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Antilisterial effects of lysozyme-nisin combination at temperature and pH ranges optimal for lysozyme activity: Test of key findings to inactivate *Listeria* in raw milk

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ABSTRACT

This study aimed to determine antilisterial potential of lysozyme (LYS)-nisin (NIS) combination at temperatures and pHs optimal for LYS activity. Tests in buffers at pH 4.5 and 6.0 showed that heating at 50 or 60 °C combined with LYS-NIS caused higher *Listeria innocua* inactivation (- 6.2 to >6.6 log) than heating alone (- 0.05 to 5.5 log), or heating combined with LYS (- 4.34 to 6.0 log) or NIS (3.9 to >6.6). The antimicrobial performance of LYS-NIS in buffer at 50 °C was not pH-dependant (5.8–5.9 logs) while heating at 60 °C with LYS-NIS at pH 6.0 (>6.6 logs) caused higher *Listeria* reduction than that at pH 4.5 (5.7 logs). Heating at 50 °C for 45 min alone or in combination with LYS-NIS caused 0 and 5.5 logs *Listeria* reduction in milk, respectively. In contrast, *Listeria* inactivation in milk at 60 °C occurred mainly by heat (5.5 logs) with limited contribution of LYS and/or NIS. Milk heated at 50 °C maintained 73% of NIS and 63% of LYS activity. Application of LYS-NIS at 50 °C provides an opportunity to improve milk safety with less destruction of milk enzyme and microbial flora necessary to obtain desired ripening periods, and aroma and flavour in traditional cheeses.

1. Introduction

Lysozyme (LYS) is an antimicrobial enzyme that hydrolyzes the β (1–4) bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan (PG) layer at the cell walls of Gram-positive bacteria (Ormus, Oulahal, Noël, Degraeve, & Gharsallaoui, 2015; Proctor & Cunningham, 1988). Therefore, LYS extracted from hen egg white has been used in the food industry mainly for the inhibition of Gram-positive spoilage bacteria in different food such as ripening cheeses, wine and unpasteurized beer (Lesnierowski & Kijowski, 2007; Liburdi, Benucci, & Esti, 2014; Silvetti, Morandi, Hintersteiner, & Brasca, 2017). LYS is also one of the most potential candidates for antimicrobial packaging since it shows good stability and activity in different films and food systems, and it inhibits the critical Gram-positive pathogenic bacteria *Listeria monocytogenes* (Mecitoğlu et al., 2006; Duan, Park, Daeschel, & Zhao, 2007; Ünalan, Korel, & Yemenicioğlu, 2011). The prevention of food contamination by *L. monocytogenes* and application of hurdles to prevent listerial growth in risky food are critically important since this bacterium may cause deadly infections in susceptible individuals such as pregnant women, old people and the immunosuppressed people (Álvarez-Ordóñez, Leong, Hickey, Beaufort, & Jordan, 2015; Vázquez-Boland et al., 2001).

A recent trend in the use of LYS in food involves the combination of this antimicrobial enzyme with the bacteriocin nisin (NIS). NIS is a well-known antimicrobial peptide that is produced commercially by certain strains of *Lactococcus lactis* spp. *lactis,* and employed in a great variety of food product such as cheeses (fresh, ripened and/or processed), pasteurized fluid egg products (egg yolk or white and whole eggs), desserts (e.g., cereal, starch, and dairy-based desserts), etc (Ahmadi, Soleimanian-Zad, & Sheikh-Zeinoddin, 2016; Kallinteri, Kostoula, & Savvaidis, 2013; Morsy, Sharoba, Khalaf, El-Tanahy, & Cutter, 2015; Oshima et al., 2014; Schuman, 1997; Silva, Silva, & Ribeiro, 2018; Smigic et al., 2018; Sobral et al., 2019). NIS shows antimicrobial activity mainly on Gram-positive bacteria, but its mechanism of antimicrobial activity involves interaction with the anionic phospholipids at the bacterial surfaces and formation of pores, and dissipation of proton motive forces at the bacterial membrane (Sudagidan & Yemenicioğlu, 2012). The addition of LYS-NIS combination directly in food with or without encapsulation or in edible coatings has been attracting growing interest since this combination shows synergetic antimicrobial activity not only

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on spoilage lactic acid bacteria, but also on *L. monocytogenes* (Chung & Hancock, 2000; Datta, Janes, Xue, Losso, & La Peyre, 2008; Bhatia & Bharti, 2015; Sozbilen, Korel, & Yemenicioğlu, 2018; Sozbilen & Yemenicioğlu, 2020; Were, Bruce, Davidson, & Weiss, 2004). The dairy industry also shows a particular interest to improve safety of milk using LYS-NIS in combination with thermal and non-thermal processing. The LYS, with generally recognized as safe (GRAS) status in the United States (FDA, 1998) and quantum satis (Q.S.) in the European Union (EPCD, 1995; Masschalck & Michiels, 2003), has already been added into milk used in the production of ripening cheeses (e.g., parmesan, edam and gouda) to prevent late blowing caused by *Clostridium tyrobutyricum* (De Roos, Walstra, & Geurts, 1998; Lesnierowski & Kijowski, 2007). The NIS, also an agent with GRAS status in the USA (FDA & Federal, 1988), is approved by WHO/CODEX as a safe food additive for milk and milk products with a level of 12.5 mg kg^{-1} (De Arauz, Jozala, Mazzola, & Penna, 2009). In the literature, different studies exist to use LYS-NIS combination in pasteurized and raw milk. For example, Saad, Ombarak, and Abd Rabou (2019) added LYS-NIS combination in milk following classical low temperature long time pasteurization at 65 °C for 30 min. These authors reported that the addition of LYS-NIS in pasteurized milk has caused an inhibitory effect on the total bacterial count, aerobic spore-formers, and psychrotrophic bacterial counts, and extended the 6-days shelf-life of the pasteurized samples to 15 days. The combination of LYS-NIS with pulsed electrical fields (PEF) with or without mild heating in milk has also been tested by some workers. Smith, Mittal, and Griffiths (2002) combined LYS-NIS with PEF at 52 °C and achieved a 7-log reduction in total plate count of milk. Sobrino-López and Martín-Belloso (2008) also achieved 6.2 logs inactivation of *Staphylococcus aureus* in milk by combining LYS-NIS with PEF. However, no studies exist related to the antilisterial effects of LYS-NIS in milk at mild heating temperatures (between 50 and 60 °C) below pasteurization temperature range, 63 °C for 30 min for batch and 72 °C for 15 s for continuous flow processes (CODEX/WHO, 2011). The milk heated at pasteurization temperatures is not preferred for the significant portion of ripening cheeses in Europe since it needs longer ripening periods and gives inferior aroma and flavor profile than cheese made from raw milk (Grappin & Beuvier, 1997; Fernández-Garcia, Serrano & Nuñez, 2002).

