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ABDULLAH DİKİCİ

OSMAN İRFAN İLHAK

MEHMET ÇALICIOĞLU

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Effects of essential oil compounds on survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in çiğ köfte

Abdullah DİKİCİ[1], Osman İrfan İLHAK[2], Mehmet ÇALICIOĞLU[2,*]

[1]Department of Food Engineering, Faculty of Engineering, Tunceli University, 62100 Tunceli, Turkey

[2]Department of Food Hygiene and Technology, Faculty of Veterinary Medicine, Fırat University, 23119 Elazığ, Turkey

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Abstract: This study was undertaken to evaluate the effects of some essential oil compounds for the inactivation of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in çiğ köfte. In experiment 1, a çiğ köfte batch was inoculated with high levels of *E. coli* O157:H7 or *L. monocytogenes* (7.00 $\log_{10}$ cfu/g) and the batches were divided into 6 groups. Each group was treated with 18 mL/kg of normal saline, cineole, limonene, carvone, linalool, or eugenol. Treatment with carvone, linalool, and eugenol resulted in a significant ($P < 0.05$) decrease in the numbers of both pathogens. In experiment 2, the çiğ köfte was inoculated with the same pathogens and each group was treated with 0 (control), 20, 22.5, and 25 mL/kg of eugenol. Increases in concentration did not cause a difference in pathogen reduction. The results showed that eugenol, carvone, and linalool can be used for the inactivation of *E. coli* O157:H7 and *L. monocytogenes* in çiğ köfte or similar foods.

Key words: Essential oil compounds, ground beef, eugenol, antimicrobial, nonthermal inactivation

1. Introduction

In Turkey, çiğ köfte is a popular traditional appetizer containing raw ground beef. Consumption in cold sandwiches is common. Çiğ köfte is simply prepared by mixing bulgur, which is a heat-processed wheat product, with lean ground beef (bulgur-to-ground beef ratio of approximately 1:0.5), salt, onion, garlic, tomato and red pepper pastes, lemon juice, and some other spices including basil, allspice, and black pepper, which may differ from recipe to recipe. The ingredients are added to the bulgur in order while thoroughly mixing by hand. No heating steps are involved in the preparation. The resulting köfte dough is formed into small balls and served. Çiğ köfte is usually consumed within a few hours of preparation.

Various studies have been conducted on the microbiological quality of çiğ köfte in the marketplace in different cities in Turkey (1–4). These studies concluded that çiğ köfte harbors pathogenic bacteria and fecal indicator bacteria, including *Escherichia coli* and coliforms. In 2001, Tuncel and Tiryaki (5) isolated *Salmonella* from 14% of çiğ köfte samples. In other studies held for the investigation of the fate of *Salmonella* (2) or *Listeria monocytogenes* (6,7) during preparation and 24 h of refrigerated storage, it was reported that these pathogens survive well in the product with no significant change in numbers. Although any ingredient in çiğ köfte may contribute to the microbial health risks that may be associated with the product, the ground beef is the major contributor because it is well known that a variety of foodborne pathogenic bacteria as well as zoonotic parasites can be found in ground beef (8,9). Therefore, practical and nonthermal methods are needed for assuring the microbial safety of çiğ köfte (10). Essential oil compounds (EOCs) can be considered as an alternative to be used for this purpose because they are natural substances with specific aroma, flavor, and various bioactivities (11). In addition, they have been known to have a variety of bioactivities, including antibacterial, antifungal, and antioxidant effects (11,12). The antibacterial effects of essential oils or their compounds have been studied extensively and have become the subject of some recent review papers (11–13). The objective of the present study was to investigate the effects of selected EOCs (cineole, limonene, carvone, linalool, and eugenol) on the survival of *L. monocytogenes* and *E. coli* O157:H7 in çiğ köfte.

