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Effects of Chitosan and Some Essential Oils (Thyme, Clove, Rosemary), Individually or in Combination, on Inactivation of *Salmonella* Typhimurium in Cig Kofte (Turkish Raw Meatball)*

Hüsnü Şahan GÜRAN[1]
Gülsüm ÖKSÜZTEPE[2]

[1]Dicle Üniversitesi,
Veteriner Fakültesi,
Besin Hijyeni ve Teknolojisi
Anabilim Dalı,
Diyarbakır, TÜRKİYE

[2]Fırat Üniversitesi,
Veteriner Fakültesi,
Besin Hijyeni ve Teknolojisi
Anabilim Dalı,
Elazığ, TÜRKİYE

This study was undertaken to determine the effects of chitosan and some essential oils on the inactivation of *Salmonella* Typhimurium bacteria in "cig kofte" (raw meatball). For this purpose, cig kofte was inoculated with *Salmonella* Typhimurium and divided into 8 portions, such that they were treated differently in 8 groups, including Groups C (control), I (250 mg/kg thyme essential oil), II (250 mg/kg clove essential oil), III (250 mg/kg rosemary essential oil), IV (500 mg/kg chitosan), V (500 mg/kg chitosan + 250 mg/kg thyme essential oil), VI (500 mg/kg chitosan + 250 mg/kg clove essential oil) and VII (500 mg/kg chitosan + 250 mg/kg rosemary essential oil). The eight cig kofte portions were stored at 4±1 °C for 24 hours, and analyses for the numbers of *Salmonella* Typhimurium, pH value, and water activity (a_w) were performed at 0, 3, 6, 12 and 24 hours of storage. At the end of the storage period (at the 24th hour), the highest level of pathogen reduction was determined in group V (1.60 $\log_{10}$ CFU/g), while the level of pathogen reduction in groups I, II, III, IV, VI and VII were determined as 0.57, 0.55, 0.54, 0.88, 0.58 and 0.68 $\log_{10}$ CFU/g respectively.

Key Words: *Chitosan, essential oils, Salmonella, inactivation, cig kofte.*

Kitosan ve Bazı Esansiyel Yağların (Kekik, Karanfil ve Biberiye) Tek Başlarına ve Kombine Halde Çiğ Köftede *Salmonella* Typhimurium Üzerine Etkileri

Bu araştırma, kitosan ve bazı esansiyal yağların çiğ köftede *Salmonella* Typhimurium bakterisinin inaktivasyonu üzerine etkilerinin belirlenmesi amacıyla gerçekleştirildi. Bu amaçla *Salmonella* Typhimurium ile kontamine edilen çiğ köfte; C (kontrol), I. grup (250 mg/kg kekik), II. grup (250 mg/kg karanfil), III. grup (250 mg/kg biberiye), IV. grup (500 mg/kg kitosan), V. grup (500 mg/kg kitosan + 250 mg/kg kekik), VI. grup (500 mg/kg kitosan + 250 mg/kg karanfil) ve VII. grup (500 mg/kg kitosan + 250 mg/kg biberiye) olmak üzere 8 gruba ayrıldı. 4±1 °C'de 24 saat muhafaza edilen gruplar, muhafazanın 0., 3., 6., 12. ve 24. saatlerinde *Salmonella* Typhimurium, pH ve su aktivitesi (a_w) yönünden analiz edildi. Muhafaza sonunda (24. saat) en fazla patojen azalması V. grupta (1.60 $\log_{10}$ CFU/g) tespit edilirken I., II., III., IV., VI. ve VII. gruplarda ise sırasıyla 0.57, 0.55, 0.54, 0.88, 0.58 ve 0.68 $\log_{10}$ CFU/g olarak tespit edildi.

Anahtar Kelimeler: *Kitosan, esansiyel yağlar, Salmonella, inaktivasyon, çiğ köfte.*

Introduction

"Cig kofte (raw meatball)" is a popular traditional appetizer in Turkey and some Middle East countries. This dish is prepared by kneading finely ground lean beef, cracked wheat, chopped onions, garlic, spices (red pepper, black pepper, cumin and pimento, etc.) salt and tomato paste. The amount and variety of additives used in the preparation of this dish varies, as no standard exists for cig kofte. Cig kofte is usually consumed shortly after preparation, but can be stored in the refrigerator for up to 24 hours (1). According to Turkish Food Codex of 2013, the use of ground meat and all food additives except citric acid is prohibited in cig kofte (2). However, some particular restaurants and some local sale points or homemade cig kofte can still be made with raw ground meat.