This study aimed to determine the antilisterial potential of lysozyme (LYS)-nisin (NIS) combination below pasteurization temperatures and pHs optimal for LYS activity. The temperature dependency for lytic enzyme activity of LYS was characterized in buffer at different pH, and its antilisterial activity was tested both in buffer and in raw milk against *L. innocua*, a frequently used surrogate for *L. monocytogenes* (Omac, Moreira, Castillo, & Castell-Perez, 2015). This work is important in that it is the first report that characterized the antilisterial effects of LYS-NIS combination below pasteurization temperatures optimal for LYS activity. Moreover, it is one of the first studies that worked *Listeria* inactivation in milk using LYS-NIS in combination with mild temperature heating below milk pasteurization temperatures. The results of this work provide a basis to minimize use of thermal methods in inactivation of food pathogens and to support more effective widespread application of biopreservation.

2. Materials and methods

2.1. Materials

Chicken egg white lysozyme (≥40,000 U mg $protein^{-1}$, ≥90%) (L6876), nisin (≥1000 IU mg^{-1}; 2.5%) from *Lactococcus lactis* (N5764), *Micrococcus lysodeikticus* ATCC No: 4698 as a substrate of lysozyme were purchased from Sigma-Aldrich Chem. Co. (St. Louis, Mo., USA). Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate dihydrate, citric acid monohydrate, nutrient broth, nutrient agar, pepton water, Oxford *Listeria* Agar and Oxford *Listeria* Selective supplement were obtained from Merck (Darmstadt, Germany). The surrogate of *Listeria monocytogenes*, *L. innocua* NRRL-B 33314 (ATCC 1915) was from the culture collection of the microbiology laboratory of the Department of Food Engineering at Izmir Institute of Technology (IYTE), Izmir. Fresh raw milk (Efeler Çiftliği, Söke, Aydın, 3.3–3.4% fat) was purchased from a market in İzmir.

2.2. Methods

2.2.1. Activity and stability of LYS at different temperature and pH

The pH stability of LYS was determined using two different activity monitoring procedures, the first one was conducted by measuring activity at pH 7.00 for all enzyme solutions stored at different pH while the second one was conducted by measuring activity directly at the pH of storage. The LYS solutions at 1.5 mg mL^{-1} (~60,000 U mL^{-1}) concentration were prepared at different pH between 2.50 and 6.50 and stored at 4 °C for 28 days. The buffers used were 0.05 M Na-phosphate buffer for pH between 5.00 and 7.00, and 0.05 M citrate-phosphate buffer for pH between 2.50 and 4.75. Activities were measured at days 0, 3, 7, 14, 21, and 28. In the first procedure, the reaction mixture was formed by mixing 0.1 mL of the enzyme at different pH with 2.4 mL of *Micrococcus lysodeikticus* solution at pH 7.00. In the second procedure, 0.1 mL of the enzyme at different pH was mixed with 2.4 mL of *M. lysodeikticus* solution prepared at the same pH with the enzyme solution. LYS activity was measured spectrophotometrically at 660 nm and 30 °C (Shimadzu Model 2450 equipped with a water circulating constant temperature cell holder) as described by Sozbilen and Yemenicioğlu (2020). The activities calculated from slopes of the initial linear portions of abs vs time curves were expressed as Units (0.001 absorbance change in 1 min) per 1 mL of enzyme solution. Results were given as averages from duplicate analysis that each performed at least with three measurements at the given pH points.

The optimal temperature of LYS activity at different pH (2.50–6.50) was determined by measuring enzyme activity at 4, 10, 30, 40, 50, and 60 °C. Lysozyme activity was calculated as U mL^{-1}. The temperature dependency of enzyme activity at different pH was analyzed by determination of activation energy (E_a) from the Arrhenius equation given below;

$$k = A \exp(-Ea / RT) \quad (1)$$

where k is the rate of enzymatic activity of lysozyme, A is the pre-exponential factor, E_a is the activation energy, T is the temperature (K), R (8.314 J mol^{-1} K^{-1}) is the universal gas constant. The E_a at different pH was calculated from the slope of the Arrhenius curve obtained by plotting the natural logarithm of k (ln k) vs reciprocal of activity determination temperatures (T^{-1}).

2.2.2. Antilisterial effect of LYS-NIS in buffer at different pH and temperature

Antilisterial effect of LYS-NIS was determined by a slight modification of the method given by Sozbilen and Yemenicioğlu (2020). Briefly, the inoculum (10^8 CFU mL^{-1}) was grown at 37 °C for 24 h. The stock solutions of antimicrobial agents were prepared either in 0.05 M citrate-phosphate buffer at pH 4.50 or in 0.05 M Na-phosphate buffer at pH 6.00. After that, 4 mL nutrient broth at pH 4.50 or 6.00, and 0.5 mL of LYS, NIS, or LYS-NIS prepared in a buffer (at pH 4.50 or 6.00) were added into sterile capped tubes. The tubes were then immersed into a water bath working at 50 °C or 60 °C and heated until tube contents reached to target heating temperature (checked with a thermometer). Then, 0.5 mL of inoculum was added into tubes, and tubes were vortexed thoroughly. The final concentrations of LYS and NIS in the tubes at this stage were 500 μg mL^{-1} and 15.6 μg mL^{-1} (~20,000 U mL^{-1} and ~15.6 IU mL^{-1}), respectively. The concentrations of LYS and NIS were selected following a preliminary based on an antimicrobial test conducted at 4 °C and pH 6.00 for 12 days. The concentrations of LYS and NIS showing considerable (~2 logs) inhibition without allowing

