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Original Research Article

Effect of nisin on the viability of *Staphylococcus aureus* in kareish cheese

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ABSTRACT

Staphylococcus aureus is a common cause of food-borne disease worldwide and food poisoning. This study reports the effect of nisin (0, 10 and 12.5 ppm) against *Staphylococcus aureus* in manufactured kareish cheese. Nisin was effective in reducing *S. aureus* count in cheese; a reduction of *S. aureus* count was observed from the 2nd day of storage period. *S. aureus* in kareish cheese decreased gradually from $4x10^8$ to (8×10^7, 6.5×10^7, 5.8×10^7 CFU/gm.) in the 1st week till reached at the end of storage period of the 4th week to (4×10^4, 1.1×10^3, 1×10^2 CFU/gm.) for cheese containing (0, 10 and 12.5 ppm) of nisin, respectively during manufacture and storage for a month in the refrigerator at 4 ˚C. The data obtained in this study suggested that the use of nisin-containing cheese can be an effective method of controlling the growth and multiplication of *S. aureus* in cheese.

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1. Introduction

Kareish cheese is one of the soft cheeses which are the most popular dairy products in Egypt and Arabian countries owing to its high protein, low fat and reasonable price (Metwalli., 2011). It is an acid coagulated fresh cheese, made from skim milk with soft composition white curd and slightly salty (Francois et al., 2004). Kareish cheese is considered one of the most food products rich in calcium and phosphorus. These elements are essential for bones and teeth formation, it is also rich in sodium and potassium, which play an important role in the formation of body fluids and muscles (Mahmoud et al., 2013). kareish cheese is a good medium for the growth of *S. aureus* and under favorable conditions, it can secrete heat resistant enterotoxins (Fadel and Ismail, 2009).

Staphylococcus aureus is an important foodborne pathogen and can cause a mild skin infection to more severe diseases, such as pneumonia and septicemia (Lowy, 1998). Some *S. aureus* strains are able to produce Staphylococcal enterotoxins (SEs) in food matrices which are responsible for food poisoning and one of the most common causes of gastroenteritis worldwide (Balaban and Rasooly, 2000). The importance of enterotoxins comes due to their heat stability and their resistance to inactivation by gastrointestinal proteases like pepsin. Although Staphylococcus can be killed at normal cooking temperature, the toxins remain active (Presscott et al., 2012). In spite of advances achieved in dairy technology, several outbreaks of Staphylococcal food poisoning have been recorded, involving large number of people throughout the world (Adams and Moss, 2000).

Food preservation is designed to enhance or protect food safety as well as maintaining or improving product quality. It aims at inactivating or inhibiting the growth of undesirable microorganisms. Traditionally, food is preserved by the use of heat, freezing, drying and/or fermentation. However, such processes may cause changes in color, flavor, texture and nutritive value of the food (Sofos and Busta, 1993). Bacteriocins are widely considered as a potential and valuable biological alternative to chemical preservatives and to improve food preservation and safety. The antimicrobial activities of bacteriocins can offer many benefits to food preservation by extension of shelf life, providing extra protection during conditions of temperature abuse, control of foodborne pathogens, reduction in economic losses associated with food spoilage, reduction in chemical preservatives addition and allows the use of less drastic heat treatment (Balciunas et al., 2013).

Nisin has been suggested as a natural antimicrobial to be used as a biopreservative in foods, including dairy products, and is generally regarded as safe (GRAS) (Adams, 2003). Also it's a biopeptide produced by *Lactococcus lactis subspecies lactis* and has been used effectively as a natural preservative in milk products such as cream, cheese and others (Sabreen and El-prince, 2001). Nisin is licensed as a food preservative (E234) and is recognized to be safe by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (Favaro et al., 2015). Nisin is a peptide composed of 34 amino acid residues, with a molecular mass of 3.5 kDa, and is classified as a class-Ia bacteriocin or lantibiotic (Hurst, 1981). The importance of nisin is due to its broad spectrum of activity against a wide range of spores, Gram-negative and positive bacteria, including Staphylococcus (Arauz et al., 2009).

