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Research Article

The Effect of Eugenol on Survival of *Listeria monocytogenes* Inoculated İnegöl Meatball [1] [2]

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Abstract

In this research, it was aimed to determine the effect of eugenol with concentrations of 0.5% and 1.0% on *L. monocytogenes* with amount of 10^3 cfu/g, 10^4 cfu/g, 10^5 cfu/g and on total aerobic mesophilic microorganisms. In the experimental samples, presence of *L. monocytogenes*, the total aerobic mesophilic microorganisms and pH values were determined in the beginning (day 0), 5th and 10th days of cold storage at 4°C and 30th and 60th days of frozen storage at -18°C. There were significant differences in the number of total aerobic mesophilic microorganisms at the beginning day (day 0) and 30th day of the experimental İnegöl meatballs between groups, statistically ($P<0.01$). Considering the storage period, it was observed that the number of initial total aerobic mesophilic microorganisms was reduced in experimental samples during whole storage time ($P<0.01$). As an overall evaluation it was determined that the initial number of *L. monocytogenes* untreated with eugenol was higher than the samples treated with eugenol 0.5% and 1% after 60 days of frozen storage. This difference probably indicates that eugenol has an inhibitory effect on *L. monocytogenes*.

Keywords: *Eugenol, İnegöl meatballs, L. monocytogenes*

Eugenolün İnegöl Köfteye İnokule Edilen *Listeria monocytogenes*'in Varlığını Sürdürmesi Üzerine Etkisi

Özet

Araştırma, %0.5 ve %1.0 eugenol uygulamalarının 10^3 kob/g, 10^4 kob/g, 10^5 kob/g *L. monocytogenes* 4b ile inokulasyon sonrası etkisi ve aynı oranlardaki eugenol uygulamalarının toplam genel canlı mikroorganizmalar üzerine etkisini belirlemek amacıyla yapıldı. Deneysel numunelerde başlangıç (0. gün), 4°C'de muhafazanın 5. ve 10. günlerinde, -18°C'de donmuş muhafazanın 30. ve 60 günlerinde *L.monocytogenes*, toplam canlı mikroorganizması sayısı ve pH değerleri belirlendi. Deneysel İnegöl köftelerin başlangıçta (0.gün) ve donmuş muhafazanın 30. gününde toplam mezofilik aerob mikroorganizma sayısında istatistiki bakımdan gruplar arası önemli farklılıklar gözlemlenmiştir ($P<0.01$). Muhafaza periyodu dikkate alındığında ise deneysel uygulamalar yapılan numunelerin başlangıçta belirlenen toplam canlı mikroorganizma sayısının muhafaza periyodu süresince azaldığı gözlemlenmiştir ($P <0.01$). Genel olarak değerlendirildiğinde eugenol uygulanmayan numunelerin başlangıçta tespit edilen *L. monocytogenes* sayısının 60. gün donmuş muhafaza sonrasında %0.5 ve %1 eugenol uygulanan numunelerde belirlenen *L. monocytogenes* sayısından daha yüksek olduğu belirlenmiştir. Bu farklılık muhtemelen eugenolün *L. monocytogenes* üzerine inhibe edici etkisinin olabileceğini göstermektedir.

Anahtar sözcükler: *Eugenol, İnegöl köfte, L. monocytogenes*

INTRODUCTION

Listeria monocytogenes, which is an important food-borne pathogen, cause widespread epidemics, pneumonia, septicemia, meningitis, central nervous system infections and approximately 30% result with deaths. Human listeriosis formed by three serotypes (4b, 1/2a, 1/2b) [1,2]. Meat and meat products are contaminated with *L. monocytogenes* in various stages of production. It was reported by various investigators that, minced meat widely

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contaminated with *L. monocytogenes*. It's stated that, up to 100 colonies of *L. monocytogenes* can be tolerated in a gram or milliliter of foods and may be inactivated with heat before consumption. However it is stated that foods with high numbers of *L. monocytogenes* needs to be removed from consumption [3].

