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Original Article

Antibacterial Properties of Whey Protein Coating and *Mentha aquatica L.* Essential Oil on Coliform Bacteria in Iranian White Cheese

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ABSTRACT

Background and Objectives: In this study, antibacterial effects of whey protein coating with *Mentha aquatica L.* essential oil on *Escherichia coli*, *Enterobacter* and *Klebsiella pneumoniae* in Iranian white cheese were investigated.

Materials and Methods: *Mentha aquatica* L. essential oil was extracted using Clevenger apparatus. *Mentha aquatica* L. essential oil was added to each whey protein coating solution at 0.5, 1 and 1.5% concentrations. Whey protein treatment included cheese, bacteria and whey protein with no *Mentha aquatica* L. essential oil. Control treatment only included cheese and bacteria with no *Mentha aquatica* L. essential oil and whey protein. Minimal inhibitory concentration and minimal bactericidal concentration were assessed for the bacterial species. Antibacterial properties of the designed active coating system against *Escherichia coli*, *Enterobacter* and *Klebsiella pneumoniae* were assessed using direct cell counting and disk diffusion methods.

Results: *Mentha aquatica L.* essential oil at 1.5% concentration at the end of Day 15 of storage at 4 °C inhibited growth of *Escherichia coli*, *Enterobacter* and *Klebsiella pneumoniae* up to 100, 64 and 18%, respectively. To achieve the highest growth inhibition of *Escherichia coli*, *Enterobacter* and *Klebsiella pneumoniae*, 1.5, 1 and 1% of the essential oil were used to preserve organoleptic properties of the cheese.

Conclusions: Use of whey protein coating with 1.5% essential oil included no adverse effects on organoleptic, sensory and appearance characteristics of the cheese. Whey protein coating with *Mentha aquatica L.* essential oil inhibited growths of *Escherichia coli* completely and *Enterobacter* partially in Iranian white cheese.

Keywords: Biopolymer coating, *Enterobacter*, *Escherichia coli*, Essential oils, Food protection, Dairy products, *Klebsiella pneumonia*

Introduction

Based on the increased world population and needs to conserve resources for the future generations, researchers have searched for novel solutions of food and food packaging, including use of antibacterial packaging technology. Recent spread of microbial contamination has fascinated researchers to look for innovative ways to inhibit microbial growth in foods without compromising food quality, freshness and safety. One solution is to use active packaging to provide margins of safety and quality (1). Active packaging is generally a type of packaging. In addition to include basic properties of conventional packaging such as gases and water vapor and mechanical stress tolerance, active packaging preserves and enhances safety, shelf-life and sensory properties of the foods. Furthermore, active packaging enables new functions such as antibacterial and antioxidant properties of the packaging materials and absorption of oxygen, moisture and ethylene by the packaging absorbent materials as well as controlled releases of flavored and ethanol substances into the foods. In this type of packaging, spread of antibacterial substances from the polymeric matrices to the surfaces of foods is carried out slowly over a specified period of time. Therefore, a relatively high concentration of the antibacterial substances is always present in the product surface, resulting in increases in shelf-life of the products (2).

Effective compounds extracted from various plant organs include anti-pain, anti-inflammatory, insecticidal, antimicrobial, antioxidant and other therapeutic properties. These compounds are collected from various parts of plants, including leaves, stems, roots, flowers, leachates

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and gums (3). Use of *Mentha* essential oil and whey protein coating showed more antibacterial effects on various bacteria, compared to that use of garlic and rosemary did (4). Antibacterial activity of whey protein coating and 5% *Mentha* essential oil was investigated in beef packaging (5) and traditional lighvan cheese (6) with the highest inhibitory effects on *Escherichia coli*, *Pseudomonas* and *Listeria monocytogenes*. Moreover, *Mentha* essential oil associated with chitosan coating was an effective antibacterial packaging system against *E. coli*, *Staphylococcus aureus* and *L. monocytogenes* in Iranian white cheese. Furthermore, *Teucrium polium* essential oil included similar effects against *E.coli* O157:H7 in Kishk (7, 8). Active packaging prepared using low-density polyethylene films loaded by titanium dioxide and zinc oxide nanoparticles showed significant antibacterial effects on *E. coli* (9) and *S. aureus* (10). Other plant extracts such as *Zataria multiflora*, *Laurus nobilis* and *Chamaemelum nobile* essential oils demonstrated good antibacterial properties against pathogenic *E. coli* (11, 12).