significant regrowth of *L. innocua* during incubation period were used in the current study (see related results of detailed preliminary tests at Figs. 1S and 2S provided in Supplementary file). The tubes with LYS, NIS, or LYS-NIS were heated at 50 and 60 °C for 0, 7.5, 15, 30, or 45 min, and for 0, 2.5, 5, 10, or 15 min, respectively, and cooled immediately in an ice water bath. Each time-temperature combination was prepared in duplicate. Tubes with only inoculum (free from antimicrobials) were used as control. The *L. innocua* count of tubes was determined by the spread plate method using nutrient agar. The colonies were enumerated after 24 h incubation at 37 °C. Results were given as averages from duplicate analysis that enumeration for each (Control, with LYS, NIS, or LYS-NIS) was carried out in triplicate.

2.2.3. Antilisterial effect of LYS-NIS in raw milk subjected to mild heating

The antilisterial effect of LYS-NIS was determined by conducting heating experiments at 50 °C or 60 °C as described in section 2.2.2 by using raw milk (pH 6.50) instead of nutrient broth. However, for microbiological analysis, heated milk samples were serially diluted with 0.1% pepton water and plated by the spread plate method on Oxford *Listeria* agar supplemented with Oxford *Listeria* Selective supplement. The enumeration of black colonies surrounded by black zones was carried out after 48 h incubation at 37 °C. The microbiological counts of *L. innocua* were expressed as log CFU mL^{-1} of raw milk. Results were given as averages from duplicate analysis that enumeration for each (Control, with LYS, NIS, or LYS-NIS) was carried out in triplicate.

2.2.4. Soluble active LYS and NIS remained in raw milk before and after mild heating

To determine % soluble active LYS or NIS left in milk after heating, acidic coagulation was applied to raw unheated and heated milk with or without LYS (500 μg mL^{-1}) or NIS (15.6 μg mL^{-1}). This procedure was preferred to eliminate caseins that cause turbidity and interfere with the activity testing methods. The acidification was conducted by bringing the pH of milk below the isoelectric point of casein (<pH 4.60) using a 25% lactic acid solution. The fat (top layer) and curd (mainly casein precipitate) were separated from whey by centrifugation at 15000g and 4 °C for 15 min. The collected whey samples were further clarified with a second centrifugation cycle, and they were filtered through cheesecloth. The pH of the whey samples was then adjusted back to the original milk pH of 6.50 using 1N NaOH. After that, the whey obtained from LYS and NIS added milk samples (W_{LYS} and W_{NIS}, respectively) were analyzed for their soluble LYS and NIS activity. The whey obtained from control milk (no LYS or NIS added) was used as a negative control. Positive control was also prepared using this whey. For this purpose, LYS (500 μg mL^{-1}) or NIS (15.6 μg mL^{-1}) at the same amounts added initially into milk was dissolved in control whey, and then these controls ($W_{LYS\text{-}CTR}$ and $W_{NIS\text{-}CTR}$, respectively) were tested for their LYS or NIS activities.

The percentage of LYS and NIS left in unheated and heated milk were calculated using the following formula:

$$\%\,\text{soluble LYS or NIS} = \frac{\text{Amount in WLYS or in WNIS}}{\text{Amount in WLYS} - \text{CTR or WNIS} - \text{CTR}} \times 100 \quad (2)$$

The measurements were conducted using raw unheated milk and milk heat treated at 50 °C for 45 min after the addition of LYS or NIS. The remaining activity of LYS was determined by the measurement of its activity at pH 6.50 as described in section 2.2.1. The NIS concentration was determined by the classical agar diffusion method given by Sozbilen et al. (2018). Results were given as averages from triplicate analysis that each were tested at least three times for LYS activity or NIS concentration.

2.2.5. Statistical analyses

The results presented are averages and standard deviations that were calculated from replicate measurements (Microsoft Excel, Microsoft Corporation, Redmond, WA). The Fisher test was used for the comparison of means, with significance assigned at $P \leq 0.05$ by using a statistical software of Minitab release 16 (Minitab Inc., State College, Pa., U.S.A.)

3. Results and discussion

3.1. Activity and stability of LYS at different pH and temperature

The pH stability of LYS was determined by 28 days cold-storage test between pH 2.50 and 6.50. The activities of LYS incubated at different pH determined directly at storage pH at 30 °C (Fig. 1) or at pH 7.00 at 30 °C (see supplementary file Fig. 3S) showed some slight to moderate fluctuations, but results clearly proved that there was no considerable enzyme inactivation due specifically to pH factor. The pH 4.50 was highly critical for enzyme since pH above this value caused dramatic increases in enzyme activity at 30 °C. It is noteworthy to report that change of pH range from 2.50 to 4.00 to 4.50–6.50 increased activities at 30 °C from 204 to 2039 to 17705–48295 U mL^{-1} range, respectively. Thus, it is clear that antibacterial applications based on LYS activity should be conducted with food having pH within 4.50–6.50 range. These results compare well with those of Nakimbugwe, Masschalck, Anim, and Michiels (2006) who showed that LYS was almost inactive between pH 2.80 and 4.60 at 25 °C while it showed an increase in activity as pH was increased gradually from 4.60 to 7.80. da Silva Freitas and Abrahão-Neto (2010) who measured LYS activity at 25 °C determined inconsiderable LYS activity at pH 4.0, but they showed that activity increased between pH 4.5 and 8.0. However, none of these studies conducted long term pH stability tests as in the current work, and they did not investigate activity-pH profiles of LYS at elevated temperatures.