Many recent studies have shown the effect of nisin against *S. aureus* and other Gram-positive bacteria (Mitra et al., 2010). In addition, the previous studies showed the effect of nisin on different types of cheeses such as Cuajada (Arqués et al., 2008), Traditional Minas Serro cheese (Pinto et al., 2011), Minas Frescal cheese (Pimentel-Filho et al., 2014), ripened cheese (Dal Bello et al., 2013), Cheddar cheese (Zottola

et al., 1994) and Damietta cheese (Amer and Ewina, 2003), but at present a few studies that worked on skimmed milk that used in manufacture of kareish cheese such as (Sobrino-López and Martín-Belloso, 2006). So our study was undertaken to throw light on the effect of nisin on the viability of *S. aureus* in kareish cheese.

2. Materials and Methods

2.1. Bacterial suspension inoculation for *S. aureus*

The isolates of *S. aureus* from a previous work confirmed by PCR and produced sea and seb toxins was maintained on tryptic soya agar slants at 4 ˚C. The strain was activated by inoculation in tryptic soya broth and incubated at 37˚C for 24 hrs, and then tenfold serial dilution was made. Inoculation level of Staphylococcus strain was determined by direct plating on Baird-Parker's medium.

2.2. Nisin preparation (Hurst, 1981)

Pure nisin was dissolved in nisin diluent that contains 0·02 N HCl and 0·75% NaCl. The activity of 1 µg of pure nisin is 40 IU. In this study, prepared nisin with 10 ppm concentration as (1 ml nisin solution per 1 kg. milk), while in case of the concentration of 12.5 ppm nisin added (1.25 ml nisin solution per 1 kg. milk).

2.3. Manufacture of kareish cheese

Cheese was manufactured according to the procedure described by Fahmi and Sharara, (1950). Fresh skimmed milk was obtained from a local producer and transferred from the farm to the laboratory within 30-40 min. Milk was heated in a water bath at 80^{o}C with agitation for 10 min. After that the milk was cooled to 40 ^{o}C, and then calcium chloride and sodium chloride were added at levels of 0.02 and 7% w/w, respectively. On the other hand, rennet was added (1.5 g/100 kg milk) as a coagulating agent and then *S. aureus* was inoculated with initial inoculation (4×10^{8} cfu/ml), the milk was divided into three batches. The first and second batches of milk were separately inoculated with 10 ppm and 12.5 ppm concentration of nisin of cheese-making milk, respectively. The last batch was maintained without any further inoculation. After coagulation, the curd of each batch was ladled and left overnight to drain. The resultant cheeses were aseptically cut and packed in plastic containers with their whey and stored in the refrigerator at 4^{o}C for 30 days. The samples were taken from milk after inoculation (0 h), curd and every 2 days from cheeses during the storage period.

The number of colony forming units (cfu/g.) was determined by surface spread technique onto Baird-Parker's agar (CM0275B, Oxoid) supplemented with egg yolk tellurite emulsion (SR0054C, Oxoid) for enumeration of *S. aureus*. Plates were incubated at 37^{o}C for 24–48 hrs. then counted for viable organisms (Roberts and Greenwood, 2003).

2.4. Determination of sodium chloride content (APHA, 1992)

In a beaker, put 2 gm. of cheese sample with 20 ml of warm solution, The particles were broken till form uniform slurry and 10 ml of warm solution were transferred to 250 ml Erlenmeyer flask, then 25 ml of 0.1 N AgNo3, 10 ml of conc. nitric acid and 50 ml of warm distilled water were added, then the solution was boiled and 15 ml of 5% KMNO3 in 5 ml portions were added till the solution became brown for at least 5 min. of gentle boiling, the heating continued until brown color disappeared and formed clear, straw-colored solution. The solution was filtered and cooled filtrate was titrated against 0.1 N kSCN using 2 ml of ferric ammonium sulfate as indicator to the first red–brown color that lasted 30 sec. The Nacl% was determined according to the following formula:

Sodium chloride (salt) % =
{ (ml of N AgNo3) – (ml of kSCN) } × 0.0585 × 100 ⁄ gm. sample.

