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Effect of activated lactoperoxidase system, nisin and their combination on raw milk quality during refrigerated storage

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Abstract
The inhibitory activity of lactoperoxidase system (LPS) and nisin (150 IU) added individually or in combination in raw milk was investigated against spoilage and pathogenic bacteria during 10 days of cold storage. The combined treatment kept lower acidity of raw milk when compared to control. A synergetic bacteriostatic activity was noted for the combined biopreservative agents especially against psychrotrophic flora, thermoresistant coliforms, coagulase positive *Staphylococcus, Listeria* spp and fungi which were an effective tool to improve raw milk quality. However, total coliforms and lactic acid bacteria were greatly reduced, respectively by the use of LPS and nisin alone.

Keywords: lactoperoxidase system, nisin, combination, raw milk, quality preservation

Introduction

Raw milk is a complete food, containing proteins, fats, carbohydrates, vitamins and mineral salts. It is considered as an ideal medium for the growth of a broad spectrum of bacteria due to its high nutritional value and can be easily contaminated mainly with bacteria during handling, transportation and processing (Yuan *et al*., 2017) [1]. In addition, it is imperative to minimise the storage temperature of raw milk at under 4 °C to relent the microorganism growth. However, under refrigeration at 4 °C, it is not sufficient to inhibit the growth of many spoilage bacteria and fungi which are able to cause the deterioration of foodstuff quality and make it inedible (Boulares *et al*., 2011) [2]. Besides, refrigerated raw milk may contain psychrotrophic microorganisms that produce thermoresistant exoproteases and lipases (Ben Moussa *et al*., 2008; Ribeiro Junior *et al*., 2017) [3, 4], undesired and pathogenic bacteria such as *L. monocytogenes* which is considered as a significant food pathogen which may compromise the quality of processed fluid milk and dairy products during storage. Thus, the quality of most dairy products is closely related to the microbial status of raw milk from which they are manufactured (Ben Moussa *et al*., 2013) [5]. Therefore, there is a global demand for dairy products with good quality and a great expectation to increase their shelf life. For this reason, it is necessary to keep contamination to a minimum and to control specific microorganisms with great spoilage potential (Ribeiro Junior *et al*., 2017) [4].

To reduce the risk of microbial growth in raw milk and dairy products during refrigerated storage, the incorporation of multiple barrier systems, including a suitable chemical food preservative, into the product is recommended. However, consumer demands have led to renewed interest in the use of natural antimicrobials for food (Arques *et al*., 2008) [6]. This in turn is creating pressure on food suppliers to consider the use of 'natural' alternatives to these chemical agents. In this context, the combination of these biopreservatives to achieve an enhanced level of product safety and stability has gained increased attention (Arques *et al*., 2008) [6].

In this regard, nisin, a well-known antimicrobial of the lantibiotic group, has GRAS (generally recognized as safe) status for use in food products in many countries (Razavi Rohani *et al*., 2011) [7]. Nisin is a bacteriocin produced by some *Lactococcus lactis* subsp. *Lactis* strains (Arques *et al*., 2008) [6]. In addition, nisin widely used as a preservative in the dairy industry and has a wide inhibitory spectrum against a wide range of Gram-positive bacteria, notably, *L. monocytogenes* and *S. aureus* (Arques *et al*., 2008) [6]. In fact, the anti-listerial properties of nisin have been well studied and applied in a variety of foods, including vegetables, dairy products and meats (Razavi Rohani *et al*., 2011) [7].

In order to enhance antibacterial activity of nisin, sensitize the resistant spoilage and food-borne microorganisms and expand its range of application, many new alternative approaches have been studied such as his combination with other antibacterial substances like the lactoperoxidase system, lactoferrin, thymol and different plant essential oils (Razavi Rohani *et al*., 2011) [7].