2. Materials and methods

2.1. Preparation of the inoculum

Five different strains each for *L. monocytogenes* and *E. coli* O157:H7 were used. The *L. monocytogenes* strains were

* Correspondence: mcalicioglu@firat.edu.tr

N7143, N7144, RSKK 472, RSKK 474, and RSKK 475, and the *E. coli* O157:H7 strains were ATCC 43890, ATCC 43894, ATCC 43895, ATCC 35250, and RSKK 232. The strains were obtained from the Refik Saydam Hıfzıssıhha Institute (RSKK), a national research institute in Turkey, and from the American Type Culture Collection (ATCC). Strains N7143 and N7144 were provided by Dr John Samelis from the Dairy Research Institute of Ioannina, Greece. The strains were grown separately in nutrient broth (Merck, Darmstadt, Germany) at 35 °C for 24 h. At the end of the incubation period, the cultures were centrifuged at 4200 rpm for 15 min at 4 °C (NF800R, Nüve, Turkey). The supernatants were removed, and the pellets were resuspended in sterile normal saline and then centrifuged to remove the residual organic material. This washing step was repeated 2 times. Finally, the resulting pellets were resuspended in approximately 3.5 mL of sterile 0.1% peptone water (PW). All of the strains of each pathogen were combined in a single beaker. The final level of the pathogens was approximately 10^9 cfu/mL in the inoculum.

2.2. Inoculation of the çiğ köfte and experimental treatments (experiment 1)

For each trial and for each pathogen, 1050 g of çiğ köfte, freshly prepared by a local restaurant, was used. The experiment for each pathogen was repeated 3 times. A 150-g sample was taken for determining the salt level and pH. The pathogen mixture was added to the remaining 900 g of çiğ köfte and this combination was mixed manually. The inoculated çiğ köfte batch was divided into 6 groups: 1) control (sterile 0.9% NaCl), 2) carvone (purity: 98%; Sigma, USA), 3) cineole (purity: 99%; Merck), 4) eugenol (purity: 99%; Merck), 5) linalool (purity: 97%; Merck), and 6) limonene (purity: 95%; Merck).

The groups were treated with 18 mL/kg of filter sterilized (0.22-µm pore size, Syrfil MF, Costar Corp., USA) EOCs. The concentration of 1.8% (18 mL/kg) was selected based on the results of the study of Çalıcıoğlu and Dikici (14), which reported a significant reduction, greater than 6 $\log_{10}$ cfu/g, in the numbers of *Salmonella* in inoculated çiğ köfte. The treated çiğ köfte was mixed by hand in a separate sterile stainless steel container and was held at room temperature for 30 min, which represents an approximate time of serving. Next, the containers were covered with stretch film and held at 4 °C for 3 h to mimic the practice in çiğ köfte restaurants.

2.3. Analysis of the samples

Duplicated samples of 25 g were taken from each treatment group at 30 and 180 min after mixing with the treatment compounds. The samples were transferred into sterile Stomacher bags and 225 mL of sterile 0.1% PW was added and homogenized with pummeling (Bagmixer 400W, Interscience, France) for 2 min. The resulting homogenate was decimally diluted and surface-plated onto sorbitol MacConkey agar (SMAC) (LabM, IDG, UK) for the enumeration of *E. coli* O157:H7 (15) and onto PALCAM agar plates (Oxoid, UK) for the enumeration of *L. monocytogenes* (16).

The SMAC plates were incubated at 35 °C for 24 h and the PALCAM plates were incubated at 30 °C for 24–48 h, and the typical colonies were then counted.

Three randomly selected colonies of each pathogen from the agar plates were analyzed for confirmation of the pathogens. The colonies were confirmed for *E. coli* O157:H7 by the Microscreen *E. coli* O157 latex agglutination test (Microgen Bioproducts Ltd., UK). Confirmation of *L. monocytogenes* was carried out by polymerase chain reaction using *L. monocytogenes*-specific primers, which are LM1 (5'- CCT AAG ACG CCA ATC GAA - 3') and LM2 (5'- AAG CGC TTG CAA CTG CTC - 3') (17).

2.4. Determining the effect of the concentration of eugenol on survival of the pathogens in çiğ köfte (experiment 2)

The second experiment was undertaken to determine the effect of eugenol concentration for a 5-log reduction in the numbers of the pathogens tested. Eugenol was chosen based on the results of experiment 1, where it was the most effective compound.

The çiğ köfte batch was prepared as indicated in the first experiment and was divided into 4 groups. Each group was treated with different levels of eugenol. These groups were 0 mL/kg (control), 20 mL/kg, 22.5 mL/kg, and 25 mL/kg. Following mixing, the çiğ köfte groups were held at 4 °C. After 60 min, 2 samples were taken and analyzed for the enumeration of *L. monocytogenes* and *E. coli* O157:H7. The experiment for each pathogen was repeated 3 times.

