

# Antimicrobial activity of *Thymbra spicata* L. essential oil in Turkish dry fermented sausages

Serhat AL<sup>a,✉</sup>, Yeliz YILDIRIM<sup>b</sup>

Erciyes University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Kayseri, Turkey  
<sup>a</sup>ORCID: 0000-0003-2721-9275; <sup>b</sup>ORCID: 0000-0001-8783-3889

✉Corresponding Author: serhatal@erciyes.edu.tr

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**Abstract:** Essential oils (EO) could address the need for alternative additives for food producers to maintain safety and quality of meat products. The aim of this study was to evaluate antimicrobial activity of *Thymbra spicata* L. subsb. *spicata* EO on *Escherichia coli* and *Salmonella* Typhimurium in Turkish dry fermented sausage (sucuk). Antimicrobial activity of EO, obtained from the collected plant, has been demonstrated *in vitro* and in sucuk matrices against selected foodborne pathogens. In the composition of the essential oil obtained in the study, total of 47 components (99.41%) were assayed including mainly carvacrol (43.6%),  $\gamma$ -terpinene (16.69%) and p-cymen (13.97%). *Thymbra spicata* L. observed to have antimicrobial effect on the related pathogens *in vitro* however, increased amount of EO use, to be antimicrobiologically effective in sucuk, negatively affected the organoleptic properties. It is concluded that natural additives could potentially be used as an alternative to chemicals in food technology to prevent foodborne diseases and to extend the shelf life of products. Further studies are needed to evaluate the combined and synergetic effects of different EOs and other preservation methods to cope with foodborne pathogens in the food matrices.

**Keywords:** Antimicrobial activity, consumer health, essential oil, fermented sausage

## Fermente Türk sucuklarında *Thymbra spicata* L. uçucu yağının antimikrobiyel aktivitesi

**Özet:** Esansiyel yağların, et ürünlerinde kalite ve güvenliğin sağlanması için gıda üreticilerinin alternatif katkı maddelerine duyduğu ihtiyacı karşılayabileceği düşünülmektedir. Bu çalışmanın amacı *Thymbra spicata* L. esansiyel yağının fermente Türk sucuklarında *Escherichia coli* ve *Salmonella* Typhimurium üzerine antimikrobiyel aktivitesini değerlendirmektir. *Thymbra spicata* L.'den elde edilen esansiyel yağın seçilen gıda patojenlerine karşı antimikrobiyel aktivitesi, *in vitro* ve sucuk matrisinde ayrı ayrı değerlendirilmiştir. Esansiyel yağın kimyasal kompozisyonu incelendiğinde, ağırlıklı olarak karvakrol (% 43,6),  $\gamma$ -terpinen (% 16,69) ve p-simen (% 13,97) olmak üzere toplam 47 bileşen (% 99,41) tespit edilmiştir. Bununla birlikte *in vitro* olarak patojenler üzerinde antimikrobiyel etkiye sahip olduğu gözlenen *Thymbra spicata* L., esansiyel yağının sucuk ortamında etkili olması için yüksek miktarlarda kullanılması gerektiği, bu durumda da ürünün organoleptik özelliklerinin olumsuz yönde etkilendiği ortaya konulmuştur. Doğal katkı maddelerinin, gıda kaynaklı patojenleri önlemek ve gıdaların raf ömrünü uzatmak için gıda teknolojisinde kullanılmakta olan kimyasallara alternatif olarak kullanılabilmesi kanısına varılmıştır. Gıda matrislerde gıda patojenleriyle başa çıkmak için; farklı esansiyel yağlar ve muhafaza yöntemlerinin kombinasyonlarına ilişkin sinerjik etkilerin değerlendirildiği yeni çalışmalara ihtiyaç vardır.

**Anahtar sözcükler:** Antimikrobiyel aktivite, esansiyel yağ, fermente sucuk, tüketici sağlığı

## Introduction

Having many hurdles to prevent the growth of pathogens, dry fermented meat products are considered to be safe products. Outbreak reports from Europe and United states of America as well as the microbiological quality determination studies reveal the concerns on the safety of fermented and cured meat products (8, 15, 24).