2.5. Experimental design and statistical analysis

The statistical analyses were performed with the SPSS pocket program for windows (version 16, 2007) One-way analysis of variance

(ANOVA), Duncan's multiple range tests and two-tailed Pearson correlation were used to determine the significant differences in the measured attributes at P value < 0.05. Results were expressed as mean ± standard error (SE).

3. Results

Table 1. Effect of Nisin on the growth and survival of *S. aureus* in kareish cheese

Storage time	Control	10 ppm	12.5 ppm
Zero time	4×10^{8}	4×10^{8}	4×10^{8}
Curd form	3.2×10^{8}	2.3×10^{8}	2.5×10^{8}
2 days	2.1×10^{8}	1.9×10^{8}	1.6×10^{8}
4 days	1×10^{8}	8×10^{7}	7×10^{7}
6 days	8×10^{7}	6.5×10^{7}	5.8×10^{7}
8 days	6×10^{7}	4.6×10^{7}	4.1×10^{7}
10 days	3.6×10^{7}	7.8×10^{6}	7.1×10^{6}
12 days	2.3×10^{7}	6.3×10^{6}	6.6×10^{6}
14 days	2×10^{7}	5.7×10^{6}	8×10^{5}
16 days	7.6×10^{6}	9×10^{5}	7.3×10^{5}
18 days	6.5×10^{6}	7.2×10^{5}	6.5×10^{4}
20 days	5.4×10^{6}	6.5×10^{5}	4.8×10^{4}
22 days	8×10^{5}	4.7×10^{4}	5×10^{3}
24 days	6.7×10^{5}	3.1×10^{4}	4.6×10^{3}
26 days	3.4×10^{5}	4.3×10^{3}	7×10^{2}
28 days	6×10^{4}	3.7×10^{3}	3.4×10^{2}
30 days	4×10^{4}	1.1×10^{3}	1×10^{2}

Table 2. The statistical analytical results between control and nisin (10&12.5 ppm)

Storage time	Control	10ppm	12.5ppm
Zero time till 10th day	$1.72\times10^{8}\pm5.35\times10^{7}$b	$1.25\times10^{7}\pm3.72\times10^{6}$a	$3.82\times10^{5}\pm1.55\times10^{5}$a
12th day - 20th day	$1.46\times10^{8}\pm5.2\times10^{7}$b	$2.85\times10^{6}\pm1.29\times10^{6}$a	$1.74\times10^{4}\pm9.18\times10^{3}$a
22th day- 30th day	$1.41\times10^{8}\pm5.33\times10^{7}$b	$1.65\times10^{6}\pm1.25\times10^{6}$a	$2.66\times10^{3}\pm1.24\times10^{3}$a

Means with different letters (a and b) within the same row are significantly different at *P* value < 0.05.