2.5. Other analysis

The salt levels of the çiğ köfte used for the experiments were determined using the Mohr method (18). In addition, the pH and water activity (a_w) of the çiğ köfte samples were also determined at the same sampling intervals as in the first experiment. The pH was measured using a digital pH meter (Selecta pH 2001, Spain). The a_w of the çiğ köfte samples was determined using a water activity meter (Testo 650, USA).

2.6. Statistical analysis

Each experiment was composed of 3 replicates. Microbiological data were converted to $\log_{10}$ cfu/g and evaluated using a 6 × 2 (treatment groups × sampling time) factorial design. Data for each pathogen were analyzed by analysis of variance (ANOVA) for main (fixed) effects (treatment, time) and 2-way interactions between treatments and time using SAS 6.1 (SAS Institute Inc., USA). For the second experiment, the concentration groups were compared using one-way ANOVA. Least squares means were separated using Fisher's least significance difference test, using the general linear models

procedure of SAS. A significance level of 0.05 was used for all of the statistical analyses. The averages and standard deviations of the salt, pH, and a_w were calculated (19).

3. Results

3.1. Antimicrobial effects of EOCs on the survival of *E. coli* O157:H7 or *L. monocytogenes* in çiğ köfte (experiment 1)

The effects of the EOCs on the numbers of *E. coli* O157:H7 and *L. monocytogenes* are shown in Tables 1 and 2, respectively. Statistical analysis of the data revealed that the main effects of the treatments and time and their interactions were significant ($P < 0.05$). The initial levels of the pathogens after inoculation were 7.35 ± 0.38 and 7.31 ± 0.33 $\log_{10}$ cfu/g for *E. coli* O157:H7 and *L. monocytogenes*, respectively (Table 1). Regardless of the sampling time during the 3 h of refrigerated storage, the numbers of both pathogens were reduced significantly ($P < 0.05$) between the treatment groups (Tables 1 and 2). The EOCs tested were ranked in an order from the most effective to the least effective as eugenol, linalool, carvone, cineole, and limonene for *E. coli* O157:H7 (30 and 180 min) (Table 1). The corresponding order for *L. monocytogenes* was eugenol, linalool, cineole, limonene, and carvone (30 min) (Table 2). After 180 min, however, the rank of the EOCs' effects on *L. monocytogenes* changed slightly, to eugenol, linalool, cineole, carvone, and limonene. Significant reductions ($P < 0.05$) compared to the control group in *L. monocytogenes* by eugenol and linalool were seen at 30 min, whereas those by cineole and carvone ($P < 0.05$) were seen after 180 min. There was a significant difference in the numbers of *L. monocytogenes* between the 30- and 180-min intervals in the carvone- and cineole-treated groups. On the other hand, there was no significant difference in the *E. coli* O157:H7 numbers between the time intervals (Table 1).

3.2. Effects of various concentrations of eugenol on viability of *E. coli* O157:H7 or *L. monocytogenes* in çiğ köfte (experiment 2)

The effects of various concentrations of eugenol on the viability of the pathogens are shown in Table 3. The numbers of *E. coli* O157:H7 and *L. monocytogenes* were significantly ($P < 0.05$) lower in all of the treatment concentrations than those in the control group.

However, the differences among the 20 mL/kg, 22.5 mL/kg, and 25 mL/kg groups were not significant. In the 20 mL/kg eugenol group, the reduction in the numbers was 4.37 $\log_{10}$ cfu/g for *E. coli* O157:H7 and 3.43 $\log_{10}$ cfu/g for *L. monocytogenes*. The reduction in the 25 mL/kg group was 6.07 $\log_{10}$ cfu/g for *E. coli* O157:H7 and 3.85 $\log_{10}$ cfu/g for *L. monocytogenes* (Table 3).

Table 1. Effects of selected essential oil compounds on the populations of *E. coli* O157:H7 inoculated in çiğ köfte ($\log_{10}$ cfu/g, N = 3, n = 2).