As cig kofte is prepared without applying any heat treatment or using any preservative, its microbiological quality depends on personal hygiene, production method, hygienic quality of ingredients used in particular of minced meat and spices (3). Several studies have been conducted on the microbiological quality of cig kofte. These studies have demonstrated that this food product can be contaminated with fecal indicator bacteria and pathogenic bacteria, including coliforms, *Salmonella* spp., *L. monocytogenes*, *S. aureus*, *E. coli*, *Y. entereocolitica* and *B. cereus* (4-6). Listed among the major food pathogens, which cause significant public health problems, *Salmonella* spp. are reported to have a prevalence ranging between 0-14% in cig kofte (4, 7, 8).

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Yazışma Adresi
Correspondence

Hüsnü Şahan GÜRAN
Dicle Üniversitesi,
Veteriner Fakültesi,
Besin Hijyeni ve Teknolojisi
Anabilim Dalı,
Diyarbakır - TÜRKİYE

sahaguran@yahoo.com

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Previous research has shown that cig kofte is a high-risk food product in terms of public health and food safety. Literature reports on how to increase the microbiological safety of cig kofte are few. The scarcity of scientific information requires the use of antimicrobial food additives apart from the application of good hygiene practices. The natural composition and low toxicity of these natural antimicrobial substances have enabled them to find common use in the food industry. Of these substances, chitosan is a natural polymer obtained by the deacetylation of chitin found in the exoskeleton of crustaceans, including crabs and shrimps (9). In the past few years, chitosan has been widely investigated by the food industry, and is categorized as "generally recognized as safe (GRAS)" by the Food and Drug Administration (10). It is reported that chitosan can prevent the growth of Gram negative bacteria, including *E. coli*, *Pseudomonas aeruginosa, Shigella dysenteria*, *Vibrio spp.* and *Salmonella* spp., and that the minimum inhibitory concentration of this substance for these bacteria ranges from 100 ppm to 10.000 ppm (11). Essential oils are another group of natural compounds with antimicrobial property. Owing to their taste, aroma and broad bioactivity profile, they constitute a significant alternative for use in cig kofte (12-14). Several essential oils, including those of cloves, thyme and rosemary, are classified as GRAS by the FDA (10). Previous research has shown that, apart from their antimicrobial property, essential oils also exhibit antibacterial and antifungal affects both *in vitro* and in food (12). The inhibitory effect of these essential oils on saprophytic and pathogenic Gram-negative and Gram-positive bacteria suggests that they may play an important role in enhancing the safety of risk food products (15).

The present study was carried out to investigate the effects of the use of chitosan and thyme, clove and rosemary essential oils, alone or in combination, on the inactivation of *Salmonella* in cig kofte.

Materials and Methods

Chitosan and Essential Oils: Chitosan (Sigma C3646, ≥75 % deacetylated) was purchased from Sigma Company (St. Louis, MO, USA). Stock solution of chitosan (1%) was prepared in 1% acetic acid (food grade). Essential oils were purchased from the Kalsec Company (Kalamazoo, Mich.) which supplies food grade oils. Undiluted 100% oil of clove, and oil of thyme, and oil of rosemary, were used.

Prerapation of Inoculums: The strains used to inoculate cig kofte were three *Salmonella* Typhimurium strains (ATCC 14028, NCTC 12416, RSKK 95091). The strains were stored at −80 °C before use. After thawing, each strain was cultured in 10 mL of tryptic soy broth (Merck, Darmstadt, Germany) and incubated at 37 ^{o}C for 18 h. Then, the cultures were centrifuged at 4.192 × g for 10 min at 5 °C (NF800R, Nüve, Turkey), and the pellets were washed with 0.1% sterile peptone water (LAB M, England) before re-centrifuging to remove organic residues. The pellets of each strain were re-suspended in an aliquot of peptone water. These suspensions were then combined in a single beaker and completed to 10 mL with 0.1% sterile peptone water. The final level of the pathogen was approximately 10^8 CFU/mL in the inoculum.