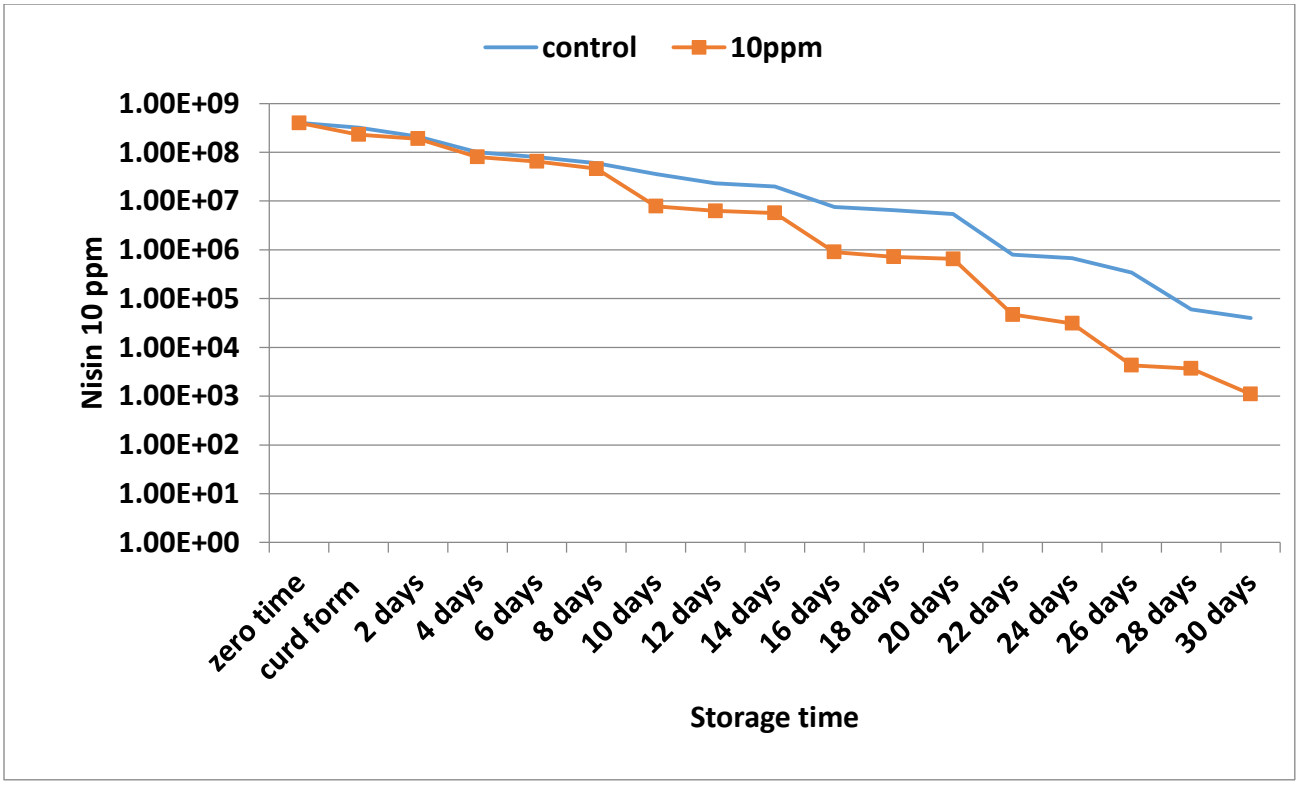

Fig. 1 Effect of nisin (10 ppm) on *S. aureus* count / gm. in kareish cheese samples during the refrigerator storage