Time (min)	Treatment groups					
	Control	Limonene	Cineole	Carvone	Linalool	Eugenol
30	7.35 ± 0.38^{Az}	7.12 ± 0.32^{ABz}	6.66 ± 0.58^{ABz}	6.15 ± 0.29^{Bz}	4.95 ± 0.66^{Cz}	3.60 ± 0.40^{Cz}
180	6.98 ± 0.26^{Az}	6.92 ± 0.41^{Az}	6.54 ± 0.49^{ABz}	5.47 ± 0.25^{Bz}	4.30 ± 0.51^{Cz}	2.82 ± 0.55^{Dz}

$^{A, B, C, D}$: Means within a row lacking a common superscript letter are different ($P < 0.05$).
$^{z, y}$: Means within a column lacking a common superscript letter are different ($P < 0.05$).

Table 2. Effects of selected essential oil compounds on the populations of *Listeria monocytogenes* inoculated in çiğ köfte ($\log_{10}$ cfu/g, N = 3, n = 2).

Time (min)	Treatment groups					
	Control	Limonene	Cineole	Carvone	Linalool	Eugenol
30	7.29 ± 0.26^{Az}	6.92 ± 0.28^{ABz}	6.53 ± 0.45^{ABz}	7.06 ± 0.23^{Az}	6.17 ± 0.36^{Bz}	4.26 ± 0.15^{Cz}
180	7.40 ± 0.23^{Az}	6.97 ± 0.32^{ABz}	6.43 ± 0.44^{Bz}	6.53 ± 0.46^{Bz}	5.99 ± 0.45^{Bz}	3.84 ± 0.31^{Cz}

$^{A, B, C}$: Means within a row lacking a common superscript letter are different ($P < 0.05$).
$^{z, y}$: Means within a column lacking a common superscript letter are different ($P < 0.05$).

Table 3. Effect of eugenol at different concentrations on the inactivation of *E. coli* O157:H7 and *L. monocytogenes* in çiğ köfte (log_{10} cfu/g, N = 3, n = 2).

Pathogens	Eugenol concentrations (mL/kg) in çiğ köfte			
	0	20	22.5	25
E. coli O157:H7	7.31 ± 0.33^{A}	2.94 ± 0.45^{B}	1.77 ± 0.29^{B}	1.24 ± 0.69^{B}
L. monocytogenes	7.73 ± 0.04^{A}	4.30 ± 0.12^{B}	3.95 ± 0.10^{B}	3.88 ± 0.21^{B}

$^{A, B, C}$: Means within a row lacking a common superscript letter are different ($P < 0.05$).

3.3. Salt, pH, and a_w in çiğ köfte

The average level of NaCl in the çiğ köfte was 2.86 ± 0.56% in the present study. The pH and a_w values of the treatment groups are provided in Table 4. No appreciable difference was observed between the groups, indicating that the addition of the EOCs did not change the pH and a_w in the çiğ köfte.

4. Discussion

Çiğ köfte can be classified as one of the most risky ready-to-eat products, since it is made from raw ground beef and lacks heat treatment. Çiğ köfte leftovers are stored in refrigerators and may be consumed the next day. Previous studies revealed that *L. monocytogenes* multiplies in çiğ köfte at 25 °C (6), *Bacillus cereus* multiplies and produces toxins (20), and *Staphylococcus aureus* can produce toxins at ambient temperatures (1). In addition, it was reported that *Salmonella* survived in çiğ köfte for at least 24 h with no reduction in numbers (2,14). The latter finding indicated that çiğ köfte provides a bacteriostatic environment, allowing *Salmonella* to survive. The data in our control group are in agreement with the bacteriostatic environment in çiğ köfte, because no significant changes in the counts of the pathogens were found in experiment 1. However, it has been reported that significant reductions were observed in the numbers of *Salmonella* Enteritidis and *E. coli* depending on the lemon juice concentration (12).

In our previous study, we reported that a 1.8% (18 mL/kg) concentration of carvone, cineole, linalool, or eugenol resulted in significant reductions in the numbers of *Salmonella* in çiğ köfte (14). Limonene was not effective against *Salmonella*, while eugenol was the most effective. In the current study, eugenol, linalool, and carvone were significantly effective in reducing the numbers of both pathogens, while cineole was effective on only *L. monocytogenes*. These results indicate that the antimicrobial effects of the EOCs tested vary depending on the species of bacteria. Limonene was not effective against *E. c*oli O157:H7 or *L. monocytogenes* in çiğ köfte. These compounds were reported to have antimicrobial effects against a variety of bacterial species (13,21). However, these studies used laboratory media rather than a food environment for testing the antimicrobial effect. It should be noted that the number of studies investigating the antimicrobial effects of essential oils in a food environment is more limited than those used laboratory media (22–24).