***Salmonella* Inoculation and the Addition of Essential Oils and Chitosan:** For each trial, freshly prepared 1500 g of cig kofte was obtained from a local restaurant. In the present study, all ingredients used by the restaurant were presented in Table 1 as g/ kg cig kofte. Each trial was repeated three times. A cig kofte sample of 100 g was taken and analyzed for pH, water activity, salt level, and *Salmonella* spp. presence. The remaining 1400 g was inoculated with *Salmonella* mixture and mixed by hand. Subsequently, the cig kofte sample was divided into 8 portions/groups, each weighing 175 g. The control group did not include any essential oil or chitosan, and Groups I, II III, IV, V, VI and VII were added 250 mg/kg of thyme essential oil, 250 mg/kg of clove essential oil, 250 of rosemary essential oil, 500 mg/kg of chitosan, 500 mg/kg of chitosan + 250 mg/kg of thyme essential oil, 500 mg/kg of chitosan + 250 mg/kg of clove essential oil, and 500 mg/kg of chitosan + 250 mg/kg of rosemary essential oil, respectively. In all of the experimental groups, including the control group, the cig kofte portions were stored in styrofoam containers covered with stretch wrap at 4±1 °C in a cooled incubator (ES 120, Nüve, Turkey) for 24 hours. At 0, 3, 6, 12 and 24 hours of storage, microbiological and chemical analyses (pH and water activity – a_w value) were performed.

Table 1. Proximate composition of the ingredients used in producing cig kofte (g /kg)

Ingredients	Amount	Ingredients	Amount
Bulgur (cracked wheat)	360	Salt	7
Ground lean beef	200	Garlic	7
Tomato paste	180	Pimento	6
Chopped onion	90	Cumin	3
Parsley	20	Black pepper powder	7
Red pepper paste	60	Cinnamon	5
Traditional paprika (isot)	5	Water (mL/kg)	50

Enumeration of *Salmonella*: In each group, at 0, 3, 6, 12 and 24 hours of storage, a 25-g portion of the sample was taken under aseptic conditions, added 225 ml of sterile peptone water, and homogenized for 2 minutes (Bagmixer 400W, Interscience, France). 1/10 serial dilutions were prepared up to $1/10^8$, and each dilution was spread in volumes of 0.1 mL onto duplicate XLD agar plates using the spread plate method, and incubated at 35 °C for 24 hours. Three colonies randomly selected from the counted plates were confirmed as *Salmonella* colonies, based on the demonstration of the "O" antigen with a polyvalent *Salmonella* antiserum (13).

Chemical Analysis: Chemical analyses were conducted in duplicate to determine the following: salt levels determined by Mohr method (16), pH was measured using a pH meter (selecta 2001 Spain) and water activity (aw) determined using a water activity meter (Testo 650 , USA).

Experimental Design and the Statistical Analysis of Data: The trials were designed as three repetitions performed at an interval of 15 days. The data converted to $\log_{10}$ CFU/g was subjected to variance analysis for fixed effects and interactions between variables according to the 3x8x5 factorial design as "replication x treatment groups x sampling time to be taken at a time from each test group". In accordance with the General Linear Model (GLM) procedures, the least square means were detected by Fisher's Least Significant Difference (LSD) test, and the level of statistical significance was set at 5%. The data was analyzed using the Statistical Analysis System software (17).

Results

Changes of the *Salmonella* numbers in cig kofte samples during storage treated with some essential oils and chitosan are shown in Table 2. Initial number of the *Salmonella* was determined to be between 7.50 and 7.60 log CFU/g in control and treatment groups. The pathogen number in the treatment groups were decreased during the storage, whereas the control group had a slight increase. However, no significant difference was observed between group I, II, III, VI, VII and the control group during the storage ($P>0.05$). A significant difference was observed between group IV, V and control group from 12th hours to the end of storage period ($P<0.05$). At the end of the storage period (at the 24th hour), highest level of the pathogen reduction was determined in group V (1.60 $\log_{10}$ CFU/g), but the other treatment groups were below the 1 log of reduction. A significant difference was found only within the group V at the end of storage ($P<0.05$) except in the other treatment groups ($P>0.05$).

Water activitiy (a_w) and pH values of treatment groups and control were evaluated during storage and are shown in Table 3. No significant difference was determined between the groups, indicating that the addition of the essential oils and the chitosan did not change the pH and aw in the cig kofte ($P>0.05$).

