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Evaluation of microbial and sensory properties of red meat sausage formulated with carmine dye and cumin essential oil as nitrate substitute

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Abstract

Nitrate and nitrite have long history of use in processed meat products and several studies have been done about their replacement with healthier alternatives. In this work, production of nitrite-free sausages containing cumin essential oil (EO) and carmine dye was studied for the first time. Concentrations of 200, 400, and 600 mg/kg carmine alone and in combination with 2 mL cumin EO were added to sausages containing 40% w/w meat. Microbial growth and sensory attributes were studied in the samples during storage. Principal component analysis (PCA) was done for better comparison of control (formulated with 12o my/kg sodium nitrite) and the treatments. The sample containing 2 mL cumin EO and 600 mg/kg carmine (T_7) could successfully suppress the microorganisms. Main concern was related to Clostridium perfringens as indicator of C. botulinum in the product. Fortunately, T_7 was superior to the control in suppression of C. perfringens. According to PCA, T_7 was similar to the control in variation of microbial contamination during storage and it was accepted by sensory panelists same as control. Furthermore, no significant differences were observed between the sample containing 2 mL cumin EO and 400 mg/kg carmine (T_6) and the control in microbial and sensory experiments. In conclusion, addition of 400-600 mg/kg carmine and 2 mL cumin EO to sausage led to development of healthier products. Given the health concerns arisen from synthetic preservatives, our selected formula can be developed as organic nitrate-free sausage to prevent side-effects of nitrosamine in the consumers.

Keywords: *Meat products, microbial contamination, natural preservative, nitrosamine, sensory evaluation*

Running title: Natural substitute of synthetic nitrite

Introduction

Modernization of countries has shifted people toward consumption of fast foods. Sausage is formulated with nitrate/nitrite salts as synthetic additive that is of main concerns globally. In technical view, the synthetic additive inhibits growth of pathogenic bacteria especially *Clostridium* species, and also delays lipid oxidation [1]. In addition, nitrite helps in development of desired color in the product [2]. Despite the mentioned benefits, frequent intake of nitrite-containing foods is harmful to health due to its reaction with secondary amines in the products. N-nitrous compounds especially nitrosamine is further formed that is known as carcinogenic and genotoxic to human [3]. Nitrosamine is classified as group 2B by International Agency for Research on Cancer that is possibly carcinogenic to human [4].

Formulation of industrial foods by natural preservatives derived from agricultural crops is of interest. In this regard, Maleki *et al*. (2017) replaced sodium nitrite with different concentrations of celery extract (20, 40, 60, 80 and 100%) in sausages containing 55% chicken meat. Their results showed that 60% of sodium nitrite can be replaced with celery extract in the product without any adverse effect on microbial growth and color [5]. Indeed, the inhibitory effect of celery extract was attributed to its nitrate/nitrite content. Nonetheless, it is a natural preservative and the formulated product is considered as "organic" compared to those containing synthetic nitrate/nitrite salt. Under controlled condition, nitrite residue in the final product formulated with natural preservatives such as celery extract may be lower than those developed by synthetic nitrite [6,7]. In this regard, protective role of bioactive compounds such as polyphenols in natural sources in suppression of microbial growth and inhibition of n-nitroso compounds cannot be ignored [8-10].

Ozaki *et al*. (2021a) added radish powder and oregano essential oil (EO) to fermented sausage instead of nitrite salt. The new formula could successfully control and decrease microbial contamination and develop acceptable sensory attributes. Even though the fermented sausage formulated with radish powder alone showed lower microbial count than control, it had more nitrite residue than control during storage [11]. Characterization of radish powder by the authors showed a high level of nitrate (16.3 g/kg) [6]. However, it is assumed that plant polyphenols can scavenge residual nitrite in the products and inhibit their conversion to precursors in formation of n-nitrous compounds under acidic environment of the stomach. Therefore, high level of nitrite residue in food products may be compensated by the positive activity of phenols. It might be the reason of higher permitted level of nitrate/nitrite in vegetables compared to meat products [12]. Role of plant sources in limiting the substrates availability in formation of n-nitrous compounds has been reported by Munekata *et al*. [13]. Nonetheless, concentration of nitrate in plant sources is determined by several factors including soil type, time of harvesting, plant species, and storage condition [14], and use of natural sources containing lower concentration of nitrate is of importance.

