Effects of alginate coating incorporated with *Bunium persicum* essential oil and Lactoperoxidase system on inoculated *Listeria monocytogenes* in chicken breast fillets

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**Abstract**

**Background:** The present study aimed to evaluate the effect of alginate coating incorporated with *Bunium persicum* essential oil (BPEO) and Lactoperoxidase system (LPOS) individually and in combination to control the growth of inoculated *Listeria monocytogenes* in fresh chicken breast stored at 4±1°C.

**Materials and Methods:** In vitro antibacterial activity of *L. monocytogenes* was evaluated using the microdilution method. Chicken breast fillets were inoculated with a cocktail of *L. monocytogenes* culture, treated with BPEO (0.5 and 1% w/v) and LPOS (5% v/v) as natural antimicrobials, and counted during 20 days of storage at 4±1°C. The data were analyzed in SPSS software (Version 21) through repeated measure ANOVA followed by Bonferroni post-hoc tests. P-value less than 0.05 was considered statistically significant.

**Results:** Both individual and combinational use of BPEO and LPOS could significantly inhibit the growth of inoculated *L. monocytogenes*. However, the combinational use of them had higher effects, compared to control. The results also indicated that the application of alginate coating with no antimicrobial agent significantly supported the growth of inoculated *L. monocytogenes* on chicken breast samples at 4±1°C.

**Conclusions:** The combinational use of BPEO and LPOS can be practically applied to food systems, especially in the meat industry to ensure the safety of the product.

**Keywords:** Alginate, *Bunium persicum*, Chicken meat, Lactoperoxidase, *Listeria monocytogenes*

**Introduction**

Food safety is critically related to public health which has attracted increased attention in recent years. The consumption of foods contaminated with pathogenic bacteria, such as *Listeria monocytogenes* has a wide economic and public health impact worldwide (1). The symptoms of listeriosis are fairly variable and range from a mild-flu like illness to more serious complications, including meningitis, septicaemia, stillbirth, and abortion. There are many reports indicating a relatively high prevalence of contaminated raw chicken products by *L. monocytogenes* (1-2).

Therefore, the management of the contamination resulted from these pathogenic bacteria is important to decrease the outbreaks of the foodborne diseases (2). Various methods have been proposed to control post-process contamination of ready-to-eat (RTE) meat and poultry products by *L. monocytogenes*. These techniques include the utilization of thermal processing (i.e., hot water, steam, and radiant heat) and the inactivation of *L. monocytogenes* in RTE meat and poultry products (3-4).

Poultry meat is a very popular food commodity...
and its consumption has increased over the last decades in many countries (3). Modern trends for industries include the application of the hurdle technology concept and the use of natural food preservatives in order to schedule the process with the shortest time and ensure protection from both spoilage and pathogenic microorganisms (5). Meat product outbreaks are often due to inadequate cooking or cross-contamination from other foods; however, contamination may occur while the meat is being processed, cut, packaged, transported, sold, or handled (6).

Edible coatings are known for preserving food quality and shelf life extension, as they are selective barriers to moisture transfer, oxygen uptake, lipid oxidation, and the loss of volatile aromas and flavors (5). Natural antimicrobial agents may be incorporated into these solutions, adding functionality to them. Antimicrobials or antioxidant compounds incorporated into the polymer matrix may prevent the growth of spoilage and pathogenic microorganisms, delay rancidity and oxidation, prevent discoloration, and even improve the nutritional quality of coated foods (7).

Phenolic compounds in essential oils (EO), such as thymol, carvacrol, terpinene, and p-cymene are widely reported to possess strong antibacterial activity and applied to food in order to control pathogenic bacteria (8). Essential oils are regarded as “natural” alternatives to chemical preservatives and their utilization in foods meets the demand of consumers for minimally processed products (9). Bunnyum persicum is a known spice originated in the Middle East and obtained from the plant seeds. Several researchers have investigated the antibacterial and antifungal effect of B. persicum EO (BPEO) which is proposed to be due to its major component, namely cumin-aldehyde (10).