The lactoperoxidase–thiocyanate–hydrogen peroxide system (LPS) occurs naturally in milk and exhibits a broad antimicrobial activity against Gram-positive and Gram-negative bacteria (Arques *et al*., 2008, Boulares *et al*., 2011) [6, 2]. The enzyme lactoperoxidase catalyzes the oxidation of thiocyanate by hydrogen peroxide, yielding short-lived oxidation products, as hypothiocyanite ions, responsible for the LPS antimicrobial effect (Boulares *et al*., 2011; Aprodou

et al., 2014) [2, 8]. Moreover, LPS is known to extend the shelf life of milk which may lead to an increase in the amount of milk that could be available for marketing with benefits for both milk producers and consumers (Aprodou *et al*., 2014)[8]. After activation of LPS using thiocyanate and hydrogen peroxide, a bactericidal and bacteriostatic effects were observed in raw milk, pasteurized milk, ultra high temperature treated milk, cheese and yogurt against Gram-negative (*Escherichia coli*, *Salmonella* spp. and *Pseudomonas* spp.), Gram-positive bacteria (*L. monocytogenes*, *Staphylococcus*) involved in food-borne illnesses and fungi (Aklilu *et al*.,, 2021; Seval and Metin, 2020; Arques *et al*., 2011; Boulares *et al*., 2011; Ben Moussa *et al*., 2013,) [9, 10, 6, 2, 5].

Hurdle technology is a multifactor procedure for food preservation, is gaining interest as a method of inhibiting the growth of microbes. However, given the difference in the modes of action of nisin and LPS system with respect to various groups of spoilage and pathogenic bacteria present in raw milk, the objective of this study was to evaluate the antimicrobial effects of nisin and lactoperoxidase system (LPS) alone or in combination on physicochemical and microbiological raw milk quality during 10 days of refrigerated storage at 4 °C.

Material and Methods

1. Milk sample collection and treatment

Refrigerated raw milk was obtained from a collection centre situated in northern Tunisia during the spring period when the lactation curve was at its maximum. Milk samples were collected in sterilised flasks and immediately refrigerated at 4 °C. At the start of experiment, raw milk was divided into four batches in sterile bottles of 1L each one. The first batch served as untreated control. Then, LP system was activated in the second batch as described by Boulares *et al.* (2011)[2]. Reactivation of LPS was performed by addition of sodium thiocyanate (NaSCN) as a source of thiocyanate (SCN^-) to a final concentration of (14 mg/L) (Fluka Chemie, Steinheim, Germany). After mixing gently raw milk sample, 30 mg/L of sodium percarbonate (Fluka Chemie, Steinheim, Germany) was added as a source of hydrogen peroxide ($2Na_2CO_33H_2O$) as recommended by the Codex Alimentarius (CAC/GL 13-1991)[11]. Reactivated raw milk was thoroughly mixed and stored at 4°C in a refrigerator until analysis. In the third milk sample, nisin (N5764; Sigma, St. Louis, MO, USA) was added at a final concentration of 150 IU/mL according to Boussouel *et al*.(2000) [12] and Arques *et al*. (2008) [6] with some modifications. Previously, a fresh solution of nisin was prepared by dissolving it in 0.02 N HCl and the solution was heated at 70 °C for 10 min. Concerning the fourth raw milk sample, it was subject to the activation of LP system and the simultaneous addition of nisin (150 IU/mL).

All raw milk samples were aseptically distributed in little bottles (150 ml) and stored at 4 °C for 10 days. Microbiological and physico-chemical analyses were performed in triplicate on days 0, 1, 2, 3, 4, 5, 7 and 10 days. All the experiment was repeated three times.

2. Chemical analysis

The pH and acidity (ºDornic) were determined according to the standard method for the examination of dairy products according to the study of Boulares *et al*. (2011) [2].

3. Microbiological analysis

Microbiological analysis was performed by homogenising 10 ml of raw milk sample in 90 mL of peptone water (Oxoid). Decimal dilutions in 1% peptone water (Oxoid) were carried out on Plate Count Agar (PCA; Oxoid, France) for mesophilic and psychrotrophic bacteria with the plates being incubated respectively at 30 °C for 72h and at 7 °C for 10 days (Boulares *et al*., 2011) [2]. Lactic acid bacteria (LAB) were counted using De Man Rogosa and Sharp Agar medium (MRSA, Oxoid, France) after incubation of plates at 37 °C for 48h. Coliforms were enumerated using desoxycholate gelose 1‰ and incubated for 24h at 30 °C for total coliforms and at 44 °C for thermo-resistant coliforms (Boulares *et al*., 2011) [2].

