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The Effects of Alginate Coatings Containing *Zataria multiflora Boiss* Essential Oil in the Forms of Coarse Emulsion and Nano-emulsion on Inoculated *Escherichia coli* O157: H7 in Beef Fillets

Seyed Hamid Alavi[1], Saeid Khanzadi[1]*, Mohammad Hashemi[2], Mohammad Azizzadeh[3]

1. Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.
2. Department of Nutrition, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran.
3. Department of Clinical Sciences, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

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ABSTRACT

The present study aimed to compare the inhibitory effects of alginate coatings containing *Zataria multiflora Boiss* essential oil (ZMEO) in the forms of coarse emulsion and nano-emulsion on the growth of inoculated *Escherichia coli*O157: H7in beef fillet during 16 days of refrigeration at the temperature of 4°C. Alginate solutions (3%) with various concentrations of ZMEO (0.25%, 0.5%, and 1%) were prepared, and the coarse emulsion and nano-emulsion forms were also prepared. The beef fillets were inoculated with *E. coli*O157: H7(1.5×10^5 log CFU/g) and immersed in various alginate treatments, and the bacterial count was performed during refrigeration on days zero, four, eight, 12, and 16.The obtained results indicated that the alginate coating containing ZMEO in both forms (coarse/nano-emulsion) was a proper candidate to control *E. coli*O157: H7at the temperature of 4°C. However, the antibacterial effects were more significant on the samples treated by the nano-emulsion form compared to the coarse emulsion form and controls. In addition, the lowest bacterial growth was observed in the samples coated with the alginate nano-emulsion containing 1% ZMEO (5.3±0.24 log CFU/g) at the end of storage. Therefore, it could be concluded that the use of alginate coatings containing ZMEO (particularly in the nano-emulsion form) could effectively decrease the growth of *E. coli*O157: H7 during storage, and this natural additive could be applied in the food industry, especially the meat industry.

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Introduction

Food-borne diseases are associated with high mortality and economic losses every year and are primarily caused due to the consumption of the products that are contaminated with bacterial pathogens (1).Considering the growing rate of various diseases and their severity across the world, the safety of meat and its products have become increasingly important.

Antibiotics have played a pivotal role in the treatment of infectious diseases over the past decades, while the complications associated with microbial resistance to antibiotic treatment have urged researchers to opt for the use of natural antimicrobial compounds in foods (1).

Enterohemorrhagic *E. coli* is an important foodborne pathogen, which is an *E. coli* subgroup producing Shiga-toxin and could cause hemolytic uremic syndrome and hemorrhagic colitis in humans. The most common serotype is of this bacterium is O157: H7, which has become epidemic, leading to high mortality in Europe, North America, and Canada. Such epidemics are associated with the consumption of half cooked meat and water and food contaminated with cattle manure. This serotype has been isolated from 3.7% (164.6%) of fresh beef samples and 1-2% of other fresh meat products, such as pork, poultry, and lamb (2).Food contamination by *E. coli* O157: H7 has been reported worldwide (3).

An effective approach to enhancing the safety of food such as fresh meat and its products is to use edible coatings. Edible coatings are thin layers of the materials that cover foods, preventing moisture, oxygen, and nutrient-soluble materials from transmission to food products (4). Among the other advantages of edible coatings are eco-friendliness, non-toxicity, non-polluting, and cost-effectiveness. Furthermore, coatings and films could be used as carriers of additives, flavors, antioxidants, and antimicrobials (5).

* *Corresponding author:* Saeid Khanzadi, Department of Food Hygiene and Aquaculture, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. Email: Khanzadi@um.ac.ir, Tel: +985138805610.

Alginate is an important edible coating, which is an alginic acid salt and a polymer of D-β-mannuronic acid (M) and L-α glucuronic (G) units. Alginate is a proper coating candidate owing to its unique colloidal properties, including stability, consistency, suspension, thin-layer coating formation, gel production, and emulsion stabilization (4). In addition, this coating is water-soluble and able to preserve the taste, color, and nutritional value of food products (e.g., vitamin and essential amino acid preservation) (6). The most prominent feature of alginates is their ability to react with multi-capacity metal cations (particularly calcium ions) to produce potent gels or insoluble polymers (7). Essential oils are natural compounds that could be used as additives in coatings and films in order to increase the shelf life and safety of food products. Essential oils are volatile compounds with potent antimicrobial and antioxidant activity and could be applied in various food products to reduce or eliminate pathogens as natural antimicrobial additives (2). *Zataria multiflora Boiss* essential oil (ZMEO) is a well-recognized essential oil containing phenol monoterpene compounds, such as carvacrol, thymol, and P-cymene in various parts of plants (8).

