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Inhibition of Staphylococcus Aureus in Hamburger Using Chitosan Film Containing the Nanoemulsion of Trachyspermum Ammi and Bunium Persicum Essential Oils

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ABSTRACT

Introduction: Antimicrobial agents such as essential oils have wide applications, and their use in edible films has been reported to enhance the shelf life of meat and its products. The present study aimed to assess the effects of chitosan films on the inhibition of Staphylococcus aureus in hamburger samples in storage conditions (temperature: 4±1°C).

Methods: The prepared films contained 0.8% nanoemulsion of Bunium persicum essential oil (NBPEO) and 1.6% nanoemulsion of Trachyspermum ammi essential oil (NTEO). The hamburger samples were inoculated with S. aureus and divided into several groups, including control (no film), chitosan with 7.5% cellulose nanofiber (Ch-CNF), chitosan with 7.5% cellulose nanofiber, 0.8% NBPEO, and 1.6% NTEO (Ch-CNF-NEO). The samples were preserved in storage conditions (temperature: 4°C), and bacterial count was carried out on days 0, 3, 6, 9, and 12. Data analysis was performed using Bonferroni post-hoc test and repeated measures ANOVA.

Results: According to the results, S. aureus count significantly decreased in the treatment groups compared to the control samples. In addition, the maximum reduction rate was observed in the Ch-CNF-NEO treatment (1.41 log CFU/g) compared to the control samples.

Conclution: According to the results, it is offered that nanocomposite film of chitosan with nanoemulsion of essential oils practically be applied in hamburger to enhance its safety against S. aureus.

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Introduction

Staphylococcus aureus is a gram-positive, anaerobic, facultative foodborne pathogen, which is a major cause of the contamination of foods with high protein content (e.g., milk, egg, meat and fish). Food poisoning due to S. aureus and other species of this bacterium is highly prevalent in humans and animals across the world (1). Evidence suggests that the ingestion of the staphylococcal enterotoxins that are secreted by approximately 10^6 CFU/g of S. aureus (0.2-1.0 g) could give rise to numerous complications. Such examples are abdominal cramps, chills and sweating, nausea and vomiting, retching, shallow respiration, prostration, shock, weak pulse, and subnormal body temperature (2).

Meat products and beef provide a favorable environment for the growth of S. aureus. In the production of ground beef, the mixing and grinding processes provide the optimal conditions for the distribution of bacteria across the surfaces of the meat products (3). Such contaminations have been prevented variably in the food industry, and most of the attempts in this regard have been focused on the use of chemical preservatives, which are also able to increase the shelf life of meat products. However, one of the key limitations of these methods is the development of antimicrobial resistance in bacterial species and various other complications due to the use of synthetic chemical preservatives (1).

In the modern era, food industries are in an urgent need for efficient techniques for food preservation, especially in the case of the food products with a short shelf life (e.g., seafood, fruits, vegetables, and red meat). A novel

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approach in this regard involves the use of antimicrobial films in food packaging, which has proven effective in improving the quality, microbial safety, and sensory properties of packaged food products (4).

Chitosan is a linear polysaccharide of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine and has been reported to have remarkable ability in film formation, biodegradability, and nontoxicity. Furthermore, evidence attests to the efficiency of chitosan as a potential material for food packaging (5). In general, antimicrobial films are produced by the mixing of various polymers (e.g., polysaccharides, proteins, lipids) with antimicrobial agents (4).

Essential oils are the aromatic compounds that are extracted from various herbs and plants. Recently, herbal essential oils have attracted the attention of researchers as natural substitutes for antibacterial agents and synthetic antiseptic drugs owing to their remarkable antibacterial properties (6). *Trachyspermum ammi* is an annual herb belonging to the Apiaceae family, originating in the Eastern Mediterranean and Egypt and extending to India from the near east. The fruit pods and leaves of *T. ammi* are appropriate for human consumption (7). The fruits contain 2-5% of a brownish essential oil, which produces the taste and odor of the plant and is known as *T. ammi* oil. Some of the main applications of *T. ammi* essential oil include the treatment of bronchial complications, gastrointestinal disorders, and loss of appetite. Thymol is the key constituent of *T. ammi* essential oil (35-60%), which has potent fungicide, germicide, and antispasmodic properties. Among the other important elements found in the essential oil of *T. ammi* are a-pinene, p-cymene, b-pinene, c-terpinene, and other negligible components, which do not have similar functions to thymol (8). Nevertheless, the p-cymene and c-terpinene contents of *T. ammi* essential oil may occasionally exceed the thymol content; otherwise, p-cymene and thymol are not considered to be the most predominant components in this herbal essential oil (9).