For enumeration of coagulase positive *Staphylococcus*, appropriate decimal dilutions were seeded onto Baird Parker Agar (Biolife, Italy) containing tellurite and egg and incubated under aerobic conditions at 37 °C for 24–48 h. Coagulase tests of five isolates, from each plate, were performed. 1 ml of an overnight culture of each pure colony, in a brain heart broth, were emulsified on rabbit plasma and the tubes were incubated at 37 °C. Readings were taken at 1, 2, 3 and 4 h and further incubated overnight at room temperature if no clot formation was observed (Arques *et al*., 2008) [6].

Listeria counts were determined on Palcam agar (Difco, France), by streaking the plates with 0.1 ml of undiluted samples. Colonies were counted after incubation at 37 °C for 48 h. *Listeria* colonies are green with grayish reflection or olive green. *Listeria* enumeration was completed with Gram staining, mobility and catalase tests (Arques *et al*., 2008) [6].

Yeasts and moulds were counted on Sabouraud (Oxoid, France) after incubation at 25 °C for 3 days (Boulares *et al*., 2011) [2].

The detection of *Salmonella* was made according to the conventional method (ISO 6579, 2002) [13]. Milk samples of 25 ml were homogenized in 225 ml of Buffer Peptone Water (Difco, France) and incubated for 16–20 h at 37 °C. A volume of 0.1 ml of the pre-enriched sample was used to inoculate 10 ml of the Rappaport–Vassiliadis medium (Oxoid, France) and 1 ml of the pre-enriched sample was used to inoculate 10 ml of the selenite–cystine medium (Oxoid, France). Samples were incubated for 7 h at 42 °C (RV) and at 37 °C (SC), respectively. Subsequently, volumes of 0.1 ml each of individual selectively enriched samples were used to inoculate broth S-S agar and desoxycholate citrate agar (Oxoid, France) and incubated at 37 °C for 18–24 h.

4. Statistical analysis

The effect of LPS, nisin and storage period, were assessed using analysis of variance (ANOVA) by the general linear model procedure of the SPSS 10.0 statistical package program.

Results and discussion

1. Effect of the activation of LPS and/or addition of nisin on pH and acidity variation during refrigerated storage of raw milk

In the present study, pH and titrable acidity variations of treated and untreated raw milk during 10 days of refrigerated

storage, were shown in Figures 1 (a, b). Raw milk had initial pH and acidity values of 6.71 ± 0.07 and 18.63 ± 1.05 °D, respectively. These findings were similar to the values (6.60 ± 0.15 and 19.33 ± 3.05 °D) found by Boulares *et al*. (2011) [2]. During refrigerated storage at 4 °C, the acidity increased and pH values decreased with the progression of storage time as described in the previous work of Mankai *et al*. (2012)[14] on refrigerated raw milk. In this study, after 4 days of refrigerated storage, the pH value of untreated control reached 6.35 ± 0.1 while acidity was about 27.50 ± 1.05 °D. We noted different results from those found (6.09 ± 0.25 and 28.00 ± 3.20 °D, respectively for pH and titrable acidity) by Mankai *et al*. (2012) [14]. Thereby, LPS, nisin and their simultaneous combination kept refrigerated raw milk suitable to industrial transformation after four days of storage; since the pH values (between 6.41 ± 0.11 and 6.43 ± 0.07) are higher than 6.4 (NT 14 -141, 2007) [15]. Moreover, pH values of control raw milk decreased significantly ($p<0.05$) to reach a value of 5.59 ± 0.08, after ten days of refrigerated storage (Figure 1a, 1b). However, at the end of storage, significant ($p<0.05$) higher pH values were observed for all treated milk samples. Similarly, a significant ($p<0.05$) increase in acidity value was observed to reach at the end of refrigerated storage the highest acidity (48.33 ± 1.39 °D) in control. This acidification of control refrigerated milk was probably due to the initial microbiological count and the proliferation of psychrotropic bacteria as reported in previous study of Ribeiro Junior *et al*. (2017) [4] and Boulares *et al*. (2011) [2]. In fact, the cooling of raw milk allows the multiplication of some mesophilic microorganisms, called psychrotrophs, adapt to refrigeration temperatures and psychrotropic bacteria responsible for the secretion of extracellular enzymes, hydrolysis of lactose producing lactic acid and acidification of raw milk and thermal instability of milk proteins as a result (Ribeiro Junior *et al*., 2017)[4]. Contrary, the lowest acidity (30.80 ± 0.8 °D) was observed when raw milk was activated by LPS and treated with nisin which is due to the great synergetic effect of LPS and nisin (Boussouel *et al*., 2000) [12].

