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The Antimicrobial Effects of Alginate Coating Containing Solid Lipid Nanoparticles of Peppermint Essential Oil against *Escherichia coli* O157: H7 and *Salmonella* Typhimurium Inoculated on Beef Fillet

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ABSTRACT

Background: The current study aimed to evaluate the effect of an alginate coating containing peppermint essential oil (PEO) and solid lipid nanoparticles (SLNs) against *Salmonella* Typhimurium and *Escherichia coli* O157: H7 inoculated on beef fillets and subsequently stored at a temperature of 4 ± 1 °C for 12 days.

Methods: Beef fillet samples were divided into four groups: CON group (without any coating solution), ALG group (coated with alginate solution), ALG + PEO group (coated with alginate solution containing 0.1 % (w/v) PEO), and ALG + SLN-PEO group (with alginate solution in combination SLN containing 0.1 % (w/v) PEO). The samples were then analyzed for the presence of inoculated *E. coli* O157: H7 and *S.* Typhimurium during refrigerated storage.

Results: The ALG + SLN-PEO coating had a larger impact in controlling the growth of pathogenic bacteria on beef fillets compared to the other treatment groups.

Conclusion: According to the obtained results, the ALG + SLN-PEO coating could potentially be used in the food industry to reduce the risks associated with contamination of beef fillets by *E. coli* O157: H7 and *S.* Typhimurium.

1. Introduction

Beef spoiling is a sophisticated process involving the interactions of microbiological, chemical, and physical changes, which occur swiftly in nature. Among the primary factors contributing to foodborne diseases and shelf-life loss in beef, lipid oxidation, and microbial contamination during processing and storage are pivotal [1]. *Salmonella* Typhimurium, a gram-negative bacterial pathogen, is recognized as a global cause of foodborne illnesses in both humans and animals. Its transmission primarily occurs through the consumption of raw or uncooked eggs, vegetables, fruits, and poultry. The presence of this microorganism in food poses a major hazard to public health [2, 3]. *Salmonella* species are well-known as important foodborne and waterborne pathogens that cause a wide range of ailments [4]. *Escherichia coli* is a bacterium belonging to the Enterobacteriaceae family, specifically the genus *Escherichia,* and it commonly resides within the gut bacteria [5]. The majority of *E. coli* species can ferment lactose. Because this bacterium is found in the human and animal digestive tracts, it is used as a marker for food

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contamination in the feces. Furthermore, *E. coli* is an opportunistic bacterium that can be easily transmitted to individuals through food, promoting extensive screening of food products for its presence [5]. Edible coatings made of proteins, polysaccharides, and lipids have demonstrated the ability to extend the shelf life of food products by serving as barriers against vapor, gases, and solutes. However, the use of edible coatings and films to protect food quality is not a new notion, research in this subject has lately increased at academic, government, and private-sector facilities. Edible coatings could be used in almost any sector of the food business if they were properly developed [6]. Alginate is a natural anionic polymer derived from brown seaweed that is low-toxicity, biocompatible, low-cost, and has moderate gelation when divalent cations such as Ca^{2+} are added. Due to the mentioned advantages, alginate has been widely studied and used for various biomedical applications [7]. Plant-based essential oils (EOs) are high in phenolic components including flavonoids and phenolic acids that have antibacterial activity against a variety of bacteria [8]. Peppermint, a hybrid mint derived from the combination of *Mentha spicata* (spicy mint), *Mentha alba*, and *Mentha aquatica* (blue mint), contains more than 40 distinct chemical components, including menthol, menthone, and menthyl acetate. Extensive toxicological studies have provided evidence of the safety of peppermint [9]. Peppermint possesses digestive, choleretic, antiseptic, antibacterial, antiviral, antispasmodic, antioxidant, expectorant, sedative, anti-inflammatory, analgesic, and tonic properties, as well as dilates blood vessels [9]. To achieve targeted and controlled delivery of therapeutic agents, solid lipid nanoparticles (SLNs) are emerging as a promising alternative to colloidal methods. These nanoparticles are comprised of biodegradable and biocompatible materials capable of integrating hydrophilic and lipophilic drugs, with sizes typically ranging from 50 to 1000 nm. SLNs incorporate the benefits of several colloidal carriers, such as liposomes and emulsions, which are well-tolerated by the body. Similar to polymeric nanoparticles, SLNs enable the controlled release of drugs from the lipid matrix [10]. Previous studies have explored the application of diverse coatings on beef, including alginate coatings against pathogenic bacteria in beef meat [11-14]. Few studies investigated the effect of essential oil-loaded solid lipid nanoparticles in different coatings [15-17]. However, to the best of our knowledge, no study has explored the impact of coating combined with nanoparticles containing essential oil against foodborne bacteria in beef. Therefore, the purpose of the current research was to evaluate the effectiveness of peppermint essential oil (PEO)-loaded SLNs in alginate coating against *S.* Typhimurium and *E. coli* O157: H7 inoculated onto beef fillet samples and stored at a temperature of 4 ± 1 °C over 12 days.