Cochineal insect is source of cochineal dye or carminic acid or carmine. The dye is extracted from female insect of *Dactylopius coccus*. It is a natural dye isolated from body of the insect. Carmine is used in formulation of cosmetics, drug, and foods [15]. It is coupled with various cations of ammonium, calcium, potassium, and sodium with different water solubility [16], and is resistant to light and oxidation [17]. These characteristics make the dye popular in food industry. Carmine is known as E120 in European additive list [18], and has been approved for food preparation by the US Food and Drug Administration [19]. Moreover, Codex Alimentarius has determined different permitted levels of carmine for each food group [20].

Cumin with scientific name of *Cuminum cyminum L.* is a plant of *Apiaceae* family. It is an endemic plant grown in Mediterranean regions and has several curative properties such as antioxidant potential. Cuminaldehyde, *p*-cymene, γ-terpinene, β-pinene, and cuminic alcohol are the main components of cumin EO [21]. Due to its favorable roles and availability of cumin EO in Iran, we examined it in formulation of sausage for inhibition of common microorganisms especially Clostridium genus.

Considering the controversial issues about use of plant extracts as nitrite substitute in formulation of meat products, we examined other natural sources to find out their effectiveness in preservation of sausage by their bioactive compounds. In this regard, we used carmine dye and cumin EO in formulation of sausage instead of nitrate salt for the first time. The physicochemical and antioxidant properties of the new formula were studied in our previous research [22]. In this study, individual and combined effects of carmine dye and cumin EO on safety and sensory attributes of the sausages were studied on days 1, 10, 20, and 30 of storage.

Experimental section

Materials

The raw materials for preparation of sausage were included to red meat (frozen meat imported from Brazil), carmine dye (Kristin Hansen, Denmark), cumin EO (Adonis, Iran), liquid oil (Golbahar, Iran), wheat flour (Derakhshan, Iran), salt, garlic powder, and soybean (Tasty, Iran), gluten (Ardineh, Iran), spices (Naderi, Iran), sodium phosphate (Stupipi, China), and sodium nitrite (Pouya Shimi Hegmatan, Iran). Microbial culture media of Plate Count Agar for total mesophilic microorganisms, Violet Red Bile Agar for coliforms, Yeast Glucose Chloramphenicol Agar for yeast/mold, Tryptose Sulfite Cycloserine agar for *Clostridium perfringens*, and Baird-Parker Agar for *Staphylococcus aureus* enumeration were used. All culture media were purchased from Merck (Germany).

Sample preparation

Samples were prepared by mixing 20 kg red meat, 14.8 kg ice, 6.5 kg liquid oil, 4 kg wheat flour, 2 kg soybeans powder, 1 kg gluten, 0.6 kg spices, 0.5 kg NaCl, 0.4 kg garlic powder, and 0.2 kg sodium phosphate in cutter (Talsa Bowl Cutter K200neo, Spain). Control sausage was produced by addition of sodium nitrite at concentration of 120 mg/kg to the formula. For preparation of other treatments, carmine dye and cumin EO were added to the formula according to Table 1. Their concentration was determined based on our initial study to achieve a desired quality.

Table 1- Synthetic and natural preservatives used in preparation of sausages formulated with 40% w/w red meat

Sample	***Synthetic preservative***	***Natural preservatives***	
	Sodium nitrite (mg/kg)	***Carmine (mg/kg)***	***Cumin essential oil (mL)***
T_1 (Control)	***120***	-	-
T_2	-	***200***	-
T_3	-	***400***	-
T_4	-	***600***	-
T_5	-	***200***	***2***
T_6	-	***400***	***2***
T_7	-	***600***	***2***

After filling in polyamide casing with 32 cm length and 5 cm diameter, all samples were transferred to cooking room and heated at 70-72 ºC for 1 h. Then, they were cooled rapidly and stored at 0-4 °C until analysis.

Microbial analysis

Microbial analysis was included to enumeration of total mesophilic microorganisms, mold/yeast, coliforms, *C. perfringens*, and *S. aureus.* Experiments were done according to the methods described in ISO no. 152147 [23], ISO no. 6888 [24], and APHA no. 0222.039 [25]. Microbial analyses were done on days 1, 10, 20, and 30 of storage.