Turkish dry fermented sausage (sucuk) is a traditional, commonly consumed meat product in Turkey (13). Beef, tail fat, salt, nitrite, sugar and various spices

are the common ingredients of sucuk. Being mostly fermented without starter culture, it may potentially harbor foodborne pathogens and pose a risk for public health.

Herb and spice extracts are used in meat industry not only for the flavor and other sensitive attributes but also to control pathogens and to extend the shelf life (5, 11). Plant essential oils (EOs) have been widely used due to their biological activity, low toxicity, lower environmental impact and high acceptance by consumers (16). *Thymbra*

*spicata* L., having carvacrol and thymol as antimicrobial components, is commonly used in foodstuffs in some Mediterranean countries (23).

Within the frame of this study, it was aimed to investigate: (i) the chemical composition of *Thymbra spicata* L. subsp. *spicata* EO (ii) the antimicrobial activity of EO both *in vitro* and in sucuk matrix and (iii) to evaluate the consumer acceptability of EO added sucuk.

## Material and Methods

### Extraction of *Thymbra spicata* L. essential oil:

*Thymbra spicata* L. plants were collected from East Mediterranean region of Turkey during May-July 2015. The plants were dried and floral forms were identified as *Thymbra spicata* L. subsp. *spicata* by Herbarium specialist of Erciyes University, Department of Biology. The extraction of air dried *Thymbra spicata* L. leaves was performed according to European Pharmacopoeia 5.0 using clevenger apparatus for 3 hours (27). The EO gained was dried over anhydrous sodium sulphate and was stored in dark glass bottle at 4 °C until analyses.

**GC-MS analyses:** The chemical composition analyses were performed by Gas Chromatography-Mass Spectrometry (GC-MS) with Thermo Trace GC ultra model gas chromatograph and Thermo Trace DSQ mass spectrometer. A HP Innowax (30 m - 0.25 mm - 0.25 µm) column was used for gas carrying in the process. High-purity grade of helium (purity of 99.995%) was used as a carrier gas. The GC oven temperature was kept at 60 °C for 1 min and programmed to 150 °C at rate of 3 °C/min, 250 °C at rate of 25 °C/min and kept constant at 250 °C for 1 min. In the MS process mass range was 40-450 m/z and ion source temperature was 200 °C. The composition of EO was listed by retention index and peak area.

**Bacterial strains:** *E. coli* (ATCC 25922), *E. coli* O157:H7 (NCTC 12900), *Salmonella* Typhimurium (ATCC 13311), *L. monocytogenes* (N 7144) and *S. aureus* (ATCC 25923) cultures were obtained from Erciyes University, Veterinary Faculty, Food Hygiene and Technology Department culture collection. In experimental fermented sucuk production, Chr. Hansen commercial preparation (Bactoform F-RM-52) containing *Staphylococcus carnosus* and *Lactobacillus sakei* lyophilized starter cultures were used.

**In vitro antimicrobial activity:** *In vitro* antimicrobial activities of EO was screened by agar disc diffusion and broth microdilution methods for selected pathogens. MIC values of EO were determined as described by Dussault et al. (12).

**Production of sucuk:** Experimental sucuk dough was traditionally prepared proportionally including cattle meat (88 kg), tail fat (12 kg), salt (2.2 kg), sugar (0.5 kg), garlic (1.5 kg), spice mix (4 kg) (red pepper, red hot pepper, black pepper and cumin) and NaNO<sub>2</sub> (0.015 kg). Briefly, refrigerated meat and tail fat was minced with

meat grinder (Fakir, Torque 1800, Germany). Starter cultures, spices and other ingredients were added into dough and mixed homogenously. Sucuk dough was divided into six experimental groups with added different amounts of EO and pathogens (8 log cfu/g) as stated below;

Group I: 300 ppm EO + *E. coli*

Group II: 500 ppm EO + *E. coli*

Group III: control for *E. coli* (without EO)

Group IV: 300 ppm EO + *Salmonella*

Group V: 500 ppm EO + *Salmonella*

Group VI: Control for *Salmonella* (without EO).

