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ORIGINAL ARTICLE

Nisin or organic acid salt mixtures for the Calabrese-type sausages in industrial-scale production

Nisina ou misturas de sais de ácidos orgânicos para produção de linguiça tipo Calabresa em escala industrial

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Abstract

This study aimed to produce Calabrese-type sausages on an industrial scale by incorporating nisin and mixtures of organic acids and their salts as preservatives. In addition, it was assessed the stability of the product during storage at 25 °C. Various formulations were investigated, each featuring distinct preservative combinations as follows: Treatment 1 (T1) included sodium lactate and sodium acetate; Treatment 2 (T2) contained sodium lactate, sodium acetate, citric acid, and nisin; Treatment 3 (T3) involved a combination of salt and nisin alongside sodium lactate; and a control group which utilized sodium lactate. The moisture values ranged from 55.84% to 57.45%, proteins from 18.53 to 19.07, and lipids from 17.04% to 19.15%, all following Brazilian legislation. The pH remained stable until 90 days of storage, with values of 6.30, 6.28, 6.05, and 6.23 for control, T1, T2, and T3, respectively. T2 presented higher acidity compared to the other formulations due to the presence of citric acid. The evaluated preservatives (T1, T2, and T3) showed lower lipid oxidation indices and inhibited microbial growth. Therefore, the evaluated preservatives have the potential to be used industrially because they maintain the characteristics of the product and ensure 120 days of storage at 25 °C.

Keywords: Preservative; Lipid oxidation; Storage; Stability; Nisin; Organic acids.

Resumo

O objetivo deste estudo foi produzir linguiça tipo Calabresa em escala industrial, incorporando nisina e misturas de ácidos orgânicos e seus sais como conservantes. Adicionalmente, avaliar a estabilidade do produto durante o armazenamento a 25 °C. Várias formulações foram investigadas, cada uma apresentando combinações

conservantes distintas: Tratamento 1 (T1) incluiu lactato de sódio e acetato de sódio; o tratamento 2 (T2) continha lactato de sódio, acetato de sódio, ácido cítrico e nisina; o tratamento 3 (T3) envolveu uma combinação de sal e nisina juntamente com lactato de sódio, e um grupo controle utilizando lactato de sódio. Os valores de umidade variaram de 55,84% a 57,45%, proteínas de 18,53 a 19,07, e lipídios de 17,04% a 19,15%, todos de acordo com a legislação brasileira. O pH se manteve estável até 90 dias de armazenamento, apresentando valores de 6,30; 6,28; 6,05, e 6,23 para T1, T2, T3 e controle, respectivamente. O T2 apresentou maior acidez em relação às demais formulações devido à presença do ácido cítrico. Os conservantes avaliados (T1, T2 e T3) apresentaram menores índices de oxidação lipídica e inibiram o crescimento microbiano. Portanto, os conservantes avaliados têm potencial para serem utilizados industrialmente desde que mantenham as características do produto e garantam 120 dias de estocagem a 25 °C.

Palavras-chave: Conservante; Oxidação lipídica; Armazenamento; Estabilidade; Nisina; Ácidos orgânicos.

Highlights

- Nisine and organic acid mixtures improved sausage preservation and quality
- Promising industrial potential of preservatives in Calabrese-type sausages

1 Introduction

Pork stands as the most extensively consumed meat globally, accounting for 36%, followed by poultry (33%), beef (24%), and goats/sheep (5%) according to the United Nations Food and Agriculture Organization (2023). This prominence is in raw or processed meat, with a great demand for ham, sausages, and bacon (Hoelscher et al., 2023; Schwert et al., 2020). Among the typical Brazilian meat products made exclusively of pigs, some sausages are traditional, including mainly Tuscan sausage, smoked loin, and Calabrese-type sausages (Munekata et al., 2021). Per capita, pork consumption has shown a steady increase, culminating in 18 kg per person in 2022, as reported by the Brazilian Animal Protein Association. Concurrently, pork production in Brazil in the year 2022 attained a noteworthy milestone, reaching a total of 5 million tons (Associação Brasileira de Proteína Animal, 2023).

Sausages are one of the oldest processed foods. They are strongly present in the gastronomy of different countries. According to Brazilian legislation (Brasil, 2000), Calabrese-type sausage is an industrialized meat product, obtained from slaughtered animals, with or without added fatty tissues, condiments, wrapped in natural or artificial wrapping, and subjected to adequate technological processes, such as smoking and cooking.

The deterioration of Calabrese-type sausage is a problem for producers, retailers, and consumers. Microbial growth, lipid oxidation, and autolytic enzyme activity are the main causes of deterioration of these products (Malik et al., 2019). To ensure preservation and extend shelf life, it is essential to implement a combination of measures, including rigorous control and best practices throughout production, as well as thermal treatment, proper packaging, and the use of suitable ingredients (Brustolin et al., 2021; Cence et al., 2023).