Many methods are used against the risks created by pathogen microorganisms in foods. Heating, freezing, antimicrobial compounds and synthetic preservatives are the most common applications among these methods. However, these methods cause changes in organoleptic properties of food and loss of nutrients. Therefore, there has been increasing interest in the obtaining of the natural antimicrobial compounds which are effective, nontoxic, constant flavour and nondecremental of nutrient value of foods instead of synthetic preservatives which has negative effects on public health [4,5]. Although spices obtained from plants is usually used as flavoring agents, it is shown by numerious researches that essential oils (EO) of various spices has antimicrobial activity and can be used as natural preservative [6,7].

Essential Oils (EO) are known to have different biological effects since the MiddleAges [8]. In addition to antibacterial [9-12] effect of EO or EO compounds, antiviral [13,14], antimycotic [15-18], antioxidant [19-22], antitoxigenic [23-25], antiparasitic [26,27] effects have been reported by several researches.

This study was then conducted to investigate the effects of eugenol on total aerobic mesophilic bacteria (TAMB) and *L. monocytogenes* in İnegöl meatballs, which has widespread ready to consumption in Turkey and has low microbiological quality.

MATERIAL and METHODS

Experimental Materials

Meat and spices, used in this study were obtained from markets in Konya. The rib cap (lower portion, LP) of lamb and brisket region of veal meats were used.

Experimental Production of İnegöl Meatballs

In this study, İnegöl meatball samples were prepared according to conventional methods. Veal (70%), lamb (12%) and tallow (18%) were passed together through a meat grinder. Then 1.5% NaCl, 10% breadcrumbs, 0.05% ascorbic acid, 1.0% caseinate, 0.15% garlic, onion 5%, 1.2% spices (pepper powder, paprika, cumin, allspice) added to this main mixture and mixed thoroughly by adding 0.7% water and passed through a grinder again. 25 g meatballs were formed of rod from meatball dough. 12 İnegöl meatball production groups were carried out for experimental studies. Procedures applied to the production groups are shown in *Table 1*.

Table1. *Experimental samples and control groups used in the study*
Tablo 1. *Araştırmada kullanılan deneysel numuneler ve kontrol grupları*

Group	Procedures
I.	- Meatball + inoculation of 10^3 CFU/g *L. monocytogenes 4b* - Meatball + inoculation of 10^4 CFU/g *L. monocytogenes 4b* - Meatball + inoculation of 10^5 CFU/g *L. monocytogenes 4b*
II.	- Meatball + 0.5% Eugenol + inoculation of 10^3 CFU/g *L. monocytogenes 4b* - Meatball + 0.5% Eugenol + inoculation of 10^4 CFU/g *L. monocytogenes 4b* - Meatball + 0.5% Eugenol + inoculation of 10^5 CFU/g *L. monocytogenes 4b*
III.	- Meatball + 1.0% Eugenol + inoculation of 10^3 CFU/g *L. monocytogenes 4b* Meatball + 1.0% Eugenol + inoculation of 10^4 CFU/g *L. monocytogenes 4b* - Meatball + 1.0% Eugenol + inoculation of 10^5 CFU/g *L. monocytogenes 4b*
P1	Production of traditional Inegöl Meatballs
P2	Meatball + 0.5% Eugenol
P3	Meatball + 1.0% Eugenol

After production and applications, all experimental İnegöl meatball groups were porsioned and placed in foam plates and covered with stretch film. Packages were stored under cold and freezing conditions at +4°C and –18°C. Analysis were carried out at 0th, 5th and 10th days for +4°C storage and 0th, 30th and 60th days for –18°C storage. Production were carried out in 3 replications.

Experimental Methods

Detection of Total Mesophilic Aerobic Bacteria: Detection of Total mesophilic aerobic bacteria count were performed according to the method suggested by Maturin and Peeler [28] in FDA Bacteriological Analytical Manual.

Detecting and Enumeration of L. monocytogenes: Detecting and enumeration of *L. monocytogenes* was performed according to the methot suggested by Hitchins ve Jinneman [29] in FDA Bacteriological Analytical Manual.

Statistical Analysis

The data obtained from the survey were analyzed using SPSS software package 21.00. Variance analysis (One-way ANOVA) of the obtained data, was subjected in accordance with the experimental design, and Duncan test was applied to detect differences between groups ($P<0.05$).

