

Antibacterial Efficacy of Essential Oils and Sodium Nitrite in Vacuum processed Beef Fillet

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Abstract

Background and Objective: Minimizing the exposure to nitrate and nitrite and therefore reducing the level of potentially carcinogenic nitrosamines is desired due to the strong public demand and political controversy around this issue in many countries. The present study was designed to investigate antibacterial activity of five different types of essential oils alone or in combination with different concentrations of sodium nitrite.

Material and Methods: Five types of essential oils *Zataria multiflora Boiss*, *Satureja bachtiarica Bunge*, *Rosmarinus officinalis L.*, *Mentha pulegium*, and *Origanum vulgare L.* were used in the experiments. NaNO₂ in concentrations of 0, 100, and 200 mg kg⁻¹ were used to study the growth inhibition of *Clostridium spp.* inoculated in vacuum processed beef fillet. Essential oils were analyzed by gas chromatography-mass spectrometry. Antimicrobial activity against vegetative cells of *Clostridium perfringens* and *Clostridium sporogenes* were primarily done by disc diffusion method and minimum inhibitory concentration of essential oils against vegetative cells were determined by broth macro dilution. Sensory evaluation of the uninoculated cooked vacuum processed beef fillet samples with three essential oils with higher antibacterial activity against the more resistant *Clostridium spp.* was done

Results and Conclusion: Among the examined Essential oils *Satureja bachtiarica Bunge* showed the most inhibition effect on *Clostridium perfringens* (4.1 mg ml⁻¹) and *Clostridium sporogenes* (5.5 mg ml⁻¹) followed by *Zataria multiflora Boiss*, *Origanum vulgare L.*, *Mentha pulegium* and *Rosmarinus officinalis L.* The antimicrobial activity of essential oils against *Clostridium spp.* was increased in combination with sodium nitrite. It can therefore be assumed that the combination of these two additives could have significant repercussion in the control of *Clostridium perfringens* and *Clostridium sporogenes* in vacuum processed beef fillet samples without compromising the organoleptic properties. The best result was achieved by the combination of 100 mg kg⁻¹ sodium nitrite and 1.1 % v w⁻¹ *Satureja Bakhtiaria Bunge* which could inhibit the growth of *Clostridium* species. The results of the current study showed that the essential oils of interest have had drastic effects on clostridium inhibition and could be used in the meat industry especially for sausages due to their impact on technological, microbiological and sensory properties.

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1. Introduction

Clostridium species as gram-positive, rod-shaped, spore-forming anaerobic bacteria that can be found in some environments such as soil as well as the intestinal tract of humans and animals. *Clostridium* species

especially *Clostridium (C.) perfringens* and *C. botulinum* can be considered as main reasons for fatal food infections [1]. *C. botulinum* produces seven different types of toxins

(A-G). Foodborne botulism is associated with both proteolytic and non-proteolytic *C. botulinum*.

Chronic diseases that afflict humans caused by *C. perfringens* are gas gangrene and food poisoning. Temperature abuse during cooling or reheating of cooked products is a major reason in *C. perfringens* spoilage [2].

Meat and meat products are the favored medium for proliferation and toxin production of Clostridium species. Vacuum processed beef fillet (VPBF) stored at abusive temperature could pose the high risk as result of growing and producing toxin of Clostridium species.

Nitrate and nitrite salts are widely used in processed meat for the inhibition of spoilage as a result of pathogen bacteria, especially *Clostridium* spp. [3]. Reducing the incorporation of nitrate and nitrite in food formulation is recommended by the European Food Safety Authority (EFSA) due to the high potential carcinogenicity of nitrosamines [4].

Essential oils (EOs) are volatile, natural and complex compounds of an aromatic plant's secondary metabolism [3]. EOs are active against a wide variety of microorganisms, including Gram-positive bacteria such as *Clostridium* spp. depending on their concentration and presence of terpenoid and phenolic compounds [5,6]. *Origanum (O.) vulgare* belongs to the Lamiaceae family and has been used for a long time as a flavoring agent in meat products [7]. The potent antibacterial activity of *O. vulgare L* and *Satureia (S.) Montana* against *C. perfringens* was reported by Kačániová et al. [6]. *S. bachtiarica Bunge* is an endemic species of the genus *Satureja* in Iran. It is an aromatic medicinal plant which belongs to the Lamiaceae family [8]. *Zataria (Z.) multiflora Boiss* grows only in Iran, Pakistan, and Afghanistan and belongs to the family of Lamiaceae. The extracted EO can be used as a flavoring agent in different foods and demonstrated the antimicrobial and anti-inflammatory effects [8]. Rosemary (*Rosmarinus (R.) officinalis L.*), a member of the Lamiaceae family, is used as a medicinal agent, functional food and additive in traditional Mediterranean cuisine [9]. *Mentha pulegium* is an aromatic and medicinal plant that belongs to Labiateae family and is used as an herbal tea and flavoring agent [10].

The *C. sporogenes* is used as a non-toxic surrogate of *C. botulinum* (due difficulties of working with *C. botulinum* as a highly potent food poisoning organism). In this context, *C. sporogenes* is widely used in studies on controlling the growth of *C. botulinum* [11]. No alternative with the same effects of nitrate has been introduced in the meat industry. Therefore, finding a good substitute to avoid adverse side effects and prevent the resulting chronic illnesses of nitrosamine is already a major need for both manufacturers and consumers.

Therefore, this research aimed to investigate the antimicrobial activity of five essential oils including *Z. multiflora Boiss*, *S. bachtiarica Bunge*, *R. officinalis L.*, *M. pulegium*, and *O. vulgare L.* on *C. perfringens* and *C. sporogenes* in vitro. Also, different levels of sodium nitrite in combination with the essential oils for the inhibition of *C. perfringens* and *C. sporogenes* growth in vacuum processed beef fillet after cooling and during storage at abusive temperature were evaluated.