Biopolymers include advantages such as biodegradability, renewability and low costs to conventional packaging materials from synthetic polymers and petroleum derivatives. Protein coatings include good capabilities such as ductility, elasticity and oxygen permeability but are highly permeable to water and moisture. These coating are good to prevent moisture from penetrating coatings of fats; however, their resistance to oxygen passage is poor (13). Mechanical properties of these materials are poor. Addition of microbial transglutaminase to whey protein-based coatings improved their physicochemical properties, including water vapor permeability, tensile strength, elongation and solubility in water (14, 15). Coliforms are a group of Gram-negative bacteria, commonly referred to as indicators of food quality and water hygiene. These bacteria are morphologically bacilli-shaped and actually immobilized that ferment lactose when exposed to acid and gas at 35–37 °C. Coliforms are detected in aquatic, soil and vegetation environments. Moreover, these bacteria exist in feces of warm-blooded animals and their natural habitats include human and warm-blooded animal gastrointestinal tract (GIT) (16). Most *E. coli* strains are harmless; however, serotypes such as O157: H7 cause food poisoning and diarrhea and act as opportunistic pathogens, causing infections such as blood poisoning, urinary tract (UT) infections, pulmonary infections in immunocompromised people and neonatal brain infections (17). *Klebsiella* causes complicated UT infections, intra-abdominal infections, cellulitis, surgical wound infections and meningitis linked to brain and nerve surgeries (18). *Enterobacter cloacae* and *E. aerogenes* are responsible for most of the infections caused by *Enterobacter* spp., producing clinical syndromes similar to those caused by *Klebsiella* spp. These species play important roles in causing hospital infections (19). The aim of the current study was to assess antibacterial effects of whey protein coating containing *M. aquatica L.* essential oil against pathogenic coliforms, *E. coli*, *Enterobacter* and *K. pneumoniae* in Iranian white cheese.

Materials and Methods

Materials

Lyophilized *E. coli* PTCC1330 (O157:H7), *Enterobacter* PTCC1291 and *K. pneumoniae* PTCC1290 from the Persian Type Culture Collection (PTCC) were provided by the Iranian Research Organization for Science and Technology, Iran. *Lactococcus lactis* subsp. *diacetylactis* and *L. lactis* subsp. *cremoris* (Chr. Hansen R 704) and rennet were purchased from Chr. Hansen (Christian Hansen, Denmark). The *M. aquatica* L. fresh leaves were collected from Mazandaran Province, Iran. Blood agar, nutrient agar and Mueller-Hinton agar were purchased from Merck, Germany. MacConkey agar and plate count agar were provided by Sigma-Aldrich, USA. Whey protein isolate powder was purchased from Alra Food Ingredient, Denmark. Food grade glycerol was provided by Merck, Germany. All other chemicals were purchased from Iranian reliable manufacturers.

Mentha aquatica L. essential oil

The *M. aquatica* L. essential oil was prepared using Clevenger apparatus and water distillation method. The *M. aquatica* L. fresh leaves were dried at 55 ^{o}C for 75 min using vacuum oven and were then powdered. Essential oil extraction process continued until the essential oil vapors were detected in cooling section of the apparatus and volume of the achieved essential oil was constant with no increases. Due to the organic nature of essential oils, a non-polar solvent of normal hexane (1 ml) was used to increase the essential oil collection. Collected aromatic water in the pipe contained a very small quantity of the essential oil. Then, essential oil was separated from the aromatic water. At each stage of the extraction process, 150 g of dried leaves with 700 ml distilled water (DW) were poured into a glass balloon and extraction was carried out at 100 °C for 150 min. Essential oil was dehydrated with dry sodium sulfate and stored at 4 ^{o}C inside dark glass containers with aluminum cover. In general, nearly 4 g of the essential oil was achieved from 150 g of powdered mint leaves. The essential oil extraction was carried out for several times and nearly 35 g of the essential oil were prepared (9, 10).

Whey protein solution

In this study, commercial whey protein powder was used. To prepare whey protein coating solution, 5 g of whey protein were dissolved in 100 ml of DW and agitated at 100 rpm for 3 h at room temperature using magnetic stirrer (IKA pH Basic 2, Germany). Then, 2 g of glycerol were added to the solution as plasticizer, mixed for 10 min

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using magnetic stirrer and centrifuged to remove impurities. Coating solution, including 5% of whey protein and 2% of glycerol, was heated at 80 ^{o}C for 30 min with agitation using water bath. Heating was carried out to form intermolecular disulfide bonds. The optimum coherence and flexibility of the polymeric coating depended on the formation of covalent and non-covalent cross-links. Whey protein coating was then cooled in an ice water bath to prevent excessive denaturation of the proteins until they reached 45 ^{o}C (20). Then, *M. aquatica* L. essential oil was added at 0.5, 1 and 1.5% concentrations to the whey protein coating solutions and mixed at 100 rpm for 30 min using magnetic stirrer. Control solution was prepared using similar protocol with no *M. aquatica* L. essential oil.

Recovering of the bacteria and preparation of fresh cultures

To recover initial suspensions from *E. coli* PTCC1330 (O157:H7), *Enterobacter* PTCC1291 and *K. pneumoniae* PTCC1290, contents of the vials were separately dissolved in three test tubes, containing 5 ml of sterile DW. Suspensions were cultivated immediately on slants in oblique directions as well as on a few Petri dishes of MacConkey agar, blood agar and nutrient agar for *E. coli*, *Enterobacter* and *K. pneumoniae*, respectively. Then, 0.1 ml of diluted bacterial suspensions petri dishes was added to the solid culture media. Petri dishes were incubated at 37 °C for 24 h until bacterial colonies appeared. These plates were then used to prepare bacterial suspensions (12). Counting of the colonies was carried out using direct cell counting method.

