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Application of Natural Antimicrobial Additives and Protective Culture to Control Aerobic Spore Forming Bacteria in Low Salt Soft Cheese

Eman K. Abo Shaala[1*], Sameh A. Awad[2], and Ashraf M. Nazem[1]

[1]*Department of Food Hygiene, Faculty of Veterinary medicine, Alexandria University, Egypt*
[2]*Department of Dairy Science and Technology, Faculty of Agriculture, Alexandria University, Egypt*
[*]Corresponding author's Email: keman718@gmail.com; ORCID: 0000-0003-4573-2056

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ABSTRACT

There is an increasing interest in the application of natural antimicrobials instead of chemical ones to enhance the microbiological quality of dairy products. The objective of this study was to assess the effect of some natural antimicrobial additives and protective culture for reducing the usage of chemical preservatives, shelf-life extension, retarding microbial spoilage in low-salt soft cheese. The antimicrobial agents (protective culture, nisin, lysozyme, and natamycin**)** were studied on the activity of 28 isolates of spore-forming bacteria. Inhibitory effect of different natural antimicrobial additives as protective culture (*Lactobacillus rhamnosus*, 40 mg kg $^{-1}$), nisin (25 mg kg $^{-1}$), lysozyme (100 mg kg $^{-1}$), combination of nisin and lysozyme (25 mg kg $^{-1}$/100 mg kg $^{-1}$), and combination of protective culture and natamycin (40 mg kg $^{-1}$/25 mg kg $^{-1}$) were studied on the growth of aerobic spore-forming bacteria in low-salt soft cheese during the storage period (30 days) at 4±1°C. The results revealed that the addition of different natural antibacterial additives with various concentrations had a significant effect on aerobic spore-forming bacteria, compared to other treatments and control. The growth pattern of aerobic spore-forming bacteria gradually decreased in all treatments along the storage period with variable reduction percentages in comparison with control cheese which was in continuous increment. The application of a combination of nisin and lysozyme had the most significant reduction of aerobic spore-forming bacteria, compared to control and other treatments.

Keywords: Aerobic spore-forming bacteria, Lysozyme, Nisin, Natamycin, Protective culture

INTRODUCTION

Soft cheese is one of the oldest dairy products with the best nutritive value and health keeping function. It is popular in many countries worldwide with palatable taste and various flavor (Awad et al., 2012). Cheese consumption has greatly increased during the past decade in the world (Elsamani et al., 2014). Considering the common consumption of cheeses, there is a growing concern regarding its safety and microbiological quality (Al-Gamal et al., 2019). Soft cheese manufacturing procedures include standardization and in many cases pasteurization of milk, acidification mainly through indigenous production of lactic acid by specific bacteria, coagulation of milk by rennet, dehydration of the curd by transforming coagulum into specific characteristic shapes; the resultant cheese could be consumed fresh or matured (After ripening of the curd, Fox et al., 2017). Salt is a vital component as it affects many aspects of cheese including shelf-life, activity of enzymes, flavor, casein hydration, and microbial proliferation during ripening. Salt is applied during cheese making for several purposes including acquiring the desirable flavor and texture, preservative action, and as a taste enhancer. Many consumers not prefer cheese with elevated salt percentage, mainly due to health risks such as hypertension, cardiovascular disease (CVD), stroke and heart attacks (Bae et al., 2017).

Aerobic spore-forming bacteria mainly Bacillus are of great importance in the dairy industry as the spores of these bacteria in raw milk can survive during pasteurization as well as other processing treatments and finally existed into final products (Coorevits et al., 2008). They are considered one of the most important genera encountered in the spoilage of milk and dairy derivatives. In addition, Bacillus is also involved in the formation of biofilms on stainless steel surface lines of dairy plant equipment (Lopez-Brea et al., 2018). The presence of Bacillus enzymes such as proteases in milk may decrease yield and shorten the required time for coagulation during cheese manufacturing due to the elevated concentration of amino acids that enhance the growth of starter culture quickly (Kumari and Sarkar, 2016). Furthermore, their germination leads to food spoilage as they threaten dairy products manufacturing due to high economic losses, impairment of equipment, and reputational damage of food companies or pathogenic cases resulted from food borne illness (Egan et al., 2016). Some Bacillus species cause two types of foodborne diseases, an emetic (vomiting) intoxication and diarrheal infection. The former has resulted from the ingestion of a preformed toxin in food, and the latter originates from eating bacterial cells/spores that release enterotoxins in the small intestine (Budka et al., 2005).