2. Materials and Methods

2.1. Bacterial strains and spore preparation

The *C. perfringens* (ATCC 13124) and *C. sporogenes* (ATCC 3584) strains were provided by Austrian Agency for Health and Food Safety. *C. perfringens* spore preparation was done according to described method by Redondo-Solano using modified Duncan-Strong medium [2]. Spore of *C. sporogenes* was produced in cooked meat medium (OXOID, UK) according to the proposed procedure by Khanipour et al. [11]. The anaerobic atmosphere for growing Clostridia was prepared using self-contained gas generating system AnaeroGen™ (Oxoid, UK). The spores were collected by centrifugation at 7012×g for 20 min at 4°C (GS-15R, Beckman, Palo Alto, USA). The pelleted spores were washed with 100 ml sterile distilled water and centrifuged at 2000 ×g (10 min). The washing process was done according to Brunt et al, and repeated three times [12]. The spore suspension was mixed with sterile phosphate-buffered saline (Sigma, UK) and heated at 80°C for 15 min to destroy the vegetative cells. Spores were suspended in sterile distilled water and stored frozen at -80°C until the day of the experiment.

2.2. Plant essential oils

Hydro-distilled EO from *Z. multiflora Boiss*, *S. bachtiarica Bunge*, *R. officinalis L.*, *M. pulegium*, and *O. vulgare L* were supplied by Barij Essence Pharmaceutical Co., Kashan, Iran and stored at 4°C in the brown sealed glass.

2.2.1. Essential oil analysis

The composition of EOs were determined by gas chromatography-mass spectrometry (GC-MS) analysis using Agilent HP-6890 gas chromatography (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS capillary column (30m×0.25 mm×0.25 mm) coupled with a HP 5973 mass spectrometer (Agilent Technologies) with electron impact ionization 115 (70 eV).

Two µl of a hexane solution of essential oil was injected with the following conditions: The initial temperature: 50°C, final temperature: 250°C, and program rate: 15°C per min. Helium was set as the carrier gas with a flow rate of 0.8 ml min⁻¹ according to the recommended procedure by Moshayedi et al. [13].

2.3. Antimicrobial activity assay

2.3.1. Agar disk diffusion method

Antimicrobial activity of essential oils against vegetative cells of *C. perfringens* and *C. sporogenes* were primarily determined by disc diffusion method. Briefly, Mueller Hinton Agar plates were inoculated with approximately 0.1 ml of 10^8 CFU ml⁻¹ vegetative cells of *C. sporogenes* and *C. perfringens*, separately. Sterile blank disks (6 mm diameter) were soaked with 15 µl of each essential oil and placed on the surface of the agar plates. Plates were incubated anaerobically using self-contained gas generating system AnaeroGen™ (Oxoid, UK) at 37°C for 24 h. Microbial inhibition was determined by measuring the diameter of inhibition zones (mm) by digital caliper [6]

2.3.2. Broth macro dilution

Minimum inhibitory concentration (MIC) of essential oils against vegetative cells of *C. perfringens* and *C. sporogenes* were determined by broth macro dilution. EOs were dissolved in Tween 80 (concentration of 0.5 %v w⁻¹) (Sigma, UK) and different concentrations of essential oils (1-50 mg ml⁻¹) were prepared. *C. sporogenes* and *C. perfringens* were cultured separately in Mueller-Hinton Broth (MHB) (Oxoid, UK) to reach a final density of approximately 10^8 CFU ml⁻¹. The tubes were incubated anaerobically using self-contained gas generating system AnaeroGen™ (Oxoid, UK) at 37°C for 24 h. Three essential oils with the higher antibacterial activity against *C. perfringens* and *C. sporogenes* were selected for further investigation in VPBF samples.

2.4. Sensory evaluation

Sensory evaluation of the treated VPBF samples with three EOs with higher antibacterial activity against the more resistant *Clostridium* spp. at MIC/2, MIC, 2 MIC, 3 MIC levels (*S. bachtiarica* Bunge at 0.275, 0.55, 1.1 and 1.65 %v w⁻¹ and *Z. multiflora* Boiss at 0.35 0.71 , 1.42 and 2.13 %v w⁻¹ as well as *O. vulgare* L. at 0.39, 0.79, 1.58 and 2.37%v w⁻¹) was done on un-inoculated cooked VPBF samples in the sensory laboratory at the National Nutrition and Food Technology Research Institute using a selected trained panel of ten judges (5 females and 5 males) according to ISO 13299, 2003 "General guidance for establishing a sensory profile" [7]. The evaluation was carried out by each panelist in private booths under white fluorescent lights. Drinking water was provided for the panelist to clean the palate between samples. The VPBF samples were served warm in dishes coded with a 3-digit random number. The panelists used a 9-point hedonic scale for scoring the odor and taste attribute that 1=unacceptable, 9=very acceptable while samples with scores below 5 were presumed unacceptable [8].

2.5. Preparation of vacuum processed beef fillet

Beef fillets were obtained from a slaughterhouse in Tehran. Outer surface of meat were removed with sterile knives and the inner part cut into 100 g pieces and were cured by injection of a solution [sodium nitrite (0, 100 and 200 mg kg⁻¹), sodium polyphosphate (0.3 %w w⁻¹), sugar (0.5 %w w⁻¹), sodium chloride (1.85 %w w⁻¹), ascorbic acid (0.05 %w w⁻¹) and various concentrations of selected essential oils based on MIC against the more resistant *Clostridium* spp. and sensory evaluation test (*S. bachtiarica* Bunge at (0.275, 0.55and 1.1 %v w⁻¹), *Z. multiflora* Boiss at (0.355 and 0.71 %v w⁻¹) and *O. vulgare* L. at (0.395 and 0.79%v w⁻¹)]. Then, injected meat pieces were transferred into the tumbler Tumbling takes place at temperatures of ≤4°C for 45 min tumbling and 2 hours resting periods.

