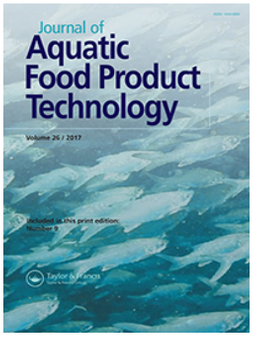

Journal of Aquatic Food Product Technology

ISSN: 1049-8850 (Print) 1547-0636 (Online) Journal homepage: http://www.tandfonline.com/loi/wafp20

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To cite this article: Fateme Sharifi, Saeid Khanzadi, Mohammad Hashemi & Mohammad Azizzadeh (2017) Control of Listeria Monocytogenes and Escherichia coli O_{157}:H_7 Inoculated on Fish Fillets Using Alginate Coating Containing Lactoperoxidase System and Zataria multiflora Boiss Essential Oil, Journal of Aquatic Food Product Technology, 26:9, 1014-1021, DOI: 10.1080/10498850.2017.1375057

To link to this article: http://dx.doi.org/10.1080/10498850.2017.1375057

Published online: 29 Sep 2017.

JOURNAL OF AQUATIC FOOD PRODUCT TECHNOLOGY
2017, VOL. 26, NO. 9, 1014–1021
https://doi.org/10.1080/10498850.2017.1375057

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Control of *Listeria Monocytogenes* and *Escherichia coli* O_{157}:H_7 Inoculated on Fish Fillets Using Alginate Coating Containing Lactoperoxidase System and *Zataria multiflora* Boiss Essential Oil

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ABSTRACT

The present study was conducted to investigate the inhibitory effect of alginate coating incorporated with *Zataria multiflora* Boiss essential oil (ZEO) and lactoperoxidase system (LPOS), individually and in combination, in order to control inoculated *Listeria monocytogenes* and *Escherichia coli* O_{157}:H_7 in rainbow trout fillets during 16days of storage at 4°C. The antibacterial activity of ZEO was evaluated against *Listeria monocytogenes* and *E. coli* O_{157}:H_7 through determination of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC). *In vivo* study was performed by inoculating four strains of *L. monocytogenes* as culture cocktail and one strain of *E. coli* O_{157}:H_7 on trout fillets for their survival evaluation during the 16-day storage. Results indicated that ZEO and LPOS, when used in combination in alginate solution, had a stronger effect on the control of mentioned bacteria in trout fillets; however, their individual use could significantly inhibit their growth, when compared to the control. Moreover, it has been implicated that alginate coating, when used with no antimicrobial agent, had a supportive effect on the growth of these pathogenic bacteria. Therefore, the application of alginate coating containing ZEO and LPOS is recommended in foods, especially fish and fish products.

KEYWORDS

Alginate coating; Avishan Shirazi; lactoperoxidase; pathogen inoculation; rainbow trout fillet

Introduction

Seafood has high nutritional value, and it is rich in proteins, minerals, vitamins, and omega-3 polyunsaturated fatty acids (n-3 PUFA) with a high bioavailability (Rebole et al., 2015). On the other hand, it is sensitive to contamination by microorganisms and notably by pathogenic bacteria such as *E. coli* O_{157}:H_7 and *L. monocytogenes* (Oussalah et al., 2007). Fish and fish products can cause outbreaks of foodborne diseases (Ozer and Demirci, 2006b). A notable level of outbreaks of food borne diseases has been reported by Center for Science in the Public Interest (CSPI) following consumption of contaminated seafood and seafood products during 1990–2002 (Ozer and Demirci, 2006b).

E. coli O_{157}:H_7 produces enterohaemorrhagic toxins and other virulence factors that could result in various forms of diarrhea (mild to severe, bloody and painful) and lead to severe complications (Djenane et al., 2011). It is responsible for many outbreaks involving various types of food products due to surface contamination mainly by post-processing contamination (Min et al., 2005a; Ozer and Demirci, 2006b), although it also can be present in seafood products due to pond water contamination. This risk can be caused by the application of animal manures, especially bovine manure as pond fertilizers (Djenane et al., 2011).

