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Spinach Extract as a source of nitrite in fresh sausages: quality attributes and stability during refrigerated storage

Extrato de espinafre como fonte de nitrito em linguiça frescal: atributos de qualidade e estabilidade durante o armazenamento refrigerado

Abstract

Introduction: Consumers express concerns about chemical additives in the diet, including preservatives, which can produce carcinogenic compounds when interacting with substances present in food. Some vegetables, such as spinach, can be natural sources of preservatives, and their application can increase the healthiness of meat products. ***Objective:*** To evaluate the effects of spinach extract as a source of nitrite for the preservation of fresh sausages during 35 days of refrigerated storage. ***Material and Method:*** Four formulations were produced: positive control, with commercial curing salt (CP), negative control, without curing salt (CN), spinach extract (EE) and spinach extract pre-converted by nitrate-reducing bacteria (EEPC). The sausages were evaluated for proximate composition, color parameters, residual nitrite content, TBARS, and microbiological characterization. ***Results:*** The curing salt (CP) led to a few changes in the color of the fresh sausages. TBARS increased approximately 2.5 times ($p<0.05$) during the 35 days for all formulations, and the lowest values were found for the formulations CP and EEPC at the end of the storage. These formulations had the highest residual nitrite levels and the lowest LAB and enterobacteria count (35 days), which proves that EEPC is an effective preservative to replace commercial curing salt in fresh sausages subjected to refrigerated storage. ***Conclusion:*** The use of EEPC can be a promising alternative for the meat industry, enabling the development of clean-label products that meet the demand for healthier products.

Keywords: Nitrate. Meat products. Healthy food. Vegetables.

Resumo

Introdução: Os consumidores têm inúmeras preocupações em relação à saúde, dentre as quais o consumo elevado de aditivos químicos, como os conservantes, que ao reagir com substâncias presentes nos alimentos podem produzir compostos cancerígenos. Alguns vegetais, como o espinafre, podem ser fontes naturais desses conservantes, e sua aplicação pode aumentar a saudabilidade dos produtos cárneos. ***Objetivo:*** Avaliar os efeitos do extrato de espinafre como fonte de nitrito para conservação de linguiças frescais, armazenadas durante 35 dias sob refrigeração. ***Material e Método:*** Quatro formulações foram produzidas: controle positivo, com sal de cura comercial (CP), controle negativo, sem sal de cura (CN), extrato de espinafre (EE) e extrato de espinafre pré-convertido por bactérias nitrato-redutoras (EEPC). As linguiças foram avaliadas através da composição centesimal, cor, teor residual de nitrito, TBARS e avaliação microbiológica. ***Resultados:*** O sal de cura (CP) resultou em menores alterações na coloração das linguiças frescais. O TBARS aumentou

aproximadamente 2,5 vezes ($p<0,05$) ao longo dos 35 dias, em todas as formulações, e os menores valores, ao final desse período, foram encontrados nas formulações CP e EEPC. Estas mesmas formulações apresentaram os maiores teores residuais de nitrito e menor contagem de BAL e enterobactérias (35 dias), o que comprova que o EEPC é tão eficiente quanto o sal de cura comercial como conservante em linguiça frescal armazenada sob refrigeração. ***Conclusão:*** O uso de EEPC pode ser uma alternativa promissora para a indústria cárnea, possibilitando o desenvolvimento de produtos *clean label* que atendam à demanda por produtos mais saudáveis.

Palavras-chave: Nitrato. Produtos da carne. Alimento saudável. Vegetais.

INTRODUCTION

Curing salts based on nitrite and nitrate are used in cured meat products to help develop color and flavor, control lipid oxidation by delaying the development of rancidity and inhibit the growth of pathogenic microorganisms such as *Clostridium botulinum*. These chemical additives are often used in combination, as nitrate is more beneficial in long-term curing processes, while nitrites act at the beginning of the process.[1]

Studies have shown that the nitrites and nitrates found in meat products can react with certain amines present in food and produce N-nitroso compounds, such as nitrosamines, which are carcinogens responsible for increasing the risk of gastric cancer, the main disease associated with the consumption of processed meats.[2-4]

In general, consumers have numerous health and food safety concerns, including the consumption of products containing a large amount of chemical additives, such as preservatives, which increases the demand for foods made with natural ingredients. Some vegetables can be natural sources of nitrite and nitrate, such as beet, grape seeds, chestnuts, organic tomato pulp, leeks, and spinach, among others. These foods have considerable amounts of nitrate in their composition, which can be used as an alternative source of this additive in meat products.[5-8]

In addition to being high in iron, spinach (*Spinacia oleracea*) contains high amounts of nitrate and nitrite.[9-11] Sebranek & Bacus[12] reported levels of 3,227 ppm of nitrate in spinach juice. In addition, easy planting and fast-growing make spinach a lucrative raw material for use in the food industry.[13]

Currently, there is a need to reduce and/or replace chemical additives in food production aimed at producing healthier foods. In turn, it is also known that spinach can serve as a natural preservative for the food industry due to its high nitrate content. Therefore, this study aimed to evaluate the physicochemical and microbiological characteristics of fresh pork sausages made with the addition of spinach extracts and spinach pre-converted by nitrate-reducing bacteria as an alternative source of nitrite during refrigeration storage.