One hundred gram portions of the processed beef fillet from each treatment were placed in vacuum bags made of laminate polyvinylidene chloride with 16µm thickness and aseptically inoculated with *C. sporogenes* and *C. perfringens* spores separately to reach spore population of ca. 3 log CFUg⁻¹ of processed meat [2]. Inoculated prepared beef fillets were vacuum packaged, massaged manually and flattened according to ascribed method Redondo-Solano et al. [2]. VPBF samples were heated in water baths at 75°C for 20 min. chilling were done in a refrigerated bath with water circulation capabilities from 54.4° C to 26.7°C within 5 h and then from 26.7°C to 7.2°C for 10 h according to recommended procedure by the United States Department of Agriculture Food Safety and Inspection Service [2] and stored at room temperature and analyzed for 30 days.

2.6. Enumeration of *Clostridium* spp. in VPBF

For count of *C. perfringens* and *C. sporogenes*, 10 g of the VPBF samples were weighed and transferred to a sterile stomaching bag, and then 90 ml of sterile peptone water (Oxoid, UK) was added to the bags and stomached for 2 min. Serial dilutions of stomached slurries were prepared with peptone water (0.1% w v⁻¹), and aliquots (100 µl) were inoculated in tryptose sulfite cycloserine agar (Oxoid, UK) supplemented with 200 mg of D-cycloserine and egg yolk emulsion according to Oliveira et al, and tryptone peptone glucose yeast extract agar (Sigma, UK) according to Young Byun et al, [3,15]. Plates were incubated for 36 h at 37°C using self-contained gas generating system AnaeroGen™ (OXOID, UK) and the colonies were counted.

2.7. Statistical analysis

The obtained results were presented as the mean± standard deviations of each treatment. Analysis of variance was done to determine the significant differences. Differences between means were tested by Duncan and values of p≤0.05 were considered as significantly different.

Friedman test was used to analyze the sensory preference ranking. All statistical tests were conducted using SPSS 10.0 software (SPSS Inc, Chicago, IL, USA).

3. Results and Discussion

The antimicrobial activity of essential oils and the possibility of using EOs in combination with sodium nitrite for inhibition of inoculated *Clostridium* spp. in VPBF after cooling and during storage at abusive temperature were evaluated.

The recorded values for the diameter of inhibition zone which primary determined the antibacterial activities of EOs against *C. perfringens* and *C. sporogenes* by discs diffusion method were shown in Table 1. The *Satureja bachtiarica* Bunge EO had the highest and *R. officinalis* L. EO had the lowest antibacterial activity. *C. perfringens* showed higher susceptibility (4.92 ± 0.38 mm) while *C. sporogenes* showed less sensitivity (4.12 ± 0.43 mm) toward all tested EOs.

The antibacterial potency of *M. pulegium* mostly has been associated with the presence of pulegone and menthone. The antibacterial activity of *R. officinalis* L EOs has been related to the presence of α -Pinene, 1, 8-Cineole and b-pinene. The antibacterial potency of *S. bachtiarica* Bunge, *Z. multiflora* Boiss, and *O. vulgare* L EOs can be attributed to the carvacrol and thymol [9]. The antibacterial activities of pinene-type monoterpene hydrocarbons are due to membrane disruption by the lipophilic compounds while carvacrol and thymol could bind to proteins of the cell membrane with hydrogen bonding and change the permeability, resulting in the release of cellular contents, as well as disruption of electron transport, nucleic acid synthesis and nutrient uptake [16]. Carvacrol and thymol are phenolic monoterpenes with similar structures that both contain a hydroxyl group on the phenolic ring. The difference between carvacrol and thymol is the position of the hydroxyl group in their phenolic ring [17]. A hydrophilic feature of functional groups and lipophilic feature of hydrocarbon skeleton are important in the antibacterial activity of essential oil components. So, phenols have the highest antibacterial activity followed by aldehydes, ketones, alcohols, ethers and hydrocarbons [18]. Thymol and carvacrol have more antibacterial activity compared to 1, 8-Cineole which belongs to the ether group and it may result in a weaker antibacterial activity of *R. officinalis*. In the current study, the amount of carvacrol and thymol in *S. bachtiarica* Bunge were the highest, 46 and 28.5 %v w⁻¹, respectively which is in agreement with the results of Falsafi et al. [19]. The quantity of carvacrol and thymol in *O. vulgare* L. in the present study were reported as 29 and 16 %v w⁻¹ while Moshayedi et al. reported 11.7 and 9.4 %v w⁻¹ [14]. In this study, the amount of carvacrol and thymol in *Z. multiflora* Boiss were recorded as 35 and 22 %v w⁻¹ while Mahboubi

and Bidgoli reported the amount of carvacrol 15 %v w⁻¹ and for thymol 38.7 %v w⁻¹ [20]. These differences correlate with a number of factors including the geographical origins, the level of macronutrients, micronutrients, hydration, the harvesting seasons, the genotype, the climate, the drying and extraction method and the distilled part of the plant.

Sensory evaluation revealed that the organoleptic properties of VPBF samples treated with essential oils of *S. bachtiarica* Bunge at 0.275, 0.55 and 1.1 %v w⁻¹ and *Z. multiflora* Boiss at 0.355 and 0.71 %v w⁻¹ and *O. vulgare* L. at 0.395 and 0.79 %v w⁻¹ were acceptable by the panelists and attribute scores of odor and taste were above 6 ($p < 0.05$) but the odor and taste of VPBF treated with EOs at 3 MIC levels against *C. sporogenes* were unacceptable (Fig. 1). In general, VPBF samples treated with *S. bachtiarica* Bunge obtained the highest scores of odor and taste in comparison with other examined essential oils and were significantly preferred ($p < 0.05$).

