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Research Article

The Synergistic Effect of pH and Sodium Citrate on the Bacteriocidal Activity of Nisin against *Staph aureus*

Zina Saab Khudhir

Department of Veterinary Public Health, College of Veterinary Medicine, University of Baghdad, Iraq
***Corresponding author:** zinasaabe@yahoo.com

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ABSTRACT

To evaluate the synergistic effects of pH (4, 7 and 10) and sodium citrate at (2, 2.5 and 3%) after 6 hrs of exposure time on the bacteriocidal activity of Nisin at (50 and 100 IU/ml) against *Staph. aureus*, Nisin inhibited the *S. aureus* at 100 IU/ml, pH_4 while pH_7 more significant effect on the viability of *Staph.aureus.* The results suggested that neutral pH had a potential antibacterial effect synergistically with sodium citrate at (3%). The study was carried out to evaluate the effect of both sodium citrate at of 3% and pH_7 on the bacteriocidal activity of Nisin and its effect on the *Staphylococcus aureus* counts after 6 hrs. Nisin was directly added to the broth previously inoculated with ($8x10^6$ cfu) of *Staphylococcus. aureus*. Nisin at 100 IU/mL was able to reduce *Staphylococcus. aureus* counts approximately (3-log) cycles after 6 hrs.

Key words: pH, Sodium citrate, Bacteriocidal activity of Nisin, *Staph aureus*

INTRODUCTION

Nisin has received a great interests because of its antibacterial activity with the broad spectrum against gram-positive bacteria as a natural preservative for improving the safety and extended the shelf-life of different kinds of foods such as meat, eggs, chicken, and dairy products (Gálvez *et al.*, 2014). In the United State and many another countries,Nisin was classified under the generally recognized as safe (GRAS) preservative. (Thomas *et al.*, 2000). There are extensive reports on the use of emulsifying salts such as sodium citrate in processed cheese and their effects on the functionality, chemistry and microbial activity against different species of pathogenic and spoilage microorganisms (Shirashoji *et al.*, 2006, Kapoor and Metzger,2008 Tamime, 2011). Many of chemical compounds can be used for alerted the bacterial outer membrane, these chemical preservatives includes acids and their salts, Lactic acid, citric acid, and their salts, sodium citrate and tri-sodium phosphate, which act as analyzing agents of the bacterial outer membrane and have demonstrated activity in the sensitization of many kinds of bacteria including pathogenic *Escherichia coli*, *Yersinia enterocolitica*, *Staph.aureus* and *Salmonella spp* (Belfiore *et al* 2007, Cobo Molinos *et al.*, 2008). The sensibility of bacterial cell can be also influenced by changes in the bacterial cell composition as a response to the different environmental circumstances (Kawasaki, 2012). Sodium salts characterized by low molecular weight, the organic acids; such as lactic, and citric have been used to hinder the microbial growth also improving the sensory properties and extend the shelf life of different kinds dairy and food products as cheese, meat, fish and poultry (Sallam, and Samejima, 2004, Boskou and Debevere 2000). The activity of different kinds of bacteriocins are influenced by pH level, many researchers reported that Nisin and bovicin HC5, are more efficient as antibacterial at low pH value (Norhana *et al.*, 2012, Prudêncio *et al.*, 2015). The main objectives of this study are to monitoring the changes in the growth of *Staphlycoccus aureus* that may be caused by Nisin and determined the effects of both pH and emulsified salt (Sodium citrate) to improving the Nisin antimicrobial effectiveness against *Staph aureus* bacteria.

MATERIALS AND METHODS

Isolation and identification of *Staphylococcus aureus* bacteria from the locally produced Buffalo's soft cheese samples

Preparation of Soft cheese samples: Fifty buffalos soft cheese samples were purchased from the locally markets in Baghdad province. All soft cheese samples were transported to the laboratory of the department of veterinary public health /University of Baghdad in ice-cooled box. The microbiological examination was