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L. monocytogenes is an important foodborne pathogen widely present in seafood products, with a contamination rate of 0% to more than 50% (Zarei et al., 2012). It can grow in a wide range of temperature (0–45°C) and pH (4.6–9.6) and can form biofilm to survive under adverse conditions and on the surface of the equipment (Min et al., 2005a). *L. monocytogenes* causes Listeriosis following the consumption of contaminated foods, which could result in serious health problems especially in the elderly, pregnant women, and people with weakened immune system (Tocmo et al., 2014). Therefore, it is important to control food contamination, especially seafood, in order to decrease the outbreak of foodborne diseases (Min et al., 2005a).

In recent years, much research has focused on the use of natural origin preservatives in food (Burt, 2004). Because preservatives can extend food's shelf life with no side effects, there is an increased tendency to apply them by food producers and researchers (Burt, 2004). In this regard, edible coatings and films are of importance; since they can be used as a carrier of food additives such as flavors, antimicrobials, antioxidants, enzymes, and colors, and they can also maintain high levels of these additives on the surface of foods (Cutter, 2006; Raeisi et al., 2016).

Essential oils (EOs) with plant origins are rich in phenolic compounds such as flavonoids phenolic acids, which have shown antimicrobial activity against a wide variety of microorganisms (Raeisi et al., 2012). Generally gram-negative bacteria compared to gram-positive bacteria are more resistant to antibacterial effects of EOs, because the hydrophilic lipopolysaccharides in the outer membrane of gram-negative bacteria create a barrier against hydrophobic compounds such as EOs (Hashemi et al., 2013); however, this was not always true (Aminzare et al., 2014; Oussalah et al., 2007). *Zataria multiflora* Boiss essential oil (ZEO) is a known spice used in food preservation due to its strong antioxidant and antimicrobial properties. It is also used in medicine to treat cases of respiratory tract infection, as an antiseptic, antitussive, as well as in treatment of irritable bowel syndrome (Tajik et al., 2015).

Lactoperoxidase (LPO) is a natural single chain poly-peptide that secretes in milk, saliva, and tears and has no antibacterial effect on its own. LPO system (LPOS) consists of 3 compounds: LPO enzyme, thiocyanate ion (SCN−), and hydrogen peroxide (H_2O_2). The enzyme oxidizes SCN− in the presence of H_2O_2, producing hypothiocyanite (OSCN−) and hypothiocyanous acid (HOSCN) that oxidize sulphydryl (−SH) groups of microorganisms. LPOS is a well-known broad spectrum antimicrobial with a bactericidal effect against gram-negative bacteria and bacteriostatic effect against gram-positive bacteria (Jasour et al., 2015), and it is generally recognized as safe (GRAS) (Elliot et al., 2004).

There have been many studies focusing on the application of natural antimicrobials in food; however, few studies have used natural antimicrobials to control food-borne pathogenic bacteria in fish (Datta et al., 2008; Ozer and Demirci, 2006b; Tocmo et al., 2014). Furthermore, few studies have used sodium alginate for coating solutions or films in fish (Datta et al., 2008; Heydari et al., 2015), and there have been no studies regarding effects of LPOS and ZEO as natural antimicrobials against food-born pathogenic bacteria including *E. coli* O_{157}:H_7 and *L. monocytogenes* in fish. Accordingly, the present study was conducted to determine the effect of alginate coating solution incorporated with natural antimicrobials (LPOS and ZEO) individually and in combination to control *E. coli* O_{157}:H_7 and *L. monocytogenes* in rainbow trout fillets and to ensure its microbiological safety and also to decrease the hazards of the presence of these pathogenic bacteria.