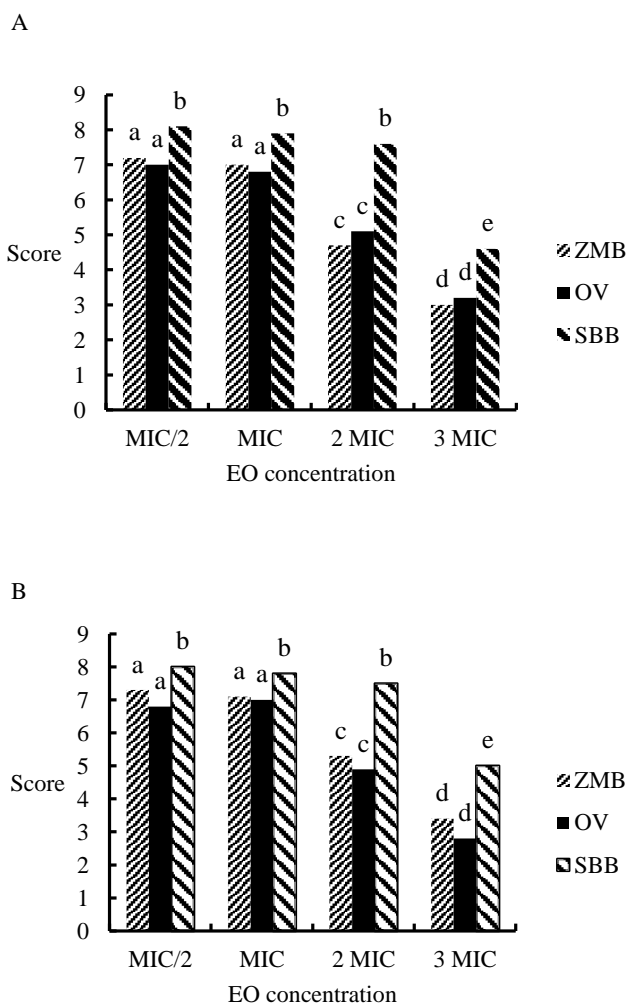


Figure 1. Sensory evaluation odor (A) and taste (B) of vacuum processed beef fillet treated with *Z. multiflora* Boiss EO (ZMB) and *O. vulgare* L EO (OV) *S. bachtiarica* Bunge EO (SBB) at MIC/2, MIC, 2 MIC and 3 MIC levels against *C. sporogenes* (values with the same letter are not significantly different at $p < 0.05$)

Table 1. Antimicrobial activity of essential oils against *C. perfringens* and *C. sporogenes*.

Plant species	<i>C. perfringens</i>		<i>C. sporogenes</i>	
	Agar disc diffusion (mm)	MIC (mg ml ⁻¹)	Agar disc diffusion (mm)	MIC (mg ml ⁻¹)
<i>Zataria multiflora</i> Boiss	6.50 ± 0.50 ^a	5.50 ± 0.50 ^A	5.20 ± 0.45 ^e	7.10 ± 0.12 ^E
<i>Origanum vulgare</i> L	6.00 ± 0.70 ^a	6.20 ± 0.20 ^A	5.50 ± 0.31 ^e	7.90 ± 0.25 ^E
<i>Mentha pulegium</i>	3.10 ± 0.12 ^b	15.00 ± 0.50 ^B	2.60 ± 0.27 ^f	16.80 ± 0.50 ^F
<i>Satureja bachtiarica</i> Bunge	7.00 ± 0.42 ^c	4.10 ± 0.10 ^C	6.20 ± 0.54 ^g	5.50 ± 0.10 ^G
<i>Rosmarinus officinalis</i> L.	2.00 ± 0.20 ^d	20.00 ± 0.60 ^D	1.10 ± 0.60 ^h	24.00 ± 0.70 ^H

*Data in the same columns followed by different letters are statistically different ($p \leq 0.05$).

Acceptability of VPBF treated with 1.1 % v w⁻¹ *S. bachtiarica* Bunge in meat does not mean that this EO does not influence the organoleptic properties, but the sensorial effect of VPBF formulated with 1.1 % v w⁻¹ *S. bachtiarica* is acceptable and could be suitable for commercial use. Van Haute et al. reported that addition of EOs to meat could positively, neutrally or negatively change the sensorial properties. As cooking improves the odor of meat, possibly due to the combination of EO odors and cooked meat odor compounds and volatilization of EO compounds [21]. The MIC values of the five EOs against *C. perfringens* and *C. sporogenes* were determined between 4.1 and 24 µg ml⁻¹ (data were shown in Table 1). *S. bachtiarica* Bunge showed the highest inhibition effects (lowest MIC values) against *C. perfringens* (4.1 mg ml⁻¹) and *C. sporogenes* (5.5 mg ml⁻¹) followed by *Z. multiflora* Boiss, *O. vulgare* L., *M. pulegium* and *R. officinalis* L. *S. bachtiarica* Bunge, *Z. multiflora* Boiss and *O. vulgare* L. were selected for investigation of antimicrobial activity against *C. perfringens* and *C. sporogenes* in VPBF.

Major chemical constituents of the EOs of *Z. multiflora* Boiss, *O. vulgare* L., *M. pulegium*, *S. bachtiarica* Bunge, and *R. officinalis* L. were demonstrated in Table 2.