Sensory evaluation

Samples with acceptable microbial growth were examined organoleptically by 10 trained evaluators on days 1, 10, 20, and 30 of storage. Sausages were cut into 3 cm pieces and randomly coded. The panelists were requested to use water and salt-free biscuit between their assessment. Attributes of color, smell, taste, and texture were tested by the evaluators and average results were reported as overall acceptance. The panelists evaluated the samples by 9-point hedonic method. Sensory scores were included to 1= dislike extremely, 2= dislike very much, 3= dislike moderately, 4= dislike slightly, 5= neither like nor dislike, 6= like slightly, 7= like moderately, 8= like very much, 9= like extremely [26].

Data analysis

In this study, a completely randomized design was used to arrange the treatments. Accordingly, seven treatments (T_1-T_7) were prepared. Microbial tests were performed in three replicates and the results are presented as CFU/g. The means were analyzed by one-way ANOVA followed by Duncan test. For Principal component analysis (PCA), Minitab software version 16 was used. Differences were significant at $P \leq 0.05$.

Results and Discussion

Microbial growth

Processed meat products are susceptible to microbial deterioration. Food additives can improve safety of products by several antimicrobial mechanisms. Nitrite is a common synthetic preservative which has been used in sausage for a long time. It shows several mechanisms to extend shelf life and provide appropriate color in the product. Following the adverse effects of nitrite and its derivatives in human health, several studies have been done to find appropriate alternatives. In this regard, natural preservatives are more popular than synthetic counterparts due to their health benefits. Essential oils are derived from herbal sources and exert their antimicrobial activity by interaction with bacterial cell membrane and interfering in electron transfer, ion gradient, protein placement, phosphorylation, and other enzyme-dependent reactions [27]. Indeed, their phenolic compounds play a pivotal role against microorganisms [28]. They have strong tendency to the lipids located in bacterial cell membrane due to their hydrophobic nature [29]. In addition, antimicrobial potency has been reported for carmine dye due to its structure composed of anthraquinone which could interfere in cell integrity and DNA synthesis [30,31]. Therefore, it is expected that carmine dye and cumin EO show synergistic effect in suppression of microorganisms in the environment owing to the activity of their potent bioactive compounds.

