

ORIGINAL ARTICLE

## Effect of nisin and lysozyme on bacteriological and sensorial quality of pasteurized milk

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### ABSTRACT

**Objectives:** The objective of this study was to evaluate the impact of the antimicrobials nisin and lysozyme to control the growth of spoilage bacteria of pasteurized milk during cold storage.

**Materials and Methods:** Nisin, lysozyme, and a mixture of them were inoculated into freshly pasteurized milk at 500 IU/ml concentrations each. The acidity, sensory evaluation, and bacteriological quality of the treated pasteurized milk samples were examined at zero time and every 3 days till the samples showed the signs of spoilage, that were checked every day.

**Results:** Obtained results showed that there was a slight increase of the titratable acidity of the control and treated samples during refrigerated storage, but the acidity increase was significantly lower in samples containing lysosomes and/or nisin than the control samples. Nisin and lysozyme at 500 IU/ml concentration possessed inhibitory effect on the total bacterial, aerobic spore-formers, and psychrotrophic bacterial counts and extended the shelf-life of the treated samples. The efficacy of nisin 500 IU/ml combined with lysozyme 500 U/ml was assessed and synergistic activity has been detected, that was expressed in the form of higher inhibitory effect and extending the shelf-life of the samples up to 15 days at cold storage. Moreover, the sensory evaluation showed that nisin and lysozyme does not affect the acceptability of the examined samples.

**Conclusion:** The obtained data indicate that nisin and lysozyme have the potential to enhance the post-process bacteriological safety of pasteurized milk during the storage period and could aid in the elimination of post-process contamination and prolong its shelf-life.

### ARTICLE HISTORY

Received June 12, 2019

Revised July 02, 2019

Accepted July 17, 2019

Published August 18, 2019

### KEYWORDS

Bacteriological quality;  
lysozyme; nisin;  
pasteurized milk



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### Introduction

Troublesome spoilage microorganisms, including psychrotrophic and spore-forming bacteria, can produce proteolytic and lipolytic enzymes and cause a variety of defects and the extent to which pasteurized milk is contaminated with these bacteria is a major determinant of its shelf-life as well as they can cause infections as they might include pathogens [1].

The dairy industry is searching for minimum processing techniques that can be utilized to extend the shelf-life and enhance milk's sensorial characteristics [2]. In the same context, customers are increasingly demanding that food products be used without chemical preservatives; consequently, there has been a constant increase in the search of

alternative and efficient compounds for food preservation. Therefore, there is increased interest in the application of natural antimicrobials that can act as preservatives to enhance the microbiological quality of food [3,4].

Many naturally occurring compounds, such as nisin, lactoperoxidase, lactoferrin, essential oils, and lysozyme, are effective potential antimicrobial agents that could be used as food preservatives to improve food quality and extend its shelf-life through inhibiting spoilage and pathogenic microorganisms [5,6].

Nisin is a natural, toxicologically safe, generally recognized as safe (GRAS) antimicrobial peptide, approved by FDA, and utilized in more than 55 countries as a food bio preservative [7]. Because it is non-toxic, flavorless, and

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**How to cite:** Saad MA, Ombarak RA, Rabou HAS. Effect of nisin and lysozyme on bacteriological and sensorial quality of pasteurized milk. *J Adv Vet Anim Res* 2019; 6(3):403–8.

heat stable, it is used commercially in various kinds of food such as dairy, meat, fish, and eggs to reduce the incidence of foodborne pathogens and spoilage microorganisms [8,9]. It is most effective against Gram-positive foodborne pathogens and spore-forming bacteria that represent a well-defined spoilage concern in the food industry [10].

Lysozyme is a GRAS natural antimicrobial protein, widely distributed in livestock and plants and used for food preservation, e.g., as food additive in milk products as it has antimicrobial activity [11,12]. It is used to extend the shelf-life and enhance the quality of various cheese types such as mozzarella, Buratta, and Coalho [13–15].

Post-pasteurization contamination of fluid milk with spoilage bacteria plays a significant role in reducing the shelf-life and quality of conventionally pasteurized fluid milk. Therefore, the objective of this research was to assess the impact of nisin and lysozyme and their combination on pasteurized milk's bacteriological and sensorial quality in cold storage and its role to extend its shelf-life.

## Materials and Methods

### *Nisin and lysozyme solutions preparation*

Nisin powder (Sigma-Aldrich, USA), 2.5% pure nisin with  $10^6$  IU/gm potency, was dissolved in 0.02 N HCl. Lysozyme powder (Sigma Aldrich, USA), from chicken egg albumen, having an activity of about 20,000 U/mg was dissolved in sterilized distilled water. The solutions were filter sterilized through a 0.22  $\mu$ m membrane and freshly used at 500 IU/ml concentrations.