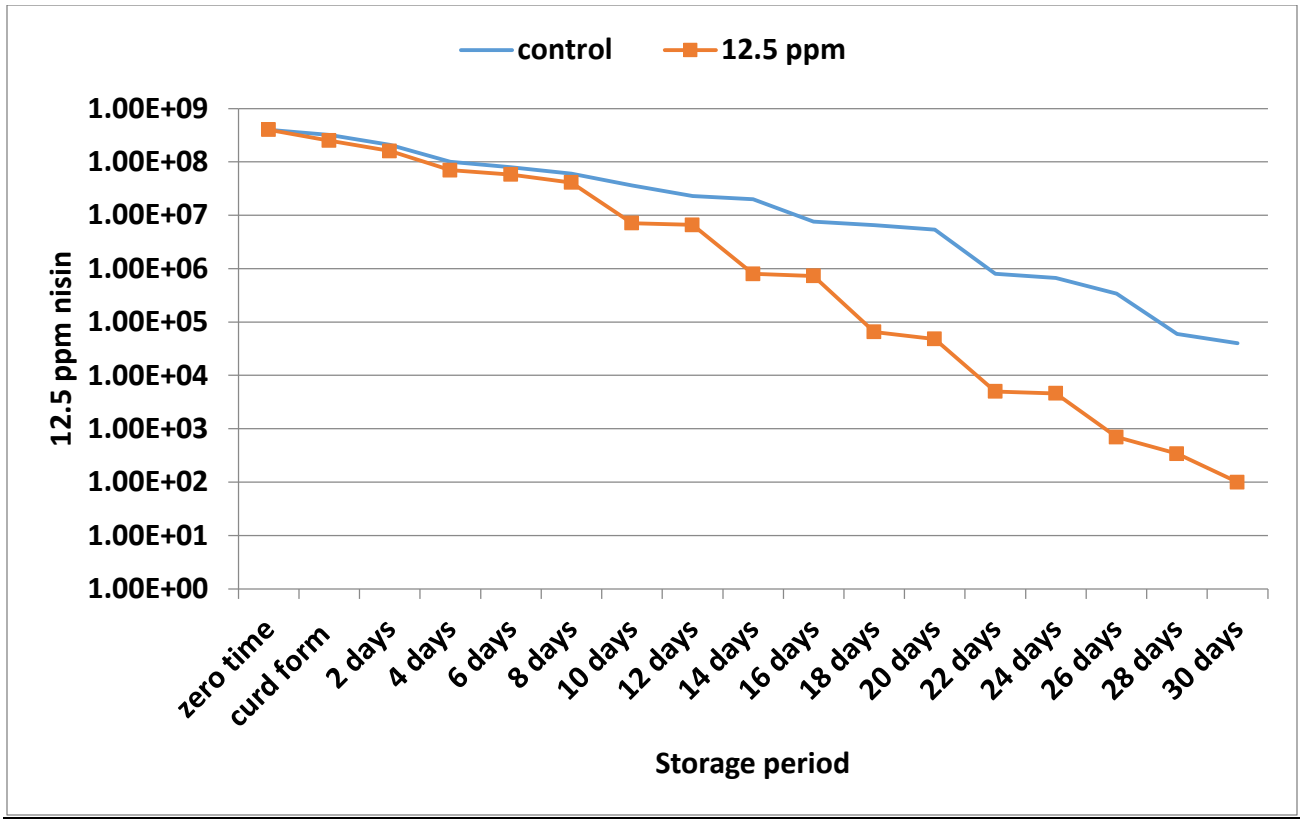

Fig. 2 Effect of nisin (12.5 ppm) on *S. aureus* count / gm. in kareish cheese samples during the refrigerator storage

Table 3. The statistical analytical results of salt (Nacl%) in control and nisin (10&12.5 ppm)

Storage time of formed curd	Control	10 ppm	12.5 ppm
Zero time	5.8±0.08a	5.8±0.08a	5.8±0.08a
Second day	5.8±0.08a	5.8±0.08a	5.8±0.08a
10th day	6.20±0.02ab	6.15±0.02a	6.25±0.03b
20th day	6.45±0.01a	6.55±0.02b	6.60±0.03b
30th day	6.90±0.03b	6.80±0.01a	6.85±0.02ab

Means with different letters (a and b) within the same row are significantly different at *p* value < 0.05.