Preparation of cheese

To ensure that milk was not contaminated with *E. coli*, *Enterobacter* and *K. pneumoniae*, samples were assessed before cheese making process. Briefly, five milk samples (each nearly 1 l) were pasteurized at 72 °C for 10 min and then cooled down to 35 °C. Microbial assessment was carried out to ensure no existence of *E. coli*, *Enterobacter* and *K. pneumoniae* in pasteurized milk. Nearly 10^5 cell ml^{-1} of each *E. coli*, *Enterobacter* and *K. pneumoniae* were inoculated in pasteurized milk. Then, 1% w/v of starter, including *Diacetylactis* and *L. lactis* subsp. *Cremoris* (Chr. Hansen R 704; Christian Hansen, Horsholm, Denmark) was added to the milk samples. After 30 min, 0.2% w/v of calcium chloride dissolved in 20 ml of sterile DW at 40 °C was uniformly added to the milk. After 24 h when pH reached to 5–5.5, 0.005% w/v rennet (Christian Hansen, Horsholm, Denmark) was added to the milk and routine cheese producing steps were carried out (8).

Minimal inhibitory concentration and minimal bactericidal concentration

To assess minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), standard microdilution method (NCCLS) was used (21). Briefly, 10 test tubes containing 10 ml of sterile nutrient broth and various dosages of *M. aquatica* L. essential oil of 0–5% were used for each bacterial strain. Inhibitory effects of ten various dosages of 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 4 and 5% v/v *M. aquatica* L. essential oil against each bacterial strain were assessed. Samples were incubated at 37 °C for 24 h and the essential oil concentration of the plates, including the most and the least bacterial colonies, were considered as MIC and MBC, respectively.

Coating the cheese samples with whey protein solution

First, small cubic pieces of cheese, weighing approximately 30 g, were prepared using sterile sharp blades. Then, whey protein coating on the samples was carried out through submerging samples in whey protein solution using two 5-min steps. Pieces of cheese were incubated at 10 °C for 5 h until the coating formed completely. Then, samples were transferred to the refrigerator, stored at 4 °C for 15 days and assessed every five days for the growth of *E. coli*, *Enterobacter* and *K. pneumoniae*. Samples were as follows:

1) 0.5% treatment contained cheese, bacteria and whey protein coating with a concentration of 0.5% *M. aquatica* L. essential oil.

2) 1% treatment contained cheese, bacteria and whey protein coating with a concentration of 1% *M. aquatica* L. essential oil.

3) 1.5% treatment contained cheese, bacteria and whey protein coating with 1.5% concentration of *M. aquatica* L. essential oil.

4) Whey protein treatment contained cheese, bacteria and whey protein coating with no *M. aquatica* L. essential oil.

5) Control treatment contained cheese, bacteria with no *M. aquatica* L. essential oil and whey protein coating.

Antibacterial survey

Antibacterial effects of whey protein coating and *M. aquatica* L. essential oil against *E. coli*, *Enterobacter* and *K. pneumoniae* in Iranian white cheese were assessed using two methods of direct cell counting and disk diffusion methods. In former method, surface cultivation was used to assess the microbial count of *E. coli*. A sample was removed from each treatment and assessed separately. For each sample, 5 g of cheese were dissolved in 45 ml of sterile peptone water completely homogenized. Dilutions of 10^{-4} and 10^{-3} were used. In general, 0.1 ml of the sample was removed with a sterile pipette and spread on a plate surface containing MacConkey agar. After 48 h of incubation at 37°C, colonies were counted. This was carried out for each treatment separately at consecutive times (every five days for 15 days). For *Enterobacter* and *K. pneumoniae*, similar protocols were used using blood agar and nutrient agar, respectively.

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In the latter method, essential oil was diluted with dimethyl sulfoxide to prepare 50, 100 and 150 mg ml^{-1} (equivalent to 0.5, 1 and 1.5%) concentrations of *M. aquatica* L. essential oil. Then, 10 µl of each solution were poured onto 6-mm blank disks for 3 min. Bacteria were transferred to Mueller-Hinton broth (Merck, Germany) and incubated at 30 °C for 24 h. Bacterial sources were prepared through culturing incubated Mueller-Hinton broth on Mueller-Hinton agar plates (for using) and slants (for storing). Suspensions of *E. coli*, *Enterobacter* and *K. pneumoniae* in sterile saline were prepared using McFarland 0.5 Standard solution of 1.5×10^8 bacterial units per ml (22). Then, a uniform culture was carried out using 100 µl of the prepared suspension on the surface of Mueller-Hinton agar media. Antibacterial assessment was carried out at 37 °C for 24 h using three discs containing three concentrations of essential oil (0.5, 1 and 1.5%, respectively) as well as one disc as negative control. After incubation, inhibition zone diameters were measured and recorded. Discs containing methyl sulfoxide with no essential oil were used as negative controls (23).

Organoleptic assessment

First, small samples of cheese (nearly 10 g) were prepared. Samples were impregnated with whey protein coating and 1.5% of *M. aquatica* L. essential oil based on the highlighted method in previous sections. A group of 40 students were selected to carry out the organoleptic assessment. Each student was given various cheese samples separately and then asked about the cheese taste, odor, appearance and color.

Results

Minimal inhibitory concentration and minimal bactericidal concentration

Based on the turbidity records and bacterial cell counting methods, the essential oil doses of 0.2 and 2% were considered as MIC and MBC for *E. coli*, respectively. For *Enterobacter*, similar findings were recorded. The MIC and MBC for *Enterobacter* were reported as 0.5 and 2%, respectively. For *K. pneumoniae*, concentrations of 1 and 2% v/v *M. aquatica* L. essential oil were reported as MIC and MBC, respectively.