Black cumin (*Bunium persicum*) is a perennial, aromatic plant, which grown in the areas from the northern India to Central Asia (10). According to the literature, the fruits of *B. persicum* contain approximately 9% essential oil (10). Several studies have evaluated the main constituents of black cumin essential oil, confirming its beneficial antibacterial and pharmacological effects (11). On the other hand, properties such as the strong flavor, odor, and color of black cumin essential oil have limited its application to the food products that could be modified in terms of sensory properties. Consequently, various alternatives have been proposed for the herbal essential oils application, such as encapsulation and the use of systems without contact with food (12).

Nanotechnologies are the novel arena of sciences, which have attracted the attention of researchers in every industrial and scientific field, including the food industry. Nanoemulsion is defined as the dispersion of oil in water with the droplet diameter of 10-100 nanometers (13). Evidence suggests that the nanoemulsion of essential oils (NEOs) could enhance the bioactive properties of essential oils, which in turn reduces the applied concentration of essential oils and their effects on the taste and odor of food products (14, 15). To date, no studies have investigated the use of nanocomposite films containing the nanoemulsions of *B. persicum* essential oil (NBPEO) and *T. ammi* essential oil (NTEO) and their effects on the inhibition of pathogenic bacteria in hamburger.

The present study aimed to assess the effects of chitosan nanocomposite films containing NBPEO and NTEO on the growth of inoculated S. aureus in the hamburger samples stored at the temperature of 4°C.

Materials and Methods

Experimental Materials

Chitosan powder (medium molecular weight: 450 kDa) was obtained from Sigma-Aldrich (USA), and cellulose nanofiber (CNF) was supplied by Nano Novin Polymer (Mazandaran, Iran). The essential oils were provided by Nader Mashhad Industry and Cultivate Company (Iran).

The applied culture media included the brain heart infusion (BHI) broth and agar and Baird-Parker agar, which were purchased from Quelab (Canada). In addition, Staphylococcus aureus ATCC 25923 and 29737 were obtained from the culture collection of the Department of Food Hygiene at the School of Veterinary Medicine of Ferdowsi University of Mashhad (Mashhad, Iran).

Preparation of the Nanoemulsions

The essential oil nanoemulsions were prepared based on the protocol proposed by Hashtjin et al. (2015) with slight modifications. The oil-in-water (O/W) nanoemulsions were prepared using the essential oils (2% w/w) as the oil phase, along with Tween 80 (2% w/w) and deionized water (96% w/w) as the aqueous phase. All the emulsions were prepared through a two-stage process. Initially, the oil and aqueous phases (total: 100 g) were placed in a glass beaker and mixed at room temperature (25°C) using a magnetic stirrer (700 rpm for 15 minutes). Afterwards, they were sonicated (SONOPULS Ultrasonic Homogenizers; BANDELIN, Germany), and the sonication process was performed at 50% amplitude for 15 minutes (pulse: 45 seconds, rest: 15 seconds). In order to control the temperature during the sonication process, a beaker was placed in an ice container (16).

Preparation of the Chitosan/ CNF/ NTEO/ NBPEO Nanocomposite Films

The chitosan-based films were prepared by dissolving chitosan in the aqueous solution of glacial acetic acid (1% v/v) to the concentration of 2% (w/v) while stirring on a magnetic stirrer/hot plate for six hours. Following that, glycerol was added to chitosan (0.75 ml/g) as the plasticizer, which was blended into the solution for 30 minutes. At this stage, 7.5% CNF was also added, and after stirring for another hour to form the second film containing the NBPEO and NTEO, the nanoemulsions of the essential oils were added to the chitosan solution to reach the final concentrations of 0.8% for NBPEO and 1.6% for NTEO (v/v). Afterwards, the film-forming solutions were casted on the center of Teflon plates and dried for 48 hours at an ambient temperature (25°C) (17).

Preparation of Staphylococcus aureus

Two strains of S. aureus were cultured in nine milliners of the BHI broth separately, and incubation was performed at the temperature of 37°C for 24 hours. Afterwards, the cultures were prepared by initially adding one milliliter to nine milliliters of sterile BHI broth, which was incubated at the temperature of 37°C for 10-18 hours, and the bacterial suspension was obtained from the 18-hour culture in order to provide the 0.5 McFarland turbidity standard (containing 1.5×108 CFU/ml).