Physical-chemical and microbiological factors that can affect food quality include microbial contamination and biochemical reactions that cause changes in color, texture, odor, and unpleasant taste (Amit et al., 2017; Galanakis, 2019; Ibrahim, 2020).

An emerging focus in food science research revolves around the exploration of food additives with antimicrobial properties. The processors use preservatives due to their widespread accessibility, comprehensive antimicrobial effectiveness, and cost-effectiveness (Sharma et al., 2021). Among the most

widely used conventional preservatives are organic acids and their derivatives. Acetic, propionic, lactic, sorbic, benzoic, citric, malic, fumaric, tartaric, and adipic acids, hydroxybenzoic acid esters (parabens), and glucono-delta-lactone (GDL). Benzoate, propionate, and sorbate are used in a wide variety of foods, while lactate and acetate are frequently used in meats and meat products (Malik et al., 2019; Matthew et al., 2020; Sharma et al., 2021). The antimicrobial activity of organic acids and their salts is mainly due to the inhibition of ATP generation, which affects the proton motive force of the membrane (Matthews et al., 2020; Sharma et al., 2021).

Nisin is a natural antimicrobial produced by certain strains of *Lactococcus lactis*. It can effectively inhibit most Gram-positive bacteria with a strong inhibitory effect on pathogenic bacteria such as *Staphylococcus aureus*, *Clostridium botulinum*, and *Streptococcus hemolyticus* as a natural antimicrobial peptide (Chen, 2019; Ray, 2019; Saltmarsh, 2020). Nisin has been confirmed as an effective preservative to extend shelf life in meat products as well (Jia et al., 2021).

According to the resolution RDC No. 272/2019 (Brasil, 2019b), new additives with a preserving function were approved for use in cooked products in Brazil, such as sodium acetate, sodium diacetate, nisin, propionic acid, and sodium propionate. By acknowledging these challenges, the study provides a foundation for further research and innovation in the application of preservatives to meats and meat products. It emphasizes the importance of finding effective antimicrobial solutions that are suitable for industrial-scale applications without compromising sensory quality (color, odor, taste, and flavor), as well as ultimately leading to the production of safer and more consumer-acceptable meat products. Thus, this study aimed to evaluate the preservative effect of nisin and mixtures of organic acids and their salts added to formulations of Calabrese-type sausage, produced on an industrial scale and vacuum-packed, during storage at 25 °C.

2 Material and methods

2.1 Preparation of Calabrese-type sausage formulations

Calabrese-type sausage was made in a pilot plant of an agroindustry in southern Brazil under the supervision of the Federal Inspection. The equipment and utensils were cleaned and sanitized following the Technical Guidelines for Identity and Quality of the Sausage of the Ministry of Agriculture of Brazil (Brasil, 2000).

Four formulations of Calabrese-type sausages were prepared using different preservatives, described as Treatments 1 (T1), 2 (T2), and 3 (T3), in addition to the control formulation (Table 1).

The control formulation contained sodium lactate as a preservative, which is traditionally used by the industry. In the other formulations studied, this preservative was replaced or used in association. The other constituents followed a standardized formulation used in the industry in which the experiments were conducted.

In Treatment 1 (T1), preservative A was used, which contained sodium lactate (53.5% w/w) associated with sodium acetate (9.6% w/w) (Corbion, Amsterdam), representing 1.79% (w/w) of the formulation. Treatment 2 (T2) used preservative B, composed of an organic acid mixture (sodium lactate, sodium acetate, and citric acid, 91% w/w) and nisin (0.0025% w/w) (Corbion, Amsterdam), used in partial replacement of sodium lactate, which was 0.5% (w/w) of the formulation in treatment 2. For Treatment 3 (T3), preservative C composed of sodium chloride (99.975%) and nisin (0.025%) (Kraki, Brazil) was used, representing 0.21% (w/w) of the formulation, and was associated with 1.55% (w/w) of sodium lactate. The amount of each preservative added was based on the supplier's indication. Sodium chloride (table salt) was obtained commercially from Salazir Ltda (Brazil). The amount of sodium chloride added to all formulations (T1, T2, and T3) and the control was 2% (w/w).

Table 1. Calabrese-type sausage formulations, based on the percentage of application indicated by each supplier.

Ingredients	Formulations			
	Control	T1	T2	T3
Lean scraps and pork bacon (%)	68.65	68.65	68.65	68.65
Mechanically separated meat (%)	19.67	19.67	19.67	19.67
Condiments (%)	9.87	9.87	9.87	9.87
Sodium Nitrite (%)	0.002	0.002	0.002	0.002
Sodium lactate (%)	1.79	-	1.29	1.55
Preservative A (%, w/w)	-	1.79	-	-
Preservative B (%, w/w)	-	-	0.50	-
Preservative C (%, w/w)	-	-	-	0.21

The Calabrese-type sausage was produced using raw pork (lean, trim and bacon) cut into pieces and ground with a meat grinder (model PS-22, Skymsen) into an 8mm disc. Mechanically separated meat (MSM) was obtained through a mechanical process of grinding and separating bones, carcasses, or parts of chicken carcasses. The condiments were not disclosed because the composition was confidential and was used for the preparation of the product by industry.