RESULTS

In this study; it was investigated that the use of eugenol essential oil and its' antibacterial effect on the total aerobic bacteria and *Listeria monocytogenes* in İnegöl meatballs which is sold and consumed commonly in Turkey and reported to have a low microbiological quality. The first group of experimental production of İnegöl Meatballs

held for 10 days at the temperatures between 0-4°C and the samples of the other groups were stored at -18°C for 60 days. After the production of experimental samples (day 0), changes in the total mesophilic aerobic and *L. monocytogenes* count was determined on 5th and 10th days of cold storage at 4°C and 30th and 60th days of frozen storage at -18°C. It was confirmed that the experimental samples do not carry any *L. monocytogenes* with analyzing of control samples before the inoculation process.

The count of total aerobic mesophilic bacteria in experimentally manufactured meatball samples obtained with inoculation of 10^3 cfu/g *L. monocytogenes* 4b and different rates of eugenol is shown in *Table 2*.

There were major statistical differences in the number of the total mesophilic aerobic microorganisms between the groups at the beginning stage (day 0) and frozen storage at 30th day of the experimental İnegöl meatballs *(Table 2*; P<0.05). When considering the storage period, it was determined that the total number of aerobic mesophilic microorganisms decreased during the storage period. The minimum number of total aerobic mesophilic microorganisms count was observed in the experimental samples stored at -18°C. These differences were found to be statistically important *(Table 2*; P<0.01).

At the beginning stage, the total mesophilic aerobic number of viable microorganisms were found between 5:46 and 6:51 in the experimental samples. Considering the eugenol application the differences was determined only 30th day of frozen storage between groups. At this period, the lowest number of microorganisms was determined in the group that 0.5% eugenol application was performed. In general consideration, it can be stated that this effect is due to the low number of microorganisms of these groups of samples at the beginning stage rather than the effective application of eugenol. Furthermore, in view of storage period, the frozen storage of the samples were considered to be more effective on the growth of total viable microorganisms *(Table 2)*.

In general, a reduction is achieved in the microflora at varying rates by freezing of food. A quite large variation of the reduction in microflora may be caused by the contamination in production process and growth of microorganisms during the thawing stage.

This can be explained by the view of Yildirim [30]. The researcher stated that the microflora in a certain period (lag phase) of cold stored food has been unchanged qualitatively and quantitatively. He stated that bacteria in food start to multiply at the end of the lag phase depending on storage conditions (cold or frozen), replication of microflora can be inhibited in the products which chilled in accordance with technological rules and has a high quality. He also indicated that, both mesophilic bacteria and psychrotrophic bacteria reduce during the cold storage for 3-5 days in meat and meat products. The microbiological results of this study support the views and ideas mentioned above.

The experimental meatball samples with different rates of eugenol which inoculated with 10^3cfu/g *L. monocytogenes* 4b displayed intergroup differences on 10th day of cold storage and 60th day of frozen storage with regard to the number of bacteria *(Table 3*; P<0.05). The highest value of bacteria on 10th day of cold storage and 60th day of frozen storage was determined in the Group I which the samples are untreated with eugenol. When considering the storage period, samples of Group III were only observed to be statistically different *(Table 3*, P<0.01).

The number of *L. monocytogenes* in the first experimental group (Group I) which is untreated with eugenol, was almost stable and maintain the level of initial stage (3.45 cfu/g). The number of the bacteria in the second experimental group (Group II) which is treated with 0.5% eugenol decreased 3.49 cfu/g to 2.92 cfu/g from the initial level to the 60th day of frozen storage, respectively. In the sample which is treated with 1.0% eugenol, it was determined that the initial count of *L. monocytogenes* detected as 3.52 cfu/g and decreased to 3.12 cfu/g on 60th day of frozen storage. Based on these results, it was thought that the application of the eugenol may have an inhibitory effect on *L. monocytogenes*. In addition, reduction in the number of *L. monocytogenes* in 1.0% eugenol treated

Table 2. *Total aerobic mesophilic bacteria count of experimental Inegol meatball obtained by the application of different rates of eugenol (log10/g)*
Tablo 2. *Farklı oranlarda eugenol uygulanan deneysel İnegöl köfte numunelerinde toplam mezofilik aerobik bakteri sayıları (log10/g)*