MATERIAL AND METHOD

Material

The reagents used in the analysis and manufacture of the sausages were sodium hydroxide, sodium tetraborate, zinc acetate, dibasic sodium phosphate, and potassium ferrocyanide from Synth (São Paulo, Brazil); sulfanilamide and N-(1-Naphthyl) ethylenediamine (NED) from Sigma-Aldrich (São Paulo, Brazil); plate count agar (PCA), MRS agar, and VRBG agar from Merck (Belo Horizonte, Brazil). Kura Tel Frescal from Conatril - SBR Foods Ltda (Rio Claro, Brazil) was used as a curing salt. All other reagents were of analytical grade. The spinach was collected in the city of Chapecó-SC, Brazil (latitude: 27º 05' 47" S, longitude: 52º 37' 06" W). The raw materials used to produce the fresh sausages were purchased at a local market in the city of Pinhalzinho-SC.

Production of spinach powder

The spinach was sanitized with a 200 ppm sodium hypochlorite solution for 15 minutes and rinsed under running water. The spinach (stalk and leaf) was dried at 80 ºC (defined in pre-tests) in an oven with a CE-205 forced air convection system (Cienlab, Brazil) until reaching a moisture content lower than 10%

(approximately 6 hours). They were then ground in a blender (Diamante 800, Britânia, Brazil) and sieved through a 32-mesh Tyler sieve (Bertel, Brazil).

Preparation and characterization of spinach extracts

To prepare the extracts, 20 g of spinach powder was mixed with 200 mL of distilled water and kept for 2 hours at 90 ºC in an SSD 5L thermostatic bath (Solidsteel, Brazil) to extract the compounds of interest, according to Gardes et al.[14] with adaptations. The extract was then divided into two parts, consisting of extract 1 (pre-converted spinach extract - EEPC) and extract 2 (spinach extract - EE). Then, 0.05% (w:v) of active nitrate reductase culture containing *Staphylococcos carnosus Staphylococcos xylosus* (Bactoferm SM-75, CHR Hansen) was added to the EEPC, and the mixture was placed in a Luca-223 shaker incubator (Lucadema, Brazil) at 30 °C for 24 hours, at 100 rpm. The solution was then centrifuged in an SL-700 centrifuge (Solab, Brazil) at 6,000 rpm for 10 minutes and the supernatant was filtered through Whatman No. 1 filter paper and stored in a drying oven (model 80/280; Lucadema, Brazil) at 90 °C for 15 hours for extract concentration and bacterial inactivation. The EE was subjected to the same analysis conditions for extract concentration. The EEPC and EE extracts were stored in amber vials at 4 °C until analysis. The determinations were carried out in triplicate, as described below.

The pH was determined according to method 981.12.[15] The residual nitrite content was determined using the spectrophotometric method as described by Oliveira et al.,[16] in which 10 g of sample were mixed with 100 mL of deionized water at 60 ºC in an Erlenmeyer flask. Then, 5 mL of 0.5% sodium tetraborate solution (w:v) was added and kept for 15 minutes under agitation in a LUCA-157/28 thermostatic bath (Lucadema, Brazil). The solution was then transferred to a 250 mL volumetric flask and 5 mL of 15% potassium ferrocyanide solution (w:v) and 5 mL of 30% zinc acetate solution (w:v) were added, and the volume was made up with deionized water. The solution was filtered through Whatman No. 1 filter paper, and a 10 mL aliquot was transferred to a 50 mL volumetric flask, with the addition of 5 mL of 0.5% sulfanilamide solution (w:v) and rest for 3 minutes. Then, 4 mL of 0.5% (w:v) NED solution was added, and the volume was made up of deionized water. The remaining solution was left to stand for 30 minutes, and absorbance readings were performed in a Cirrus 80SA spectrophotometer (Femto, BRA) at 540 nm. The standard curve was previously prepared with sodium nitrite concentrations of 0.16 to 1.12 $mg.kg^{-1}$, subjected to the same procedures as the samples. The results were expressed in $mg.kg^{-1}$ of sample.