Materials and methods

Materials

ZEO was purchased from Iranian Institute of Medicinal Plants, Karaj, Alborz province, Iran, and all culture media were purchased from Merck (Darmstadt, Germany). The lyophilized cultures of four strains of *L. monocytogenes* (ATCC: 7644, 7834, 10671, 82119) were purchased from Iranian Biological Resource Center, Tehran, Iran, and *E. coli* O_{157}:H_7 NCTC 12900 was prepared from Department of Food

Hygiene, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran. All the applied reagents were of analytical grade and purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Determination of minimum inhibitory concentration and minimum bactericidal concentration values of the ZEO

The micro-dilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of ZEO by 96-well micro-plates (Ehsani et al., 2016). Bacterial suspensions (four strains of *L. monocytogenes* and one strain of *E. coli* O_{157}:H_7) were prepared by culturing the bacteria in 9 mL of brain heart infusion (BHI) broth and incubating at 37°C for 24 h. The suspensions were adjusted to 0.5 McFarland standard turbidity (1.5×10^8 CFU/mL) and diluted to the desired bacterial density (1.5×10^7 CFU/mL). ZEO was dissolved in 10% dimethyl sulfoxide in a concentration range of 40–0.31 mg/mL. Then, 20µl of the inoculums with 20µL of different concentrations of ZEO were added to the wells containing 160 µL of BHI broth. Wells without any bacteria (180 µL of BHI broth and 20 µL of the ZEO) and wells without ZEO (180 µL of BHI broth and 20 µL of the inoculums) were considered as negative and positive control, respectively. The final volume of each well was 200 µL; the final concentration of the inoculums was approximately 1.5×10^6 CFU/mL; and the final concentration of ZEO was in range of 0.031 to 4 mg/mL. The micro-plates were incubated at 37°C for 18–24 h under constant shaking (50–100 rpm) by a shaker incubator (GFL 3031), and the lowest concentrations with no visible bacterial growth were regarded as MIC values of ZEO against the tested bacteria. For determination of MBC values, non-turbid wells were inoculated on BHI agar and incubated at 37°C for 24 h; the lowest concentrations with no visible bacterial growth on the agar were regarded as MBC values of ZEO against tested bacteria.

Preparation of the LPOS

LPOS was prepared according to the method previously described (Shokri et al., 2015). Briefly, LPOS components (weight ratios: 1.00, 0.35, 108.70, 1.09, and 2.17 for the LPO, glucose oxidase, D-(α)-glucose, potassium thiocyanate, and H_2O_2, respectively) were dissolved in phosphate buffer (50mL, pH 6.2) based on 15.5 mg of LPO. The solution was filtered using a filter paper and incubated at 23°C for 24 h under constant shaking (160 rpm) by a shaker incubator (GFL 3031) in order to boost antimicrobial activity of LPOS.

Preparation of trout fillets and inoculation of the bacteria

Fresh rainbow trout fish (*Oncorhynchus mykiss*) with an average weight of 300 ± 50 g were purchased from a local fish farm (Mashhad County, Iran) in summer, 2015, filleted and immediately transported to the laboratory. The fillets were then washed to remove blood and slime and then dried. The fillets were cut into pieces weighing 25 ± 1 g, sprayed with ethanol (95% v/v), then burnt and trimmed to eliminate the surface microorganisms (Raeisi et al., 2016). Aliquots of 125 µL of a 10^8 CFU/mL dilution of 4 strains of *L. monocytogenes* as the culture cocktail along with one strain of *E. coli* O_{157}:H_7 were inoculated (using adjustable volume micropipettes) on each side of separate fillets to a final concentration of ~ 10^6 CFU/g (Raeisi et al., 2016).

Preparation of coating solutions and treatments

Alginate solutions were prepared by dissolving the alginate powder (3% w/v) in sterilized distilled water containing 2% glycerol as plasticizer at a controlled temperature (70°C), and they were constantly stirred for 30 min to become clear. Calcium chloride was dissolved (2% w/v) in distilled water and sterilized by autoclaving at 121°C for 15 min. Then, the boosted LPOS (5%) and ZEO (0.5 and 1%) were added to the solutions. ZEO was dissolved in alginate solutions using tween 80 (0.2 g/g $_{EO}$) as emulsifier at a controlled temperature (40°C) and stirred for 30 min to create a uniform, stable, and clear solution. Inoculated trout fillets were divided into seven groups as treatments, as described in Table 1. Then, they were immersed in

Table 1. List of treatments in the present study.

