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Effect of Chitosan Coating Nano-emulsion Containing *Zataria multiflora and Bunium persicum* Essential Oils on *Escherichia Coli* O_{157}:H_7 in Vacuum-packed Rainbow Trout Fillet

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ABSTARACT

Background: Active antimicrobial packaging is a novel method for increasing the safety and shelf life of food products. The present study aimed to investigate the inhibitory effects of chitosan coating nano-emulsion incorporated with *Zataria multiflora* and *Bunium persicum* essential oils at the concentrations of 0.5% and 1%, respectively on *E. coli* O_{157}:H_7 in vacuum-packed fish samples during 12 days of refrigeration.
Methods: The samples were divided into various groups, including control (no coating), 2% chitosan, 2% chitosan nano-emulsion, and chitosan coating nano-emulsion containing *Zataria multiflora* and *Bunium persicum* essential oils at the concentrations of 0.5% and 1%, respectively. The samples were vacuum-packed and stored at refrigeration temperature, and bacterial counting was performed on days zero, one, two, four, six, eight, and 12.
Results: The mean bacterial count had a significant difference between the study groups during 12 days of storage ($P < 0.001$). The most significant inhibitory effect on the growth of *E. coli* O_{157}:H_7 was observed with nano-emulsion of chitosan containing 1% of *Bunium persicum.*
Conclusion: According to the results, using the nano-emulsion of chitosan coating with essential oils could effectively decrease the growth of *E. coli* O_{157}:H_7 in food products, especially fish.in food especially fish.

1. Introduction

Fish is most commonly consumed by humans and has several health benefits. However, fish is considered to be the most vulnerable meat and a common source of disease outbreaks across the world, which also affects its shelf life and safety [1]. The high rate of foodborne diseases outbreaks was reported by the Center for Science in the Public Interest (CSPI) following the consumption of contaminated seafood products during 1990-2002 [2].

Seafood products could be a major source of foodborne pathogens, such as *E. coli* O_{157}:H_7, which has been reported to cause various diseases and severe health complication. This highlights the need for a novel approach to the thorough control and reduction of bacterial growth in these food products. These bacteria are prone to various outbreaks involving different food products due to cross-contamination, particularly through post-processing contamination [3].

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Today, use of natural preservatives (mainly medical plants) has been on the rise since individuals are more conscious of the lateral adverse effects of chemical preservatives on health. In this regard, use of edible coatings and antimicrobial agents such as chitosan and essential oils has been notable [4]. Essential oils (EOs) could be extracted from the flowers, buds, seeds, leaves, bark, herbs, fruits, and roots of various plants [5]. These secondary metabolites could restrain the growth of foodborne pathogens and food spoilage bacteria [6]. Terpenoids and phenylpropanoids are the major constituents of EOs, which exert variable biological effects on food products, including antibacterial, anti-fungal, and antioxidant effects [7].

Zataria multiflora is a member of the Lamia¬ceae family, and its EO is a natural additive used in food preservation owing to its remarkable antioxidant and antimicrobial properties. Thymol and carvacrol are the two main constituents of this herbal essential oil [8]. *Bunium persicum* is a member of the Apiaceae family, which grows in different regions of Asia, such as central Asia, Iran, Pakistan, Afghanistan, and India [9]. The EO of this plant contains high concentrations of various antibacterial agents due to the high levels of oxygenated monoterpenes, mainly γ-terpinene, cuminaldehyde, ρ-cymene, and limonene [10].

Active, edible coatings increase the quality of coated food products and their nutritional value. Chitosan is the deacetylated form of chitin with remarkable properties, such as biodegradability and ability to form coating films, which could be used in agriculture and food preservation [11]. Several hypotheses have been proposed regarding the antibacterial activity of chitosan, and a hypothesis has been linked to its structure [12]. Chitosan could be used for the formation of films and gels so as to improve the organo¬leptic properties of food products in terms of permeability to humidity, oxygen, and functional substances, such as flavoring additives, antioxidants, vitamins, and coloring agents. On the other hand, chitosan has been extensively applied in the biomedical membrane [13].

Nano-emulsions are among the most promising systems to improve the solubility, bioavailability, and functionality of hydrophobic compounds. Nano-emulsions have numerous applications since they could act as delivery systems for lipophilic compounds, such as nutraceuticals, drugs, flavors, antioxidants, and antimicrobial agents [14]. The nano-emulsion of coating solutions and Eos has been reported to offer higher antibacterial activity compared to conventional emulsions [15]. Nano-emulsions are produced by multiple approaches, such as low-energy and high-energy techniques. One of the high-energy methods in this regard is ultrasonic emulsification, which could be effectively applied to prepare nano-emulsions with small droplet diameters and low size distribution [16].

