EXPLORING LYSOZYME-NISIN ANTIMICROBIAL SYNERGY AT DIFFERENT CONDITIONS FOR NOVEL FOOD APPLICATIONS

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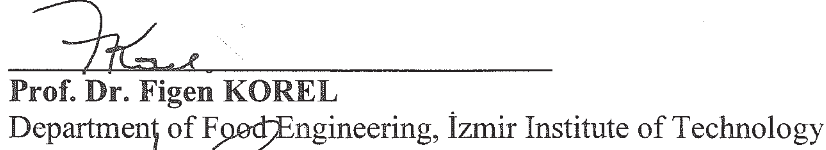

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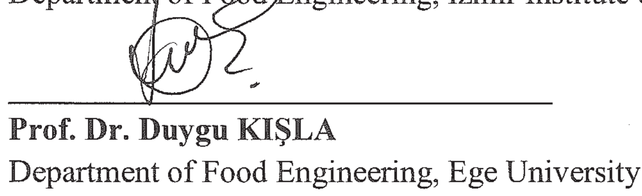

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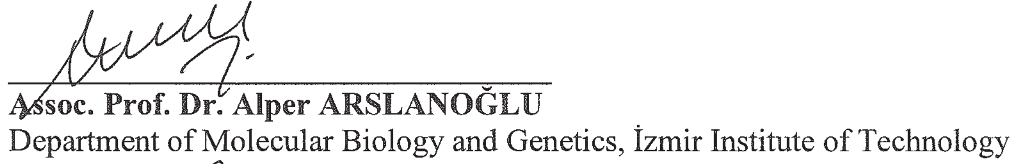

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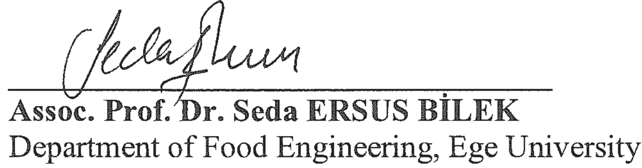

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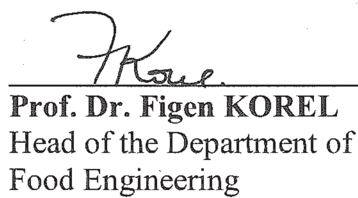

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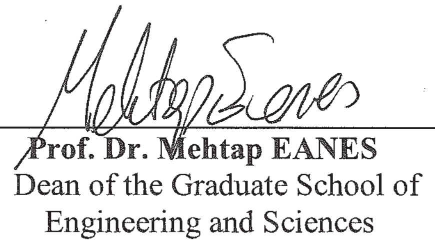

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ABSTRACT

EXPLORING LYSOZYME-NISIN ANTIMICROBIAL SYNERGY AT DIFFERENT CONDITIONS FOR NOVEL FOOD APPLICATIONS

The main objective of this thesis is to explore lysozyme-nisin antimicrobial synergy in combination with refrigeration, mild heating or edible packaging for novel food applications. The thesis is based on the following four chapters: (1) Characterization of biochemical properties of lysozyme at different conditions; (2) Lysozyme-nisin synergy in combination with mild heating against *Listeria innocua*: test of findings in inoculated raw-milk (3) Lysozyme-nisin synergy in combination with chitosan coating against *Listeria innocua*: test of findings on coated inoculated sprouting seeds; (4) Lysozyme-nisin synergy in combination with refrigeration against lactic acid bacteria (LAB): test of findings in boza, a traditional fermented beverage. The results of this thesis clearly showed that the use of lysozyme-nisin synergy in combination with mild heating at 50 °C or in combination with edible chitosan coating could be employed to reduce risk of listeriosis from raw-milk and sprouted seeds, respectively. It is also clearly demonstrated that the lysozyme-nisin synergy in combination with refrigeration could be used to delay acidic spoilage of boza without reducing LAB below 10^6 CFU/mL. This thesis clearly showed the high potential of using lysozyme-nisin synergy against pathogenic or spoilage bacteria as an effective hurdle.

ÖZET

LİSOZİM-NİSİN ANTİMİKROBİYAL SİNERJİSİNİN FARKLI KOŞULLAR ALTINDA ORTAYA KONMASI VE BUNUN YENİLİKÇİ GIDA UYGULAMALARI

Bu tezin başlıca amacı, lisozim-nisin antimikrobiyal sinerjisinin soğukta depolama, ılımlı ısıtma veya yenilebilir paketleme ile kombine edilerek yenilikçi gıda uygulamalarının araştırılmasıdır. Bu tez çalışması temelde dört bölümden oluşmakta olup bunlar şu şekilde sıralanmaktadır: (1) Lisozimin farklı koşullardaki biyokimyasal özelliklerinin karakterizasyonu; (2) Lisozim-nisin sinerjisinin ılımlı ısıtma ile kombine edilerek *Listeria innocua*'ya karşı etkisi: bulguların inoküle edilmiş çiğ sütte test edilmesi; (3) Lisozim-nisin sinerjisinin kitosan kaplama ile kombine edilerek *Listeria innocua*'ya karşı etkisi: bulguların inoküle edilerek kaplanmış çimlendirilecek tohumlarda test edilmesi; (4) Lisozim-nisin sinerjisinin buzdolabı koşullarında laktik asit bakterilerine (LAB) karşı etkisi: bulguların geleneksel fermente bir içecek olan bozada test edilmesi.

Bu tezde elde edilen sonuçlar, lisozim-nisin sinerjisinin 50 °C'de ılımlı ısıtma veya yenilebilir kitosan kaplama ile kombine edilerek sırasıyla çiğ süt ve çimlendirilecek tohumlar gibi riskli gıdalardan kaynaklanacak listeriosis riskini azaltmak amacıyla kullanılabileceğini açık bir şekilde göstermiştir. Ayrıca, lisozim-nisin sinerjisinin buzdolabı koşulları ile kombine edilerek, LAB sayısını 10^6 KOB/mL sayısının altına düşürmeden boza gibi fermente bir üründe asidik bozulmayı geciktirebileceği de gösterilmiştir. Bu tez çalışması, lisozim-nisin sinerjisinin hem patojenik hem de bozulma yapan bakterilere karşı kullanılabilecek yüksek potansiyelli bir engel yöntemi olduğunu göstermiştir.

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CHAPTER 1

INTRODUCTION

Foods are spoiled by their enzymes, spoilage microorganisms, and microbial destructive enzymes. Preventing the spoilage and extending the shelf life of foods are the main concerns of manufacturers and thermal processing of food is the mostly applied conventional method to overcome these issues. However, during thermal processing, the excess amount of heat energy is transferred into the food which is exposed to undesirable reactions stimulated by the heat, such as nutrient degradation and the formation of undesirable sensory changes. Thus, increasing interest of foods that are preserved by less severe methods has gained much more attention due to health concerns of the consumer. Moreover, the health concerns of consumers prompt them to search for foods that include natural additives instead of chemical additives which are effective enough to prevent spoilage and prolong the shelf life of the product. Therefore, manufacturers and scientists have much more focused on the replacement of the chemical additives with natural alternatives in order to meet consumer demands.

Nature is rich for antimicrobials that are produced from plants (herbs, spices, polyphenols), microorganisms (bacteriocins and enzymes) and polysaccharide like chitosan. These compounds are used in biopreservation of the foods. Among the biopreservation agents, lactic acid bacteria (LAB) and their metabolites, especially an antimicrobial bacteriocin called as nisin, and a lytic enzyme, lysozyme are the most studied ones due to their Generally Recognized as Safe (GRAS) status.

The former antimicrobial agent, nisin, is a bacteriocin synthesized by certain strains of *Lactococcus lactic* subsp. *lactis*. It has antimicrobial action against Gram-positive bacteria and the spores of Gram-positive bacteria like *Bacillus* spp. and *Clostridium* spp., whereas Gram-negative bacteria, viruses and fungi are not sensitive to the antimicrobial activity of nisin. However, its antimicrobial activity is also restricted among Gram-positive bacteria that are able to synthesize nisin specific protease called as nisinase (de Arauz et al., 2009). Certain strains of *Streptococcus thermophilus*, *Lactobacillus plantarum* and *Bacillus* species were reported as nisinase producer strains (Zhou et al., 2014). Nisin is the only bacteriocin approved as a food additive. Because it

is a harmless peptide that can be ingested into amino acids by the enzymatic activity of α-chymotrypsin in the intestine. Moreover, when it is added into food, no sensory change occurs due to the presence of nisin (de Arauz et al., 2009). Moreover, it has many applications in food products due to its heat stability, in addition to its GRAS status as well. Therefore, nisin remains anti-microbially efficient after thermal processing such as pasteurization and sterilization (Santos et al., 2018). In this context, several studies were performed to increase the efficiency of nisin by combining with heat treatment (Budu-Amoako et al., 1999: Wirjantoro et al., 2001; Penna and Moraes, 2002). Thereby, it can be exploited as antimicrobial agent in canned foods to inhibit the growth of spore former and heat resistant bacteria (Santos et al., 2018). But, one of the main area of nisin use in the food industry is dairy products to hamper late blowing in cheeses by inhibiting *Clostridium tyrobutyricum* that is the responsible for that damage in cheeses (Silva et al., 2018).

Lysozyme is an enzyme that may present in cow milk, papaya, cauliflower, egg from hen, goose, and duck and also in much human tissues such as the spleen, blood serum, saliva, tears. It has antimicrobial action against Gram-positive bacteria by lysis of their peptidoglycan layer in the cell wall. But, it is inefficient to Gram-negative bacteria due to their protective layer on their peptidoglycan layer in the cell walls (Lesnierowski and Kijowski, 2007). In the food industry, lysozyme is mainly used in semi- or hard-cheese production to prevent undesirable flavors and late blowing caused by the growth of a bacterium called *Clostridium tyrobutyricum.* It is also used in cheeses to accelerate the ripening of cheese by stimulating the lysis of the starter bacteria whose cytoplasmic enzymes are released as a result of lysozyme's action (Lesnierowski and Kijowski, 2007). In addition, lysozyme can be added into unpasteurized beers to inhibit the growth of spoilage LAB without changing the sensory properties of beers. Therefore, its use in beer is beneficial to extend the shelf life without causing chill haze and affecting foam stability of beers (Silvetti et al., 2010). Moreover, the use of lysozyme in wine was permitted as a replacement or complement of sulfur dioxide by the International Organization of Vine and Wine in 1997. Also, it is used to retard malolactic fermentation and to prevent the growth of spoilage LAB such as *Pediococcus* spp., *Lactobacillus* spp., and *Leuconostoc mesenteroides* (Silvetti et al., 2017).

Different food applications of nisin and lysozyme have still been heavily investigated by researchers. Recently, the synergistic effect of using lysozyme in combination with nisin has been demonstrated (Chung and Hancock, 2000). Accordingly,

several studies have also been initiated to combine these antimicrobial agents in food systems (Datta et al., 2008; Mangalassary et al., 2008; Antolinos et al., 2011; Takahashi et al., 2012; Sozbilen et al., 2018). But, no study focused on the optimum pH condition for their synergistic interaction. On the other hand, numerous studies have been presented about the incorporation of nisin (Padgett et al., 1998; Teerakarn et al., 2002; Pranoto et al., 2005; Li et al., 2006; Ye et al., 2008b; Jin et al., 2009) or lysozyme alone (Mecitoğlu et al., 2006; Duan et al., 2007; Arcan and Yemenicioğlu, 2013; Boyacı et al., 2016) into a biopolymer film. However, studies to exploit the synergistic action of these natural antimicrobials in food packaging systems are scarce (Datta et al., 2008). Exploiting them as antimicrobial agents to extend shelf life of food and improve sensory characteristics of food by incorporating them into the biopolymers are considered as a novel approach to maintain food safety through active packaging. The concept of active packaging has emerged from several necessities. These include consumers' demand for healthier foods, new practices in global trading, new practical habits of consumers, distribution of the products via the Internet and strict legislation concerning the benefit of consumers (Suppakul et al., 2003). Active packaging is a novel and ever-growing research area of food science. It can be defined as a kind of packaging that prolongs the shelf life of the food, improves food safety and food quality and also keeps the desirable sensory properties of food through packaging material itself (Quintavalla and Vicini, 2002). Moreover, antimicrobial films enable protection for antimicrobial agents by preventing the direct interaction of antimicrobials with food components like proteins and lipids (Wu et al., 2019).

In the light of above-mentioned knowledge, this doctoral thesis study is designated to comprise of following four chapters: (1) Biochemical characterization of lysozyme at different pH and temperature conditions; (2) The exploiting the antimicrobial synergy between lysozyme and nisin against *L. innocua* in raw bovine milk treated with mild heating; (3) The development of an antimicrobial chitosan based coating solution incorporated with lysozyme and nisin to apply on legume seeds that are germinated to produce sprouts; (4) The application of lysozyme and/or nisin in biopreservation of boza as a model fermented food system.

CHAPTER 2

IMPORTANCE OF LYSOZYME AND NISIN AS FOOD ADDITIVES

2.1. Antimicrobial Active Agents as Food Additive

Increasing demand for healthy foods, the regulatory restriction of the use of chemicals in food production and concern of consumers about antibiotics usage in agriculture has led to the development of new alternatives like usage of antimicrobial agents in the food industry (Smid and Gorris, 2007; Hintz et al., 2015). Antimicrobial agents are defined as “substances used to preserve food by preventing the growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors” by the FDA (21CFR 170.3 (o) (2)). However, antimicrobial agents are generally used with other processing methods that could result in a detrimental effect on the food quality when they are used alone. In addition, many of the antimicrobial agents are not sufficient to stimulate cellular inactivation, thereby using them in combination with other compounds or processing methods yields better antimicrobial effects. This combinational use of preserving techniques is the basis of the hurdle concept as well (Gabriel, 2015). Antimicrobial agents can be classified into two groups as follows: traditional and naturally occurring antimicrobials (Davidson and Branen, 2005). Three requirements are needed to define an antimicrobial as a traditional antimicrobial that (i) have been used for many years, (2) have been approved to be used in food processing in many countries by legislation (eg, lysozyme), (3) have been chemically synthesized (nisin production by fermentation process from *Lactococcus lactis* subsp. lactis) (Gabriel, 2015).

