Evaluation of the bactericidal effect of Nisin and /or Potassium sorbate and Sodium chloride on the viability of *Staphylococcus aureus* in soft cheese

Zina Saab Khudhir and Adnan Jawad Ahmed
Department of Veterinary Public health, College of Veterinary Medicine, Baghdad University, Iraq.
E-mail: adnanjawad@yahoo.com

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Summary

Effect of different concentrations of Nisin, Potassium sorbate and Sodium chloride on the keeping quality of the locally produced soft cheese samples against the *Staphylococcus aureus* viability was conducted during November 2015/ May 2016. *Staphylococcus aureus* were isolated from 20(73.33%)out of 30 bovine and ovine locally produced soft cheese samples and their identification were confirmed based on the morphological, rapid biochemical reactions and serological properties. *Staphylococcus aureus* isolates obtained by plating on the Chromogenic agar were further tested serologically. The highest significant (P<0.05) prevalence levels of *Staph aureus* were found in the cow's soft cheese samples 8/10(80%) followed by both ewe's and buffalo's soft cheese samples7/10 (70%) respectively. Three concentrations for each of Nisin, Potassium sorbate and Sodium chloride (50, 25 and 12.5 IU/ml), (0.4, 0.2 and 0.1%) and (10, 5 and 2.5) were used respectively. The minimum inhibitory concentrations of the Nisin and both Sodium chloride and Potassium sorbate against *Staphylococcus aureus* that were determined by the turbid metric technique were 50 IU/ml, 10% and 0.4% respectively. Mean values (log c.fu/ml) of the starting initial *Staph aureus* counts in the experimentally manufactured soft cheese samples decreased significantly (P<0.05) from 7.00±0.002 (log c.fu/ml) at 0 hour to 5.11±0.039 (log c.fu/ml) after 24 hrs of exposure to action of Nisin and Potassium sorbate inside the brine solution at 37°C. The bactericidal activities of Nisin and Potassium sorbate were more effective than either of them alone after 24 hrs. of exposure inside the brine solution at 37°C (reduction level approximately 3 log cycles), but the bacterial counts still were above the safe limit, which is required for the production of *Staph aureus* enterotoxin.

Keywords: *Staphylococcus aureus*, Nisin, Soft cheese, Potassium sorbate, Sodium chloride.

Introduction

Staphylococcus aureus food poisoning is a pathogen that is often transmitted to human by improperly handling, storage and distribution of traditional dairy products, in the numerous countries (1). Food-borne Staphylococcal poisoning is caused by the consumption of enterotoxins in foods contaminated with *Staphylococcus aureus* (2). Preservatives such as the natural organic acids (Lactic, Malic, Citric, Propionic, Sorbic and Benzoic) and their salts, sodium chloride, sugars and sulfur dioxide are generally recognized as safe "GRAS" for addition to the foods and can act separately or in combination with another antimicrobial preservative agents such as Nisin which is considered as the only the bacteriocin that has been certified as (GRAS) by the Food and Drug Administration and the World Health Organization (FDA/ WHO) (3-5). The antimicrobial agents were used as foods additives to prevent the food-borne poisoning and to extend the products shelf life by reducing the microbial load in foods during handling, preparation, processing and the storage (6 and 7). Hurdle technology was regarded as a new technology for producing safe nutritious, tasty and economical products. Combination of natural preservatives such as the herbal, essential oils and bacteriocin were used to achieve a high quality and hygienic food products (8).The objectives of the current study were to isolate and identify of *Staph. aureus* from the locally produced bovine and ovine soft cheese samples and studying the bactericidal effects of Nisin, Potassium sorbate and Sodium chloride against *Staph. aureus* viability in the soft cheese that manufactured experimentally and stored for 24 hours at 37°C.

