



Evaluation the Antibacterial Activity of the Brine, Nisin Solution, and Ozonated Water Against *E. coli* O157:H7 in the Experimentally Local Produced Soft Cheese

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A B S T R A C T

This study aimed to evaluate the antibacterial efficacy of brine solution in compared to nisin and ozonated water treatments against *E. coli* O157:H7 in experimentally local produced soft cheese (ELPSC). A total of 25 raw cow milk samples were collected from farmers and local markets in Baghdad city and utilized to manufacturing 25 of ELPSC samples following the traditional procedure in rural Iraq without salting or heat treatment. Bacteriological analysis for potential *E. coli* O157:H7 contamination was performed and confirmed by cultural, biochemical, and serological tests. Antibacterial activity of brine solution (7% w/v), nisin solution (100 IU/mL), and ozonated water (0.6 ppm) was performed for positive samples after 2 h contact time at ambient temperature. Positive isolates of *E. coli* O157:H7 were detected and confirmed as 3 out of 25 cheese samples (12%), with initial average bacterial count of 6.146 log₁₀ CFU/g. The initial bacterial count was significantly (P<0.05) reduced to 3.778, 4.380 and 4.544 log₁₀ CFU/g when subjected to brine salting, nisin solution, and ozonated water, respectively. Cheese samples exposed to brine had a significantly (P<0.05) higher reduction in the bacterial growth than those exposed to nisin or ozonated water treatments for each alone. More effective reduction in *E. coli* O157:H7 bacterial growth was not observed when brining exposure time elongated at ambient temperature to 6 h. These findings may be useful in raising the public knowledge about the use of brine solution as a domestic antibacterial approach for minimizing the possible risk of *E. coli* O157:H7 contamination in the local produced soft cheese.

Keywords: soft cheese, *E. coli* O157:H7, brine, nisin, ozonated water

INTRODUCTION

To enhance food quality and extend shelf life, different preservation techniques are designed to control the growth of undesirable pathogenic microorganisms. These techniques include heating, salting, freezing, drying, and fermentation process (1). Cheeses are ready-to-eat food without any heat treatment before consumption (2). Many enteric pathogens can multiply in dairy products made from unpasteurized raw milk, which are highly marketable and the most common route for

transmission of these pathogens from farmer dairy animals to humans (3).

Numerous studies have reported that both pathogenic and non-pathogenic strains of *E. coli* bacteria can contaminate the cheese products (2). *E. coli* is found in the gastrointestinal tract of man and animals, but some species are known as pathogenic such as *E. coli* O157:H7. This pathogenic microorganism can cause many serious clinical symptoms such as bloody diarrhea, fever, and hemolytic uremic syndrome which could lead to death in both children and elderly (4). Hurdle technology, when two or more preservation methods are used simultaneously, can

be used to limit the growth and survival of pathogenic microbes in food (5).

Nisin is a polycyclic antibacterial peptide of 34 amino acids, including the uncommon residues such as lanthionine, methyllanthionine, produced by some strains of *Lactococcus lactis* subsp. *lactis* (6). According to the US Food and Drug Administration (US FDA), nisin is generally recognized as safe (GRAS) for use as a preservative in processed cheeses (Federal Register: 53 FR 11247, April 6, 1988). Currently, this bacteriocin is licensed in many countries as a natural food biopreservative mainly for dairy products (6). Antibacterial activity of nisin against Gram-positive bacteria is superior to that of Gram-negative bacteria; nisin is difficult to penetrate through the outer membrane of Gram-negative bacteria, this may result to low activity of nisin against these microorganism (7).

Ozone is a powerful oxidizing substance composed form oxygen gas which is quickly gives up nascent oxygen molecule, used as antimicrobial agent to kill bacteria, fungi and inactivated the viruses (8). Ozone regarded as a promising preservative method with no chemical-residues and powerful antibacterial agent specially in the food industry, water treatment, and medical uses, as the most Gram-positive and -negative bacteria, spores of bacteria, fungi, spores of fungi, viruses, and protozoans are vulnerable to ozone (10).