### *Pasteurized milk samples preparation*

Four, 250 ml screw-cap, sterilized flasks were filled with 200 ml pasteurized milk, subjected to laboratory pasteurization at 65°C for 30 min, each. In the first flask, lysozyme at a concentration of 500 IU/ml (T1) was added, nisin 500 IU/ml (T2) was added to the second flask, to the third one, (T3) nisin and lysozyme were added, 500 IU/ml each, while the last flask was kept as control (C), to which no lysozyme or nisin was added. All flasks, either control or treated, were subjected to acidity determination and bacteriological examination at day zero (within 2 h after treatment) then stored at 4°C  $\pm$  1°C and examined every 3 days till the signs of spoilage were detected. The samples were checked for the signs of the spoilage every day. Each treatment was prepared in triplicate.

### *Determination of titratable acidity*

Titratable acidity% (TA%) for each sample was determined according to Kirk and Sawyer [16]. Briefly, 40 ml of boiled and cooled distilled water and 1 ml of phenolphthalein (prepared at 1% in 95% ethanol) were added to 10 ml

of milk sample. The blend was titrated with 0.1 N NaOH solution until the color changed to faint pink and persisted for 30 sec. The volume of alkali used was noted and TA% was calculated.

### *Bacteriological evaluation*

Samples were opened on each sampling day and 10 ml were aseptically transferred into 90 ml of 0.1% buffered peptone water (Biolife Italiana, Italy). Ten-fold serial dilutions were prepared and 0.1 ml aliquots of the appropriate dilutions were plated on plate count agar (Oxoid, UK) in triplicates and aerobically incubated at 37°C for 24–48 h and 7°C for 7–10 days for the enumeration of total bacterial count (TBC) and psychrotrophic bacteria, respectively [17].

For enumeration of aerobic spore-formers, dextrose tryptone agar medium (HiMedia, India) was used and the plates were incubated at 37°C for 24–48 h as described by APHA [18]. After incubation, the colonies were counted, and data were demonstrated as  $\log_{10}$  cfu/ml.

### *Sensory evaluation*

Sensory evaluation, appearance, odor, and flavor were evaluated by nine panelists according to ISO 22935-2:2009/ IDF 99-2:2009 [19] criteria.

### *Statistical analysis*

The statistical analysis was conducted using one-way analysis of variance. Statistical Package for Social Science software was used to analyze the data. The results were considered significantly different with a significance level of  $p \leq 0.05$  [20]. The experiments were performed three times and the indicated values were the average of triplicate  $\pm$  standard error.

## Results and Discussion

New concepts to reduce contamination as well as control the growth of spoilage microorganisms in milk and dairy products using natural compounds are being researched. Most of the available studies were focused on milk products as cheese and yogurt [13,14,15,21]. In the current study, the antimicrobials nisin, lysozyme, and their mixture were studied to determine their ability to control the growth of spoilage bacteria of pasteurized milk during cold storage. Moreover, the impacts of nisin and lysozyme on pasteurized milk's titratable acidity and sensorial quality were investigated.

### *Determination of titratable acidity*

Titratable acidity is a good indicator of milk quality. In this study, the TA% of the control and treated pasteurized milk

samples were evaluated and the findings showed that the TA% of the samples was slightly increased during the storage period but the rise was significantly lower in samples containing nisin and/or lysosomes than the control samples during the same storage period as shown in Table 1.

At the 9th day of storage, the control samples showed signs of spoilage and were no longer suitable for examination, while the samples treated with 500 IU/ml nisin or lysozyme remained apparently normal till the day 14th. On the other hand, samples treated with combined nisin and lysozyme (500 IU/ml each) remained apparently normal till the day 17th (Table 1). This indicated that the increase of TA% in control samples was developed faster than those of T1, T2, and T3.

The obtained results might be due to the inhibitory effect of nisin and lysosomes on the lactic acid bacteria and subsequently on their ability to produce acid [22], which leads to the slow rate of acid development.

### Bacteriological evaluation

Nisin and lysozymes are natural additives known for their inhibitory action on microorganisms [5]. In the current study, TBC, aerobic spore-formers, and psychrotrophic bacterial counts of the pasteurized milk samples containing lysosomes and/or nisin were analyzed.