All the ingredients were homogenized for 10 minutes (mixer model MT-96, Incomaf). The samples were stuffed into natural wrapping, previously hydrated, with an approximate caliber of 40 mm and sausage link length of 20 cm. The sausage links were hung on stainless steel rods and placed in carts of the same material for cooking and smoking.

For the drying stage, thermal exchange with dry air (i.e., without direct steam injection) at an average temperature of 65 °C was employed. Natural smoke from the burning of eucalyptus sawdust was used, with an average temperature of 70 °C. The drying, smoking, and direct steam application occurred in 10 stages in an oven (model AG D-78479, Maurer). The seven initial stages were spaced apart by 15 minutes of drying, followed by 15 minutes of smoking. In stages 8, 9, and 10, the samples were submitted to smoking, drying, and direct steam, respectively (Table 2). The direct steam application involved the injection of humid steam until it reached 72°C in the middle of the product. The cumulative cooking duration amounted to 4 hours (240 minutes). The formulations were weighed to calculate the cooking loss.

Table 2. Stages of operations for drying, smoking, and direct steam application in the Calabrese-type sausage.

Stages	Description	Time (min)
1	Drying	15
2	Smoking	30
3	Drying	15
4	Smoking	30
5	Drying	15
6	Smoking	30
7	Drying	15
8	Smoking	30
9	Drying	30
10	Direct steam application	30
Total time		240

The samples were then cooled in a chamber to 12 °C. Subsequently, the product was packed in nylon/polyethylene bags (with an oxygen permeability rate lower than 50.00 CC/m^2 /day) and subjected to vacuum sealing. The product was stored at 25 °C for 120 days to evaluate its stability. An industrial-scale production of 50 kg was executed for every formulation, followed by packaging into 400 g units. The industrial process stages of mixing, stuffing, cooking, and packaging of Calabrese-type sausage are shown in Figure 1.

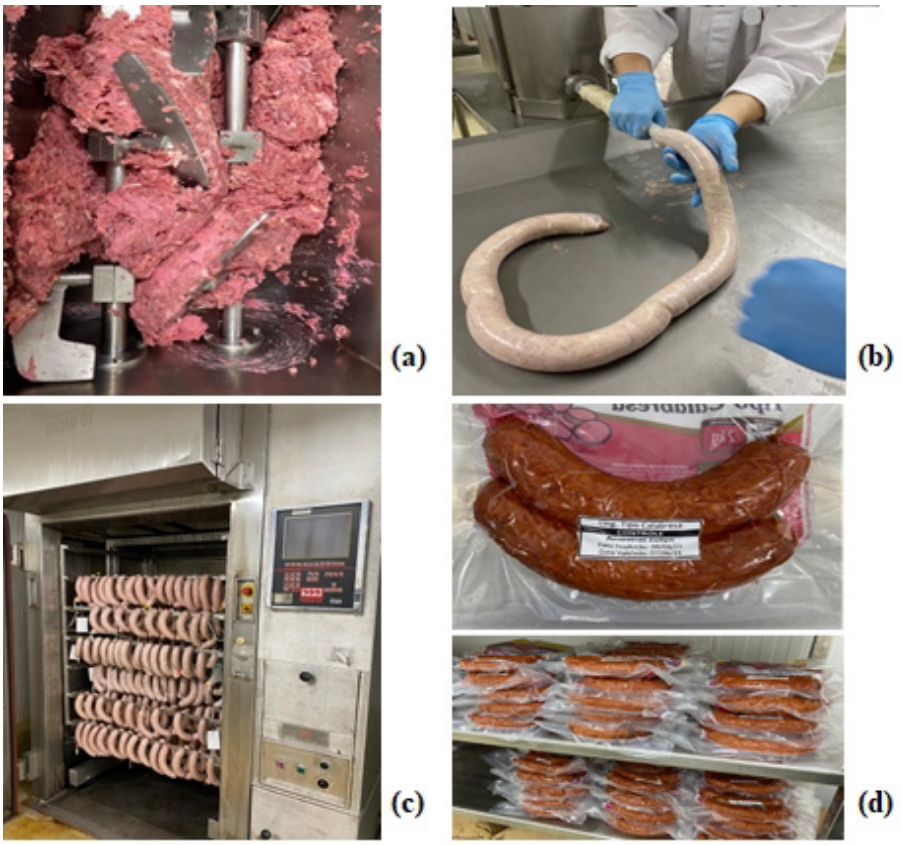

Figure 1. Illustration of industrial processes of mixing (a), stuffing (b), cooking (c), and packaging (d) of Calabrese-type sausages.

