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Antimicrobial Activity of Olive Leaf Extract on Selected Foodborne Pathogens and its Effect on Thermal Resistance of *Listeria monocytogenes* in Sous Vide Ground Beef

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Abstract

Antimicrobial activity of a commercial olive leaf extract (OLE) against *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Escherichia coli* Biotype I, *Salmonella* Enteritidis and *Salmonella* Typhimurium was tested by disc diffusion assay. The Gram negative bacteria tested in this study were more sensitive to OLE than Gram negatives. The highest antimicrobial activity was on *L. monocytogenes* which yielded the largest inhibition zone. Effect of OLE on thermal resistance of *L. monocytogenes* was tested both in Tryptic Soy Broth supplemented with 0.6% yeast extract (TSBYE) and sous vide packed ground beef. OLE added TSBYE (0, 0.5, 1%) tubes and ground beef (0, 1%) samples were inoculated with *L. monocytogenes* (7-8 log cfu/ml-g) and heated at 55, 60 and 65°C up to 30, 20 and 7.5 minutes, respectively. Total reductions in TSBYE tubes added with 0.5 and 1% OLE were slightly higher than control tubes (0% OLE) for all temperatures. Counts of *L. monocytogenes* in sous vide packed ground beef samples added with 1% OLE and then cooked at 55°C (30 min), 60°C (20 min) and 65°C (7.5 min) were 0.31, 1.04 and 0.73 log cfu/g lower than those control samples, respectively. The results indicate that OLE included in formulation may be an additional hurdle to control *L. monocytogenes* in heat processed ground beef.

Keywords: Olive leaf extract, Sous vide, Antimicrobial activity, *Listeria monocytogenes*, Ground beef

Introduction

In recent years, the plant extracts those rich in phenolic compounds have gained increasing attention due to their GRAS (Generally Recognized as Safe) status. Accordingly, they have been preferred as food additives for their antimicrobial and antioxidant functions (Perumella and Hettiarachy, 2011). The olive tree (*Olea europaea* L.) has been cultured for ages in Mediterranean region for not only its fruits but also its oil. Olive leaf extract (OLE) is a byproduct used mainly for medicinal purposes (Erdohan and Turhan, 2011). The content of phenolic compounds in olive leaf is as high as in olive fruit and derived products (Rahmanian et al., 2015). Olive leaf contains fourteen different compounds and among these compounds concentrations of oleuropein, hydroxytyrosol, luteolin-7-glucoside, epigenin-7-glucoside and verbascido are higher than the others (Benaventa-Garcia et al., 2000; Hayes et al., 2011). These bioactive compounds have biological activities including antimicrobial, antioxidant and antiproliferative (Rahmanian et al., 2015). The antimicrobial activity of OLE against foodborne pathogens has been shown by several studies (Pereira et al., 2007; Markin et al., 2003; Sudjana et al., 2009; Aliabadi et al., 2012; Gökmen et al., 2014; Hussain et al., 2014). Gökmen et al. (2014) determined antimicrobial activity of a commercial OLE against *L. monocytogenes* with >32 mg/ml MIC (Minimum Inhibitory Concentration) value. Liu et al. (2017) reported that olive leaf extract reduced cell motility in *L. monocytogenes*.

Sous vide is a cooking method in which food is

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subjected to heat process in vacuum sealed pouches. *Listeria monocytogenes*, a facultatively anaerobic bacterium, can be assumed a risk for sous vide cooked meats considering its heat resistance and ability to grow at refrigerated temperatures (Baldwin, 2012). Natural antimicrobials may contribute to reduce thermal resistance of *L. monocytogenes*. Juneja et al. (2013) has mentioned that the commercial apple powder rich in phenolic compounds added into ground beef reduced thermal resistance of *L. monocytogenes*. Therefore, the plant extracts rich in phenolic compounds may help to assure microbial safety by reducing thermal resistance of foodborne pathogens, when food is undercooked unintentionally or subjected to mild heat treatment intentionally to keep its fresh characteristics.