The antibacterial effects of the *S. bachtiarica* Bunge, *Z. multiflora* Boiss, and *O. vulgare* L. on *C. perfringens* and *C. sporogenes* were significantly lower in VPBF in compare to in vitro (Fig. 2). For example, adding *S. bachtiarica* Bunge, at the MIC level against *C. sporogenes* to meat reduced *C. sporogenes* population by 0.84 (log

CFU g⁻¹) compared to the control sample after one-week storage. It could be due to the protection of the bacteria by meat components such as lipids and proteins. Cui et al. suggested nutrients in food matrix promote the repair of the bacterial cell and reduce the efficiency of antibacterial agents. Moreover, phenolic compounds may bind to amino acids, and proteins with hydrogen bonding resulted in reduced antibacterial effect in beef medium [22]. VPBF can be considered as a suitable medium for the growth of *Clostridium* bacteria due to the high amount of protein and minimum required moisture as well as anaerobic conditions. Although heat processing and sanitizing treatment could destroy the vegetative cells of *Clostridium* spp., their spores can survive and grow in prepared and stored food at room temperature. Temperature abuse during distribution, handling, and storage of food products not only leads to a reduction in shelf-life and decreasing the quality but also allows for the spore germ-ination and rapid growth of bacteria such as *Clostridium* ssp. and consequently, foods spoilage and increasing the risk of food poisoning. The optimal temperature for preparation, storing and transportation of fresh meat and meat products is between +2°C to +7°C depending on the type of meat [22]. In our study preparation and packaging of VPBF was done at ≤4°C but the used heat treatment was appropriated for activation of *Clostridium* spp. to investigate temperature abuse due to processing disruption. VPBF then cooled in 15h to 7.2°C and stored at room temperature.

Table 2. Major chemical constituents of the essential oils determined by GC-MS

Plant species	Composition (% v w ⁻¹)
<i>Z. multiflora</i> Boiss	Carvacrol (35.5%), Thymol (22%), p-Cymene (13.02%), γ-Terpinene (6.32%), α-Pinene (4%)
<i>O. vulgare</i> L	Carvacrol (29%), γ-Terpinene (20%), Thymol (16%), β-Pinene (7%), p-Cymene (5.3%)
<i>M. pulegium</i>	Pulegone (50%), Menthone (21%), Piperitenone (11%)
<i>S. bachtiarica</i> Bunge	Carvacrol(46%),thymol(28.5%), p-cymene(4.4%),γ-terpinene(4.0%)
<i>R. officinalis</i> L.	α-Pinene (26%), 1, 8-Cineole (21%), b-pinene (19%) Camphor (13%), limonene (8%)

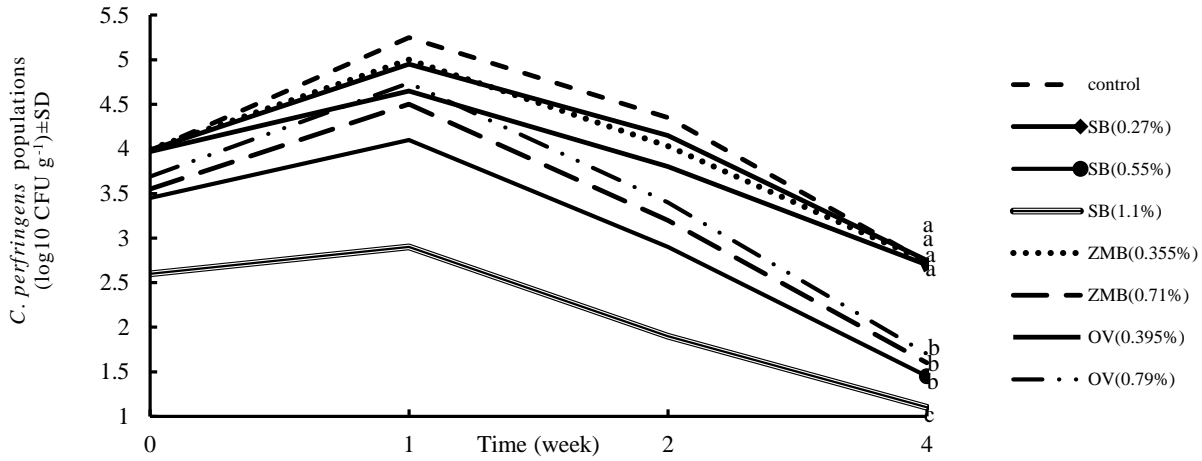


Figure 2a. Number of *C. perfringens* in vacuum processed beef fillet formulated with different levels of *S. bachtiarica* Bunge EO (SB), *Z. multiflora* Boiss EO (ZMB), *O. vulgare* L. EO (OV) and without sodium nitrite during storage at room temperature for 30 days.

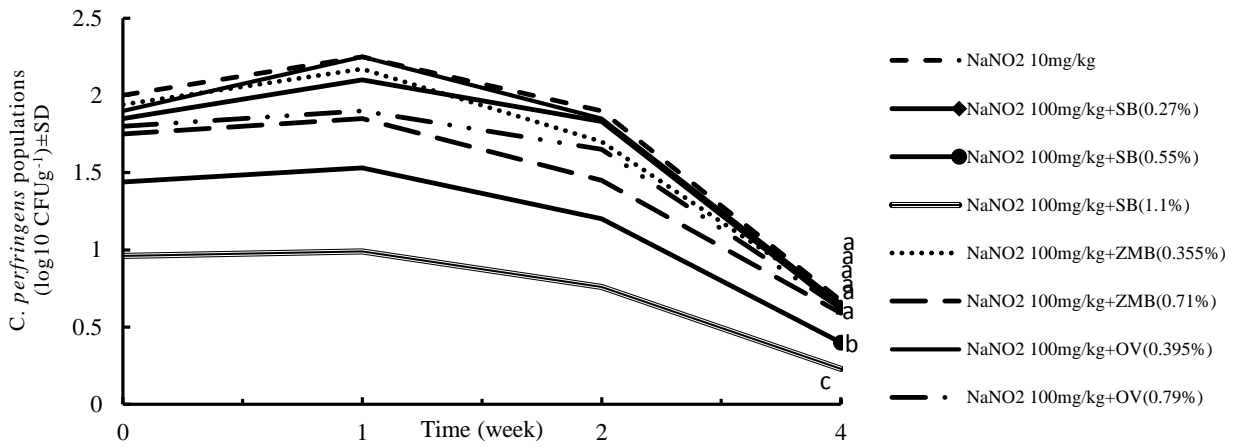


Figure 2b. Number of *C. perfringens* in vacuum processed beef fillet formulated with different levels of *S. bachtiarica* Bunge EO, *Z. multiflora* Boiss EO, *O. vulgare* L. EO and (100 mg kg⁻¹) sodium nitrite during storage at room temperature for 30 days