2. Materials and Methods

2.1 Experimental Materials

The growth media used in the study were sourced from Merck (Darmstadt, Germany). PEO was obtained from the Iranian Institute of Medicinal Plants in Karaj, Alborz Province, Iran. The two-way lyophilized culture of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC: 14028, 29629) was purchased from the Iranian Biological Resource Center in Tehran, Iran. The *E. coli* O157: H7 (NCTC 12900) was obtained from Ferdowsi University of Mashhad, Mashhad, Iran. All the reagents were of analytical quality and were acquired from Sigma-Aldrich (St. Louis, MO, USA) Chemical Company.

2.2 GC-MS Analysis of Peppermint Essential Oil

The chemical composition of the PEO was analyzed using GC-MS. The analysis was performed using a chromatograph (Agilent 6890 UK) equipped with an HP-5MS capillary column. The dimensions of the columns were 30 × 0.2 mm ID × 0.2 μm film thickness. the method used for the analysis of PEO was described by Nasseri *et al.* (2016). To confirm the obtained results, PEO was also analyzed by gas chromatography-mass spectrometry (Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass-selective detector; Agilent UK) and was applied the same capillary column and analytical conditions [18]. The identification was made by comparison of the obtained mass spectrums of the relevant chromatographic peaks with spectrums of the NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) and Wiley libraries.

2.3 Preparation of solid lipid nanoparticles- peppermint essential oil

To prepare the lipid phase, glycerol monostearate was melted at a concentration of 1 % w/v at 75 °C. Once the glycerol monostearate reached its melting point, PEO was added to the melted lipid phase at a concentration of 0.5 % w/v. Simultaneously, tween 80 was dissolved in double distilled water to create an aqueous phase. The aqueous phase was then heated to the melting point of the lipid phase and added to the lipid phase. Finally, the emulsion was provided using a bath sonication (Power sonic 505 W; Hwashin Technology, Gyeonggi-do, South Korea) in three 10-minute cycles with 5 s intervals between each cycle [18].

2.4 Bacterial inoculation in beef fillets

Fresh beef was purchased from a local butcher shop and immediately transferred to the laboratory under the cold chain. Then, 10 g samples of beef were prepared. In order to remove the existing microbial flora, the beef fillets were sprayed with ethanol (70 % v/v). After drying the samples, 100 µl of bacterial suspension prepared (at a concentration of 10^7 CFU/g) from one strain of *E. coli* O157: H7 or two strains of *S.* Typhimurium (as a cocktail) were inoculated to their surface to reach a final concentration of 10^5 CFU/g. For bacterial adhesion, the samples were then left at 25 °C for 30 min [8].

