Slov Vet Res 2016; 53 (1): 5-12
UDC 579.67.08:615.331:637.513.14:636.5

Original Scientific Article

EFFECTS OF OREGANO ESSENTIAL OIL, GRAPEFRUIT SEED EXTRACT AND THEIR COMBINATION ON THE GROWTH AND SURVIVAL OF *Salmonella* Typhimurium AND *Listeria monocytogenes* IN POULTRY FILLETS UNDER MODIFIED ATMOSPHERE PACKAGING

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Summary: The antimicrobial effect of oregano essential oil (OEO), grapefruit seed extract (GSE) and their combination on the growth and survival of foodborne pathogens (*Salmonella* Typhimurium and *Listeria monocytogenes*) were determined in poultry fillets under modified atmosphere packaging (30% CO_2/70% N_2). In a preliminary experiment, OEO and GSE were used at concentrations of 0.05%, 0.1%, 0.5%, 0.8%, 1.0%, 1.5%, 2.0% and of 0.01%, 0.02%, 0.04%, 0.08%, 0.1%, respectively. Paper disc diffusion testing showed that OEO at 0.05%, 0.1% and GSE at 0.01%, 0.02%, 0.04%, 0.08% had weak antibacterial activity. In addition, due to the very strong odour and taste, poultry samples treated with OEO at 1.0%, 1.5%, 2.0% and the combinations were assessed with scores below the limit of acceptance. Thus, the levels of 0.5% and 0.8% of OEO and 0.1% of GSE were further used in poultry fillets. In this study, the pathogens were affected by OEO and GSE. *L. monocytogenes* was the most sensitive pathogen. In conclusion, the results of this study confirmed the possibility of using natural products with MAP in food production to prevent the growth of foodborne bacteria.

Key words: oregano essential oil; grapefruit seed extract; poultry; foodborne pathogens

Introduction

Poultry is a very popular food commodity worldwide, and its consumption has increased over the last decades in many countries due to its relatively low cost of production, low fat content and its high nutritional value (1). Increased demand for fresh poultry and a desire to transport to more distant markets have increased the need to extend the shelf life of poultry products (2).

Received: 1 October 2013
Accepted for publication: 13 May 2015

Nowadays, consumers increasingly demand the use of natural products as alternative preservatives in foods, as the safety of synthetic additives has been questioned. The most abundant groups of natural compounds are represented by essential oils and plant extracts (3). Oregano and Grapefruit Seed Extract (GSE) have frequently been used successfully for food preservation (4, 5). The practical application of several essential oils in foods is limited due to the strong flavour they impart to foods and also to their interaction with some food ingredients. The preservative effect of essential oils and extracts may be achieved by

using lower concentrations of essential oils in combination with other preservation technologies, such as modified atmosphere packaging (MAP) (6).

The aim of this study was to determine the effect of oregano essential oil (OEO), GSE and their combinations on the survival of pathogens (*S.* Typhimurium and *L. monocytogenes*) in poultry fillets under MAP.

Material and methods

Extraction of OEO and Preparation of GSE

Oregano (*Origanum vulgare*) leaves from Aegean part of Turkey, samples were air dried at room temperature (23±1 °C), and their essential oils were obtained by continuous steam distillation, using a Clevenger-type apparatus for 3 h. The essential oil was collected, dried over anhydrous sodium sulphate and stored at 4 °C until analysis (7).

GSE (Nutribiotic, Lakeport, USA) was dissolved in double distilled water with 0.05% (v/v) Tween-80 (Merck 822187, Darmstadt, Germany) as a surfactant. A 10% (v/v) stock solution prepared and filter sterilized through with a 0.22 µm filter.