Brining method is immersing the cheese completely inside the sodium chloride solution. Brined soft cheeses are commonly consumed in rural and urban locations throughout the Middle East, including Iraq. Local soft cheese in Iraq is manufactured by raw bovine milk that is coagulated by a commercially available rennet and usually not exposed to heat treatments. Because local soft cheese is produced following traditional methods, there is a greater risk of infection with pathogenic food-borne bacteria. The main objective of this study was to evaluate the effect of brining process under ambient storage temperature to recommend the most suitable salting temperature/time for improving the quality of the locally produced soft cheese compared to nisin solution and ozonated water as other preservative methods to hinder the survival of pathogenic *E. coli* in this kind of local product that made in the rural areas in Iraq.

MATERIALS AND METHODS

Soft Cheese Manufacturing

A total of twenty-five fresh raw cow milk samples (three liters each) were obtained from farmer houses and local markets in Baghdad city and immediately transferred in cooling using an icebox to the Milk Hygiene Laboratory, Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Iraq. Experimentally local produced soft cheese (ELPSC), 25 samples, was manufactured according to the traditional procedure used in Iraqi's rural areas. Without salting or heat treatment, a commercial rennet powder was blended with sterile water and added to the milk. The milk was

allowed to coagulate for 40 to 60 minutes before being squeezed through sterile cotton cloths to remove the whey from the cheese.

Antibacterial Activity

The antibacterial activity of different solutions was evaluated after immersing the cheese samples in the nisin solution that prepared from commercial standard stock solution (100 mL, Sigma), 2.5% (w/w) of nisin was prepared sterilized by Millipore filter paper (No. 0.02 μm) then transferred into 1000 mL of sterile distilled water (12).

The final concentration of antibacterial solutions of nisin, brine solution, and ozonated water were 100 IU/mL (7% w/v) 7 g of sodium chloride in the sterile distal water and 0.6 ppm respectively with contact time for 2 h at ambient temperature ($\sim 30^\circ\text{C}$). All the solutions used in this study were prepared daily as demand.

Viability of *E. coli* O157:H7 was evaluate in the experimentally local produced soft cheese as one hundred g of each cheese samples was placed into three closed plastic containers that were filled with (7% w/v) brine solution, nisin solution at 100 IU/mL, and dipping the cheese pieces in the third container during the bubbling by the ozonated water (aqueous solutions) at 0.6 ppm by insert the stone of ozone generator inside the plastic container containing 1000 mL of sterile distilled water, all the containers were maintained at ambient conditions before and after each of the above treatments, the plastic containers fleshed by ozone as disinfected method.

Ozone Equipment

Ozone was generated using Aqua-6 Ozone Generator a commercially available from A2Z, Ozone Inc., USA, with generation limit between 0-0.6 and 0.6-3 ppm. The ozone concentration that used in this current study was at 0.6 ppm with contact time for 2 h at ambient temperature, all the containers that used in the current study were disinfected by flushed with the ozonated water and dried well before each treatment.

Microbiological Assay

After immersion of cheeses samples at ambient temperature in the different antibacterial solutions, each of cheese pieces were sampled for bacteriological analysis, the bacteria that selected in the current study was *E. coli* O157:H7, bacterial contamination were determined by using the spreading plate assay with triplicates (11). The cheese samples were cut by a sterile knife to smaller pieces and weighed (100 g) then cheese pieces (10 g) were homogenized in the sterile polyethylene lab stomacher bags for 5 min with 90 mL mixture of warmed ($40\text{-}45^\circ\text{C}$) sodium citrate (2%), and sterile peptone water (0.1%) by using the Laboratory Stomacher to provide the dilution of 10^{-1} , tenfold serial dilutions were made (10^{-2} to 10^{-7}) precisely and 0.1 mL of 10^5 and 10^6 dilutions was spread on the surface of the selective culture media (Chromo-agar™ *E.*

coli O157:H7, Paris). The isolates were subjected to the biochemical and serological tests such as Gram stain, potassium cyanide (KCN) test, indole, catalase, oxidase, and motility. Additionally, the *E. coli* O157:H7 latex test (Remel) was performed serologically for both O157 and flagellar antigen (Table 1). All analysis were conducted in triplicate.