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Contamination of milk and dairy derivatives with spore-forming bacteria involves two routes of the entrance, namely the raw milk route and the post pasteurization route. Raw milk in the farm tank is contaminated through cattle's teats and inefficiently cleaned milking facilities contaminated by soil, faeces and bedding material. Soil is the primary and direct contamination origin of spore-forming bacteria into foods, since it is a major source of these microorganisms. On the other hand, the post pasteurization contamination of milk with spores is associated with the dairy industry or to the biofilms of spore-forming bacteria growing in processing lines that can finally be widely spread by release into the system of milk production (Heyndrickx, 2011). Improperly cleaned facilities and poor handling within the dairy plant is also considered as main sources and shedders of the contamination of spore formers at dairy processing equipment (Faille et al., 2014).

There is an increasing interest in the application of bio-preservation methods that utilize natural antimicrobial compounds such as nisin, lysozyme and protective culture (Sudagidan and Yemenicioğlu, 2012). Nisin has been evidenced as (Generally Regarded as Safe) by the US Food and Drug Administration (FDA) since 1988 as it has been long applied in food protection (E234) without being associated with health problems (Gharsallaoui et al., 2016). Application of nisin alone, or combined with other treatments as lysozyme, could provide a desirable advance for the microbiological safety and keeping of sensory criteria in milk and dairy products (Sobrino-López and Martín-Belloso, 2008). The functional characteristics of lysozyme as a natural antiviral and antibacterial substance have been implicated in industrial applications, especially in the food, pharmaceutical and medical industries (Arabski et al., 2015). Most food fermentation processes include mixed cultures in which various microbial species interact with each other. These interactions may have a neutral, positive or negative impact on the activity of the strains performing the fermentation (Arioli et al., 2016). Protective cultures have strong antagonistic activity against food-spoilage and pathogenic aerobic spore-forming bacteria as a result of the production of bacteriocins, inhibitory enzymes, organic acids and hydrogen peroxide (Ibrahim and Awad, 2018). Consumers have an increasing knowledge of health risks caused by the usage of chemical preservatives. Therefore, there is an increasing requirement in the dairy industry to prolong product shelf-life and inhibit spoilage by natural preservatives and/or new methods of conservation (Silva et al., 2018). Therefore, this research aimed to study the application of some natural antimicrobial additives (Nisin, lysozyme, protective culture and natamycin) to enhance the quality, shelf-life and safety of low-salt soft cheese.

MATERIALS AND METHODS

Materials

The employed materials in the current study included fresh buffalo milk obtained from a small dairy farm at Abis, Alexandria governorate. Microbial rennet: Fromase, France, Nisin: manufactured in the Netherlands by Siveele B.V, imported by AWA Food Solution. 4th Industrial area, New Borg Al Arab City. Alexandria, found in the market in form of powder with commercial name Nisaplin® and has the EU food additive number E234. Natamycin: from Spain, Imported by AWA Food Solution. 4th Industrial area, New Borg Al Arab City. Alexandria. Lysozyme: Lysozyme from chicken egg white was from Merck, Germany. Protective culture (Lyofast LRB), SACCO Co, Italy. It consists of a selected strain of *lactobacillus rhamnosus* (Used in dairy derivatives as non-starter lactic acid bacterial culture, which provides a slight acidity and smell from slow fermentation). Spore forming isolates included 28 strains isolated from commercial low-salt soft cheese collected from Egyptian markets. These isolates were purified and classified as aerobic spore-forming bacteria (study under publication).