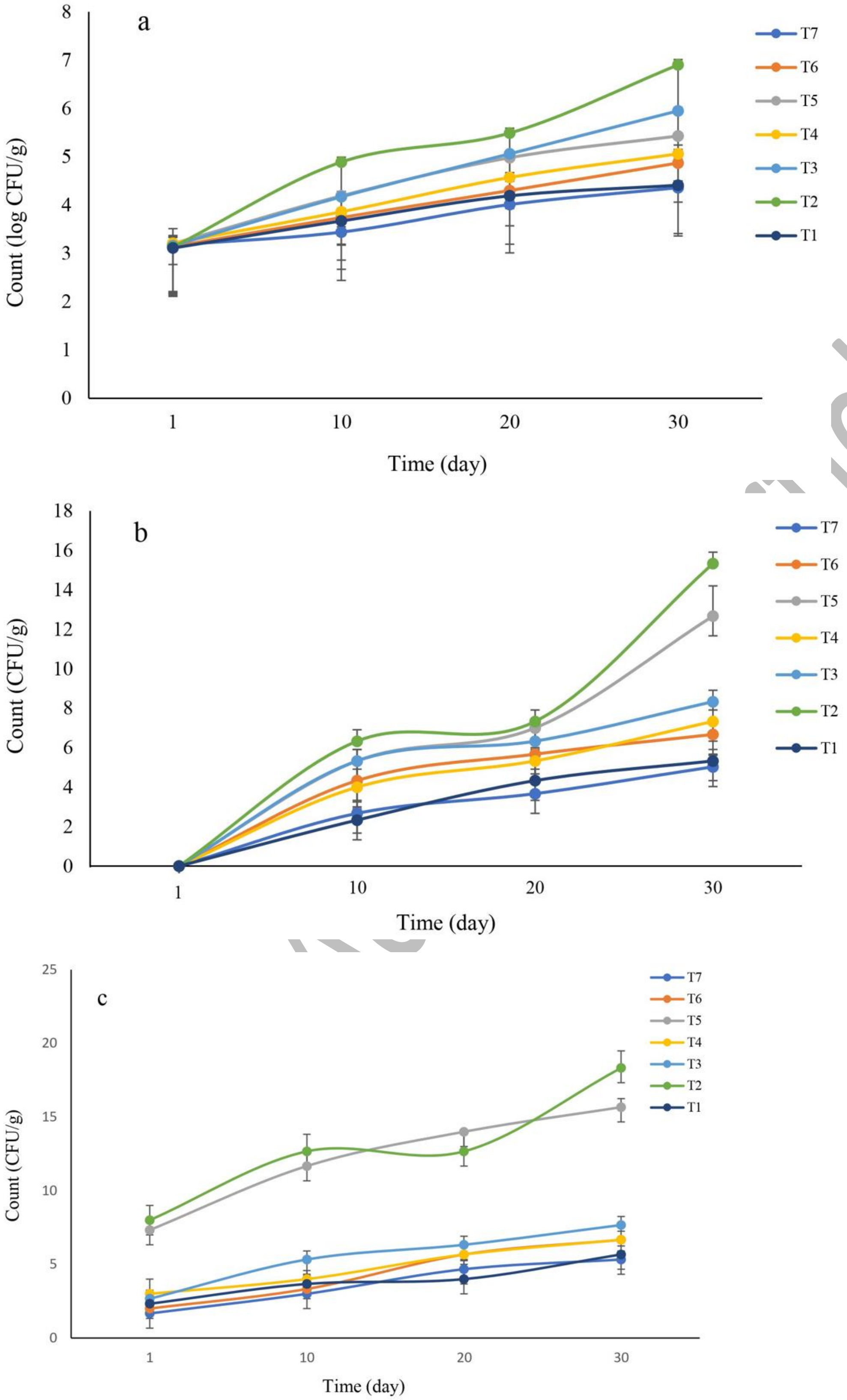
Growth of total mesophilic microorganisms in the samples is presented in Figure 1a. As seen, number of microorganisms increased significantly during storage. Replacement of sodium nitrite with carmine dye and cumin EO had a significant effect in this regard and the best result was observed for T_7 compared to the control. Microbial count had increasing trend in all treatments, but the highest growth was observed in T_2 (200 mg/kg carmine dye) and T_3 (400 mg/kg carmine) at the end of storage. The highest inhibition was seen in T_7 so that no significant differences were observed between T_1 (control) and T_7 during storage. Similar changes of T_7 and T_1 (control) is clearly depicted in Figure 1a. According to Iran regulation, maximum microbial count of 10^5 CFU/g is acceptable in sausages [32]. Therefore, T_1 (control), T_6 (400 mg/kg carmine + 2 mL cumin EO), and T_7 (600 mg/kg carmine + 2 mL cumin EO) were hygienically accepted after 30 days of storage.

Enumeration of fungi showed significant increasing during 30 days of storage. As observed, use of cumin EO and increased concentration of carmine could significantly suppress fungi in the sausages. The highest and the least number of fungi was respectively observed in T_2 (200 mg/kg carmine) and T_7 (600 mg/kg carmine + 2 mL cumin EO) at the end of storage. Non-significant differences of T_7 and T_1 are clearly seen in Figure 1b. In Iran regulation, maximum number of 100 CFU/g of mold/yeast is permitted in sausages [32]. Fortunately, growth of fungi was within the acceptable range in all samples despite its increasing trend over time. Antifungal activity of cumin EO was also reported by other studies [33,34].

According to the results, coliforms increased significantly during 30 days of storage. Similar to the other microorganisms, no significant difference was observed between T_1 and T_7 all the time. Addition of cumin EO could significantly decrease number of microorganisms especially when it was used with the highest concentration of carmine dye (Figure 1c). Except for T_2 (200 mg/kg carmine) and T_5 (200 mg/kg carmine + 2 mL cumin EO), the other samples were acceptable within 30 days of storage (coliforms were less than 10 CFU/g) according to Iran regulation [32]. Anti-coliform activity of cumin EO was also reported by Alizadeh Behbahani *et al*., which observed its inhibitory effect against *Escherichia coli* in vitro [35].

