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Original Article

Effect of Dry Red Grape Pomace as a Nitrite Substitute on the Microbiological and Physicochemical Properties and Residual Nitrite of Dry-cured Sausage

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ABSTRACT

Background and Objectives: Sodium nitrite and potassium nitrite have been traditionally used for inhibition of Clostridium botulinum and also as an agent to stabilize the color of meat products; however, usage of these additives at high levels could lead to toxicity and cancer originating from the formation of nitrosamines. Nowadays, application of natural preservatives in order to reduce the nitrite content in meat products is increasing. Thus, we used dry red grape pomace (DRGP) as a natural alternative to sodium nitrite.

Materials and Methods: The effect of two levels of DRGP (1 and 2%) on the proximate composition, microbial counts, pH values and residual nitrite level of the samples formulated with two levels of sodium nitrite (30 and 60 mg/kg), as well as the comparison of these sausages with the blank (nitrite-free) and control (full nitrite added) samples on the 1rst, 10th, 20th and 30th days of storage at 3-5 °C were evaluated.

Results: The results showed that all chemical compositions were in the ranges reported by other researchers, and nitrite was very effective in preventing the microbial growth. Also about 50 % of the ingoing nitrite could be analyzed in the samples after processing. Moreover, the residual nitrite level declined both during the storage of sausage and after the addition of DRGP.

Conclusions: The use of DRGP in combination with nitrite for sausages was more effective in keeping the quality and safety of the refrigerated consumer products as indicated by the lower nitrite levels, microbial count and similar composition as compared to the samples treated with nitrite and without nitrite.

Keywords: Dry red grape pomace (DRGP), Sausage, Nitrite, Microbial count

Introduction

Grape pomace, as a by-product, constitutes 25% of the grape weight whose seed is about 38–52% on dry basis (1). It is usually burned, causing environmental pollution or much of it is discarded without any type of treatment, causing great environmental impacts such as increasing biochemical and chemical oxygen demands (2). Grape pomace is a natural product rich in dietary fibers and polyphenols. Grape by-products have drawn increased attention in recent years for their potential health benefits. Flavanols are the most abundant phenolic compounds in grape peel and grape seeds, being rich in monomeric phenolic compounds (3). These compounds act as antimutagenic and antiviral agents; they can also be used to preserve food because of their protective effects against microorganisms (4). As sources of natural antioxidants, these substances could be reused as additive substitutes or new ingredients in the food and pharmaceutical industries (5, 6).

Nitrite is a well-known ingredient used as a curing agent in sausage, It is one of the most effective means of controlling pathogenic bacterial growth, mainly from *Clostridium botulinum;* however, in addition to

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acting as a strong antioxidant, it can significantly affect the color, odor and flavor of sausage (7).

During cooking, nitrite can combine with secondary amines and amino acids in meat products and form N-nitroso compounds(e.g. nitrosamines) having health risks including allergic effects, vasodilator effects, metmyoglobin production, and production of carcinogenic nitrosamines (8). Recently, consumers have developed a stronger negative view towards artificial additives; therefore, the industry, researchers and official bodies are trying to respond to current consumer demands.

Meat and meat products are essential components in the human diets of the developed countries. Their consumption is affected by various factors such as paying attention to nutritional properties and safety. Many studies have been done about adding various plant materials instead of nitrite because of having effective antioxidant and antimicrobial properties. There has been some research dealing with a strategy for using natural ingredients/additives instead of nitrite in without/low added nitrite products to reproduce the functions (color, antioxidant activity, antimicrobial property, etc.) of nitrite like citrus fruits by-products (9), green tea (10), bearberry (11) grape seed (12) rosemary and oregano (13). The aim of this study was to determine the individual and cumulative effects of sodium nitrite and DRGP powder on the TBARS, color change and sensory evaluation of cooked sausages after 30 days of storage at 4°C.

Materials and Methods

Preparation of grape pomace: The pomace (nitrite level: 3.2±1.2 µg/ml) of the variety *Vitis vinifera* L. var. Siahe Sardasht a by-product of red grape juice manufacturing process, was supplied by Azarkam Juicing Company (Urmia, Iran). The grape pomace studied was composed of stems, seeds and skins (without any separation of their components). The samples were dried in an industrial counter current dryer at 40-60°C for 6-12 h, They were dehydrated to a moisture content of approximately 11.15%, milled by a grinder and passed through a 60-mesh standard sieve to obtain a uniform particle size distribution. In order to eliminate all microbial contaminations, they were exposed to gamma irradiation at the dose of 25 kGy in the Atomic Energy Organization (Tehran, Iran). They were stored at 4°C in a dark place for several weeks before use.