The aims of this study were to investigate the antimicrobial activity of OLE on selected foodborne pathogens, and to evaluate if the combination of OLE with mild heat treatment could eliminate *L. monocytogenes* in TSBYE and sous vide cooked ground beef.

Materials and Methods

Materials

Olive leaf extract (OLE) in liquid form was obtained from Tariş Zeytin Inc. (İzmir, Turkey). *Listeria monocytogenes* ATCC 7644, *Bacillus cereus*, *Escherichia coli* O157:H7, *Escherichia coli*, *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium and *Staphylococcus aureus* were from culture collection of Engineering Department of Engineering Faculty, Sakarya University. Ground beef (85% lean) was purchased from a local market, brought to laboratory in cooled conditions and frozen. Frozen ground beef was thawed in refrigerator (4±1°C) overnight before experiments.

Antimicrobial activity assay

Disc diffusion method was used to determine antimicrobial activity of OLE against selected bacterial cultures. *Listeria monocytogenes* ATCC 7644, *Bacillus cereus*, *Escherichia coli* O157:H7, *Escherichia coli*, *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium and *Staphylococcus aureus* were activated twice in Tryptic Soy Broth supplemented with 0.6% yeast extract (TSBYE, Merck, Darmstadt, Germany). *L. monocytogenes* culture was incubated at 30°C and the other cultures at 37°C for 24 h. From each bacterial culture, a 50 ml-portion was spread on Tryptic Soy Agar supplemented with 0.6% yeast extract (TSAYE, Merck, Darmstadt, Germany). OLE was prepared at different concentrations (5, 10, 20, 30, 40, and 50%; v/v) using distilled water or 1% dimethyl sulfoxide (DMSO). The sterile paper discs (6 mm in diameter) were placed on culture inoculated TSAYE and then discs were impregnated with 50 ml OLE. Same amounts of distilled water or DMSO were transferred to control discs. Plates were incubated at 37°C for 18-24 h and then the inhibition zones larger than 7 mm were measured. Tests were repeated twice and mean values were presented.

Preparation of *Listeria monocytogenes* inoculum

L. monocytogenes stock culture was activated twice in TSBYE by incubating at 30°C for 18-24 h. Active culture was inoculated into TSBYE and incubated for 18-24 h at 30°C. Following incubation, TSBYE culture was centrifuged at 4000 rpm at 4°C for 10 minutes. After discarding supernatant, the pellet was washed twice by adding sterile peptone water (PW; 0.1% peptone) and centrifuging at 4000 rpm for 10 min at 4°C. To finish the supernatant was removed and the pellet was completed to the original volume by sterile PW.

Effect of olive leaf extract on heat resistance of *L. monocytogenes* in TSBYE

To determine the effect of OLE on thermal resistance of *L. monocytogenes* in vitro conditions, one ml *L. monocytogenes* inoculum was transferred into 9 ml TSBYE added with olive leaf extract (0, 0.5 and 1%) (Skandamis et al., 2008). The tubes were placed into a water bath adjusted to 55, 60 and 65°C. Water level was 4 cm higher than then the liquid media in the tubes. The tubes were shaken every 3-5 min during heating. Three tubes were removed at each sampling time and cooled rapidly by immersing in ice slurry.

Inoculation and cooking of ground beef samples

The frozen ground beef was thawed in a refrigerator overnight and then divided into two batches. One batch was added with 1% olive leaf extract and the other with same amount of sterile distilled water. Each batch was mixed by hand for three minutes. Then, each batch was inoculated with *L. monocytogenes* at the level of 7-8 log cfu/g and mixed well for even distribution of the pathogen. Ground beef was shaped using sterile glass petri plates (6 cm in diameter and 1 cm in thickness) and each portion was 25-30 g. Samples were placed into bags (EVP-SV1520-100, 15×20 cm, Elektrola, ÖRKA, Istanbul, Turkey) and the bags were evacuated with 99% vacuum (CromPack Vacuum Systems VM48, İstanbul, Turkey). Samples were heat treated using a temperature controlled sous vide cooker with a circulator (PolyScience, CRC-AC2E, ÖRKA, Istanbul, Turkey) adjusted to 55, 60 and 65°C. Inner temperatures of samples were monitored by a thermocouple inserted into an uninoculated ground beef sample. The total cooking times were 30, 20 and 7.5 min at 55, 60 and 65°C, respectively. At each sampling time two samples were removed and cooled to 4°C by immersing in ice slurry.