As depicted in Figure 1d, *C. perfringens* increased significantly during 30 days of storage. Combination of cumin EO and the highest concentration of carmine dye successfully reduced *C. perfringens* in the samples. Growth of *Clostridium* species is of great concern in meat products. Interestingly, T_7 showed the least growth of *C. perfringens* and it was superior to the control in this regard. Nevertheless, maximum number of 50 CFU/g *C. perfringens* is acceptable in sausages according to the national regulation [32], and all the treatments were within the range during 30 days.

S. aureus increased significantly during storage (Figure 1e). The best result was observed in T_7 which was not different significantly from the control all the time. Antibacterial effect of cumin EO against *S. aureus* was reported in other studies [36,37]. Similar results were observed in our experiments and cumin EO could significantly reduce number of *S. aureus* in the products. Nonetheless, same as coliforms, carmine dye showed a determining role in suppression of *S. aureus* so that T_2 (200 mg/carmine) and T_5 (200 mg/kg carmine + 2 mL cumin EO) showed the highest contamination by the bacterium. They were contaminated by more than 10 CFU/g *S. aureus* at the end of storage that is the maximum permitted level determined by the national regulation [32].


a
Count (log CFU/g)
Time (day)
b
Count (CFU/g)
Time (day)
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Count (CFU/g)
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T1