Sausage preparation**:** The sausages were manufactured according to a traditional formulation (Table 1) in the pilot plant of Solico Co. (Tehran, Iran). Five different formulations (all with 55% meat) were studied. The control sample was prepared traditionally with chemical nitrite added (120 mg/kg), and the blank sample was prepared without any chemical nitrite added. The reformulated samples were prepared with different percentages of grape pomace (1-2%) and nitrite (30-60 mg/kg). Materials including beef meat (55% comprising moisture: 69.98% ± 0.8, protein: 19.08% ± 0.4, lipid: 9.97% ± 0.4, and ash: 0.79% ± 0.01), water (in the ice form 15.87%), sugar (0.7%), soybean oil (15.3%), NaCl (0.9%), starch (3%), soybean isolate (3%), $Na_5P_3O_{10}$ (0.3%), frozen garlic powder (1%), ascorbic acid (1.25%) and spice (1.25%, mixture of black pepper, mace, coriander, ginger and cardamom) were transferred to a chilled cutter (Seydelmann, Aalen, Germany), and the following preservatives (nitrite/grape pomace) were added in different levels to each treatment described in Table 1 in the pilot cutter. After complete homogenization, all samples were stuffed into 50 mm-diameter polyamide casings, previously humidified with lukewarm water. The sausages were heat-processed for 1 h until the core of the product reached 75°C. The internal temperature was monitored by using thermocouples (Omega Engineering, Inc. Stamford, CT) inserted in each sample (thermal center), and when heating was complete, the sausages were chilled in a cold water bath (15°C) and stored at 4°C until further analysis for 30 days. All batches were produced in duplicate.

Table 1. DRGP (%) and sodium nitrite (mg/kg) content of beef sausage

Treatment	Sodium nitrite (mg/kg)	DRGP(%)
Control	120	0
Blank	0	0
T_1	60	1
T_2	30	1
T_3	60	2
T_4	30	2

Chemical composition: Proximate analysis of the meat and sausage samples was conducted by the methods of AOAC (14) . The results were expressed as moisture%, protein%, fat%, ash% and

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carbohydrate%. Feder value was calculated as the moisture/protein ratio according to Pearson (1981)(15).

Determination of pH: The pH of the raw sausage was determined using a pH-meter (Zagchemi, Iran) by direct measurement with a glass electrode calibrated with the phosphate buffers 4.0 and 7.0 at room temperature (21°C) for all samples. The sample was blended and homogenized in distilled water at a ratio of 10:90 (w/v) for 2 min, and its pH was measured (16).

Microbiological analysis: The microbiological characteristics of the samples were assessed on the days 0, 10, 20 and 30 after manufacture, according to the methodology described by Viuda-Martos M *et al*. (2010) in triplicate. For each sample, 25 g was separated from the interior of the sausage with a sterile scalpel and forceps. They were then homogenized with 225ml of 0.1% peptone water in a Stomacher 400. Appropriate decimal dilutions were pour-plated on plate count agar for total viable count, and incubated at 35 °C for 48 h. Psychrotrophic microbes were determined on Plate Count Agar, and the plates were incubated at 7 °C for 10 days (17).

Presumptive identification of Clostridium *perfringens*: For the identification of *C. perfringens*, the dilutions were poured into Sulfite Polymyxin Sulfadiazine Agar (SPS agar), placed in an anaerobic jar (BBL GasPak System, USA) with an anaerobic kit (Oxoid), and incubated at 37 °C for 48-72 h. Microbial counts in this study were expressed as the logarithms of colony forming units (CFU) per gram of sample (Log cfu/g). All microbiological analyses were performed in duplicate (18).

Residual nitrite: Residual nitrite level (mg $NaNO_2$/kg sample) was determined through the following standard ISO (2918). First, potassium ferrocyanide, zinc acetate, acetic acid and saturated borax solution were added to precipitate proteins. Then sulfanilamid, N-1 naphthyl ethylene diamine dihydro chloride and acid chloride were added to the filtrate, and red color was developed because of their interaction with nitrite. The absorbance value was measured at 538 nm using a UV-visible spectrophotometer (Unicam, Cambridge, UK)(19).