TBC is an indication of the sanitary status of the food [23]. Results showed that TBC ( $\log_{10}$  cfu/ml) in the examined pasteurized milk samples treated with nisin at concentration 500 IU/ml (T1) or lysozyme at concentration 500 IU/ml (T2) was decreased than the control samples during the same storage period. TBC of treated samples (T1 and T2) reached to  $2.17 \pm 0.25$  and  $2.15 \pm 0.25 \log_{10}$  cfu/ml at 12th day of storage, respectively (Fig. 1). Moreover, the samples treated with nisin combined with lysozyme, at concentration 500 IU/ml each (T3), showed the lowest

**Table 1.** The mean of the titratable acidity values of the examined pasteurized milk samples during their refrigerated storage.

Storage (days)	Titratable acidity (Mean $\pm$ S.E)			
	Control	T1	T2	T3
0th	0.13 $\pm$ 0.01	0.13 $\pm$ 0.01	0.12 $\pm$ 0.01	0.12 $\pm$ 0.01
3rd	0.17 $\pm$ 0.01	0.14 $\pm$ 0.02	0.13 $\pm$ 0.01	0.13 $\pm$ 0.01
6th	0.19 $\pm$ 0.04	0.15 $\pm$ 0.01	0.14 $\pm$ 0.02	0.13 $\pm$ 0.02
9th	S***	0.15 $\pm$ 0.01	0.15 $\pm$ 0.02	0.14 $\pm$ 0.01
12th	S	0.17 $\pm$ 0.03	0.18 $\pm$ 0.01	0.15 $\pm$ 0.01
15th	S	S	S	0.17 $\pm$ 0.03
18th	S	S	S	S

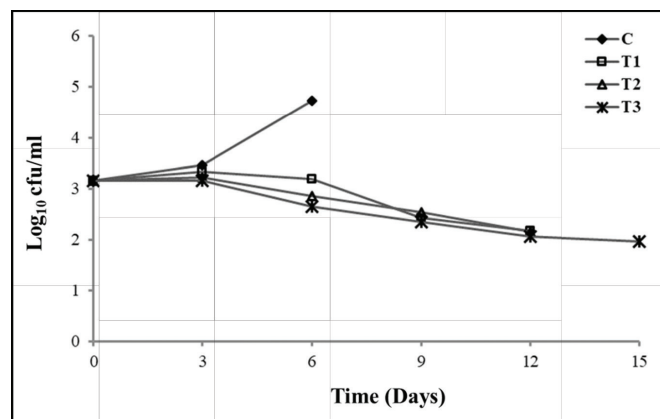
S.E = Standard error; Control = pasteurized milk without any treatments; T1: pasteurized milk with 500 IU/ml lysozyme; T2: pasteurized milk with 500 IU/ml nisin, and T3: pasteurized milk with nisin and lysozyme (500 IU/ml each); S = spoiled sample

TBC,  $1.97 \pm 0.03 \log_{10}$  cfu/ml at 15th day of storage, and their shelf-life extended till the samples spoiled at the 18th day (Fig. 1).

In fact, the shelf-life of commercially pasteurized milk is around 14 days in intact containers and chilled at 4°C [24], while after opening its container, the shelf-life becomes few days. Our study shows the potential of nisin and/or lysozyme in extending the shelf-life of pasteurized milk, even after opening its container, due to their effect on TBC. According to the limits proposed by Egyptian Standards [25], TBC of pasteurized milk must not exceed  $3 \times 10^4$  cfu/ml milk. Our study showed that the addition of nisin and/or lysozyme to pasteurized milk samples kept the TBC under that limit.

In the dairy industry, aerobic spore-forming bacteria are a particular concern as they can form biofilms within pipes and stainless-steel machinery and are able to survive industrial pasteurization [26]. The addition of nisin (T1) and lysozyme (T2) to pasteurized milk had significantly lowered the aerobic spore former counts compared with control samples. The main count reached to  $2.00 \pm 0.15$  and  $1.01 \pm 0.05 \log_{10}$  cfu/ml at 12th day of storage; respectively. Samples treated with nisin combined with lysozyme (T3) showed decreased counts during the storage period till the aerobic spore-forming bacteria were not detected after the 12th day of storage (Fig. 2). These findings come to an agreement with those reported by Ávila et al. [27] who observed that nisin and lysozyme combinations were active against Gram-positive foodborne spores such as *Bacillus* spp. and *Clostridium* spp.