	Treatment	Description
1	CON	Control: Samples without any coating solution
2	ALG	Samples coated with alginate solution
3	EO 0.5%	Samples coated with alginate solution containing 0.5% (w/v) *Zataria multiflora* EO
4	EO 1%	Samples coated with alginate solution containing 1% (w/v) *Zataria multiflora* EO
5	ENZ	Samples coated with alginate solution containing 5% (v/v) Lactoperoxidase system
6	ENZ+EO 0.5%	Samples coated with alginate solution containing 0.5% (w/v) *Zataria multiflora* EO in combination with 5% (v/v) Lactoperoxidase system
7	ENZ+EO 1%	Samples coated with alginate solution containing 1%% (w/v) *Zataria multiflora* EO in combination with 5% (v/v) Lactoperoxidase system

alginate solutions (1 min), drained (30 s), immersed in $CaCl_2$ solution (30 s), and stored at 4 ± 1°C for 16 days to be analyzed at 4-day intervals: 0, 4, 8, 12, and 16 (Raeisi et al., 2016; Shokri et al., 2015).

Enumeration of L. monocytogenes and E. coli O_{157}:H_7

For enumeration of the inoculated bacteria, the fillets (25 g) were brought to a final volume of 250 mL with 0.1% sterile peptone water and then homogenized by a stomacher (Seward Medical, London, UK) for 3 min. Decimal dilutions were prepared, and 10 µL (Naghili et al., 2013; Shokri et al., 2015) of serial dilutions of homogenates were plated on PALCAM agar (Ozer and Demirci, 2006a) and on Cefixime Tellurite Sorbitol MacConkey (CT-SMAC) agar (Shin et al., 2004) for enumeration of *L. monocytogenes* and *E. coli* O_{157}:H_7, respectively. PALCAM agar plates were incubated at 30°C for 48 h, while CT-SMAC agar plates were incubated at 37°C for 24 h.

Statistical analysis

All the tests of the present study were performed in triplicate. Statistical analysis was carried out using SPSS ver. 21 software (SPSS, Inc. Chicago, IL, USA). Repeated measure analysis of variance (ANOVA) followed by Bonferroni post- hoc test or Dunnette T3 tests were used to determine the significant differences at $P < 0.05$ level.

Results and discussion

MIC and MBC values of ZEO

The results showed significant sensitivity of the tested bacteria to the antibacterial effect of ZEO (Table 2). MIC values of *L. monocytogenes* were similar in all four examined strains and were lower than MIC values of *E. coli* O_{157}:H_7, indicating higher sensitivity of *L. monocytogenes* than *E. coli* O_{157}:H_7. Similar results were obtained in previous studies (Aliakbarlu et al., 2013; Ehsani et al., 2014; Elizaquível et al., 2013; Tajik et al., 2015). Based on this result and the results of former studies on LPOS (Min et al., 2005a; Shokri et al., 2015), the best concentrations (0.5 and 1% for the ZEO and 5% for the LPOS) were selected to be added to the coating solutions.

Enumeration of L. monocytogenes and E. coli O_{157}:H_7

Figures 1 and 2 represent the effect of the treatments on the growth of *L. monocytogenes* and *E. coli* O_{157}:H_7 during the 16 days of storage. The initial count of *L. monocytogenes* was ca. 6.23 ± 0.17 log CFU/g, which significantly increased during the storage time in all groups. This result is completely consistent with the results of former studies (Datta et al., 2008; Elliot et al., 2004). The respective growth rate was significantly more rapid in ALG and CON samples than in other samples ($P < 0.05$) and reached 9.14 ± 0.11 and 8.8 ± 0.35 log CFU/g, respectively, at the end of the storage time. The

Table 2. Antibacterial properties (MIC, MBC) of *Zataria multiflora* EO against the tested bacteria by micro-dilution method.