Statistical analysis: Data were analyzed using the SPSS software (ver. 15.0). Descriptive analysis was performed first in order to determine means ± standard deviations of the three determinations of each parameter. Analysis of variance (one-way ANOVA) and Duncan`s multiple range test were carried out to evaluate the statistical significance ($p<0.05$) of the effect of each formulation to establish statistical differences between the samples within each group. The analysis of the obtained data concerning the effects of time during the storage was carried out through the repeated measure analysis of variance (ANOVA). The data mean comparison was conducted the Bonferroni`s multiple range test also in the probability level of 0.05. All experiments were triplicated.

Results

The proximate analysis and the Feder values of the sausage samples are presented in Table 2. The results indicated that the control sample comprised 56.05% moisture, 14.65% protein, 21% lipid and 2.03% ash in the first day of storage. In the case of measured factors except for ash, no significant ($p>0.05$) difference was observed between all the produced samples and the control one.

Table 2. Proximate analysis and Feder values of the sausage samples (on fresh weight basis)

Treatment	Moisture	Protein	Carbohydrate	Fat	Ash	Feder value
Control	56.05^{A}±1.20	14.65^{A}±0.77	3.75^{A}±0.21	21.00^{A}±1.27	2.03^{C}±0.04	3.83^{A}±0.28
Blank	55.75^{A}±0.36	15.10^{A}±0.56	3.80^{A}±0. 14	21.45^{A}±0.35	2.01^{C}±0.02	3.69^{A}±0.11
T_1	56.31^{A}±0.01	14.81^{A}±0.28	3.82^{A}±0.10	20.85^{A}±0.35	2.23^{B}±0.04	3.80^{A}±0.07
T_2	56.70^{A}±0.56	14.85^{A}±0.49	3.85^{A}±0.50	20.65^{A}±0.49	2.19^{B}±0.01	3.81^{A}±0.08
T_3	57.25^{A}±1.35	15.50^{A}±0.84	4.37^{A}±0.49	21.35^{A}±0.77	2.41^{A}±0.02	3.69^{A}±0.11
T_4	57.05^{A}±0.80	15.45^{A}±0.91	3.90^{A}±0.15	20.80^{A}±1.69	2.39^{A}±0.01	3.69^{A}±0.23

The same letters in each column show the non-significant difference in the probability level of 0.05 ($p>0.05$) in Duncan's test (n=3 replications) in one-way (ANOVA). Control and blank samples with 120 and 0 mg/kg nitrite, respectively, T_1(60mg/kg nitrite,1%DRGP), T_2(30mg/kg nitrite,1%DRGP), T_3(60mg/kg nitrite, 2%DRGP), T_4(30mg/kg nitrite, 2%DRGP).

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As observed in Table 3, as the nitrite level increased, the microbial load of the produced samples decreased significantly ($p<0.05$). In general, the population of the viable bacteria of the manufactured samples was less than that of the blank sample (Table 3). The effect of DRGP was not significant ($p>0.05$) for the produced samples. The microbial population followed an incremental trend significantly ($p<0.05$) in all samples during 30 days of storage. The blank sample had the highest microbial population on all days of storage as compared with the other samples. The obtained results revealed that all samples, except for the blank one, had a microbial population lower than the allowed limit at the end of storage. No *C.perfringens* was observed in any of the samples during storage, and the psychrotrophic bacteria were uncountable until the 30th day of storage.

Addition of different amounts of DRGP created a significant ($p<0.05$) difference between the pH values of the DRGP-containing samples and those of the other samples (Figure 1). In the first day of storage, the pH values of all samples were in the range of 6.0-6.2. As observed, the pH values of all samples decreased gradually after 10 days of storage. They increased after 20 days of storage and even reached the values a little higher than the initial ones. The pH value of the blank sample was significantly ($p<0.05$) higher than those of the other samples.

The residual nitrite increased with the rise in the nitrite level ($p<0.05$). Apart from the treatments, the residual nitrite level significantly ($p<0.05$) decreased gradually during storage (Figure 2), and as DRGP increased, the residual nitrite level decreased significantly ($p<0.05$).