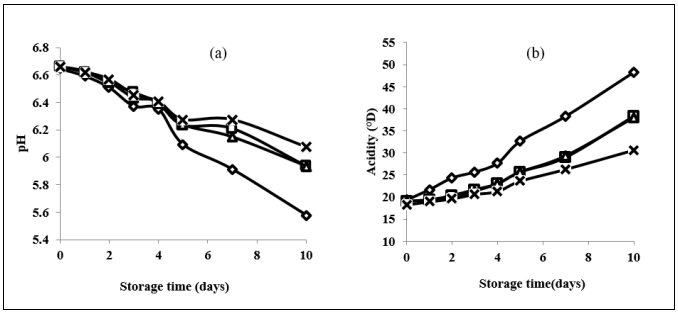

Fig 1: pH (a) and Lactic acidity values (b) of refrigerated raw milk (control) --◇--, with activated LPS system --□--, with nisin added (150 IU) --△--, and with activated LPS system and nisin added simultaneously --X—

Effect of the activation of LPS and/or addition of nisin on the microbiological quality of raw milk during refrigerated storage

Mesophilic and psychrotrophic total flora growth

Mesophilic (MBC) and psychrotrophic (PBC) bacterial counts in raw milk activated by LPS, added with nisin (150 IU/mL) individually or treated with their simultaneous combination were shown in Figure 2 (a, b). Initial counts, in control milk, were about 9.12 ± 0.18 log CFU/mL and 8.41 ± 0.37 log CFU/mL, respectively for MBC and PBC flora. These counts were higher to those (4.18 ± 0.31 log CFU/mL and 3.04 ± 0.42 log CFU/mL) reported by Ribeiro Junior *et al*. (2017) [4]. This differences and diversity between different raw milk samples may be linked to the hygienic condition of milking environment, animal wellness, refrigerated conditions before processing, packaging and handling (Yuan *et al*., 2017)[1]. Our findings demonstrated the low microbiological quality of Tunisian raw milk since the PBC count was higher than 5 log CFU/mL (Ribeiro Junior *et al*., 2017) [4]. This study supported the observations of previous works suggesting that PBC is an important determinant of raw milk quality and ultimately the quality of final dairy products because of their enzymatic spoilage potential and biofilm forming ability (Yuan *et al*., 2017)[1]. Throughout 7 days of storage time, the growth of these flora was significantly ($p<0.05$) reduced in treated milks when compared with untreated control with the lowest counts (10.80 ± 0.65 log CFU/mL and 9.91 ± 0.31 log CFU/mL, respectively for MBC and PBC) were observed after combining LPS and nisin in milk. Thus, storage time may be a great factor affecting the MBC and particularly the PBC and even the quality of final dairy products while it is related to raw milk collection intervals and transport distances which affected the microbial quality of raw milk (Yuan *et al*., 2017) [1]. However, after 10 days of refrigerated storage, no significant ($p>0.05$) differences were observed between all raw milk samples with always the highest counts observed in untreated control. This result could be explained by the spontaneous emergence of bacteria resistant to nisin and LPS. Our findings showed that the great antimicrobial effect of tested preservative agents was shown with the simultaneous use of LPS activation and nisin addition. Besides, the inhibitory effect of added nisin and activated LPS individually was found to be lower than the combination. In fact, both antimicrobial agents enhanced antibacterial effect, cause damage to the cytoplasmic membrane, which could explain their synergistic action (Boussouel *et al*., 2000) [12]. Primary reaction product of the LPS, hypothiocyanite, was known to react with the thiol groups of various proteins and inactivate crucial enzyme and protein systems (Boots and Floris 2006) [16]. Similarly, nisin can be transported through the lipopolysaccharidic layer and create pores in the cytoplasmic membrane (Juncioni de Arauz *et al*., 2009) [17]. In this connection, both antimicrobials play an important role in the depletion of proton motive force and the leakage of intracellular nutrients, since the bacterial respiration chain is targeted by the LPS (Boots and Floris 2006) [16], and nisin pores lead to a collapse of the membrane electrical potential and the pH gradient (Juncioni de Arauz *et al*., 2009) [17].