Application	Storage					
	4°C			-18°C		P
	Day 0	Day 5	Day 10	Day 30	Day 60	
A1	6.51 ± 5.66^{aA}	5.49 ± 4.53^{B}	5.47 ± 4.60^{B}	5.35 ± 4.21^{bB}	4.20 ± 3.18^{B}	0.001
A2	5.46 ± 4.86^{bA}	5.50 ± 4.73^{A}	5.52 ± 4.69^{A}	4.36 ± 3.13^{aB}	4.20 ± 3.35^{B}	0.001
A3	6.21 ± 5.86^{bA}	5.54 ± 4.65^{B}	5.51 ± 4.57^{B}	5.35 ± 4.18^{bB}	4.21 ± 3.27^{B}	0.001
P	0.002	0.877	0.858	0.001	0.982	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***A,B,C:*** *The differences between the mean values with different letters on the same line are important (P<0.05).* ***A1.*** *Traditional Inegöl Meatball production,* ***A2.*** *Meatball + 0.5% Eugenol addition,* ***A3.*** *Meatball + 1.0% Eugenol addition*

samples were determined to have statistically significant *(Table 3*; P<0.01).

The experimental meatball samples with different rates of eugenol which are inoculated with 10^4 cfu/g *L. monocytogenes* 4b displayed intergroup differences only on 5th day of storage with regard to the number of bacteria. The number of *L. monocytogenes* of the groups II and III were found lower rather than the group I which was untreated with eugenol *(Table 4*, P<0.05) Considering the storage period, the groups II were found to contain lower numbers of *L. monocytogenes* only in 60th days of -18°C storage *(Table 4*, P<0.05).

The experimental meatball samples with different rates of eugenol which are inoculated with 10^5 cfu/g *L. monocytogenes* 4b displayed intergroup differences only on 10th day of storage with regard to the number of bacteria *(Table 5*, P<0.05). When the groups were compared the groups II and III which were treated with eugenol displayed a lower number of *L. monocytogenes* rather than the group I which was untreated with eugenol. Considering the storage period, the samples including group II and III were found to contain lower numbers of *L. monocytogenes* on 30th and 60th days.

On the 60th day of the experiment, the initial number of *L. monocytogenes* in the experimental samples which were untreated with eugenol, treated with 0.5% eugenol and treated with 1.0% eugenol was determined to decrease from 5.35 cfu/g to 5.11 cfu/g, 5.26 cfu/g to 4.30 cfu/g and 5.24 cfu/g to 4.69 cfu/g, respectively.

DISCUSSION

An overall evaluation of the results displayed that the number of *L. monocytogenes* in the experimental samples of group II and III which are treated with eugenol was determined to reduce at the duration of the storage period. This finding of the study was suggested that the eugenol may possess a growth inhibitory effect on *L. monocytogenes* as well as in other studies. While a large number of studies [31-47] have reported that eugenol has an inhibitory effect against *L. monocytogenes*, in contrast several researchers [48] claimed that the eugenol has no effect against *L. monocytogenes*.

It was observed that the eugenol was more effective on *L. monocytogenes* strains in comparison with other essential oils (cinnamaldeyhd, thymol, citral, citronellol, limonenes) by Balch and Deans [31]. Filgueiras and Vanetti [35]

Table 3. *Number of L. monocytogenes in experimental samples of Inegol meatballs which inoculated different rates of eugenol and 10^3 cfu/g L. monocytogenes 4b (log10/g)*

Tablo 3. *10^3 kob/g oranında L. monocytogenes 4b (log10/g) inokule edilen deneysel İnegöl köfte numunelerinde L. monocytogenes sayısı*

Application	Storage					P
	4°C			-18°C		
	Day 0	Day 5	Day 10	Day 30	Day 60	
I	3.45±3.10	3.55±2.85	3.72±3.10^{a}	3.62±2.97	3.83±3.20^{a}	0.292
II	3.49±2.72	3.32±3.11	3.33±3.10^{b}	3.33±3.11	2.92±2.24^{b}	0.102
III	3.52±2.50^{A}	3.31±1.96^{B}	3.45±2.19^{abA}	3.30±2.46^{B}	3.12±2.11b^{C}	0.001
P	0.828	0.122	0.028	0.069	0.002	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***I.*** *Meatball + Inoculation of 10^3 cfu/g L. monocytogenes 4b.* ***II.*** *Meatball + 0.5% Eugenol + Inoculation of 10^3 cfu/g L. monocytogenes 4b.* ***III.*** *Meatball + 1.0% Eugenol + Inoculation of 10^3 cfu/g L. monocytogenes 4b*