Despite the strong antimicrobial activity of EOs, their practical application is currently limited due to changing food product flavor (11) and their interaction with some food ingredients (8). Therefore, the desirable preservative effect of essential oils may be achieved using lower concentrations of essential oils in combination with other preservation ingredients, such as Lactoperoxidase system (LPOS) (11) or preservation technologies, such as low temperature (12).

The LPO is a natural single chain polypeptide with no antibacterial effect on its own which is secreted in milk, saliva, and tear. The LPOS consists of three compounds, namely the LPO enzyme, thiocyanate ion, and hydrogen peroxide (H2O2) (13).

With this background in mind, the aim of the present study was to determine phytochemical constituents of the BPEO and characterize antilisterial potency of the BPEO by determination of MIC and MBC values. Moreover, this study aimed at investigating the effect of alginate coating incorporated with B. persicum essential oil and the LPOS individually and in combination on growth inhibition of L. monocytogenes in fresh chicken breast fillets stored at 4 °C.

Materials and Methods

Microorganisms and materials

B. persicum essential oil obtained through steam distillation was purchased from the Iranian Institute of Medicinal Plants, Karaj, Iran. Four lyophilized strains of Listeria monocytogenes (ATCC: 7644, 7834, 10671, 82119) were purchased from Iranian Biological Resource Center, Tehran, Iran. All culture media were purchased from Merck (Merck, Darmstadt, Germany). All used reagents were of analytical grade and purchased from Sigma (Sigma-Aldrich Chemical Co. St. Louis, USA). The study protocol was approved by the Ethics Committee of Mashhad University of Medical Sciences, Mashhad, Iran (IR.MUMS.fm.REC.1395.155).

Gas chromatography/mass spectrometry analysis

The EO composition was determined by gas chromatography-mass spectrometry (GC-MS) (14). According to the provider, GC-MS apparatus was a Varian star 3400 GC equipped with a fused-silica column (DB-5, 30 m × 0.25 mm i.d., film thickness 0.25 μm), interfaced with a mass spectrometric detector. The operating conditions were as follows: oven temperature: 60-240°C with a rate of 3°C/min, injector temperature: 280°C, injector mode: split injection, carrier gas: Helium, flow rate: 2 ml/min, mass spectra: electronic impact, ionization potential 70 eV, ion source temperature: 250°C, ionization current 1000 IA, resolution 1000, and mass range of 40-300 u.

Preparation of bacteria

Four strains of L. monocytogenes were plated on PALCAM agar and incubated at 37 °C for 24 h and typical colonies of L. monocytogenes were then confirmed by biochemical tests.

Determination of minimum inhibitory concentration and minimum bactericidal concentration values

The microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration
(MBC) of the BPEO against four strains of L. monocytogenes (14). Bacterial suspension of each strain was prepared in 9 ml of BHI broth and incubated at 37 °C for 24 h. The suspensions were adjusted to 0.5 McFarland standard turbidity (1.5×10⁶ CFU/ml) using a spectrophotometer (Optizen 2120 plus, South Korea) and diluted to the desired bacterial density (1.5×10⁶ CFU/ml). The BPEO was dissolved in 10% dimethyl sulfoxide in a concentration as a stock solution and later serial two-fold dilutions were made in a concentration range from 40 to 0.31 mg/ml in nutrient broth. Subsequently, 20μl of each inoculum with 20μl of different concentrations of the BPEO were added to the wells containing 160 μl of BHI broth to achieve a final volume of 200 μl. Wells without any bacteria (180 μl of BHI broth and 20 μl of the BPEO) and wells without any antibacterial agent (180 μl of BHI broth and 20 μl of each inoculum) were considered as a negative and positive control, respectively. The final concentration of the inoculums was approximately 1.5×10⁵ CFU/ml and the final concentration of the BPEO was in the range of 4 to 0.031 mg/ml. The microplates were incubated at 37 °C for 18- 24 h under constant shaking (50-100) rpm by a shaker incubator (GFL 3031, Burgwedel, Germany) and the lowest concentrations with no visible bacterial growth were regarded as the MIC values. The MBC values were determined by inoculation of non-turbid wells on BHI agar and incubation at 37 °C for 24 h. The lowest concentrations with no visible bacterial growth on the agar were regarded as the MBC values.