On the other hand, the naturally occurring antimicrobials are originated from animal, plant and microbial sources (Tiwari et al., 2009). Animal originated antimicrobials are proteins (lactoferrin, poly-L-lysine and etc.) and enzymes (lysozyme, lactoperoxidase, etc) (Carocho et al., 2015). The other animal originated antimicrobial agents are antimicrobial peptides (AMPs) which are small-sized, cationic and heat stable peptides produced by almost all living organisms and if the producers are bacteria they

are known as ‘bacteriocin’. They are not only effective on bacteria but also exhibit their antimicrobial activities against parasites, fungi, and viruses. Although, the main application is of AMPs can be considered as the pharmaceutical industry, they have the potential to be used in the food industry as a food additive (Li et al., 2012). For instance, activated lactoferrin isolated from milk is capable to bind iron and can be used as food preservatives particularly in meat and beef products in the USA (Rai et al., 2016). Furthermore, ovotransferrin is likewise able to bind iron and its degradation, along with ovomucin and ovodefensin, which causes egg white thinning that is a sign of the diminished quality of eggs which become more vulnerable to microbial spoilage (Liu et al., 2018). Lysozyme which will be discussed later is one of the animal-derived antimicrobials is the sole permitted natural antimicrobial derived from a animal source in the USA and the EU (Carocho et al., 2015).

On the other hand, plant-derived antimicrobial compounds mainly include organic acids, phenolic compounds, essential oils and derivatives, and phytoalexins. The latter is produced in the case of microbial infestation or treatment of the plant tissue and generally effective on phytopathogenic fungi, but antimicrobial activity against bacteria has also been reported. The well-known phytoalexins are isoflavonoids that are produced by the legumes such as *Glycine max* and *Phaseolus vulgaris*, and disease-associated proteins like chitinase, zeamatin, and thionins. The former protein targeted to chitin that is the cell wall component of most fungi. However, phytoalexins as food additives do not exhibit sufficient antimicrobial activity at low concentration. In contrast with low levels of phytoalexins, they can be cytotoxic at a high concentration that is needed to achieve a desired antimicrobial activity in a food matrix (Smid and Gorris, 2007). Similarly, phenolic compounds are also secondary metabolites synthesized to protect from predators and are produced generally when the plants are exposed to stress factors (Hintz et al., 2015). Moreover, they contribute to the taste, odor and color of fresh plants. Phenolic compounds are characterized by the presence of benzene ring bonded with one or more hydroxyl groups and functional groups as well (Smid and Gorris, 2007). Phenolic compounds of plants can be classified into ten classes in regard to their chemical structure. But, major plant phenolics are simple phenols and flavonoids (Bravo, 1998). Their antimicrobial activities are dependent on their concentration. When they are present at low concentrations, energy production related enzymes are affected. On the other hand, they result in the denaturation of proteins at high concentrations (Tiwari et al., 2009). The other plant originated antimicrobial compound is essential oils which are generally

obtained by the extraction from roots, leaves, stems, and fruits of plants. They are composed of a complex volatile mixture of different chemical compounds like esters, terpenes, ketones and aldehydes (Smid and Gorris, 2007). They help to the pollination of plants by attracting the flies and insects and majorly contribute to characteristic flavor and aroma of the herbs and spices, thereby attracting animals to seed distribution as well (Smid and Gorris, 2007; Hintz et al., 2015). The main essential oils are carvacrol, eugenol, thymol, cinnamon oil, and oregano oil. Their antimicrobial effects have been exploited in foods such as meat, fish, vegetable, etc. to prolong shelf life and inhibit spoilage microorganisms (Carocho, 2015). Besides, they are more effective against Gram-positive bacteria compared to Gram-negative bacteria (Tiwari et al., 2009; Hintz et al., 2015). It is reported that highly hydrophobic nature of the essential oils facilitates the penetration of hydrophobic compounds of essential oils into the Gram-positive bacteria's cell membrane where the lipophilic site of lipoteichoic acids are abundant, while the outer membrane of Gram-negative bacteria renders them as resistant to the essential oils (Tongnuanchan and Benjakul, 2014). Lastly, plant-based organic acids are mainly citric, malic, tartaric and succinic acids and can be present in a vast variety of fresh fruits and vegetables. Moreover, microbial originated organic acids such as lactic, acetic and propionic acids could also be synthesized as a result of the fermentation process (Smid and Gorris, 2007). Antimicrobial activities of hydrophilic plant-based organic acid emerge primarily by acidifying the cytoplasm and then, lowering the external pH (Stratford and Eklund, 2003).

Finally, microbial originated antimicrobials are bacteriocins that are produced by bacteria (Carocho et al., 2015). They are peptides having antimicrobial action and bacteriocins produced by LAB gained great attention due to the nontoxic effect and many LAB are regarded as safe (Deegan et al., 2006). However, their spectrum is restricted to closely related species that is the main limitation bacteriocin application (Carocho et al., 2015).

In this Ph.D. thesis, lysozyme and nisin were exploited as antimicrobial agents in different food applications that comprise the main structure of this thesis. The detailed information about these antimicrobial agents will be given below.

2.2. Lysozyme as a Food Additive

The lysis action of egg white on organisms was observed by Laschtshenko in 1909 and then, Fleming discovered lysozyme in human nasal secretions in 1922 (D'Amato et al., 2010; Davidson and Branen, 2005). Lysozyme has been approved for many applications in the food industry, especially for its antimicrobial activity. This has been attributed to the GRAS status of lysozyme approved by the Food and Drug Administration (FDA) and good stability and activity in different food systems (Arcan and Yemenicioğlu, 2013).

Many functional and physiological properties are possessed by lysozyme. These are the role in surveillance of membranes of mammalian cells, enhancement of the phagocytic activity of polymorphonuclear leukocytes and macrophages and stimulation of antitumor function and proliferation of monocytes and antimicrobial activity which provides the great interest of scientist to practice for novel applications in the food industry (D'Amato et al., 2010). Moreover, owing to its stability over a wide range of pH and temperature, the variety of potential food applications are available (Park et al., 2004).

Apart from the food industry, the pharmaceutical industry also focuses on the various application of this versatile enzyme because of its anti-inflammatory, antiviral, antiseptic, antihistamine and antineoplastic activities (Wu et al., 2017b). Moreover, Zheng et al., (2016) have profited from the binding affinity of lysozyme to the cell walls of Gram-positive bacteria to produce a lysozyme-based biosensor to detect bacterial infection.

2.2.1. Sources of Lysozyme

It has been reported to isolation of lysozyme from various plants like cauliflower, papaya, and cabbage, bacteria, human tissues such as salivary acinar cells, lactating mammary tissue, Paneth cells, renal tubular cells, myeloid cells and histiocytic cells, and animal tissues that are hen, duck and goose egg whites, cow milk (Liburdi et al, 2014). On the other hand, lysozyme content in its sources can be given in descending order as follows: Human tears (3000-5000 ppm FW (fresh weight), hen egg white (2500-3500 ppm FW, duck egg white (1000-1300 ppm FW), goose egg white (500-700), human

spleen (50-160 ppm FW), human thymus (60-80 ppm FW), human milk (55-75 ppm FW), human pancreas (20-35 ppm FW), cauliflower juice (25-25 ppm FW) and cow milk (10-15 FW) (Syngai and Ahmed, 2019). In addition, lysozyme was also purified from an oyster species (Xue et al., 2004).

Lysozymes present in nature can be separated into three groups that are (i) c-type (chicken type), (ii) g-type (goose type) and (iii) i-type (invertebrate type). But, hen egg white lysozyme (HEWL) (EC 3.2.1.17) from c-type that is small sized (14.3 kDa) hydrolytic enzyme with high isoelectric point around 11 and composed of 129 amino acids in a single polypeptide chain as depicted in Figure 2.1, has received attention of food industry due to abundance in nature, high solubility and stability (Silvetti et al., 2017; Wu et al., 2019). In the structure of lysozyme, four disulfide bonds are present and responsible for its compact conformation and thermal stability (Silvetti et al., 2017) which is also stabilized by six helical regions (Barbiroli et al., 2016). In addition, it is the only lysozyme permitted to use as a food additive in the food industry (Wu et al., 2019), thanks to its feasible and low-cost purification methods and abundance in egg white protein of whose 3.5% constitutes the enzyme (Barbiroli et al., 2016).

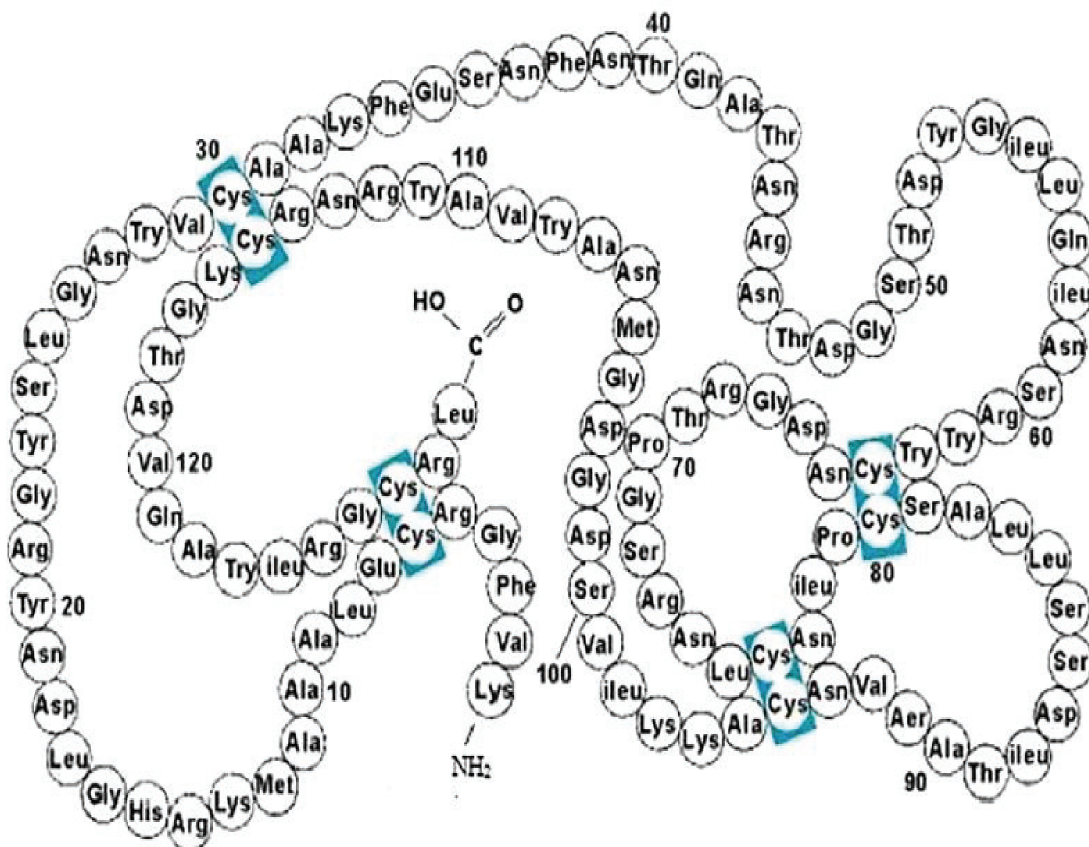

Figure 2 1. Lysozyme structure (Source: Wu et al., 2019)

2.2.2. Antimicrobial Mechanism of Lysozyme

Lysozyme exhibits antimicrobial activity on Gram-positive bacteria due to the ability to lyse the cell wall by splitting the bond between N-acetylglucosamine and N-acetylmuramic acid of the peptidoglycan layer (Arcan and Yemenicioğlu, 2013). By virtue of this ability, lysozyme is not effective on Gram-negative bacteria when it is applied alone, because of their lipopolysaccharide layer which surrounds the peptidoglycan layer (D'Amato et al., 2010; Gemili et al., 2007). However, the combination of lysozyme with ethylenediaminetetraacetic acid (EDTA) is an alternative method to overcome this drawback by destabilizing the outer membrane of Gram-negative bacteria (Arcan and Yemenicioğlu, 2013).

Yet the inefficient antimicrobial activity of lysozyme has been revealed against some of the Gram-positive bacteria, some of which have been reported as the strains of *Staphylococcus aureus* isolated from raw milk and cheeses. Neither the growth of bacteria nor the formation of biofilm was affected by lysozyme, while the formation of biofilms was also stimulated by lysozyme in some the strains (Sudagidan and Yemenicioğlu, 2012). This resistance among staphylococci could be attributed to the *O*-acetylation in their peptidoglycan layer. On the contrary, a non-acetylated peptidoglycan layer was found in sensitive strains of staphylococci. However, the resistance mechanism of Gram-positive bacteria to lysozyme can be varied depending on the strains or species as well (Barbiroli et al., 2016).

2.2.3. Food Applications of Lysozyme

HEWL has been ruled as a GRAS compound by FDA since 1998 (Arcan and Yemenicioğlu, 2013). Thus, it can be employed in several food products such as wine, dairy, meat, fish, fruit, and vegetable products (Gemili et al., 2007). The maximum permitted levels of lysozyme according to Codex Alimentarius and Turkish Food Codex (TFC) were given in Table 2.1 and Table 2.2, respectively.