Psychrotrophs are the most frequently isolated organisms that caused the spoilage of the heat-treated milk and



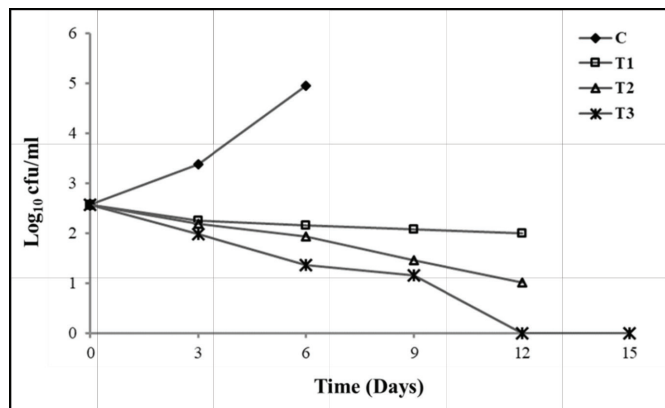
**Figure 1.** The mean of total bacterial counts ( $\log_{10}$  cfu/ml) in the examined pasteurized milk samples during their refrigerated storage. C: pasteurized milk without any treatments; T1: pasteurized milk with 500 IU/ml lysozyme; T2: pasteurized milk with 500 IU/ml nisin, and T3: pasteurized milk with nisin and lysozyme (500 IU/ml each).

dairy products; they usually enter the products through post-pasteurization contamination [28]. During refrigerated storage of the pasteurized milk, psychrotrophs can grow that require special attention, as they can cause spoilage as well as they can cause infections as they might include pathogens [29].

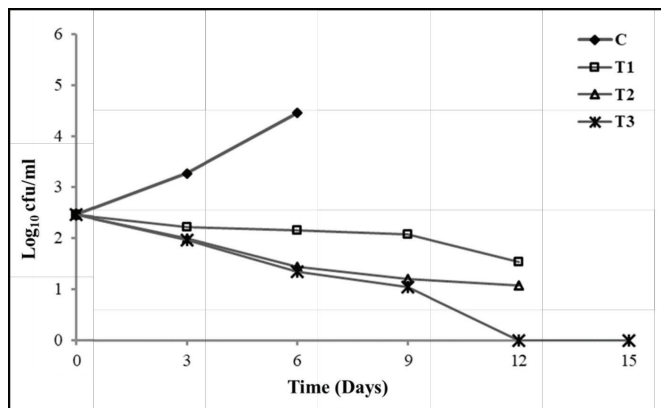
In the current study, the addition of nisin and/or lysozyme to pasteurized milk samples inhibited the growth of psychrotrophs. Samples treated with nisin combined with lysozyme (T3) showed higher inhibition rate and

psychrotrophic count were not detectable after the 12th day of storage. On the other hand, samples those treated by 500 IU/ml concentration of either nisin (T1) or lysozyme (T2) showed significant inhibitory effect on psychrotrophic counts with the mean counts of  $1.54 \pm 0.12$  and  $1.08 \pm 0.05$  after the 12th day of storage, respectively (Fig. 3).

Moreover, the sensory properties including appearance, odor, and flavor of the pasteurized milk samples treated with nisin (T1) or lysozyme (T2) or both (T3) were tested and as shown in Table 2, the sensory quality of the samples



**Figure 2.** The mean counts of aerobic spore formers bacteria ( $\text{Log}_{10}$  cfu/ml) in the examined pasteurized milk samples during their refrigerated storage. C: pasteurized milk without any treatments; T1: pasteurized milk with 500 IU/ml lysozyme; T2: pasteurized milk with 500 IU/ml nisin, and T3: pasteurized milk with nisin and lysozyme (500 IU/ml each).



**Figure 3.** The mean counts of psychrotrophic bacteria ( $\text{Log}_{10}$  cfu/ml) in the examined pasteurized milk samples during their refrigerated storage. C: pasteurized milk without any treatments; T1: pasteurized milk with 500 IU/ml lysozyme; T2: pasteurized milk with 500 IU/ml nisin, and T3: pasteurized milk with nisin and lysozyme (500 IU/ml each).