**Preparation of Lactoperoxidase system**

The LPOS was prepared according to the method previously described (15). Briefly, LPOS components (weight ratios: 1.00, 0.35, 108.70 and 1.09 for the LPO, glucose oxidase, D-(α)-glucose, potassium thiocyanate, and H2O2, respectively) were dissolved in phosphate buffer (50ml, pH 6.2) based on 15.5 mg of the LPO. The solution was filtered and incubated at 23 °C for 24 h under constant shaking (160 rpm) by a shaker incubator (GFL 3031, Burgwedel, Germany) in order to boost the antimicrobial activity of the LPOS.

**Preparation of chicken breast fillets and bacterial inoculation**

The fresh chicken breast meat was purchased from a local poultry market in Mashhad, Iran, in summer during 2016. Subsequently, the breast meat was filleted, washed, and dried. Afterward, the fillets were cut to pieces weighing 10±1 g, sprayed with ethanol, burnt, and trimmed to eliminate surface microorganisms. Aliquots of 100 μl of (~10⁷ CFU/ml) culture cocktail of 4 strains of L. monocytogenes were inoculated on each side of separate fillets to a final concentration of ~ 10⁵ CFU/g (16).

**Preparation of coating solutions and treatments**

Alginate solution was prepared by dissolving the alginate powder (3% w/v) (16) in sterilized distilled water. The solution was stirred until alginate was dissolved. Glycerol was applied as a plasticizer and added (2%) at a controlled temperature (70 °C) to coating solution and constantly stirred for 30 min to become clear (16). Sterilized calcium chloride (2% w/v, in distilled water) was utilized for gel-forming and cross-linking reactions. The activated LPOS (5%) and different concentrations (0.5 and 1%) of the BPEO were incorporated into the alginate-based edible coatings according to described treatments in Table 1. All formulations were mixed with a magnetic stirrer for 5 min to form emulsions. The BPEO were dissolved in the alginate solutions using tween 80 (0.2 g/g EO) at a controlled temperature (40 °C) and stirred for 30 min to create a uniform, stable, and clear solution. Inoculated chicken fillets were dipped in alginate-based formulations for 1 min and excess coating materials were allowed to drip off (20 min). They were then dipped in calcium chloride solution for

| No | Treatment        | Description                                                                 |
|----|------------------|-----------------------------------------------------------------------------|
| 1  | CDN              | 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) Bunium persicum essential oil |
| 4  | EO 1%            | Samples coated with alginate solution containing 1% (w/v) Bunium persicum essential oil |
| 5  | LPOS             | Samples coated with alginate solution containing 5% (v/v) Lactoperoxidase system |
| 6  | LPOS + EO 0.5%   | Samples coated with alginate solution containing 0.5% (w/v) Bunium persicum essential oil and 5% (v/v) Lactoperoxidase system |
| 7  | LPOS + EO 1%     | Samples coated with alginate solution containing 1% (w/v) Bunium persicum essential oil and 5% (v/v) Lactoperoxidase system |
30 s, packed in UV-sterilized (45 min) plastic zip pack, stored at 4±1 °C for 20 days and analyzed over 5-day intervals (i.e., days 0, 5, 10, 15, and 20) (15, 16).

**Enumeration of L. monocytogenes**

Chicken samples (10 g) were brought to a final volume of 100 ml of sterile buffered peptone water (0.1% w/v) and homogenized in a stomacher (Seward Medical, UK) for 3 min. For each sample, appropriate serial dilutions (1:10) were prepared and 10μl (15, 17) of each sample was plated on PALCAM agar (16). PALCAM agar plates were incubated at 30 °C for 48 h (16).