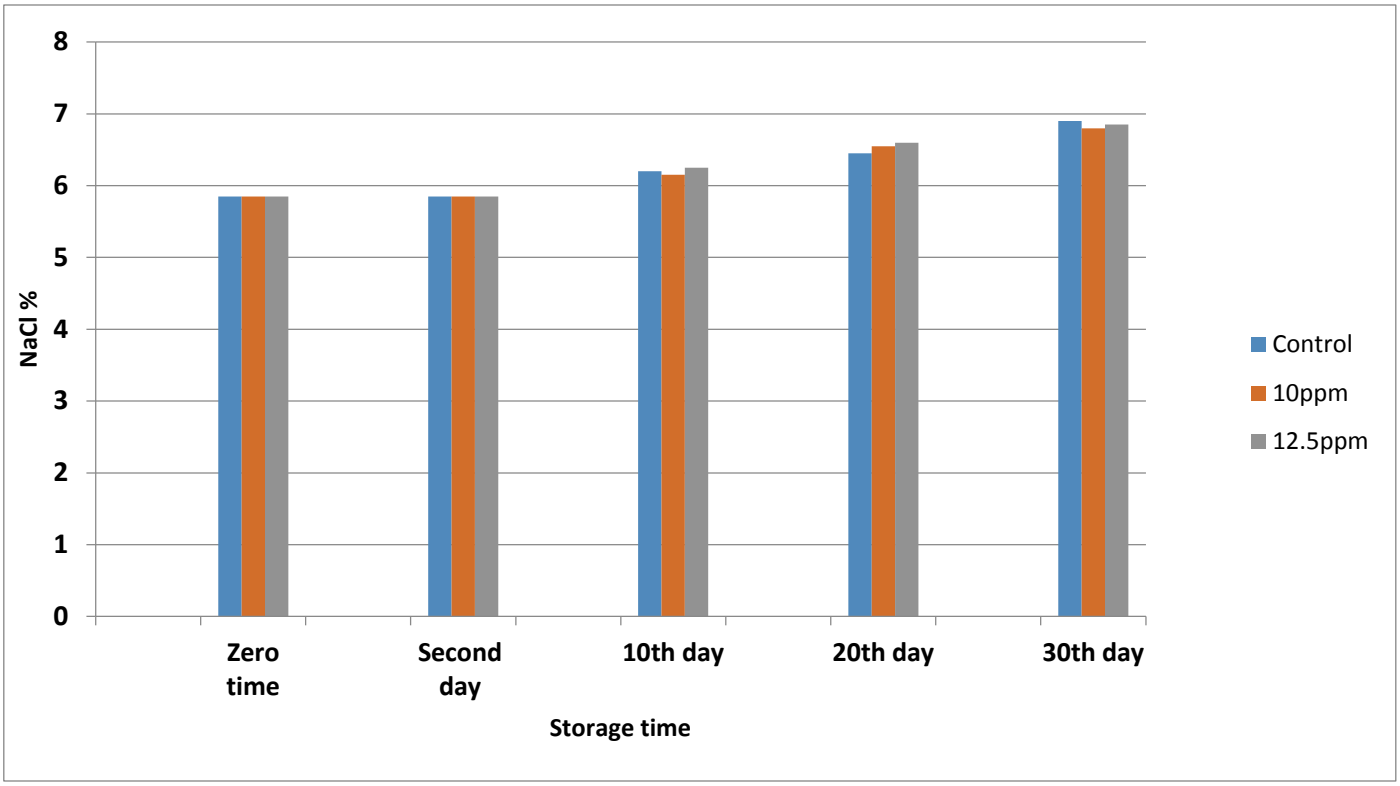

Fig. 3 Changes in salt (Nacl%) during the manufacture and storage of kareish cheese

4. Discussion

4.1. Effect of nisin (10 ppm) on growth and survival of *S. aureus* during manufacturing and storage of kareish cheese

Data illustrated in Table (1) and Figure (1) revealed that the count of *S. aureus* in the manufactured kareish cheese decreased gradually from $4x10^8$ to 1.1×10^3 CFU/gm. at 30th day, while in control ones decreased to 4×10^4 CFU/gm. The results showed that the population of the pathogen was $6.5x\ 10^7$ at the 1st week then became $5.7x10^6$ at the 2nd week, $6.5x10^5$ at 3rd week and finally at the end of storage period of the 4th week was 1.1×10^3 CFU/gm, less than the control; this attributed to the inhibitory effect of nisin (10 ppm) against the growth of *S. aureus* in kareish cheese.

The statistical analysis of results in Table (2) approved that there was significant difference (P value < 0.05) between the count of *S. aureus* in control and cheese treated with nisin which indicated that the addition of 10 ppm of nisin concentration to cheese diminished the development of *S. aureus* in cheese. Nearly similar results were recorded by (Amer and Ewina, 2003), while (WHO, 1969) gave nisin an international acceptance as food preservative at 4 units of nisin per gram food that is about 10 ppm.