The residual nitrate content was determined as proposed by Cataldo et al.[17] with adaptations. For that, 0.2 mL of the extract was mixed with 0.8 mL of 5% (v/v) salicylic acid solution in sulfuric acid in a beaker and kept for 20 minutes at room temperature for the reaction. Then, 19 mL of 2 N sodium hydroxide was added, and after temperature stabilization for color development (25 °C) absorbance readings were performed at 410 nm in a Cirrus 80SA spectrophotometer (Femto, Brazil). The standard curve was prepared with sodium nitrate concentrations of 0.002 to 0.06 $mg.mL^{-1}$. The results were expressed in $mg.kg^{-1}$ of sample

Manufacture of fresh sausages with spinach extract

The fresh sausages were made according to the formulations described in Table 1: negative control (NC) containing only raw meat material; positive control (PC) containing nitrate/nitrite curing salt; EEPC containing 4% EEPC extract (pre-converted by nitrate-reducing bacteria); and EE containing 4% EE extract. The sausages were produced from pork meat and fat previously ground into an 8 mm disk in an MSI-10 industrial grinder (Becker, Brazil), followed by the addition of the other ingredients, which were mixed by hand

for 15 minutes and then stuffed into 36 mm natural pork casings. The control formulation was produced with a lower salt content, due to the presence of salt in the sodium nitrite/nitrate condiment

Table 1. Fresh pork sausage formulations made with different types of preservatives. Pinhalzinho, SC, 2022

Raw materials and ingredientes	CN (%)	CP (%)	EEPC (%)	EE (%)
Pork leg	85	85	85	85
Pork fat back	15	15	15	15
Salt	2	1.575	2	2
Sodium erythorbate	0.1	0.1	0.1	0.1
Sugar	0.1	0.1	0.1	0.1
Garlic powder	0.05	0.05	0.05	0.05
Onion powder	0.05	0.05	0.05	0.05
Black pepper powder	0.025	0.025	0.025	0.025
Water	4	4	-	-
Spinach extract (E1)	-	-	4	-
Spinach extract (E2)	-	-	-	4
Curing salt	-	0.425	-	-

Fresh pork sausages: CN - negative control without curing salt; CP - positive control with the addition of curing salt; EE - spinach extract; EEPC - pre-converted spinach extract.

The sausages were vacuum-packed (200 S sealing machine; Selovac, Brazil) and stored at 4 ºC (Solid Steel, Brazil). All treatments were replicated three times. For each replication, 13 sausages were produced per treatment. The samples were characterized for proximate composition on day 1, and pH, color parameters, thiobarbituric acid reactive substances (TBARS), residual nitrite contents, and microbiological analysis on days 1, 7, 14, 21, 28, and 35 of refrigerated storage (4 ºC), as described below.

Characterization of fresh sausages with spinach extract

The proximate composition of the sausages was determined 24 hours after manufacture, as described by AOAC.,[15] for moisture (method 925.45 (b)), fat (Soxhlet method 920.39 (c)), ash (method 940.26), and protein contents (method 920.152), in triplicate. The pH and residual nitrite contents were evaluated in triplicate, according to the methodologies described for the characterization of spinach extracts.

The color measurements were performed in triplicate using a MiniScan EZ portable colorimeter (Hunter Lab, Brazil) for the parameters L* (luminosity) (black (0) to white (100)), a* (green (-a*) to red (+a*)), and b* (blue (-b*) to yellow (+b)), according to the manufacturer's instructions. The color coordinates a* and b* were used to calculate the chroma values (C*) and Hueangle.[18]

To evaluate the lipid oxidation, thiobarbituric acid reactive substances (TBARS) assay was performed as described by Marangoni & Moura,[19] with modifications. For that, 5 g of the sample was homogenized with 30 mL of 7.5% trichloroacetic acid solution, in triplicate. The mixture was filtered through qualitative filter paper and a 2 mL aliquot of the filtrate was transferred to a test tube with 2 mL of 0.02 M thiobarbituric acid, which was immersed in an SSD 5L digital thermostatic bath (Solidsteel, Brazil) at 100 ºC for 20 minutes. The tubes were then cooled to room temperature and absorbance readings were performed at 532 nm in a Cirrus 80SA spectrophotometer (Femto, Brazil). A standard curve was made with concentrations of 0.00 to 0.9 µg.mL^{-1} of 1,1,3,3-tetraethoxypropane (TEP). The results were expressed in mg of malonaldehyde (MDA).kg^{-1} of sample.

