).

ical evaluation of freshly processed, cooked Calabrese sausages (24 h postheat treatment, Table 7) revealed that all tested microbial groups, *L. monocytogenes*, LAB, *C. perfringens*, and coagulase-positive staphylococci, were below detectable limits (<1.0 log CFU/g or < 2.0 log CFU/g). *Salmonella* spp. was absent in all samples, confirming the microbiological safety of the products for subsequent challenge studies.

Challenge testing for *L. monocytogenes* revealed significant main effects for treatment, inoculum level, and storage duration ($P < 0.01$), along with significant two-way interactions between treatment × time ($P < 0.01$) and inoculum × time ($P < 0.05$) (Fig. 4). No significant three-way interaction was observed ($P > 0.05$).

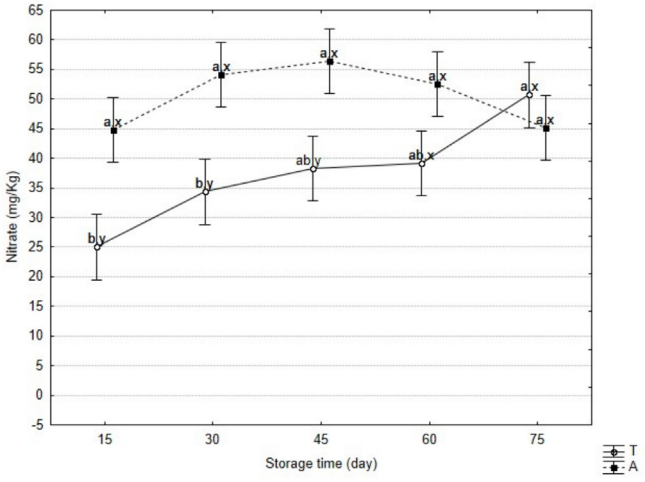

Figure 3. Least-squares means of residual nitrate in sliced cooked Calabrese sausages during storage at 4 °C. Error bars represent ± SEM (=2.729). Significant differences over time within the same treatment are indicated by different letters (a through b), and differences between treatments are indicated by different letters (x through y) ($P < 0.05$).

Table 6
P values for fixed factors and their interaction effects on the counts of *L. monocytogenes* and LAB in cooked Calabrese sausage during storage at 4 °C

Factors	Dependent variable	
	L. monocytogenes	LAB
Treatment	0.000000	0.000000
Inoculum concentration	0.000000	0.000000
Time	0.000000	0.000000
Treatments * Inoculum concentration	0.015743	0.495
Treatments * Time	0.000000	0.000000
Inoculum concentration* Time	0.007657	0.1043181
Treatments * Inoculum concentration * Time	0.425	0.448

Table 7
Mean microbiological counts of freshly processed, cooked Calabrese sausage slices

Bacteria counts (log CFU/g) [a]	Treatment		
	T	A	C
L. monocytogenes	<1.0	<1.0	<1.0
LAB	<1.0	<1.0	<1.0
C. perfringens	<1.0	<1.0	<1.0
Coagulase-positive staphylococci	<2.0	<2.0	<2.0
Salmonella spp. (absence in 25 g)	absent	absent	absent

[a] colony – forming unit.

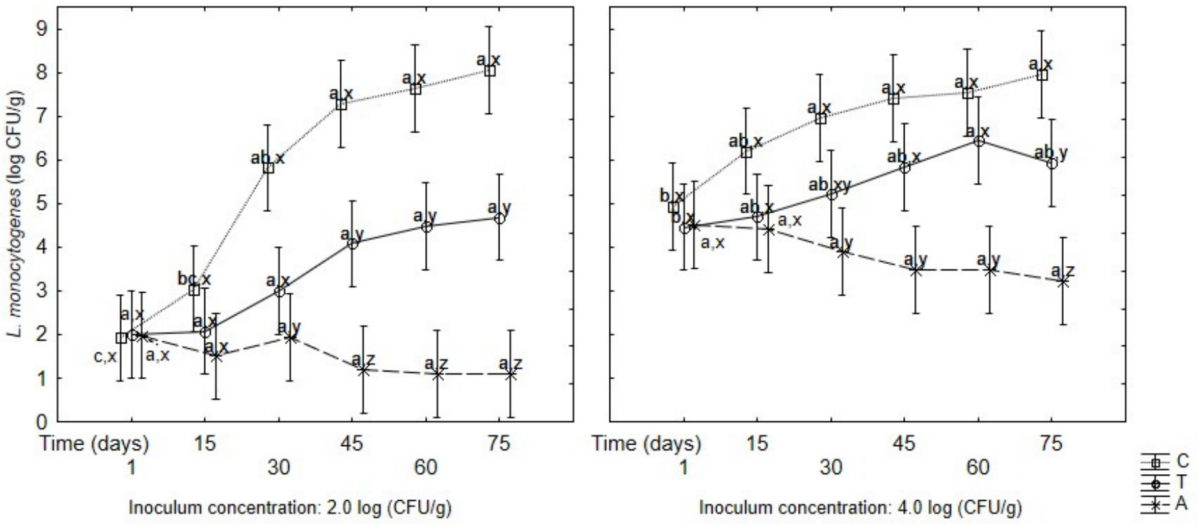

Figure 4. Least-squares means of *L. monocytogenes* counts (log CFU/g) in sliced, cooked Calabrese sausages inoculated with 2.0 log CFU/g (left) or 4.0 log CFU/g (right) and stored under refrigeration (4 °C) for 75 days. Error bars represent ± SEM (=0.503). Significant differences over time within the same treatment are indicated by different letters (a through c), and differences between treatments are indicated by different letters (x through z) ($P < 0.05$).