Nanotechnology has become a priority field in today's world trade. The production of nano-emulsions for plasticization and controlling the release of bioactive compounds (e.g., drugs, colors, essential oils, and vitamins) has attracted the attention of researchers in the food industry. Nano-emulsions are submicron emulsions with the droplets size of 50-1,000 nanometers. Nano-emulsions are thermodynamically and synthetically stable, and owing to their special size, they may be transparent or semi-transparent through the naked eye. Moreover, they are stable and resistant against sedimentation and becoming creamy. These properties have rendered nano-emulsions proper options for fundamental and applied studies in chemical, health, pharmaceutical, food sciences, and several other fields (9).

Since beef is a major ingredient in the diet of individuals, its contamination during processing may lead to the survival and growth of *E. coli* O157: H7, which could be easily transmitted to humans, thereby causing severe human epidemics, especially in children, the elderly, and pregnant women. Therefore, the control and prevention of food contamination by this bacterium is of utmost importance.

The present study aimed to enhance food safety against *E. coli* O157: H7 using natural antimicrobial agents as functional coatings in the form of coarse emulsion and nano-emulsion.

Materials and Methods

Experimental Material

Sodium alginate was obtained from Sigma-Aldrich (USA), and sorbitol-MacConkey agar, glycerol, and calcium chloride were provided by Merck (Darmstadt, Germany). The bacterial strain of *E. coli* O157: H7 (NCTC: 12900) was obtained from the microbial collection of the Laboratory of Food and Aquaculture at the School of Veterinary Medicine at Ferdowsi University of Mashhad in Mashhad, Iran.

Preparation of the Bacteria

The bacterial strain was cultured on sorbitol-MacConkey agarcontaining potassium tellurite and cefixime (CT-SMAC) and incubated at the temperature of 37°C for 24 hours. In addition, typical colonies were cultured in 10 milliliters of sterilized brain heart infusion (BHI) broth and incubated at the temperature of 37°C for 24 hours. Afterwards, one milliliter of the sample was collected, re-cultured in 10 milliliters of sterile BHI broth, and incubated at the temperature of 37°C for 18 hours in order to achieve the logarithmic phase of bacterial growth. The bacterial suspension was adjusted to 0.5 McFarland standard turbidity using a spectrophotometer (optical density: 0.85-0.1) at the wavelength of 600 nanometers in order to attain 1.5×10^8 CFU/ml and diluted (1:10) to $1/5\times 10^7$ CFU/ml as the desired bacterial density.

Preparation of the Beef Fillets and Bacterial Inoculation

Fresh beef was purchased from a local market and transferred to the laboratory in an ice box, and 10-gram fillets were prepared. In order to remove the normal microbial flora, the samples were immersed in 70% ethanol solution for 3-5 minutes and sterilized until ethanol was evaporated and the samples were dried. Following the sterilization and drying of the samples, 100 microliters of *E. coli* O157: H7 was inoculated with 1.5×10^7 of the bacteria per milliliter (final dose: 1.5×10^5) on each 10-gram beef fillet, which was finally spread onto the samples using a sterilized L-shaped rod.

Preparation of the Alginate Coating Containing ZMEO in the Form of Simple Emulsion and Nano-emulsion

At this stage, three grams of sodium alginate was dissolved in 100 milliliters of distilled water in order to provide 3% alginate coating. In addition, two grams of calcium chloride was dissolved in 100 milliliters of distilled water in order to prepare 2% calcium chloride solution. Afterwards, 2% glycerol (v/v) was added to the coating solution as the plasticizer and mixed using a heater stirrer. The essential oils were added to the solution at the concentrations of 0.25%, 0.5%, and 1% (w/v). In order to distribute the essential oil in the coating solution, 0.5% of Tween 80was used as the emulsifier.The alginate nano-emulsion containing the essential oil was prepared using the high-energy ultra-sonication method. To this end, the 3% alginate solution was initially prepared, and the nano-emulsion was prepared afterwards using DIAX 900 (Heidolph, Germany) for six minutes and an ultrasound device (200 W HF-power, Bandelin, Germany). At the next stage, the size of the particles was evaluated using DLS (Nano S, Malvern, UK).

Preparation of the Experimental Treatments

All the samples (eight groups) were placed in 3% alginate solution for one minute. After coating, the samples were drained for 30 minutes and re-suspended in 2% calcium chloride solution for 30 seconds (Table 1). Following that, each sample was refrigerated in sterile zipper packs at the temperature of 1±4°C for further analysis on days zero, four, eight, 12, and 16.