Gas Chromatography – Mass Spectroscopy (GC-MS) Analysis

GC-MS analysis was conducted in MERLAB, Research Institute of Istanbul University. The analysis was performed using a Trace GC Ultra (Thermo Electron Corporation, Milan, Italy) equipped with MS DSQ II detectors (30 m × 0.25 mm, Zebron) with 0.25 µm film thickness was used. For GC-MS detection, the sample was concentrated at 43 °C/1 min with the rotavapor and then was flushed. OEO in the amount of 0.4 µl was subjected, and electron ionization energy of 70 eV was used. The temperature program, starting from 60 °C for 8 min and then gradually increased to 240 °C at 3 °C/min, held for 10 min and finally raised to 325 °C at 10 °C/min. The injector, interface and ion source temperature were 200 °C, 275 °C, and 200 °C, respectively. Helium was used as a carrier gas, the flow through the column was 1.0 ml/min, and the split ratio was set to 100:1. The components were identified with the comparison of retention time (RT) and mass spectra with standard compounds (carvacrol, ρ-cymene, thymol, linalool, caryophyllene, cineole, α-pinene, y-terpinene, borneol, phellandrene, and 4-terpineol) by using NIST and the Wiley mass spectral library of the GC-MS system and literature data.

Reference Bacteria

S. Typhimurium (ATCC 14028) and *L. monocytogenes* (ATCC 7644) strains were obtained from Microbiologics® (Minnesota, USA). All strains were maintained in glycerol (30%) at -80 °C. They were streaked on Tryptone Soya Agar (Oxoid CM131, Basingstoke, England) plates and incubated at 35 °C overnight (18–24 h). Working cultures of the selected strains were made by inoculation from stock cultures into 10 ml Tryptone Soya Broth (TSB; Oxoid CM129) and incubating for 20 h at 37 °C.

Antibacterial Assay Using the Disc Diffusion Method

OEO, GSE, and their combination were tested for antibacterial activity with the paper disc diffusion method. Sterilized filter paper discs (Whatman No 1, 0.6 cm in diameter) were placed on the surface of Nutrient Agar (Oxoid CM309) that *S.* Typhimurium and *L. monocytogenes* were individually seeded by spreading 0.1 ml from TSB incubated at 37 °C. Fifteen microliters of dilutions of OEO (0.05%, 0.1%, 0.5%, 0.8%, 1.0%, 1.5%, and 2.0%) and GSE (0.01%, 0.02%, 0.04%, 0.08%, and 0.1%) were applied to sterile filter paper discs. The inhibition zone diameter was measured after 24 h (8). All analyses were performed in triplicate.

Poultry Inoculation

S. Typhimurium and *L. monocytogenes* strains individually were prepared in 10 ml TSB and incubating at 30 °C for 24 h. Strains were sub-cultured twice in TSB before use. The strains were centrifuged (8000 x g) at 4 °C for 10 min, washed with sterile phosphate buffered saline (PBS) and serially diluted with PBS to a concentration capable of giving approximately 10^4 CFU/g of poultry samples.

Poultry breast meat (totalling 30 kg) was supplied from a poultry (broiler) processing plant within 12 h after slaughter. Immediately after delivery, the meat was filleted in small pieces (20 g). The poultry fillets were divided into three equal groups

and portion was placed in a polyethylene sachet. Samples in the first group were contaminated with only *S.* Typhimurium and the second group was contaminated with only *L. monocytogenes*. The non-inoculated group was used for sensory analysis. Poultry fillets were placed in stomacher bags and inoculated with single-strain pathogens by dipping. The inoculated samples were manually massaged for 10 min at room temperature (23±1 °C) to ensure proper distribution of the pathogen. Prior to the inoculation, oils and fillets were also examined for any contamination by tested pathogens.

Following homogenization, the inoculated and non-inoculated groups (*S.* Typhimurium, *L. monocytogenes*, and no bacterial cultures) were treated with six different applications. Treatments were (1) the addition of OEO at 0.5%, (2) the addition of OEO at 0.8%, (3) the addition of GSE at 0.1%, (4) additions at the combinations of 0.5% OEO plus 0.1% GSE, (5) additions at the combinations of 0.8% OEO plus 0.1% GSE, and (6) untreated control. OEO at 0.05%, 0.1% and GSE at 0.01%, 0.02%, 0.04%, and 0.08% were not examined for screening because of weak antibacterial activity against pathogens. In addition, OEO at 1.0%, 1.5%, 2.0%, and their combinations were not used because of unacceptable organoleptic properties in the poultry meat.