All sucuk groups were filled in collagen sucuk casings after being rested for 24 h at 4 °C and were ripened under the following conditions: 1 day at 95% relative humidity (RH) at 22±2 °C, 2 days at 90% RH at 22±2 °C, 1 day 83% RH at 22±2 °C and finally 2 days 80% RH at 18±2 °C. After the ripening process all groups were stored at 4 °C until the analyses as unpacked.

**Microbiological analyses:** Experimental sucuk groups were microbiologically analyzed on 0, 1, 3, 6, 10, 15 and 30<sup>th</sup> days. The production day of the sucuks was accepted as the zero-day. *E. coli*, *Salmonella* spp., Lactic acid bacteria (LAB) and *Micrococcus/Staphylococcus* counts were determined on MacConkey Agar (Merck 1.05465), XLD agar (Merck 1.05287), Man Rogosa Sharp Agar (Merck 1.10660) and Baird Parker Agar (Merck 1.05406) supplemented with Egg Yolk Tellurite (Merck 1.03785), respectively. For *E. coli*, *Salmonella* spp. and *Micrococcus/Staphylococcus*, the plates were incubated at 37 °C for 24-48 hours. For Lactic acid bacteria (LAB), plates were incubated at 30°C for 48-72 hours in anaerobic jars with AnaeroGen sachet (Oxoid, UK).

**Chemical analyses:** pH and dry matter measurements were carried out on days 0, 1, 3, 6, 10, 15 and 30<sup>th</sup> of processing (3). After ripening, Thiobarbituric acid reactive substances (TBARS) values were determined by spectrophotometric method (31).

**Sensory analyses:** Colour, odor, texture (chewiness), flavour properties and overall appreciation of sucuk samples with added 300 ppm and 500 ppm EO were revealed by 47 panelists, who gave a score for each sample according to their perceptions of each attributes, using a 5 point hedonic scale as the worst to the best.

**Statistical analyses:** *E. coli*, *Salmonella*, LAB and *Micrococcus/Staphylococcus* counts (log cfu/g) and pH and dry matter measures were tested by repeated measures analysis of variance for the statistical significance. Kruskal Wallis test was used to control the significance between groups in respect to sensory properties. Binary comparisons in evaluation of sensory analysis were tested with Mann-Whitney U test after Bonferroni correction. All of the statistical analyses were done using the SPSS package program version 14.01 (SPSS Inc., USA). Microbiological and chemical analyses in the study were carried out in triplicate with two parallels.

**Table 1.** Chemical composition of *T. spicata* L. subsp. *spicata* EO

Compounds	RT*	Yield (%)
Thujene	2.83	1.47
Camphene	3.32	0.08
$\alpha$ -Pinen	3.92	0.20
3-Carene	4.64	0.11
$\alpha$ -Myrcene	4.96	4.16
$\alpha$ -Terpinene	5.27	2.98
Limonene	5.69	0.38
Sabinene	5.90	0.34
2-Hexenal	6.27	0.33
$\gamma$ -Terpinene	6.91	16.69
p-Cymene	7.61	13.97
3-Hexen-1-ol	11.25	0.07
3-Octanol	11.57	0.30
p-Cymenene	13.02	0.58
1-Octen-3-ol	13.64	0.53
trans-Sabinene hydrate	14.00	0.10
Benzaldehyde	16.16	0.08
cis-Sabinene hydrate	17.05	0.05
Linalool	17.27	0.08
Caryophyllene	18.45	6.31
Aromadendrene	18.73	0.39
1-4-Terpineol	19.00	0.69
Junipene	19.73	0.08
$\alpha$ -Humulene	21.02	0.28
Ledene	22.02	0.31
Borneol	22.56	0.24
$\gamma$ -Elemene	23.36	0.06
L-Carvone	23.57	0.35
Benzoik asit 2-(acetyloxy)-	23.98	0.06
$\delta$ -Cadinene	24.33	0.10
Benzoik asit 2-hidroksi-	25.16	0.25
Benzene	26.41	0.13
Benzenemethanol	28.05	0.03
p-Isopropylphenetole	30.06	0.06
Caryophyllene oxide	31.83	0.23
Spathulenol	36.69	0.18
Eugenol	38.30	0.04
2-Methyl-3-butyn-2-ol	39.09	1.04
4-ethyl-2,6-xilenol	39.61	0.10
Carvacrol	39.96	43.60
Trimethyl(phenyl)silane	41.29	0.04
p-(2methylallyl)-	45.92	1.65
2-pentadecyl ester	49.05	0.16
Hexadecanoic acid	58.24	0.09
1,4-dimethoxy-2,6dimethylbenzene	62.07	0.22
Octadecanoic acid	63.09	0.16
12-Methoxy-3-methylcholanthrene	65.82	0.06