Table 1. This study applied treatments

	Treatments	Description
1	Control	Sample without alginate coating
2	Alginate control	Sample with alginate coating
3	0.25% emulsion	Sample with alginate coating containing 0.25% Thymus essential oil as simple emulsion
4	0.5% emulsion	Sample with alginate coating containing 0.5% Thymus essential oil as simple emulsion
5	1% emulsion	Sample with alginate coating containing 1% Thymus essential oil as simple emulsion
6	0.25% Nano-emulsion	Sample with alginate coating containing 0.25% Thymus essential oil as Nano-emulsion
7	0.5% Nano-emulsion	Sample with alginate coating containing 0.5% Thymus essential oil as Nano-emulsion
8	1% Nano-emulsion	Sample with alginate coating containing 1% Thymus essential oil as Nano-emulsion

Counting of E. coli O157: H7

Initially, 10-gram samples were mixed in zipper packs with 90 milliliters of sterilized peptone water and placed in a bag mixer for three minutes in order to obtain a homogeneous suspension (dilution: 10^{-1}). Afterwards, one milliliter of the supernatant was collected by a sampler and poured into a tube containing nine milliliters of sterilized peptone water in order to obtain 10^{-2} dilution. After the preparation of the serial dilutions, they were drop-cultured on the sorbitol-MacConkey agar containing potassium tellurite and cefixime and incubated at the temperature of 37°C for 24 hours of storage.

Statistical Analysis

Data analysis was performed in SPSS version 21, and the data of each group was expressed in the mean, standard deviation, minimum, and maximum of the total bacterial counts during the storage period. Moreover, the bacterial growth of the study groups was analyzed for 16 days using repeated measures ANOVA, and Bonferroni's post-hoc test was also used to compare the treatments. The P-value of less than 0.05 was considered statistically significant.

Results and Discussion

Table 1 shows the growth patterns of the inoculated *E. coli* O157: H7 in various treatments during storage. Accordingly, the number of the bacteria decreased in all the groups during this period as the studied strains were mesophile bacteria. The highest and lowest microbial load were observed in the alginate group (4.46 log CFU/g) and nano-emulsion group with the ZMEO concentration of 1% (2.11 log CFU/g), respectively after the storage period, which confirmed the significant antimicrobial properties of the nano-emulsion of the alginate coating containing the essential oil. In another study, Moghimi et al. (2016) evaluated the effects of the pure form and nano-emulsion of *Thymus daenensis* on *E. coliin-vitro*, reporting that the antibacterial properties of the nano-emulsion form were more significant compared to the pure form due to its increased ability in destroying the bacterium membrane; this is consistent with the results of the present study (10). In addition, Sorino et al. (2015) investigated the antibacterial activity of chitosan-based modified coatings containing nano-treated essential oils, gamma

rays, and modified atmosphere packaging independently and in combination with green beans inoculated with *E. coli* O157: H7 during 13 days at the temperature of 4°C. According to the findings, the nano-treated carvacrol had the highest antibacterial activity against *E. coli* O157: H7. Since the antibacterial activity of *Zataria multiflora Boiss* depends on carvacrol as its major component, the mentioned finding is in line with the current research (11).

According to the results of the present study, the alginate coating containing the essential oil (0.25%, 0.5%, and 1%) was more effective in the reduction of bacterial growth compared to the alginate group alone. In another research, Heydari et al. (2015) assessed the impact of sodium alginate coating containing 0.5% and 1% of oregano essential oil on the quality of carp fillets maintained at the temperature of 4°C (12). According to the obtained results, the samples treated with the sodium alginate containing the essential oil could reduce bacterial growth more effectively compared to the control and alginate groups, which is consistent with our findings (13).

Table 2. Average logarithmic reduction of *E. coli* O_{157}: H_7 in different study groups

Mean Difference I-J / Group (I)	Group (J) Alginate	AL+EO 0.25%	AL+EO 0.5%	AL+EO 1%	AL+EO 0.25% nano	AL+EO 0.5% nano	AL+EO 1% nano
Control	-0/0052	0/51***	0/63***	0/83***	0/82***	1/06***	**1/41*** **
Alginate		0/52***	0/64***	0/87***	0/83***	1/07***	**1/42*** **
AL+EO 0.25%			0/12	0/35***	0/31***	0/55***	**0/90*** **
AL+EO 0.5%				0/22*	0/18***	0/42***	**0/77*** **
AL+EO 1%					-0/04	0/19***	**0/54*** **
AL+EO 0.25% nano						0/24*	**0/58*** **
AL+EO 0.5%nano							**0/34*** **

Description: The values being entered at the intersection of the two groups show logarithmic mean difference (I-J) during the study period. Duplicate values were not written here. *** : 0/001< .p and* :0/05<p،<

Table 2 shows the reduction of *E. coli* O157: H7 counts and their comparison during 16 days of refrigeration. Accordingly, the most significant difference was observed between the alginate group and nano-emulsion at 1% concentration (1.42 log CFU/g). Furthermore, the comparison between the alginate coating treatments containing the essential oil in the form of simple emulsion and nano-emulsion revealed that the nano-emulsion treatments were more effective compared to the simple emulsion treatments at similar concentrations, indicating the enhanced antimicrobial properties of the essential oil in the nano-emulsion treatments, which increased significantly at the higher concentrations of the essential oil.