Since the lysozyme adversely affects the growth of Gram-positive bacteria, it is used in the alcoholic fermentation for cider production without affecting the yeast proliferation (Mainente et al., 2017) and in wine to control LAB which may cause spoilage by malolactic fermentation. The permitted maximum limit of lysozyme in wine

is 500 mg/L which was allowed by Regulation (EC) No 607/2009 (Weber et al., 2009). Therefore, lysozyme is an alternative to sulfites whose maximum limit got reduced gradually because it causes allergic symptoms like nausea, headache, difficulties in breathing in patients who suffered from asthma (Santos et al, 2012; Liburdi et al., 2014). Moreover, lysozyme addition was reported to delay the growth of *Alicyclobacillus acidoterrestris* in apple juice (Molva and Baysal, 2017), *Lactobacillus brevis* and *Pediococcus damnasus* that result production of lactic acid and diacetyl which are undesirable aromas and flavor and also turbid appearance in beer (Silvetti et al., 2017) and to control the growth of *Shigella Typhimurium* in fruit juices (Liburdi et al., 2014). Although the addition of lysozyme into wine has been approved by the International Organisation of Vine and Wine (OIV) for almost a decade ago, lysozyme may produce an allergic reaction in individuals who are sensitive to hen's egg. So it is mandatory to label lysozyme in the label of a wine bottle (Weber et al., 2009; Maitente et al., 2017).

Meat products are one of the food groups that may be preserved by the use of lysozyme. In combination with other preservative compounds, lysozyme produces a reduction in the microbial population that causes spoilage. Some of the examples of these meat products are fresh meat, buffalo meat and minced meat; nisin, EDTA and chitooligosaccharides are used in addition to lysozyme, respectively, for the extension of products' shelf life (Liburdi et al, 2014).

On the other hand, dairy products are the major application area of lysozyme (Barbiroli et al., 2016). Its use in butter or milk to produce Italian cheeses was also patented in the United Kingdom for the control spoilage microorganism like *Cl. tyrobutyricum* which is the main reason of the late blowing in semi- or hard-cheeses. This spoilage is associated with the contamination of milk by *Cl. tyrobutyricum* whose sources are soil, silage used for feeding animals. When the animals are fed by contaminated silage and the milking conditions are not hygienic, the spores of above-mentioned bacteria can contaminate milk used in cheese production. Since the bacterium spores are resistant to heat applied during cheese production, they can survive and cause fermentation of lactate into the butyric acid. This spoilage is characterized by undesirable texture and eye formation in cheese because of large quantities of CO_2 and H_2 production by bacteria (D'Amato et al., 2010).

Table 2.1.The maximum permitted levels of lysozyme in food products

Food product	Maximum level	Reference
Ripened cheese	GMP*	Codex alimentarius
Cider and perry	500 mg/kg	Codex alimentarius
Grape wines	500 mg/kg	Codex alimentarius

* indicates that the quantity of the additive added to food shall be limited to the lowest possible level necessary to accomplish its desired effect

Table 2.2. The maximum permitted levels of lysozyme according to TFC

Food product	Maximum level	Reference
Ripened cheese	GMP*	TFC
Beers subjected to neither pasteurization nor sterile filtration	GMP*	TFC
Wines	500 mg/kg	TFC
Distilled alcoholic drinks having maximum 15% of alcohol and containing the mixture of both non-alcoholic drinks and alcoholic drinks	GMP*	TFC

* indicates that the quantity of the additive added to food shall be limited to the lowest possible level necessary to accomplish its desired effect

2.2.4. Use of Lysozyme in Antimicrobial Packaging

Lysozyme is one of the frequently studied antimicrobial agents in food biopreservation and antimicrobial food packaging that used to suppress, delay or inhibit the growth of the target microorganisms (Appendini and Hotckiss, 2002) that are principally proliferate at the surface of the food in solid and semi-solid foods. The antimicrobial active agents that are incorporated into packaging materials that can also be in the form of edible films or coatings as well are released into the food to maintain food safety and quality throughout the desired shelf life (Corrales et al., 2014). Because, the direct addition of antimicrobial agents into food yields short terms protection, while antimicrobial packaging achieves the release of antimicrobial agents from antimicrobial

film or coating onto food contact surface at a rate of the required amount for a longer period (Syngai and Ahmed, 2019).

Lysozyme can be incorporated into a film or coating matrix which serves as a carrier for lysozyme through the methods of absorption, immobilization or entrapment. Chitosan, whey protein, zein from corn, carrageenan, bovine or fish gelatin are the examples of biodegradable carrier matrices for lysozyme (Silvetti et al., 2017). Duan et al. (2007) reported that chitosan film incorporated with lysozyme (60% w/w chitosan) was efficient to reduce the growth of *Listeria monocytogenes, Escherichia coli and Pseudomonas fluorescens* and to inhibit completely the growth of molds namely, *Cladosporium* spp., in chitosan-lysozyme film coated mozzarella cheeses during the storage for 14 days at 10 °C. Besides, the growth of *L. monocytogenes* was suppressed by using an antimicrobial zein based film incorporated with lysozyme in fresh Kashar cheese stored for 8 weeks at 4 °C (Ünalan et al., 2013). Moreover, fresh eggs coated with chitosan coating incorporated with lysozyme have been maintained their internal quality 3 weeks longer compared to uncoated eggs (Yuceer and Caner, 2014). On the other hand, the lysozyme incorporated biodegradable films can also be employed in fish products such as fresh salmon fillets on where collagen-lysozyme was coated and preserved the quality attributes by controlling the growth of total viable count and limiting the enzymatic activities of endogenous proteases. Additionally, the texture of the product was enhanced and weight loss was reduced thanks to the collagen-lysozyme coating during the storage of fresh salmon fillets at 4 °C for 15 days (Wang et al., 2017). Boyacı et al. (2016) also developed an antimicrobial whey protein-oleic acid composite film incorporated with lysozyme whose release was controlled through acidification during storage of smoked salmon. This film yielded almost 0.6 decimal (D) reductions in *L. innocua* at the end of a week storage of smoked salmon slices at 4 °C. Furthermore, Min et al. (2008) also applied whey protein film incorporated with lysozyme to control the growth of *L. monocytogenes* in smoked salmon as well. Researchers of that study aimed to release the free form of the enzyme into food by pH adjusting of whey protein film solution to eliminate charge-charge interaction takes place between whey protein and lysozyme. In order to enhance antimicrobial efficiency against Gram-negative bacteria, lysozyme can also be combined with EDTA as performed by Ünalan et al. (2011a) who incorporated lysozyme-EDTA (700 $\mu g/cm^2$:300 $\mu g/cm^2$) into zein films to control *L. monocytogenes, E. coli* O157:H7, and *S.* Typhimurium in ground beef patties.

On the other hand, lysozyme was also incorporated into polymers used in food packaging. Appendini and Hotchkiss (1997) immobilized lysozyme on different polymers that were polyvinylalcohol (PVOH), pellets of nylon 6,6 and cellulose triacetate (CTA) films. In addition, the lysozyme immobilization on PVOH films cross-linked by glyoxal and glutaraldehyde was successfully performed by also Conte et al. (2006) who tested the antimicrobial effectiveness of the films against *Micrococcus lysodeikticus.*

2.3. Nisin as a Food Additive

Nisin was discovered in 1928. The certain strains of *Lactococcus lactic* subsp. *lactis* are responsible for the production of nisin. This 34 amino acids length peptide (3.5 kDa) has an isoelectric point above 8.5 (D'Amato and Sinigaglia, 2010). It is included in the bacteriocin class Ia which is called also as lantibiotics due to the presence of unusual amino acids that are lanthionine, methyl-lanthionine, dehydrobutyrine and dehydroalanine (Figure 2.2) (Cleveland et al., 2001).

The antimicrobial action of nisin is affected by several factors including pH, temperature and solubility. Nisin's solubility and stability are much better in an acidic environment. For example, its solubility is 12 wt % at pH 2.5, while it is 4 wt % at pH 5.0. Similarly, the stability of nisin is better at lower temperatures but this is also related to pH conditions of the food environment. Increasing pH values with increasing temperatures cause less solubility; thus the decrease in antimicrobial action of nisin occurs (Gharsallaoui et al., 2016a). Food having a pH below 7.0 is compatible with nisin incorporation due to better solubility and stability throughout the processing and storage period (Balciunas et al., 2013). As exemplified by the study of Ananou et al. (2007), at pH 2.0, nisin is 228 times more soluble compared to at pH 8.0 and retains its stability and activity at pH 2.0 after autoclaving at 121 °C for 15 min (Penna et al., 2005). However, the activity of nisin is lost after 30 min at 63 ºC - pH 11 (Gharsallaoui et al., 2016a).

The use of bacteriocins alone does not ensure food safety since Gram-negative bacteria, yeast and molds are not sensitive to nisin. Insufficiency of bacteriocins to ensure food safety has prompted to food industry to apply hurdle technology such as the use of non-thermal treatments like high hydrostatic pressure (HHP), pulsed electric field (PEF) with bacteriocins, bacteriocin usage with organic acids or chelating agents like ethylene diamine tetraacetate (EDTA) (Deegan et al., 2006).

Nisin (E234) is the most preferred bacteriocin in the food industry for its high antibacterial activity. Since it has been ruled as a GRAS component by FDA since 1988, its usage as a food additive is widely popular over 50 countries around the world.

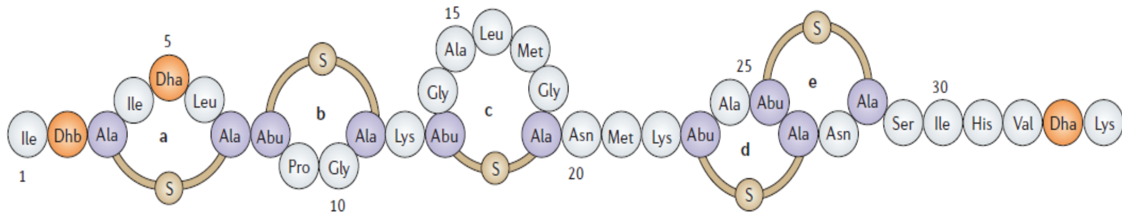

Figure 2.2. Structure of nisin (a-e denotes the lantionine ring) (Source: Breukink and Kruijff, 2006)

2.3.1. The Source of Nisin

The certain strains of *Lactococcus lactis* subsp. *lactis* are responsible for the production of nisin (D'Amato and Sinigaglia, 2010). This lactic acid bacterium has also a GRAS status and potential to be used in the same way as nisin against pathogenic bacteria that can be controlled through the outgrowth of *Lc. lactis* (Dygico et al., 2019).

Bacteriocins can be employed in a food product in three different forms. These include viable cells of bacteriocin-producing strains, crude and pure form of the bacteriocin (Jamaluddin et al., 2018). To purify bacteriocins, bacteriocin-producing strains should be growth in a medium rich in nutritional value that makes the process difficult and costly. Purification of a bacteriocin can be performed in three different ways. Firstly, conventional methods including sequential stages of ammonium sulfate precipitation, ion exchange, hydrophobic interaction, gel filtration, and reverse-phase high pressure liquid chromatography can be applied (Balciunas et al., 2013). But, these subsequent stages of purification have many drawbacks such as low recovery rate, high-cost and time-consuming (Jamaluddin et al., 2018). Secondly, ammonium sulfate precipitation, chloroform/ methanol extraction and reversed-phase high- pressure liquid chromatography can be performed respectively as a three-stage procedure. Thirdly, one of the unit operations such as expanded bed adsorption chromatography, macroporous monolith, aqueous two-phase systems can be employed as well (Balciunas et al., 2013; Jamaluddin et al., 2018). The latter two ways of purification are time-saving and high-yielded methods (Balciunas et al., 2013).

2.3.2. Antimicrobial Mechanism of Nisin

Nisin is characterized by the presence of cationic and hydrophobic peptides and their mechanism on targeted cells generally occurs by the formation of pores in their cytoplasmic membrane (Deegan et al., 2006). In other words, the adsorption of nisin in the target cell is initiated by the electrostatic interaction that takes place between cationic nisin and anionic phospholipids of the cell membrane (Gharsallaoui et al., 2016a). This interaction facilitates the bound between nisin and peptidoglycan precursor -Lipid II- and then, nisin-lipid II has primary role in the pore formation through which essential cellular materials are leaked and consequently, the integrity of the membrane and proton motive force are disrupted and eventually, death of the cell occurs (Figure 2.3.) (Gharsalloui et al., 2016a; O'Bryan et al., 2018).

Nisin has antimicrobial action against a wide range of Gram-positive bacteria such as *L. monocytogenes*, spore-forming species of *Clostridium, Alicyclobacillus* and *Bacillus* and some species of *Streptococcus, Staphylococcus, Lactobacillus,* and *Micrococcus.* But Gram-negative bacteria, yeast and molds are not sensitive to nisin (Delves-Broughton et al., 1996). However, its antimicrobial activity is also restricted among Gram-positive bacteria that are able to synthesize nisin specific protease called as nisinase (de Arauz et al., 2009). Certain strains of *Streptococcus thermophilus*, *Lactobacillus plantarum* and *Bacillus* species were reported as nisinase producer strains (Zhou et al., 2014).

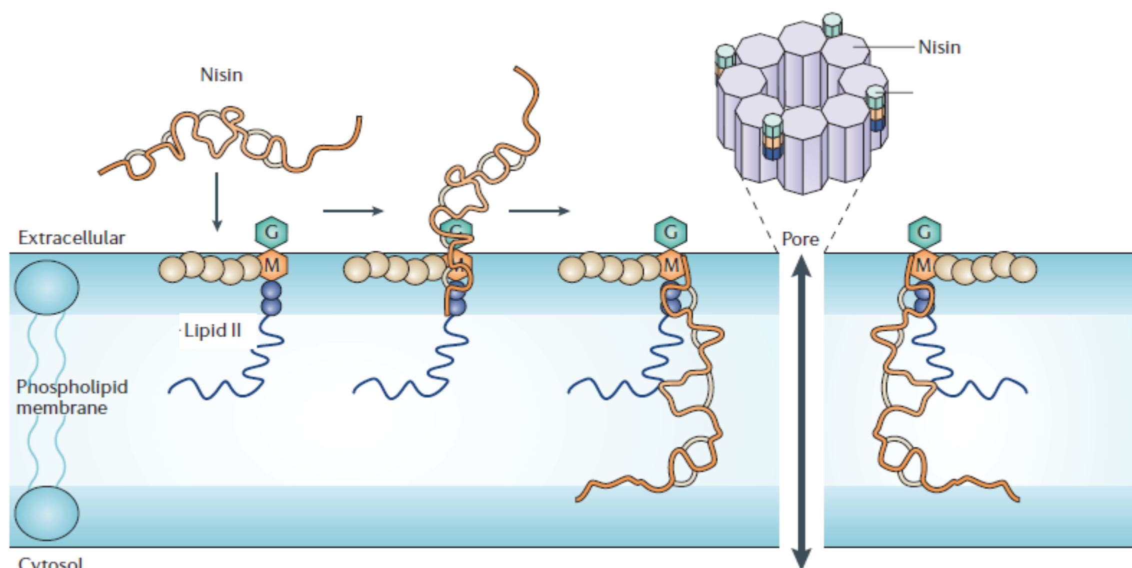

Figure 2.3. Antimicrobial mechanism of nisin via lipid-II target (Source: Breukink and Kruijff, 2006)

2.3.3. Food Applications of Nisin

Nisin is the most commonly used bacteriocin since it is the only bacteriocin confirmed as a food preservative. The safety of nisin for humans was proven with studies of acute, subchronic, chronic toxicity and reproduction, sensitization, in-vitro and cross-resistance studies (Cleveland et al., 2001). The Acceptable Daily Intake (ADI) of nisin was determined as 2.9 mg/person/day (U.S. FDA, 1988). These properties of nisin have attributed to be used in the food industry as the most common bacteriocin. Therefore, it has many applications in foods like meat, dairy products, vegetables, fish, etc. The maximum permitted levels of nisin in certain food products given in Codex Alimentarius and TFC were also presented in Table 2.3 and Table 2.4, respectively.