2.5 Preparation of alginate coating solutions and treatments

To prepare the alginate solution, sterilized distilled water was employed to dissolve alginate powder at a concentration of 3 % w/v. The solution was subjected to repeated stirring under a controlled temperature (70 °C). Then, 2 % glycerol as a plasticizer was added. PEO was dissolved in alginate solutions with tween 80 (0.2 g/g EO) as an emulsifier and agitated for 30 min at a controlled temperature (40 °C) to produce a homogenous, stable, and clear solution. Alginate solutions were supplemented with adding SLNs (0.1 % v/v) containing PEO (0.1 % w/v). Calcium chloride was dissolved in distilled water (2 % w/v) and autoclaved for 15 min at 121 °C. Table 1 shows how inoculated beef fillets were separated into four treatment groups. The beef samples were then immersed in the $CaCl_2$ solution for 30 s. Finally, the treated samples were stored at a temperature of 4 ± 1 °C for 12 days. The evaluation of the samples was conducted at specific time intervals, namely 0, 1, 2, 4, 6, 8, and 12 days [8].

Table 1. List of treatments in the present study

Treatment	Description
CON	Control: Beef fillets without any coating solution
ALG	Beef fillets coated with alginate solution
ALG + PEO	Beef fillets coated with alginate solution containing 0.1 % (w/v) peppermint essential oil
ALG + SLN-PEO	Beef fillets coated with alginate solution in combination with 0.1 % (v/v) solid lipid nanoparticles containing 0.1 % (w/v) peppermint essential oil

2.6 Enumeration of S. Typhimurium and E. coli O157:H7

The samples (10 g) were put into sterile bags with 90 mL of peptone water and shaken for 2 min at 240 rpm with a stomacher (Seward Medical, London, UK). Then, serial dilutions (1: 10) were prepared and 100 µl of the suitable dilutions were cultured using the surface culture method on a plate containing Xylose Lysine Deoxycholate and Sorbitol MacConkey agar for *S.* Typhimurium and *E. coli* O157: H7, respectively. Characteristic colonies were then counted after the plates were incubated for 24 h at 37 °C [8].

2.7 Statistical analysis

The obtained data, collected in triplicate, were expressed as the mean ± standard deviation (Mean ± S.D.). Repeated measure ANOVA was used to examine the trend of logarithmic changes in the growth rate of bacteria in different groups over a 12-day period. The Dunnett post-hoc test was used to compare the groups pairwise. All statistical analyses were carried out using SPSS software version 21. A significance level of $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 GC-MS Analysis of PEO

The chemical compounds identified in PEO are presented in Table 2. All of the identified chemical compounds represented 92.80 % of total PEO. The most compounds included iso-menthol (24.69 %), carvone (16.98 %), and menthone (12.35 %). These findings were in line with the results of Laein *et al.* (2022) [15]. Similarly, in the study conducted by Yilmaztekin *et al.* (2019), peppermint (*Mentha piperita* L.) EO was analyzed by GC-MS method and they reported that the main chemical compounds were menthol and *p*-menthone [19]. Zhao *et al.* (2022) reported menthol, menthone, iso-menthone, and neomenthol as important and effective chemical components of PEO [20]. The differences in the results of different studies could be due to different extraction methods, plant organs, and distillation conditions [15].

Table 2. Chemical compounds of peppermint essential oil by GC-MS

NO	RT	Components	%
1	11.74	Alpha-Pinene	0.73
2	14.09	Beta-Pinene	0.90
3	15.29	3-Octanol	0.24
4	16.70	para-Cymene	1.12
5	16.88	Limonene	10.88
6	17.05	1,8-Cineole	3.81
7	18.37	Gama-Terpinene	0.21
8	20.61	Linalool	0.26
9	23.69	Menthone	12.35
10	24.08	iso-Menthone	2.59
11	24.32	Menthol	2.33
12	24.91	iso-Menthol	24.69
13	25.30	neoiso-Menthol	0.59
14	25.85	trans-Dihydrocarvone	4.06
15	26.86	trans-Carveol	0.40
16	27.71	Pulegone	0.55
17	28.21	Carvone	16.98
18	28.53	Piperitone	0.79
19	29.83	neo-Menthyl acetate	2.88
20	30.38	Thymol	0.53
21	31.44	iso-Dihydrocarveol acetate	0.34
22	32.47	Piperitenone	2.11
23	33.97	Beta-Bourbonene	0.81
24	42.27	Spathulenol	0.59
25	42.45	Caryophyllene oxide	1.65
26	42.95	Veridiflorol	0.42
Total			92.80