Enumeration of *L. monocytogenes*

Serial dilutions from the cooled TSBYE tubes were prepared using MRD (Maximum Recovery Diluent; 1 g/l peptone, 8.5 g/l NaCl) and 0.1 ml portions from appropriate dilutions were spread on Tryptic Soy Agar supplemented with 0.6% yeast extract (TSAYE, Merck, Darmstadt, Germany) and plates were incubated at 30°C for 24-48 h. After incubation colonies were counted manually and the results were expressed as log cfu/ml of TSBYE.

A ten gram ground beef sample was transferred to stomacher bag added with 90 ml MRD and homogenized for 2 min. Serial dilutions were prepared using MRD and 0.1 ml portions from dilutions were spread plated on TSAYE. Plates were kept at 25°C for 3 hours. Then, 10 ml PALCAM Agar (Merck, Darmstadt, Germany) added with selective supplement at 45°C was poured on TSAYE (Miller et al., 2010). Plates were incubated at 30°C for 48 h. After incubation the typical grey-green colonies with black zone were counted as *L. monocytogenes* and the results were expressed as log cfu per gram of ground beef (log cfu/g).

Water activity measurement

Water activity values of ground beef samples were determined by Aqualab Water Activity Meter (Model Series 3, Decagon Devices, Pullman, WA).

Results and Discussion

Antimicrobial activity of OLE

The disc diffusion assay results were presented in Table 1. *L. monocytogenes* and *S. aureus* were more sensitive against OLE. In other words, OLE prepared DMSO or distilled water yielded larger inhibition zones against these two bacteria than the other bacteria. The largest zone diameters were 13.67 and 14 mm obtained by 50% OLE (DMSO) for *S. aureus* and *L. monocytogenes*, respectively. Twenty percent of OLE prepared with DMSO did not yield any inhibition zones for all bacterial cultures, while that prepared with distilled water yielded inhibition zone only against *L. monocytogenes*. On the other hand, 5 and 10% concentrations of OLE whether prepared with DMSO or distilled water did not show antimicrobial activity on any of the bacterial cultures tested in current study.

Table 1. Antimicrobial activity of OLE at different concentrations (Inhibition zones in mm)

Bacterial cultures	OLE concentrations (v/v)							
	DMSO				Distilled water			
	20%	30%	40%	50%	20%	30%	40%	50%
E. coli O157:H7	-*	-	7.75	9.00	-	7.50	7.50	7.67
E. coli	-	-	8.00	8.50	-	7.33	7.33	7.33
S. Enteritidis	-	8.00	8.50	9.33	-	7.00	7.33	7.50
S. Typhimurium	-	-	7.50	8.00	-	7.00	7.50	8.00
B. cereus	-	7.00	8.50	9.00	-	-	7.75	8.00
S. aureus	-	10.25	12.67	13.67	-	8.75	10.50	11.50
L. monocytogenes	-	8.17	10.67	14.00	7.83	10.33	11.83	12.67