The effect of temperature on LYS activity between 4 and 60 °C within 2.50–6.50 pH range was presented in Fig. 2. The results clearly proved that LYS activity was not considerably affected by temperature change below pH of 4.50 (Fig. 2). It seems that pH below 4.50 causes enzyme to undergo some reversible conformational changes that interfere with its ability to interact with bacterial cell walls. The reaction temperature at 30 °C increased activity between pH 5.50 and 6.50, but enzyme activity at this temperature reduced 2–3 fold when pH was reduced below 5.50. The activity measurements at 4 and 10 °C gave the lowest LYS activities that were not affected by the change of pH through 4.50–6.50 range. In contrast, at pH above 4.50, LYS activity starts to show significant changes depending on the severity of reaction temperature. In particular, the temperatures ≥40 °C caused dramatic increases in LYS activity between pH 4.50 and 6.50 range. The most effective temperature on LYS activity was 60 °C followed by 40 and 50 °C. The reaction temperature at 60 °C caused significantly higher enzyme activities at pH 4.75, 5.00, 5.50, and 6.50 than the other temperatures. However, it is interesting to note that LYS showed quite similar lytic activities at 40, 50, and 60 °C when the pH was 6.0. The temperatures at 40 and 50 °C gave comparable activity profiles at pH 4.50, 4.75, 5.00, and 6.00, but 40 °C is more effective than 50 °C at pH 5.25, 5.50, 5.75, and 6.50. However, 40 °C could not be applicable in food preservation since it is within the range of optimal growth temperatures of mesophilic pathogenic bacteria resistant to lytic activity of LYS (e.g., *Staphylococcus aureus*) (Sudagidan & Yemenicioğlu, 2012). The overall results showed that mild temperatures at 50 and 60 °C, and pHs between 4.50 and 6.50 range could be exploited to maximize the antimicrobial activity of LYS.

The activation energies (Ea) that show temperature dependency of LYS activity at different pH were also given in Fig. 3 (please see also reaction rates provided at Table 1S and Arrhenius curves at Fig. 4S in Supplementary file). The results proved that the Ea increases from 2.7 kJ mol^{-1} to 32.8 kJ $^{-1}$ mol as the pH value of the reaction mixture increases from 2.50 to 4.50. However, the Ea values of enzyme between pH 4.50 and 6.50 varied at a narrow range between 28.9 and 33.1 kJ mol^{-1}. This result once more proved that the pH range between 4.50 and 6.50 is the most suitable range for thermal activation of LYS activity. In the literature studies related to the temperature dependency of LYS activity at different pH are scarce. However, Matsuura et al. (2002)

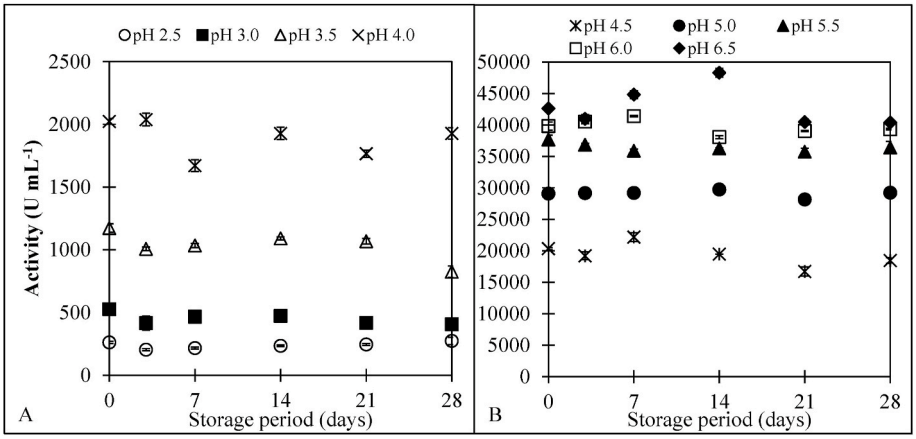

Fig. 1. The stability of LYS enzyme activity at different pH (Note: Activities were determined at respective storage pH).

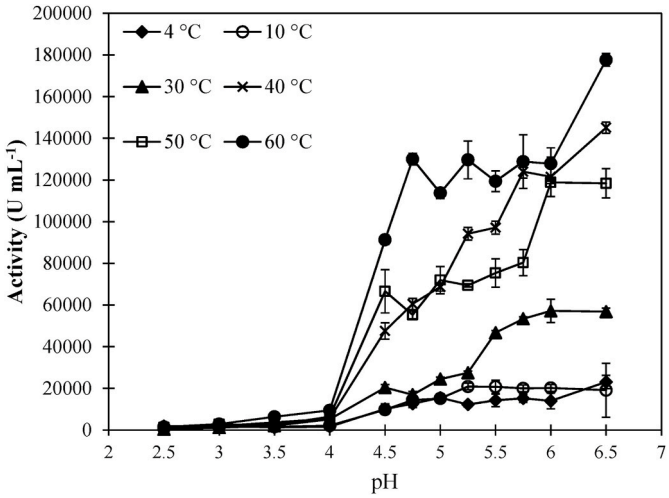

Fig. 2. Effect of temperature on pH-activity profiles of LYS.

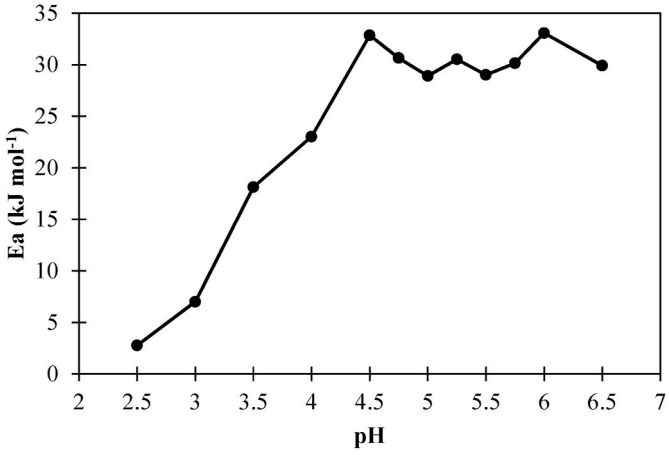

Fig. 3. The Ea for temperature dependency of LYS activity at different pH.

determined the Ea of LYS activity at pH 7.00, between 5 °C and 65 °C as 42.7 kJ mol^{-1}, a value 1.3–1.5 fold higher than that determined in the current work at pH range between 4.5 and 6.50 due possibly to differences in pH and temperature ranges studied.