Escherichia coli growth inhibition

In this study, number of live bacteria in each treatment was compared to the number of bacteria in control sample. To assess decreases in live bacterial number in treatment of 1% essential oil after 10 days of storage, number of live bacteria in this treatment was compared to the number of live bacteria in control sample on Day 1. Similarly, all samples and controls were compared to control sample on Day 1. Number of live *E. coli* in control on Day 1 was 5.6×10^5 CFU/g. After 5 days of storage at 4°C, number of *E. coli* per gram of cheese sample decreased as 23.83, 34.75 and 41% and respectively reached 4.25×10^5, 3.64×10^5 and 3.30×10^5 CFU/g in treatments of 0.5, 1 and 1.5% essential oil, compared to control. On Day 10 of storage at 4 °C, decreases of 47.14, 61.1 and 83% were recorded for the treatments of 0.5, 1 and 1.5% essential oil, respectively. Number of *E. coli* cells respectively reached 2.96×10^5, 2.18×10^5 and 0.95×10^5 CFU/g in treatments of 0.5, 1 and 1.5% essential oil. After 15 days of storage at 4 °C, number of *E. coli* per gram of cheese sample decreased as 59.1, 75 and 98% and respectively reached 2.29×10^5, 1.4×10^5 and 0.11×10^5 CFU/g in treatments of 0.5, 1 and 1.5% essential oil, compared to control (Fig. 1). The initial *E. coli* population in control was 5.65×10^5 CFU/g, which increased to 5.88×10^5 CFU/g after 5 days. At the end of Day 10, number of *E. coli* in control reached 5.15×10^5 CFU/g and decreased to 4.82×10^5 CFU/g at the end of the storage time (Day 15), decreasing of almost 14% compared to Day 1 (Fig. 1). Figure 1 shows the inhibitory growth rate of *E. coli* in each treatment at various time intervals, compared to control as well as antibacterial properties of whey protein coating and *M. aquatica* L. essential oil against *E. coli* PTCC1330 (O157:H7).

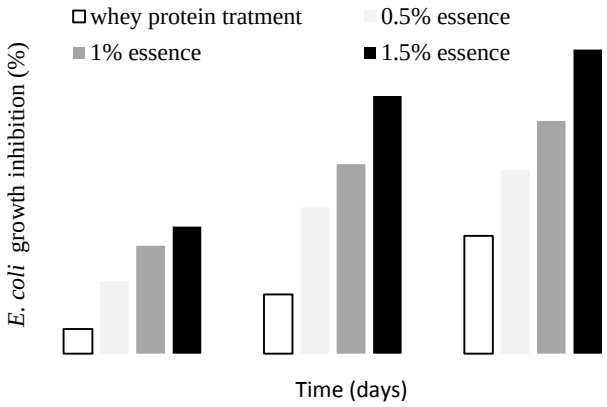

Figure 1. *Escherichia. coli* PTCC1330 (O157:H7) growth inhibition in Iranian white cheese using various doses of *Mentha aquatica* L. essential oil and whey protein coating within 15 days of storage at 4 °C

Enterobacter growth inhibition

Number of the live bacteria in each treatment in each time interval was compared to the number of *Enterobacter* in control on Day 1. Similarly, all samples and controls were compared to control on Day 1. The approximate number of live *Enterobacter* on Day 1 was 6.2×10^5 CFU/g. After 5 days of storage at 4 °C, number of *Enterobacter* decreased as 15, 18.06 and 23.55% and respectively reached 5.27×10^5, 5.08×10^5 and 4.74×10^5 CFU/g in treatments of 0.5, 1 and 1.5% essential oil, compared to control. On Day 10 of storage at 4 °C, decreases of 42.26, 59.2 and 62.1% were recorded for the treatments of 0.5, 1 and 1.5% essential oil, respectively.

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Number of *Enterobacter* cells in treatments of 0.5, 1 and 1.5% essential oil reached 3.58×10^5, 2.53×10^5 and 2.35×10^5 CFU/g, respectively. After 15 days of storage at 4 °C, number of *Enterobacter* per gram of cheese sample decreased as 44, 61.3 and 64% and respectively reached 3.47×10^5, 2.4×10^5 and 2.23×10^5 CFU/g in treatments of 0.5, 1 and 1.5% essential oil, compared to control (Fig. 2). For whey protein treatment, number of *Enterobacter* cells reached 5.86×10^5 CFU/g and recorded a 5.48% decrease after 5 days of storage at 4 °C, compared to control. On Day 10, the mean number of *Enterobacter* in whey protein treatments decreased by 13%, compared to control and the number of live bacteria reached 5.39×10^5 CFU/g. This reached 4.28×10^5 CFU/g on Day 15, which showed a decrease of 31% (Fig. 2). The initial live cells of *Enterobacter* in control included 6.2×10^5 CFU/g, which increased to 6.68×10^5 CFU/g after five days. However, the initial live cells of *Enterobacter* decreased to 5.87×10^5 CFU/g at Day 10 and 5.64×10^5 CFU/g at the end of the storage time (Day 15) with a decrease of almost 9%, compared to Day 1 (Fig. 2). Figure 2 represents the antibacterial effects of whey protein coating and *M. aquatica* L. essential oil against *Enterobacter* PTCC1291 in various treatments and time.