* No inhibition zone

According to the antimicrobial activity assay results *L. monocytogenes* and *S. aureus* were determined as more susceptible than the other bacteria. The previous studies on antimicrobial activity of OLE on different bacteria revealed that OLE is effective on both Gram-positive and Gram-negative bacteria including those tested in the current study (Pereira et al., 2007; Markin et al., 2003; Sudjana et al., 2009; Aliabadi et al., 2012; Gökmen et al., 2014; Hussain et al., 2014). However susceptibility of tested microorganisms shows variation. Markin et al. (2003) reported that *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were the most susceptible against water extract of olive leaf while *B. cereus* was the least susceptible. According to Pereira et al. (2007) *B. cereus* was more susceptible with the extract concentration of 0.63 mg/ml that inhibits 25% of microbial growth (IC_{25}) than the other bacteria including *E. coli* and *S. aureus*. Sudjana et al. (2009) determined that commercial OLE was the most active against *Campylobacter jejuni, Helicobacter pylori* and *S. aureus* (MIC value 0.31-0.78% v/v), while the extract showed little activity against other microorganisms including *L. monocytogenes, B. cereus, E. coli* and *Salmonella.* In a study by Aliabadi et al. (2012) the aqueous extract of olive leaf (15-50 mg/ml) was effective on *S. aureus*, *S. typhimurium, E. coli, K. pneumoniae* and *B. cereus* while *S. typhimurium* was the most susceptible. These variations may arise from several reasons such as composition of OLE, extraction method, the solvent type used for extraction as well as strain differences and test conditions. One of the most possible reasons seems to be OLE composition. Lee and Lee (2010) evaluated individual and combined activities of phenolic compounds found in olive leaf and determined that caffeic acid was effective on *B. cereus*, *E. coli* and *Salmonella* Enteritidis, while oleuropein inhibited only *S.* Enteritidis. Therefore, the differences in OLE compositions may result in variations of antimicrobial activity test results. In current study the most susceptible bacterial cultures were *L. monocytogenes* and *S. aureus* which are Gram-positive. Sudjana et al. (2009) suggested that one or more components within OLE may specifically be active against the Gram-positive cell wall.

Reduced heat resistance of *L. monocytogenes* by OLE

Use of mild processes in food preservation may help to produce natural healthy foods. In other words the low doses of one or more treatments can keep the sensory characteristics and nutritional value of the products. However, it is vital to inhibit the pathogenic bacteria for ensuring microbial safety of mildly processed foods.

The reductions of *L. monocytogenes* counts in TSBYE added with 0, 0.5 and 1% OLE were shown in Figure 1. The total reduction in *L. monocytogenes* counts in TSBYE after heating at 55°C for 30 min was 0.64 and 0.84 log cycle for 0.5 and 1% OLE added tubes, while it was 0.39 log cycle in control tubes (0% OLE). Heating at 60°C for 20 min reduced the number of pathogen by 3.63, 4.03 and 3.83 log cfu/ml in TSBYE tubes added with 0, 0.5 and 1% OLE, respectively. The total reductions in 0.5 or 1 % OLE added TSBYE tubes were approximately 0.90 log cycle higher than control tubes after 7.5 min heating at 65°C.