**Statistical analysis**

All tests of the present study were performed in triplicate. The data were analyzed in SPSS software (Version. 21, IBM; Armonk, N. Y, USA). Moreover, all data were checked regarding normality using the Shapiro-Wilk test. Repeated measure ANOVA followed by Bonferroni post-hoc tests were used to determine the significant differences. P-value less than 0.05 was regarded statistically significant.

**Results**

**GC-MS analysis**

The chemical constituents of the BPEO are summarized in Table 2. The EO content was about 2.1% (v/w) based on dry weight and 17 compounds were characterized representing 94.54% of the total content of the BPEO. Its major components were P-Cumic aldehyde (38.39%), P-Cymene (18.36%), and 2-Caren-10-al (13.26%).

**Table 2.** The results of *Bunium persicum* essential oils by GC/MS

| NO  | RT     | Compound            | %     |
|-----|--------|---------------------|-------|
| 1   | 11.937 | Cumene              | 4.69  |
| 2   | 14.355 | Beta-Pinene         | 3.89  |
| 3   | 14.975 | (+)-Camphene        | 0.50  |
| 4   | 15.478 | Alpha-Terpine       | 0.36  |
| 5   | 16.059 | P-Cymene            | 18.36 |
| 6   | 17.222 | Gamma-Terpine       | 7.38  |
| 7   | 22.739 | R-(+)-Pulegone      | 2.73  |
| 8   | 23.560 | Alpha-Terpineol     | 0.34  |
| 9   | 24.033 | 3-Caren-10-al       | 0.14  |
| 10  | 25.737 | P-Cumic aldehyde    | 38.39 |
| 11  | 26.171 | Alpha-Thujenal      | 1.27  |
| 12  | 26.964 | Phellandral         | 0.35  |
| 13  | 27.070 | Isogeraniol         | 0.59  |
| 14  | 27.442 | 2-Caren-10-al       | 13.26 |
| 15  | 28.984 | P-Cymene-7-ol       | 1.33  |
| 16  | 30.557 | Carvacrol           | 0.91  |
| 17  | 35.307 | Caryophyllen        | 0.05  |
|     |        |                     | 94.54 |

**Minimum inhibitory concentration and minimum bactericidal concentration of the Bunium persicum essential oil**

Table 3 summarizes the notable sensitivity of tested bacteria to the antibacterial effect of the BPEO. The MIC and MBC values of all tested strains of *L. monocytogenes* were 2 mg/ml and 4 mg/ml, respectively.

**Enumeration of L. monocytogenes**

Fig 1 represents the effect of the treatments on the growth of *L. monocytogenes* during 20 days of storage.

The initial mean count of *L. monocytogenes* was 5.24±0.02 log CFU/g which increased during the storage time in all groups. The lowest final counts were observed in LPOS+EO 1% (6.35±0.06 log CFU/g) and LPOS+EO 0.5% (6.56±0.09 log CFU/g) samples, respectively. The highest average reduction rate of *L. monocytogenes* count (0.94 log CFU/g) was also observed in the comparison of LPOS+EO 1% and ALG samples (P<0.05) when the average-throughout the storage-reduction rate of *L. monocytogenes* count for each treatment was compared to another (Table 4).

The results also revealed that the bacterial count in ALG samples was significantly (P<0.05) higher than that in CON samples. This indicates the protective effect of alginate coating with no antibacterial agents against cold storage condition for the growth of inoculated *L. monocytogenes* on the surface of chicken breast fillets stored in refrigeration condition.

**Table 3.** Antibacterial properties of *Bunium persicum* essential oil against four strains of *Listeria monocytogenes* using micro-dilution method

| Strains             | MIC (mg/ml) | MBC (mg/ml) |
|---------------------|-------------|-------------|
| *L. monocytogenes*  |             |             |
| 7644                | 2           | 4           |
| 7834                | 2           | 4           |
| 10671               | 2           | 4           |
| 82119               | 2           | 4           |

![Fig 1](image) Changes in the bacterial count (Log CFU/g) of chicken fillet samples inoculated with *L. monocytogenes* during storage (Means±SD)
Discussion

The major component of BPEO was P-Cumic aldehyde (38.39%). Several researchers investigated the phytochemicals of the BPEO (10, 14). According to the results, there is minor variation in the reported findings of the chemical composition of the BPEO which is due to the effect of several factors, such as climatic, seasonal, and geographical conditions (14).