Total and thermoresistant coliforms growth

Counts of total and thermoresistant coliforms in treated and control raw milks were presented in Figure 2 (c, d). These data showed significant differences ($p<0.05$) between treated and control raw milk throughout 10 days of refrigerated storage for total coliforms. However, reducing effect wasn't significant ($p>0.05$) at the end of storage for thermoresistant

coliforms. Our results were partially in agreement with those reported by other researchers. Indeed, LP system and nisin had separately wide spectrum of bactericidal activity against Gram-negative bacteria in dairy products (Boulares *et al*., 2011) [2], the combined LPS with nisin in order to improve the preservation of sardines was found to be more effective than LPS alone against Gram-negative strains that are insensitive to nisin because of its large size (1.8-4.6 kDa) which restricts its passage across the outer membrane (Boots and Floris 2006) [16]. This result can be explained by the capacity of some coliforms bacteria to produce nisinase, enzyme which can inactivate nisin.

Contrary, the simultaneous antibacterial effect of LPS and nisin against thermoresistant coliforms was higher than that of LPS and nisin alone (Figures 2 d). In fact, it has been shown that nisin Z had antibacterial activity against *E. coli* by use a cationic amino acid mediated mechanism (Kuwano *et al*., 2005) [18] and the loss of bacterial viability of *E. coli* was correlated to inhibition of the succinate-dependent respiration system of the bacterium caused by LP system (Boots and Floris 2006)[16]. In this regard, both antimicrobials play an important role in the depletion of proton motive force, since the bacterial respiration chain is targeted by the LPS and nisin pores lead to a collapse of the membrane electrical potential and the pH gradient.

Lactic acid bacteria growth

Figure 2 (e) indicates the growth of lactic acid bacteria (LAB), during 10 days of storage at 4 °C, in untreated control, activated LPS, added nisin and simultaneous treatment by LPS and nisin raw milks. As shown in the Figures 2 (e), LAB counts in treated raw milks were significantly ($p<0.05$) lower than their count in the untreated control milk, until 7 days of storage. They reduction reached respectively 38.7%, 51.4% and 57% in activated LPS, added nisin and combined LPS and nisin treated raw milks. The effect of this later treatment was higher than antibacterial activity of activated LPS alone against LAB. Our findings were in line with those of Juncioni de Arauz *et al*. (2009) [17] suggesting that nisin is an effective bactericidal agent against Gram-positives bacteria including strains of *Lactococcus*, *Streptococcus*, *Staphylococcus*, Micrococcus, *Pediococcus* and *Lactobacillus* because his effect on the target bacteria in vegetative cells is exerted at the cytoplasmatic membrane. In this study, we observed that activated LP system had a bacteriostatic effect against LAB as reported before by Boulares *et al*. (2011) [2] when activating LPS in raw milk. However, the highest effect of added nisin alone on LAB proliferation when compared to the combined treatment was attributed to the competition between different floras on nutriments particularly LAB and coliforms which were competing on lactose. Thus, when LPS was activated coliforms decreased significantly. Consequently, the growth of lactic acid bacteria would be favoured.

Coagulase positive *Staphylococcus* and *Listeria* spp. growth

Staphylococcus aureus and *Listeria monocytogenes* are pathogens of great concern for the dairy industry. *Staphylococcus aureus* is capable of producing enterotoxins responsible for one of the most prevalent causes of gastroenteritis (Arques *et al*., 2008) [6]. *L. monocytogenes* is ubiquitous found in dairy processing environments which can grow in refrigerated milk and can survive for long periods under adverse conditions. Besides, some psychrotrophic pathogenic microorganisms, such as *Staphylococcus* and *Listeria* have also demonstrated high potential to form biofilms on food contact surfaces, which may contribute to foodborne outbreaks (Yuan *et al*., 2017) [1].