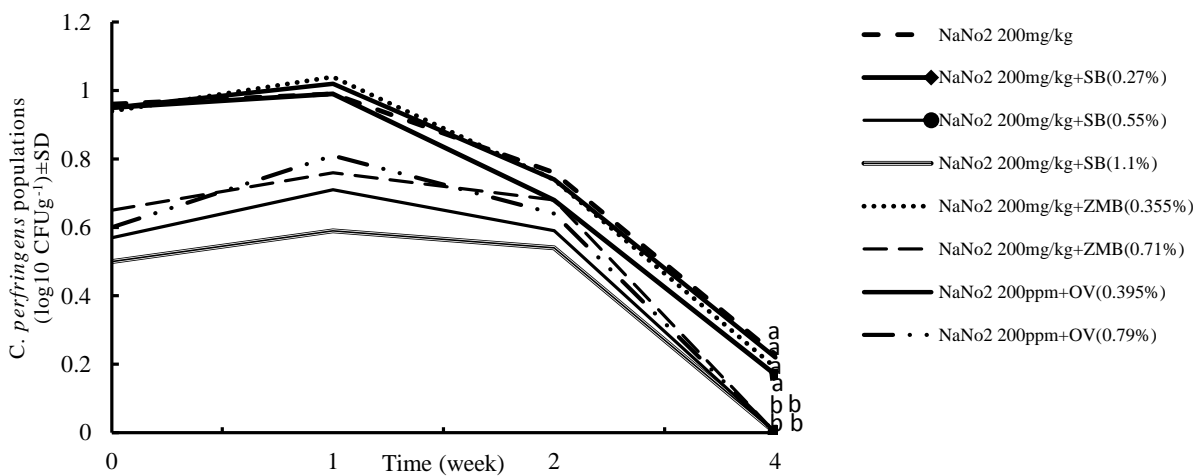


Figure 2c. Number of *C. perfringens* in vacuum processed beef fillet formulated with different levels of *S. bachtiarica* Bunge EO, *Z. multiflora* Boiss EO, *O. vulgare* L. EO and (200 mg kg⁻¹) sodium nitrite during storage at room temperature for 30 days.

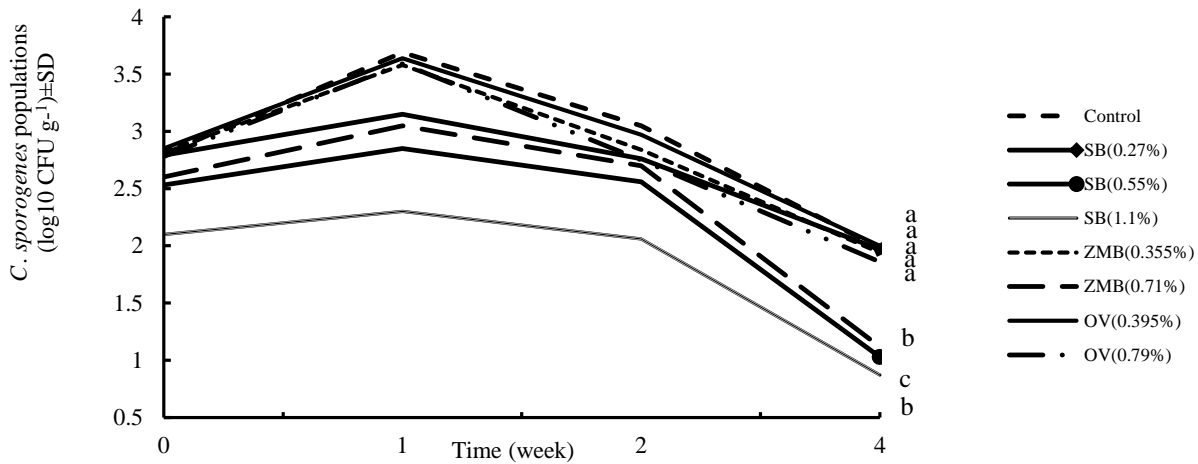


Figure 2.d. Number of *C. sporogenes* in vacuum processed beef fillet formulated with different levels of *S. bachtiarica* Bunge EO, *Z. multiflora* Boiss EO, *O. vulgare* L. EO and without sodium nitrite during storage at room temperature for 30 days.