Strains	MIC (mg/mL)	MBC (mg/mL)
E. coli O157H7	1	1
L. monocytogenes (ATCC: 7644)	./5	1
L. monocytogenes (ATCC:7834)	./5	1
L. monocytogenes (ATCC:10671)	./5	1
L. monocytogenes (ATCC:82119)	./5	1

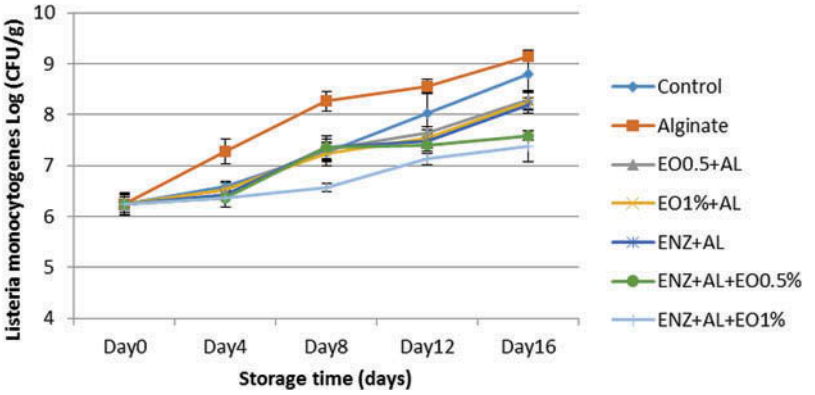

Figure 1. Changes in bacterial count (CFU/g) of trout fillet samples inoculated with *L. monocytogenes* during storage (M ± SD).

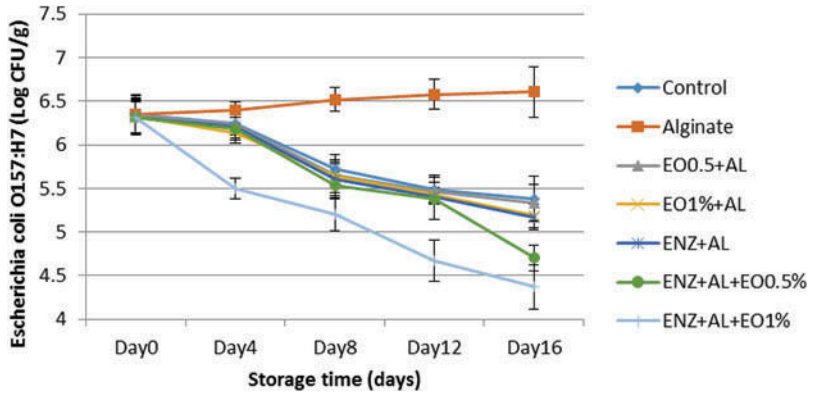

Figure 2. Changes in bacterial count (CFU/g) of trout fillet samples inoculated with *E. coli* O_{157}:H_7 during storage (M ± SD).

lowest final counts were observed in ENZ+EO 1% and in ENZ+EO 0.5% samples (7.37 ± 0.3 and 7.59 ± 0.09 log CFU/g), respectively, showing the highest antibacterial effects of ZEO and LPOS when used in combination. Heydari et al. (2015) indicated that alginate coating enriched with two levels (0.5 and 1%) of horsemint (*Mentha longifolia* EO) could decrease microbial growth (total viable count and total psychrotrophic count) in bighead carp fillets. Min et al. (2005b) reported that whey protein films and coatings incorporated with LPOS could decrease the growth of *L. monocytogenes* in media culture as well. Several previous studies also reported that combinational use of antimicrobial agents is more effective against microbial growth than their individual use (Aminzare et al., 2014; Ehsani et al., 2014; Raeisi et al., 2016); however, they may have antagonistic, synergistic, or additive effects according to the type of antimicrobial agent and microorganism (Fu et al., 2007). These mentioned results are in agreement with results of the present study.

Table 3. Average reduction rate of the *L. monocytogenes* counts among treatments when compared together during study period.

Mean Difference I-J Group(I)	Group(J)	ALG	EO 0.5%	EO 1%	ENZ	ENZ+EO 0.5%	ENZ+EO 01%
CON		−0.51*	0.18	0.23*	0.25*	0.39*	0.65*
ALG			0.67*	0.74*	0.76*	0.91*	1.16*
EO 0.5%				0.04	0.06	0.21	0.46*
EO 1%					0.02	0.16	0.42*
ENZ						0.14	0.40*
ENZ+EO 0.5%							0.25

Table 4. Average reduction rate of the *E. coli* O_{157}:H_7 counts among treatments when compared together during study period.