Immediately after treatment, all subgroups were individually (300-350 g) packaged under MAP conditions (30% CO_2/70% N_2). MAP was carried out using Ponapack (VTK 40 SC) packaging machine (Ponapack, Istanbul, Turkey) in low O_2 permeable (8–12 $cm^3/m^2/24$ h at STP) polystyrene/ethyl vinyl alcohol (EVOH)/polyethylene (PE) trays and were over-wrapped with oxygen permeable (6000–8000 $cm^3/m^2/24$ h at STP) polyvinyl-chloride film (Wrap Film Systems Ltd., Shropshire, England). Packages were prepared by placing samples into 400 mm^3 inner volume trays to obtain 1:1 (v/v) headspace ratios and were stored at 4 °C for 7 days. Sampling was carried out at predetermined time intervals: 0, 1, 3, 5 and 7 days of storage for microbiological and sensory analysis. On each sampling date, six packs from each group were examined.

Gas Analysis of Package Atmospheres

Gas analyses of the internal package atmosphere were done in duplicate at 1, 3, 5 and 7 days of storage. Analyses for CO_2, O_2, and N_2 within the packages were monitored by injecting 0.5 ml of gas removed from the headspace with a syringe (B. Braun, Melsungen, Germany) into a PDI gas chromatograph (PBI-Dansensor A/B, Ronnedevaj 18, Ringsted, Denmark) fitted with a thermal conductivity detector.

Microbiological Analysis

Samples (25 g) were combined with 225 ml buffered peptone water (Oxoid CM509) in sterile stomacher bags (Seward, Worthing, England) and homogenized for 2 min in a stomacher (Interscience, St. Nom la Breteche, France). Following homogenization, 10-fold serial dilutions were made in sterile Maximum Recovery Diluents (Oxoid CM317) and sample dilutions (0.1 ml) were streaked onto Xylose Lysine Deoxycholate Agar (Oxoid CM469) and Chromogenic Listeria Agar (Oxoid CM1080) supplemented with Listeria Selective Supplement (Oxoid SR227) and Listeria Differential Supplement (Oxoid SR228) for enumeration of inoculated *S.* Typhimurium and *L. monocytogenes*, respectively (9, 10). Microbiological analyses were carried out in triplicate.

Sensory Evaluation of Poultry Fillets

Non-inoculated poultry fillets were examined, and sensory analysis was used only for determining the concentration in terms of acceptability by consumers. Sensorial attributes were evaluated by eight well-experienced panelists, ranging in age between 26 and 45 years (2 females and 6 males), trained according to ISO 1993 (11). Prior to the analysis, vocabularies of the sensory attributes (odour-odour intensity (sour, sweet, and spicy) and taste-flavour intensity (spicy taste, salty taste, sweet taste, and acidic taste)) were developed by the panelists, using a standardized procedure (12).

The panel members were seated in individual booths in a temperature and light-controlled room (fluorescent lighting of 2000 lx; Philips 40W Cool White), receiving a set of six samples in a completely randomized order. Before evaluation, poultry samples were wrapped in aluminium foil and cooked individually in an oven (220 °C) for 20 min. Each sample was served warm in dishes coded with three-digit code numbers. Unsalted crackers and water were served to panelists to freshen their mouth between each sub-samples assessment.

Statistical Analysis

Analysis of variance was conducted for each variable to investigate the effect of the antibacterial activities of OEO, GSE, and their combination during storage time. The trial was performed in triplicate, and the General Linear Model procedure (PROC GLM) of SPSS 13.0 was used to analyse the data (13). The microbiological analysis and sensory characteristics were evaluated, and significant differences were defined as $P < 0.05$. Microbial counts were expressed as log CFU/g and mean separations was obtained using Duncan's multiple range tests.

Results

The main volatile components of OEO used in poultry fillets were characterized by prominent (>1%) concentrations of carvacrol (68.97%), ρ-cymene (11.47%), thymol (4.85%), linalool (2.21%), caryophyllene (2.85%), cineole (1.14%), α-pinene (1.04%), y-terpinene (2.37%) and 4-terpineol (1.57%). In the present study, carvacrol was detected as a major constituent. The composition of OEO may be due to the variety of the plant, origin, extraction modality and agronomic practices (14).

The antibacterial activities of OEO, GSE and the combination determined by the paper disc diffusion are shown in Table 1.