In our previous study, the addition of eugenol at a level of 1.8% (18 mL/kg) resulted in a >6.5 log_{10} cfu/g reduction in *Salmonella* numbers within 15 min in çiğ köfte, indicating an immediate killing effect (14). However, in the current study, reductions by 18 mL/kg eugenol became limited with 3.75 log_{10} cfu/g for *E. coli* O157:H7 and 3.03 log_{10} cfu/g for *L. monocytogenes*. Increasing the concentration of eugenol (20, 22.5, and 25 mL/kg) in çiğ köfte resulted in a proportional decrease in the numbers of both *E. coli* O157:H7 and *L. monocytogenes*, although the differences between the eugenol groups were not significant. However, reductions in *L. monocytogenes* were more limited compared to those in *E. coli* O157:H7 (Table

Table 4. The average pH and water activity (a_w) values of çiğ köfte treated with selected essential oil compounds after inoculation with *E. coli* O157:H7 and *L. monocytogenes* (N = 3, n = 2).

	Treatment groups					
	Control	Limonene	Cineole	Carvone	Linalool	Eugenol
pH	4.88 ± 0.16	4.94 ± 0.12	4.89 ± 0.13	4.92 ± 0.14	4.94 ± 0.11	4.93 ± 0.13
a_w	0.875 ± 0.021	0.881 ± 0.016	0.906 ± 0.021	0.873 ± 0.019	0.883 ± 0.013	0.877 ± 0.020

3). It has been reported that gram-negative bacteria are generally more resistant against the antimicrobial effects of EOCs due to the protective effect of lipopolysaccharides on the outer membrane (11,25). Our results are not in agreement with this finding because *L. monocytogenes*, a gram-positive pathogen, was more resistant to the effects of the EOCs than *Salmonella* (14) and *E. coli* O157:H7. Likewise, *L. monocytogenes* was more resistant to some plant extracts than some gram-negative bacteria (11,24,25). Such a discrepancy can be explained by the difference in food matrices and the effect of other ingredients in the food environment on the survival of the pathogens.

There is limited information about the mechanism of the antimicrobial effect of EOCs. In a recent study, eugenol was shown by scanning electron microscope to have caused tears in the outer membrane of the cell walls of *E. coli* O157:H7, leading to the leakage of intracellular content. (26). In the same study, limonene, carvacrol, thymol, and cinnamaldehyde were shown to cause structural changes in the outer membrane of the pathogen.

In general, low pH, low protein and fat levels, high levels of moisture, and low levels of oxygen by packaging method have been reported to increase the antimicrobial effect of essential oils (11). However, in a more recent study, an increased protein level was found to increase the antimicrobial effect of essential oils, while the increased starch level in the tryptic soy broth resulted in a decrease (27). Low pH was claimed to increase the hydrophobicity of essential oils, allowing them to dissolve the fatty acids in the cell wall of bacteria (28). Although this information in the literature about the factors influencing the antimicrobial effects of EOCs has shed some light on interpreting our results, they are still too general to explain why the effects of various EOCs are different against different species of bacteria in the same food. We suggest that specific molecular effects of individual EOCs and their complex interactions with the surrounding environment determine the level of antimicrobial effects of EOCs in foods.

In summary, linalool, carvone, and eugenol were effective for reducing the numbers of *E. coli* O157:H7 and *L. monocytogenes* in çiğ köfte. *L. monocytogenes* seemed more resistant to the effects of these EOCs than *E. coli* O157:H7. Eugenol at 25 mL/kg was able to reduce more than 6.0 $\log_{10}$ cfu/g of *E. coli* O157:H7 in çiğ köfte, whereas a 5-log reduction could not be achieved in *L. monocytogenes*. For foods that require nonthermal treatment such as çiğ köfte, the use of EOCs can be an alternative method if the overall acceptance of the food is not influenced. Based on our results, it can be stated that unlike most antimicrobials that are commonly used in processed foods, EOCs provide rapid reductions in bacterial numbers, provided that the food environment is suitable to show their antimicrobial effect. It is obvious that çiğ köfte, as a raw meat-containing product, cannot be banned. However, preventative measures must be taken to protect the public from the microbiological hazards that might be associated with çiğ köfte.

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