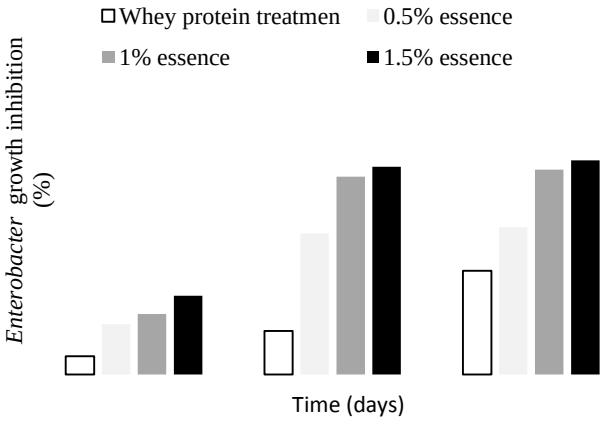

Figure 2. *Enterobacter* PTCC1291 growth inhibition in Iranian white cheese using various doses of *Mentha aquatica* L. essential oil and whey protein coating within 15 days of storage at 4 °C

Klebsiella pneumoniae growth inhibition

The initial number of live bacteria of *K. pneumoniae* in control on Day 1 was 4.78×10^5 CFU/g, which increased to 5.26×10^5 CFU/g after five days. However, this decreased to 4.42×10^5 CFU/g on Day 10 and 4.32×10^5 CFU/g at the end of the storage time (Day 15), a decrease of almost 9.6% compared to Day 1 (Fig. 3). The reason was exactly similar to that previously stated for *E. coli* and *Enterobacter*. Figure 3 shows the antibacterial properties of various doses of *M. aquatica* L. essential oil with whey protein coating against *K. pneumoniae* PTCC1290 within 15 days of storage at 4 °C.

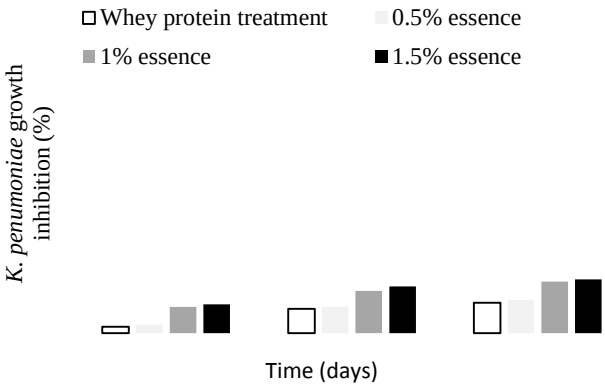

Figure 3. *Klebsiella pneumoniae* PTCC1290 growth inhibition in Iranian white cheese using various doses of *Mentha aquatica* L. essential oil and whey protein coating within 15 days of storage at 4 °C

After a 5-day storage of the whey protein treatment at 4 °C, number of *K. pneumoniae* cells reached 4.67×10^5 CFU/g and recorded a 2.2% decrease, compared to control. On Day 10, the mean number of *K. pneumoniae* in the whey protein treatment decreased by 8.32%, compared to control and the number of live bacteria reached 4.38×10^5 CFU/g. This reached 4.28×10^5 CFU/g on Day 15, which showed a decrease of 10.4% (Fig. 3).

Disk diffusion experiments

Mean diameters of the inhibition zones of *E. coli*, *Enterobacter* and *K. pneumoniae* using only whey protein coating with no essential oil (treatment no. 4) were reported as 5.24, 3.01 and 2.14 mm, respectively. Using 0.5% of the essential oil could cause increases of 98.85% in *E. coli* inhibition zone. A 153.82% increase in the inhibition zone was recorded for *Enterobacter*, using 0.5% essential oil with whey protein coating. For *K. Pneumoniae*, no enhancement was seen when 0.5% of the essential oil was used in the antibacterial coating system (Table 1). At the concentration of 1% essential oil, values increased by 152.48, 245.18 and 65.42% for *E. coli*, *Enterobacter* and *K. pneumoniae*, respectively. Using essential oil at a concentration of 1.5%, maximum mean diameters of the inhibition zones were achieved and 261.07, 261.46 and 68.22% increases were respectively reported for *E. coli*, *Enterobacter* and *K. pneumoniae*, compare to control (Table 1).

Organoleptic and sensory characteristics of the treatments

As data show in Table 2, 85% of acceptance were achieved for taste and odor and 100% of acceptance for appearance and color of the products.