Table 3. The effect of DRGP and nitrite on total viable counts (CFU/g) during chilling storage of raw cured sausages (on fresh weight basis) at the level of 0.05

	Days of storage			
	1	10	20	30
Control	$2.672^{Ad}\pm0.021$	$3.703^{Dc}\pm0.014$	$4.152^{Db}\pm0.035$	$4.491^{Da}\pm0.014$
Blank	$2.671^{Ad}\pm0.026$	$4.970^{Ac}\pm0.049$	$5.446^{Ab}\pm0.035$	$5.792^{Aa}\pm0.021$
T_1	$2.677^{Ad}\pm0.028$	$3.910^{Cc}\pm0.021$	$4.374^{Cb}\pm0.014$	$4.716^{Ca}\pm0.028$
T_2	$2.658^{Ad}\pm0.021$	$4.165^{Bc}\pm0.021$	$4.633^{Bb}\pm0.035$	$4.977^{Ba}\pm0.035$
T_3	$2.653^{Ad}\pm0.012$	$3.902^{Cc}\pm0.028$	$4.364^{Cb}\pm0.028$	$4.702^{Ca}\pm0.021$
T_4	$2.644^{Ad}\pm0.002$	$4.152^{Bc}\pm0.028$	$4.619^{Bb}\pm0.014$	$4.940^{Ea}\pm0.036$

The same capital letter indicates no significant statistical difference in each column is at 5%.
The same small letter indicates no significant statistical difference in each row at 5%.
The numbers are the average of three replicates, and were reported as Mean ± Standard Deviation. Control and blank samples with 120 and 0 mg/kg nitrite, respectively, T_1(60mg/kg nitrite,1%DRGP), T_2(30mg/kg nitrite,1%DRGP), T_3(60mg/kg nitrite, 2%DRGP), T_4(30mg/kg nitrite, 2%DRGP).

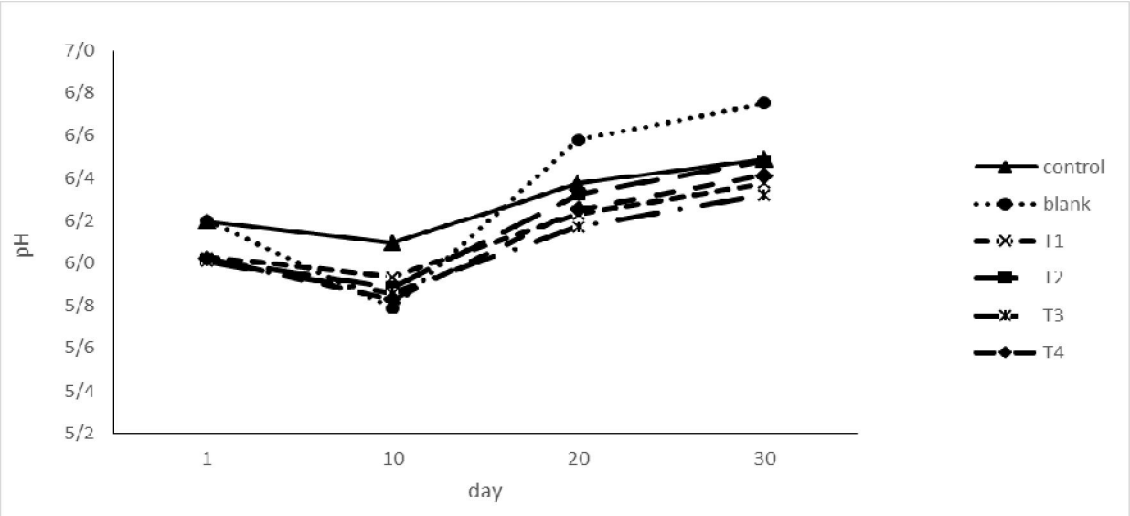

Fig. 1. pH value changes during chilling storage of raw cured sausages (on fresh weight basis) at the level of 0.05.
Control and blank samples with 120 and 0 mg/kg nitrite, respectively, T_1(60mg/kg nitrite,1%DRGP), T_2(30mg/kg nitrite,1%DRGP), T_3(60mg/kg nitrite, 2%DRGP), T_4(30mg/kg nitrite, 2%DRGP).