Preparation of the Treatments

Beef meat and fat were purchased from a local market, and the meat was ground using a three-millimeter plate (MK-ZG1500, Panasonic). The hamburger samples were prepared by mixing the ground lean beef with beef fat (12.5%), potable water (10%), salt (1%), onion powder (1%), garlic powder (1%), and black pepper (0.5%) based on the weight of the beef. Following that, the ground meat and ingredients were mixed. A bacterial suspension (100 µl of ~10^8 CFU/ml of a culture cocktail of two *S. aureus* strains) was used to directly inoculate the ground meat, and the final bacterial cell concentration was estimated at 10^6 CFU/g (18). In order to prepare the hamburgers, 50 grams of the described mixture was pressed onto a plastic wrap using a manual hamburger press machine. The prepared hamburgers had the diameter and thickness of 11 and 0.8 centimeters, respectively. Both sides of the hamburgers were coated with various film formulations, and the hamburgers with no coating were considered as the controls, which were stored at the temperature of 4°C for 12 days and assessed microbiologically on days 0, 3, 6, 9, and 12 (Table 1).

Table 1. List of Treatments

No.	Treatment	Description
1	CON	Control (hamburgers without film)
2	Ch-CNF	Hamburgers with Chitosan Film Containing 7.5% CNF and No NEO
3	Ch-CNF-NEOs	Hamburgers with Chitosan Film Containing 7.5% CNF, 0.8% NBPEO, and 1.6% NTEO

Microbial Analysis

To carry out the microbial analysis of the meat samples (10 g), 0.1% sterile peptone water was utilized to achieve the final volume of 100 milliliters. The samples were homogenized using a stomacher (Seward Medical, London, UK) for three minutes. After the preparation of the decimal dilutions, the drops method was used to culture 10 microliters of the homogenate serial dilutions onto the Baird-Parker agar with egg yolk tellurite emulsion. The obtained dilution was incubated at the temperature of 37°C for 24 hours. In addition, the bacterial counts were expressed as $\log_{10}$ colony-forming units (CFU) per gram of the samples (19).

Statistical Analysis

Data analysis was performed in SPSS version 21 (SPSS Inc., Chicago, USA) using the analysis of variance (ANOVA), Bonferroni post-hoc test, and Dunnett test. All the experiments were carried out in triplicate, and the observed differences were considered significant at the P-value of less than 0.05.

Results

S. aureus is a common pathogen that extensively grows in meat and its products (20). In the current research, the initial *S. aureus* count was estimated at 6 and 5.7 log CFU/g in the control and treatment hamburger samples, respectively (Figure 1). According to the obtained results, *S. aureus* had a higher growth rate in the control samples compared to the treatment samples, with the rate reaching 7.5 log CFU/g after the storage period. The final bacterial counts in the Ch-NCF and Ch-CNF-NEO film treatments were estimated at 5.5 and 5.1 log CFU/g, respectively. The findings of the current research demonstrated significant differences between the coated and non-coated hamburger samples in terms of the Ch-NCF and Ch-CNF-NEO films ($P<0.05$).

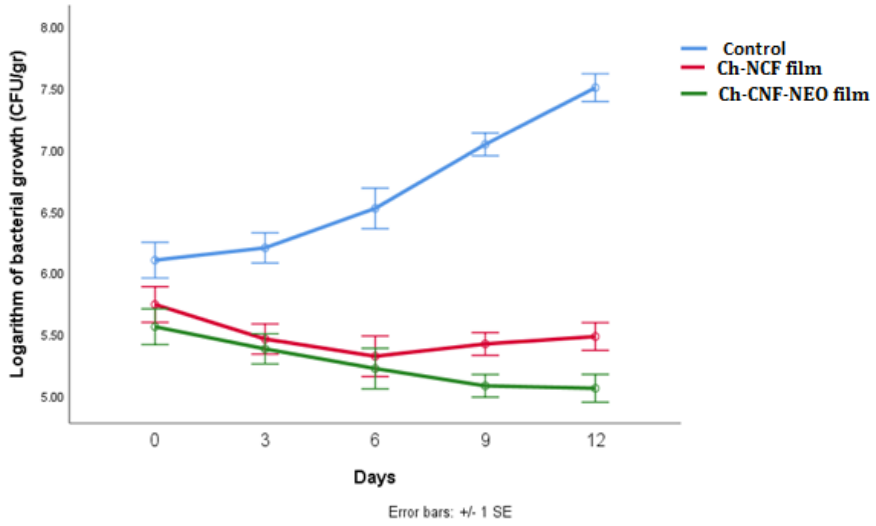

Fig 1. Effect of films on *S. aureus* in hamburger during storage at 4 °C for 12 days. Each point represents the mean ± SD.