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Table 1. Mean diameters of the inhibition zones of *Escherichia coli* PTCC1330 (O157:H7), *Enterobacter* PTCC1291 and *Klebsiella pneumoniae* PTCC1290 using various *Mentha aquatica* L. essential oil doses and whey protein coating

Treatment Number	Mean diameter of the inhibition zone for *E. coli* (mm)	Mean diameter of the inhibition zone for *Enterobacter* (mm)	Mean diameter of the inhibition zone for *K. pneumoniae* (mm)
1	10.42±0.43	7.64±0.27	2.06±0.13
2	13.23±0.06	10.39±0.12	3.54±0.08
3	18.92±0.04	10.88±0.71	3.60±0.33
4	5.24±0.65	3.01±0.18	2.14±0.20
5	0	0	0

Table 2. Organoleptic assays of the Iranian white cheese containing 1.5% of *Mentha aquatica* L. essential oil

Organoleptic factor	Positive comments No. (from 40 tester)	Positive comments (%)	Negative comments No. (from 40 tester)	Negative comments (%)
Taste	34	85%	6	15%
Odor	34	85%	6	15%
Color	40	100%	0	0
Appearance	40	100%	0	0

Discussion

For MIC and MBC of *E. coli*, Carvalho et al. (2018) reported MIC of 0.39% for pepper mint essential oil against *E. coli* ATCC25922 (24). In the present study, 0.2% essential oil dose was reported as MIC value. Based on the achieved values of MIC and MBC for the bacterial strains, concentrations of 0.5, 1 and 1.5% of *M. aquatica* L. essential oil were considered for the antibacterial assessment in this study. Due to the numerous reports on cheese contamination with *E. coli*, effects of *M. aquatica* L. essential oil on the bacteria from the Iranian white cheese were studied in the present study. Hygiene of the foods in terms of their microbial load and shelf-life depends on the decrease of the primary food contamination and preventing or limiting growth of the bacterial population. Based on the results, *M. aquatica* L. essential oil showed acceptable antibacterial effects at various doses. In treatment of 1.5% *M. aquatica* L. essential oil, growth of *E. coli* was almost stopped completely on Day 15. In whey protein treatment, the mean number of *E. coli* cells decreased by 8% after five days of storage at 4 °C, compared to control. Moreover, number of the bacteria reached 5.15×10^5 CFU/g. On Day 10, the mean number of *E. coli* in whey protein treatment decreased by 19.1%, compared to control. Furthermore, number of the live bacteria reached 4.53×10^5 CFU/g. This reached 3.47×10^5 CFU/g on Day 15, which showed a decrease of 38% (Fig. 1). The major reason for increases in number of the bacteria in control sample after five days was linked to growth and proliferation of the bacteria in cheese samples and process of the bacterial logarithmic growth. After five days, number of the live bacteria gradually decreased, which was a natural phenomenon in bacterial cell population. The most important reason could be linked to changes in the cheese pH.

Based on the results, use of 0.5% *M. aquatica* L. essential oil with whey protein coating could inhibit *E. coli* growth in Iranian white cheese by up to 60% after 15 days of storage at 4 °C. However, only up to 38% of the bacterial growth were inhibited during this time using whey protein coating with no *M. aquatica* L. essential oil. In control sample (with no whey protein coating or *M. aquatica* L. essential oil), inhibition of *E. coli* growth after 15 days of storage at 4 °C was normally seen up to 14%. Therefore, it can be concluded that the presence of whey protein coating and *M. aquatica* L. essential oil included significant antibacterial effects against *E. coli* PTCC1330 (O157:H7) in Iranian white cheese. By increasing the concentration of *M. aquatica* L. essential oil from 0.5 to 1.5%, inhibition rate of the bacterial growth increased significantly. As the concentration of essential oil increased from 0.5 to 1%, inhibition rate of the bacterial growth increased from 59 to 75%. With the increase of essential oil concentration from 1 to 1.5%, inhibition rate of the bacterial growth increased from 75 to 98%. At 1.5% of the essential oil, growth of *E. coli* was completely inhibited. Antibacterial properties of α-pinene, one of the most important chemicals of *M. aquatica* L., against *E. coli* have been reported by the researches (25). Karim and Bonyadian (2004) studied antimicrobial effects of volatile oils of thyme (*Thmus Vulgaris*), tarragon (*Arlemisia dracunculus*), caraway seed (*Caram Carvi*), penny royal (*M. pulgium*) and peppermint (*M. piperita*) on *E. coli* in Iranian white cheese (26). They reported that thyme included the highest antimicrobial effects on *E. coli*. After seven days of incubation with 0.3 and 0.4% concentrations of *M. piperita* essential oil, 40 and 46% decreases were respectively achieved in *E. coli* cell number; similar to those achieved in the present study. Moreover, significant antibacterial effects of *M. aquatica* L. extract (27) and M. piperita L. essential oil (28) on *E. coli* were reported.

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Results of culturing and counting live *Enterobacter* PTCC1291 in various treatments showed that use of whey protein coating with *M. piperita* essential oil in various doses included antibacterial effects in inhibiting growth of the coliformic bacteria such as *E. coli*. In treatment of 1.5% *M. piperita* essential oil on Day 15, growth of *Enterobacter* was inhibited by 64%. Results showed no significant differences between the concentrations of 1 and 1.5% of *M. piperita* essential oil in inhibiting growth of *Enterobacter* on Days 5, 10 and 15. Moreover, no significant differences were observed in effects of storage time on decreasing number of live bacteria between Days 10 and 15 in each of the essential oil doses. In general, it could be concluded that the antibacterial effect of *M. piperita* essential oil against *Enterobacter* PTCC1291 was much less than that against *E. coli*. The initial live cells of *Enterobacter* in control increased after five days. However it decreased at the end of the storage time (Day 15) by almost 9%, compared to Day 1 (Fig. 2). The reason of this was quite similar to that of *E. coli*.