3.2 Microbial changes in the beef fillets

3.2.1 Enumeration of S. Typhimurium

The effects of the treatments on the growth rate of *S.* Typhimurium during the 12 days of storage are depicted in Figure 1. The amount of *S.* Typhimurium bacteria in the CON samples declined from 5.48 to 3.62 log CFU/g after 12 days of storage following inoculation, which is attributable to the bacterium's mesophilic nature. The data obtained are in line with those of McLay *et al.* (2002), who investigated

the suppression of food pathogens, including *E. coli*, by the lactoperoxidase system, as well as the reduction of mesophilic bacteria at refrigerator temperatures [21]. During the 12 days, the mean logarithm of *S.* Typhimurium in the alginate therapy group declined from 5.27 to 3.51 log CFU/g, which is considerably lower than the CON group ($p < 0.05$). These findings were in agreement with the results of Alavi *et al.* (2020) [12]. This reduction in bacterial numbers could be due to alginate's strong capacity to store water in its molecules, which keeps water out of the bacteria [12]. During the storage period, the average logarithm of *S.* Typhimurium in the ALG + PEO treatment declined from 5.17 to 3.31 log CFU/g, which was considerably lower than the alginate group. These findings were in line with the research performed by Hashemi *et al.* (2023) who indicated that coating alginate with EO was more effective than declining the number of bacteria in the samples during storage [16]. During the 12-day storage period, the average logarithm of *S.* Typhimurium in ALG + SLN-PEO declined from 4.93 to 2.96, which was considerably lower than the ALG + PEO group. Because the colloidal delivery systems are enclosed and thus the evaporation rate is lowered, solid lipid nanoparticles inhibit essential oil evaporation due to nanosorption of their particles. As a result, they have more opportunities to connect with microbes, Therefore, they are more effective than essential oils alone, according to Naseri *et al.* (2016) [18]. In another research, Hashemi *et al.* (2023) reported that alginate coating combined with *Carum copticum* EO-SLN was more effective than alginate + *carum copticum* EO coating in reducing the growth rate of bacteria and increasing the shelf life of hamburgers during a 12-day storage period at 4 ± 1 °C that these results were in agreement with findings of this study [16].