Table 2. Inactivation of *Salmonella* in cig kofte samples, treated with alone or combination of some essential oils and chitosan, stored at 4 °C for 24 hours ($\log_{10}$ CFU/g).

Groups	Storage time (hours)				
	0	3	6	12	24
C	7.60 ± 0.16	7.58 ± 0.11	7.60 ± 0.18	7.60 ± 0.17^{a}	7.68 ± 0.15^{a}
I	7.54 ± 0.15	7.35 ± 0.34	7.26 ± 0.23	7.12 ± 0.09^{ab}	7.11 ± 0.12^{a}
II	7.61 ± 0.23	7.56 ± 0.15	7.39 ± 0.09	7.21 ± 0.14^{a}	7.13 ± 0.22^{a}
III	7.53 ± 0.18	7.41 ± 0.32	7.30 ± 0.69	7.20 ± 0.27^{ab}	7.14 ± 0.28^{a}
IV	7.55 ± 0.16	7.43 ± 0.28	7.35 ± 0.12	7.08 ± 0.19^{b}	6.80 ± 0.25^{bc}
V	7.50^{x} ± 0.05	7.32 ± 0.35^{x}	7.06^{x} ± 0.17	6.77 ± 0.22^{bxy}	6.08 ± 0.04^{cy}
VI	7.59 ± 0.10	7.48 ± 0.12	7.33 ± 0.11	7.24 ± 0.24^{a}	7.10 ± 0.32^{ab}
VII	7.56 ± 0.17	7.45 ± 0.31	7.35 ± 0.09	7.24 ± 0.36^{a}	7.00 ± 0.25^{ab}

^{a,b,c} : Means with the different superscripts within the same column are significantly different ($P<0.05$).
^{x,y}: Means with the different superscripts within the same row are significantly different ($P<0.05$).
C- Control, I- 250 mg/kg thyme oil, II- 250 mg/kg clove oil, III- 250 mg/kg rosemary oil, IV- 500 mg/kg chitosan, V- 500 mg/kg chitosan + 250 mg/kg thyme oil, VI- 500 mg/kg chitosan + 250 mg/kg clove oil, VII, 500 mg/kg chitosan + 250 mg/kg rosemary oil.

Table 3. Mean values and standard errors (SEs) of pH and a_w in cig kofte samples treated with alone or combination of some essential oils and chitosan stored at 4 °C for 24 hours

Parameter	Groups	Storage time (hours)				
		0	3	6	12	24
pH	C	5.53 ± 0.11	5.47 ± 0.13	5.50 ± 0.06	5.56 ± 0.11	5.52 ± 0.10
	I	5.52 ± 0.15	5.53 ± 0.21	5.54 ± 0.05	5.53 ± 0.13	5.54 ± 0.12
	II	5.59 ± 0.01	5.57 ± 0.12	5.54 ± 0.01	5.58 ± 0.06	5.57 ± 0.03
	III	5.56 ± 0.11	5.58 ± 0.14	5.53 ± 0.10	5.58 ± 0.08	5.59 ± 0.10
	IV	5.35 ± 0.10	5.38 ± 0.10	5.30 ± 0.16	5.38 ± 0.09	5.30 ± 0.13
	V	5.40 ± 0.25	5.34 ± 0.12	5.35 ± 0.13	5.36 ± 0.12	5.27 ± 0.09
	VI	5.32 ± 0.10	5.33 ± 0.16	5.35 ± 0.08	5.35 ± 0.10	5.29 ± 0.05
	VII	5.31 ± 0.10	5.34 ± 0.17	5.32 ± 0.17	5.34 ± 0.12	5.34 ± 0.13
a_w	C	0.896 ± 0.10	0.887 ± 0.13	0.874 ± 0.06	0.883 ± 0.11	0.861 ± 0.10
	I	0.905 ± 0.15	0.918 ± 0.21	0.936± 0.05	0.923 ± 0.13	0.902 ± 0.12
	II	0.897 ± 0.01	0.891± 0.12	0.885 ± 0.01	0.874 ± 0.06	0.863 ± 0.03
	III	0.889 ± 0.11	0.886 ± 0.14	0.874 ± 0.10	0.851 ± 0.08	0.843 ± 0.10
	IV	0.877 ± 0.10	0.848 ± 0.10	0.831 ± 0.16	0.803 ± 0.09	0.772 ± 0.13
	V	0.865 ± 0.25	0.847 ± 0.12	0.831± 0.13	0.815 ± 0.12	0.809 ± 0.09
	VI	0.883 ± 0.10	0.867 ± 0.16	0.859 ± 0.08	0.821 ± 0.10	0.807 ± 0.05
	VII	0.872 ± 0.10	0.850 ± 0.17	0.832 ± 0.17	0.811 ± 0.12	0.793 ± 0.13