Table 1: Inhibition zones of OEO and GSE against the pathogens (mm)

	Different concentrations of oils and extracts														
Test Bacteria	OEO (%)							GSE (%)				OEO (%) + GSE (%)			
	0.05	0.1	0.5	0.8	1.0	1.5	2.0	0.01	0.02	0.04	0.08	0.1	0.5+0.08	0.5+0.1	0.8+0.1
S. Typhimurium	11	17	22	24	26	28	30	ND	3	8	14	15	23	25	28
L. monocytogenes	12	19	24	26	27	28	29	3	6	10	15	19	25	27	28

OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract; ND: Not Detected.

Table 2: Effect of OEO and GSE on *S.* Typhimurium in poultry fillets under MAP (log CFU/g)

Storage Days	Control	OEO (%)		GSE (%)	OEO (%) + GSE (%)	
		0.5%	0.8%	0.1%	0.5% +0.1%	0.8% + 0.1%
0	4.32*±0.04^{aE**}	3.98±0.05^{bA}	3.94±0.06^{bcA}	4.24±0.07^{aA}	3.93±0.06^{bcA}	3.90±0.07^{cA}
1	4.36±0.10^{aD}	3.60±0.12^{cB}	2.81±0.15^{eB}	4.15±0.11^{bB}	3.52±0.10^{dB}	2.75±0.12^{eB}
3	5.05±0.14^{aC}	2.98±0.14^{cC}	1.07±0.10^{eC}	3.65±0.23^{bC}	2.08±0.15^{dC}	1.00±0.10^{eC}
5	5.15±0.13^{aB}	2.22±0.27^{cD}	ND^{eD}	3.50±0.22^{bD}	1.40±0.21^{dD}	ND^{eD}
7	5.22±0.19^{aA}	1.30±0.22^{cE}	ND^{eD}	3.47±0.17^{bE}	1.12±0.20^{dE}	ND^{eD}

a, b, c, d, e: Means with different lowercase letters in the same row are significantly different (P < 0.05)
A, B, C, D, E: Means with different capital letters in the same column are significantly different (P < 0.05)
OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract; ND: Not Detected
*log CFU/g
** Standard Error (S.E.).

Table 3: Effect of OEO and GSE on *L. monocytogenes* in poultry fillets under MAP (log CFU/g)

Storage Days	Control	OEO (%)		GSE (%)	OEO (%) + GSE (%)	
		0.5%	0.8%	0.1%	0.5% +0.1%	0.8% + 0.1%
0	4.61*±0.83^{aE**}	4.20±0.05^{bcA}	4.18±0.12^{bcA}	4.38±0.15^{abA}	4.14±0.15^{bcA}	4.08±0.14^{cA}
1	4.71±0.23^{aD}	3.56±0.14^{bB}	3.21±0.15^{cB}	4.05±0.21^{aB}	3.02±0.80^{cdB}	2.98±0.25^{dB}
3	4.83±0.27^{aC}	3.02±0.10^{cC}	2.12±0.12^{dC}	3.56±0.23^{bC}	2.16±0.22^{dC}	1.75±0.21^{eC}
5	5.42±0.20^{aB}	2.35±0.28^{cD}	ND^{dD}	2.60±0.20^{bD}	ND^{dD}	ND^{dD}
7	5.61±0.28^{aA}	ND^{cE}	ND^{cD}	2.18±0.13^{bE}	ND^{cD}	ND^{cD}

a, b, c, d, e: Means with different lowercase letters in the same row are significantly different ($P < 0.05$)
A, B, C, D, E: Means with different capital letters in the same column are significantly different ($P < 0.05$)
OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract; ND: Not Detected
*log CFU/g
** Standard Error (S.E.).

Table 4: Headspace gas compositions of packages during storage time

Group	Storage time (day)											
	1			3			5			7		
	O_2	CO_2	N_2	O_2	CO_2	N_2	O_2	CO_2	N_2	O_2	CO_2	N_2
Control	0.13	29.80	70.07	0.55	28.20	71.25	0.75	27.60	71.65	0.92	26.40	72.68
0.5% OEO	0.12	29.80	70.08	0.55	28.30	71.15	0.76	27.80	71.44	0.88	26.45	72.67
0.8% OEO	0.11	29.75	70.14	0.54	28.10	71.36	0.75	27.45	71.80	0.87	26.40	72.73
0.1% GSE	0.10	29.80	70.10	0.50	28.50	71.00	0.78	27.85	71.37	0.93	26.35	72.72
0.5% OEO + 0.1% GSE	0.12	29.70	70.18	0.56	28.00	71.44	0.78	27.70	71.52	0.90	26.30	72.80
0.8% OEO + 0.1% GSE	0.12	29.85	70.03	0.59	28.10	71.31	0.79	27.70	71.51	0.91	26.50	72.59