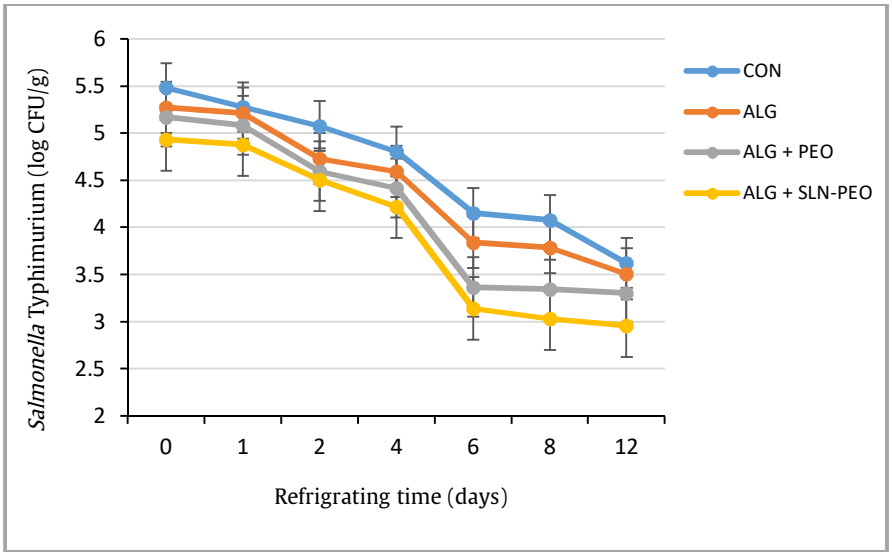

Figure1. Changes in *Salmonella* Typhimurium count (log CFU/g) of beef fillet samples in different treatments during 12 days of storage at 4 ± 1 °C (CON, control; ALG, alginate; ALG + PEO, alginate + 0.1 % (w/v) peppermint essential oil; ALG + SLN + PEO, alginate + 0.1 % (v/v) solid lipid nanoparticles containing 0.1 % (w/v) peppermint essential oil)

* Despite the significant decrease in the number of *S.* Typhimurium in different groups compared to the control group, the ALG + SLN - PEO group had the most significant logarithmic decrease in the number of bacteria when compared to the CON group, on day zero.

Table 3. The difference between the mean logarithm of *S.* Typhimurium in various groups

mean difference I-J	Group (J)	ALG	ALG+PEO	ALG + SLN - PEO
Group (I)				
CON		0.22*	0.46*	0.69*
ALG			0.24*	0.47*
ALG + PEO				0.23*

*Indicate a statistically significant difference ($P < 0.05$)

3.2.2 Enumeration of E. coli O157:H7

Figure 2 shows the changes in E.coli O157: H7 counts of beef fillet samples during 12 days of storage at 4 ± 1 °C. The quantity of *E. coli* O157: H7 bacteria in the control samples reduced to 3.5 CFU/g log during 12-day storage at refrigerator temperature, and its logarithm decreased from 5.21 to 3.54 on day 12 after inoculation, according to the findings of this study (zero-day). It was discovered that this is related to the bacterium's mesophilic nature. The findings are in line with those of McLay *et al.* (2002), who investigated the suppression of food pathogens, including *E. coli*, by the lactoperoxidase system, as well as the reduction of mesophilic bacteria at refrigerator temperatures [21]. During the 12-day period, the mean logarithm of *E. coli* O157: H7 in the alginate treatment group declined from 5.03 to 3.41, which is considerably lower than the control group. Alavi *et al.* (2020) compared the impacts of alginate coatings containing *Zataria multiflora* Boiss EO in two kinds of coarse emulsion and nanoemulsion inoculated in beef fillets at 4 ± 1 °C for 16 days [12]. The high capacity of alginate to store water in its

molecules, which keeps water out of the bacteria, was discovered to be helpful in suppressing bacterial growth in the study [12]. During the storage period, the mean logarithm of *E. coli* O157: H7 in the ALG + EO treatment declined from 4.88 to 3.13, which was considerably lower than the alginate group. According to Alavi *et al.* (2020), alginate coatings + *Zataria multiflora* EO in both forms (coarse/nanoemulsion) were a suitable option to control *E. coli* O157: H7 at 4 ± 1 °C. It efficiently inhibits *E. coli* O157:H7 growth during storage [12]. During the 12-day storage period, the mean logarithm of *E. coli* O157: H7 in ALG + SLN-PEO declined from 4.73 to 2.77, which was considerably lower than the ALG + PEO group. This finding was in line with the study performed by Laein *et al.* (2022). They found that the PEO-loaded SLN in gelatin coating was more effective than the gelatin + PEO coating in reducing *E. coli* O157: H7 in samples during storage at 4 ± 1 °C for 12 days [15]. Tables 3 and 4 show the mean difference in the logarithm reduction of *S.* Typhimurium and *E. coli* O157: H7 bacteria in the study groups after a 12-day storage period at refrigerator, respectively. During the storage period, all treatments differ from the control. The most significant distinction was between the control and ALG + SLN-PEO treatments (0.69 for *S.* Typhimurium and 0.67 for *E. coli* O157: H7). ALG + SLN-PEO treatment showed a decrease of ~ 0.7 log CFU/g of *S.* Typhimurium and *E. coli* O157: H7 during 12 days compared to the control group.