For the enumeration of aerobic mesophilic bacteria, lactic acid bacteria (LAB), enterobacteria, and psychrotrophic bacteria, 10 g of the sample was aseptically transferred to sterile plastic stomacher bags, and 90 mL of 0.1% peptone saline solution was added. Then, 0.1 mL of the dilutions were inoculated on to Petri plates containing plate count agar (PCA) and incubated at 37 ºC for 48 hours for aerobic mesophilic bacteria counts; in Man Rogosa & Sharpe agar (MRS) at 37 ºC for 48 hours for LAB counts; in Violet Red Bile Glucose agar (VRBG) at 37 ºC for 24 hours for total enterobacteria counts, according to Pintado et al.,[20]. Psychrotrophic bacteria counts were performed using PCA, incubated at 10 ºC for 10 days, according to Schilling et al.[21] The plates with the presence of colonies were counted, and the results were expressed as log CFU.g^{-1}.

Statistical analysis

Data were analyzed for analysis of variance (ANOVA) and comparison of means using Tukey's test at a 5% significance level using STATISTICA 14 Trial software (Statsoft).

RESULTS AND DISCUSSION

Characterization of spinach extract

Significant differences ($p<0.05$) were observed for the residual nitrite and nitrate contents of the spinach extracts EEPC and EE. EEPC had residual nitrate and nitrite contents of 596.99±0.75 and 1162.68±0.44 mg.kg^{-1} respectively, while the spinach extract (EE) had residual nitrate and nitrite contents of 923.22±0.37 and 528.34±0.65 mg.kg^{-1}, respectively. The nitrate reductase culture was able to convert nitrate into nitrite in the spinach extract, resulting in nitrite values twice as high in EEPC. For pH, no significant differences ($p>0.05$) were found between the samples EEPC and EE (5.72±0.05 and 5.77±0.01, respectively).

Characterization of fresh sausages made with different preservatives

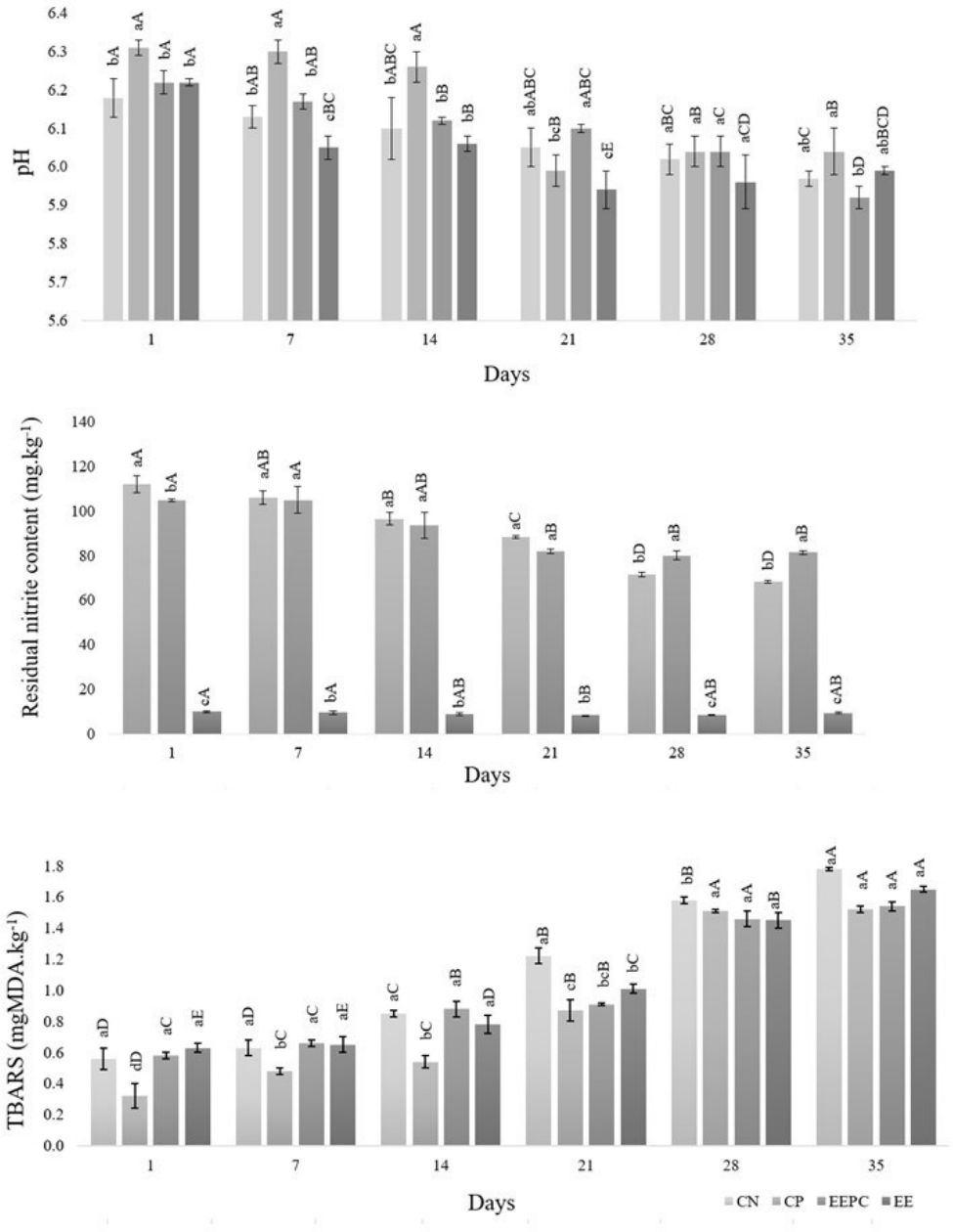
There were no significant differences between the formulations ($p>0.05$) for the physicochemical parameters moisture, fat, protein, and ash (Table 2). The sausages met the identity and quality standards required for the product, according to Brazilian legislation,[22] which establishes max 30% fat, min 12% protein, and max 70% moisture. These results confirm that the addition of spinach extract did not alter the physicochemical characteristics of the samples, indicating that it is possible to use the extract in fresh pork sausage formulations at the concentration studied.