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performed on arrival to determine the average of *Staphylococcus aureus* counts (cfu/ gm), tenfold serial dilutions (10^{-1}to10^{-7}) were prepared in a sterile solution 0.1% (wt /v) of peptone water. Ten grams of each soft cheese sample were removed aseptically and transferred into a sterile stomacher plastic bag containing (90 ml) of sterile warmed sodium citrate at different concentrations (2,2.5 and 3%). The contents were homogenized for (1-3) minutes by a laboratory stomacher to provide a dilution of 10^{-1}, then the tenfold serial dilution (10^{-2} to 10^{-7}) were prepared in the sterile 0.1% (wt/v) peptone water and then spreading techniques were used with duplicates (Cutter *et al.*,1995), then 0.1 mL of the 10^4, 10^5 dilutions were spreaded on the surface of the selective Chrom -agars and incubated at temperature of 37±2ºC for 24 to 48 hours. Typical colonies of *Staphylococcus aureus* were appeared as-mauve and black color colonies on the Chrom-agar and Baird-Parker agar with egg yolk emulsion respectively and were selected for inoculating in the tubes containing brain heart infusion broth then incubated at 37±2ºC for 24 -48 hours for further conventional,rapid biochemical and serological tests (Butz and Tauscher 2002). Biochemical identifications tests were including the Gram stain, Catalase, Coagulase activity and DNase production tests. The appearance of yellow golden zone surrounding the *Staphylococcus aureus* colonies was regarded as a positive result. rapid biochemical tests were used as, Dry spot staphytect plus for detection of *Staphlyococcus aureus* clumping factor and Electronic Rapid ™ Staph plus system, (ERIC) with standard colors chart and specific ATCC codes online Oxoid – Remel (2013).

Preparation of Nisin solutions Each of 50 and 100 ml of Nisin standard stock solution that sterilized by filter paper number 0.02 µm was accurately pipetted and transferred into 1000 ml of autoclaved distal water to get the final concentrations of Nisin solutions at levels of 50 and 100 IU/ml and each of standard solution was prepared freshly when needed to use (Al-Holy *et al.*, 2016). The pH level of broth with and without Nisin were measured by using the digital pH meter which was calibrated before each experiment in standard buffer solutions of pH 4 and pH 7. All experiments were done in duplicate.

Determination the growth of *Staph. aurus* bacteria in the brain heart infusion broth in the presence of Nisin and sodium citrate at favorable pH values was done at 37°C for 24-48 hrs of incubation.The activity assay was determined by using the surface plating technique and the Nisin activity was recorded as (IU/ml). The antibacterial effect of different pH levels (Cobo Molinos *et al.*,2008) and sodium citrate at concentrations of (2, 2.5 and 3%) on the viability of pure cultures of *Staph.aurus* was determined by broth culture assay after 6 hrs of exposure time and then spreaded on the surface of the sterile brain heart infusion agar (APHA, 2002)). Broth culture assay-tests were statistically analyzed, the significant differences obtained on th*e Staph. aureus* reduction growth rate between different treatments with main actions of sodium citrate and/or pH on the antibacterial activity of Nisin were examined by analysis of differences (ANOVA) test that used to compare the means of treatments, This, statistical test was done with the aid of a computer program package (Statistical, version 6).

RESULTS

In the current study, a total of 50 locally produced soft cheese samples from the retail stores were analyzed microbiologically to determine the levels of contamination with *Staph.aureus* bacteria. The prevalence rate is shown in Table 1. The laboratory studies of *Staph. aureus* culturing from the soft cheese samples revealed that the isolation percentage of *Staph. aureus* bacteria were (90%), where out of 50 locally produced buffalos soft cheese samples examined 45 samples were positive for *Staph.aureus* bacteria with mean log counts of 6.930 as shown in Table 1. such high prevalence rate of contamination with *Staph. aureus* pointed out the very high contamination level of locally produced buffalos soft cheese samples which can lead to the potential Iraqi consumers public health hazarded.

The *Staph aureus* populations (counts) were proved as an indicator for good or poor sanitation practices during different stages of locally produced cheese production and handling. The average mean values of *Staph aureus* counts (cfu/gm) in the soft cheese samples after subjecting to Nisin at concentrations of 0 as (zero-time), 50 and 100

Table 1: Cultural, biochemical, serological characteristics and numbers of positive isolates with mean log counts of *Staph aureus* bacteria isolated from buffalo's locally produced soft cheese samples in Baghdad city

Source of locally produced soft cheese samples	Numbers of positive isolates with mean log counts of Staph *aureus bacteria*				Cultural characteristics		Biochemical and Serological characteristics	
	No of samples	Positive isolates	The percentage of positive samples (%)	Mean log counts Per gm	Chromo--agar	Mauve colonies	Biochemical B-hemolysis pattern	Serological Dryspot *staph. aureus* positive by agglutination
Buffalos	50	45	90	6.930	Baird-Parker agar with egg yolk emulsion	Black colonies	D Nase test positive by yellow golden zone surrounding the colonies	Latex mast staph positive by agglutination
					Mannitol salt agar	Golden Yellow colonies	Catalase positive by bubbles of oxygen	
					Blood agar	B-hemolysis pattern	Coagulase positive by agglutination	