Table 4. The mean difference of logarithm reduction of *Escherichia coli* O157: H7 in different groups

mean difference I-J Group (I)	Group (J)	ALG	ALG+PEO	ALG + SLN - PEO
CON		0.19*	0.44*	0.67*
ALG			0.25*	0.49*
ALG + PEO				0.23*

*Indicate a statistically significant difference ($P < 0.05$)

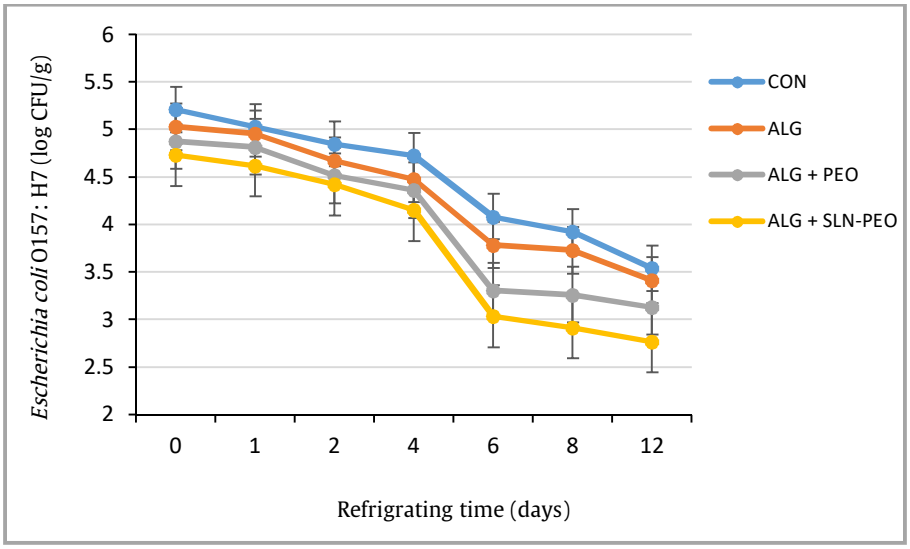

Figure 2. Changes in *Escherichia coli* O157: H7 counts (log CFU/g) of beef fillet samples in different treatments during 12 days of storage at 4 ± 1 °C (CON, control; ALG, alginate; ALG + PEO, alginate + 0.1 % (w/v) peppermint essential oil; ALG + SLN + PEO, alginate + 0.1 % (v/v) solid lipid nanoparticles containing 0.1 % (w/v) peppermint essential oil)
* Despite the significant difference in the number of bacteria between the different groups with control on day zero, the largest difference was found between the CON group and the ALG + SLN-PEO group on day zero, which shows the effect of this treatment on bacteria at time zero.

4. Conclusion

Our findings revealed that all the treatments reduced bacterial counts as compared to the control group. Moreover, ALG + SLN - PEO had the strongest antibacterial activity against *E. coli* O157: H7 and *S.* Typhimurium. ALG + SLN - PEO treatment reduced *S.* Typhimurium and *E. coli* O157:H7 by roughly 0.7 logs over the refrigeration period, when compared to the CON group. In addition, the quantity of bacteria was reduced in two alginate and alginate + essential oil treatments. Therefore, this investigation indicated that the use of alginate coating containing SLN of PEO leads to a considerable reduction in the population of the examined bacteria in the beef fillet sample.

Authors' Contributions

Atefeh Nazari Yazdi: Investigation; Methodology; writing-original draft. Saeid Khanzadi: conceptualization; supervision; project administration; funding acquisition; validation; writing-review & editing. Mohammad Hashemi: methodology; resources; visualization; writing-review & editing. Mohammad Azizzadeh: data curation; software; formal analysis; validation. Fatemeh Mollaei: writing-original draft; writing-review & editing.

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Conflicts of Interest

The authors report no conflict of interest.

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Ethical considerations

Project No: 3/54501.

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