Table 4. *Number of L. monocytogenes in experimental samples of Inegol meatballs which inoculated different rates of eugenol and 10^4 cfu/g L. monocytogenes 4b (log10/g)*

Tablo 4. *10^4 kob/g oranında L. monocytogenes 4b (log10/g) inokule edilen deneysel İnegöl köfte numunelerinde L. monocytogenes sayısı*

Application	Storage					P
	4°C			-18°C		
	Day 0	Day 5	Day 10	Day 30	Day 60	
I	4.27±3.30^{a}	4.30±3.73^{a}	4.40±3.43	4.14±3.39	4.52±4.20	0.110
II	4.39±3.27^{A}	4.11±3.40^{bA}	4.23±3.82^{A}	4.24±3.86^{A}	4.17±3.95^{B}	0.009
III	4.35±3.48	4.13±3.18^{b}	4.19±3.38	4.10±3.22	3.71±3.22	0.236
P	0.116	0.046	0.067	0.251	0.087	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***I.*** *Meatball + Inoculation of 10^4 cfu/g L. monocytogenes 4b.* ***II.*** *Meatball + 0.5% Eugenol + Inoculation of 10^4 cfu/g L. monocytogenes 4b.* ***III.*** *Meatball + 1.0% Eugenol + Inoculation of 10^4 cfu/g L. monocytogenes 4b*

Table 5. *Number of L. monocytogenes in experimental samples of Inegol meatballs which inoculated different rates of eugenol and 10^5 cfu/g L. monocytogenes 4b (log10/g)*

Tablo 5. *10^5 kob/g oranında L. monocytogenes 4b (log10/g) inokule edilen deneysel İnegöl köfte numunelerinde L. monocytogenes sayısı*

Application	Storage					
	4°C			-18°C		P
	Day 0	Day 5	Day 10	Day 30	Day 60	
I	5.35±4.33	5.32±5.11	5.41±4.54^{a}	4.92±4.31	5.11±4.39	0.185
II	5.26±4.26^{A}	5.16±4.60^{A}	5.16±4.63^{bAB}	4.36±4.11^{AB}	4.30±4.10^{B}	0.035
III	5.24±4.33^{A}	5.16±4.52^{A}	5.17±4.55^{bA}	4.48±4.14^{B}	4.69±4.23^{B}	0.018
P	0.083	0.315	0.021	0.206	0.143	

a,b,c: *The differences between the mean values in the same column with different letters are important (P<0.05).* ***I.*** *Meatball + Inoculation of 10^5 cfu/g L. monocytogenes 4b.* ***II.*** *Meatball+ 0.5 %Eugenol + Inoculation of 10^5 cfu/g L. monocytogenes 4b.* ***III.*** *Meatball + 1.0% Eugenol + Inoculation of 10^5 cfu/g L. monocytogenes 4b*

investigated the growth of *L. monocytogenes* and listeriolysin O (LLO) production. They stated that eugenol promoted a delay on the growth of *L. monocytogenes* at concentrations of 100, 300 and 500 mg mL^{-1} and above 800 mg mL^{-1} the effect was bactericidal. In addition, they argued that production of LLO by *L. monocytogenes* was reduced 80-100% in the presence of eugenol.

It was suggested that Gram (-) bacteria are more resistant to volatile oils [45]. Indeed Bežić et al.[32] stated that lipopolysaccharide (LPS), a structure of Gram (-) bacteria cell wall, inhibited the interaction of the volatile oil cell to bacteria membrane. However, Kim et al.[42] argued that *L. monocytogenes* is more resistant to the volatile oil although it's Gram (+).

Blaszyk and Holley [33] stated that 500 ppm eugenol has an inhibitory effect on *L. monocytogenes*. Chen et al.[34] argued that the forms of eugenol and thymol are more effective on *L. monocytogenes* than other forms. The bactericidal activity of clove on food-borne pathogens such as *L. monocytogenes* had been also reported by Ting and Deibel [46].