Table 2. Proximate composition of fresh pork sausages during 35 days of refrigeration storage (4 ºC). Pinhalzinho, SC, 2022.

Samples	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
CN	64.20±1.58	16.01±0.08	11.08±0.41	2.51±0.07
CP	64.01±2.09	15.98±0.07	11.40±0.23	2.68±0.07
EEPC	64.98±1.24	15.92±0.10	11.36±0.29	2.65±0.20
EE	63.65±1.19	15.94±0.06	11.37±0.54	2.68±0.13

Concerning the pH (Figure 1), a decrease ($p<0.05$) was observed during 35 days of storage for all formulations, due to the increase in lactic acid bacteria counts (Table 4). These bacteria have lactic acid as their metabolic product, which leads to a decrease in pH.[23] Djeri & Williams[11] reported a reduction in the pH of mortadella made with celery juice powder as a substitute for nitrite from the third week of storage, due to an increase in the number (log CFU.g^{-1}) of lactic acid bacteria, which is similar to that found in this study.

Figure 1. Results of pH, residual nitrite content and lipid oxidation by the TBARS assay of fresh pork sausages during 35 days of refrigerated storage (4 °C)



Fresh pork sausages produced with different preservatives: CN - negative control without curing salt; CP - positive control with the addition of curing salt; EE - spinach extract; EEPC - pre-converted spinach extract. Different lowercase letters within the same storage time show a significant difference ($p<0.05$). Different uppercase letters between storage times show a significant difference ($p<0.05$).

Concerning the color parameters L*, C*, and Hue angle (Table 3), there was an increase ($p<0.05$) in the L* values for the treatments CN, EEPC, and EE from day 1 to 35 of storage, which was not expected since during the storage of meat products under vacuum, myoglobin oxidizes to metmyoglobin, which is brown, resulting in the darkening of the samples. However, some factors such as protein denaturation and water exudation, which are intensified by the pH reduction (Figure 1), may have led to an increase in L* values.[24]

Similar results were also reported by Sucu & Turp,[7] who studied beet powder as a substitute for nitrite in beef sausages after 56 days of storage.

Regarding the L* values between the samples for the same day, there was no significant difference on day 1 ($p>0.05$); however, CP showed a lower L* value ($p<0.05$) on the last day of storage, i.e., they were darker. This formulation also showed no variation in this parameter over the 35 days studied, indicating that the commercial curing salt can maintain color stability, which was not observed for the other formulations.

Table 3. Color parameters luminosity (L*), hue (ºHue), and chromaticity (C*) of fresh pork sausages during 35 days of refrigerated storage (4 ºC). Pinhalzinho, SC, 2022.