Statistical Analysis

Data were statistically analyzed using the SPSS software version 22.00 (IBM SPSS Inc., Chicago, IL, USA). Data were subjected to one-way ANOVA and means deemed significant were separated by Fisher's least significant differences (LSD) at $P \leq 0.05$. Data were illustrated as the means of three replicates with standard error (Mean \pm SE). The bacterial count results were converted to \log_{10} CFU/g and analyzed.

RESULTS AND DISCUSSION

The percentage of *E. coli* O157:H7 that isolated from the soft cheese samples was 12%; out of 25 examined samples, 3 were positive to cultural, biochemical, and serological tests (Table 1). Results of antibacterial activity of different solutions against *E. coli* O157:H7 in the ELPSC that immersed inside the solutions at ambient temperatures at zero time (control) and after 2 h of exposure time are shown in Table 2. The initial average values of *E. coli* O157:H7 count in the soft cheese samples was 6.146 \log_{10} CFU/g. Bacterial population in the cheese samples decreased during the immersion process in the brine, nisin solution and ozonated water to 3.778, 4.380, and 4.544 \log_{10} CFU/g, respectively. There was no significant ($P > 0.05$) difference decrease in the bacterial population when compared the antibacterial activity of nisin solution and ozonated water.

Immersed the cheese pieces in the sodium chloride solution at concentration of (7 %w/v) for 2 h had significant ($P < 0.05$) decrease in the bacterial viability compared to the nisin and ozonated water for each treatment alone as shown in (Table 2).

The average values of the viability of *E. coli* O157:H7 in the cheese samples that subjected to activity of brine solution (7 % w/v) after 2 h and 6 h of contact time at ambient temperature were 3.778 and 3.376 \log_{10} CFU/g, respectively (Table 3).

Table 1. Prevalence, cultural, biochemical, and serological tests of *E. coli* O157:H7 isolates in the experimentally local produced soft cheese (ELPSC)

No. examined samples	Tests	Result	No. (%) positive isolates
25	Gram stain	Gram negative	3 (12)
	Selective media ¹	Mauve colonies	
	KCN test ²	No growth	
	indole	Red ring	
	Oxidase	Negative	
	Catalase	positive	
	Motility	Motile	
Latex test ³	Agglutination		

¹Chromagar™. ²Potassium cyanide. ³Performed serologically for both O157 and flagellar antigen

Table 2. Antibacterial activity of different solutions separately against *E. coli* O157:H7 that isolated from experimentally local produced soft cheese (ELPSC) after 0 and 2 h of contact time at ambient temperature

Antibacterial solutions	Contact Time (h)	
	0 \log_{10} CFU/g	2 \log_{10} CFU/g
Brine solution (7%)		3.778 \pm 0.087 ^c
Nisin (100 IU/mL)	6.146 \pm 0.073 ^a	4.380 \pm 0.042 ^b
Ozonation (0.6 ppm)		4.544 \pm 0.010 ^b

Mean \pm SE. Values with different letters revealed significant ($P < 0.05$) differences in the *E. coli* O157:H7 counts 2 h after storage at ambient temperature

There are many factors that influence the survival and multiplication of pathogenic microorganisms in cheese, such as acidity degree, type of preservatives, methods of preservation, water activity, temperature of storage competing flora, and salt levels (13). *E. coli* and *Salmonella* bacteria, for example, belong to the Enterobacteriaceae family, do not tolerate high salt concentrations (14). Raw milk is the crucial factor that contributing to cheese-associated outbreaks, and local homemade cheese is a popular product usually made from raw cows' milk. This kind of unaged cheese is produced without salt addition and marketed in Iraq's rural and urban markets. Due to many factors interfering during the local cheese making process, such as traditional milking procedures, abuse temperatures during storage, and transportation to local cheese markets using equipment polluted from farm conditions, this local cheese is signified as a health risk for the people and farmers' families. All these variables could be deemed post-contamination stages, particularly in the case of this homemade product that is traditionally produced on farms and consumed without any heat treatments.