In treatment A, at both inoculum levels (2.0 and 4.0 log CFU/g), no growth of *L. monocytogenes* was observed, and counts remained at or below the detection limit throughout storage. In contrast, treatment T exhibited bacterial growth from day 30 (2.0 log CFU/g inoculum), reaching 4.7 log CFU/g by day 75. The treatment C showed earlier and more rapid growth, beginning at day 15 and peaking at approximately 8.0 log CFU/g by day 75, representing net increases of 2.7 log CFU/g (T) and 6.1 log CFU/g (C), respectively.

With the higher inoculum level (4.0 log CFU/g), treatment T exhibited a slower growth trend, peaking at 6.4 log CFU/g by day 60. The control group again showed more aggressive growth, reaching nearly 8.0 log CFU/g by day 45. Final net increases were 2.0 and 3.0 log CFU/g for treatments T and C, respectively.

Regarding LAB (Fig. 5), a significant treatment × time interaction was observed ($P < 0.05$). No LAB growth was detected in treatment A throughout the storage period. In contrast, treatment T exhibited an increase in LAB counts starting at day 60 (2.0 log CFU/g inoculum) and day 30 (4.0 log CFU/g inoculum). The absence of a significant three-way interaction indicates that the initial *L. monocytogenes* inoculum concentration did not significantly influence LAB growth dynamics, as confirmed by statistical groupings.

The sliced, cooked Calabrese sausage produced using the treatment A system exhibited an average a_w of 0.95 and pH of 5.8, values that, according to the European Commission (2005) and Brazil (2009), may support the growth of *L. monocytogenes*. Therefore, challenge tests are necessary to ensure that, if the product is contaminated at the industrial level, *L. monocytogenes* will not proliferate during its shelf life. According to current safety criteria, only products with pH ≤ 4.4, $a_w \leq 0.92$, or pH = 5.0 and $a_w = 0.94$ are exempt from such testing requirements. These considerations highlight the importance of understanding *L. monocytogenes* behavior in new meat products of this category.

The increase in *L.* monocytogenes counts in treatment T after 60 days of storage coincided with a decline in residual nitrite levels, suggesting that nitrite depletion reduced microbial inhibition. The addition of a sodium lactate/acetate blend, compounds well recognized for their anti-Listeria activity (Glass et al., 2002; Weaver & Shelef, 1993), likely contributed to pathogen control in treatment T, particularly compared with treatment C, which showed higher *L.* monocytogenes counts. The pH of treatment T remained stable at approximately 6.0 throughout storage, indicating little influence of acidity. In contrast, treatment A exhibited substantially lower residual

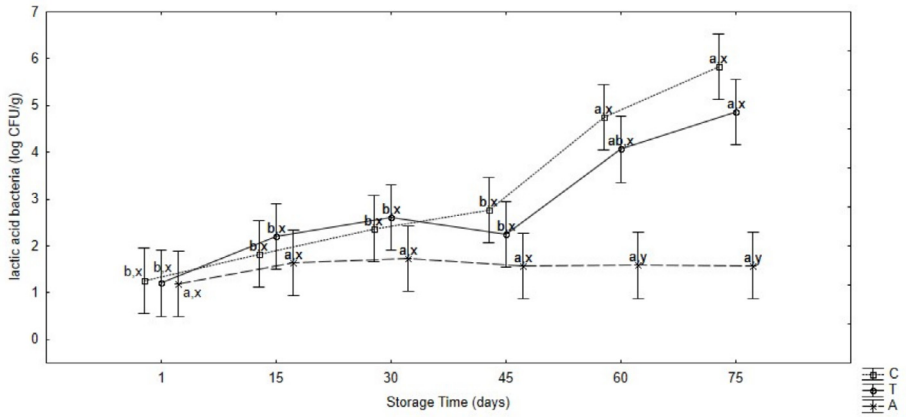

Figure 5. Least-squares means of LAB counts (log CFU/g) in sliced, cooked Calabrese sausages during 75 days of refrigerated storage (4 °C). Error bars represent ± SEM (=0.355). Significant differences over time within the same treatment are indicated by different letters (a through c), and differences between treatments are indicated by different letters (x through z) ($P < 0.05$).

nitrite levels, suggesting that nitrite alone did not account for pathogen inhibition. Instead, the combination of lower pH and aw values, together with stable nitrate levels, likely acted synergistically to enhance microbial suppression.