Methods

Low-salt soft cheese manufacture

Low-salt soft cheese was made according to the method adopted by Fahmi and Sharara (1950). Fresh buffalo`s milk was used for manufacturing six treatments of low-salt soft white cheese. Raw milk was pasteurized at 63ºC for 30 min, cooled to 37 ºC, then calcium chloride and sodium chloride was added in 0.02% and 3% (w/v). Cheese milk was divided into six portions, then, the additives for each treatment were added individually. The commercial rennet was added to the milk and incubated at 37 ºC for coagulation within 90 min. The treatments of low-salt soft white cheeses using pasteurized milk were as following: pasteurized milk with no additives as a control (Treatment 1), pasteurized milk with adding protective culture (40 mg/kg, Treatment 2), pasteurized milk with adding lysozyme (100 mg/kg, Treatment 3), pasteurized milk with adding nisin (25 mg/kg, Treatment 4), pasteurized milk with adding nisin and lysozyme (25 mg/kg +100 mg/kg, Treatment 5), pasteurized milk with adding protective culture and natamycin (40 mg/kg + 25 mg/kg, Treatment 6). In treatments of protective culture (T2 and T6), the protective culture (lyofast LRB) 200 mg were added to 5 kg milk according to the recommendation of suppliers followed by incubation at 37 °C for 1 hour then adding NaCl (3 %), $CaCl_2$(0.02%) and finally the addition of rennet (0.002%(w/v)) to coagulate milk within 90 min using the same procedure as in other treatments.

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Microbiological evaluation of laboratory manufactured low-salt soft cheese

Using aseptic technique, 5 grams of low salt laboratory manufactured soft cheese were transferred by sterile spatula to a sterile polyethylene bag then adding 45 ml sterilized sodium citrate 2%, bags were placed in a stomacher for shaking at 160 rpm for 5 min, then serial dilutions using sterilized sodium citrate 2% were performed (Wehr and Frank, 2012).

Enumeration of aerobic spore-forming bacteria

All previous prepared serial dilutions were heated in a water bath at 80 ºC for 10 min then cooled suddenly to the 30 ºC before transferring one ml aliquots into sterilized Petri dishes containing nutrient agar. The duplicate plates were incubated at 32 ºC for 48 hours. Mesophilic aerobic spore-forming bacteria were enumerated on nutrient agar after incubation (Wehr and Frank, 2012).

Isolation of aerobic spore-forming bacteria

Colonies suspected as Bacillus species based on colony morphology, spread with usual features e.g slimy, crusty, dry, embedded or forming skin-like pellicles, were sub-cultured in nutrient broth at 32°C for 48 hours, then purified on non-selective medium nutrient agar plates for another 48 hours at 32°C. The isolates were inoculated into the nutrient broth and incubated at 32°C for 48 hours and then stored in Eppendorf tubes containing nutrient broth with 15-20% glycerol at -20 °C for further examinations (Wehr and Frank, 2012).

Studying the antibacterial activity of natural additives against isolated spore-forming bacteria

Natural antimicrobial agents such as nisin, lysozyme, protective culture and natamycin were evaluated to inhibit the growth of all isolates of spore-forming bacteria. Each of the tested isolated strains was inoculated in nutrient agar medium in Petri plates, After solidification of agar, four-wells in each plate were performed, each natural antibacterial additives such as nisin, lysozyme, protective culture and natamycin with identified concentration and quantity as follow 100 µl of nisin (25% concentration),100 µl of natamycin (concentration 25%), 100 µl of protective culture (concentration 0.04%) lysozyme 100 µl (0.1% concentration) was placed in the wells .The plates were incubated at 37 ºC for 24 hours and then recording of inhibition zone to determine their effectiveness against isolated aerobic spore-forming bacteria. This was carried out according to Performance Standards for Antimicrobial Susceptibility Testing (CLSI, 2018).

Statistical analysis

Statistical analysis of the data was performed using ANOVA, F-test, and LSD procedures available within the SAS software package (version 9.13 2008). Means with a significant difference were compared by Duncan's multiple range tests according to Steel and Torrie (1980). All physicochemical and microbiological analyses were performed in duplicate.