The antimicrobial activity of nisin diminishes when it is used in free forms because nisin interacts with food components and may be degraded by enzymes inherent in food (Lopes et al, 2017, Wu et al., 2017a, Cui et al, 2017). The use of nisin in meat product has been of great interest due to the consideration of it as a healthier alternative to nitrite addition into the processed meat products. Davies et al. (1999) showed the effectiveness of nisin to control the growth of LAB in Bologna-type sausages with low-fat content. However, there are concerns about the direct use of nisin due to the reduction in its stability and antibacterial activity. In particular, meat products are not sufficiently protected from bacterial spoilage when nisin is directly used (Khan and Oh, 2016). One of these concerns is phospholipids that existed in meat prevent the homogenous distribution of nisin and render nisin as ineffective for food preservation especially in meat products with a high-fat content (Delves-Broughton et al., 1996; Cleveland et al., 2001; Deegan et al., 2006). The other concerns are the poor solubility of nisin at high pH of meat (D’Amato and Sinigaglia, 2010) and the binding of nisin with glutathione found in raw meat by glutathione S-transferase (Cleveland et al., 2001). As a result, using nisin at high concentrations is the only way to control the growth of *Clostridium botulinum* in meat products, but this is not economical. Thus, the combination of nisin with other preservation methods such as modified atmosphere and vacuum packaging has been proposed to exert the antimicrobial action using a mixture of different type of bacteriocins together or combining with non-thermal treatments (Cleveland et al., 2001; D’Amato and Sinigaglia, 2010, Khan and Oh, 2016).

Processed cheeses were the first food product in which nisin was added. The efficiency of nisin and nisin producing strains against *Clostridium* and *Listeria* in cheeses has been confirmed in many studies (Ananou et al., 2007). Besides *L. monocytogenes, S. aureus* and *Cl. botulinum* are some of the examples of Gram-positive bacteria that have been studied to be inhibited by nisin in cheese and milk products (Sudagidan and Yemenicioğlu, 2012). Nisin can be employed to inhibit the growth of post-germinated spores and toxins of *C. botulinum* in pasteurized-milk cheeses (Balciunas et al., 2013). In addition, in soft cheeses like ricotta and cottage cheeses, the main bacterial target of nisin is *L. monocytogenes* (Gharsallaoui et al., 2016a), while *Cl. tyrobutyricum* is the main target of nisin in Gouda and Emmental cheeses (Delves-Broughton et al, 1996). Moreover, the fresh cheeses made from unpasteurized milk mainly get spoiled by LAB which is adversely affected by nisin (Delves-Broughton et al, 1996; Cleveland et al., 2001). Cui et al. (2017) showed that the use of polyethylene oxide nanofiber with nisin loaded poly-γ-glutamic acid/chitosan nanoparticles as a cheese packaging material had an antilisterial activity. Besides, some studies are reporting nisin addition in milk, chilled dairy desserts, clotted cream, and mascarpone cheeses to extend shelf life (D'Amato and Sinigaglia, 2010).

Nisin was also reported as an effective antimicrobial agent in wine to control the growth of LAB (Santos et al., 2012). Since yeasts are not susceptible to nisin, it has potential use in other alcoholic beverages like beer (D'Amato and Sinigaglia, 2010).

The other food products that have the potential to be extended their shelf life by nisin are hot baked flour products, canned products, salad dressing, pasteurized liquid egg, yogurt, fish products and kimchi (Delves-Broughton et al., 1996; D'Amato and Sinigaglia, 2010). Moreover, nisin delays the lag phase of *Alicyclobaciluus acidoterrestris* DSM 3922 in apple juice as the storage temperature of apple juice increases from 27 °C to 43 °C (Molva and Baysal, 2017).

2.3.4. Use of Nisin in Antimicrobial Packaging

Nisin was approved as GRAS almost 30 years ago (D'Amato and Sinigaglia, 2010) and thus, it has been using as a food additive around the world since then. But, similar to the immobilization of lysozyme or any antimicrobial agent into food packaging

Table 2.3. The maximum permitted levels of nisin in food products

Food product	Maximum level	Reference
Clotted cream (plain)	10 mg/kg	Codex Alimentarius
Unripened cheese	12.5 mg/kg	Codex Alimentarius
Ripened cheese	12.5 mg/kg	Codex Alimentarius
Cheese analogues	12.5 mg/kg	Codex Alimentarius
Whey protein cheese	12.5 mg/kg	Codex Alimentarius
Dairy based dessert	12.5 mg/kg	Codex Alimentarius
Cereal an starch based dessert	3 mg/kg	Codex Alimentarius
Fine bakery wares and mixes	6.25 mg/kg	Codex Alimentarius
Heat treated processed meat, poultry, and game products in whole pieces or cuts	25 mg/kg	Codex Alimentarius
Heat treated processed comminuted meat, poultry, and game products in whole pieces or cuts	25 mg/kg	Codex Alimentarius
Edible casing	7 mg/kg	Codex Alimentarius

Table 2.4. The maximum permitted levels of nisin according to TFC

Food product	Maximum level	Reference
Clotted cream	10 mg/kg	TFC
Mascarpone	10 mg/kg	TFC
Ripened cheese	12.5 mg/kg	TFC
Processed cheese	12.5 mg/kg	TFC
Ripened and processed cheese	12.5 mg/kg	TFC
Pasteurized fluid egg (eggwhite, eggyolk or whole egg)	6.25 mg/kg	TFC
Semolina and tapioca puddings	3 mg/kg	TFC

material, the incorporation of nisin into a food packaging material is also important in terms of release kinetics that is the most significant parameter that should be controlled. Because the excess release of nisin into the food may result in the resistance development of the target bacteria. Therefore, the controlled release of nisin in small amounts into food is essential to maintain long term inhibition of the bacteria. Besides, three factors that are the properties of film, food nature and conditions of storage are closely associated with the release rate of nisin (Gharsallaoui et al., 2016b). This can be underlined with the study of Mauriello et al., (2005) who coated nisin on low-density polyethylene (LDPE) film in which TSB inoculated with *Micrococcus luteus* were placed and stored at 4 °C or 25 °C

for 2 days. The dramatic decrease in microbial growth occurred at 25 °C showed the sudden release of nisin from packaging film into TSB, while nisin was released at a slower rate at 4 °C.

One of the alternative usages of nisin in packaging technologies can be considered as paperboard that was coated with nisin by using vinyl acetate-ethylene as a binder for packaging of milk and orange juice. At low-temperature storage, it showed effectiveness against the growth of total aerobic bacteria and yeast, in milk and orange juice, respectively (Irkin and Esmer, 2015). The other usage of nisin in packaging technologies is that incorporating it into a polymer that can be listed as follow: sodium caseinate, alginate, methylcellulose/hydroxypropyl methylcellulose, polyvinyl chloride, nylon, soy protein, whey protein, zein, and wheat gluten. Antimicrobial activity of polylactic (PLA) film incorporated with nisin (25% w/w PLA) on *L. monocytogenes* and *Salmonella* Enteritidis in liquid egg white and *E. coli* O157:H7 in orange juice was monitored and, 3 D, 2 D and 2.5 D reductions were observed compared to the control group at 24 °C for 3-day storage, respectively (Jin and Zhang, 2008). Similarly, frankfurter contaminated with *L. monocytogenes* was packaged through cellulose film on which nisin was adsorbed at both 625 IU/mL and 2500 IU/mL concentrations. Only the higher concentration of nisin leads to significant antimicrobial activity around 2 D reductions after 14 days of storage at 4 °C (Nguyen et al., 2008). Besides, a vacuum bag treated with antimicrobial nisin solution that also includes EDTA has been shown to control spoilage microflora in freshly cut beef samples. The spoilage microflora composed of *Carnobacterium* spp. lactic acid bacteria and *Brochotrix thermosphacta* was suppressed up to 11th days of storage at 1 °C (Ercolini et al., 2010). In addition, Wu et al. (2017a) has enhanced the quality attributes and limited the microbial growth in yellow croaker which was immersed into chitosan-nisin microparticles and stored at -3 °C for 30 days. The common conclusion of all the studies mentioned above is the potential of nisin as an antimicrobial agent in food packaging materials.

On the other hand, the use of nanofibers in food packaging is of great interest as an emerging technology due to their relatively large surface area, biodegradability, and fine pores. In order to maintain the stability of nanoparticles, the electrospinning technique can be employed to embed nanoparticle into nanofibers. For instance, Cui et al. (2017) successfully employed the polyethylene oxide nanofibers embedded with nisin loaded poly-γ-glutamic acid chitosan nanoparticles to control *L. monocytogenes* with 5.3 D reduction in model cheese system stored at 4 °C for 15 days without affecting sensory

properties of cheese. Moreover, nanoencapsulation and immobilization of nisin into hydroxypropyl methylcellulose films both reduced the loss of activity of nisin and the growth of *L. monocytogenes* (Imran et al., 2012).

2.4. Synergetic Effects and Current Combined Food Applications of Lysozyme and Nisin

Lysozyme and nisin, both are effective on Gram-positive bacteria, their mechanisms of action are quite different from each other. The existed synergy between each other against LAB was proven by in vitro study which was conducted by Chung and Hancock (2000). Another in vitro study was conducted by Chai et al. (2015) to inhibit *Clostridium difficile* growth by the 30 min incubation with nisin (20 mM) and lysozyme (0.2 mM). However, the combined use of nisin and lysozyme is not sufficient to inactivate the spores of *C. difficile* unless the germination is triggered. Chai et al. (2017) reported the inactivation of *C. difficile* spores by a solution including 1% w/v sodium taurocholate, 1% w/v tryptose and 1% w/v NaCl, 20nM nisin and 0.2mM lysozyme in which former three compounds were used to stimulate the germination of the spores. Therefore, their combined usage has invoked the interest of food manufacturers for the extension of the shelf life of food, such as meat and dairy products (Nattress et al., 2001). Some of the studies reporting the combined action of lysozyme and nisin application in food systems were given in Table 2.3. Contrary, a few studies given in Table 2.4 have been focused on the inhibitory effect of the various hurdle treatments including the combination of lysozyme and nisin on spoilage microorganisms in food systems. In addition, the combined action of lysozyme, nisin, and HHP on *E. coli* was demonstrated by an *in vitro* study in which six log reductions in *E. coli* numbers were observed as a result of their combined inhibitory effect (Masschalck et al., 2000). Similarly, Smith et al. (2002) showed the synergistic inhibition of total viable counts in raw milk by the combination of lysozyme and nisin with pulsed electric field treatment at 52 °C. Moreover, a study was performed by using milk in which antimicrobial effect of the combination of lysozyme (50 µg/mL), nisin (either 50 IU/mL or 150 IU/mL) and heat treatment at 85 °C against the spores of *Bacillus atrophaeus* has been reported (Sikin et al., 2017). However, the incorporation of these antimicrobials together into a packaging material is as yet limited to only one study in which lysozyme and nisin were incorporated into a calcium alginate coating that was employed to exert antimicrobial activity against *L.*

monocytogenes and *Salmonella anatum* in smoked salmon (Datta et al., 2008). Therefore, the promising results obtained from this study could be adapted to establish new researches focusing on synergistic antimicrobial effects of lysozyme and nisin in new food applications.