In this study, results of coagulase positive *Staphylococcus* counts were shown in Figure 2 (f). Relative reduction, after 24 h of cooling at 4 °C, were 22%, 71.4% and 61%, respectively, in activated LPS, added nisin and combined treated raw milk samples (Figure 2f). After 5 days of cold storage, the reduction of this flora reached respectively 19%, 64% and 51%. However, no significant differences ($p>0.05$) were observed from the seventh day of refrigerated storage between treated samples. Although, it is well known that nisin and LPS separately had respectively bactericidal and bacteriostatic effect against coagulase positive *Staphylococcus* (Buys 2011) [19], their real simultaneous antimicrobial effect was the highest at the end of storage. In fact, antibacterial effect of nisin alone was shown to be the most important during all storage period and became significantly similar to the combined effect of LPS and nisin at the end of storage. In this regard, combinations of the two biopreservatives resulted in a greater inhibitory effect on *Staphylococcus* than when applied individually. This finding was in line with the result of Arques *et al*. (2008) [6] suggesting that synergism has been reported between LAB bacteriocins and traditional and novel treatments such as their combination with other antibacterial compounds which may enhance their antibacterial effect.

Concerning *Listeria* spp, his growth in treated and untreated raw milk samples was shown in Figure 2 g. We observed, in this study, that the most pronounced increase of *Listeria* count was generally at untreated control during all the storage period. Initial count of *Listeria* in control without biopreservatives was log 2.08 ± 0.19 log CFU/mL. After 5 days, individually and simultaneous added biopreservatives led to a slight delay in the growth of the pathogen, although no statistically significant differences ($p>0.05$) were observed between all tested milk samples. These results were in line with those of Arques *et al*. (2008) [6] and could be due to the nisin-resistance of *L. monocytogenes* (Martinez *et al*., 2005) [20]. In this way, Juncioni de Arauz *et al*. (2009) [17] found that many Gram-positive bacteria have been shown to be resistant to nisin due their ability to synthesize an enzyme, nisinase, which could inactivate nisin. After that, *Listeria* count decreased until the end of storage and reached 2.42 ± 0.13 log CFU/mL, 2.32 ± 0.18 log CFU/mL, 2.52 ± 0.11 log CFU/mL and 2.45 ± 0.19 log CFU/mL, respectively for untreated control, added nisin, activated LPS and combined treated raw milks. Our results were partially in agreement with those of Arques *et al*. (2008) [6] reporting that nisin and the LPS, added individually, caused a delay in the growth of the pathogen during storage at 10 °C and that *Listeria monocytogenes* could not be detected on day 12 when nisin was combined with the LPS.