	Days					
Sample	1	7	14	21	28	35
L*						
CN	38.21±0.41^{aC}	41.99±0.77^{aB}	41.87±0.28^{abB}	48.04±0.40^{aA}	48.27±0.66^{aA}	48.31±1.00^{aA}
CP	38.64±1.54^{aA}	40.97±0.77^{aA}	40.58±0.93^{bcA}	40.25±1.37^{cA}	39.58±0.16^{cA}	41.04±0.51^{bA}
EEPC	37.65±0.45^{aC}	37.93±0.56^{bC}	38.90±0.65^{cC}	43.68±0.92^{bB}	47.07±0.77^{abA}	48.74±0.38^{aA}
EE	40.16±1.05^{aE}	42.27±0.19^{aD}	42.83±0.59^{aCD}	44.57±0.86^{bBC}	45.91±0.45^{bAB}	47.23±0.65^{aA}
ºHue						
CN	57.11±1.42^{aA}	53.13±0.06^{cA}	54.84± 1.61^{bA}	60.60±0.61^{bA}	55.89±1.96^{bA}	60.90±1.72^{cA}
CP	53.56±0.78^{bC}	61.84±1.62^{aAB}	60.77±1.85^{aBC}	65.58±1.28^{aAB}	67.57±0.52^{aAB}	68.09±1.68^{abA}
EEPC	55.35±0.78^{abB}	54.46±0.58^{bcB}	64.12±1.42^{aA}	67.44±1.56^{aA}	65.84±1.54^{aA}	71.35±1.70^{aA}
EE	57.29±1.12^{aC}	55.58±0.68^{bC}	60.84±1.48^{aB}	65.10±1.42^{aA}	65.19±1.64^{aA}	65.84±0.72^{bA}
C*						
CN	13.37±0.12^{bcABC}	13.95±0.79^{aAB}	13.35±1.16^{aABC}	14.56±0.36^{aA}	12.73±0.28^{bBC}	12.04±0.14^{bC}
CP	12.91±0.38^{cAB}	14.14±0.50^{aA}	13.78±0.54^{aA}	13.45±0.57^{aA}	12.01±0.42^{bB}	12.95±0.68^{abAB}
EEPC	14.56±0.40^{aA}	14.45±1.16^{aA}	15.02±0.25^{aA}	14.35±0.52^{aA}	14.47±0.94^{aA}	13.99±0.46^{aA}
EE	14.15±0.29^{abABC}	14.84±0.39^{aA}	14.36±0.61^{aAB}	13.41±0.54^{aBC}	13.14±0.25^{abC}	13.46±0.25^{aBC}

Mean ± standard deviation. Fresh pork sausages produced with different preservatives: CN - negative control without curing salt; CP - positive control with the addition of curing salt; EE - spinach extract; EEPC - pre-converted spinach extract. Different lowercase letters in the same column show a significant difference between the samples ($p<0.05$). Different uppercase letters in the same row show a significant difference between the samples ($p<0.05$).

The color measurements of the sausages ranged from 53.56º to 71.35º, i.e. they remained between the red (0º) and yellow (90º) color.[25] A significant increase ($p<0.05$) in ºHue was observed for CP during 35 days of storage. In turn, EEPC showed an increase followed by stabilization after day 14 of storage. The same behavior was observed for EE, with a significant increase ($p<0.05$) up to day 21 of storage. For CN, no significant differenceswere observed throughout the 35 days of storage ($p>0.05$). The reddish color of the sausages, especially on days 1 and 7, is due to the formation of oxymyoglobin.[26] The increase in ºHue observed in CP, EEPC, and EE, which indicates an increase in the yellowish hue, is associated with lipid oxidation reactions leading to the formation of yellowish polymers,[27] which corroborates the results of lipid oxidation in this study (Figure 1). Furthermore, the increase in °Hue may be due to the pigment from the spinach extract, which is naturally green. The destruction of myoglobin by bacterial growth can also lead to

an increase in Hue angle since the microorganisms use myoglobin as a nutrient, developing a greenish and/or yellowish color.[23]

Saturation is directly associated with the concentration of the coloring element and represents a quantitative attribute. The higher the C*, the greater the color saturation perceptible to humans.[25] Overall, no significant differences ($p>0.05$) were observed for the C* values of the samples during the 35 days of storage. It is worth mentioning that changes in color during longer storage times can be minimized by using natural dyes such as carmine red, which is a common practice in the meat products industry, especially fresh sausages, which can minimize the sensory rejection resulting from the use of spinach extract.

The values for lipid oxidation increased approximately 2.5 times ($p<0.05$) for all sausages evaluated during the period studied (Figure 1). After 35 days of storage, there was no significant difference ($p>0.05$) between CP and EEPC, thus the pre-converted extract was as effective as the commercial curing salt in controlling lipid oxidation in fresh pork sausages. Studies using spinach extract in cured pork loin showed a similar protective effect in controlling lipid oxidation when compared to synthetic nitrite.[28] CN also showed greater oxidation on days 21 and 35 of storage ($p<0.05$), showing that the addition of nitrate/nitrite in the form of spinach extract or commercial curing salt is capable of inhibiting the oxidation process in meat products.[1] Rosa et al.[29] reported that values of up to 1.59 mg of MDA.kg^{-1} in meat are not perceived by sensory analysis and do not cause damage to consumer health. In this study, only the positive control samples (PC) and the samples made with pre-converted spinach extract (EEPC) showed values below 1.59 mg of MDA.kg^{-1} for the 35 days of storage, thus they may be suitable for consumption from a sensory point of view.