2.2 Characterization and chemical stability of the formulations

To characterize the Calabrese-type sausage samples, moisture, protein, and lipid analyses were carried out. To evaluate the product stability, pH, water activity (Aw), residual sodium nitrite and nitrate, lipid oxidation, and total acidity were monitored during storage at 25 °C for 1, 15, 30, 60, 75, 90, 105, and 120 days. A destructive sample was randomly selected for each analysis.

The determinations of moisture, protein, and lipids were performed on the first day of storage using the analytical methodologies proposed in the official methods manual for the analysis of animal-origin foods (Brasil, 2019a). Moisture was obtained by drying in an oven with air recirculation (Ethik Technology, model 500-5TS 150 L) at 103 ± 2 °C. The protein content was determined using the Kjeldahl method after sulfuric acid digestion in a digestor block (Kjeldatherm) and distiller (Vapodest VAP 450, Gerhartd). The nitrogen content was calculated from the amount of ammonia produced, and the protein content was determined by multiplying the nitrogen content by a factor of 6.25. The lipids were determined by the Soxhlet method using a Soxtherm apparatus (416, Gerhardt).

The pH was measured using a pHmeter (AKSO, model AK 96) for solids. The total acidity was determined according to the methodology described by the Adolfo Lutz Institute (Instituto Adolfo Lutz, 2008) and expressed in g of lactic acid per 100 g of sample. The water activity (Aw) was measured by AquaLab equipment (model 4TE).

The analysis of residual sodium nitrite and nitrate was carried out according to Brasil (2019a) which is based on the extraction of the sample with hot water, protein precipitation, and filtration, followed by the development of the red color by the addition of sulfanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride and determination in a spectrophotometer (Hitachi, model U-5100) at 538 nm. For sodium nitrate, an additional step occurred in the reduction of nitrate (NO_3) to nitrite (NO_2) from the addition of metallic cadmium.

To evaluate lipid oxidation in the samples, the test of substances reactive to 2-thiobarbituric acid reactive substances (TBARS) was used, according to Raharjo et al. (1992), concerning the interference of sugar in the reaction. The concentration was calculated by spectrophotometry (Parkin Elmer, Lambada EZ150 model) at 531 nm using a standard curve with TEPC (1 x 10-8 to 1 x 10-7 mol/mL). The results were expressed in milligrams of malondialdehyde (MDA) per kilogram of sample (MDA mg/kg of sample).

2.3 Microbiological analyses of formulations

The microbiological analyses of aerobic and facultative anaerobic mesophilic bacteria, molds, yeasts, *Salmonella* sp., *Clostridium* sulfite reducer, *Staphylococcus* coagulase positive and thermos-tolerant coliforms were evaluated on the first day, while the lactic acid bacteria (LAB) were monitored during storage for 120 days. The aerobic and facultative anaerobic mesophilic bacteria, molds, yeasts and LAB were evaluated according to the methodologies described by the Association of Official Analytical Chemists (2012). Microbiology analyses of *Salmonella* sp., *Clostridium* sulfite reducer, *Staphylococcus* coagulase positive and thermos-tolerant Coliforms were performed according to the recommendation and requirements of the Normative Instruction N° 60 of December 26, 2019 of the National Health Surveillance Agency (Brasil, 2019c). For the evaluation of *Salmonella* ssp., *Clostridium* sulfite reducers and *Staphylococcus* coagulase positive were determined by the method described in Normative Instruction nº 62 (Brasil, 2003), and thermo-tolerant coliforms according to American Public Health Association (2001).

2.4 Statistical analysis

All experiments were conducted in triplicate. The results for moisture, protein, and lipids underwent statistical analysis using one-way analysis of variance (One-Way ANOVA) and were compared using Tukey's test at a 95% confidence level ($p < 0.05$). The SASM-Agri program was utilized for this analysis.

For the results of Aw, pH, total acidity, TBARS, sodium nitrite, and nitrate, a Two-Way ANOVA was applied, and comparisons were made using Tukey's test at a 95% confidence level ($p < 0.05$). The Past 4.32 program was employed for this analysis.

3 Results and discussion

3.1 Chemical characteristics and stability of formulations

Table 3 presents the moisture, protein, and lipid contents of the Calabrese-type sausage formulations. The moisture content ranged from 55.84 to 57.45%, proteins from 18.53 to 19.07, and lipids from 17.04 to 19.15%. The protein values did not show a statistical difference ($p > 0.05$) between formulations. However, the moisture and lipid values showed significant differences ($p < 0.05$) between the formulations, which is common in the industrial environment and justified by the variability that occurs in the process of product elaboration, from the stage of preparation of the raw materials to the mixing and cooking of the Calabrese-type sausages. However, all values found are following Brazilian legislation for cooked sausages, which is up to 60% for moisture, a minimum of 14% protein, and a maximum of 35% for lipids (Brasil, 2000).

Table 3. Moisture, protein, and lipid contents of Calabrese-type sausage formulations with the addition of different preservatives.