Results of this study indicated that *Enterobacter* PTCC1291 was more resistant than *E. coli* PTCC1330 (O157:H7). Results of this section showed that an inhibition factor of 44% could be achieved for *Enterobacter* proliferation rate after 15 days of storage at 4 °C using 0.5% of *M. aquatica* essential oil with whey protein coating. Without the presence of *M. aquatica* essential oil and by use of whey protein alone, growth of the bacteria was inhibited up to 31%. Antibacterial effects of *M. aquatica* essential oil and whey protein coating were milder for *Enterobacter* than *E. coli*. In control samples, inhibition of the bacterial growth was naturally observed up to 9% after 15 days of storage at 4 °C. Therefore, it could be concluded that the presence of whey protein coating included significant effects on inhibition of *Enterobacter* growth in Iranian white cheese. However, effects of *M. aquatica* essential oil at 0.5% on inhibiting the growth of these bacteria were not significant. With increases in concentration of the essential oil from 0.5 to 1%, inhibition rate of the bacterial growth increased significantly from 44 to 61%. However, increasing concentration of the essential oil from 1 to 1.5% did not include significant effects on increasing antibacterial properties of the essential oil. The essential oil ability to inhibit the bacterial growth increased by only 3% from 61 to 64%. Use of whey protein and *M. aquatica* essential oil up to 60% could inhibit growth of *Enterobacter* in cheese samples. Although antibacterial effects of the whey protein and *M. aquatica* essential oil on *Enterobacter* were not as significant as their effects on *E. coli*, comparing growth inhibition of *Enterobacter* in control (no whey protein coating and essential oil) with the coated sample with 5% of the essential oil, use of whey protein coating and 1.5% of essential oil inhibited growth of *Enterobacter* in cheese samples by nearly seven times. The bridged bi-cyclic monoterpenes such as α-pinene and β-pinene as two important ingredients of *M. aquatica* essential oil included significant antibacterial activities (29). Studies by Ferhat et al. (2017) showed considerable antibacterial properties of *M. aquatica* L. extract on *E.* **aerogenes (27).**

Studies on the effects of various doses of *M. aquatica* essential oil with whey protein coating on *K. pneumoniae* growth inhibition indicated that the designed antibacterial system included a little antibacterial effects against the bacteria. In principle, *K. pneumoniae* is one of the most resistant bacteria that are resistant to several antibiotics (30). Results of this study showed that *M. aquatica* essential oil could not inhibit growth of the bacteria in cheese samples to acceptable levels. Based on the results of storage at 4 °C on Day 5, viable cells of *K. pneumoniae* decreased as 2.93, 8.99 and 9.8% and reached 4.64×10^5, 4.35×10^5 and 4.31×10^5 CFU/g respectively in treatments of 0.5, 1 and 1.5% essential oil, compared to control. After 10 days of storage at 4 °C, decreases of 8.99, 14.4 and 15.9% were recorded for the treatments of 0.5, 1 and 1.5% essential oil, respectively. Moreover, number of *K. pneumoniae* cells reached 4.35×10^5, 4.09×10^5 and 4.02×10^5 CFU/g, respectively in treatments of 0.5, 1 and 1.5% essential oil. After 15 days storage at 4 °C, number of *K. pneumoniae* decreased as 11.2, 17.57 and 18.41% and reached 4.24×10^5, 3.94×10^5 and 3.90×10^5 CFU/g respectively in treatments of 0.5, 1 and 1.5% essential oil, compared to control (Fig. 3).

In various treatments with various doses of *M. aquatica* L. essential oil and whey protein coating, no significant differences were seen in number of live *K. pneumoniae* and its inhibition growth rate. These results suggested that use of whey protein and *M. aquatica* L. essential oil did not include significant effects on growth inhibition of *K. pneumoniae*. In fact, it seems that the presence of whey protein coating and various concentrations of *M. aquatica* L. essential oil mildly accelerated decreases in number of live bacteria in consecutive days of storage, compared to decreases in number of live bacteria in control. Furthermore, a small decreases were reported in number of live cells of *K. pneumoniae* at the end of Day 15 of incubation. At the concentrations of 1 and 1.5% essential oil, decreases in number of live bacteria respectively were 17.57 and 18.41% after 15 days, which were almost twice the decreases of live bacteria in control. Therefore, results of the experiments in this section demonstrated that whey protein coating and *M. aquatica* L. essential oil were not good options for inhibiting growth of *K. pneumoniae* in Iranian white cheese. A review of results achieved in this section revealed that using a concentration of 0.5% essential oil with whey protein coating resulted in only 11% inhibition of the bacterial growth in cheese samples after 15 days of incubation at 4 °C. However, if essential

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oil was not used and only whey protein coating was used, the bacterial growth was inhibited up to 10.4%. Presence of 0.5% essential oil did not include overall antibacterial effects on the bacteria. Since inhibition of the bacterial growth in control (with no coating and essential oil) was nearly 10% after 15 days of storage at 4°C (natural inhibition), it could be concluded that use of whey protein coating included no effects on the bacterial growth inhibition. By increasing concentration of the essential oil from 0.5 to 1 and 1.5%, inhibition rate of the bacterial growth did not increase significantly, reaching from 11 to 18%. Comparison of the bacterial growth inhibition of *K. pneumoniae* in control with whey protein coated sample and 1.5% of the essential oil indicated that use of these techniques caused only a two-time inhibition in the bacterial growth. However, other researchers reported good antibacterial properties of *M. aquatica* L. extract (27) and **M. piperita** L. essential oil (28) on *K. pneumonia*.