Vacuum packaging is packaging in rigid or flexible containers, from which substantially all the air has been eliminated before the sealing of the package. It is considered to be an efficient system for the distribution and long-term storage of fresh meat. Vacuum packaging is commonly used owing to the high public demand for fresh fish. This type of packaging could decrease bacterial growth and fat oxidation [17].

Several recent studies have been focused on increasing the shelf life and safety of food products using natural antimicrobial agents, such as edible coatings and EOs [18]. Previous studies have denoted the use of chitosan-based edible coatings and EOs in various food products [19, 20]. However, few studies have investigated the use of nano-chitosan solutions with EOs in vacuum seafood [21, 22].

The present study aimed to evaluate the effects of nano-chitosan solutions with *Zataria multiflora* and *Bunium persicum* EOs on vacuum-packed rainbow trout fillet.

2. Materials and Methods

2.1. Experimental Materials

The EOs of *Zataria multiflora* and *Bunium persicum* were purchased from the Iranian Institute of Medicinal Plants in Karaj, Alborz province, Iran. All the culture media were purchased from QUELAB (Quelab Laboratories Inc., Montreal, Canada). Chitosan with low molecular weight (LMW; 1.03×10^5) and 91% deacetylation degree was also obtained from Sigma-Aldrich Company (St. Louis, MO, USA), and *E. coli* O_{157}:H_7 (NCTC 12900) was obtained from the Department of Food Hygiene at the School of Veterinary Medicine at Ferdowsi University of Mashhad in Mashhad, Iran.

2.2. Preparation of the Chitosan Coatings

The chitosan solution was prepared with 2% (w/v) in 1% (v/v) acetic acid. In this process, two grams of chitosan were blended with 100 milliliters of distilled water, and the solution was mixed by a hotplate magnetic stirrer at the temperature of 40 °C for 10 minutes in order to become transparent. Afterwards, glycerol was added to chitosan at the concentration of 0.75 ml/gr as a plasticizer and stirred for 10 minutes [23].

2.3. Preparation of Chitosan Coating Nano-emulsion Containing Zataria multiflora and Bunium persicum Eos

At this stage, the EOs of *Zataria multiflora* and *Bunium persicum* (concentrations of 0.5% and 1%, respectively) were added to the prepared chitosan solution using Tween 80 (0.2 g) as an emulsifier, and the solution was stirred for 30 minutes to form a transparent solution. Following that, the coating solutions were subjected to ultra turrax for three minutes at 3,000 rpm and ultrasonic emulsification sonicator (50 °C, pulse, 45 seconds, rest, and 15 seconds) for six minutes. Afterwards, the particle size of the solution was measured using the DLS device (Nanophox Sympatec GmbH, Clausthal, Germany) [24].

2.4. Preparation of Rainbow Trout Fillets and Inoculation of the Bacteria

Fresh rainbow trout fish (Oncorhynchus mykiss) with the mean weight of 700 ± 50 grams were purchased from a local fish farm (Mashhad, Iran) in summer 2017. The fish were filleted and transferred to the laboratory aseptically in a cool box. Following that, the fillets were washed, slimed, dried, cut to pieces (weight: 10 g), and burnt to exterminate

the surface microorganisms. *E. coli* O_{157}:H_7 were inoculated using adjustable volume micropipettes on each side of the fillets separately to the final concentration of ~10^6 CFU/g [4].

2.5. Treatments

The samples that were inoculated with bacteria were divided into seven groups (Table 1). The treatment of the samples involved immersion in the chitosan solution (one minute), drainage (15 minutes), drying, and vacuum packaging, which was performed manually or automatically by placing the samples in a plastic film package, removing air from inside the package (low oxygen concentration: <1%), and sealing the package using a vacuum device (Henkelman, Hertogenbosch, Netherlands). Afterwards, the samples were stored at the temperature of 4 ± 1°C for 12 days to be analyzed at seven-day intervals (zero, one, two, four, six, eight, and 12) [18].

2.6. Enumeration of E. coli O_{157}:H_7

Initially, the fillets (10 g) were brought to the final volume of 90 milliliters with 0.1% sterile peptone water and homogenized using a stomacher (Seward Medical, London, UK) for three minutes. Following that, decimal dilutions were prepared, and 10 microliters of the serial dilutions of the homogenates were plated on Sorbitol-MacConkey agar (SMAC) (Quelab Laboratories Inc., Montreal, Canada) for the enumeration of *E. coli* O_{157}:H_7. Afterwards, the SMAC agar plates were incubated at the temperature of 37 °C for 24 hours [25].