Formulations	Moisture (%)	Protein (%)	Lipids (%)
Control	$56.98^{a} \pm 0.52$	$19.07^{a} \pm 0.26$	$17.74^{b} \pm 0.06$
T1	$55.84^{b} \pm 0.14$	$18.53^{a} \pm 0.16$	$19.15^{a} \pm 0.13$
T2	$56.79^{ab} \pm 0.55$	$18.99^{a} \pm 0.12$	$18.82^{a} \pm 0.52$
T3	$57.45^{a} \pm 0.35$	$19.02^{a} \pm 0.44$	$17.04^{b} \pm 0.09$

Means (± standard deviations) with different lowercase letters in the same column differ significantly at 5% level (Tukey's test). Control (sodium lactate), T1 - Treatment 1 (sodium lactate and sodium acetate), T2 - Treatment 2 (sodium lactate, sodium acetate, citric acid, and nisin), and T3 - Treatment 3 (nisin and sodium lactate).

The values of Aw, pH, total acidity, TBARS, residual sodium nitrite, and nitrate were evaluated during 120 days of storage (Figure 2). The results of these variables showed significant differences ($p < 0.001$) for treatment, storage time, and interaction between the variables, with the exception for the nitrite of the treatment and interaction (Table 4). The Aw in T1 and the control presented similar values ($p > 0.05$) during 120 days of storage. T3 showed lower values than the other treatments throughout the first 60 days of storage. During storage, the Aw of the control formulation decreased after 105 days ($p < 0.05$), reaching 0.947 in the last evaluation at 120 days. Similar behavior was observed for T1. This reduction may be associated with an increase in LAB counts (Figure 3) and proteolytic reactions in the product during storage. The cooking loss of all formulations evaluated was around 12 to 14%. These factors triggered greater water loss in the Calabrese-type sausage samples and a consequent reduction in Aw.

The results of the present study corroborate those obtained by Silva et al. (2014), who evaluated the effect of the addition of 3% sodium lactate and a combination of 3% sodium lactate and 0.5% nisin in pork sausage and obtained Aw values of 0.952 and 0.955, respectively.