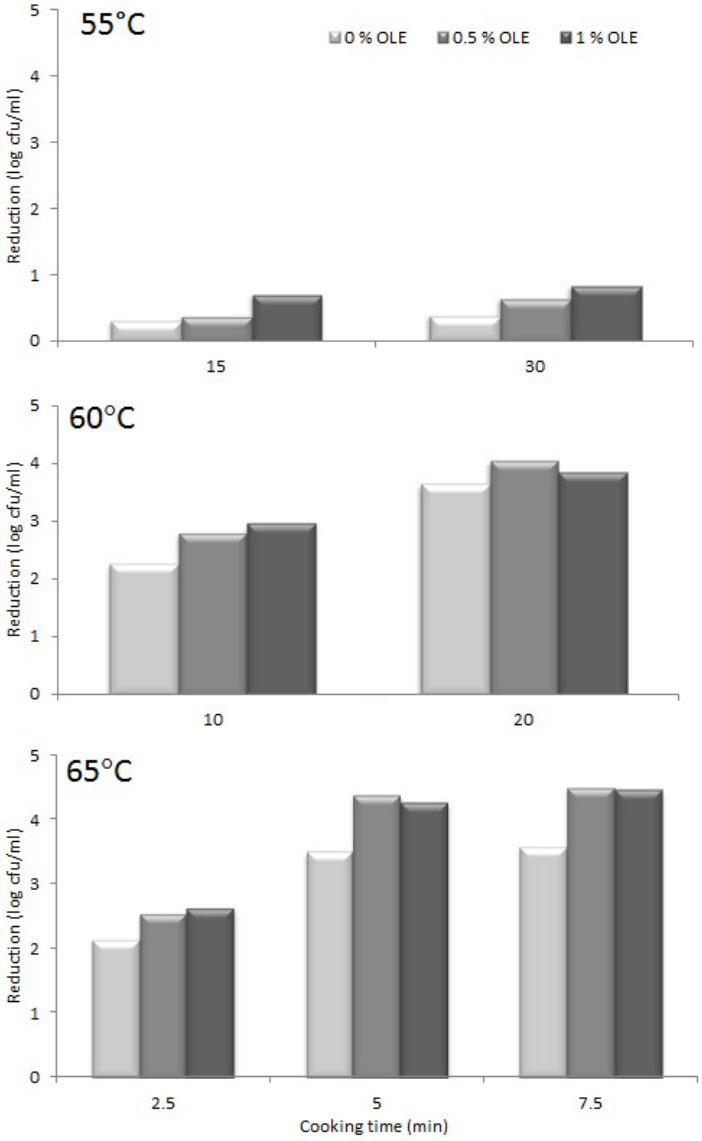

Figure 1. Reduction of *Listeria monocytogenes* in TSBYE with or without OLE at 55, 60 and 65°C (log cfu/ml)

Water activity (a_w) affects thermal resistances of bacteria; accordingly increasing a_w may result in increased thermal sensitivity (Syamaladevi et al., 2016). The a_w values of control ground beef samples (0% OLE) were 0.974 ± 0.003, while that was 0.973 ± 0.002 in 1% OLE added ground beef samples. Therefore, 1% OLE addition did not affect a_w value of ground beef.

The inner temperatures of ground beef samples during sous vide cooking increased gradually and slightly lower than the target temperature (Table 2). Figure 2 shows the reductions in *L. monocytogenes* inoculated in ground beef added with 0 or 1% OLE. When 0 and 1% OLE added ground beef samples were heated at 55°C for 30 min, the counts of *L. monocytogenes* reduced by 0.96 and 1.27 log cfu/g, respectively. Supplementation with 1% OLE resulted in an additional 1.04 log reduction following heat treatment at 60°C for 20 min when compared to non-treated control samples. On the other hand, *L. monocytogenes* counts were 0.73 log cfu/g lower in 1% OLE added ground beef than control samples heated at 65°C for 7.5 min.

Reduction in *L. monocytogenes* counts heated at 55°C were similar both in TSBYE and ground beef. In contrast, when the heating temperature was 60 or 65°C, less reduction was observed in ground beef samples than in TSBYE. These differences may arise from the lower thermal conductivity of ground beef than liquid growth medium. It is well known that composition of the heating medium may affect the heat resistance of *L. monocytogenes* (Doyle et al., 2001).

Table 2. Inner temperatures of ground beef samples during sous vide cooking at 55, 60 and 65°C

Water temperature (°C)	Time (minute)	Inner temperature (°C)
55	0	20.0
	15	51.7
	30	53.7
60	0	26.1
	10	52.4
	20	57.2
65	0	28.3
	2.5	49.9
	5	55.4
	7.5	58.8