According to Table 3, the MIC values of L. monocytogenes were similar regarding four tested strains. Based on this result and results of former studies on the LPOS (15, 18), the best concentrations were 0.5 and 1% for the BPEO and 5% for the LPOS to be added to the coating solutions.

The increment rate of L. monocytogenes count of all samples during storage is completely consistent with the results of former studies (15, 16, 19). The respective growth rate was significantly more rapid in ALG and CON samples, compared to other samples (P< 0.05) and reached 8.01±0.18 and 7.75±0.23 log CFU/g, respectively, at the end of storage time.

Higher antibacterial effects were observed in treatments with combinational use of BPEO and LPOS. Min et al. (2005b) reported similar results on the effect of the LPOS on growth decrement of L. monocytogenes (19). Moreover, several previous studies reported that the combinational use of antimicrobial agents was more effective against microbial growth than their individual use (16, 20, 21). However, the combinational use of antimicrobial agents may have antagonistic, synergistic, or additive effects based on the kind of antimicrobial agent and microorganism (22). Datta et al. (2008) conducted a study on the control of inoculated L. monocytogenes on the surface of smoked salmon coated with alginate coating containing oyster lysozyme and nisin. According to the results, the protective effect of alginate coating was reported with no antibacterial agents at cold storage condition for the growth of inoculated L. monocytogenes. The obtained result was completely consistent with the results of the present study (23). Similarly, Sharifi et al. (2017) conducted a study regarding the control of inoculated L. monocytogenes and Escherichia coli O157: H7 on the rainbow trout fillets coated with alginate coating containing the LPOS and Zataria multiflora EO and reported similar results (24).

Conclusion

The obtained results of this study indicated the potential of alginate coating enriched with the LPOS and the BPEO for control of L. monocytogenes in chicken breast fillets at 4°C. The utilization of these natural antimicrobial agents in alginate coating could significantly reduce the growth of L. monocytogenes in chicken breast fillets during storage time. In addition, the results revealed that LPOS+E0 1% had the greatest effect on growth inhibition of L. monocytogenes. However, alginate coating with no antibacterial agent had a supportive effect on its growth at 4°C, compared to control. Therefore, considering the producer and consumer preferences in terms of the utilization of natural additives in food, it is suggested that alginate coating solution containing the LPOS and BPEO can be practically applied to chicken breast fillets. This results in an increase in its safety against pathogenic bacteria, such as L. monocytogenes contamination. It should be noted that its effect would be greater if it is employed with other food preservation techniques.

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Conflicts of Interest

The authors declare no conflict of interests.

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### Table 4. Mean reduction rate of the L. monocytogenes counts (Log CFU/g) regarding treatments when compared to each other during storage time (days: 0-20)

| Mean Difference | Group (I) | ALG | EO 0.5% | EO 1% | LPOS | LPOS + EO 0.5% | LPOS + EO 1% |
|-----------------|-----------|-----|---------|-------|------|---------------|--------------|
| Group (I)       | -0.32*    | 0.21*| 0.36*   | 0.18* | 0.52*| 0.62*         |              |
| ALG             | 0.54*     | 0.69*| 0.51*   | 0.84* | 0.94*|              |              |
| EO 0.5%         | 0.14      | -0.03| 0.30*   | 0.40* | 0.25*|              |              |
| EO 1%           | -0.17     | 0.15 | 0.33*   | 0.43* |      |              |              |
| LPOS            | 0.69      | 0.25 | 0.40    | 0.62  |      |              | 0.10         |
| LPOS + EO 0.5%  | 0.36      | 0.18 | 0.52    | 0.62  |      |              |              |

Asterisk (*) shows significant difference at the P<0.05.
Antibacterial effects of alginate coating

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