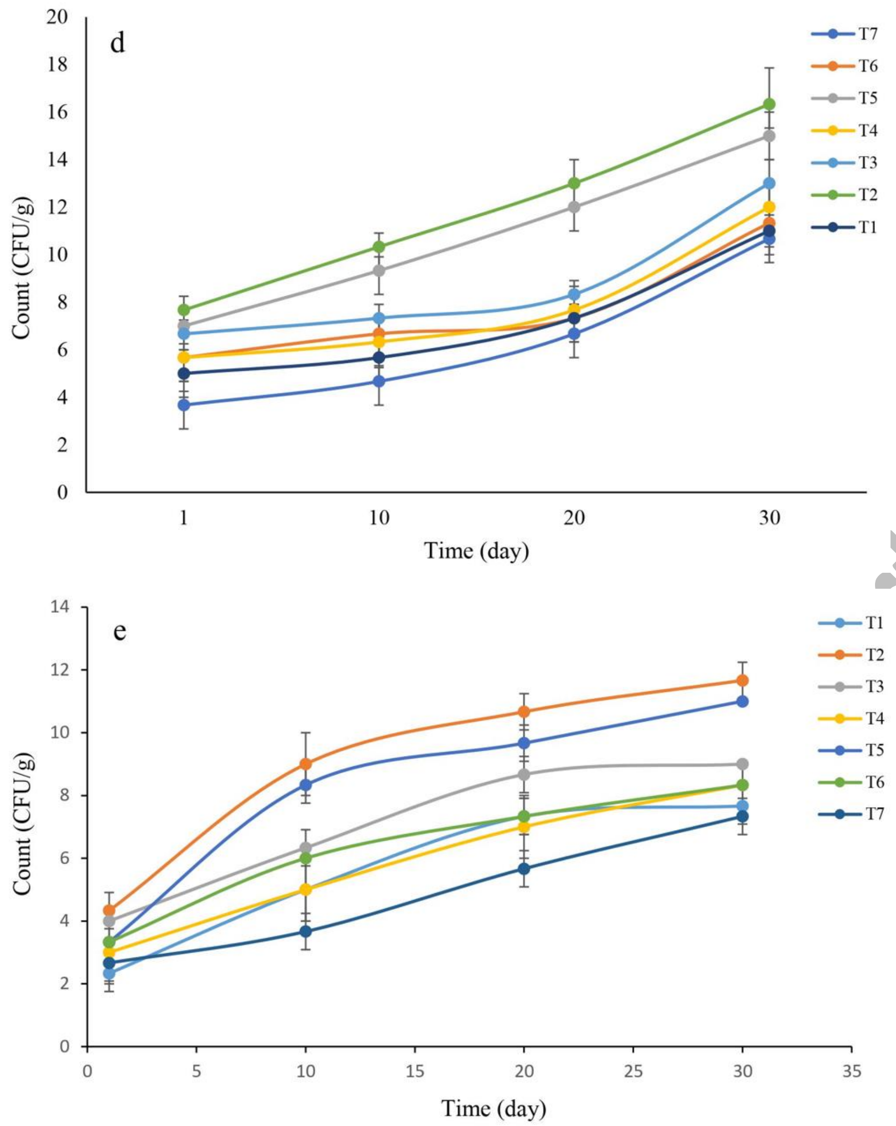

Figure 1- Growth curve of a) total mesophilic microorganisms, b) mold/yeast, c) coliforms, d) *Clostridium perfringens*, and e) *Staphylococcus aureus* in red meat sausages formulated with 120 mg/kg sodium nitrite (T_1: control), 200, 400, and 600 mg/kg carmine dye (T_2, T_3, T_4, respectively), 200, 400, and 600 mg/kg carmine dye and 2 mL cumin essential oil (T_5, T_6, T_7, respectively) during storage.

Results of microbial growth in the products were also examined by PCA. As seen in Figure 2, PC1 shows 94.95% of variation and PC2 shows 4.66% of variation in data. In PCA score plot, distribution of T_1 (control) and T_7 sample was similar, while the other samples were differently distributed. This plot confirmed the results presented before, and showed that T_7 sample had the most similarity with the control prepared by sodium nitrite. On the other hand, 120 mg/kg sodium nitrite had same antimicrobial activity as combination of 600 mg/kg carmine + 2 mL cumin EO in sausage, while the other treatments had lower similarity with the control in suppression of microorganisms in sausage during 30 days.

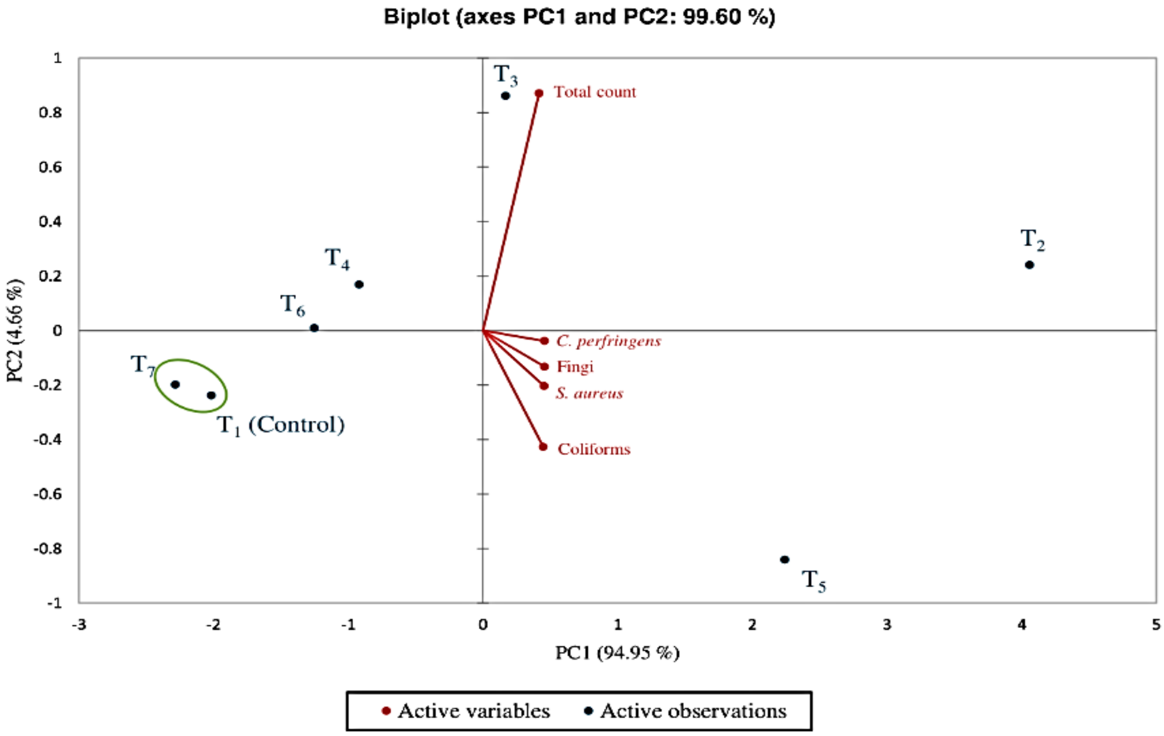

Figure 2- Principal component analysis of red meat sausages formulated with 120 mg/kg sodium nitrite (T_1: control), 200, 400, and 600 mg/kg carmine dye (T_2, T_3, T_4, respectively), 200, 400, and 600 mg/kg carmine dye and 2 mL cumin essential oil (T_5, T_6, T_7, respectively)