RESULTS

As can be seen in Table (1), the reduction percentage of aerobic spore-forming bacteria in laboratory manufactured low-salt soft cheese treated with different concentrations of natural antimicrobial additives during the storage period (30 days) at 4±1°C. The addition of protective culture (40 mg/kg) reduced aerobic spore- forming bacteria on first day of analysis by 7.41 %, reduction percentage increased on the day 14 of storage to reach 83.10% then the reduction percentage was at a high rate (90.94 %) on day 30 of storage. Aerobic spore- forming bacteria in cheese treated with lysozyme (100 mg/kg) reduced by 33.68% on first day of manufacturing then reached 97.82% on the day 30 of storage. Losing percent of aerobic spore-forming bacteria in cheese treated with nisin (25 mg/kg) was 31.60%, 90.32%, 96.54% on days 1, 14 and 30 of storage, respectively. Reduction percentage of aerobic spore-forming bacteria in cheese treated with combined nisin and lysozyme (25 mg/kg and 100 mg/kg) was at the highest rate among other additives on first day of analysis as was 51.50% then increased to 93.97% on the day 14 of storage then reduction percentage elevated sharply on the day 30 of storage to reach 97.82%. The addition of a combination of protective culture plus natamycin (40 mg/kg and 25 mg/kg) to cheese led to a reduction percentage of 21.18% at the first analysis then elevated to 92.78% at the second analysis then finally increased greatly on the day 30 of storage to reach 93.08% (Figure 1). Results recorded in Table 2 showed that the mean value of inhibition zone (mm) of nisin against isolated spore-forming bacteria was 25.30 ± 11.9 mm, while the mean value of inhibition zone diameter of lysozyme against isolated spore-forming bacteria was 32.90±19.30. The mean value of inhibition zone diameter of protective culture against isolated aerobic spore-forming bacteria was 26.10 ± 10.20. Natamycin had no inhibitory effect against spore-forming bacteria.

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Table 1. Growth pattern and reduction percentage of aerobic spore-forming bacteria (cfu/g) in laboratory manufactured soft cheese treated with different concentrations of natural additives during storage period (30 days) at 4±1°C.

Storage / Period time (day)	Control		Natural additives									
			Protective culture (40 mg kg $^{-1}$)		Lysozyme (100 mg kg $^{-1}$)		Nisin (25 mg kg $^{-1}$)		Nisin (25 mg kg $^{-1}$) + lysozyme (100 mg kg $^{-1}$)		Protective culture (40 mg kg $^{-1}$) + Natamycin (25 mg kg $^{-1}$)	
	Mean ± SD	R%	Mean ± SD	R%	Mean± SD	R%	Mean ±SD	R%	Mean ± SD	R%	Mean ± SD	R%
1	864 ± 0.71^{a}	0	800 ± 0.71^{b}	7.41	573 ± 0.71^{e}	33.68	591 ± 0.71^{d}	31.60	419 ± 0.71^{f}	51.50	681 ± 1.41 ^{c}	21.18
14	1509 ± 0.71^{a}	0	255 ± 0.71^{b}	83.10	119 ± 0.71^{e}	92.11	146 ± 0.71^{d}	90.32	91 ± 0.71^{f}	93.97	109 ± 0.71^{c}	92.78
30	2109 ± 0.71 ^{a}	0	191 ± 0.71^{b}	90.94	46 ± 0.71^{e}	97.82	73 ± 0.71^{d}	96.54	46 ± 0.71^{f}	97.82	146 ± 0.71 ^{c}	93.08

SD= Standard deviation, R%=Reduction percentage, *Means carrying a different superscript small letter on the same row are significantly different (P≤0.05).