**Table 2.** Sensory evaluation results of the examined pasteurized milk samples.

	0th day	3rd day	6th day	9th day	12th day	15th day	18th day
Appearance							
Control	$4.5 \pm 0.18^{\text{A}}$	$2.75 \pm 0.13^{\text{C}}$	$1.98 \pm 0.03^{\text{C}}$	S	S	S	S
T1	$4.5 \pm 0.18^{\text{A}}$	$3.5 \pm 0.08^{\text{B}}$	$2.5 \pm 0.18^{\text{AB}}$	$2 \pm 0.13^{\text{C}}$	$1.5 \pm 0.05^{\text{C}}$	S	S
T2	$4.5 \pm 0.18^{\text{A}}$	$4 \pm 0.1^{\text{A}}$	$3 \pm 0.2^{\text{B}}$	$2.75 \pm 0.1^{\text{B}}$	$2 \pm 0.02^{\text{B}}$	S	S
T3	$4.5 \pm 0.18^{\text{A}}$	$4 \pm 0.1^{\text{A}}$	$3.75 \pm 0.05^{\text{A}}$	$3.5 \pm 0.05^{\text{A}}$	$2.75 \pm 0.2^{\text{A}}$	$2.5 \pm 0.8^{\text{A}}$	S
Odor							
Control	$4 \pm 0.17^{\text{A}}$	$2.09 \pm 0.15^{\text{C}}$	$0.97 \pm 0.2^{\text{C}}$	S	S	S	S
T1	$4 \pm 0.17^{\text{A}}$	$3 \pm 0.05^{\text{B}}$	$2 \pm 0.13^{\text{BC}}$	$1.5 \pm 0.05^{\text{C}}$	$1 \pm 0.11^{\text{C}}$	S	S
T2	$4 \pm 0.17^{\text{A}}$	$3.5 \pm 0.1^{\text{AB}}$	$2.75 \pm 0.05^{\text{B}}$	$2 \pm 0.13^{\text{B}}$	$1.5 \pm 0.1^{\text{B}}$	S	S
T3	$4 \pm 0.17^{\text{A}}$	$3.75 \pm 0.1^{\text{A}}$	$3.4 \pm 0.2^{\text{A}}$	$3 \pm 0.02^{\text{A}}$	$2.75 \pm 0.05^{\text{A}}$	$2.25 \pm 0.05^{\text{A}}$	S
Flavor							
Control	$4 \pm 0.02^{\text{A}}$	$3 \pm 0.1^{\text{C}}$	$1.5 \pm 0.01^{\text{C}}$	S	S	S	S
T1	$4 \pm 0.02^{\text{A}}$	$3.5 \pm 0.02^{\text{B}}$	$2.75 \pm 0.05^{\text{B}}$	$2.5 \pm 0.3^{\text{C}}$	$2 \pm 0.12^{\text{C}}$	S	S
T2	$4 \pm 0.02^{\text{A}}$	$3.75 \pm 0.12^{\text{AB}}$	$3.25 \pm 0.1^{\text{AB}}$	$3 \pm 0.05^{\text{B}}$	$2.5 \pm 0.02^{\text{B}}$	S	S
T3	$4 \pm 0.02^{\text{A}}$	$4 \pm 0.05^{\text{A}}$	$3.75 \pm 0.02^{\text{A}}$	$3.5 \pm 0.02^{\text{A}}$	$3 \pm 0.1^{\text{A}}$	$2.75 \pm 0.13^{\text{A}}$	S

\*The same letters in columns express no difference statistically, different letters express a difference statistically.



treated with nisin and or lysozyme was acceptable for longer periods compared with control samples. There were some statistically significant variations between the nisin added and lysozyme added samples. The samples with the highest scores were the samples with nisin combined with lysozyme (T3). This indicates that nisin and lysozyme could maintain the appearance, odor, and flavor of the pasteurized milk during its shelf-life.

In this study, as reflected in Figures 1–3, synergistic inhibition was observed when 500 IU/ml of nisin were used in conjunction with 500 IU/ml of lysozyme. Nisin showed significant inhibition than lysozyme. These results are in line with Chung and Hancock [22] findings that indicated synergy between nisin and lysozyme as their combination causes more severe bacterial cell damage.

## Conclusion

Nisin and lysozyme at 500 IU/ml concentration possessed inhibitory effect on the total bacterial, aerobic spore-formers, and psychrotrophic bacterial counts and extended the shelf-life of the treated samples and synergistic activity has been detected, that was expressed in the form of higher inhibitory effect and extending the shelf-life of the samples up to 15 days, in opened container, at cold storage without affecting the acceptability of the examined samples. The obtained data indicate that nisin and lysozyme have the potential to enhance the bacteriological safety of pasteurized milk during the storage period and thus extending its shelf-life even after opening its container.

## Conflict of interests

None of the authors have any conflict of interest to declare.

## Authors' contribution

All authors listed on this paper have made significant contributions to design the study, analysis, or interpretation of data; and to drafting the manuscript or revising it critically.

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