The highest residual nitrite content ($p<0.05$) was found for the sample CP (Figure 1), on day 1 of storage, due to the composition of the curing salt, which contains nitrate and sodium nitrite. At 7, 14, and 21 days of storage, the sample EEPC showed values similar to the positive control ($p<0.05$). However, at the end of storage (28 and 35 days), EEPC reached higher values than CP, thus the nitrite content of the samples made with curing salt decreased more quickly.

A sharper decrease in nitrite in CP was also reported by Bertol et al.[30] in dry fermented sausages made with rosemary and celery extract. A decrease of residual nitrite was observed for CP and EEPC ($p<0.05$) throughout the storage. During the curing stage, nitrate is reduced to nitrite and the latter to nitric oxide, which reacts with the iron in the meat's heme pigment molecule, resulting in nitrosomyoglobin, which is responsible for the formation of the pink color typical of cured meat products.[31]

Fresh sausage made with spinach extract that was not pre-converted (EE) had a low residual nitrite content throughout the storage. This extract has high nitrate contents and therefore requires more time (since the action of nitrate-reducing bacteria can be slow) for conversion to nitrite. The sample CN is not shown in Figure 1, as no residual nitrite content was detected in this formulation.

Concerning the microbiological characterization, the sausages showed initial (day 1) mesophilic bacteria counts of 3.5 log CFU.g^{-1} (Table 4), which are within the parameters determined by Normative Instruction 161, of July 1, 2022, which establishes the microbiological parameters for food.[32] On day 35 of storage, no significant differences were observed for bacterial counts of the treatments CP, EEPC, and EE ($p>0.05$), while CN showed higher mesophilic bacteria counts ($p<0.05$). These results indicate that spinach and pre-converted spinach extracts may have antimicrobial activity similar to commercial curing salt in chilled fresh pork sausages.

Table 4. Count of mesophilic bacteria, psychrotrophic bacteria, lactic acid bacteria, and enterobacteria (log CFU.g^{-1}) in fresh pork sausages during 35 days of refrigerated storage (4 °C). Pinhalzinho, SC, 2022.

	Storage time (days)					
Samples	1	7	14	21	28	35
Mesophilicbacteria						
CN	3.55±0.09^{aD}	6.25±0.10^{aBC}	6.55±0.17^{aB}	6.33±0.07^{aBC}	6.04±0.06^{aC}	7.96±0.15^{aA}
CP	3.51±0.12^{aD}	3.89±0.09^{bCD}	4.61±0.33^{cC}	5.53±0.41^{bB}	5.82±0.46^{aAB}	6.63±0.33^{bA}
EEPC	3.42±0.14^{aE}	4.38±0.43^{bD}	4.90±0.15^{bcCD}	5.15±0.12^{bBC}	5.76±0.21^{aB}	7.04±0.14^{bA}
EE	3.52±0.12^{aC}	4.29±0.24^{bC}	5.46±0.46^{bB}	6.67±0.15^{aA}	6.61±0.18^{aA}	7.05±0.11^{bA}
Psychrotrophic bacteria						
CN	4.29±0.03^{aC}	7.47±0.15^{aA}	7.29±0.01^{aAB}	7.35±0.20^{abAB}	7.45±0.04^{aA}	7.61±0.04^{cA}
CP	4.17±0.14^{aD}	4.44±0.11^{cD}	5.21±0.27^{bC}	6.55±0.25^{cB}	7.05±0.05^{bAB}	7.51±0.05^{bA}
EEPC	4.27±0.21^{aC}	6.81±0.04^{bAB}	7.19±0.19^{aA}	7.09±0.05^{bA}	6.63±0.17^{cB}	7.42±0.09^{bA}
EE	4.28±0.09^{aC}	7.39±0.13^{aB}	7.60±0.14^{aAB}	7.68±0.19^{aAB}	7.41±0.05^{aB}	7.92±0.11^{aA}
Lacticacid bacteria						
CN	3.14±0.14^{aC}	5.44±0.36^{aB}	5.92±0.14^{bB}	6.25±0,07^{bB}	6.31±0.16^{aB}	7.26±0.15^{aA}
CP	3.04±0.12^{aD}	3.41±0.23^{bD}	4.68±0.16^{cC}	5.66±0.29^{cB}	6.40±0.23^{aA}	6.50±0.12^{bA}
EEPC	2.70±0.09^{aD}	3.65±0.09^{bC}	4.37±0.09^{cB}	4.97±0.09^{dB}	4.86±0.09^{bB}	6.28±0.10^{bA}
EE	2.62±0.15^{aD}	5.85±0.13^{aC}	6.70±0.08^{aB}	6.73±0.09^{aB}	5.23±0.27^{bE}	7.37±0.04^{aA}
Enterobacteria						
CN	1.91±0.13^{aC}	3.30±0.25^{aB}	4.15±0.19^{aA}	3.33±0.05^{aB}	4.29±0.45^{aA}	4.59±0.35^{aA}
CP	2.00±0.13^{aBC}	1.68±0.33^{cC}	2.02±0.16^{cBC}	2.31±0.07^{bAB}	2.45±0.22^{bAB}	2.75±0.03^{bA}
EEPC	2.08±0.26^{aB}	2.23±0.06^{bcAB}	2.54±0.23^{cAB}	2.62±0.32^{bAB}	2.62±0.11^{bAB}	2.93±0.54^{bA}
EE	2.20±0.06^{aD}	2.76±0.10^{abC}	3.28±0.37^{bB}	3.72±0.21^{aAB}	3.81±0.11^{aA}	4.19±0.06^{aA}