Mean Difference I-J Group(I)	Group(J)	ALG	EO 0.5%	EO 1%	ENZ	ENZ+EO 0.5%	ENZ+EO 01%
CON		−0.65*	0.034	0.094	0.094	0.21*	0.62*
ALG			0.687*	0.748*	0.748*	0.867*	1.28*
EO 0.5%				0.06	0.06	0.17	0.59*
EO 1%					−0.00	0.11	0.53*
ENZ						0.11	0.53*
ENZ+EO 0.5%							0.41*

The initial count of *E. coli* O_{157}:H_7 was ca. 6.32 ± 0.18 log CFU/g, which decreased during the storage in all samples except for ALG samples. The highest final count of *E. coli* O_{157}:H_7 was observed in ALG and CON samples (6.6 ± 0.28 and 5.37 ± 0.25 log CFU/g), and the lowest was observed in samples with combinational use of ZEO and LPOS (ENZ+EO 1%: 4.37 ± 0.25 log CFU/g and ENZ+EO 0.5%: 4.70 ± 0.14 log CFU/g, respectively). Similar results were obtained in previous studies (Elliot et al., 2004; Kennedy et al., 2000; Yener et al., 2009). Consistent with results of this study, Min et al. (2005a) also reported that whey protein films incorporated with LPOS could decrease the growth of *E. coli* O_{157}:H_7 in culture media.

Tables 3 and 4 represent the average reduction rate of *L. monocytogenes* and *E. coli* O_{157}:H_7 counts in different treatments. As can be seen, the highest reduction rate of *L. monocytogenes* (1.16 log CFU/g and 0.65 log CFU/g) was observed in ENZ+EO 1% samples when compared to ALG and CON samples, respectively; and the highest reduction rate of *E. coli* O_{157}:H_7 (1.28 log CFU/g and 0.62 log CFU/g) was observed in ENZ+EO 1% samples when compared to ALG and CON samples, respectively.

Results also indicated that the bacterial count in ALG samples was significantly higher than CON samples. This may be due to the protective effect of alginate coating with no antibacterial agents for the growth of inoculated *L. monocytogenes* and *E. coli* O_{157}:H_7 against cold storage condition. A similar result was previously reported by Datta et al. (2008) in controlling inoculated *L. monocytogenes* on the surface of smoked salmon with alginate coating containing oyster lysozyme and nisin.

Conclusion

Results of this study demonstrated the potential use of alginate coating enriched with LPOS and ZEO to control *L. monocytogenes* and *E. coli* O_{157}:H_7 in trout fillets at 4°C. The application of these antimicrobial agents in alginate coating could preserve their antibacterial activity in trout fillets during the storage time and could significantly reduce the growth of *L. monocytogenes* and *E. coli* O_{157}:H_7. Results also indicated that ENZ+EO 1% had the best effect on growth inhibition of *L. monocytogenes* and *E. coli* O_{157}:H_7 in trout fillets, but alginate coating when used alone had a supportive effect for the growth of these pathogenic bacteria at 4°C, when compared to the control. Finally, it should be considered that the treatments could not ensure the safety of refrigerated trout

fillets when contaminated with *L. monocytogenes* at doses as high as 10^6 CFU/g due to its ability for growth at refrigeration temperature; however, they could effectively decrease its growth rate by about 1.5 log CFU/g at the end of storage time. The treatments had an acceptable effect even on high doses (10^6 CFU/g) of *E. coli* O_{157}:H_7 and could effectively accelerate its reduction rate in contaminated trout fillets stored in refrigeration. Therefore, considering the producer and consumer preference for the use of natural additives in food, it is suggested that alginate coating solution with LPOS and ZEO to be practically applied in fish fillets in order to increase its safety against pathogenic bacteria; nevertheless, to highly ensure its safety, it should be applied together with other techniques.

Acknowledgments

The authors would like to thank Ferdowsi University of Mashhad, Mashad, Iran, for financial support and also Mrs. S. Khajenasiri for her kind cooperation.

Conflict of interests

The authors declare no conflict of interests.

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