Table 2: Effect of different concentrations of sodium citrate on the antibacterial activity of Nisin against *Staph aureus* bacteria after 6 hrs of exposure time

Sodium citrate concentration %	*Staph aureus* counts (mean Log $_{10}$ /cfu) ± SE		
	Nisin concentrations		
	0 IU/ml (control)	50 IU/ml	100 IU/ml
2	6.930	6.732±0.011Aa	5.139±0.054Ab
2.5	6.930	6.528±0.012Aa	5.112±0.019Ab
3	6.930	6.080±0.017Aa	4.364±0.014Bb

*The capital different letters at same column denoted that significant differences between sodium citrate concentrations (P≤0.05). *The small different letters at same row denoted that significant differences between different Nisin concentrations (P≤0.05).

Table 3: Effect of different levels of pH on the antibacterial activity of Nisin against *Staph.aureus* after 6 hrs of exposure time

Different levels of pH	*Staph aureus counts* (mean Log $_{10}$/cfu)) ± SE		
	Nisin concentrations		
	0 IU/ml	50 IU/ml	100 IU/ml
4	6.930	6.819±0.007Aa	6.078±0.045Ba
7	6.930	5.378±0.020B	5.376±0.202C
10	6.930	6.715±0.014A	6.515±0.021A

*The small different letters at same row denoted that significant differences between different pH levels (P≤0.05); *The capital different letters at same column denoted that significant differences between Nisin concentrations (P≤0.05).

Table 4: Combined actions of sodium citrate at concentration 3% and pH7 on the antibacterial activity of Nisin against *Staph aureus* bacteria after 6 hrs of exposure time

Staph aureus counts (mean Log $_{10}$/cfu)) ± SE	
Nisin concentrations	
pH 7 and sodium citrate at concentration 3%	
0 IU/ml	100 IU/ml
6.930	4.324±0.088

IU/ml with sodium citrate at different concentrations 2,2.5 and 3% are shown in Table 2 and 3. Data revealed that there was a significant (P<0.05) differences in the means values of viable *Staph.aureus* bacteria in the locally produced samples. The locally produced soft cheese samples that were collected from retail markets in Baghdad city recorded significantly (P<0.05) the highest bacterial counts of *Staph.aureus* bacteria (6.930 log. cfu/gm). The average log values of survival *Staph.aureus* bacteria in locally produce d cheese that subjected to the action of different levels of pH over two Nisin concentrations of 50 and 100 IU/ml are shown in Table 2. There was a significant (P<0.05) decrease of *Staph.aureus* counts after subjecting to 2% sodium citrate for 6 hrs of ambient storage temperature at 50 and 100 IU/ml. The average log of the starting initial counts of *Staph.aureus* counts (6.930log cfu/gm) in the control samples decreased significantly (P<0.05) to 6.732±0.011 and 5.139±0.054 log cfu/gm respectively.while further decreasing in *Staph aureus* bacterial counts after subjecting to 2.5 and 3% of sodium citrate at 100 IU/ml of Nisin to 5.112±0.019 and 4.364±0.014 log cfu/gm respectively.

The average log values of survival *Staph.aureus* bacteria in the locally produced samples cheese samples that subjected to the action of different levels of pH over the two Nisin concentrations 50 and 100 IU/ml are shown in Table 3. There was a significant (P<0.05) decrease of *Staph.aureus* counts after subjecting to pH at 4 and 7 for 6 hrs of ambient storage temperature at 50 and 100 IU/ml of Nisin where the average log of the starting initial counts of *Staph.aureus* counts (6.930log cfu/gm) in the control samples decreased significantly (P<0.05) to 6.819±0.007 and 6.078±0.045 log cfu/gm respectively. Further significant decreasing in *Staph.aureus* bacterial counts after subjecting to pH 7 at 100 IU/ml of Nisin to 5.376±0.202 log cfu/gm. While *Staph. aureus* counts increase significantly (P<0.05) to 6.515±0.021 after subjecting to pH 10 for 6 hrs of ambient storage temperature at 100 IU/ml of Nisin.

The effect of sodium citrate at concentration of 3% in combination with pH $_7$ on the *Staph.aureus* bacterial counts that isolated from buffalos locally produced soft cheese is shown in Table 4. *Staph aureus* counts that regarded as an indicator test for the hygienic production practices for raw milk and other dairy products that produced from raw milk was monitored for the antibacterial activity of Nisin at 100IU/ml. There was a significant (P<0.05) decrease in the *Staph. aureus* bacterial counts over the above mentioned concentrations of Nisin at pH $_7$ and sodium citrate concentration of 3% in locally produced soft cheese samples where the counts decreased significantly (P<0.05) from the starting initial count of 6.930 log cfu/gm at 0 hr to 4.324±0.088 log cfu/gm after 6 hrs of exposure to the action of sodium citrate at pH$_7$.