Table 2. 5. Some researches of the combined application of lysozyme and nisin

Test food	***Target microorganisms***	***Results***	***References***
Pork cores of fat and lean tissue	*Brochotrix thermosphacta B2, Carnobacterium sp. 845*	LYS:NIS (1:3) mixture produced a reduction in *Carnobacterium sp.* for 21 days storage in fat tissue and supressed the growth of *B. thermosphacta B2* in lean tissue for 6 weeks in vacuum at 2 ºC.	Nattress et al., (2001)
Pork loin	*LAB, pseudomonads, B. thermosphacta, Enterobacteriaceae*	No effect on *Enterobacteriaceae* and pseudomonads. LAB and *B. thermosphacta* growth were reduced.	Nattress et al., (2003)
Skim milk	LAB	The addition of NIS alone or LYS:NIS showed a remarkable inhibitory effect against the non-nisin producing lactococci strains.	Kozáková et al. (2005)
Smoked salmon	*Salmonella anatum, L. monocytogenes*	Lysozyme incorporated into calcium alginate coating containing nisin reduced *L. monocytogenes* counts ca. 2.7 log CFU/g and *S. anatum* was reduced by ca. 1 log CFU/g	Datta et al. (2008)
In-package pasteurized low fat turkey bologna	*L. monocytogenes*	Population was reduced below the level of detection by 2-3 weeks of storage	Mangalassary et al. (2008)
Liquid egg	*Bacillus cereus*	The growth of *B. cereus* was retarded up to 10 h at 16 ºC by the concentration of 0.13µmol/L	Antolinos et al. (2011)
Ready-to-eat seafood products	*L. monocytogenes*	The count kept at low level in first 3 d of storage at 10 ºC. The population remained under detectable level for 6-8 h at 25 ºC.	Takahashi et al. (2012)
Boza	LAB	The use of NIS (250µL/g) and LYS:NIS (500:250µl/g) in boza as antimicrobial agents provided 0.67 and 0.87 D reductions in LAB count at the end of 28 day storage while enhancing quality attributes and shelf-life period of boza.	Sozbilen et al. (2018)

Table 2.6. Some researches of the combined application of lysozyme and nisin with additional hurdle system

Test food	***Target microorganisms***	***Additional hurdle***	***Results***	***References***
Milk	*E. coli*	HHP	Effectiveness of HHP against *E. coli* was increased by the addition of lysozyme (400 g/ml) and nisin (400 IU/ml) to the milk before pressure treatment. A 15-min treatment at 550 MPa and 50°C in skim milk with antimicrobial lead to decrease around 4.5 to 6.9 log CFU/mL in different strains.	García-Graells et al. (1999)
Ham and bologna	*B. thermosphacta, E. coli* O157:H7, *Lb. curvatus, Lc. mesentreoides, L. monocytogenes,*	EDTA	1:3 LYS:NIS (500 mg/kg) and EDTA (500 mg/kg) combination lead to decrease in the counts of *B. thermosphacta* and *Lc. mesenteroides* on both products. Antimicrobial treatment avoided proliferation of *B. thermosphacta* and *Lb. curvatus* for 4 and 3 weeks, respectively. Treatment of bologna reduced growth of *Lc. mesenteroides* and *L. monocytogenes* for 2 weeks. Treated ham showed reduction in the growth of *E. coli* O157:H7 for 4 weeks.	Gill and Holley (2000)
Skim milk	Total viable count	PEF and mild heat treatment	50 pulses of 80kV/cm application at 52 ºC with the addition of lysozyme and nisin (1638 IU/mL:38 IU/mL) resulted in 7 log reduction in skim milk microflora	Smith et al. (2002)
Red and white grape juice (GJ)	Yeast	PEF	In red GJ, 20 pulses of 65 kV/cm application at 50 ºC with 1:3 lysozyme:nisin (0.4g/100mL) caused 5.9 log reduction, while in white GJ microbial reduction was found as 4.4 log CFU/mL	Wu et al. (2005)
Ostrich meat patties	*L. monocytogenes*	EDTA	The *L. monocytogenes* counts decreased in both vacuum and air packaged samples below the limit (<2 log CFU/g) in 1d.	Mastromatteo et al. (2010)
Milk	*B. atrophaeus*	High pressure nitrous oxide (HPN_2O) and heat treatment at 85 °C (H85)	2.4 and 2.5 log reductions in the spores of *B. atrophaeus* were obtained in milk by the combined treatment of HPN_2O, H85, nisin (50 IU/mL) and lysozyme (50 µg/mL), and HPN_2O, H85, nisin (150 IU/mL) and lysozyme (50 µg/mL), respectively.	Sikin et al. (2017)

CHAPTER 3

BIOCHEMICAL CHARACTERIZATION OF LYSOZYME AT DIFFERENT CONDITIONS

3.1. Introduction

In order to develop a new application of lysozyme in food, the characterization studies are needed to elaborate its effectiveness under various pH and temperature conditions. In literature, information related to the biochemical characterization of lysozyme is scarce and they are also not updated. To expand the knowledge about the biochemical behavior of lysozyme makes this enzyme much more efficient under different preserving and processing conditions.

Therefore, the objective of this chapter is to determine the enzymatic activities and kinetic parameters of lysozyme under different pH and temperature conditions that generally represent food pH and processing temperatures.

3.2. Experimental Study

In the following sections, both materials and methods performed for biochemical characterization of lysozyme were given in detail.

3.2.1. Materials

Chicken egg white lysozyme (L6876), *Micrococcus lysodeikticus* ATCC No: 4698 as a substrate of lysozyme were purchased from Sigma-Aldrich Chem. Co. (St. Louis, Mo., USA). Disodium hydrogen phosphate and sodium dihydrogen phosphate monohydrate were obtained from Merck (Darmstadt, Germany). Citric acid monohydrate was obtained from Sigma-Aldrich (Taufkirchen, Germany).

3.2.1.1 Buffers

0.05 M sodium phosphate buffer for pH range from 5.0 to 7.0 and 0.05 M citrate-phosphate buffer for the pH range from 2.5 to 4.75 were used in the characterization of lysozyme experiments during this study.

3.2.2. Methods

The methods given below were performed to characterize the biochemical properties of lysozyme at different pH and temperature conditions.

3.2.2.1. Measurement of Lysozyme Activity

The lysozyme activities were measured at 660 nm by spectrophotometer (Shimadzu Model 2450, Japan) equipped with a cell holder that keeps the temperature constant at 30 °C. The reaction mixture for the determination of the enzymatic activity of lysozyme was prepared by mixing 2.4 mL *Micrococcus lysodeikticus* cell suspension kept at 30 °C and 0.1 mL of enzyme solution that was kept in an ice bath and heated to 30 °C in a waterbath just before the experiment. The cell suspension of *Micrococcus lysodeikticus* (0.26 mg/mL) was prepared freshly in 0.05 M sodium phosphate buffer at pH 7.0. The slope of the initial linear part of the reduction in absorbance vs time curve was used in the calculation of lysozyme activity which was expressed as unit (U). The enzymatic activity of lysozyme was calculated using the following formula:

$$Activity\left(\frac{U}{mL}\right)=\frac{\Delta Abs}{1000}*10*2.5*DF$$

where ΔAbs is the change in absorbance which is divided by 1000 because the 0.001 change in absorbance in 1 min is defined as 1 unit. 10 is the correction factor for 0.1 mL enzyme solution, 2.5 is the volume of the reaction mixture and DF is the dilution factor of the enzyme solution. The activity of lysozyme measurements was conducted in triplicate.

3.2.2.2. Determination of the pH Stability of Lysozyme

The pH stability of lysozyme was determined at two different conditions. Firstly, activities of lysozyme were measured by using enzyme solutions (1.5 mg/mL) prepared at different pH ranged from 2.5 and 6.5 and stored at 4 °C for 28 days. Measurements were conducted spectrophotometrically on 0th, 3rd, 7th, 14th, 21st and 28th days of storage with the reaction mixture containing 0.1 mL of enzyme solution and 2.4 mL of *Micrococcus lysodeikticus* solution at pH 7.0. Secondly, the pH stability of lysozyme was determined at pH ranging between 2.5 and 6.5 by using the reaction mixture containing 0.1 mL of enzyme solution prepared at different pH (2.5 to 6.5) and 2.4 mL of *Micrococcus lysodeikticus* solution at different pH (2.5 to 6.5). Each lysozyme solution having different pH was mixed with substrate solution having the same pH, as well. The activity of lysozyme was expressed as U/mL. Each analysis was performed in duplicate and at least three measurements were performed for each pH point in each analysis.

3.2.2.3. Determination of Optimum Temperature of Lysozyme at Different pH

The optimal temperature for lysozyme activity was determined spectrophotometrically at different pH values. For this purpose, *Micrococcus lysodeikticus* cell suspensions prepared in different pH values (2.5 to 6.5) was mixed with lysozyme solution (1.5 mg/mL) and at least triplicate measurements were conducted for each pH values at 4, 10, 30, 40, 50 and 60 °C using a spectrophotometer (Shimadzu Model 2450) equipped with a cell holder that keeps the temperature constant at desired temperatures. The activity of lysozyme was expressed as U/mL. Each analysis was performed in duplicate and at least three measurements were performed for each pH-temperature point in each analysis.

3.2.2.4. Determination of Arrhenius Parameters

To identify the catalytic mechanism of lysozyme at various temperatures and pHs, the Arrhenius equation was used.

Accordingly, activation energies, Ea, were calculated using the Arrhenius equation which is as follows:

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

where k is the rate of enzymatic activity of lysozyme, A is the pre-exponential factor, Ea is activation energy, T is temperature, R is the universal gas constant, was used in the calculation of the activation energies of lysozyme at each test pH values. The natural logarithm of lysozyme's enzymatic activities (ln k) for each pH-temperature measurement vs the reciprocal of heating temperatures (1/T) was plotted. Ea values were computed by multiplying the slope of the best fit line by a universal gas constant that is 8.314 J/mol K.

Q_{10}, as another Arrhenius parameter, can be briefly defined as the effect of a 10 °C increase in temperature on the rate of the reaction. Q_{10} was calculated using the formula provided by Cemeroğlu (2015):

$$Q10 = \left({k2}/{k1}\right)^{\frac{10}{T2-T1}}$$

where k_2 and k_1 are the reaction rates at the temperatures T_2 and T_1, respectively.

3.2.2.5. Determination of Thermal Inactivation Kinetics of Lysozyme

Thermal inactivation studies were carried out as described by Gemili et al. (2007) at 85, 90 and 95 °C with thermal inactivation tubes which have 9 mm of diameter and 1 mm wall thickness. The tubes containing 0.9 mL buffers at pH 3.5, 4.0, 4.5, 5.5 or 6.0 were heated up to 85, 90 and 95 °C until the 0.1 mL of lysozyme solution (15 mg/mL) dissolved in buffers at pH 3.5, 4.0, 4.5, 5.5 or 6.0 was added into the corresponding tubes. Then, the tubes were incubated during 60, 120, 180, 240 and 300 mins. At the end of each incubation period, the spectrophotometric measurements were conducted by the reaction mixtures containing 0.2 mL of enzyme solution and 2.3 mL of *Micrococcus lysodeikticus* cell suspension at pH 7.0. The residual activities of lysozyme were calculated in terms of unheat treated lysozyme samples. The unheat-treated lysozyme activity was assumed as 100%. The activities of lysozyme were used in the determination of D-values which is the time required for the 1 log reduction in the activity of enzyme at a constant

temperature, and Z-value that refers the temperature required for the change in D value to transverse by 1 log and is expressed by the equation of $Z = (T_2 - T_1)/\log(D_1/D_2)$. The log of residual activities of lysozyme versus heating time in min gives the heat inactivation curve. Each analysis was performed in duplicate and at least three measurements were performed for each pH-temperature point in each analysis.

3.2.2.6. Statistical Analyses

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

3.3. Results and Discussion

The detailed results and discussion will be given in the following sections.

3.3.1. pH Stability of Lysozyme

The pH stability of lysozyme was observed both at the neutral pH (Figure 3.1) and at pH ranged from 2.5 and 6.5 (Figure 3.2). As depicted in Figure 3.1A and Figure 3.1B, almost all lysozyme activities increased until the 14th day of storage and then it started to fluctuate slowly in neutral pH. Regardless of the pH of the buffers in which lysozyme was dissolved and stored for 28 days, the enzymatic activities showed no obvious variation each day of sampling.

On the other hand, the activities of lysozyme dissolved in different pH buffers showed discrimination between each pH as depicted in Figure 3.2. Briefly, the increase in pH yielded a significant increase in the activity of lysozyme as well. Therefore, the greatest activities were observed at pH 6.5. Moreover, lysozyme's activities at each pH condition remained stable during the storage period. However, the activities of lysozyme measured at the pH ranging from 2.5 to 4.0 (Fig. 3.2A) were dramatically low compared

to lysozyme samples stored at the pH ranging from 4.5 to 6.5 (Fig. 3.2B). Xue et al. (2004) have stated that oyster lysozyme maintained its stability in a wide range of pH and incubating the lysozyme in 2-13 pH ranged buffers for 10 min and 30 min did not affect the stability of itself. On the other hand, Nakimbugwe et al. (2006a) also evaluated the pH dependence of the enzymatic activity of HEWL in the pH range between 2.8 and 7.8 at 25 °C, the results showed that HEWL was inactive up to pH 4.6, while progressively increasing between pH 4.6 and 7.5. These findings are in line with the results presented in Figure 3.2, particularly in the pH range from 4.5 to 6.5.