3.2. Antilisterial effect of LYS-NIS in buffer at different pH and temperature

The antilisterial effect of LYS-NIS was studied at 50 or 60 °C at pH 4.50 and 6.00 which are within the optimal activity range of LYS (Table 1). The heating alone at 50 °C and pH 4.50 is not effective on inactivation of *L. innocua* up to 15 min, but it caused significant reductions in initial bacterial load at 30 (0.6 logs) and 45 min (1.65 logs). However, no significant *Listeria* inactivation was observed with heating alone at 50 °C and pH 6.00 within 45 min. In contrast, heating alone at 60 °C at pH 4.50 and 6.00 caused 5.5 and 4.1 logs bacterial reduction at the end of 15 min, respectively. The application of mild heating at 50 or 60 °C in the presence of LYS at pH 4.50 or 6.00 accelerated the inactivation of *Listeria* significantly. The reductions determined at pH 4.50 and 6.00 by heating at 50 °C for 45 min in the presence of LYS reached to 4.3 and 4.1 logs, respectively. Moreover, heating at 60 °C for 15 min in the presence of LYS at pH 4.50 and 6.00 increased bacterial reductions to 6.0 and 5.9 logs, respectively. These results clearly showed that in the presence of LYS, the differences in pH at 50 or 60 °C did not considerably affect the *Listeria* inactivation. Moreover, it is also evident that LYS showed higher *Listeria* inactivation at 60 °C than at 50 °C. However, this should be related mainly to heat inactivation effect at 60 °C since LYS characterization studies revealed that the enzyme showed similar lytic activities at 50 and 60 °C when pH is 6.00 (see Fig. 2).

The presence of NIS also increased inactivation of *Listeria* during mild heating. The inactivation caused by NIS on *Listeria* at 50 °C and pH 6.00 within the first 15 min was more rapid compared to that it caused initially at pH 4.50. However, the extended heating at 50 °C for 45 min in the presence of NIS at pH 4.50 caused significantly greater (5 logs) *Listeria* reduction than that at pH 6.00 (3.9 logs). This result suggested that NIS was more stable at 50 °C and low pH than that at pH close to neutrality. The greater heat stability of NIS at acidic than neutral pH was reported by different workers (Penna, Jozala, Novaes, Pessoa, & Cholewa, 2005; Rollema, Kuipers, Both, De Vos, & Siezen, 1995). However, the declined antilisterial effect of NIS by extended heating at 50 °C and pH 6.00 could also be related to neutralizing interactions it underwent with wounded or death cells' components (cell walls, membranes, cytoplasmic solutes, etc.). On the other hand, in the presence of NIS, heating at 60 °C for 5 min at pH 6.00 and heating at 60 °C for 15 min at pH 4.50 caused almost similar *Listeria* reductions (~6.5–6.6 logs). Thus, it appears that heating at 60 °C enhanced the antimicrobial effect of NIS at pH 6.00, and helped it to act within a very short time before initiation of denaturing/neutralizing interactions. These results showed that the antilisterial capacity of NIS is highly pH and temperature-dependent. Further evaluation of results also showed that when the pH was 4.50, heating at a given temperature (at 50 or 60 °C) with LYS or NIS caused almost similar *Listeria* reductions ($P > 0.05$). However, inactivation of *Listeria* at 60 °C was greater than that at 50 °C in the presence of LYS or NIS at pH 4.50. The NIS showed more rapid bacterial inactivation than LYS at 50 °C and pH 6.00 within the first 30 min of heating, but final *Listeria* counts in the presence of LYS and NIS at 50 °C and pH 6.00 after 45 min were not significantly different from each other ($P > 0.05$). In

Table 1
Change in *L. innocua* count in buffers at different pH and temperature conditions.