DISCUSSION

Bacteriocins specially Nisin have a great attention as antibacterial agent due to many properties such as non-toxicity to consumer, unique antibacterial mechanism with low propensity to produced resistance (Hammami, 2013). Emulsifying salts such as sodium citrate are used for pH adjustment where the adequate pH value during the food processing affects in both the protein conformation and the hydration the textural, melting properties of food products and calcium sequestration. Some phosphate-based emulsifying salts show buffering capacity and are able to stabilize the pH of the food system against many of the surrounding effects (Fox, *et al.*,2004). Emulsifying salts that are usually used for the production of different kinds of dairy products such as processed cheese is phosphate- or citrate-based salts and these salts, can hinder the microbial growth (Fox *et al.*,2004). Each bacterial species has an optimum pH value for the growth and multiplication. Bacteria are preferred to grow at a neutral pH (6.0-8.0). The effective action of the antimicrobials is limited by the pH value the interactions with food components such as organic acids are only effective at low pH value due to the fact that their antimicrobial activity is attributed to the un-dissociated acid form (Davidson *et al*., 2004). *Staph aureus* required growth conditions such as temperature ranging from 7°C to 46 °C, pH ranging from 5.2 to 9.0, water activity higher than 0.86. and ability to grow with higher sodium chloride (NaCl) levels from 10 to 20% (Glass and Doyle, 2005). Many factors can affect in the antibacterial activity of Nisin in food such as pH which is usually reduced or hinder the growth of different kinds of microbial species.

The reduction rate of bacterial cells and the loss in the viability depend on the extent of pH that may be used in the food products. The antibacterial effect of pH can occur due to both the respiring bacterial cell and the functions of its vital enzymes (RAY, 2004). The pH level of the food products can also shorten the sterilization times where the resistance of different kinds of microorganisms in the foods product's systems was lowered when the acidity of the food was increased. In addition to that, the success potency of the antibacterial agents such as sodium citrate and the pH for reducing the viability of different kinds of microorganisms was varied according to the bacterial species, concentration of the anti- bacterial agents, exposure time and the buffering capacity of the food products which were regarded as hindering factors against many types of pathogenic bacteria (Fox *et al.,* 2004). Both Nisin solubility and stability depend on the different pH levels in the food system. The Nisin molecule is characterized as a closed polypeptide ring with cationic properties. Many reports suggested that increasing the pH of Nisin molecules caused polymerizing, or in-folding of Nisin single molecules. Polymerizing or in-folding of Nisin molecules will lead to blocking the active groups. Raising pH in the solution caused reduction in the Nisin activity and losses are more pronounced at both high pH and high temperatures. The information about Nisin stability indicated that reduction of Nisin activity was depending on different heat processing, pH, substrate, and likely temperature and length of shelf life (Anonymous, 1988). In the current study sodium citrate at concentration of 3% exhibited a significant antibacterial activity against *Staph. aureus* bacteria. The effect of different levels of pH at (4,7and10) combined with sodium citrate at different concentrations (2%,2.5% and 3%)) were evaluated after 6 hrs of exposure time on the bacteriocidal activity of Nisin at concentrations of (50 and 100 IU/ml) against *Staph. aureus* bacterial growth. The antibacterial activity of Nisin is usually inversely proportional to the different levels of pH. The use of alkaline pH did not increase in the Nisin antibacterial activity compared to pH 4. In the current study the neutral pH at 7exhibited more inhibitory activity than at acidic pH, these results were in disagreement with pervious results of Vasseur *et al*, who recorded that at lower pH with excessive acid the un-dissociated kind may accelerate the bacterial cell membrane penetration. Since the inhibitory effect at the higher pH value may be due to the protons, as opposed to the formation of un-dissociated acid. (Vasseur *et al.*, 1999). The results revealed that pH $_4$ was effective for inhibition of *S. aureus* while the pH $_7$ showed more significant inhibitory effect on the viability of *Staph aureus* bacteria. these results in agreement with one study of Lee *et al*. (Lee *et al.*, 2001), who reported that sodium citrate at 25mg for each ml / or less under the neutral pH circumstances can caused killing of both the *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Conclusions

An overall conclusion of the current results pointed out that the locally produced buffalos soft cheese samples could be preserved and extended their shelf life by improvement the antibacterial activity of Nisin by a combination of controlling pH with sodium citrate against the pathogenic bacteria contaminated the dairy products as *Staph aureus* in Iraq.