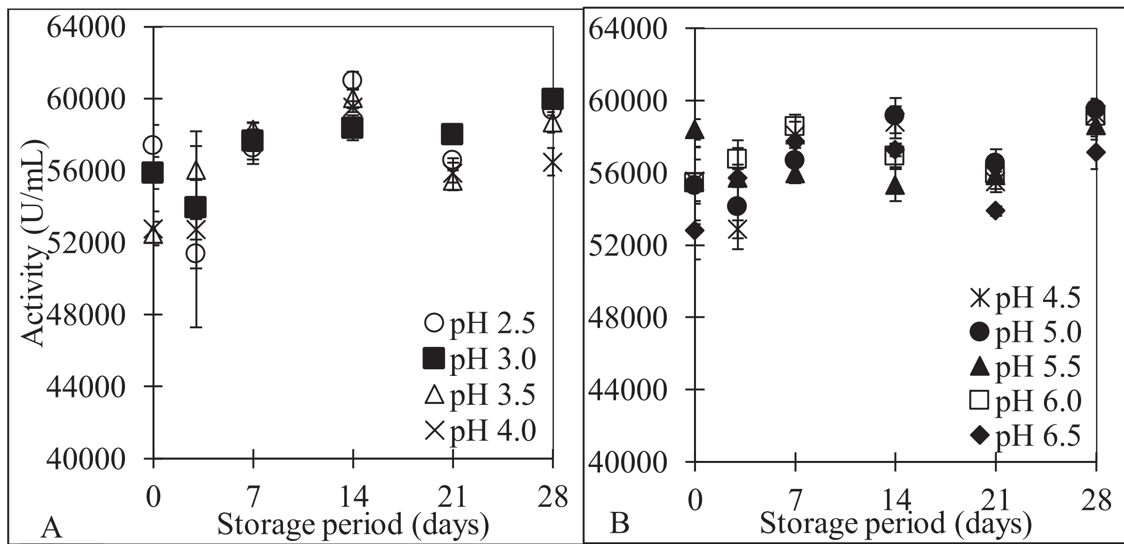

Figure 3. 1. Activities of lysozyme measured at neutral pH

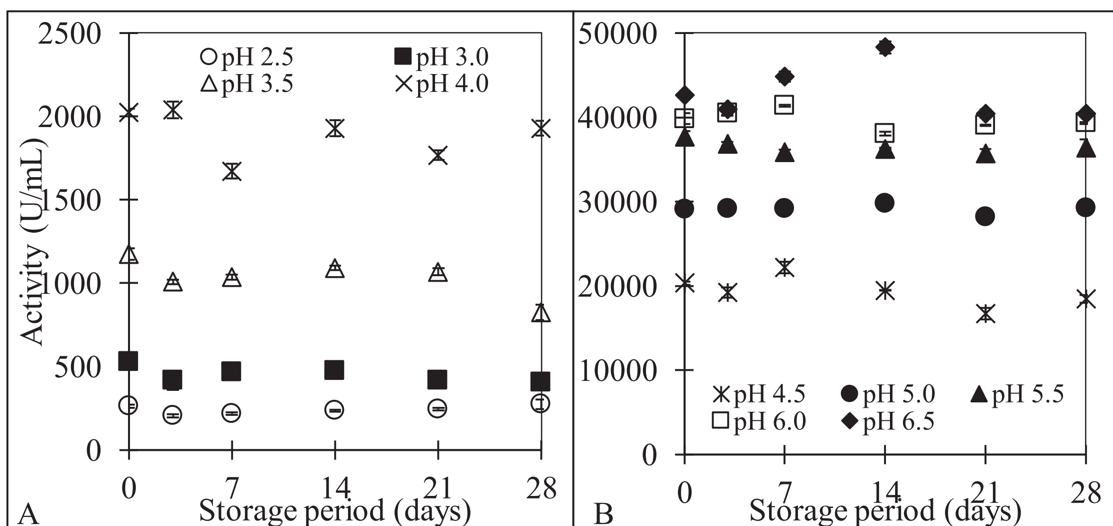

Figure 3. 2. The activity of lysozyme at pH values varying from 2.5 to 4.0 (A) and 4.5 to 6.5 (B) respectively.

3.3.2. Optimum Temperature of Lysozyme Activity at Different pH

The temperature effect on lysozyme activity at varying pH between 2.5 and 6.5 was examined at 4, 10, 30, 40, 50 and 60 °C, and its result was given in Figure 3.3. Regardless of temperature increase, the enzymatic activity of lysozyme was observed as very low in the pH range of 2.5 and 4.0. But, the activities increased dramatically with increasing temperatures as of pH 4.5. The substantial increase in enzymatic activity was more obvious at 60 °C compared to those at 40 and 50 °C. However, the activities were not affected too much with the change of pH in the range of 4.75 and 6.0 at 60 °C. On the other hand, the activities measured at the highest three temperatures were observed pretty close to each other at pH 6.0. The result obtained is in line with the study of Smolelis and Hartsell (1952) who indicated that the activity of lysozyme is greater at elevated temperatures. They observed the higher activity of lysozyme at 52 °C compared to activities of lysozyme at 37 °C and 25 °C. Moreover, Matsuura et al. (2002) determined the lytic activities of HEWL at pH 7.0 in the temperature range of 5 °C and 65 °C and observed the maximum activity of HEWL at 60 °C which is consistent with the result presented here. Besides, the maximum activity of oyster lysozyme was observed at 40 °C and pH 5.5 (Xue et al., 2004). But the variation in the optimal temperature could be attributed to the difference in the source of lysozyme.

These results suggest that lysozyme has the potential to exhibit its' enzymatic activity in food which undergoes to mild heating during processing or before consumption.

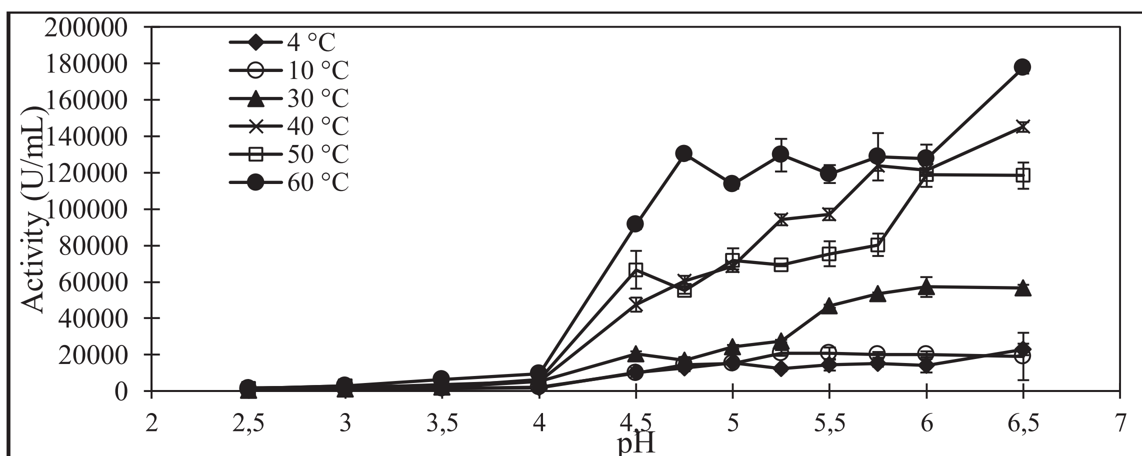

Figure 3.3. Temperature stability of lysozyme

3.3.3. Activation Energy for Lysozyme's Enzymatic Activity

Data belong to the optimum temperature of lysozyme given in Figure 3.3 were replotted in the form of the Arrhenius plot as seen in Figure 3.4. The activation energies of lysozyme at different pH conditions were calculated by the slope of best-fit lines passing through all ln k vs temperature data at each pH tested. The estimated activation energies (Ea) at each pH tested were given in Figure 3.5 and Table 3.1 in which Q_{10}, k, and R^2 values were also given. The estimated Ea values were gradually augmented from 2.7 kJ/mol to 32.8 kJ/mol with increasing pH from 2.5 to 4.5. Therefore, the temperature dependencies of Ea values and thus, the reaction rate in the pH range of 2.5 and 4.5 has considerable significance. However, in the pH range of 4.5 and 6.5, Ea values varied between 28.9 kJ/mol and 33.06 kJ/mol and thus, the temperature dependency in this pH range is substantially limited compared to those in the pH range of 2.5 and 4.5. Studies that have determined the Arrhenius parameters for lysozyme activity are limited in the literature (Makki and Durance, 1996; Matsuura et al., 2002). Makki and Durance (1996) determined Ea of the thermal inactivation of lysozyme in beer between 73 °C and 100 °C in the pH range of 4.2 and 6.24 and monitored substantially higher Ea values ranging between 110.45 kJ/mol and 151.04 kJ/mol. These higher Ea values could be attributed to higher temperatures tested. In addition, Matsuura et al. (2002) determined the Ea value of lysozyme at pH 7.0 between 5 °C and 65 °C as 42.7 kJ/mol which is nearly 1.5 times greater than Ea value found at pH 6.5 in the present study. This difference between Ea values can be related to the difference in the source of lysozyme and pH values tested.

On the other hand, Q_{10} values that show the acceleration ratio of reaction rate at each 10 ° rise in temperature were also determined in the temperature range between 30 °C and 60 °C at each pH (Fig. 3.6). According to monitored Q_{10} values, each 10° rise in temperature between 30 °C and 60 °C resulted in a higher reaction rate changing between 1.21 and 1.97 at pH 2.5 and 6.5. Particularly when the pH was 4.75, reaction rate accelerated almost 2 times. Unfortunately, there is no available literature to compare the Q_{10} values. Additionally, k values at each pH value increased by rising temperatures. Matsuura et al., (2002) have been emphasized that higher k values indicate the catalytic efficiency which is acquired by proper folding flexibility.

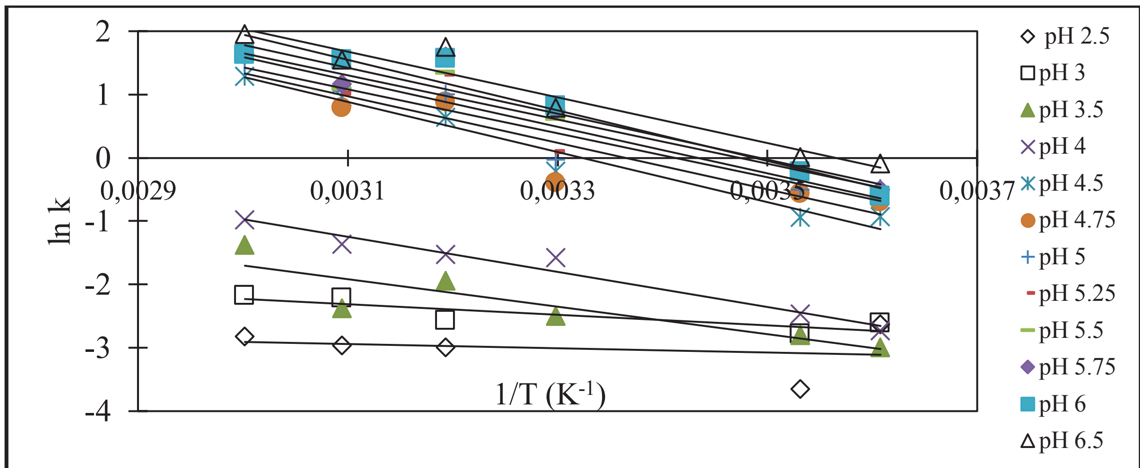

Figure 3. 4. Arrhenius plot for temperature dependence of lysozyme activity at pH ranging from 2.5 to 6.5

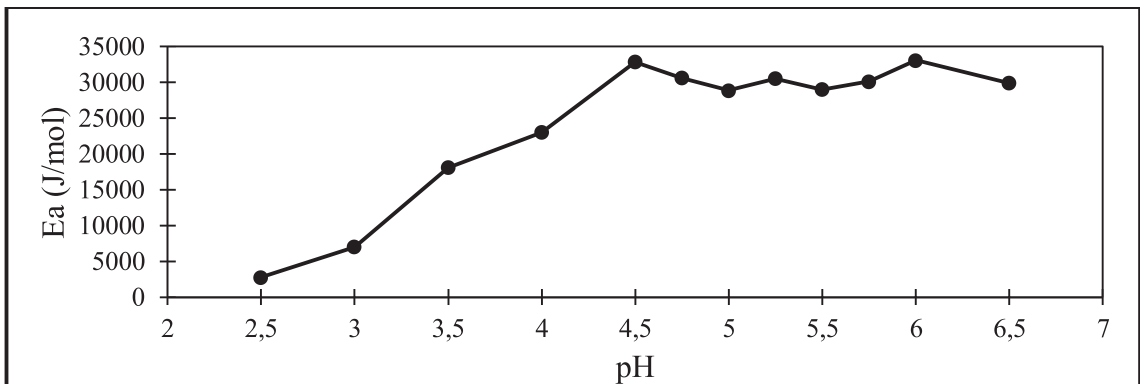

Figure 3. 5. The Ea values of lysozyme vs pH

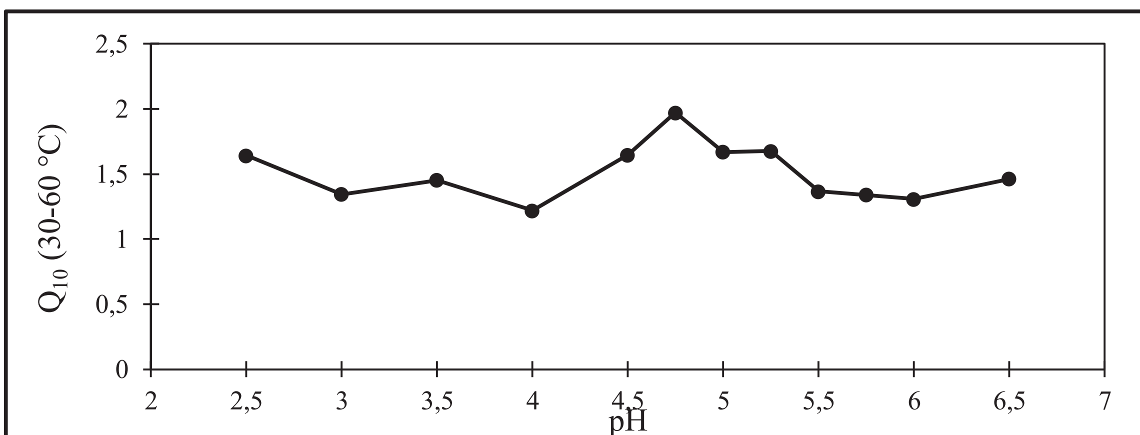

Figure 3. 6. The Q10 values of lysozyme vs pH

Table 3. 1. Arrhenius parameters for lysozyme activity at varying temperatures between 4 °C to 60 °C

pH	T (°C)	k (ΔAbs. min^{-1})	Ea(J/mol) (R^2)	Q_{10} (30-60 °C)	pH	T (°C	k (ΔAbs. min^{-1})	Ea(J/mol) (R^2)	Q_{10} (30-60 °C)	pH	T (°C)	k (ΔAbs. min^{-1})	Ea(J/mol) (R^2)	Q_{10} (30-60 °C)
	4	0.074				**4**	0.080				**4**	0.051		
	10	0.028				**10**	0.062				**10**	0.061		
2.5	**30**	0.013	2777.707 (0.055)	1.641	**3**	**30**	0.047	7002.217 (0.7481)	1.346	**3.5**	**30**	0.083	18123.69 (0.7832)	1.453
	40	0.052				**40**	0.077				**40**	0.145		
	50	0.052				**50**	0.110				**50**	0.095		
	60	0.060				**60**	0.114				**60**	0.254		
	4	0.066				**4**	0.404				**4**	0.506		
	10	0.085				**10**	0.391				**10**	0.575		
4	**30**	0.208	23023.13 (0.9673)	1.219	**4.5**	**30**	0.815	32850.28 (0.9646)	1.648	**4.75**	**30**	0.677	30647.07 (0.8583)	1.972
	40	0.218				**40**	1.903				**40**	2.422		
	50	0.257				**50**	2.664				**50**	2.211		
	60	0.377				**60**	3.653				**60**	5.200		
	4	0.620				**4**	0.491				**4**	0.572		
	10	0.606				**10**	0.836				**10**	0.831		
5	**30**	0.977	28903.62 (0.9223)	1.670	**5.25**	**30**	1.100	30520.69 (0.8972)	1.676	**5.5**	**30**	1.868	29010.04 (0.9437)	1.367
	40	2.748				**40**	3.768				**40**	3.886		
	50	2.878				**50**	2.777				**50**	3.015		
	60	4.551				**60**	5.186				**60**	4.776		
	4	0.610				**4**	0.560				**4**	0.922		
	10	0.799				**10**	0.808				**10**	1.020		
5.75	**30**	2.136	30144.07 (0.9092)	1.341	**6**	**30**	2.288	33068.1 (0.9424)	1.307	**6.5**	**30**	2.273	29908.78 (0.9392)	1.462
	40	4.960				**40**	4.861				**40**	5.806		
	50	3.215				**50**	4.754				**50**	4.736		
	60	5.153				**60**	5.113				**60**	7.106		