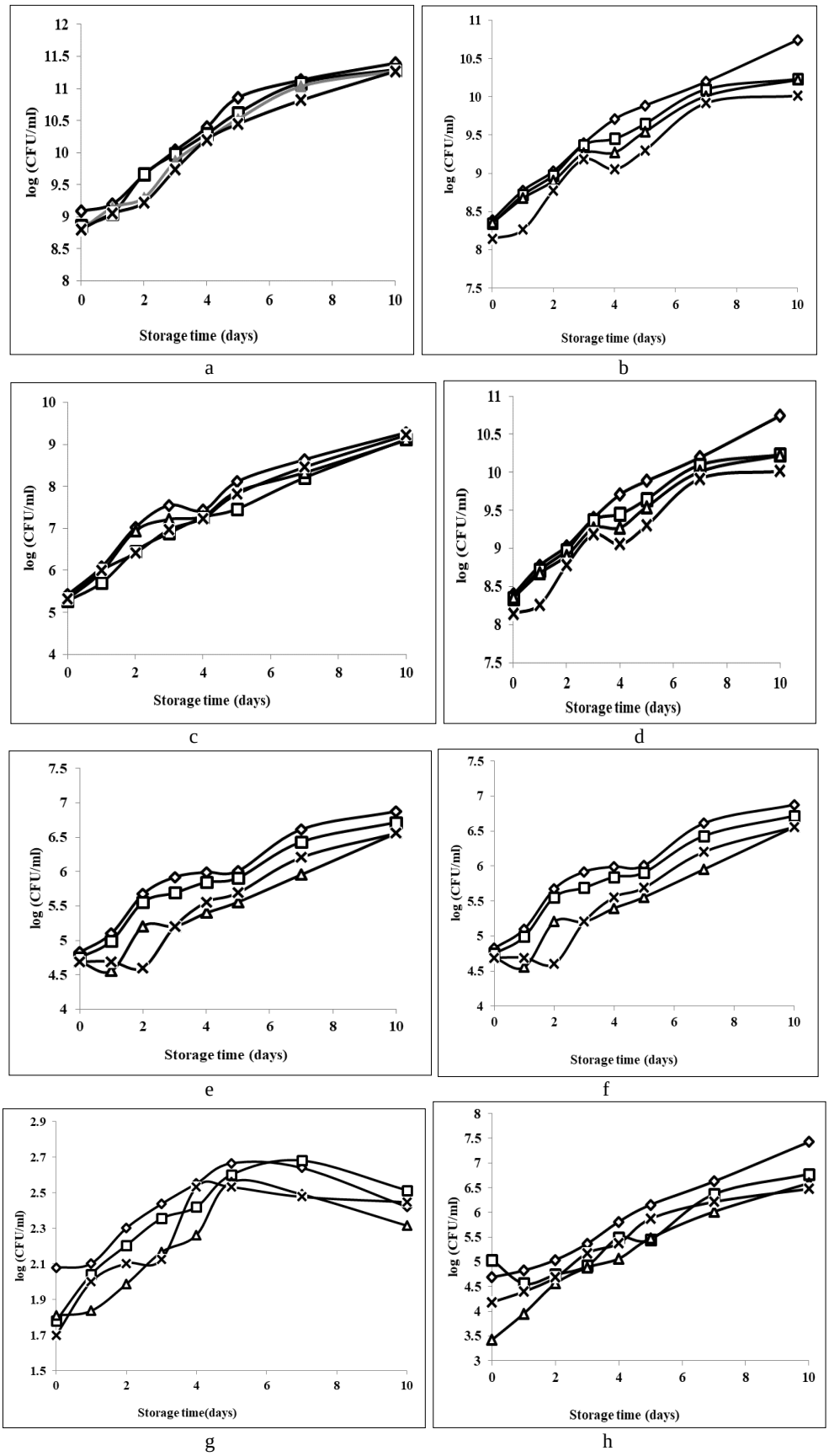

Fig 2: Changes in mesophilic (a) and psychrotrophic (b) total floras, total (c) and thermoresistant (d) coliforms, lactic acid bacteria (e), coagulase positive *Staphylococcus* (f), *Listeria* spp. (g), and mould and yeast (h) in refrigerated raw milk (control) --◇--, with activated LPS system --□--, with nisin added (150 IU) --△--, and with activated LPS system and nisin added simultaneously --X—

Yeasts and moulds growth

During 10 days of refrigerated storage, yeasts and moulds counts (Figure 1 h) were not significantly different ($p>0.05$). However, at the end of storage, the highest (6.96 ± 0.34 log CFU/mL) count was registered in untreated control. Thus the two biopreservatives applied individually or in combination resulted in a greater antagonistic effect on fungi but the differences weren't significant ($p>0.05$). Our results were in disagreement with those suggesting that the LP system was found to inhibit the growth and proliferation of many fungal and yeast species, such as *Penicillium* spp., *Candida albicans* and *Aspergillus niger* and that nisin is not generally active against fungi (Juncioni de Arauz *et al*., 2009; Buys 2011) [17, 19]. In fact, fungal resistance could be explained by the nature of their cell wall, rich in cellulose, pectin or chitin which forms a barrier for various proteinaceous and non proteinaceous molecules.

***Salmonella* spp growth**

It should be noted that *Salmonella* spp. was absent in 25 g of all analyzed samples. This finding asserts D'Amico and Donnelly (2010) [21] results. Otherwise, Yee Chye *et al*. (2004) [22] noted that the incidence of *Salmonella* spp. in raw milk was low (1.4%).

Conclusion

The results of the current study indicated that preservation of refrigerated raw milk by LP system, nisin individually or their simultaneous combination might be a useful tool to control the growth of undesired and pathogenic microorganisms in dairy products. Moreover, LPS system and nisin had a synergic bactericidal effect on milk microflora. Consequently, the combination of activated LPS and added nisin in raw milk was effective for improving the safety and the storage period of refrigerated raw milk by enhancing his physicochemical and microbiological qualities.

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