\*RT: Retention time

**Table 2.** Antibacterial activity of *T. spicata* L. subsp. *spicata* EO

Microorganisms	Disc Diffusion <sup>a</sup>		MIC Value <sup>b</sup>
	EO	Gentamicin (N, 10 $\mu$ g)	
<i>E. coli</i> ATCC 25922	21	19	3.16
<i>S. Typhimurium</i> ATCC 13311	18	20	5.53
<i>E. coli</i> O157:H7 NCTC 12900	25	22	5.44
<i>S. aureus</i> ATCC 25923	19	20	4.54
<i>L. monocytogenes</i> N 7144	16	33	3.56

<sup>a</sup>: Diameter of the inhibition zones in mm (include 5 mm disc)<sup>b</sup>: Minimal inhibitory concentrations (Values in mg mL<sup>-1</sup>)

## Results

**Chemical composition of essential oil:** The mean yield obtained in EO by hydrodistillation was 2.41% (v/w). Forty seven different constituents were identified in *T. spicata* EO as a result of GC-MS analysis. The identified compounds were found to constitute 99.41% of total EO. The major compound was carvacrol (43.6%) followed by  $\gamma$ -terpinene (16.69%) and p-cymene (13.97%). Chemical composition of the EO is presented Table 1.

**In vitro antimicrobial activity:** Results of *in vitro* antimicrobial activities of EO detected by agar disc diffusion and broth microdilution methods were shown in Table 2.

**Microbiological analysis:** The changes in *E. coli*, *Salmonella*, LAB and *Micrococcus/Staphylococcus* counts during ripening and storage of experimental sucuk groups were given in Figure 1 and *E. coli* and *Salmonella* counts in experimental sucuk groups were shown in Table 4.

**Chemical analysis:** pH and dry matter changes during ripening and storage period of experimental groups were given in Figure 1. Mean TBARS values (mg/kg) of experimental groups varied between 0.89 $\pm$ 0.004 and 1.05 $\pm$ 0.055 mg/kg. In the study, no statistically significant difference was found between the TBARS values of sucuk groups. There was also no difference in TBARS values between the sucuk groups containing *E. coli* and *S. Typhimurium* (P>0.05).

**Sensory analysis:** The sensory evaluations of 300 and 500 ppm EO added sucuks are shown in Table 3. Concerning the sensory analysis; the differences between the experimental groups were statistically significant (P<0.05). Color, odor, texture (chewiness), flavor properties and overall appreciation of sucuk samples were evaluated by 47 panelists. In respect to overall rating of the sucuk, each group was found to be different and it was seen that the group with no EO was accepted as the best and the group with 500 ppm of EO was the worst.