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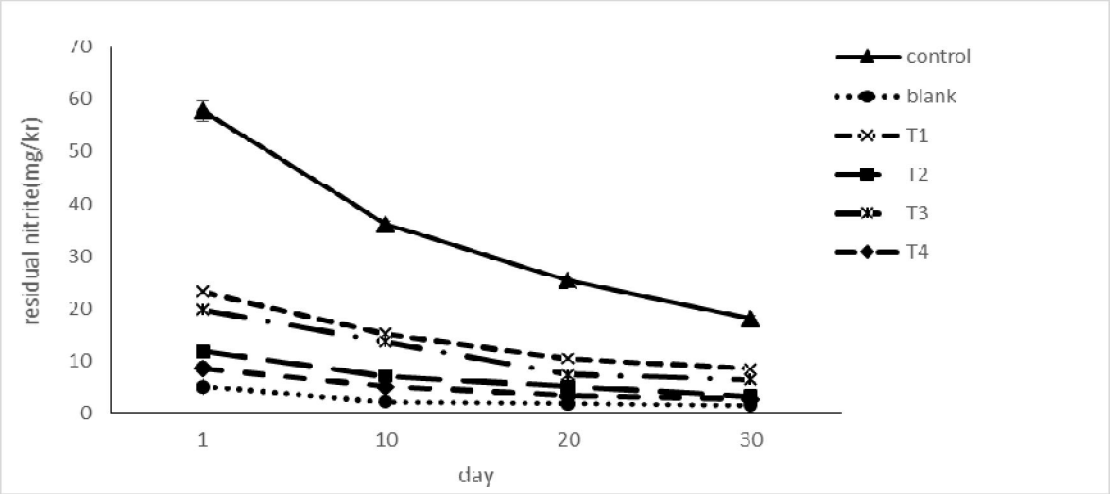

Fig. 2. Residual nitrite level (ppm) during the chilling storage of raw cured sausages (on fresh weight basis) at the level of 0.05.

Control and blank samples with 120 and 0 mg/kg nitrite, respectively, T_1(60mg/kg nitrite,1%DRGP), T_2(30mg/kg nitrite,1%DRGP), T_3(60mg/kg nitrite, 2%DRGP), T_4(30mg/kg nitrite, 2%DRGP).

Discussion

Regarding the chemical analysis, Pereira *et al*. (2000) reported that most sausage formulas fall within the following specification: moisture 50-70%, protein 11-15%, fat 15-30%, and ash contents 1.5-2.8% (on fresh weight basis). In the present research, the sausage samples' compositions were in the expected range (Table 2) (20).

Some special microbial species, causing the contamination of meat and meat products through environment, are considered as the microbial spoilage index during storage which are investigated in this study (21, 22). The antimicrobial power of nitrite reduced the microbial load of the manufactured samples. The remarkable effect of nitrite and DRGP was observed in reducing the number of aerobic bacteria. DRGP and herbal fibers have lower antimicrobial effect than herbal essential oils and extracts (23). Viuda-Martos et al. (2010) ascribed the antimicrobial activity of orange dietary fiber in sausage to its bioactive compounds, especially polyphenols and terpenes (22). The blank sample had the largest microbial population than the other samples due to the absence of preservatives. According to Iranian National Standard (No. 2303), the maximum microbial load is equal to 5 $\log_{10}$ cfu/g (24). Since the pH value was adjusted to 6, the cooking conditions were appropriate and the samples were carefully stored at 4°C, the produced samples did not spoil during the 30 days of storage. However, as observed in Figure 1, as the pH value was elevated, the microbial population increased too, which was much more pronounced, particularly on the 30th day. There is a close relationship between the pH value and the total microbial population. pH is one of the effective internal factors on microbial growth (25). Owing to its low pH (3.81), DRGP reduced the pH values of the samples, thus inhibiting the microbial growth to some extent via pH reduction. The antimicrobial activity of DRGP was not observed likely because of its low levels. *C.perfringens* was not observed, probably due to the low microbial population of the raw meat, cooking conditions and proper storage. Psychrotrophic bacteria that are able to grow at 7°C are the most important bacterial group in the putrefaction of meat products. Nevertheless, due to the predominance of gram-negative bacteria in this group and the more pronounced effect of the cooking temperature (pasteurization) on gram-negative bacteria, in addition to decrease in the thermal resistance of microorganisms by compounds such as phosphate and glutamate present in the formulation, the psychrotrophic bacteria were not countable during the storage time. The results of the present study conform to those of Viuda-Martos et al.