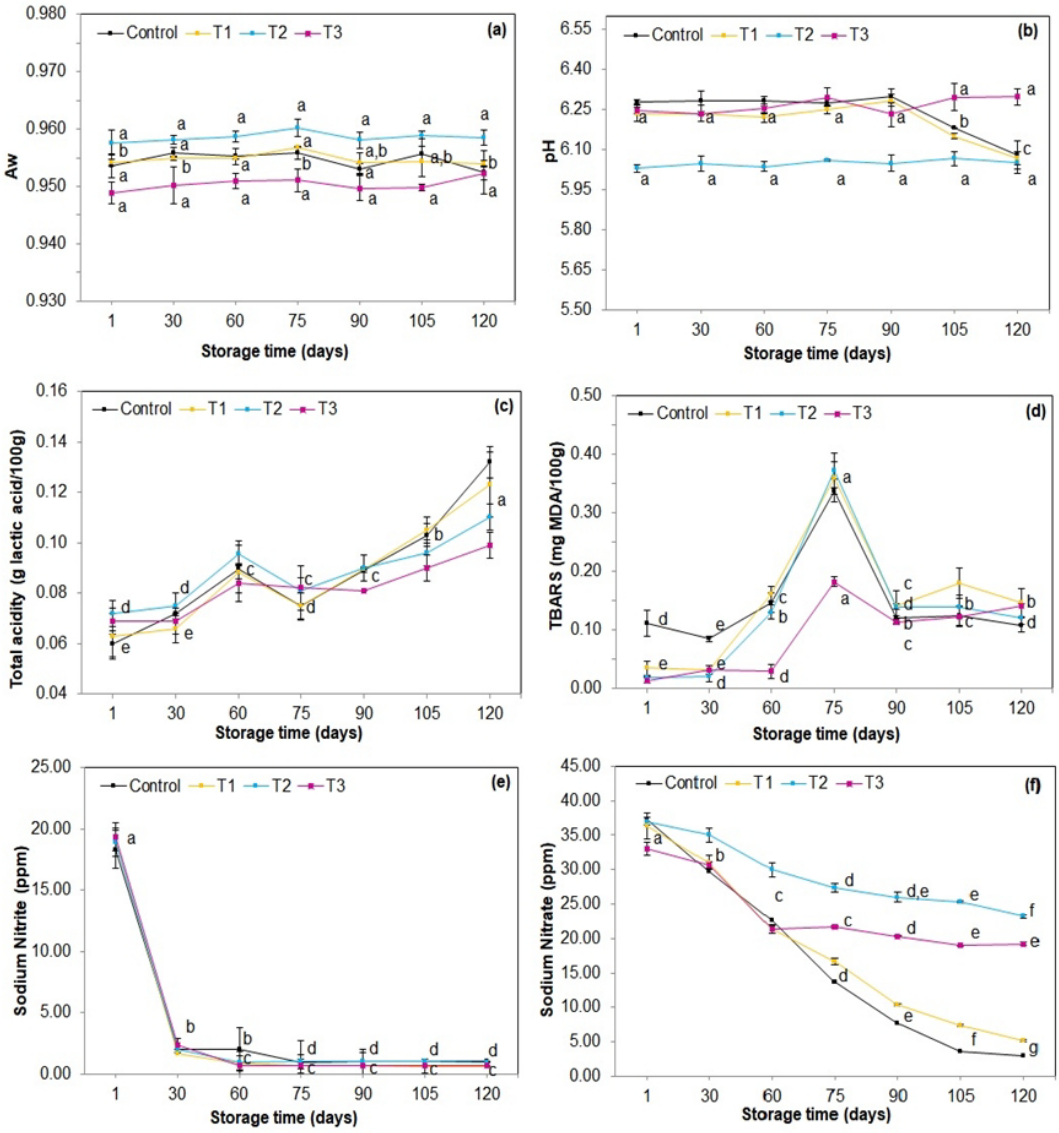

Figure 2. Values of Aw, pH, total acidity, TBARS, sodium nitrite, and nitrate of the Calabrese-type sausage formulations with the addition of different preservatives during storage. Means ± standard deviation followed by the same lowercase do not differ significantly ($p > 0.05$) according to Tukey's test during the storage. The bars represent the standard deviations. Control (sodium lactate), T1 - Treatment 1 (sodium lactate and sodium acetate), T2 - Treatment 2 (sodium lactate, sodium acetate, citric acid, and nisin), and T3 - Treatment 3 (nisin and sodium lactate).

In terms of pH, T2 showed a significant difference ($p < 0.05$) compared to the other treatments for up to 90 days of storage. This behavior can be explained based on the composition of the preservative B, which is composed of sodium lactate, sodium acetate, citric acid, and nisin. The citric acid in the applied amount caused a significant reduction in the product's pH, with the highest value being 6.07.

Throughout the storage, the pH of each formulation remained constant for up to 90 days, showing no significant difference ($p > 0.05$). Sodium lactate, sodium acetate, and citric acid additives act as buffers and regulate the acidity of meat products in addition to their preservation functions (Saltmarsh, 2020).

The kinetic profile of the acidity evolution of the Calabrese-type sausage samples shows no significant difference ($p > 0.05$) between the treatments and the control until the 75th day of storage. The gradual increase in total titratable acidity (TTA) observed after 90 days, primarily in the control and T1, can be attributed to microbial metabolism, as the bacteria present multiply and produce lactic, acetic, and succinic acids as a result (Gänzle, 2015).

To determine the extent of lipid oxidation in Calabrese-type sausage formulations, the TBARS test was performed for substances that are reactive to 2-thiobarbituric acid, indicating the preservation state of the products. The results obtained from the TBARS analysis (Figure 2) show that T1, T2, and T3 were effective in the first 30 days of storage, all showing significantly lower results ($p < 0.05$) when compared to the control, thus delaying lipid oxidation. This low TBARS value suggests that these treatments were successful in delaying the process of lipid oxidation in the product. At 60 and 75 days of storage, all treatments increased TBARs levels except T3, which remained lower than the others up to 120 days, indicating that the addition of nisin associated with sodium lactate contributed to a lower oxidation. The combination of nisin and sodium lactate offers a multifaceted approach to slowing down oxidative processes in food products. Their antimicrobial properties, pH-lowering effects, formation of stable complexes, and potential metal ion chelation collectively contribute to inhibiting the enzymatic and chemical reactions that lead to lipid oxidation, thereby extending the shelf life and maintaining the quality of the product.

Silva et al. (2014) evaluated the effect of sodium lactate and nisin applied to Toscana sausage and observed an increase in TBARS throughout storage at 4 °C with a value of 1.745 mg of MDA/kg at 42 days, also indicating that the association of additives was more effective in reducing the oxidative process.

After 90 days of storage, all TBARS values decreased and remained between 0.108 and 0.146 mg MDA/kg until 120 days of storage. Some authors presented characteristic TBARS indices of lipid oxidation. Olivo & Shimokomaki (2002) could cite that TBARS indices lower than 1.0 mg MDA/kg usually do not have residual flavors and odors of rancidity characteristic of lipid oxidation. Warriss (2000) reported that TBARS values between 0.5 and 2.0 mg MDA/kg are characteristic of oxidized meat. According to Terra et al. (2006), TBARS values above 1.59 mg MDA/kg of a sample can cause damage to the consumer's health.

In the present study, all samples were below the minimum limit of 0.5 mg MDA/kg, so no appearance of off-flavors was identified. The variations in TBARS values during storage are due to the fact that malonaldehyde reacts with a wide range of compounds or can form dienes or trienes, thus decreasing the amount of available malonaldehyde to react with thiobarbituric acid. As a consequence, the TBARS values evaluated can vary throughout storage (Schwert et al., 2020). These variations, whose values usually increase during storage, reaching a maximum value and then decreasing or stabilizing due to secondary reactions of the malonaldehyde, are probably associated with increasing concentrations of highly polar products resulting from the polymerization of secondary oxidation products (Savoldi et al., 2021).