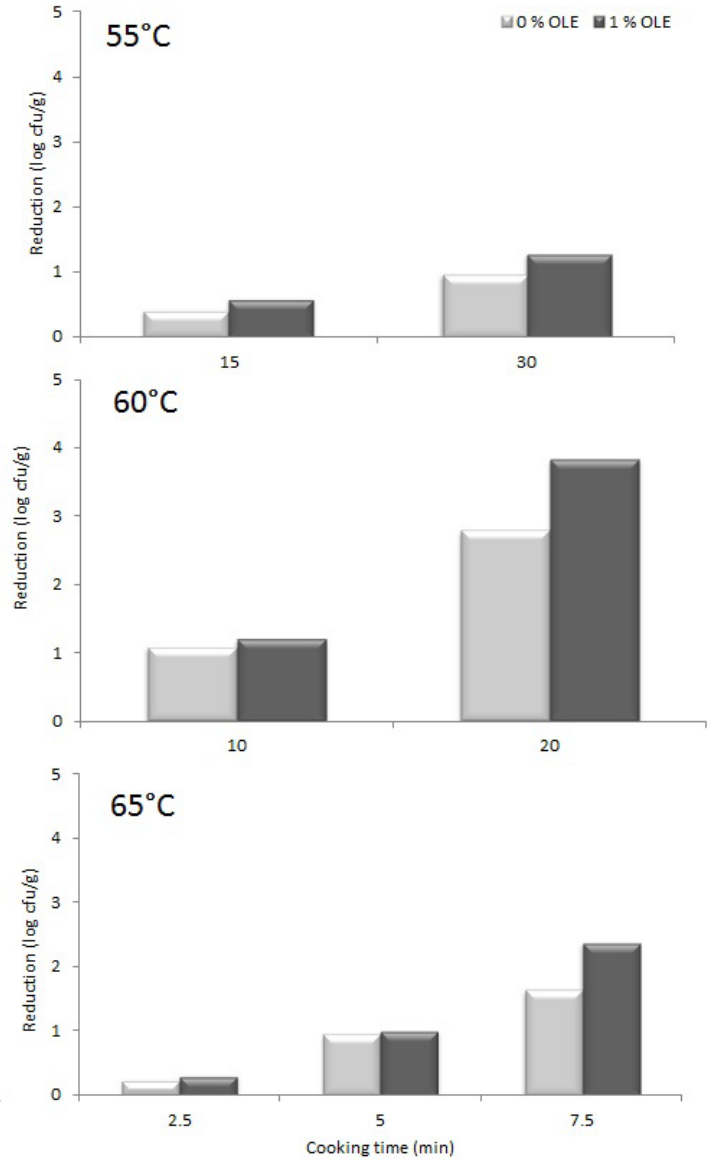

Figure 2. Reduction of *Listeria monocytogenes* in ground beef with or without OLE at 55, 60 and 65°C (log cfu/g)

These results demonstrated that OLE could contribute the inactivation of *L. monocytogenes* depending on the temperature and time. As far as we have known that this is the first report on effect of OLE on thermal resistance of *L. monocytogenes*. The previous studies have shown that OLE may retard growth of spoilage flora and then extend shelf life of animal based food products. Ahmed et al. (2014) found counts of aerobic bacteria and total coliforms were lower at least one log when raw peeled undeviened shrimps than those non-treated samples. Baker (2014) reported lower coliform, psychrophilic bacteria and aerobic plate count in 1-3% OLE added lamb patties than control samples during storage at 4°C for 12 days. Recently, Liu et al. (2017) have mentioned that OLE, at a concentration of 62.5 mg/ml, almost completely inhibited the growth of *L. monocytogenes* and reduced its cell motility demonstrated by the absence of flagella as shown by scanning electron microscope. OLE is rich in phenolic compounds (Pereira et al., 2007). It is well known that phenolic compounds damage the structure of cell membrane proteins, thus change the semipermeable nature of cytoplasmic membrane (Ultee et al., 1999). Juneja et al. (2013) reported that *L. monocytogenes* was more sensitive to lethal effect of heat when ground beef was supplemented with apple polyphenols. Similarly, Char et al. (2010) have reported that vanillin and citral addition to orange juice shortened the inactivation time for *Listeria inocua* (surrogate for *L. monocytogenes)* at 52 and 57°C.

Conclusion

The results of current study revealed OLE has antimicrobial activity on foodborne pathogens, while the Gram positive bacteria were more sensitive than Gram negatives. The combination of stress factors, i.e. OLE addition and thermal treatment, increased inactivation of *L. monocytogenes* reducing the time to destroy the pathogens at temperatures from 55 to 65°C. Although further studies are needed to understand the thermal death kinetics of *L. monocytogenes* in presence of OLE, it is obvious that OLE addition may help to control *L. monocytogenes* in heat processed meat products.

Compliance with Ethical Standards

Conflict of interest

The author declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contribution

Serap Coşansu designed the study and Özlem Kıymetli collected the data. Serap Coşansu wrote the article. All the authors read and approved the final manuscript.

Ethical approval

Not applicable.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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