REFERENCES

Al-Holy MA, AL Nabulsi A, O Saili, TM, Ayyah, MM and Shaker, RR, 2012. Inactivation of *Listeria innocua* in brined white cheese by a combination of Nisin and heat Food control 23(1): 48-53.

Anonymous, 1988. Preservation of milk and milk products with nisaplin. Tech. Info. Leaflet No. 11/88. Aplin and Barret Ltd.

APHA, 2002. Standard Methods for the Examination of Dairy Products: American Public Health Association, 16th Edition, Washington DC.

Belfiore C, Castellano P and Vignolo G, 2007. Reduction of *Escherichia coli* population following treatment with bacteriocins from lactic acid bacteria and chelators. Food Microbiol 24: 223–229.

Boskou G and Debevere J, 2000. Shelf life extension of cod fillets with an acetate buffer spray prior to packaging under modified atmosphere. Food Additives and Contaminants; 17: 17–25.

Butz P and Tauscher B, 2002. Emerging technologies: chemical aspects. Food Res Int, 35: 279-284.

Cobo Molinos A, Abriouel H, López RL, Valdivia E, Omar NB and Gálvez A, 2008 Combined physico-chemical treatments based on enterocin AS-48 for inactivation of Gram-negative bacteria in soy- bean sprouts. Food Chem Toxicol 46(8): 2912–2921.

Cutter CN and Siragusa GR, 1995. Treatments with Nisin and chelators to reduce *Salmonella* and *Escherichia coli* on beef. Journal of Food Protection, 58: 1028-1030.

Davidson PM, Sofos JN and Branen AL, 2005. Antimicrobials in Food. CRC Press, p:706.

Fox PF, Mcsweeney PLH, Cogan TM and Guinee TP , 2004. Cheese: Chemistry, Physics and Microbiology. Elsevier Applied Science, pp:434.

Gálvez A, Burgos MJG, López RL and Pulido RP, 2014. Food Bio preservation. Springer, London.

Glass K and Doyle ME, 2005. Safety of processed cheese. FRI Briefings, Food Research Institute, University of Wisconsin.

Hammami R, Fernandez B, Lacroix C and Fliss I, 2013. Anti- infective properties of bacteriocins: an update. Cell Mol Life Sci 70: 2947–2967.

Kapoor R and Metzger LE, 2008. Process cheese: Scientific and technological aspects-A review. Comprehensive Reviews in Food Science and Food Safety, 7: 194-214.

Kawasaki K, 2012. Complexity of lipopolysaccharide modifications in Salmonella enterica: its effects on endotoxin activity, membrane permeability, and resistance to antimicrobial peptides. Food Res Int 45: 493-501.

Lee YL, Thrupp L, Owens J, Cesario T and Shanbrom, E. 2001. Bactericidal activity of citrate against Gram-positive cocci. Lett Appl Microbiol 33: 349-351.

Norhana MN, Poole SE, Deeth HC and Dykes GA, 2012. Effects of nisin, EDTA and salts of organic acids on *Listeria monocytogenes, Salmonella* and native

microflora on fresh vacuum packaged shrimps stored at 4°C. Food Microbiol 31: 43-50.

Oxoid-Remel, 2013. Laboratory Manual for Media and Diagnostic Kits.

Prudêncio CV, Mantovani HC, Cecon PR and Vanetti MCD, 2015. Differences in the antimicrobial activity of nisin and bovicin HC5 against *Salmonella Typhimurium* under different temperature and pH conditions. J Appl Microbiol 118: 18–26.

RAY B, 2004. Fundamental Food Microbiology. CRC Press. 608p.

Sallam, KhI and Samejima K, 2004. Microbiological and chemical quality of ground beef treated with sodium lactate and sodium chloride during refrigerated storage. Lebensmittel-Wissenschaft und- Technologies LWT-Food Science and Technology, 37: 865–871.

Shirashoji N, Jaeggi J and Lucey J, 2006. Effect of trisodium citrate concentration and cooking time on the physicochemical properties of pasteurized process cheese. J Dairy Sci, 89: 15-28.

Tamime AY, 2011. Processed Cheese and Analogues. John Wiley and Sons. Thomas LV, Clarkson MR and Delves-Broughton J, 2000. Nisin. In Natural Food Antimicrobial Systems, Naidu, AS, Ed. CRC Press, Boca Raton. pp: 463-524.

Vasseur CL, Baverel M, Hebraud and Labadie J, 1999. Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes*. J Appl Microbial, 86: 469–476.