The antimicrobial efficacy of sodium nitrite against *L.* monocytogenes remains debated, as it depends on several interacting factors, including pH, temperature, salt concentration, hydrostatic pressure, and packaging atmosphere (Buchanan & Phillips, 1990; Lavieri et al., 2014; Pelroy et al., 1994). Although some authors argue that nitrite alone provides limited inhibition (Majou & Christieans, 2018), others have shown that it delays exponential growth by extending the lag phase in a dose-dependent manner (Lavieri et al., 2014; Myers et al., 2013). Nitrite and NO act as bacteriostatic agents targeting metabolic and respiratory enzymes with redox-active centers, such as heme and iron-sulfur clusters (Guo & Gao, 2021; Stern & Zhu, 2014). Their activity is maximized under acidic, high-salt conditions and depends largely on the residual nitrite concentration. The rise in *L.* monocytogenes counts following nitrite depletion reflects this dose–response relationship, in which NO effectively disrupts respiration and DNA at sufficient levels but allows growth to resume once detoxification mechanisms prevail. Residual nitrite thus represents a critical control factor where microbial safety, sensory quality, and toxicological limits converge (Fraqueza et al., 2021).

Schrader et al. (2009) reported that RTE meat products containing celery juice powder as a natural nitrite source were less effective at suppressing *L. monocytogenes* during 35 days of storage at 10 °C than conventionally cured counterparts, showing final populations 2.0–5.0 log CFU/g higher than controls. In that study, additional antimicrobial interventions were required to achieve safety levels comparable to conventional products. Conversely, ham produced with natural curing methods and clean-label antimicrobials (cultured sugar and vinegar blend; lemon, cherry, and vinegar powder blend) supported *L. monocytogenes* growth similar to that observed in traditionally cured controls. In contrast, reduced-sodium uncured turkey treated with buffered dry vinegar inhibited *L. monocytogenes* for 12 weeks, whereas untreated controls exhibited significant growth (Badvela et al., 2016). Collectively, these findings underscore both the limitations of natural nitrite sources and the potential of vinegar-based hurdles, indicating that complementary antimicrobial strategies are essential to ensure microbial safety equivalent to that of conventional curing.

The study evaluated the behavior of a *L. monocytogenes* strain cocktail composed of isolates originating from both the natural microbiota of meat products and reference culture collections. This approach likely provides a realistic contamination scenario, as wild strains are already adapted to the raw materials, ingredients, and processing conditions of the product.

Previous studies have reported the inhibitory effects of cultured sugar-vinegar blends containing various organic acids on *L. monocytogenes* growth (Glass et al., 2002; McDonnell et al., 2013; Weyker et al., 2016), which is consistent with the formulation of treatment A. Within the typical pH range of cured meats (5.0–6.0), acidity is a key factor (Majou & Christieans, 2018) by influencing both the nitrite-nitrate equilibrium and microbial homeostasis. Furthermore, the reduced a_w observed in treatment A may have contributed to additional inhibition of microbial growth.

In comparison with McDonnell et al. (2013), who reported no *L. monocytogenes* growth in roast beef treated with 3.0% of a cultured sugar-vinegar blend, our study likewise observed no growth despite using a lower concentration (1.5%) of these compounds. The Calabrese sausage evaluated here exhibited lower a_w (0.94 vs. 0.97) and pH values (5.78 vs. 6.31) than the roast beef, which may explain the higher antimicrobial efficacy at reduced concentrations. Additionally, differences in *L. monocytogenes* strains used between studies may have also contributed to the observed variations in growth behavior.

Regarding LAB, treatment A inhibited their growth throughout the 75-day storage period. This contrasts with the findings of Weyker et al. (2016), who observed no inhibition of *Leuconostoc mesenteroides*. Although a_w values were not reported in their study, it can be inferred that the higher salt concentration in our formulation, together with the lower pH and the presence of nitrite, likely contributed to this difference.

Biological control using LAB represents a promising strategy to inhibit *L. monocytogenes* through the production of antimicrobial metabolites (Arena et al., 2016; Camargo et al., 2018; Moradi et al., 2020). However, since these same compounds may also contribute to meat spoilage, successful industrial application requires careful balancing of antimicrobial efficacy with the preservation of product quality and shelf life.

In conclusion, the incorporation of 0.45% fermented celery juice powder (150 ppm nitrite), 0.16% cherry powder (400 ppm ascorbic acid), and 1.5% cultured dextrose-vinegar blend in cooked Calabrese sausages improves microbiological safety by lowering a_w and pH, effectively inhibiting *L. monocytogenes* and LAB throughout 75 days of refrigerated storage.

CRediT authorship contribution statement

Maria Stella de Azevedo Gonçalves: Resources, Methodology, Investigation. **Ana Lúcia da Silva Correa Lemos:** Writing – review & editing, Visualization, Validation, Resources, Formal analysis, Conceptualization. **Marcia Mayumi Harada Haguiwara:** Resources, Investigation. **Beatriz Thie Iamanaka:** Writing – review & editing, Validation. **Renata Bromberg:** Writing – review & editing, Visualization, Validation, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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