OEO: Oregano Essential Oil; GSE: Grapefruit Seed Extract

The essential oils and plant extracts showed strong activity by producing a clear inhibition zone ≥ 20 mm (8). In this study, *S.* Typhimurium and *L. monocytogenes* were inhibited by OEO at 0.5%, 0.8%, 1.0%, 1.5%, and 2.0%. Our results also showed that *L. monocytogenes* were the most susceptible microorganism. In contrast, it was detected that GSE at 0.1% had moderate activity (inhibition zone < 12-20 mm) against bacteria. Therefore, GSE was used in combination with OEO.

Sensory properties (odour and taste) of treated fillets with OEO at 0.5%, 0.8% and GSE at 0.1% were assessed by the panelists with scores above ($P < 0.001$) the rejection limit (score of 5) whereas samples treated with OEO at 1.0%, 1.5%, 2.0% and the combinations were assessed with scores below the rejection limit ($P < 0.001$). Based on sensory scores, OEO at 0.5%, GSE at 0.1% and the combinations of 0.5% OEO plus 0.1% GSE had the highest acceptability scores between 6 and 8 during storage. The odour and taste of poultry fillets treated with OEO at 0.8% and OEO at 0.8% plus 0.1% GSE was found distinctive but pleasant, and scores were slightly higher than the acceptable limit. Due to the very strong odour and taste of OEO at the concentration of 1.0%, 1.5%, 2.0% and the combinations had lowest scores for sensory evaluation. According to Skandamis and Nychas (15), OEO at 1.0% in beef gave a more acceptable odour and colour as compared to the untreated samples. However, Chouliara *et al.* (1) and Solomakos *et al.* (16) stated that OEO at 1.0% and 0.9% gave adverse organoleptic properties in chicken and beef, respectively.

The inhibitory effects of OEO, GSE, and combinations on *S.* Typhimurium and *L.*

monocytogenes in poultry fillets under MAP are shown in Tables 2 and 3.

Discussion

There is a relationship between the chemical composition of the tested oil and the antimicrobial activity (17). The phenolic compounds particularly thymol and carvacrol widely reported to possess high levels of antimicrobial activity (18). In another study by Govaris *et al.* (19), the sum of thymol, carvacrol, ρ-cymene, and y- terpinene were found to be important for screening antibacterial activity. Baranauskiene *et al.* (20) found that the bacteriostatic properties of OEO are suspected to be associated with the carvacrol content. Our results were supported by this aspect.

The measured mean headspace compositions of the packaging at 7 days of storage were 72.7 ± 3.1% N_2, 26.4 ± 1.5% CO_2 and 0.9 ± 0.4% O_2 (Table 4). The gas compositions of each package were almost constant during storage. It may be the result of the permeability of packaging material and respiration of the product. It was reported that reduction in CO_2 in packages was due to the solubility of CO_2 in the poultry meat aqueous phase (21).

Extracts of volatile compounds from plants are widely used in the food industry because of their antimicrobial properties for the inhibition of growth and reduction in numbers of the foodborne pathogens (22). In this study, the tested pathogens were affected by OEO and GSE ($P < 0.001$). The use of OEO and GSE resulted in a reduction in *S.* Typhimurium population ($P < 0.001$). The concentrations of 0.8% OEO and 0.8% OEO + 1.0% GSE completely inhibited the *S.* Typhimurium at 5 days of storage. At 7 days, the population of *S.* Typhimurium was reduced by 2.68 log CFU/g (0.5% OEO), 0.77 log CFU/g (0.1% GSE) and 2.81 log CFU/g (0.5% OEO + 0.1% GSE) ($P < 0.001$). The similar results are in accordance with those of Ahn *et al.* (23) and Xu *et al.* (24).