Antibacterial effects of essential oil extracted from *M. aquatica* L. leaves with biopolymer coating of whey protein on *E. coli* PTCC1330 (O157:H7), *Enterobacter* PTCC1291 and *K. pneumoniae* PTCC1290 were studied using disk diffusion method. The inhibition zone diameters demonstrated that *M. aquatica* L. essential oil negatively affected all the three bacterial strains at all concentrations. The *K. pneumoniae* was reported as the most resistant strain against doses of the essential oil with growth on the culture media. The *M. aquatica* L. essential oil at a concentration of 1.5% was able to inhibit growth of *E. coli* on surface of the culture media. The *M. aquatica* L. essential oil showed a good antibacterial ability to decrease cheese contamination loads of *E. coli* and *Enterobacter*. For *K. pneumoniae*, antibacterial effects of *M. aquatica* L. essential oil on inhibition of the bacterial species were not significant. These results verified results from the previous section (culturing and counting bacteria in various treatments). These data showed that *M. aquatica* L. essential oil included significant antibacterial effects on *E. coli*. However, *Enterobacter* was more resistant to the antibacterial effects of this essential oil than that *E. coli* was. Antibacterial effects of *M. aquatica* L. essential oil on *K. pneumoniae* were not significant.

Ingredient analysis of *M. aquatica* L. essential oil using mass spectrometry showed that the major effective ingredients of this essential oil included 78.8% of carvone (methyl-5-(1-methylethenyl)-2-cyclohexenone), 11.5% of limonene (dioxo-716-dideoxylimondiol-716), 1.43% of dihydrocarveol (2-methyl-5-prop-1-en-2-ylcyclohexan-1-ol), 1.23% of β-bourbonene ((1*S*,2*R*,6*S*,7*R*,8*S*)-1-methyl-5-methylidene-8-propan-2-yltricyclo[5.3.0.$0^{2,6}$]decane), 1.04% of trans-caryophyllene (4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene), 1% of menthone (2-isopropyl-5-methylcyclohexanone-2S5R) and 1% of terpinene (4-methyl-1-(1-methylethyl)-1,3-cyclohexadiene) with no special odor, color or taste (31). Bayan and Kusek (2018) reported 11.63% of limonene, 56.94% of carvone, 4.06% of caryophyllene, 3.4% of dihydrocarvone, 2.49% of terpinene and 1.93% of β-bourbonene in *M. spicata* L. volatile oil (32). Silveira et al. (2012) reported 86.05% of menthol and 3.44% of menthone for *M. arvensis* L. essential oil and 25.67% of β-caryophyllene, 12.55% of germacrene D, %9.37 of menthol, 6.86% of germacrene B and 5.61% of menthone for *M. pulegium*, L. which revealed significant differences in the essential oil compositions depending on *Mentha* varieties and cultivation regions (33). After carrying out organoleptic assessments, smell, color, taste and appearance of *M. aquatica* L. essential oil were reported popular, based on the Iranian taste.

Conclusion

Results of this study showed that use of *M. aquatica* L. essential oil with whey protein coating significantly decreased number of the highlighted bacteria in treatment groups and in groups including whey protein coating alone. Antibacterial effects of *M. aquatica* L. essential oil on growth inhibition of the bacteria showed that whey protein coating with *M. aquatica* L. essential oil was able to inhibit growth of *E. coli* and *Enterobacter* in Iranian white cheese and could play roles of a natural preservative in controlling these bacteria. Using 1.5% of the essential oil in Iranian white cheese, cheese could be provided to the market after the end of Day 15 of storage at 4°C. Although smell of *M. aquatica* L. in cheese with 1.5% of essential oil was mildly felt; however, it seemed that this type of cheese included an acceptable taste for the Iranian consumers. The optimal concentration of *M. aquatica* L. essential oil was estimated to achieve the highest inhibitory growth rates of *E. coli*, *Enterobacter* and *K. pneumoniae*, preserving organoleptic and sensory properties of the cheese. In all treatments, approximately 1 and 1.5% concentrations of the essential oil included more antibacterial effects than that 0.5% concentration of the essential oil did. For *E. coli*, antibacterial effects of 1.5% of the essential oil were significantly higher than those of 1% of the essential oil. Therefore, 1.5% concentration of the essential oil was considered as the optimal concentration to inhibit growth of *E. coli* in cheese samples. No significant differences were seen between the antibacterial effects of *M. aquatica* L. essential oil at 1 and 1.5% concentrations on *Enterobacter* and *K. pneumoniae*. For *Enterobacter* and *K. pneumoniae*, 1% concentration of the essential oil was reported as the optimal dose of the essential oil. In conclusion, this essential oil can be used in Iranian white cheese and the essential oil extracted from the leaves of this plant can be suggested to use in dairy industries as an alternative, natural safe preservative.

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Authors' contributions

The present study was resulted from S. R.'s master thesis. S. R. and F. A. contributed to experimental investigations and data collection. M. K. prepared necessary facilities for the experiments. F. A. contributed to the study as supervisor and M. K. as advisor. F. A. prepared the manuscript draft and all authors approved the final version of the manuscript.

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