Table 3. Antibacterial activities of brine solution (7% w/v) against *E. coli* O157:H7 in the ELPSC after 2 and 6 h of contact time at the ambient temperature

Antibacterial solution	Contact time (h)		
	0 \log_{10} CFU/g	2 \log_{10} CFU/g	6 \log_{10} CFU/g
Brine (7%)	6.146 \pm 0.073 ^a	3.778 \pm 0.087 ^b	3.376 \pm 0.058 ^b

Mean \pm SE. Values with different letters reveal significant ($P < 0.05$) differences in the *E. coli* O157:H7 counts directly examined after 2 and 6 h of storage inside the brine solution

The prevalence of *E. coli* O157:H7 in the ELPSC were 3 out of 25 samples (12%). This isolation percentage was high when compared with another study conducted in the Scotland on the twenty-eight samples of Artisanal farmhouse cheeses, the *E. coli* O157:H7 was not detected in this kind of cheese (15). Another study illustrated that the isolation percentage of *E. coli* was 62% from herby cheese samples subjected for sale in both of Van and Hakkari provinces, while *E. coli* O157:H7 was not detected from the same samples (16). Dairy products were implicated in seven outbreaks, four outbreaks from consuming raw milk while other three outbreaks were implicated as cheese, raw butter, and ice cream by post-contamination of these dairy products (16). The prevalence of *E. coli* O157:H7 in the soft cheese samples may attributed to the initial bacterial load of raw milk samples that collected from the rural area. Another study (17) illustrated that from 1998 to 2011

cheeses made from unpasteurized milk caused 11% of outbreaks of Shiga toxin-producing *E. coli*.

In the current study, the data revealed that the count of *E. coli* O157:H7 in the ELPSC was decreased from 6.146 to 3.778 and 3.376 log₁₀ CFU/g, respectively, after 2 and 6 h of storage in the brine solution (Table 3). Due to increasing demand for natural and unpreserved foods many of people in all over the world consuming the unpasteurized products (18). Sodium chloride as brine solution can be used as familiar rural preservative for extends the shelf-life of local produce soft cheese, in the current study the cheese pieces were maintained in the sodium chloride solution as domestic antibacterial methods, *E. coli* O157:H7 isolates were sensitive to brining process (7 % w/v) for 2 h as short term contact time but without complete inhibition at this concentration, there was no increase in the inhibition level when the cheese dipping in the brine solution for longer exposure time (6 h) at the same concentration. Previous studies revealed that increases in the cheese content of salt occurs faster in the first hours of salting process and slower after 24 h of the maintenance in the salt solution, the absorption of salt decreases with the increases of salting time, that may attributed to the decreases in the brine and the cheese moisture due to the differences in the salt content (19).

Khudhir and Hammad (20) reported that the *E. coli* O157:H7 was insensitive to the antibacterial action of nisin at concentration of 50 IU/mL while *E. coli* O157:H7 became more susceptible to its action after subjecting to activation of lactoperoxidase system these result gives an indication that antibacterial potency of nisin can increase due to the synergistic activity of nisin with another natural inhibitor. Another study recorded that high nisin concentration (470 mM) alone can eliminate the viability *E. coli* O157:H7 (21).

Aqueous solution of ozone is used as disinfectant in the food applications specially for extend the shelf life many kinds of food (22). The activity of ozonated water as disinfection agent has been reported for food according to the type and texture of food, ozone concentrations and the contact time (23). The advantages of ozone treatment are usually proposed for various kinds of food, such as meat products as well as fresh vegetables and fruits. The half-life of ozone in distilled water at 20 °C is reported as 20 to 165 min (24). Because ozone is an unstable gas at room temperature, it quickly degrades. It is highly unstable when dissolved in the water and the solubility of ozone in the water is 13 times more than of oxygen at temperature 0–30 °C. Many studies illustrated that bactericidal activity of ozone was lost with the present of organic matter in water with increase in another substances includes dissolved organic carbon, amines, lipids, proteins, and carbohydrates (25).