3.3.4. Thermal Inactivation Kinetics of Lysozyme

The thermal inactivation study of lysozyme was conducted at 85, 90 and 95 °C during incubation period ranging from 60 min to 300 min at the pH values of 3.5, 4.0, 4.5, 5,5 and 6.0. The D-values that is the time required to inactivate 90% of the enzyme and Z-value which is the temperature required for the change in D value to transverse by 1 log were given the Table 3.2. On the other hand, thermal inactivation of lysozyme followed first-order kinetics for all temperatures tested with the quite high correlation coefficients (R^2) as given in Figure 3.7. Lysozyme was very heat stable especially at the acidic condition and temperature range of 85-95 °C. Although D-values changed by the pH and temperatures, Z-values remained in a narrow limit varying between 17.57 °C and 19.26 °C. It is noteworthy to indicate that the heat stability of lysozyme is not pH dependent in the tested range of pH and temperature. On the other hand, the thermal stability of commercial lysozyme has been reported in the literature (Makki and Durance, 1996; Okanojo et al., 2005; Kudou et al., 2003; Manas et al., 2006; Ghochani et al., 2013; Ormus et al., 2015). In addition, Gemili et al. (2007) reported the thermal inactivation kinetics for partially purified lysozyme. In the latter study, $D_{90\,°C}$ and $Z_{80\text{-}90°C}$ for partially purified lysozymes from three batches were determined between 8.8- 21 min and 17.4-22.3 min, respectively. Contrary, the heat stability of commercial lysozyme was much higher than partially purified lysozyme as reported in the studies of Makki and Durance (1996) and Okanojo et al. (2005). Nevertheless, Makki and Durance (1996) found the $D_{95\,°C}$ of the enzyme dissolved in citrate-phosphate buffer at pH 6.24 as 83.2 min, while $D_{91\,°C}$ of the same enzyme was determined as 556 and 429 min at pH 4.2 and 5.2, respectively. These findings are similar to our data obtained at pH 4.0 and 5.5. Okanojo et al. (2005) and Ormus et al (2015) reported $D_{90°C}$ and $D_{95°C}$ of the commercial lysozyme around 30 min in Na-phosphate buffer (pH 6.5) and 63 min in an imidazole-acetate buffer (pH 7.0), respectively. This difference between D-values at 90 °C may be attributed to higher heat stability of lysozyme at acidic pH due to its high isoelectric point which is 11.35 (Makki and Durance, 1996), and the source of the lysozyme (Ormus et al., 2015). Moreover, our results are in accordance with the findings of Manas et al. (2006) who reported that lysozyme is very heat stable in Na-phosphate buffer (0.1 M) at pH 6.2 and temperatures between 90 °C and 130 °C. They showed that $D_{90°C}$ and $D_{95°C}$ of lysozyme are around 40

min and 13 min, respectively and $Z_{90\text{-}130°C}$ is 16 °C which is quite comparable to our findings of Z-value in the range of 85 °C and 95 °C.

The results obtained from thermal inactivation kinetics of lysozyme apparently showed that lysozyme has high thermal stability at pH and temperature ranges between 3.5 and 6.0 and 85 °C and 95 °C, respectively. Thereby, it has the potential to be added in food undergo thermal treatments.

Table 3. 2. D- and Z values of lysozyme at various pH

pH	D-value (min) 85 °C	90 °C	95 °C	$Z_{85\text{-}95}$ °C (°C)
3.5	1000	588.23	303.03	19.26
4.0	1250	714.28	357.14	18.38
4.5	1666.67	1000	476.19	18.38
5.5	1000	416.66	285.71	18.38
6.0	588.23	200	158.73	17.57

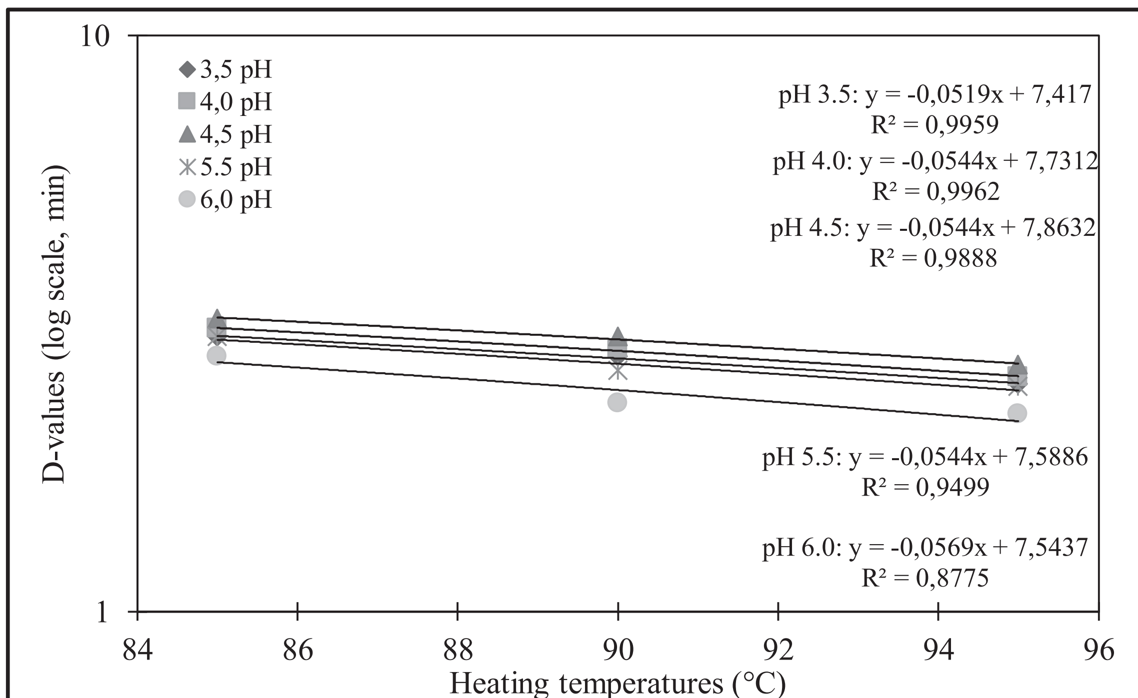

Figure 3. 7. Phantom thermal death time (TDT) curve for lysozyme

3.4. Conclusion

This is the most detailed study in the literature about the kinetic characterization of lysozyme at various pH and temperature conditions. Lysozyme showed very low enzymatic activity between pH 2.5 and 4.0 while its activity suddenly boosted and showed a dramatic increase by reaching the critical pH at 4.5 (≥10 folds) and further increasing the pH through pH 6.5 (≥15 folds). The detailed pH-activity and pH-stability profiles tested also showed that the enzymatic activity of lysozyme is extremely stable between pH 2.5 and 6.5. Thus, it appeared that the low enzymatic activity of lysozyme between pH 2.5 and 4.5 is related to reversible changes in its conformation that affect its active site accessibility to bacterial cell walls. In contrast, it seemed that the enzyme gained an optimal conformation at pH 4.5 and maintained this configuration through pH 6.5. The Ea showing the temperature dependency of lysozyme activity increased linearly between pH 2.5 and 4.5, but it remained constant between pH 4.5 and 6.5. Moreover, temperature-activity profiles of the enzyme showed that temperatures between 40 °C and 60 °C could be exploited to boost the enzymatic activity of lysozyme. The thermal inactivation kinetics of lysozyme clearly showed its high thermal stability at pH and temperature ranges between 3.5 and 6.0 and 85 °C and 95 °C, respectively. The D values of lysozyme showed that it had the highest thermal stability at pH 4.5. Moreover, z values of lysozyme indicated that its thermal stability between pH 3.5 and 4.5 was slightly higher than that between pH 4.5 and 6.0. This characterization study clearly showed that a preservation system depending on the enzymatic activity of lysozyme should have a pH range between 4.5 and 6.5. Moreover, it is also clear that mild heating temperatures between 40 °C and 60 °C and pasteurization temperatures between 85 °C and 95 °C could be combined with lysozyme to boost its potency and increase the effectiveness of preservation system's total lethality, respectively.

CHAPTER 4

LYSOZYME-NISIN SYNERGY IN COMBINATION WITH MILD HEATING AGAINST *LISTERIA INNOCUA*: TEST OF FINDINGS IN INOCULATED RAW-MILK

4.1. Introduction

In recent years, consumers are on the hunt for healthy food to live in a better and healthier way. Therefore, less processed, chemical additive free, fresh and raw foods have gained attraction by the consumers who take care of extreme attention to their diet. The 3% of the US population that doubled compared to the previous decade have been reported as raw milk drinkers in 2007. In addition, the consumers of raw milk have shifted from lower income earners and at much high school educated people to highly educated and high-income earners in recent years. Besides, 26% of the raw milk drinkers are children who are under the age of 18 and thus, they are probably not aware of the risk of raw milk consumption (Meunier-Goddik and Waite-Cusic, 2019).

On the other hand, the consumption of unpasteurized milk is considered as moderate risky in terms of listeriosis cases whose rate has been reported as 3.1 per year in the USA (Swaminathan and Garner-Smidt, 2007). Moreover, the presence of *L. monocytogenes* in raw milk and dairy products produced from raw milk such as cheese and butter has been confirmed in 11.7% of 300 samples collected from the northeastern part of Turkey (Aksoy et al., 2018). Besides, the second highest prevalence of *L. monocytogenes* among the food facilities in Europe and North America was reported as 23% in milk processing facilities (Warriner and Namvar, 2009). The first outbreak of *L. monocytogenes* associated with raw milk consumption was reported in 1979 (Bhatti et al., 2004), while the last one occurred in 2016 in the USA (CDC, 2016a). In addition, raw-milk cheese was also detected as a source of *L. monocytogenes* (CDC, 2017), whose threshold of the presence in certain RTE food products during their shelf life has been set at 100 CFU/g by EU Regulation 2073/2005 (Auvolat and Besse, 2016), whereas the zero-tolerance regulation was adopted by the FDA in the USA (Archer, 2018). As a result of

these outbreaks reported and risk of the bacterium in milk, several researches have been performed to control *L. monocytogenes* in milk in the presence of nisin (Jung et al., 1992; da Silva Malheiros et al., 2010) and in combination with moderate heat (Maisnier-Patin et al., 1995), a bacteriocin called as reuterin (Arqués et al., 2004) or poly-lactic acid incorporated into a coating material (Jin, 2010). Moreover, lysozyme has also been employed as an antimicrobial agent to control *L. monocytogenes* in milk exposed to high-pressure homogenization (Iucci et al., 2007) and in heat-treated milk (Kihm et al., 1994). However, the combinational use of lysozyme and nisin with the additional hurdle technology like PEF and HHP were studied before in milk. The combinational use of lysozyme (1638 IU/mL) and nisin (38 IU/mL) in skim milk with the presence of mild heating at 52 °C during the application of PEF achieved a 7D reduction in total viable counts (Smith et al., 2002). When these antimicrobials are employed in combination with HHP treatment at 600 MPa for 15 min, their effectiveness was reversely correlated with the milk fat content. In addition, partial inactivation of pressure resistant mutant *E. coli* strains and the complete destruction of parent strain of *E. coli* were demonstrated by the application of the combined use of lysozyme (400 µg/mL), nisin (400 IU/mL) and HHP (500 MPa at 50 ºC) (García-Graells et al., 1999). Even if the combinational use of lysozyme and nisin as antimicrobial agents was studied by many research groups (Chung and Hancock, 2000; Datta et al., 2008; Mangalassary et al., 2008; Antolinos et al., 2011; Takahashi et al., 2012), no study has been presented about the antilisterial activity of the combined use of lysozyme and nisin in milk with the presence of moderate heat application, alone in the current literature.

In this chapter, it was firstly aimed to show antilisterial activities of lysozyme and/or nisin under different treatment conditions that represent different processing temperatures ranges from 4 °C to 60 °C and pH (4.5 and 6.0). Secondly, raw milk which is a moderate risky food in terms of *L. monocytogenes* was tested as a model food against *L. innocua* NRRL B-33314 in the presence of lysozyme and/or nisin under moderate heating conditions. Thus, the risk of outbreaks originated from *L. monocytogenes* due to raw milk or raw-milk cheese consumption was aimed to be avoided.