Overall acceptance

Sensory evaluation was performed for T_1, T_6, and T_7 samples which showed the least and acceptable microbial growth during storage. Interestingly, same sensory scores were observed for all three treatments all the time (Table 2).

Table 2- Results of overall acceptance of red meat sausages during storage

Sample	*Day 1*	*Day 10*	*Day 20*	*Day 30*
T_1 (Control)	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***
T_6	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***
T_7	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***	***8.00 ± 0.00^{aA}***

*Different small letters indicate significant differences in the columns.

**Different capital letters indicate significant differences in the rows.

It is obvious that storage time had no significant effect on overall acceptance of the samples. Importantly, replacement of nitrite with carmine dye and cumin EO did not induce significant change in sensory attributes of the developed formula. Interesting results about addition of natural preservatives to food products were reported by other scientists. For example, Sharma *et al*. evaluated sensory attributes of chicken sausages containing Cassia EO, and no significant difference was observed between overall acceptability of the sample formulated with 0.125% EO and the control [38]. In study of Maleki *et al*., the most desirable sample in term of a* was chicken cocktail sausage containing celery extract as nitrite substitute. The sample prepared by reduced concentration of nitrite (up to 60% substitution) and celery extract was not significantly different from the control in sensory attributes of taste and smell [5]. Nateghi *et al*. also reported that replacement of sodium nitrate (up to 60%) with

Monascus Purpureus pigment did not compromise sensory attributes of German sausage [39]. Therefore, addition of low concentration of natural preservatives to formula in development of functional food products has no significant effect on the consumer's acceptance similar to the result observed in our study.

With regard to safety, we evaluated our preferred product by considering the average daily intake of carmine through consumption of sausage in population. According to a national report, Iranians consume 15 g/day sausage [40]. By considering average weight of about 50 and 75 kg for people younger and older than 18 years old, respectively [41,42], carmine daily intake through a product containing 600 mg/kg carmine dye would be 0.18 mg/kg body weight/day for people younger than 18 and 0.12 mg/kg body weight/day for those older than 18 years old. Compared to the permitted range of carmine (0-5 mg/kg body weight/day) [16], our evaluation showed that the selected formulation (T_7) does not induce any possible adverse effect in human. However, there is a challenge about halal status of carmine in populations especially among Islamic countries, and there is no consensus on appropriateness of carmine as a food ingredient [43]. For example, carmine is not considered as non-halal additive in Iran because it is used at low quantities in the products; but it is forbidden in some other countries [44]. Despite the fact that edible use of carmine is restricted in some societies based on religion, carmine can be used as a safe alternative for some harmful chemicals such as nitrite due to its verification by several health agencies.

Conclusion

In this study, synthetic additive of sodium nitrite was replaced with carmine dye and cumin EO in 40% red meat sausage. To find out effectiveness of these natural additives in development of safe product, microbial load of the formula was evaluated in the laboratory. In addition, sensory panelists examined the overall acceptance of the products. The results showed that cumin EO had significant effect in reduction of microbial load. However, carmine dye showed a pivotal role so that the least microbial count was observed in T_7 (600 mg/kg carmine + 2 mL cumin EO) and T_6 (400 mg/kg carmine + 2 mL cumin EO). Specifically, the most similarity with the control was observed for T_7 sample in PCA score plot. Furthermore, no significant differences were observed in overall acceptance of the control (containing 120 mg/kg sodium nitrite), T_6, and T_7. Given the safe level of carmine used in our formulas, our selected samples (T_6 and T_7) can be considered as functional sausage with desired qualitative attributes. Further studies about use of these natural preservatives in other meat products are recommended.

Conflict of Interest

The authors declare that there is no conflict of interest.

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