It is noteworthy that the legislation does not present a maximum limit of MDA/kg in samples of meat products, so all the treatments studied are suitable for consumption during the storage period evaluated. Industrially produced and commercially distributed in Brazil, Calabrese-type sausages have a shelf life of 90 days when stored at room temperature and vacuum packed. Taking into account this index, the shelf life of these products could be extended for another 30 days, keeping the product in adequate conditions for consumption concerning lipid oxidation.

The levels of sodium nitrite showed a significant reduction ($p < 0.05$) from the 1st to the 30th day of storage (Figure 2), and after 75 days, they remained stable ($p > 0.05$). It should be noted that there was no significant difference ($p > 0.05$) between the formulations on each evaluated day.

In terms of residual sodium nitrate levels (Figure 2), a decrease in values was observed for all formulations ($p < 0.05$), with a significant decrease in T1 and control. The values found for sodium nitrate may be due to this compound being present in the raw material, seasonings, and even water since they are not part of the formulation. Another factor that also explains the nitrate levels in the formulations is the oxidation of nitrite to nitrate (Honikel, 2008).

Table 4. Results of significant differences for Aw, pH, total acidity, TBARS, sodium nitrite, and nitrate of the Calabrese-type sausage formulations with the addition of different preservatives according to storage time and treatment.

Variables	Factors	Sum of squares	DF	Mean square	F	*p*-value
Aw	Treatment	0.0006	3	0.0002	61	<0.0001
	Storage time	8.7089E^{-05}	6	1.4514E^{-05}	4.148	0.0016
	Interaction:	0.0002	18	9.7589E^{-06}	2.789	0.0017
pH	Treatment	0.7080	3	0.2359	148.9	<0.0001
	Storage time	0.0502	6	0.0083	5.272	0.0003
	Interaction:	0.1417	18	0.0078	4.968	<0.0001
Total acidity (g lactic acid/100 g)	Treatment	0.00164	3	0.0005	12.39	<0.0001
	Storage time	0.0195	6	0.0032	73.53	<0.0001
	Interaction:	0.0027	18	0.00015	3.421	0.0002
TBARS (mg MDA/100 g)	Treatment	0.0335	3	0.0111	37.52	<0.0001
	Storage time	0.5992	6	0.0999	335.6	<0.0001
	Interaction:	0.1263	18	0.0070	23.59	<0.0001
Sodium Nitrite (ppm)	Treatment	0.6512	3	0.2170	0.2941	0.8295
	Storage time	3280.89	6	546.814	740.9	<0.0001
	Interaction:	14.9681	18	0.831559	1.127	0.3528
Sodium Nitrate (ppm)	Treatment	2191.94	3	730.647	44.5	<0.0001
	Storage time	5443.50	6	907.249	55.26	<0.0001
	Interaction:	1310.04	18	72.7802	4.433	<0.0001

The decrease in nitrate concentrations can be affected by pH, cooking temperature, and storage conditions (Honikel, 2008). Another possible cause of this reduction may be associated with an increase in the microbial activity responsible for the reduction of sodium nitrate. According to Manahan (2016), in the absence of free oxygen, nitrate can be used by certain bacteria as an alternative electron acceptor. Chen (2019) described that the LAB group has strong nitrate reductase activity. It is then assumed that T2 and T3 were more effective in inhibiting the growth of LAB (Figure 3) in the samples and consequently reducing the rates of sodium nitrate over storage. The reduction in microbial load, preservation of an environment less conducive to nitrate-reducing microorganisms, pH effects, metal ion chelation, and stability of relevant enzymes collectively contribute to preserving the levels of sodium nitrate during storage (Furbeck et al., 2022).

Brazilian legislation (Brasil, 2019b) states that the sum of nitrites and nitrates, expressed as sodium nitrite, must not exceed 150 ppm; thus, all evaluated formulations meet the established limit.

3.2 Microbiological analyses

The counts of aerobic and facultative anaerobic mesophilic bacteria obtained in Calabrese-type sausage formulations in the initial evaluation for all treatments were lower than 1 log CFU/g (data not shown).

Regarding the count of molds and yeasts, coagulase positive *Staphylococcus*, and *Escherichia coli* no growth was observed in any of the formulations as well as absence of *Salmonella* (data not shown).

Figure 3 presents the results of the LAB counts for Calabrese-type sausage samples in the different formulations stored at 25 °C for 120 days. In the first 15 days of follow-up, no significant counts ($p > 0.05$) of LAB were identified in all treatments except the control.