In a similar research, Hashemi et al. (2017) investigated the antimicrobial properties of the nano-emulsion of *Zataria multiflora Boiss* as an additive to polymer-based packaging materials, and the obtained results indicated that the increased concentration of the nano-treated essential oil was associated with more significant antimicrobial effects, which is consistent with the results of the present study (12).

According to the findings of the current research, the bacterial changes in *E. coli* O157: H7 decreased from 5.36% to 4.46% in the alginate treatment during 16 days of storage, while no significant difference was observed with the control group. On the same note, Fatih et al. (2009) reported that the alginate group with no antimicrobial agents had no impact on the reduction of the *E. coli, Listeria Ivanova*, and *Pseudomonas fluorescens*, which is in line with our findings (15).

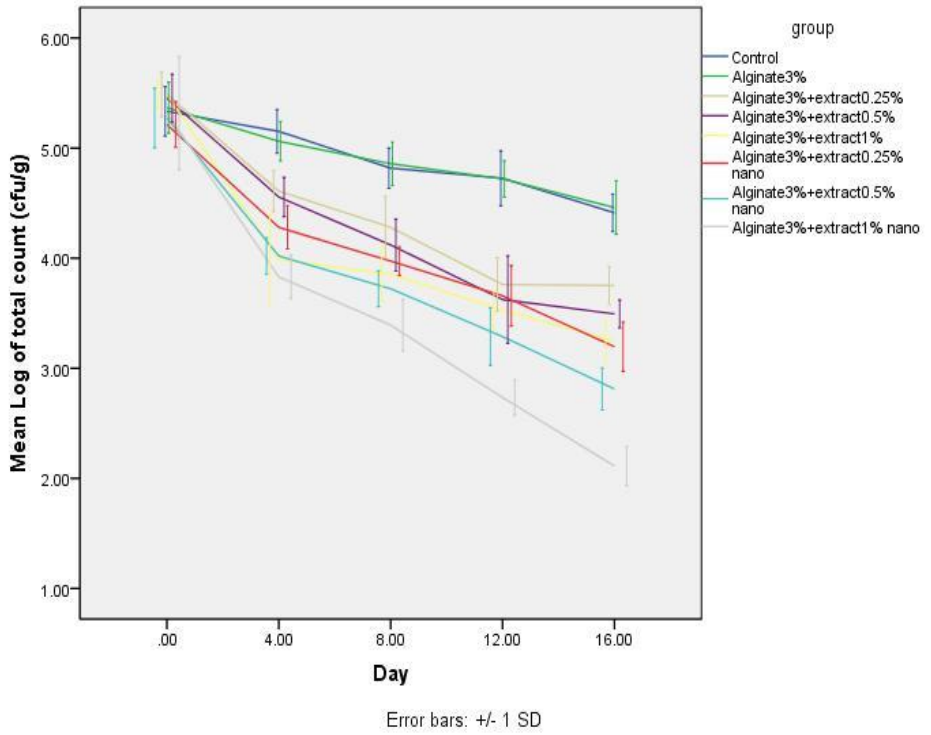

Figure 1. Logarithmic variation of the number of E. coli O_{157}: H_7 of different groups during the study period

Conclusion

The growth inhibition of *E. coli* O157: H7 was investigated in alginate treatments containing the ZMEO in the form of simple emulsion and nano-emulsion at various concentrations (0.25%, 0.5%, and 1%). According to the results, the use of alginate coating containing ZMEO in the nano-emulsion form could reduce the *E. coli* population on the refrigerated beef fillets more effectively compared to the simple emulsion for at identical concentrations. Moreover, significant results were observed regarding the effects of the treatment on the growth of *E. coli* O157: H7during storage.Finally, the obtained results demonstrated that the nano-emulsion group at 1% concentration had the optimal outcomes regarding the growth inhibition of *E. coli* O157: H7 in the beef fillets. Considering the priority of using natural additives in food products by manufacturers and consumers, it is recommended that alginate coating solution containing *Zataria multiflora Boiss* be applied in beef fillets in order to increase its safety against pathogenic bacteria. It is also notable that such treatments alone cannot entirely eliminate food contamination, and hurdle technology and other storage techniques (e.g., heating) should also be applied to effectively remove various contaminants.

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Conflict of Interests

None declared.

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