Materials and Methods

The isolation and identification of *Staphylococcus aureus* was based on the cultural Chromagar, conventional biochemical

tests (Gram stain, Catalase, Coagulase and DANase with and without toluidine blue) and rapid biochemical systems. The isolates were further tested serologically by using commercial agglutination kit (9). Stock solution of Nisin was prepared in hydrochloric acid solution (HCL) 0.02 mol at pH_3 (10). The concentrations of Nisin and both of Potassium sorbate solutions and sodium chloride were (50, 25 and 12.5 IU/ml), (0.4, 0.2 and 0.1%) and (10, 5 and 2.5%) respectively. For preparation of *Staphlococcus aureus* culture, five pure colonies of *Staphlococcus aureus* were transferred from the overnight old culture (18-24 hours) on brain heart infusion agar to a tube containing 5 ml of sterile brain heart infusion broth and the count of approximately 10x 10^6 Cfu/ml was determined after aerobic incubation for 24 hours at 37^{o}C. The bacterial counts were confirmed by preparing serial dilutions of an inoculum in sterile peptone water (0.1wt/v) and pour plated (11). The antimicrobial activity of Nisin, Potassium sorbate and Sodium chloride against *Staph. aureus* was determined by using the brain heart infusion broth (Minimium inhibitory concentration MIC) (12). The antibacterial activity of Nisin and Potassium sorbate against *Staph. aureus* were at the concentrations of 50IU/ml and 0. 4% respectively, in the experimentally manufactured contaminated soft cheese was investigated after 24 hrs. of storage inside the brine solution (10%) at 37^{o}C.

Statistical analysis was performed by using the Chi-square test. Also the data were analyzed using SPSS (Version 18 Statistical soft were Chicago, USA) Unpaired *t*. test was used to assess the differences between means. P<0.05 considered significant (12).

Results and Discussion

The cultural morphological, rapid biochemical and serological characteristics of *Staphylococcus aureus* are shown in (Table, 1) *Staph. aureus* isolates were gram positive, spherical bacterial cells, positive for both the catalase and coagulase. The isolates were DNAase producer with and with toluidine blue. Typical *Staph. aureus* colonies were appeared on chromgenic agar as mauve in color. The Presumptive *Staph. aureus* isolates were subcultured on both the blood and brain heart infusion agar for further rapid biochemical tests (Dryspot *Staph. aurues* DR0100 and Rapid *Staph* Plus system) and serological test by using the agglutination kit.

Table, 1: The Cultural, Biochemical and serological properties of *Staphylococcus aureus* isolates from soft cheese.

Media/tests	Results	Positive results
Chromogar™ *Staph. aureus*	Positive	Mauve colonies
Mannitol salt agar	Positive	yellow colonies
Blood agar	Positive	β - hemolysis
Gram stain	Gram Positive	Grape like irregular clusters
Catalase	Positive	bubbles of oxygen
Commercial latex Mast Staph	Positive	Agglutination
DNase test without Toluidine blue	Positive	purple color media with Clearance around bacterial line or bacterial Growth
DNase test with Toluidine blue		Blue color media with clearance around bacterial line or bacterial Growth
Coagulase test	Positive	Agglutination
Dryspot *Staph. aurues* (DR0100)	Positive	Agglutination
Rapid *Staph* Plus system	The Isolates were comfired as *Staph. aurues* according to ERIC online	

The laboratory studies of the cultural isolation revealed that 22 (73.33%) out of 30 soft cheese samples were positive for *Staph. aureus*, the highest significant (P<0.05) isolation percentage was recorded in cow's soft cheese samples were 8/10 (80%), out of 10 cows soft cheese samples were identified positive for *Staph. aureus* while7/10 (70%) out of 10 of each of ewe and buffalos soft cheese samples were confirmed positive for *Staph. aureus* (Table, 2).

Table, 2: The prevalence of *Staphylococcus aureus* in bovine and ovine soft cheese samples that were collected from Baghdad city.

Types of cheese samples	number of examined samples	number of positive samples	The percent of positive samples %
Cows	10	8	80 A
Ewes	10	7	70 B
Buffaloes	10	7	70 B
Total	30	22	73.33

X^2=0.2. Different capital letters in a column revealed significant differences (P<0.05) in the prevalence's of *Staph. aureus* between the different animal species.

The MICs values of Nisin, Potassium sorbate and Sodium chloride against *Staph. aureus* by the broth dilution method were 50IU/ml, 0.4 % and 10% respectively. (Table, 3). The antibacterial activities of Nisin with Potassium sorbate at concentrations values 50 IU/ml and 0.4% respectively were more effective, than either of them alone after 24 hrs of exposure inside the brine solution (10%) at 37^{o}C (reduction level a proximally 3 log cycles) but the bacterial counts still above the safe limit which is required for the production *Staph. aureus* enterotoxins. (Table, 4).