The contamination with *E. coli* O157:H7 in the experimental soft cheese can be related to the contamination of local raw milk that used for made such this local product. Brining process (7% w/v for 2 h) was the most effective as demotic antibacterial solution for reducing *E. coli* O157:H7 counts in the soft cheese, with no significant increase in the antibacterial activity after longer

exposure for 6 h at ambient temperature, followed by the nisin solution and ozonated water at 100 IU/mL, and 0.6 ppm, respectively. These results will be helpful to increase the people awareness to use brine solution at concentration 7% as an effective antibacterial washing method to reduce potential *E. coli* O157:H7 contamination facilitated by production local produced soft cheese.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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تقدير الفعالية البكتيرية للمحلول الملحي، النيسين، والماء المعامل بالأوزون ضد بكتريا الايشيريشيا القولونية المعوية النزفية في الجبن الطري المحلي المصنع مختبريا

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الخلاصة

هدفت الدراسة إلى تقييم الفعالية المضادة للبكتريا للمحلول الملحي بالمقارنة مع محلول النيسين والمياه المعاملة بالأوزون ضد الإشريكية القولونية H7: O157 في الجبن الطري المنتج تجريبياً (ELPSC). جمعت 25 عينة من حليب البقر الخام من المزارعين والأسواق المحلية في مدينة بغداد واستخدمت لتصنيع 25 عينة من الجبن الطري المنتج مختبرياً باستعمال الإجراءات التقليدية المستخدمة في ريف العراق دون التملح أو المعالجة الحرارية. اجري التحليل البكتريولوجي للتلوث المحتمل للإشريكية القولونية H7: O157 واكد من خلال الاختبارات الزرع بالواسط الزرعية والاختبارات الكيموحيوية والمصلية. اجري الكشف عن الفعالية المضاد للبكتريا للمحلول الملحي (7% وزن / حجم) ، محلول النيسين (100 وحدة دولية / مل) ، والماء المعامل بالأوزون (0.6 جزء في المليون) للعينات الإيجابية بعد ساعتين من وقت التلامس في درجة الحرارة المحيطة. كشف عن العزلات الإيجابية من الإشريكية القولونية H7: O157 واكدت انها 3 من 25 عينة جبن (12%) ، بمتوسط العد البكتيري الأولي 6.146 لو غاريتم 10 وحدة تكوين المستعمرة / جم. انخفض العد البكتيري الأولي بشكل ملحوظ ($P < 0.05$) إلى 3.778 و 4.380 و 4.544 لو غاريتم 10 وحدة تكوين المستعمرة / جم على التوالي عند تعرضها للمحلول الملحي ومحلول النيسين والماء المعامل بالأوزون . اظهرت عينات الجبن المعرضة للمحلول الملحي انخفاض معنوي ($P < 0.05$) في نمو البكتيريا أكثر من تلك التي تعرضت للمعاملة بالنيسين أو المياه المعاملة بالأوزون كلا على حدا. لم يلاحظ انخفاضاً أكثر في النمو البكتيري للإشريكية القولونية H7: O157 عندما يطول وقت التعرض للمحلول الملحي عند درجة الحرارة المحيطة لمدة 6 ساعات. ان مثل هذه النتائج قد تكون هذه مفيدة في رفع مستوى المعرفة العامة حول استخدام المحلول الملحي كمحلول مضاد للبكتريا لتقليل من المخاطر المحتملة للتلوث بالإشريكية القولونية H7: O157 في الجبن الطري المصنع محلياً .

الكلمات المفتاحية: جبن طري، محلول ملحي، الايشيريشيا القولونية المعوية النزفية، النيسين، الماء المعامل بالأوزون