C- Control, I- 250 mg/kg thyme oil, II- 250 mg/kg clove oil, III- 250 mg/kg rosemary oil, IV- 500 mg/kg chitosan, V- 500 mg/kg chitosan + 250 mg/kg thyme oil, VI- 500 mg/kg chitosan + 250 mg/kg clove oil, VII, 500 mg/kg chitosan + 250 mg/kg rosemary oil.

Discussion

In recent years, the demand for ready-to-eat products, including meat and meat products, has increased and is on the rise. Of such products, cig kofte has a particular significance due to its risk of being contaminated with pathogens, including *Listeria monocytogenes*, *Salmonella* spp., and *E. coli* 0157:H7, and thus because of the risk it poses for food safety and public health. In this context, cig kofte belongs to the group of ready-to-eat food products with the highest risk, as it is consumed without any prior heat treatment. The very few studies conducted on the viability of *Salmonella* bacteria in cig kofte have demonstrated that these bacteria may remain viable within the consumption period (up to 24 hours) of the product (13, 14). Uzunlu and Yıldırım (6) determined that in cig kofte samples contaminated with *Salmonella enteritidis* and stored at 4 °C for 24 hours, the *Salmonella* levels at 0 and 24 hours of storage were 3.57 log CFU/g and 3.54 log CFU/g, respectively. Similarly, in the present study, the *Salmonella* Typhimurium level determined as 7.60 log CFU/g in the control group at 0 hours was ascertained to have increased to 7.68 log CFU/g by the end of the 24th hour of storage (Table 2). These results clearly suggest that the ingredients of cig kofte have bacteriostatic effect on *Salmonella* bacteria. On the other hand, these results also show that the spices added to cig kofte did not suffice in eliminating *Salmonella* Typhimurium from the product. However, essential oils (i.e. thyme essential oil, clove essential oil, rosemary essential oil, sage essential oil) obtained from aromatic plants (i.e. spices) and compounds found in aromatic plants (i.e. thymol, eugenol, carvacrol) display strong antibacterial effect against foodborne pathogens (12). In the present study, when added to cig kofte samples at a concentration level of 250 mg/kg, thyme, clove and rosemary essential oils (in Groups I, II and III) did not produce any significant inhibitory effect on *Salmonella* Typhimurium throughout the storage period, yet it was shown that the addition of these essential oils to cig kofte reduced the bacterial growth rate ($P>0.05$). In previous laboratory studies carried out on the antibacterial effect of spice extracts, although these extracts were determined to significantly reduce pathogen levels even when applied at concentrations below 0.1%, it has been underlined that, to ensure such an effect in food, depending on the particular essential oil or compound used, much higher concentrations are required to be applied (18). In a study in which eugenol, linalool, carvone, cineole and limonene oil were each added separately to cig kofte samples at a concentration level of 1.8%, Çalıcıoğlu and Dikici (13) determined the reduction in the number of *Salmonella* bacteria, enabled by these compounds, at 3 hours as 6.5, 2.54, 1.81, 0.92 and 0.25 log CFU/g, respectively. Both these results and the observation of the addition of eugenol at concentrations of 0.5%, 1% and 1.5% resulting in a reduction of approximately 1, 2 and 3 log CFU/g; respectively, are in support of the need for higher concentrations in food. In general, literature reports indicate that Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria (12). While the resistance of Gram-negative bacteria has been explained by these bacteria possessing an outer membrane composed of lipopolysaccharides and proteins, some other research has shown that Gram-positive bacteria may also be resistant to the effects of essential oils (14, 19).