Table, 3: The minimum inhibitory concentrations of Nisin, Potassium sorbate and Sodium chlorid against *Staph. aureus* by the broth dilution method.

Nisin			Potassium sorbate			Sodium chlorid		
Tube number	Nisin concentration IU/ml	Growth detection by streaking	Tube number	Potasium Sorbate concentration %	Growth detection by streaking	Tube number	Nacl concentration %	Growth detection by streaking
1	100	(-)ve control	1	0.8	(-)ve control	1	20	(-)ve control
2	50	(+) ve	2	0.4	(+) ve	2	10	(+) ve
3	25	(-) ve	3	0.2	(-) ve	3	5	(-) ve
4	12.5	(-) ve	4	0.1	(-) ve	4	2.5	(-) ve
5	0	(+)ve control	5	0	(+)ve control	5	0	(+)ve control

Negative Control= antibacterial test solutions (Nisin, potassium sorbate and sodiun chloride). Positive Control=broth Medium+*Staph. aureus*. (+) ve= inhibitory activity for the bacterial growth. (-) ve= No inhibitory activity for the bacterial growth

The locally produced soft cheeses were manufactured inside the farmer's-houses (homemade), in the villages then distributed all-over the local retail markets inside Baghdad city. Such soft cheeses were contaminated with different kinds of bacteria gaining access to the raw cheese from different sources, therefore represented a health risk for the consumers**.** *Staph. aureus* was an important food born pathogen that caused massive losses in both dairy herds and the dairy industry (13). The collected soft cheese samples were cultured on the selective media such as Chromagar *Staph. aureus* (Chromagar, Paris, France) and the formation of mauve-colonies was regarded as a positive results.

Table, 4: The combining antibacterial activities of Nisin and Potassium Sorbate against *Staph. aureus* in the experimentally manufactured soft cheese after 24 hrs. of storage inside the brine solution at 37˚C .

Storage time (hours)	Concentrations of Chemical Preservatives		*Staph. aureus* counts (Log. cfu/gm) Mean±SE
0	Sodium Chloride Nisin Potassium Sorbate	10% 50 IU/ml 0.4%	7.00±0.020 A
24	Sodium Chloride Nisin Potassium Sorbate	10% 50 IU/ml 0.4%	5.11±0.039 B

Different capital letters in a column revealed significant (P<0.05) differences in the *Staph. aureus* counts between the storage times.

In current study all of the positive soft cheese samples for *Staph aureus* which were 22 (73.33%) out of 30 samples exhibited typical growth on Chromagar™ *Staph. aureus.* A similar result was reported by (14) who concluded that Chromagar *Staph. aureus* was a reliable media for the rapid isolation and identification of *Staph. aureus* and the detectable sensitivity after 24 hours of incubation at 37°C was 99%. Researchers (15) reported that the Chromagar for *Staph. aureus* isolation and identification had better sensitivity and specificity compared with the other conventional culture media (Mannitol salt and blood agar). All the presumptive *Staph. aureus* isolates were confirmed as *Staph. aureus* by using conventional (Catalase, Coagulase and DNAase tests, in addition to the rapid biochemical tests with the latex agglutination test. The β-hemolysis on the blood agar was considered an important feature for the rapid identification of *Staph. aureus* bacteria. (16). Due to the increasing concern of consumers related to safety of food various preservatives and synthetic compounds such as antibiotics have been used to eliminate foodborne pathogens, several attempts have been done to use the natural antimicrobial agent such as Nisin and Sodium chloride for keeping quality of food. Nisin in the food industries such as meat and milk products, must be examined separately and/or in