Antimicrobial concentration (μg mL^{-1})				*L. innocua* count (Log CFU mL^{-1})				
				Time (min)				
LYS	NIS	pH	T (°C)	0	7.5	15	30	45
–	–	4.5	50	$7.86 \pm 0.09^{a,A,A\prime}$	$7.71 \pm 0.12^{a,A,A\prime}$	$7.62 \pm 0.14^{a,A,A\prime}$	$7.24 \pm 0.30^{b,A,A\prime}$	$6.21 \pm 0.55^{c,A,B\prime}$
500	–	4.5	50	$7.86 \pm 0.09^{a,A,A\prime}$	$7.33 \pm 0.43^{b,A,A\prime}$	$5.36 \pm 0.88^{c,B,C\prime}$	$3.52 \pm 0.29^{d,B,C\prime}$	$3.52 \pm 0.25^{d,B,CD\prime}$
–	15.6	4.5	50	$7.86 \pm 0.09^{a,A,A\prime}$	$5.54 \pm 0.48^{b,B,B\prime}$	$5.01 \pm 0.53^{b,B,C\prime}$	$3.33 \pm 1.28^{c,B,C\prime}$	$2.88 \pm 0.70^{c,B,D\prime}$
500	15.6	4.5	50	$7.86 \pm 0.09^{a,A,A\prime}$	$2.09 \pm 0.59^{b,C,D\prime}$	$1.23 \pm 0.25^{c,C,E\prime}$	$1.15 \pm 0.16^{bc,C,D\prime}$	$1.00 \pm 0.00^{c,C,E\prime}$
–	–	6.0	50	$7.74 \pm 0.09^{a,A,B\prime}$	$7.72 \pm 0.14^{a,A,A\prime}$	$7.75 \pm 0.13^{a,A,A\prime}$	$7.73 \pm 0.13^{a,A,A\prime}$	$7.69 \pm 0.17^{a,A,A\prime}$
500	–	6.0	50	$7.74 \pm 0.09^{a,A,B\prime}$	$7.47 \pm 0.39^{ab,A,A\prime}$	$6.46 \pm 1.01^{b,B,B\prime}$	$4.65 \pm 1.40^{c,B,B\prime}$	$3.65 \pm 1.36^{c,B,C\prime}$
–	15.6	6.0	50	$7.74 \pm 0.09^{a,A,B\prime}$	$3.66 \pm 1.33^{b,B,C\prime}$	$3.57 \pm 0.98^{b,C,D\prime}$	$3.55 \pm 1.18^{b,C,C\prime}$	$3.83 \pm 0.71^{b,B,C\prime}$
500	15.6	6.0	50	$7.74 \pm 0.09^{a,A,B\prime}$	$1.83 \pm 1.22^{b,C,D\prime}$	$1.84 \pm 1.23^{b,D,E\prime}$	$1.59 \pm 1.00^{b,D,D\prime}$	$1.56 \pm 0.96^{b,C,E\prime}$
				0	**2.5**	**5**	**10**	**15**
–	–	4.5	60	$7.69 \pm 0.12^{a,A,A\prime}$	$4.52 \pm 0.62^{b,A,A\prime}$	$4.38 \pm 0.44^{b,A,A\prime}$	$3.74 \pm 0.55^{c,A,A\prime}$	$2.17 \pm 0.37^{d,A,B\prime}$
500	–	4.5	60	$7.69 \pm 0.12^{a,A,A\prime}$	$3.97 \pm 0.49^{b,AB,AB\prime}$	$2.84 \pm 0.26^{c,B,B\prime}$	$2.35 \pm 0.62^{d,B,BC\prime}$	$1.68 \pm 0.54^{a,AB,B\prime}$
–	15.6	4.5	60	$7.69 \pm 0.12^{a,A,A\prime}$	$3.33 \pm 0.22^{b,B,B\prime}$	$2.72 \pm 0.06^{c,B,B\prime}$	$1.52 \pm 0.73^{d,B,C\prime}$	$1.15 \pm 0.21^{d,B,B\prime}$
500	15.6	4.5	60	$7.69 \pm 0.12^{a,A,A\prime}$	$2.03 \pm 0.39^{b,C,C\prime}$	$1.00 \pm 0.00^{c,C,C\prime}$	<1	<1
–	–	6.0	60	$7.55 \pm 0.14^{a,A,B\prime}$	$4.08 \pm 1.04^{b,A,AB\prime}$	$3.71 \pm 0.88^{b,A,A\prime}$	$3.28 \pm 1.02^{b,A,AB\prime}$	$3.44 \pm 0.93^{b,A,A\prime}$
500	–	6.0	60	$7.55 \pm 0.14^{a,A,B\prime}$	$3.47 \pm 0.70^{b,A,B\prime}$	$2.44 \pm 1.10^{cd,B,B\prime}$	$2.81 \pm 1.23^{bc,A,ABC\prime}$	$1.67 \pm 0.70^{d,B,B\prime}$
–	15.6	6.0	60	$7.55 \pm 0.14^{a,A,B\prime}$	$2.15 \pm 0.78^{b,B,C\prime}$	<1	<1	<1
500	15.6	6.0	60	$7.55 \pm 0.14^{a,A,B\prime}$	<1	<1	<1	<1

a-d values within each row followed by the same letter are not significantly different ($P > 0.05$).
A-D values within each column at same pH and temperature followed by the same letter are not significantly different ($P > 0.05$).
A'-E' values within each column at same temperature followed by the same letter are not significantly different ($P > 0.05$).

contrast, NIS showed significantly higher antilisterial activity than LYS at 60 °C and pH 6.00 within shorter time periods.

The combination of LYS-NIS caused greater *Listeria* inactivation than LYS or NIS alone at all heating conditions. The microbial counts in buffers with LYS-NIS heated at 50 °C and pH 4.50 for 7.5–45 min were not significantly different than those obtained at 50 °C and pH 6.00 ($P > 0.05$). Thus, it is clear that the antilisterial activity of LYS-NIS was not affected by the change of pH at 50 °C. The log reductions achieved at 50 °C within 45 min at pH 4.50 and 6.00 were 6.9 and 6.2, respectively. However, it is noteworthy to report that the increase in heating temperature from 50 to 60 °C in the presence of LYS-NIS at pH 4.50 and 6.00 buffers caused ≥6.7 and ≥ 6.6 logs *Listeria* inactivation within 10 and 2.5 min, respectively. This result showed that LYS-NIS was much more effective at pH 6.00 than pH 4.5 when the heating temperature was 60 °C. The same pH-temperature dependency profile was also observed during heating studies with NIS alone. Thus, it appears that NIS is the main active agent determining the pH-temperature dependency of LYS-NIS combination.

3.3. Antilisterial effect of LYS-NIS in raw milk at different temperature

The antilisterial effect of LYS-NIS in raw milk was determined at 50 and 60 °C (Table 2). The milk pH of 6.5 was slightly higher than buffer pH of 6.00 used as model medium above. Heating at 50 °C for 45 min with or without the presence of LYS did not cause a significant reduction in *Listeria* counts of raw milk ($P > 0.05$). In contrast, heating at 50 °C for 15, 30, and 45 min in the presence of NIS caused 2.3, 3.8, and 4.6 logs reductions of *Listeria*, respectively. This result clearly proved that NIS alone maintained its antilisterial activity in raw milk at 50 °C while LYS alone lacked to show considerable lytic activity against *Listeria* in milk at this mild temperature. On the other hand, the combinational application of LYS-NIS at 50 °C for 15, 30, and 45 min caused 3.2, 4.7, and 5.5 logs reductions of *Listeria*, respectively. It is clear that LYS-NIS caused significantly higher *Listeria* inactivation (0.9–1 log) at 50 °C than NIS alone. These findings suggested that when LYS was applied alone, the conformational changes in the enzyme and/or bacterial surface at 50 °C blocked its lytic activity. The overall results clearly showed that heating at 50 °C alone or in combination with LYS did not cause any *Listeria* inactivation in milk, but effective inactivation of this critical bacterium was achieved by applying mild heating in combination with NIS or LYS-NIS. The exact mechanism of LYS-NIS synergy is still unclear. However, it has been proved that the presence of NIS in the raw milk and possible damages by this bacteriocin on cytoplasmic membranes of *Listeria* paved the way for LYS to show more effective lytic activity on sugar linkages at the peptidoglycan layer in the *Listeria* cell walls. Chung and Hancock (2000) proposed that nisin could be inhibiting energy-dependent processes that repair lysozyme damage. The synergy between LYS-NIS was also reported for *Staphylococcus aureus* with increased strength at low pH

Table 2
Antilisterial effect of LYS and/or NIS in raw milk heat treated at 50 °C and 60 °C.