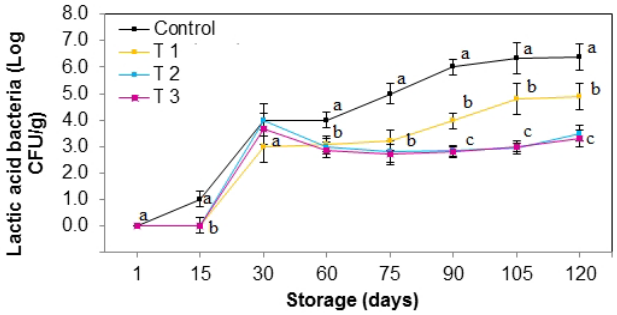

Figure 3. Lactic acid bacteria (LAB) count (log CFU/g) in Calabrese-type sausage formulations with different preservatives during storage. Means ± standard deviation followed by the same lowercase do not differ significantly ($p > 0.05$) according to Tukey's test during the storage. The bars represent the standard deviations. Control (sodium lactate), T1 - Treatment 1 (sodium lactate and sodium acetate), T2 - Treatment 2 (sodium lactate, sodium acetate, citric acid, and nisin), and T3 - Treatment 3 (nisin and sodium lactate).

On the 30th day, the counts of LAB increased in all treatments, reaching values between 3.0 and 4.0 log CFU/g and not differing from each other ($p > 0.05$). After this period, the control continued to increase fluctuations in the counts, reaching values around 6 log CFU/g after 120 days of storage. This behavior was not evident in the other treatments, which after 30 days of storage showed fluctuations and reductions in the counts of LAB. T1 (composed of sodium lactate and sodium acetate) had intermediate results, showing values in the order of 3 log CFU/g on the 75th day, reaching a maximum count of 4.9 log CFU/g at the end of the storage period evaluated. Until 120 days of storage, the T2 and T3 presented counted around 3 log CFU/g.

The use of nisin in combination with other preservatives in Calabrese-type sausage provided a barrier approach to controlling LAB growth. These combinations may increase the spectrum of antimicrobial activity because nisin and the combined substances act on different targets within the cell. Also, the combination of three organic acids associated with 10 times lower concentrations of nisin is similar to nisin treatment and more effective than combinations of two organic acids because it is more difficult for the target organism to adapt and resist a simultaneous attack on multiple targets on its cell wall (Dai et al., 2010).

It was found that T2 and T3 controlled the growth of LAB, which is desirable since microbial growth results in the degradation of structural components of meat, discoloration, formation of gases, pH change, and formation of sliminess (Malik et al., 2019).

In a study with cooked, vacuum-packed cured sausages superficially treated with commercial acid regulators containing lauric, citric, lactic, acetic, ascorbic acids, and sodium chloride at a dilution of 5% in soybean oil, it was possible to identify a considerable increase in shelf life, reaching an average life of 95 days. The acid regulator has proven its bactericidal and bacteriostatic properties, mainly in relation to lactic acid bacteria, which are responsible for the deterioration of the evaluated samples, making the appearance of the product unpleasant, which is a factor in its rejection by the consumer (Freiberger et al., 2016).

Silva et al. (2014) evaluated the effects of sodium lactate and nisin on vacuum-packed Toscana sausage storage at 4 °C. In the LAB count, the addition of 3% sodium lactate and 0.5% nisin was the most effective, obtaining 4 $\log_{10}$ CFU/g on the 40th day of storage, increasing the commercial shelf life of the sample by at least five days when compared to the others. The results highlight the synergism between the two additives used (sodium lactate and nisin), corroborating the results of this study.

Lee et al. (2020), evaluated the antimicrobial effects of EcoCal® (calcium oxide) and GF Bactostop® (a mixture of organic acids composed of salt, ascorbic acid, sodium acetate, calcium lactate, trisodium citrate, and citric acid) in sausages during storage at 10 °C. The formulation combining these two antimicrobials showed lower LAB counts ($p < 0.05$) compared to the control. In this treatment, the LAB counts were below the detection limit until the 64th day, presenting a count of 1.9 $\log_{10}$ CFU/g only on the 71st day. These results showed that the addition of 0.1% EcoCal® and 0.5% GF Bactostop® can be effective in inhibiting the growth of lactic bacteria in cooked sausages.

Generally speaking, the treatments applied in the formulations of Calabrese-type sausage affected the control of LAB after 30 days of storage. This result was satisfactory, since deterioration of cooked meat products usually occurs through the development of this group of bacteria (genera *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*), since they cause deterioration by acidification, greening, increased viscosity, and changes in the appearance and taste of the product (Mataragas et al., 2006).

4 Conclusion

The results showed that the evaluated preservatives, in particular T3, should be used as an alternative to the control treatment that presented only sodium lactate in the formulation, as they contributed to the maintenance of oxidative stability and control of LAB in Calabrese-type sausage produced on an industrial scale and stored at room temperature for 120 days. It is suggested that these preservatives have the potential to be used industrially in meat products marketed under room temperature storage (~ 25 °C).

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