4.2. Effect of nisin (12.5 ppm) on growth and survival of *S. aureus* during manufacturing and storage of kareish cheese

The results recorded in Table (1) and Figure (2) revealed that the viability of *S. aureus* in kareish cheese was sharply decreased from $4x10^8$ to 1×10^2 CFU/gm through the storage period. The count of *S. aureus* decreased gradually as following to $5.8x\ 10^7$ at the 1st week then became $8x10^5$ at the 2nd week, $4.8x10^4$ at the 3rd week and lastly at the end of storage period reached to 1×10^2 CFU/gm. during storage in refrigerator at 4°C for month; this is due to the more inhibitory effect of nisin (12.5ppm) against the growth and multiplication of *S. aureus* in kareish cheese.

Statistical analysis of the results in Table (2) approved that there was significant (*P* value < 0.05) between the count of *S. aureus* in control and cheese mixed with nisin which indicated that the addition of nisin (12.5 ppm) decreased the growth of *S. aureus* in cheese. These results are parallel to those obtained by Zottola et al. (1994) and Davies et al. (1997). The inhibitory effect of nisin on gram positive bacteria (*S. aureus*) was recorded by Maximiliano et al. (2011). In addition, The CAC, (2000) recommended 12.5 ppm nisin to preserve soft cheese.

The data showed that there were significantly (*P* value < 0.05) between count of *S. aureus* in control and cheese with nisin (10 ppm and 12.5 ppm), therefore, they were used for preservation of cheese due to their inhibitory effect against food borne pathogens, but the data indicated that nisin (12.5 ppm) had higher antimicrobial effect than nisin level of 10 ppm concentration against *S. aureus* growth in cheese during storage at 4°C, and this is due to the higher concentration that gave higher effect.

From the above results, It advisable to add nisin to milk used for soft cheese manufactured especially if such milk isn't subjected to any type of heat treatment; as nisin has an inhibitory effect on various microorganisms (Benech et al., 2002). In addition, the *S. aureus* count was reduced in the curd originating from nisin-treated milk, probably due to the bactericidal activity of nisin (Chalier et al., 2009 and Cotter et al., 2005). On the other hand, other factors could decrease the efficacy of nisin during the long storage period, such as PH (Kramer et al., 2004 and Martinez et al., 2008) and the temperature (Thomas and Wimpenny, 1996).

From the data presented in Table (3) and Figure (3) it is evident that there was no significant difference (*P* value > 0.05) between control and cheese with nisin at zero time and second day of storage period, while there was significant difference (*P* value < 0.05) between concentrations 10 ppm &12.5 ppm nisin, between control and both nisin levels (10 ppm &12.5 ppm) and between control and nisin of 10 ppm concentration only at 10th, 20th and 30th days., respectively during the storage period at 4°C for month.

Firstly, the results in Figure (3) revealed that there was fixed concentration of Nacl % during the first days of storage period, then appeared a slight increase in salt content that was observed in cheese of all treatments during the storage period and this may be attributed to the loss of weight due to loss of moisture (Walstra et al., 1999), whereas it reached 6.90, 6.80 and 6.85% on day 30 for control, 10 and 12.5 ppm nisin concentrations of cheeses., respectively. Although the initial salt levels of the cheeses were similar and the changes during storage were slight, the inactivation time for *S. aureus* was different. The salt values in cheese were close to the tolerance limit for growth of *S. aureus* (Nunez et al., 1986). This demonstrated that the salt levels hadn't any effect on *S. aureus* inactivation during cold storage of kareish cheese.