Concentrations (μg mL^{-1})			*L. innocua* count (Log CFU mL^{-1})			
LYS	NIS	T (°C)	Time (min)			
			0	15	30	45
–	–	50	$7.61 \pm 0.13^{a,A}$	$7.62 \pm 0.18^{a,A}$	$7.63 \pm 0.20^{a,A}$	$7.60 \pm 0.17^{a,A}$
500	–	50	$7.61 \pm 0.13^{a,A}$	$7.57 \pm 0.19^{a,A}$	$7.67 \pm 0.17^{a,A}$	$7.66 \pm 0.26^{a,A}$
–	15.6	50	$7.61 \pm 0.13^{a,A}$	$5.28 \pm 0.34^{b,B}$	$3.83 \pm 0.48^{c,B}$	$3.02 \pm 0.49^{d,B}$
500	15.6	50	$7.61 \pm 0.13^{a,A}$	$4.46 \pm 0.41^{b,C}$	$2.89 \pm 0.49^{c,C}$	$2.15 \pm 0.82^{d,C}$
			0	**5**	**10**	**15**
–	–	60	$7.81 \pm 0.06^{a,A}$	$3.42 \pm 0.36^{b,A}$	$2.87 \pm 0.37^{c,A}$	$2.30 \pm 0.00^{c,A}$
500	–	60	$7.81 \pm 0.06^{a,A}$	$3.56 \pm 0.21^{b,A}$	$2.40 \pm 0.09^{c,AB}$	$1.30 \pm 0.00^{d,B}$
–	15.6	60	$7.81 \pm 0.06^{a,A}$	$3.10 \pm 0.72^{b,A}$	$2.16 \pm 0.44^{b,AB}$	<1
500	15.6	60	$7.81 \pm 0.06^{a,A}$	$2.71 \pm 0.72^{b,A}$	$2.00 \pm 0.00^{b,B}$	<1

a-d values within each row followed by the same letter are not significantly different ($P > 0.05$).
A-C values within each column at the same temperature followed by the same letter are not significantly different ($P > 0.05$).

(Moshtaghi, Rashidimehr, & Shareghi, 2018) and for *Lactobacillus plantarum* (Sozbilen et al., 2018). The synergetic action of LYS with organic acids such as succinic and malic acids against *L. monocytogenes* was also reported by Oh, Lee, Jeong, and Kim (2016). It was reported that the reduction of pH from neutrality to around 5.5 did not reduce the LYS activity considerably while it caused some significant lag periods in the growth of *L. monocytogenes*. Thus, it appears that combination of LYS with reduced pH might also be an effective tool to suppress or inactivate *Listeria* during storage of food (Johansen, Gram, & Meyer, 1994). A synergy similar to LYS-NIS was also observed between LYS and lipase against *L. monocytogenes* by Liberti, Franciosa, Gianfranceschi, and Aureli (1996). These authors attributed the increased antilisterial activity in presence of lipase to its action on some extracellular cell wall components that contribute resistance against LYS action. However, addition of lipase in food might be questioned due to its negative effects on food lipids. Finally, the combination of LYS with chelating agent EDTA also enhanced LYS action against *L. monocytogenes* in inoculated fresh fish at 20 °C, but EDTA showed no contribution in antilisterial activity of LYS at 5 °C (Wang & Shelef, 1992). The combination of LYS with EDTA is also beneficial to increase sensitivity of Gram-negative bacteria against LYS since this chelating agent removes protective lipopolysaccharide (LPS) layer surrounding their PG (Ünalan et al., 2011).

The heating at 60 °C for 5–15 min alone was highly effective on the inactivation of *Listeria* and it caused 4.4 to 5.5 logs inactivation of bacteria in raw milk. The application of heating with LYS at 60 °C for 5 and 10 min did not cause a significantly lower *Listeria* load than heating at this temperature alone for 5 and 10 min. However, heating in the presence of LYS at 60 °C for 15 min caused 6.5 logs inactivation that is significantly higher (1 log) than similar heating applied without LYS ($P < 0.05$). Thus, this finding proved that LYS alone caused some lytic activity against *Listeria* in milk at 60 °C. The log reductions achieved in *Listeria* by the application of heating at 60 °C for 5 or 10 min in presence of NIS alone were not significantly different than that of LYS alone ($P > 0.05$). However, the reduction in *Listeria* (≥6.8 logs) count of milk at 60 °C within 15 min in the presence of NIS was slightly more effective than that at the same temperature within 15 min in the presence of LYS. On the other hand, it is obvious that NIS highly effective on *Listeria* at 50 °C showed more limited contribution to the inactivation of *Listeria* at 60 °C. As a result, the combination of LYS-NIS at 60 °C did not cause significantly higher *Listeria* inactivation in milk than combination with LYS or NIS at 60 °C. Thus, overall results showed that heat is the main factor causing *Listeria* inactivation at 60 °C. The contributions of LYS, NIS, or LYS-NIS in inactivation at 60 °C were limited and it remained ≤1 log at the test conditions. In contrast, the benefits of natural antimicrobials in milk appeared dramatically at 50 °C in the presence of NIS and LYS-NIS.