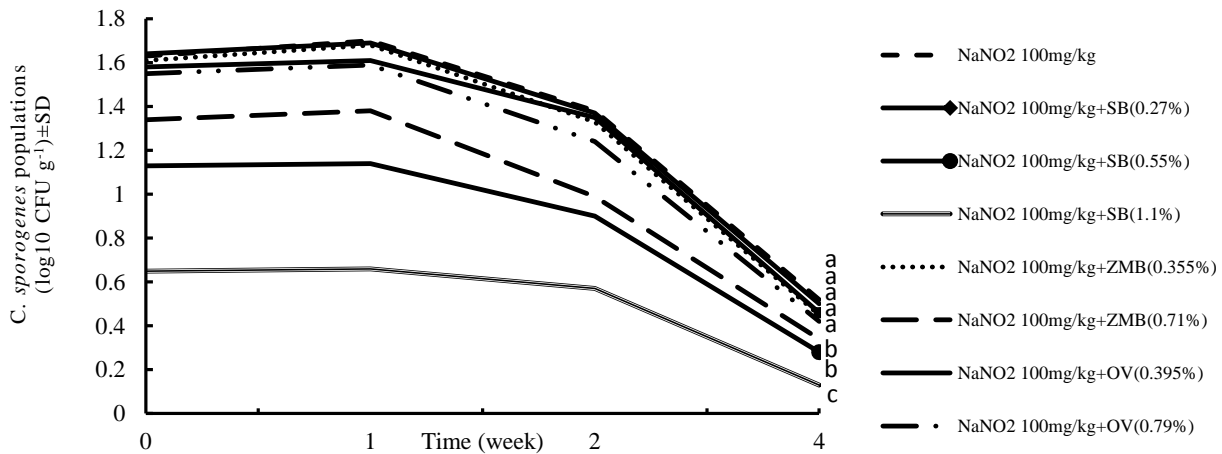


Figure 2.e. Number of *C. sporogenes* in vacuum processed beef fillet formulated with different levels of *S. bachtiarica* Bunge EO, *Z. multiflora* Boiss EO, *O. vulgare* L. EO and (100 mg kg⁻¹) sodium nitrite during storage at room temperature for 30 days.

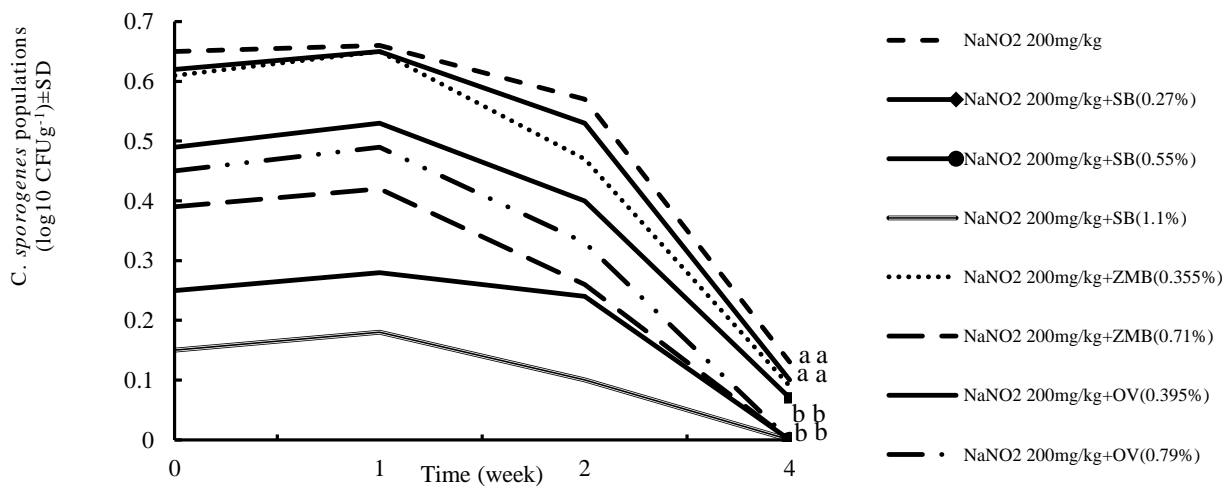


Figure 2f. Number of *C. sporogenes* in vacuum processed beef fillet formulated with different levels of *S. bachtiarica* Bunge EO, *Z. multiflora* Boiss EO, *O. vulgare* L. EO and (200 mg kg⁻¹) sodium nitrite during storage at room temperature for 30 days.

Although vacuum packing techniques are used to increase the shelf-life of VPBF by removing air and limits the growth of aerobic bacteria but *Clostridium* bacteria including *C. perfringens* and *C. botulinum* are still able to grow and proliferate and produce toxins. Curing meat by nitrate and nitrite salts provide protection against pathogenic bacteria, especially *C. botulinum* [5]. Our result show that *C. perfringens* is more sensitive than *C. sporogenes* to the sodium nitrite and the antibacterial activity of sodium nitrite against *C. perfringens* and *C. sporogenes* inoculated in VPBF was increased by using higher levels of nitrite. Antibacterial potency of sodium nitrite against *Clostridium* spp. can be attributed to the reaction of nitrite and nitrous acid with SH-groups in bacterial cells and the interference in enzymatic activities, DNA and gene expression and membrane and cell wall damage as the primary effect of nitrite [3].

The combined addition of tested EOs at the concentration of MIC/2 and MIC with sodium nitrite at 100 mg kg⁻¹ or 200 mg kg⁻¹ in VPBF resulted in better antimicrobial activity against *C. perfringens* and *C. sporogenes* inoculated in VPBF during cooling and storage. Our result is in agreement with the results of Cui et al. that observed synergy effect between plant extracts and NaNO₂ on *C. botulinum* resulting in growth inhibition or inactivation [21]. De Oliveira et al. reported the synergetic effect between *S. montana* L. EOs and reduced sodium nitrite against *C. perfringens* in mortadella-type sausages [3]. In our study, the use of *S. bachtiarica* Bunge at the level of 1.1 % v w⁻¹ with 100 mg kg⁻¹ NaNO₂ showed the similar antibacterial effect against *Clostridium* spp. compared to using 200 mg kg⁻¹ NaNO₂ alone, and according to our result, *C. perfringens* sensitivity was more than *C. sporogenes* in treatments containing EOs and nitrite. Since the permitted added levels of nitrite in meat formulations vary from 120 to 200 mg kg⁻¹ in different standards; Iran maximum 120 mg kg⁻¹ [24] and USA 200 mg kg⁻¹ [25], this treatment could be used in different countries to reduce the amount of nitrite and control the outgrowth of *Clostridium* bacteria in vacuum cured meat with acceptable sensory properties and using temperature management systems could help to monitor and control the temperature and avoid temperature abuse. There are some reports for reducing the level of nitrite in meat products by using essential oils. Doolaege et al. reported adding rosemary extract to liver pate, could reduce the amount of nitrite from 120 to 80 mg kg⁻¹ [26]. Moarefian et al. reported the amount of nitrite in sausage could be decreased to 50 % by adding *Cinnamomum zeylanicum* essential oil [27,28].