combination with the other antimicrobial preservatives such as potassium sorbate to insure the safety of food (5). The MICs is defined as the lowest concentration of antibacterial agents that inhibits the growth of microorganism after 18- 24 hrs. of incubation time at 37°C. This method contained an identical volume of the broth media which inoculated with both the identical number of test bacteria and the antibacterial agents. The broth dilution method depended on the turbid metric test, which widely used for the quantitative measurement of antibacterial agents. The mechanism that used for measuring the inhibitory action was based on the changes in the visible turbidity and was carried out by liquid medium test (17). In the current study the antibacterial assay was done by using the broth dilution test against *Staph. aureus*. For Nisin and both Sodium chloride and Potassium sorbate the concentrations that used were (50, 25 and 12.5 IU/mL), (10, 5 and 2.5%) and (0.4, 0.2 and 0.1%) respectively. Results demonstrated that there was a wide range of antibacterial activities of Nisin and both, the sodium chloride and potassium sorbate against *Staphylococcus aureus* at the concentrations of 50 IU/ml, 10% and 0. 4% respectively. In the current study the growth behaviors of *Staph. aureus* in the experimentally manufactured soft cheese was done in the laboratory according to the traditional procedure of cheese- making by the farmers. Another method that based on the dipping application in natural antibacterial agents such as sodium chloride was used and regarded as a good choice to use in meat industry because the dipping application in some plants extract reduced the growth of *Staph. aureus* on the meats products and on the other kinds of foods (18).

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تقييم التأثير القاتل للجراثيم لكل من النيسين مع/ أو سوربات البوتاسيوم وملح الطعام على حيوية المكورات العنقودية الذهبية في الجبن الطري

زينة صائب خضير وعدنان جواد أحمد
فرع الصحة العامة البيطرية، كلية الطب البيطري، جامعة بغداد، العراق.
E-mail: adnanjawad@yahoo.com

الخلاصة

تاثير تراكيز مختلفة من النيسين وسوربات البوتاسيوم وكلوريد الصوديوم في حفظ نوعية الجبن الطري المصنع محليا ضد بقاء وحيوية المكورات العنقودية الذهبية، أجريت للمده من تشرين الثاني 2015 وحتى مايو 2016. عزلت (73.33%)22 عزلة من المكورات العنقودية الذهبية من أصل ثلاثين عينة من الجبن الطري المصنع محليا من كلتا الفصيلتين الغنمية والبقرية بالاعتماد على الخصائص الزراعية والفحوصات البايوكميائية السريعة والخصائص المناعية. حصلت المكورات العنقودية الذهبية بالزرع على الوسط الانتقائي الصباغي وفحصت باستعمال الاختبارات المصلية وجد مستوى تلوث عالٍ بجراثيم المكورات العنقودية وعلى مستويات معنوية ($P<0.05$) في عينات الجبن الطري المصنع من حليب الأبقار وبنسبة 8 (80%) تليها كلاً من عينات الجبن الطري المصنع من حليب الأغنام والجاموس بنسبة 7 (70%) لكل منهما. كان اقل تركيز مثبط للمكورات العنقودية الذهبية لمادة النيسين وكلاً من كلوريد الصوديوم وسوربات البوتاسيوم والتي حددت بوساطة تقنية قياس العكرة هي 50 (وحدة دولية لكل مل) 10% و0.4% وعلى التوالي بعد 24 ساعة وعلى درجة 37 درجة مئوية. انخفض معدل القيمة اللوغارتمية للعدد الابتدائي للمكورات العنقودية الذهبية في عينات الجبن الطري المصنع مختبرياً وبصورة معنوية وعلى مستوى ($P<0.05$) من 7.00±0.020 الى 5.11±0.039 بعد 24 ساعة من التعرض لفعل النيسين وسوربات البوتاسيوم وحفظها داخل المحلول الملحي عند درجة حرارة 37 درجة مئوية، الفعالية الجرثومية لكلا من النيسين وسوربات البوتاسيوم اكثر تأثيراً مقارنة بفعاليتها كلاً على حده بعد 24 ساعة من التعرض وعند درجة حرارة 37 درجة مئوية، حيث كان معدل الانخفاض في القيمة اللوغارتمية (3 لوغارتم) ولكن ظل العد الجرثومي أعلى من الحد الآمن الذي نحتاج إلية لتجنب حيوية الجراثيم وإفرازها للسموم المعوية.

الكلمات المفتاحية: المكورات العنقودية الذهبية، النيسين، الجبن الطري، سوربات البوتاسيوم، كلوريد الصوديوم.