2.7. Statistical Analysis

Data analysis was performed in SPSS version 21. The process of the changes in the logarithmic bacterial count was analyzed using repeated measures ANOVA within a 12-day period. The paired comparison of the study groups was carried out using Bonferroni post-hoc test. In all the statistical analyses, P-value of less than 0.05 was considered significant.

3. Results and Discussion

3.1. Enumeration of E. coli O_{157}:H_7

Figure 1 depicts the effect of the treatments on the growth of *E. coli* O_{157}:H_7 during 12 days of storage. The initial count of *E. coli* O_{157}:H_7 was 6.69 ± 0.13 log CFU/g, which decreased during storage in all the samples, especially in the nano-chitosan+1% *Zataria multiflora* EO samples (4.06 ± 0.15 log CFU/g) and nano-chitosan+1% Bunium persicum EO samples (3.81 ± 0.06 log CFU/g). This finding is consistent with the results of the previous studies in this regard [18,26]. In another research, Ehsani and Hashemi reported that the combined treatment of antimicrobial coating, gamma irradiation, and MAP led to the reduction of microbial populations to undetectable levels [27]. In the present study, the reduction of the bacterial counts was considered significant in nano-chitosan+1% Zataria multiflora EO and nano-chitosan+1% Bunium persicum EO samples due to great impact of the nano-chitosan solution and EOs.

During the 12-day period in the current research, the bacterial count in the vacuum samples in the control group decreased from 6.69 ± 0.13 to 5.30 ± 0.15 since this bacterium is mesophilic, and the vacuum conditions restrained its growth [28]. Comparison of the chitosan and nano-chitosan treatments in the present study indicated the more significant reduction of the bacterial count in the nano-chitosan treatment compared to the chitosan treatment [29]. In general, it could be stated that the use of combinational antimicrobial agents was more effective against microbial growth compared to their separate use. This finding has been confirmed in several previous studies [27,30] confirmed; however, antagonistic, synergistic or additive effects may be involved depending on the type of antimicrobial agents and microorganisms.

Table 2 shows the mean reduction rate of *E. coli* O_{157}:H_7 counts in the comparison of the treatments. In fact, almost all the treatments caused a significant difference in the mean rate of *E. coli* O_{157}:H_7 counts in comparison ($P < 0.001$).

Table 1: List of treatments in the present study

Treatment		Description
1	CON	Control: vacuum Samples without any coating solution
2	CS	Vacuum Samples coated with chitosan solution
3	Nano-CS	Vacuum Samples coated with Nano-chitosan solution
4	Nano-CS+ ZMEO0.5%	Vacuum samples coated with Nano-chitosan solution containing 0.5% (w/v) *Zataria multiflora* essential oil
5	Nano-CS+ BPEO0.5%	Vacuum Samples coated with Nano-chitosan solution containing 0.5% (w/v) *bunium persicum* essential oil
6	Nano-CS+ ZMEO1%	Vacuum Samples coated with Nano-chitosan solution containing 1% (w/v) *Zataria multiflora* essential oil
7	Nano-CS+BPEO1%	Vacuum Samples coated with Nano-chitosan solution containing 1% (w/v) *bunium persicum* essential oil

Table 2: Average reduction rate of *E. coli* O_{157}:H_7 counts among treatments when compared together during storage

Mean Difference	CS	Nano-CS	Nano-CS+ZMEO0.5%	Nano-CS+BPEO0.5%	Nano-CS+ZMEO1%	Nano-CS+BPEO1%
CS	0.23*	0.45**	0.71**	0.97**	1.20**	1.40**
Nano-CS		0.22*	0.47**	0.74**	0.97**	1.17**
Nano-CS+ZMEO0.5%			0.25	0.51**	0.75**	0.95**
Nano-CS+BPEO0.5%				0.26*	0.49**	0.69**
Nano-CS+ZMEO1%					0.23*	0.43**
Nano-CS+BPEO1%						0.19

* P < 0.05, ** P < 0.001.