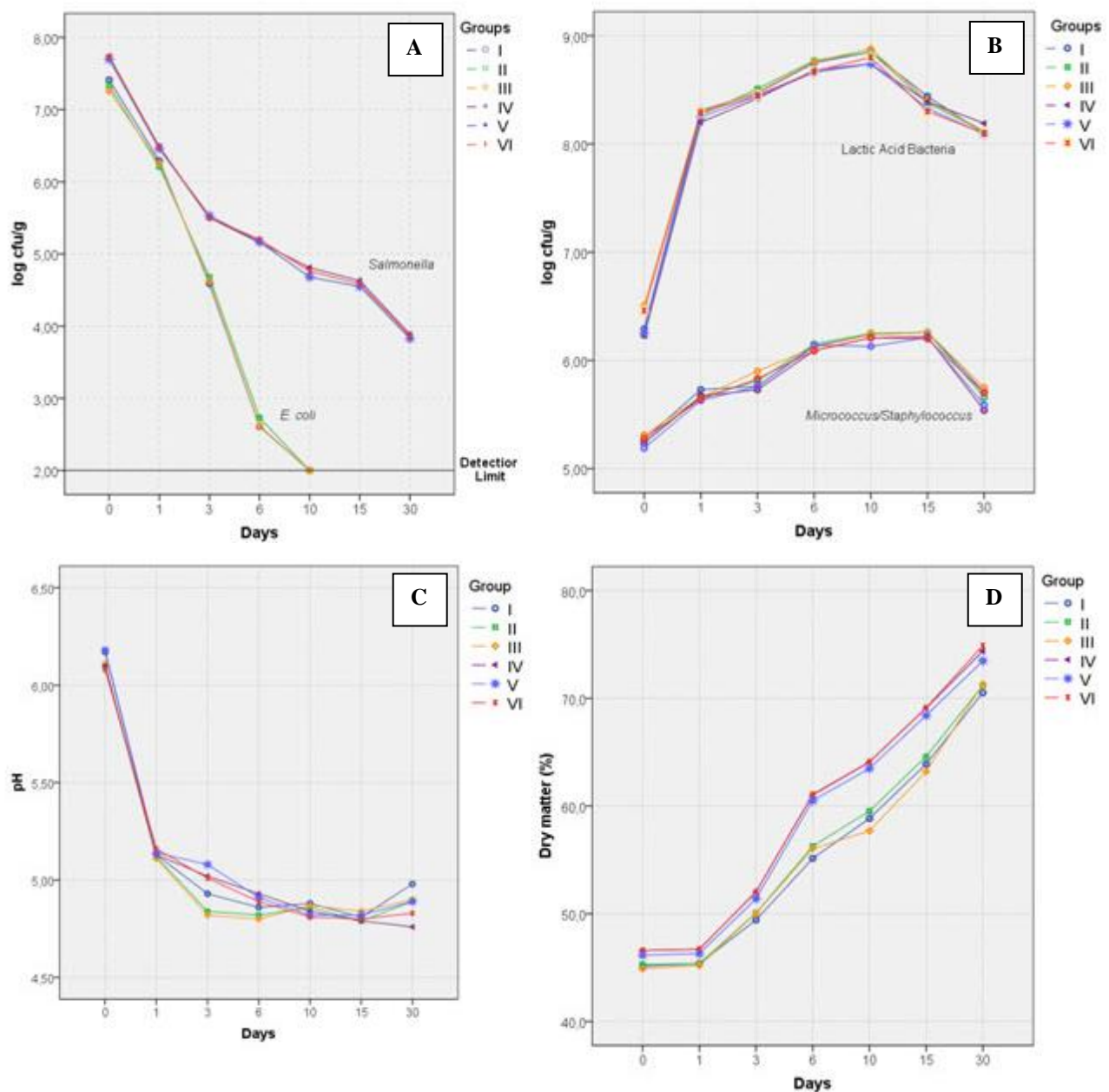
**Table 3.** Organoleptic analysis results of experimental sucuk groups after fermentation

	Control	300 ppm added	500 ppm added	P*
	Median (%25; %75)	Median (%25; %75)	Median (%25; %75)	
Color	4 (4; 4) <sup>a</sup>	4 (3; 4) <sup>a</sup>	3 (3; 4) <sup>b</sup>	0.004
Odor	4 (3; 4) <sup>a</sup>	3 (3; 4) <sup>b</sup>	2 (2; 3) <sup>c</sup>	<0.001
Texture	4 (3; 5) <sup>a</sup>	4 (3; 4) <sup>a</sup>	3 (3; 4) <sup>b</sup>	0.006
Flavor	4 (3; 5) <sup>a</sup>	3 (3; 4) <sup>b</sup>	2 (2; 3) <sup>c</sup>	<0.001
Overall Rating	4 (3; 5) <sup>a</sup>	3 (3; 4) <sup>b</sup>	3 (2; 3) <sup>c</sup>	<0.001

<sup>a,b,c</sup>: Means within same column with different letters are significantly different.