Therefore, inactivation of *S. aureus* may be due to the activity of nisin. It is well recognized that nisin can cause inhibition of the pathogen via the effect of nisin on the target bacteria in vegetative cells which exerted at the cytoplasmic membrane. Nisin forms pores that

disrupt the proton motive force and the pH equilibrium causing leakage of ions and hydrolysis of ATP resulting in cell death (Arauza et al., 2009). In addition, the biosynthesis of nisin occurs during the exponential growth phase and completely stops when cells enter the stationary growth. On the other hand, nisin is innocuous, sensitive to digestive proteases and it does not influence sensory properties of the food products. For these reasons, it has been proved to be an effective natural food biopreservative (Pongtharangkul and Demirci, 2004). The present study showed that cheese with nisin was significantly more effective in inactivation of *S. aureus* compared with control cheese. Inactivation by 12.5 ppm. nisin was significantly higher than that attained with 10 ppm nisin. Finally, the results clarified the importance of nisin addition to cheese during manufacturing for the health of the consumers when ingested such dairy products.

5. Conclusion

Nisin proved to be an efficient antimicrobial agent against *S. aureus* in kareish cheese resulting in the inhibition of growth of *S. aureus* in the cheese. The use of nisin doesn't imply that acceptable levels of *S. aureus* in cheese milk have been attained because bacteriocin (nisin) efficacy depends on the initial contamination of the milk as well as cheese handling practices. The results obtained in this study clearly showed the antibacterial effect of nisin on kareish cheese during manufacture. The obtained results in this study also indicated the remarkable effect of 10 and 12.5 ppm. nisin concentrations in preventing growth and survival of *S. aureus* during cheese storage. The inactivation rate was more pronounced with 12.5 ppm. than 10 ppm. nisin concentration; as it was dependent on concentration amount. Nisin reduced *S. aureus* populations in cheese gradually from the first week till the end of the storage period, respectively compared with the control cheese. From the above study, It's preferred to add nisin to cheese during manufacturing as a biopreservative and to extend the shelf life of product.

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تأثير النيسين علي حيوية الميكروب المكور العنقودي الذهبي في الجبن القريش

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نظرا لأهمية ميكروب المكور العنقودي الذهبي ولما يسببه من اثار سلبية على صحة الانسان في اللبن ومنتجاته، لذا وجدنا عند تصنيع الجبن القريش من اللبن منزوع الدسم ان نضيف النيسين كمادة حافظة للمنتج وتثبط نمو الميكروب وايضا تزود فترة صلاحية المنتج. وفي هذه الدراسة أشارت النتائج بمدى تأثير مادة النيسين ذو التركيزات المختلفة (١٠ و ١٢,٥ جزء من المليون) بالترتيب ضد ميكروب المكور العنقودي الذهبي في الجبن القريش المصنع معمليا واثناء تصنيع الجبن القريش تم حقن هذه التركيزات المختلفة من النيسين على الترتيب في الجبن مع وجود جزء غير محقون بالنيسين وقد تم تسجيل النتائج من بداية تصنيع الجبن وحتى الثلاثون يوما من فترة التخزين ووجدنا انه يقلل عدد الميكروب بالتدريج من ٤ x $١٠^{٨}$ الى ٦.٥ x $١٠^{٧}$ و ٨ .٥ x $١٠^{٧}$ و الى ٨ x $١٠^{٧}$ في الأسبوع الأول وفي نهاية فترة التخزين من الأسبوع الرابع قل العدد الى هذا المستوى من القراءات وهي ١.١ x $١٠^{٣}$ و ١ x $١٠^{٢}$ و الى ٤ x $١٠^{٤}$ لكل جرام في حالة التركيزات الاتية (١٠ و ١٢,٥ جزء من المليون) مقارنة بالجزء الغير محقون بالنيسين ومن هنا بدأ تاثير النيسين المثبط على نمو الميكروب على الترتيب اثناء تصنيع وتخزين الجبن القريش لمدة شهر في الثلاجة. وهنا وضحت النتائج على أهمية اضافة النيسين في الجبن القريش والذي له تأثير مضاد على حيوية الميكروب المكور العنقودي الذهبي.