Producing VPBF with 1.1 % v w⁻¹ *S. bachtiarica* Bunge essential oils in combination with reduced sodium nitrite by 100 mg kg⁻¹ will increase the cost of products slightly, but it would be negligible because the product meets the

demands of the consumers and food authorities for using natural additives and reducing the level of nitrite in meat products.

4. Conclusion

Ensuring the safety of VPBF is an important issue due to high risk of the growing and producing toxin of *Clostridium* species. The results of this study support the synergistic effects between EOs and NaNO₂ on *C. perfringens* and *C. sporogenes*. Curing the fillet with 1.1 % v w⁻¹ *S. bachtiarica* Bunge essential oils in combination with reduced sodium nitrite by 100 mg kg⁻¹ could significantly decrease the risk of *Clostridium* spp. Outgrowth. To develop and produce safe food with acceptable organoleptic properties is among food safety goals and the proposed method appears to be feasible in financial and technological terms. Further studies are needed to demonstrate the possibility of using this treatment for controlling psychro-tolerant clostridia which could cause blown pack spoilage in vacuum-packed meat resulting in high amounts of drip, gas and unpleasant odor as well as appearance of discoloration and tender meat.

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6. Conflict of Interest

The authors declare that there is no conflict of interest.

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کارایی ضد باکتریایی اسانس‌های روغنی و سدیم نیتريت در فیله گاو فرآیند شده تحت خلأ

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چکیده

سابقه و هدف: به حداقل رساندن در معرض نیترات و نیتريت قرار گرفتن و در نتیجه کاهش میزان نیتروز آمین‌های بالقوه سرطانزا به دلیل تقاضای رو به افزایش عمومی و مناقشات سیاسی، در بسیاری از کشورها مدنظر است. مطالعه حاضر برای بررسی فعالیت ضد باکتریایی پنج نوع گوناگون اسانس روغنی، به تنهایی یا در ترکیب با غلظت‌های مختلف سدیم نیتريت طراحی شد.

مواد و روش‌ها: پنج نوع اسانس روغنی *Satureja bachtiarica Bunge*، *Zataria multiflora Boiss*، *Origanum vulgare L.* و *Mentha pulegium*، *Rosmarinus officinalis L.* گرفتند. برای مطالعه مهار رشد گونه‌های کلستریدیوم تلقیح شده به فیله‌های گوشت گاو فرآیند شده تحت خلأ، سدیم نیتريت در غلظت‌های صفر، ۱۰۰ و ۲۰۰ mg kg⁻¹ مورد استفاده قرار گرفتند. اسانس‌های روغنی با طیف‌سنجی جرمی کروماتوگرافی گازی مورد آنالیز قرار گرفتند. فعالیت ضد میکروبی در برابر سلول‌های رویشی کلستریدیوم پرفرنجنس و کلستریدیوم اسپروجنس در ابتدا با روش انتشار بر روی دیسک و سپس تعیین حداقل غلظت بازدارندگی اسانس‌های روغنی در برابر سلول‌های رویشی با رقیق‌سازی در لوله آزمایش حاوی محیط کشت مایع انجام شد. ارزیابی حسی نمونه‌های فیله گوشت گاو فرآیند شده تحت خلأ و تیمار شده با سه اسانس روغنی دارای بیشترین فعالیت ضد باکتریایی در برابر گونه‌های مقاوم‌تر کلستریدیوم انجام شد.

یافته‌ها و نتیجه‌گیری: در میان اسانس‌های روغنی مورد آزمون، *Satureja bachtiarica Bunge* بیشترین اثر مهاری بر کلستریدیوم پرفرنجنس (۴/۱ mg ml⁻¹) و کلستریدیوم اسپروجنس (۵/۵ mg ml⁻¹) را نشان داد و *Zataria multiflora Boiss*، *Origanum vulgare L.*، *Mentha pulegium* و *Rosmarinus officinalis L.* در مرتبه‌های بعدی قرار داشتند. فعالیت ضد میکروبی اسانس‌های روغنی در برابر گونه‌های کلستریدیوم در ترکیب با سدیم نیتريت افزایش یافت. از این رو می‌توان فرض کرد که تلفیق دو افزودنی در کنترل کلستریدیوم پرفرنجنس و کلستریدیوم اسپروجنس در نمونه‌های فیله گاو فرآیند شده تحت خلأ اثر قابل توجه می‌تواند داشته باشد، بدون همخوانی با ویژگی‌های حسی. بهترین نتیجه در تلفیق ۱۰۰ mg kg⁻¹ سدیم نیتريت و ۱/۱ % v w⁻¹ *Satureja Bakhtiarica Bunge* به دست آمد، که می‌توانست رشد گونه‌های کلستریدیوم را مهار کند. نتایج تحقیق حاضر نشان داد اسانس‌های روغنی مورد نظر اثرات شدید بر مهار کلستریدیوم داشته‌اند و می‌توانند در صنعت گوشت، به ویژه سوسیس به علت اثراتشان بر ویژگی‌های فناورانه، میکروبی و حسی مورد استفاده قرار گیرند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ تعارض منافی وجود ندارد.

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واژگان کلیدی

- فعالیت ضد باکتریایی
- گونه‌های کلستریدیوم
- اسانس‌های روغنی
- سدیم نیتريت
- فیله گاو فرآیند شده تحت خلأ

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