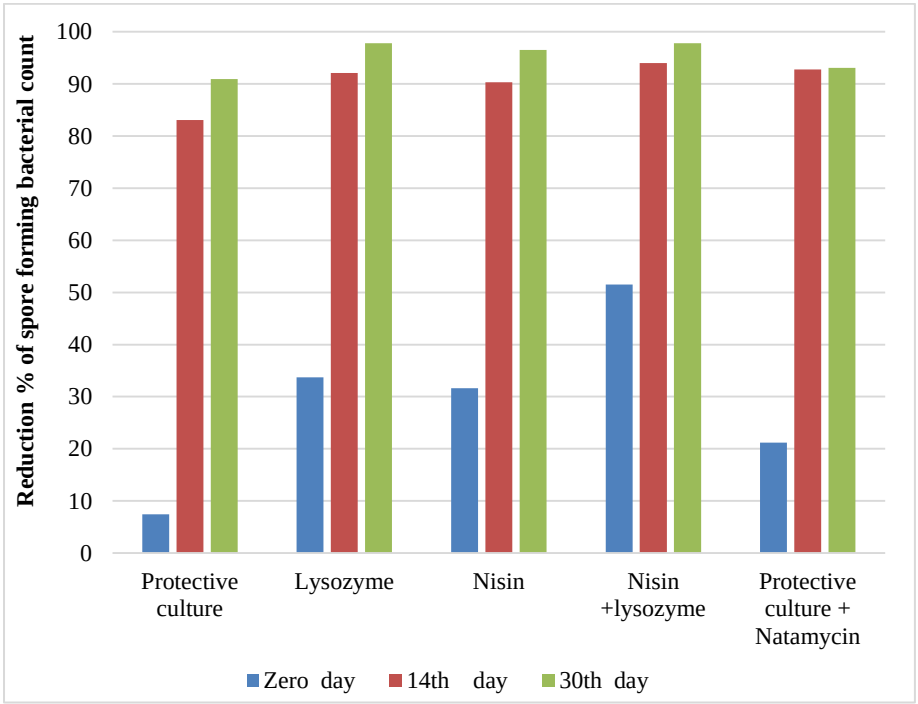

Figure 1. Effect of protective culture and some Natural antimicrobial additives on the reduction count of spore- forming bacteria in low-salt soft cheese

Table 2. Antibacterial activity of natural additives against isolated aerobic spore-forming bacteria (inhibition zone mm)

Number of Tested Isolates	Nisin	Lysozyme	Protective culture	Natamycin
	Inhibition Zone (mm)			
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
28	25.30±11.9	32.90±19.30	26.10±10.20	Not Detected

SD=Standard deviation

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DISCUSSION

Reduction percentage of aerobic spore forming bacteria in laboratory manufactured soft cheese treated with different concentrations of natural additives during storage period (30 days) at 4±1°C

Natural antimicrobial additives are a magic substitute for chemical preservatives for the maintenance of dairy product safety and microbiological quality (Lopes et al., 2019). Spore-forming bacteria cause significant economic losses and health problems in the dairy industries (Coorevits et al., 2010). They are commonly distributed in the environment and are associated with varieties of dairy products. Their existence in foods constitutes great problematic issues because the formation of their spores enables them to be resistant to thermal processing, freezing, chemical agents, and other difficult conditions that the food faces during processing operations. However, the vegetative cells are destroyed by these factors, traditionally these microorganisms have been related to the spoilage of dairy products; recently they have been associated with potential food poisoning cases (Rodriguez-Lozano et al., 2010). Multiplication of aerobic spore formers in raw milk liberates extracellular lipase enzyme, which is attached to milk fat globules and concentrated in the manufactured cheese in the course of making, the enzyme adversely causes bitter flavor by hydrolysis of fat into fatty acids and glycerides. This enzyme could be inactivated by pasteurization but thermal treatment could not destroy it (Beresford et al., 1998). The reduction percentage of aerobic spore-forming bacteria exerted by protective culture LAB strains reached 90.94% at the last analysis since some types of *lactobacillus* (such as *lactobacillus rhamnosus*) can produce antimicrobial factors such as organic acids (lactic acid and acetic acid), ethanol, hydrogen peroxide, diacetyl, acetoin, acetaldehyde, carbon dioxide and bacteriocins that could develop in various ways a potent inhibitory action against many microorganisms involving pathogenic and spoilage ones of aerobic spore formers (Al-Gamal et al., 2019). Lysozyme hydrolyzes 1,4-β-bond between N-acetylmuramic acid, and N-acetylglucosamine were found in the peptidoglycan leading to the suppression of growth, multiplication and prevalence of aerobic spore-forming bacteria which finally decreased from 573±0.71 on the first day of analysis to 46±0.71 on the day 30 of storage (Abdou et al., 2013). With the final reduction percentage of 97.82%, lysozyme showed strong bactericidal action against aerobic spore formers mainly Bacillus species (Abdou et al., 2007). Cheese is considered one of the well-known food applications of lysozyme where it governs spoilage microorganism's growth. Application of lysozyme in soft cheese can result in extending shelf-life and prevention of total bacteria including spore formers, yeast and mold (Doosh and Abdul-Rahman, 2014). Lysozyme content in cheese usually ranged between 50 and 350 mg/kg of cheese weight (Ávila et al., 2014). Nisin exerts its effect by attacking the cell wall so destroy the target microorganisms (Gharsallaoui et al., 2016). Loss of potassium ion of the bacterial cell, depolarization of the cytoplasmic membrane, depression of respiration, and partial efflux of cellular ATP are the mechanisms of action of nisin (Omardien et al., 2016). Nisin exhibited its inhibitory action after the initiation of germination, where nisin attached to lipid II in the spore after outgrowth and inhibited it from becoming metabolically active by interfering with the formation of membrane potential and oxidative metabolism. Germination initiation was required for this lipid II binding to happen as nisin cannot bind to the dormant spore due to the absence of lipid II on the external of the spore (Gut et al., 2011). The reduction percentage of aerobic spore formers in cheese treated with nisin elevated from 31.60 % at first analysis to 96.54 % on the day 30 of storage. It has been noticed that the action of nisin is elevated when it is in combination with other additives such as lysozyme (Chung and Hancock, 2000). Application of lysozyme and nisin in soft cheese can lead to the extension of shelf-life and prevention of spoilage (Zottola et al., 1994; Doosh and Abdul-Rahman, 2014). Application of combination between nisin and lysozyme has the most significant reduction of spore-forming microorganisms in comparison with control and other treatments, which decreased from 419±0.71 on the first day of analysis to 46±0.71 on the day 30 of storage. These findings are in line with those reported by López-Pedemonte et al. (2003), Meruvu et al. (2011) and Ávila et al. (2014) who found that nisin and lysozyme combination were the most effective against gram-positive aerobic spore formers such as Bacillus species.