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(2010) who worked on the effects of orange fiber, oregano essential oil and rosemary essential oil on Mortadella sausage during storage at 4°C. In that study, thermal processing, aseptic cutting, and the occurrence of salt in the product were also stated as the reasons behind the growth inhibition of psychrotrophic bacteria during storage (24 days) (17).

The reason for reduction in the pH values of the DRGP-containing samples is associated with the pH value of DRGP (3.81), and the low pH of DRGP is possibly because of the presence of organic acids and other acidic compounds in grape, and consequently, in its pomace (26). The pH reduction during the first 10 days of storage was caused by the lactic acid resulted from the metabolism of meat as well as the sugar present in the formulation by microorganisms (22, 27). Viuda-Martos et al. (2010) attributed the pH reduction to the production of lactic acid by lactic acid bacteria (22). The increase in pH value on the 20^{th} and 30^{th} days was probably due to the production of compounds such as ammonia resulted from the activity of microorganisms, in addition to the denaturation of proteins and accumulation of the raw materials (28, 29). As shown in Table 3, as the number of the microorganisms increases, the pH value increases on the 20^{th} and 30^{th} days, which could be brought about by the production of secondary metabolites. This conclusion could be justified considering its large microbial population. With a gradual rise in the pH values of the samples, all samples showed pH values lower than the critical limit (pH=7) determined for sausage during the chilling storage (15). This could be because of the ability of nitrite and DRGP to reduce and inhibit the growth of microorganisms, and hence, reducing spoilage (18, 30). Nitrite is changed into nitrite oxide in meat, where it combines with myoglobin and creates the pink pigment of nitrosomyoglobin. This pigment is the reason behind the pink color of cured meats. The preliminary reduction of nitrite could be ascribed to the fact that only 50% of the added nitrite can be detected as sodium nitrite in the product. The majority of nitrite, which has changed into nitric oxide, is combined with myoglobin (5-15%), sulfhydryl groups (5-15%), lipids (1-5%) and proteins (20-30%), and only a minority of nitrite remains in the form of nitrate (<10%) and nitrite (10-15%)(31). As a result, less than 50% of the added amount can be chemically analyzed after processing (32). The decrease in the nitrite content during storage may be associated with the dynamic deformation of nitrogen compounds in the muscle matrix (33). The content of the remaining nitrite decreased gradually during the first 10 days of storage, which was then bottomed out. After 20 days, all samples reached a bottom with a relatively constant ratio (i.e. there was not a large difference between the nitrite level of the 20^{th} and 30^{th} days)(16, 34).

The reduction in nitrite level during storage depends on different factors, including type of the raw meat, pH, initial nitrite level, manufacture and storage temperature, and presence of the reducing factors (35). The concentration of the added nitrite has dramatic effect; the higher the nitrite level, the higher the remaining nitrite content (36). The high reactivity of nitrite enables it to react with bioactive compounds (DRGP polyphenols) (37). Some polyphenolic compounds show a strong protection against the nitrite ion, and prevent from the formation of nitrosamine (38). The reduction in the residual nitrite level and its reaction with the polyphenols occurred in DRGP led to a decreased formation of N-nitroso compounds, which are very carcinogenic and mutagen (17). These results are similar to those obtained in the study by Fernández-Ginés et al. (2004) where the residual nitrite level of Bologna sausage reduced significantly through the addition of lemon albedo (39), and in the study ofViuda-Martos et al. (2010) where the addition of orange fiber and the essential oils of spices led to the reduction in the residual nitrite level of Mortadella (17). It also seems that besides the phenolic compounds, the decrease in nitrite was due to the low pH of the DRGP-containing samples because pH is one of the influential factors on the residual nitrite level. The residual nitrite level was acceptable and less than the critical limit of 100 ppm.

Addition of DRGP to sausage inhibited the microbial growth during 30 days of refrigeration storage, decreased residual nitrite level and pH, and increased the shelf life of the sausage. Moreover, addition of DRGP to the meat product enhanced health-promoting properties and improved the stability of the produced samples. Due to the concerns

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regarding the safety and toxicity of nitrite, DRGP may be proved to be useful as a safe, natural, and health-promoting nitrite substitute in the meat industry.

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