(P\* < 0,017 \*: Bonferroni correction was applied.)

Hedonic scale from 1 (the worst) to 5 (the best)

**Figure 1.** Microbiological and chemical changes in experimental groups.

**A:** *E. coli* and *Salmonella* counts; **B:** LAB and *Micrococcus/Staphylococcus* counts; **C:** pH values; **D:** Dry matter (%)

Group I (Control, *E. coli*); Group II (300 ppm EO+*E. coli*); Group III (500 ppm EO+*E. coli*); Group IV (Control, *Salmonella*); Group V (300 ppm EO+*Salmonella*); Group VI (500 ppm EO+*Salmonella*).

**Table 4.** *E. coli* and *Salmonella* counts in experimental groups (log cfu/g)

Groups	Analyses							Statistical significance in repeated measures	
	Fermentation period				Storage (4 °C)				
	Day 0	Day 1	Day 3	Day 6	Day 10	Day 15	Day 30		
	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$	$\bar{X} \pm S_{\bar{x}}$		
Group I	7.41±0.103	6.29±0.039	4.59±0.009	2.61±0.064	ND	ND	ND	Groups: P=0.065; F=4.39	
Group II	<i>E. coli</i> Counts	7.33±0.048	6.21±0.026	4.68±0.032	2.63±0.012	ND	ND	Time: P<0.001; F=3226.7	
Group III		7.26±0.071	6.26±0.070	4.62±0.018	2.62±0.038	ND	ND	Group-Time: P=0.283; F=1.37	
Group IV		7.74±0.042	6.47±0.032	5.50±0.076	5.16±0.015	4.81±0.042	4.63±0.031	3.89±0.013	Group: P=0.131; F=2.91
Group V	<i>Salmonella</i> Counts	7.70±0.051	6.46±0.030	5.53±0.030	5.17±0.007	4.70±0.048	4.55±0.049	3.83±0.033	Time: P<0.001; F=3047.8
Group V		7.73±0.022	6.49±0.012	5.50±0.076	5.20±0.039	4.76±0.024	4.59±0.021	3.86±0.023	Group-Time: P=0.825; F=0.415

$\bar{X} \pm S_{\bar{x}}$ : Mean  $\pm$  Standard error

ND: Not detected

Means from three replicates with double parallel analyses.

## Discussion and Conclusion

Due to their natural structures, EOs of aromatic and medicinal plants has attracted increased attention by conscious consumers who question the reliability of synthetic food additives. Certain synthetic chemicals have been reported to transform some ingested food materials into toxic substances or carcinogens (14). However, some plants known to have antimicrobial activities might be used as natural food additives having causing no health problems to consumers (10, 14).

In this study, the mean yield of EO was 2.41% (v/w) similar to those previously obtained as 3.1% (19). In another study, higher extraction efficiency was obtained as 4.3% of mean yield from the *Thymbra spicata* L. (1).

The chemical profile identified from *Thymbra spicata* L. EO in this study was similar to that obtained in some studies with differences between the component ratios, probably due to the extraction efficiency, harvest season, geographical region, variation in distillation technique and storage conditions (19, 20, 21, 32).

In respect to in vitro antimicrobial activity of EOs, the disc diffusion results revealed 25, 21, 19, 18 and 16 mm zone diameters on inoculated agar for *E. coli* O157:H7, *E. coli*, *S. aureus*, *S. Typhimurium* and *L. monocytogenes*, respectively. Most detrimental effect was observed on *E. coli* O157:H7 and the weaker effect on *L. monocytogenes*.

Sagdic and Ozcan (28) reported the zone diameters of *Thymbra spicata* L. EO on *E. coli* and *S. aureus* as 13 and 10 mm while no zone formation was reported for *S.*

*Typhimurium* and *E. coli* O157:H7. On the contrary, it is founded that 26, 24, 24 and 26 mm inhibition zones, for *E. coli*, *S. Typhimurium*, *S. aureus* and *L. monocytogenes* respectively (32). Differences in studies may be due to seasonal and geographical differences affecting the chemical composition.