Studying the antibacterial activity of natural additives against isolated spore-forming bacteria

The mean value of inhibition zone of nisin against isolated spore-forming bacteria was 25.30 ± 11.9 mm, which was in the same range as those reported by Pirttijärvi et al. (2001) showing that the application of diluted nisin led to an inhibition zone around 3-30 mm against many species of aerobic spore-forming bacteria. Nisin effectively suppresses the counts of aerobic spore-forming bacteria during 24hour incubation in the lab that is sufficient for industrial application. Additionally, our results are higher than the data mentioned by Morsy et al. (2018) reporting that the inhibition zone of nisin against aerobic spore-forming bacteria was 15±1.32. Nisin has been used to prevent spore out growth in many types of cheese especially soft cheese also is useful for inhibiting different types of aerobic spore formers (Komitopoulou et al., 1999). The mean value of inhibition zone diameter of lysozyme against isolated spore-forming bacteria was 32.90±19.30 that is higher than results obtained by Morsy et al. (2018) who illustrated that the inhibition zone of lysozyme against aerobic spore formers was 14±1.11 and lower than the data presented by Ramanauskiene et al. (2009) who found that lysozyme produced a clear zone of inhibition of 38.52 ± 0.17 against aerobic spore formers. The mean

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value of the inhibition zone diameter of the protective culture against isolated aerobic spore- forming bacteria was 26.10 ± 10.20. These results are higher than data reported by Tharmaraj and Shah (2009) who found that *lactobacillus rhamnosus* produced an inhibition zone of 0-15 mm against aerobic spore-forming bacteria and also higher than data mentioned by Coman et al. (2014) who illustrated that *lactobacillus rhamnosus* showed inhibition zone of 11.80 ± 0.71 against aerobic spore-forming bacteria. The findings did not support the study conducted with Hawaz (2014) who reviewed that the addition of protective culture *(lactobacillus rhamnosus)* produced no inhibition zone against aerobic spore-forming bacteria. Spore-forming bacteria were reduced by the protective culture (*lactobacillus rhamnosus*) organisms to a greater extent than the non-spore formers, the inhibitory action of protective culture was strongest against aerobic spore-formers (Tharmaraj and Shah, 2009). Natamycin had no inhibitory effect against spore-forming bacteria. It destroys yeasts by specifically attaching to ergosterol and without permeabilizing the plasma membrane. It prevents vacuolar fusion by the definite interaction with ergosterol so, it is active against fungi but not against bacteria (Te Welscher et al., 2010).

CONCLUSION

The low-salt soft cheese has short shelf life due to the presence of aerobic spore-forming bacteria which are resistant to pasteurization temperature, therefore, the producers may use some chemical preservatives. From the above mentioned results and discussion, it could be concluded that some natural antibacterial agents could be used to inhibit the growth of aerobic spore-forming bacteria, which among them addition of lysozyme (100mg/kg) in combination with nisin (25mg/kg) had a great inhibitory effect on aerobic spore-forming bacteria followed by application of lysozyme alone.

DECLARATIONS

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Competing interests

The authors declare that they have no competing interests.

Consent to publish

All authors agree on article publication.

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