Mean ± standard deviation. Fresh pork sausages produced with different preservatives: CN - negative control without curing salt; CP - positive control with the addition of curing salt; EE - spinach extract; EEPC - pre-converted spinach extract. Different lowercase letters in the same column show a significant difference between the samples ($p<0.05$). Different uppercase letters in the same row show a significant difference between the samples ($p<0.05$).

Regarding the psychrotrophic bacteria (Table 4), a significant increase ($p<0.05$) was observed for CN, EEPC, and EE from day 1 to 7, which remained stable or increased slightly until the last day of storage. The CP showed a significant increase ($p<0.05$) after 14 days of storage, with no difference from the EEPC at 35 days of storage ($p>0.05$), indicating that EEPC had a similar effect to commercial curing salt on these microorganisms.

Although the current legislation has no limit established for psychrotrophic bacteria counts, they are indicators of quality and shelf life since they develop well at refrigeration temperatures and are capable of deteriorating the product, reducing the shelf life of refrigerated foods.[33] Miyagusku et al.,[34] evaluated irradiated chicken breast cuts and reported the formation of surface slime on the product and an unpleasant odor for psychrotrophic bacteria counts above 6 log CFU.g^{-1}.

No significant differences ($p>0.05$) were observed for lactic acid bacteria countsof the formulations on day 1 (Table 4), with an increase throughout the shelf life for all formulations. At 35 days of storage, the sausages CP and EEPC had the lowest counts of this microorganism and did not differ statistically from each other ($p>0.05$).

The high LAB counts in CN and EE at 35 days of storage may be due to the low nitrite content or absence of this preservative in these formulations.[35] According to Sukumaran et al.,[36] meat products with lactic acid

bacteria counts of more than 7 log CFU.g^{-1} are unfit for consumption. Therefore, CP and EEPC can be considered safe for consumption after 35 days of storage.

Similar behavior was observed for the enterobacteria counts (Table 4). Initially, no significant difference was observed between the formulations, with an increase ($p<0.05$) throughout the storage for all formulations, with lower values observed for CP and EEPC, and no difference between them at the end ofthe refrigerated storage (day 35) ($p<0.05$).

High enterobacteria counts may be due to the microbial quality of the raw material[37] and are generally associated with meat spoilage.[38] These results confirm that pre-converted spinach extract is as effective as commercial curing salt in inhibiting pathogenic microorganisms.

CONCLUSION

Fresh pork sausages made with spinach extract showed similar physicochemical characteristics for moisture, protein, fat, and ash contents when compared to sausages made with commercial curing salt. The use of pre-converted spinach extract proved to be more effective in inhibiting lipid oxidation and controlling the growth of mesophilic microorganisms, lactic acid bacteria, and enterobacteria in fresh pork sausages over 35 days of refrigerated storage, when compared to the formulation containing unconverted spinach extract (EE).

Thus, even though spinach is an excellent source of nitrate, a pre-conversion step is necessary before its use as a substitute for sodium nitrite. Spinach can be considered a promising alternative for the meat industry, enabling the development of products with a lower content of chemical additives and meeting a growing demand for clean-label products and, consequently, greater healthiness. However, it is worth noting that, according to the National Health Surveillance Agency, a safety assessment must be carried out to authorize the use of new food additives to guarantee safe consumption within the limits established by law.

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Contributors

Cherobin AK participated in the conception and design, analysis and data interpretation, review, and approval of the final version; Amaral AMP participated in the conception and design; Moroni LS participated in the analysis and data interpretation; Cavalheiro D participated in the review and approval of the final version; Sehn GAR participated in the review and approval of the final version.

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