MIC value determination of antimicrobials has critical importance to compare the data from different studies in a healthy way. In the current study, MIC values for *S. Typhimurium*, *E. coli* O157:H7, *S. aureus*, *L. monocytogenes* and *E. coli* were detected as 5.53, 5.44, 4.54, 3.56 and 3.16 mg ml<sup>-1</sup>, respectively. Lower MIC values of *Thymbra spicata* EO were reported by Markovic et al. (21) and Unlu et al. (32) on *E. coli*, *S. Typhimurium*, *S. aureus* and *L. monocytogenes*.

Most of studied EOs were observed to have greater effects against Gram-positive bacteria and their action mechanism are reported to be associated to their hydrophobicity that causes breakup of cell membrane permeability and consequent leakage of cell component (17). Therefore Gram-negative bacteria are thought to be more resistant to the EO actions as they have an outer membrane acting as a protective barrier. Antimicrobial effect of *Thymbra spicata* L. EO is substantially associated to the presence of carvacrol, its main chemically active compound. In addition, the presence of  $\gamma$ -terpinene and p-cymene as well as caryophyllene and  $\alpha$ -myrcene pointing to a large variability of chemical composition may contribute for larger antibacterial spectrum which may explain the EO to be effective both

on Gram positive and negative bacteria. Action mechanisms of EOs may also be associated to interference on protein synthesis, energetic pathways and disruption of DNA or ATP synthesis arising from various components of *Thymbra spicata* L. (17). Cytoplasmic membrane is thought to be interacted by carvacrol, causing passive transport of ions across the membrane (9). *p*-cymene is reported to not to be an antimicrobiologically effective compound alone however synergism could take place when combined with carvacrol as well as the other components with possible interactions. Being hydrophobic in nature, *p*-cymene is reported to cause swelling of the cytoplasmic membrane and to effect on the synthesis of protein in *E. coli* (2, 4). Carvacrol is considered as one of the fast acting EO compounds as it inactivates *E. coli* and *Salmonella* in about five minutes. When used with antibiotic, carvacrol shown to display synergism with penicillin against *E. coli* and *S. Typhimurium* as well as with ampicillin and nitrofurantoin against *Klebsiella* (25, 33).

Regarding the possible effects of EO use on microbiological quality of sucuk, no difference was observed in the *Salmonella* and *E. coli* reduction in EO added sucuks compared to the controls. The effectiveness of EOs is known to be reduced by fats, carbohydrates, proteins, salts and pH in food system (6). Higher concentrations are required for the EOs to exhibit antimicrobial activity in the food matrix, than *in vitro* (29, 30). It has been claimed that approximately two-fold, 10 fold and 25-100 fold EOs in semi-skimmed milk (18), pork liver sausage (26) and soft cheese (22) should be applied respectively to achieve the antimicrobial activity.

In this study, no negative EO effect was observed on LAB and *Micrococcus/Staphylococcus* in groups. The desired fermentation was successfully achieved in EO added groups at the end of the ripening period.

The use of EO caused no significant change ( $P>0.05$ ) on the chemical parameters like pH, dry matter and TBARS in the experimental groups. pH change could be attributed to the starter culture activity which was observed to not to be affected by experimental levels of EO (300, 500 ppm).

The sensory analyses revealed the fact that consumer acceptance is inversely correlated to the concentration of EO used in the sucuk as also indicated in other studies (7, 15). Despite the sensory concerns related to the EO concentrations required for the inhibition of pathogens in the applied food, the expected synergies with other hurdles in the food process could contribute to safety of the product.

As a conclusion, the use of *Thymbra spicata* L. EO use, as an alternative to synthetic food additives, seems to be promising for sucuk manufacture when well balance in product safety and consumer acceptability is considered.

Further studies are needed for *Thymbra spicata* L. EO use in meat product manufacture focusing on the equilibrium both on antimicrobial and acceptability aspects. In addition, combined and synergetic effects of different EOs and other preservation methods could also be evaluated to cope with antibiotic-resistant microorganisms in the food matrices.

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### Ethical Statement

This study does not present any ethical concerns.

### Conflict of Interest

The authors declared that there is no conflict of interest.

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