The studies related to the application of LYS-NIS in combination with mild heating against *Listeria* in milk below pasteurization temperatures (≥63 °C) are scarce. However, there are some studies that combine LYS-NIS with pulsed electrical fields (PEF) or pasteurization. For example, Smith et al. (2002) combined LYS-NIS with PEF and mild heating at 52 °C and achieved a 7-log reduction in total plate count of milk. Moreover, Sobrino-López and Martín-Belloso (2008) employed LYS-NIS with PEF and achieved 6.2 logs inactivation of *Staphylococcus aureus* in milk. The use of LYS-NIS in combination with PEF for the preservation of milk is promising, but its economic feasibility cannot be compared with that of mild heating. The combination of LYS-NIS with heating has also been tested as a more advanced strategy to inactivate *L. monocytogenes* in meat products. However, these studies employed higher heating temperatures within the classical pasteurization temperature range. For example, Gill and Holley (2000) added LYS-NIS combination to sausages and then applied pasteurization based on reaching internal temperature of 69 °C in samples to achieve inactivation of *L. monocytogenes*. These authors did not determine any positive contribution of LYS-NIS combination to pasteurization of ham sausages, but the use of LYS-NIS

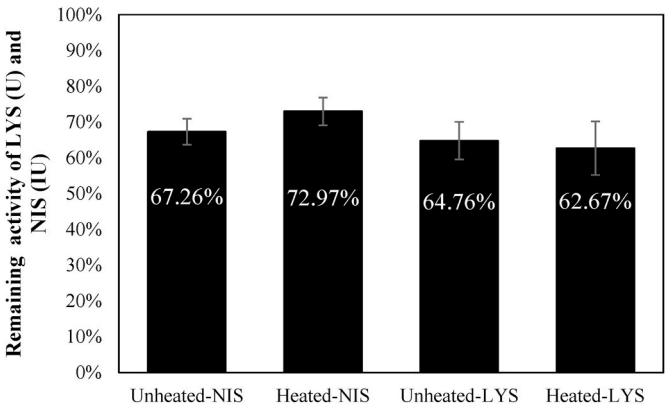

Fig. 4. Percentages of soluble LYS or NIS in heated and unheated milk samples.

improved the effectiveness of *Listeria* inactivation during pasteurization of Bologna sausages. Mangalassary, Han, Rieck, Acton, and Dawson (2008) also applied LYS-NIS in combination with pasteurization at 65 °C to inhibit *L. monocytogenes* in Bologna sausages.

3.4. Soluble active LYS and NIS left in raw milk and milk subjected to mild heating

The positively charged LYS (pI: 11.40) and NIS (pI: 8.80) in raw milk could be bind by negatively charged caseins (pI: 4.60) (Boyacı, Korel, & Yemenicioğlu, 2016; Fagan, O'Callaghan, Mateo, & Dejmek, 2017; Schneider, Becker, & Pischetsrieder, 2010; Wang et al., 2015). Moreover, the adsorption of NIS on the fat globules in milk has been demonstrated by different workers (Bajpai, Yoon, Bhardwaj, & Kang, 2014; Jung, Bodyfelt, & Daeschel, 1992; Zapico, de Paz, Medina, & Nuñez, 1999). Thus, the amount of free active LYS and NIS left in raw milk and acted effectively on *Listeria* was estimated by activity measurements in whey obtained from defatted unheated control raw milk and raw milk heated at 50 °C for 45 min (Fig. 4). The results clearly showed retention of 65 and 63% of LYS, and 67 and 73% of NIS in whey obtained from unheated and heated raw milk, respectively. Thus, it is clear that LYS and NIS remained the majority of their activity in raw milk and heated milk. Further studies are needed to show possible preservative effects of remained LYS-NIS in heat treated milk during cold storage. However, the recent study of Saad et al. (2019) is promising since they showed that the addition of LYS-NIS combination in milk following classical low temperature long time pasteurization at 65 °C for 30 min helped to control spoilage bacteria for 15 days. Finally, it also appeared that almost one-third of LYS and NIS left entrapped or bound within the curd. The binding of LYS by curd is well-known, and this is exploited during cheese making to prevent late blowing of cheeses (De Roos et al., 1998; Iaconelli et al., 2008; Schneider et al., 2010). However, further studies are also needed to investigate the amounts of LYS and NIS left in the curd and resulting cheeses, and their potential synergy against other bacteria such as *Clostridium tyrobutyricum* which causes gaseous type spoilage (late blowing) in maturated semi-hard or hard cheeses (D'Amato, Campaniello, & Sinigaglia, 2010).

4. Conclusions

The results of characterization studies in buffers clearly showed ranges of temperature (50–60 °C) and pH (4.50–6.50) conditions optimal for the lytic activity of LYS. The information about lytic activity of LYS was used to understand temperature and pH dependency of antilisterial activity for LYS-NIS combination and to maximize its effectiveness. The inactivation of *Listeria* in milk by 5.5 log at 50 °C within 45 min in the presence of LYS-NIS clearly showed the great potential of combining synergetic mixtures of natural active compounds

with mild heating to increase safety of milk. The pasteurized milk (heated at $\geq$ 63 °C) is not preferred for the significant portion of traditional ripening cheeses in Europe due to longer ripening periods, and inferior aroma and flavor profile of its cheeses than those made from raw milk. Thus, the developed mild treatment was quite promising to minimize loss of original aroma and flavour of milk as well as to increase retention of milk enzyme and bacterial flora that contribute positively to the cheese ripening process. Further studies are needed to evaluate the effect of developed treatment on other pathogenic bacteria and spoilage bacteria such as *C. tyrobutyricum* that is critical for the quality of ripening cheese obtained from treated milk.

CRediT authorship contribution statement

Gozde Seval Sozbilen: Funding acquisition, Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Conception and design of study, Acquisition of data, Drafting the manuscript, Revising the manuscript critically for important intellectual content. **Ahmet Yemenicioğlu:** Conceptualization, Formal analysis, Writing - original draft, Writing - review & editing, Conception and design of study, Analysis and/or interpretation of data, Drafting the manuscript, Revising the manuscript critically for important intellectual content.

Declaration of competing interest

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110447.

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