Table 2 shows the mean reduction rate of the *S. aureus* count in various treatments. Correspondingly, the maximum reduction rate was observed in the Ch-CNF-NEO treatment (1.41 log CFU/g) compared to the controls.

Discussion

Previous reports have also denoted the inhibition of *S. aureus* by antimicrobial coating containing chitosan in ready-to-cook meat products (21). also, it is mentioned that the antibacterial activity of chitosan against *S. aureus* occurred as a result of the interactions between polycation and cell membranes, which in turn increased membrane permeability. The subsequent changes in the membrane structures led to the leakage of enzymes, proteins, nucleotides, and ions from *S. aureus* (21). In this regard, the study conducted by Chhabra et al. (2006) demonstrated that chitosan coating (concentrations: 0.5%, 1%, and 2%) could effectively inhibit inoculated *S. aureus* in raw oyster (22).

Table 2. Comparison of Mean Reduction Rate of *S. aureus* Counts (Log CFU/g) between Various Treatments during Study Period

Mean Difference (I-J)	Group (J)	CON	Ch-CNF	Ch-CNF-NEOs
Group (I)				
CON			1.19 *	1.41 *
Ch-CNF				0.22
Ch-CNF-NEOs				

Several studies have confirmed the antibacterial effects of *T. ammi* (23-26) and *B. persicum* essential oils (26, 27) against *S. aureus*. For instance, Moghadam et al. (2018) investigated the effects of chitosan film containing various concentrations of *T. ammi* essential oil on foodborne pathogenic bacteria, such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli O157:H7*, and *Vibrio parahaemolyticus* using the disc-diffusion method. According to the results of the mentioned study, the increased concentration of the essential oils was associated with the significant expansion of the diameters of the bacterial inhibition zones. Furthermore, the essential oils exerted maximum antimicrobial effects against *S. aureus* (28).

Several studies have indicated that thymol, p-cymene, and γ-terpinene are the main compounds of *T. ammi* essential oil (29-31), which could destroy membrane integrity and induce antibacterial effects (32). In this regard, Gandomi et al. (2014) reported that the antibacterial activity of thymol is due to the presence of a phenolic-OH group (25). In another research, cumic aldehyde (38.39%) and ρ-cymene were reported to be the main components of *B. persicum* essential oil (33). Various studies have also confirmed the potent antimicrobial activity of cuminaldehyde, which is the main compound in *B. persicum* essential oil (34, 35). According to the literature, gram-positive bacteria have significantly higher sensitivity to essential oils and herbal extracts compared to gram-negative bacteria. This is due to the fact that the straight contiguity of hydrophobic compositions occurs due to the presence of this phospholipid in gram-positive bacteria (36).

One of the prominent features of *S. aureus* is the growth and production of toxins in a wide array of food products (37). Throughout the storage period in the present study (12 days), the logarithmic *S. aureus* bacterial count in the Ch-NCF and Ch-CNF-NEO treatment samples was lower than 10^6 CFU/g (Table 3). Therefore, it could be inferred that the experimental treatments could optimally influence bacterial inhibition since enterotoxin production was detected at the colony count concentration of 10^6 CFU/g (38).

Table 3. Variations in Bacterial Counts (Log CFU/g) of Hamburgers Inoculated with *S. aureus* during Storage (M±SD)

Day	CON	Ch-NCF Film	Ch-NCF-NEO Film
0	6.10±0.10	5.74±0.50	5.56±0.23
3	6.20±0.14	5.46±0.28	5.38±0.36
6	6.52±0.27	5.32±0.26	5.22±0.51
9	7.04±0.32	5.42±0.04	5.08±0.15
12	7.50±0.16	5.48±0.25	5.06±0.32

Conclusion

The obtained results demonstrated remarkable antimicrobial activity induced by the Ch-NCF and Ch-CNF-NEO films against *S. aureus* in the hamburger samples. In addition, the most significant inhibitory effects on bacterial growth were observed in the Ch-CNF-NEO film. Due to the low bacterial concentration ($<10^6$ CFU/g) in the Ch-NCF and Ch-CNF-NEO samples, the antimicrobial films in the hamburgers could effectively control the growth of *S. aureus*. These promising findings could be exploited in the meat industry to optimize the safety and shelf life of meat products through the effective inhibition of pathogenic foodborne bacteria.

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Conflicts of interest

None declared.

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