The results of the present study demonstrated that, the addition of 500 mg/kg of chitosan to cig kofte (Group IV) stored at 4 °C, yielded a reduction of 0.88 log CFU/g in *Salmonella* numbers when compared to the control group ($P<0.05$). The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of chitosan for *Salmonella* bacteria under *in vitro* conditions have been reported as 288 ppm and 300 ppm, respectively (20). Furthermore, in nutrient broth, chitosan concentrations of 1.0%, 1.5%, 2.0%, and 2.5% have been reported to produce a reduction greater than 3 log in the *Salmonella* population, during an 8-day period at a pH value of 5.5 and a temperature of 30 °C (21). While Simpson et al. (22) reported that the addition of chitosan at a concentration level of 0.01% to shrimps was sufficient to inhibit the growth of *Salmonella* Typhimurium, Chhabra et al. (23) reported that the addition of chitosan at concentrations of 0.5%, 1.0%, and 2.0% to raw oysters had no effect on the inactivation of *Salmonella* Typhimurium throughout a 12-day storage period at 4 °C. The differences between these two studies, apart from several possible variables (storage period, matrices used in modelling, etc.), are attributed to differences in the concentration, deacetylation degree and molecular weight of the chitosan used (24, 25). Some research has shown that chitosan of medium molecular weight exhibits strong antibacterial effect on *S. enteritidis* at low temperatures (9, 25). Marques et al. (26) reported the MIC value of chitosan against *S. enterica* at 10 °C as 0.05% and indicated that chitosan had no inhibitory effect on this pathogen at 20 °C. The use of chitosan with a medium molecular weight and the storage temperature being adjusted to 4 °C in the present study, also suggest that these factors may be influential on the bioactivity of chitosan.

In the present study, the greatest reduction in the numbers of *Salmonella* by the end of the storage period (1.42 log CFU/g) was treated in Group V, which was administered with chitosan plus thyme essential oil ($P<0.05$, Table 2). This suggests that thyme essential oil and chitosan may exhibit a synergistic effect when applied in combination. This is explained by the breakdown of the outer protective membrane of the *Salmonella* bacteria by both thyme essential oil and chitosan, and the inhibition of bacterial growth as a result of the interference with mRNA and protein synthesis by chitosan (27). Similarly, some other studies have shown that, in terms of the extension of the shelf life of food products and the inactivation of pathogens in food products, the combined use of chitosan and essential oils enhances antimicrobial effect (28, 29). Georgantelis et al. (30) reported that chitosan and rosemary essential oil

showed a synergistic effect in increasing the microbial quality of pork sausages, while Kanatt et al. (31) reported that the addition of 0.1% of mint oil and chitosan mixture to lamb meat enabled a reduction of 1 log in the number of Gram-negative bacteria, including *E. coli*, *P. fluorescens*, and *Salmonella* Typhimurium, and up to 2-3 log in Gram-positive bacteria, including *S. aureus* and *B. cereus*.

In the present study, the pH value was determined to range between 5.52 and 5.59 in the control group and the groups applied essential oils alone, and between 5.27 and 5.40 in the groups applied chitosan alone and chitosan in combination with essential oils ($P>0.05$, Table 3). Furthermore, the average NaCl (salt) level of the cig kofte samples was ascertained to be 1.87±0.43%. The pH values of the groups, which contained chitosan, being lower than that of the control group and the groups that were applied essential oils was attributed to the use of a chitosan solution prepared in acetic acid. The results of the present study contradict with those reported by Georgantelis et al. (30), indicating a higher pH value in Greek-style sausages added chitosan and rosemary extract, and comply with those reported by Giatrakou et al. (28) indicating a lower pH value in chicken-pepper kebab added chitosan and thyme essential oil. Generally, low pH values are associated with an increased antimicrobial activity of essential oils and chitosan (11, 18). However, in the present study, of the groups, which had a low pH value and were applied chitosan, the group that was treated wiht chitosan in combination with thyme essential oil displayed the greatest reduction in *Salmonella* bacteria ($P< 0.05$).

The essential oil and the chitosan concentrations used in the present study were subjected to a sensory evaluation prior to their use (data not shown). Based on the panel results, the flavor, odor and taste of the samples, which contained 500, 750 and 1000 mg/kg of thyme, clove, rosemary essential oils and 750 and 1000 mg/kg of chitosan were disliked, while the samples contained 250 mg/kg of these oils and 500 mg/kg of chitosan were considered to be acceptable by the panelists.

The present study demonstrated that the combined use of chitosan and thyme essential oil in cig kofte samples, presenting with a high risk of cross contamination and a risk of production under unhygienic conditions, and consumed without any prior heat treatment, produced a synergistic effect against *Salmonella* without any adverse effect on the organoleptic properties of the product.

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