Garcia-Garcia et al.[36] reported that 350 mg/kg^{-1} of eugenol did not inactivate *L. innocua*. However, a 450 mg /kg^{-1} concentration of this antimicrobial agent inactivated the microorganism in the first few hours, and this condition prevailed after 72 h. So they argued that 450 mg/ kg^{-1} eugenol concentration was the minimal bactericidal concentration for *L. innocua*.

Gaysınsky et al.[37] stated that Eugenol encapsulated in Surfynol 485W micelles was most efficient in inhibiting of the growth of the pathogens. They argued that 1.0% Surfynol 485W and 0.15% eugenol was sufficient to inhibit the growth of all strains of *E. coli* O157:H7 and three of four strains of *L. monocytogenes* (Scott A, 310 and 108).

Gill et al.[38] stated that, eugenol and carvacrol lead to degradation in *E. coli* and *L. monocytogenes*' in cell membrane, also they caused to increase extracellular ATP concentrations and reduce to intracellular ATP concentration. In addition, Gill et al.[39] stated that eugenol and carvacrol inhibited the membrane ATPase activity of *E. coli* and *L. monocytogenes*.

Gill and Holley [40] suggested that eugenol was a more effective bactericidal agent than Cinnamaldehyde in same concentration. They stated that eugenol has a dose-dependent bactericidal effect on log-phase cells of *L. monocytogenes* within 15 min.

Hao et al.[41] stated that eugenol was slow down the growth of *L. monocytogenes* in cooked beef while it was maintained at 5°C and 15°C. Smith-Palmer et al.[45] argued that the clove essential oil could be implemented to control of *L. monocytogenes* and it has low bacteriostatic and bactericidal effects at 4°C.

Perez-Conesa et al.[43] reported that *L. monocytogenes* strain Scott A was more sensitive to eugenol than to Carvacrol after 2 min of exposure, as eugenol led to a 3.3 log cfu/cm^2 reduction compared with the 1.9 log cfu/cm^2 reduction achieved by carvacrol and they observed that viable cells were below detectable levels for *L. monocytogenes* strain ScottA was exposed to 0.7% eugenol-loaded micelles for 10 and 20 min.

Santiesteban-L'opez et al.[44] evaluated the effects of antimicrobial agents on *S. aureus*, *L.innocua*, *E. coli* and *S. typhimurium* and they determined that the most effective antimicrobial agent was carvacrol followed by thymol and eugenol.

Upadhyay et al.[47] investigated that the effects of generally recognized as safe (GRAS), plant-derived anti-microbials (PDAs); trans-cinnamaldehyde (TC 0.50, 0.75 mM), carvacrol (CR 0.50, 0.65 mM), thymol (TY 0.33, 0.50 mM) and eugenol (EG 1.8, 2.5 mM) on *L. monocytogenes* (LM) biofilm formation. When applied at subinhibitory concentrations, they were considerably effective in killing mature LM biofilms and has an inhibitory effect on biofilm synthesis.

Despite advances in food technology, food poisoning continues to maintain an increasing importance in terms

of public health. Pathogenic microorganisms such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* found in meat products threaten public health even today.

L. monocytogenes is considered as an important pathogen that causes food-borne epidemia, pneumonia, septicemia, meningitis, central nervous system infections and death about 30% of cases. Meat and meat products are contaminated with *L. monocytogenes* at different stages of the production. Many methods are used in food against the risks generated to pathogenic microorganisms. Heating, freezing, preservatives and synthetic antimicrobial compounds are the most frequently used methods among these. However, these methods cause changes in the organoleptic properties of food and loss of nutrients. Synthetic preservatives are known to affect negatively to public health; because of this reason the natural antimicrobial compounds which are effective, non-toxic, constant flavour and nondecremental of nutrient value of the product are use in food production. It has been shown in the studies, spices obtained from plants are use as flavorer in food products, many spices has essential oils (EO) which can be used as a natural preservative for their antimicrobial activity.

Thus, using essential oils of plant origin as an alternative to chemical compounds in the manufacturing of the meat products, particularly İnegöl meatball would be beneficial for protecting the public health. It was concluded that eugenol can show inhibitory activity especially against *L. monocytogenes* and other pathogenic microorganisms, but for a certain opinion, new experimental models and new researches need to be done.

Conflict of Interest

All the authors declare that there is no conflict of interests regarding the publication of this research article.

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