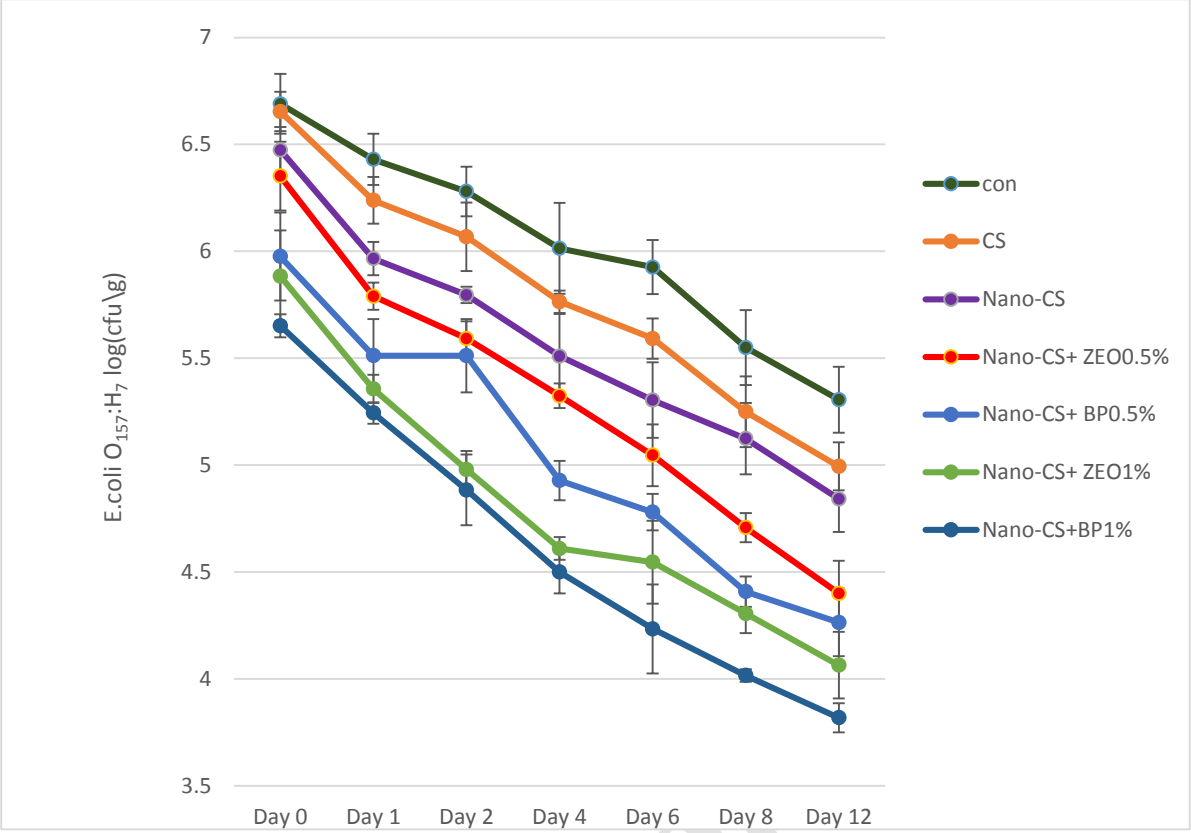

Figure 1: Effect of treatments on the growth of *E. coli* O_{157}:H_7 during storage

As can be seen, the highest reduction rate of *E. coli* O_{157}:H_7 (1.4 log CFU/g) was observed in the nano-chitosan+1% Bunium persicum EO samples compared to the control samples.

Comparison of the EOs used in the present study indicated that Bunium persicum exerted significant antibacterial effects against *E. coli* O_{157}:H_7 at the concentration of 1%, while the EO of *Zataria multiflora* had similar effects against *E. coli* O_{157}:H_7 at the concentration of 1% ($P > 0.05$). Therefore, it could be concluded that the higher concentration of the EOs was associated with their increased antimicrobial effects.

4. Conclusion

According to the results, use of the nano-emulsion of chitosan solution with the EOs of *Zataria multiflora* and *Bunium persicum* had potential antimicrobial effects against foodborne pathogens, such as *E. coli* O_{157}:H_7. Moreover, this effect could be improved by using the higher concentration of the EOs (1%). Our findings also demonstrated that the treatments with nano-chitosan+1% *Zataria multiflora* EO and nano-chitosan+0.5% *Bunium persicum* EO exerted the optimal effects against bacterial growth. In the control group, the bacterial counts were also observed to decrease, which could be due to the application of vacuum packaging to control the growth of *E. coli* O_{157}:H_7 in the fish fillets.

Authors' Contributions

F.R., performed laboratory works, S.Kh., designed the study as M.H., revised the manuscript, and M.A., performed statistical analysis.

Conflict of Interest

The authors report no conflict of interest.

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