Our results showed that *L. monocytogenes* was the most sensitive pathogen. Our results are in agreement with the results of Shelef (25) and Marino *et al.* (26), who reported that gram-negative bacteria are less sensitive to OEO than gram-positive bacteria due to their cell wall composition. Conversely, Kotzekidou *et al.* (22) indicated that gram-positive bacteria are more resistant. The volume of inocula, culture media, detection method and pH of the medium can be considered to be the reason for the difference.

The addition of OEO, GSE and their combinations with MAP showed a significant effect on the reduction and inhibition of *L. monocytogenes* ($P < 0.001$). All OEO concentrations inhibited the growth of *L. monocytogenes*; while 0.1% GSE resulted in a reduction by 2.20 log CFU/g. The efficacy of OEO against *L. monocytogenes* has been shown on beef (16) and cheese (19). OEO between 0.25% and 0.8% was used by these authors. Conversely, Ting and Deibel (27) reported that 1.0% OEO on meat did not reduce the *L. monocytogenes*. The differences could be attributed to several factors including the composition of OEO, bacterial strain, pH of food and storage temperature. In contrast and, Ahn *et al.* (23) and Xu *et al.* (24) examined the GSE against *L. monocytogenes*, and significant reductions were determined ($P < 0.01$). Jayaprakasha *et al.* (28) stated that GSE would be mainly effective against gram-positive bacteria, with gallic acid as the main active component.

The results of this study revealed the possibility of using OEO, GSE, and their combinations against foodborne pathogens on poultry meat under MAP stored at 4 °C. The usage of OEO and GSE concentrations in foods as preservatives is limited by the adverse sensorial properties. Further studies are needed to explore the efficacy of suitable concentrations of OEO and GSE in foods.

Acknowledgements

This study was obtained from the project (3223): "Antimicrobial Effect of Thyme Essential Oil and Grapefruit Seed Extract on Poultry Breast Meat Contaminated with Foodborne Pathogens and Packaged with Different Atmosphere" supported by the Research Fund of the University of Istanbul.

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UČINKI ETERIČNEGA OLJA ORIGANA, IZVLEČKA SEMEN GRENIVKE TER NJIHOVE KOMBINACIJE NA RAST IN PREŽIVETJE *Salmonelle* Typhimurium IN *Listerie monocytogenes* V FILEJIH PERUTNINE V SPREMENJENEM ATMOSFERSKEM PAKIRANJU

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Povzetek: V perutninskih filejih pakiranih v spremenjenih atmosferskih pogojih (30% CO_2 / 70% N_2), smo ugotavljali protimikrobne učinke eteričnega olja origana (OEO), izvlečka semen grenivke (GSE) ter njihove kombinacije na rast in preživetje patogenov, ki se prenašajo s hrano (*Salmonella* Typhimurium in *Listeria monocytogenes*). V poprejšnjem poskusu je bil OEO uporabljen v koncentracijah 0,05 %, 0,1 %, 0,5 %, 0,8 %, 1,0 %, 1,5 %, 2,0 %, GSE pa v koncentracijah 0,01 %, 0,02 %, 0,04 %, 0,08 %, 0,1 %. Testiranje s pomočjo difuzije s papirnatih diskov je pokazalo, da OEO pri koncentracijah 0,05 %, 0,1 % in GSE pri koncentracijah 0,01 %, 0,02 %, 0,04 %, 0,08 % slabo antibakterijsko delujeta. Poleg tega so bili zaradi zelo močnega vonja in okusa vzorci perutnine obdelani z OEO 1,0 %, 1,5 %, 2,0 % ter v kombinacijah ocenjeni z rezultati pod mejo organoleptične sprejemljivosti. Zato smo v nadaljnjih raziskavah uporabili koncentracije OEO 0,5 % in 0,8 % ter GSE 0,1 %. V tej raziskavi sta na patogene mikrorganizme vplivala tako OEO kot GSE, *L. monocytogenes* pa je bila občutljiv patogen kot *S.* Typhimurium. Rezultati te raziskave so potrdili možnost uporabe naravnih izdelkov pri proizvodnji hrane in pakiranju v spremenjenih atmosferskih pogojih za preprečevanje rasti bakterij, ki se prenašajo s hrano.

Ključne besede: eterično olje origana; ekstrakt semen grenivke; perutnina; patogeni, ki se prenašajo s hrano