4.1.1. The Importance of *Listeria monocytogenes* in Food Industry

Listeria monocytogenes is a Gram-positive, non-spore former, catalase positive, facultative anaerobe, a rod-shaped pathogenic bacterium that is widely distributed in the environment, especially in soils that are high in moisture (Buchanan et al., 2017). Therefore, the agricultural environment could harbor this pathogenic bacterium and the other contamination sources of *L. monocytogenes* could be water, fields, food processing facilities, plants, feed of livestock and the gastrointestinal tract of the animals as well (Lakicevic and Nastasijevic, 2017). Besides, 2-10% of human populations are carriers of this bacterium which is a temporary resident of the gastrointestinal tract of humans who have no symptoms of illness (Buchanan et al., 2017). *L. monocytogenes* causes mild illness such as gastrointestinal disorders and influenza-like symptoms in a healthy individual. However, the symptoms of invasive listeriosis resulted from *L. monocytogenes* infection are more severe like sepsis and meningitis in immunocompromised individuals like the elderly, pregnant women and those suffering from a chronic disease, and premature birth, stillbirth and abortion risk in pregnant women. This infection is associated with a high hospitalization rate and even death at a 20-30% range (Vijayakumar and Muriana, 2017). For these reasons, it has been considered as a notifiable disease in the USA since 2001 (Buchanan et al., 2017).

The growth temperatures of *L. monocytogenes* range between 2 and 52 °C, while it could survive at high salt concentration up to 14%, high water activity (0.92 and above), pH conditions between 4.3 and 9.4 and can withstand cooking temperatures below the 65 °C (Iannetti et al., 2016; Lakicevic and Nastasijevic, 2017). Even though, the last outbreaks of listeriosis were linked to mainly dairy products such as raw milk cheese (CDC, 2017), raw milk (CDC, 2016a), soft cheeses (CDC, 2015) and cheeses (CDC, 2014a; CDC 2013), the presence of *L. monocytogenes* in ready-to-eat (RTE) foods having prolonged storage period is the main concern because most of the listeriosis outbreaks are emanated from RTE foods such as meat products (Vijayakumar and Muriana, 2017), smoked salmon (Rørvik, 2000), packaged salads (CDC, 2016b) and prepackaged caramel apples (CDC, 2014b) due to the growth ability of *L. monocytogenes* at refrigeration temperatures (Kramarenko et al., 2016). Interestingly, cantaloupe melons caused 19 outbreaks and 37 deaths between 1983 and 2011 in the USA (Walsh et al., 2014) and the recent outbreak caused 7 deaths and 1 miscarriage has emerged in Australia (WHO,

2018b). Cantaloupes that are grown on the ground are likely to be exposed to food pathogens such as *L. monocytogenes* that is ubiquitously found in soil or the post-harvest washing in cantaloupes may favor the growth of the bacteria (Macarisin et al., 2017). In addition, the deadliest and largest outbreak of *L. monocytogenes* was recently reported in South Africa. This outbreak was confirmed with 978 individuals, of whom 674 of them had developed clinical symptoms of listeriosis which resulted in 183 deaths among 674 cases between 1st of January 2017 and 14th of March 2018. The source of this deadliest outbreak was identified as ready-to-eat processed meat product known as 'Polony' and moreover, the pathogen identified as *L. monocytogenes* sequence type 6 (ST6) was also isolated from the processing environment (WHO, 2018a). Therefore, to eliminate the risk of *L. monocytogenes* in food, FDA stated *L. monocytogenes* as an adulterant microorganism and thus, the presence or the probability of the presence of this organism in any food product or food contact surface forces the manufacturers a compulsory recall of the product (Warriner and Namwar, 2009). According to the rule of FDA (9 CFR, Part 430), *L. monocytogenes* can be controlled in three different ways in RTE meat and poultry products, as follows: (1) the combined application of post-lethality treatment and hurdle treatment that suppress the growth of the bacterium, (2) the application of *Listeria* sanitation program along with post-lethality treatment or a hurdle treatment and (3) *Listeria* sanitation program is efficiently applied alone right before the test of end products (Warriner and Namwar, 2009).

4.2. Experimental study

In the following sections, both materials and methods performed for antilisterial activity determination of lysozyme and/or nisin are given in detail.

4.2.1. Materials

Chicken egg white lysozyme (L6876) and nisin from *Lactococcus lactis* (N5764) were purchased from Sigma-Aldrich Chem. Co. (St. Louis, Mo., USA). To evaluate the antilisterial effect of lysozyme and nisin, *Listeria innocua* NRRL B-33314 was used as a test bacterium that was provided from the United States Department of Agriculture, Microbial Genomics and Bioprocessing Research Unit, Peoira, Illinois. Microbiological

enumerations were performed using Oxford Listeria selective agar (Merck, 107004) supplemented with Oxford Listeria selective supplement (Merck, 107006) and nutrient agar that was prepared by mixing nutrient broth (Merck) and agar for microbiology use (Merck). Raw milk (Efeler Çiftliği, Söke, Aydın, 3.3-3.4% fat) used as a test food was purchased from the market in İzmir.

4.2.2. Methods

The methods given below were performed to the monitor antimicrobial activity of the antimicrobial agents against *L. innocua* NRRL B-33314 in both liquid media having pH 4.5 or pH 6.0 and in raw milk.

4.2.2.1. Antilisterial Activity Determination of LYS and/or NIS in a Liquid Medium

The pH values tested in this study were selected as 4.5 and 6.0 to represent critical pH values in terms of *Cl. botulinum* and meat, fish or dairy products, respectively. Antilisterial activities of lysozyme and/or nisin were determined by the dynamic flask method. Initially, the bacterial inoculum was grown at 4 °C for 24 h subsequent to grown at 37°C for 24 h in order to adapt refrigeration temperature. This inoculum grown at 2-step yields 10^7 CFU/mL concentration in nutrient broth and it was used as a stock solution of inoculum. The initial concentration of culture was adjusted to 10^3 CFU/mL by diluting the stock solution of the inoculum. The serial stock solutions of lysozyme and/or nisin were prepared separately in 0.05M citrate-phosphate buffer at pH 4.5 or 0.05M Na-phosphate buffer pH 6.0 at the concentration ranging between 9.76 µg/mL and 156.25 µg/mL which were determined by a preliminary experiment conducted in a wider range of antimicrobials at 4.88 and 2000 µg/mL. 5mL of culture, 40 µL of nutrient broth at desired pH and 5mL of antimicrobial solution were distributed into the sterile capped Erlenmeyer flask. The samples were grouped as lysozyme (LYS), nisin (NIS), lysozyme-nisin (LYS:NIS) and control group; they were stored at 4 °C while shaking at 80 rpm. The samples including the only inoculum without an antimicrobial solution were assumed as the control group. The change in *L. innocua* count throughout storage at 4 °C was

controlled by the spread plate method on nutrient agar. The colonies were enumerated after 24 h incubation at 37 °C. This experiment was conducted in duplicate and the enumeration for each sample group was carried out in triplicate.

4.2.2.2. Determination the Effect of Mild Heating and pH on Antilisterial Synergy between Lysozyme and Nisin

This analysis was performed at both 50 and 60 °C with regard to the preliminary test conducted at 40, 50 and 60 °C. Since 40 °C is quite close to the optimum growth temperature of the bacterium, it was eliminated for further analyses. This analysisis was conducted as follows: The stock solution of inoculum (10^8 CFU/mL) was grown at 37 °C for 24 h. The stock solutions of lysozyme (5000 and 10000 µg/mL), nisin (156.25 µg/mL and 312.5 µg/mL) were prepared in either 0.05 M citrate-phosphate buffer at pH 4.5 or 0.05M sodium-phosphate buffer at pH 6.0. After that, 4 mL nutrient broth (at pH 4.5 or 6.0) and 0.5 mL of the stock solution of lysozyme or nisin (at pH 4.5 or 6.0) were added into sterile capped tubes which were then placed in a water bath and their temperature was checked by a thermometer before the analysis. For the mixture of antimicrobials, 0.25 mL of each stock solution (lysozyme at 10000 µg/mL and nisin at 312.5 µg/mL) were added into the 4 mL nutrient broth. After tubes reached to determined temperature, 0.5 mL of stock solution of inoculum was added into tubes and vortexed thoroughly. Therefore, the final concentration of lysozyme and nisin in the samples were adjusted to 500 and 15.625 µg/mL, respectively. Then, the tubes were stored for the determined period at each incubation temperature that were 50 °C (0, 7.5, 15, 30, 45 min) and 60 °C (0, 2.5, 5, 10, 15 min). Each time-concentration sample was prepared in duplicated tubes. The enumeration of the bacteria was performed as described in Section 4.2.2.1.

4.2.2.3. Determination of Soluble and Bound Fractions of Lysozyme or Nisin in Milk

To determine the % of soluble and bound insoluble lysozyme or nisin in milk, firstly lysozyme (500 µg/mL) or nisin (15.625 µg/mL) was added in raw milk and its curd, and whey was obtained through acidic coagulation. The acidification was conducted

by bringing the pH of milk below the isoelectric point of casein (< pH 4.6) using a 25% lactic acid solution. To separate the whey solution and fat layer from the curd, the coagulated milk sample was centrifuged at 15000 g and 4 °C for 15 min. After the fat layer was removed, the whey solution was collected in a new tube and recentrifuged at 15000 g and 4 °C for 10 min and filtered through cheesecloth. The pH of whey samples was adjusted to 6.5 using 1N NaOH to reach the same pH as raw milk. Secondly, lysozyme (500 µg/mL) or nisin (15.625 µg/mL) was added in a previously separated whey solution and then, pH of the samples was adjusted to 6.5 using 1N NaOH (this sample represents 100% passage of lysozyme and nisin into whey).

The % of soluble lysozyme or nisin and the % of bound lysozyme or nisin were calculated using the following formulas:

$$\text{\% of soluble LYS or NIS} = \frac{\text{Amount added into milk}}{\text{Amount determined in whey}} \times 100$$

$$\text{\% of bound LYS or NIS} = 100 - \text{\% soluble LYS or NIS}$$

All the measurements were conducted using raw unheated milk and heated milk at 50 °C for 45 min after the addition of lysozyme or nisin.

The concentration of lysozyme was determined from its enzymatic activities in whey solutions as described in section 3.2.2.1. The NIS concentration was determined by the agar diffusion method (Teerakarn *et al*., 2002) using *Lactobacillus plantarum* NRRL-B4496 as a test microorganism. MRS test agar used in this method was prepared and the analysis was performed as given by Sozbilen et al. (2018). Briefly, 50 µL of each sample was placed into the wells in MRS test agar and the petri dishes were incubated in an anaerobic jar with Aerocult C (Merck, Darmstadt, Germany) at 37 °C for 16–18 h to grow the test bacteria and observe formation of visible clear zones formed around wells by nisin. The diameter of each well and clear zone were measured from three different points using a digital caliper (Mitutoyo IP67, Japan). The standard curve was obtained by plotting the logarithm of NIS concentration *vs*. diameter of the clear zones. The retention of nisin in either whey or milk samples were expressed % retention. Samples were triplicated in each treatment group and at least three measurements were performed for each sample.

4.2.2.4. Antilisterial Activities of LYS and /or NIS in Raw Milk Exposed to Mild Heating

This analysis was performed at the same concentration and temperature conditions as described in section 4.2.2.2. But, raw milk (pH 6.52) was used as a growth medium instead of nutrient broth. No antimicrobial added raw milk inoculated with *L. innocua* at 8 log CFU/mL concentration was assumed as the control group. Milk samples were serially diluted in 0.1% peptone water and plated by spread plated method on Oxford Listeria agar supplemented with Oxford Listeria Selective supplement. Samples were taken at the 0th, 15th, 30th and 45th, and 0th, 5th, 10th and 15th min of incubation in the water bath at 50 °C and 60 °C, respectively. 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 of raw milk. This experiment was conducted in duplicate and the enumeration for each sample group was carried out in triplicate.

4.2.2.5. Statistical Analyses

The Analysis of Variances (ANOVA) was applied to determine the effects of different treatments and concentrations of antimicrobial agents tested by using MINITAB release 16 (Minitab Inc., State College, Pa., U.S.A.). Moreover, the Fisher test was used for the comparison of means, with significance assigned at $P < 0.05$.

4.3. Results and Discussion

The detailed results and discussion will be given in the following sections.

4.3.1. Antilisterial Activity of LYS and/or NIS Based on the pH

The used concentrations of lysozyme and nisin were selected to not preclude the observation of the effect of the antimicrobials. The efficient concentrations of lysozyme

and/or nisin against *L. innocua* were determined in broth medium at 4 °C and at two different pH conditions that mimic the targeted food pH's that are pH 4.5 and pH 6.0. The antilisterial activity of lysozyme and/or nisin at these pH conditions were given in Table 4.1, Table 4.2 and Figure 4.1. The growth of *L. innocua* exposing to variable concentrations of lysozyme and/or nisin was affected by the pH at 4 °C.

At pH 4.5, both NIS and LYS:NIS at a concentration of 15.62 µg/mL resulted in more than 2.88 D reduction that was the highest within 12-day storage. These highest reductions were followed by LYS:NIS at 7.81 µg/mL and NIS at 7.81 µg/mL with 2.77 D and 2.48 D reductions, respectively. The gradual decreases in the bacterial counts throughout refrigerated storage were achieved at all NIS and LYS:NIS concentrations tested (1.95 to 15.62 µg/mL). Besides, the bacterium was antagonistically affected by the mixture of LYS:NIS a few days earlier than NIS. However, LYS did not result in a remarkable reduction ($P > 0.05$) in the count of the bacteria during the storage (Fig. 4.1A to 4.1E) due to the slightly acidic pH of the medium. Moreover, the bacterial count in the control group was monitored as quite stable during 12-day storage at pH 4.5 due to prolonged lag phase that was also reported by Gallo et al. (2007) who showed the rapid growth of *L. innocua* at pH 6.0, whereas the growth occurred after a lag phase at pH 5.5. Although LYS:NIS did not exhibit a synergistic effect on *L.innocua* at pH 4.5, it was observed as more promising than using LYS alone.

At pH 6.0, the highest two concentrations of LYS:NIS yielded the highest inhibition levels as expected and achieved a synergistic inhibition on *L. innocua* more than 5.95 D reduction within 12-day storage. Furthermore, LYS:NIS at 3.90 µg/mL resulted in a 5.69 D reduction. But, its antilisterial effect was as significant as LYS at 7.81 or 15.62 µg/mL. On the other hand, the inhibition effect of nisin was monitored at the early stages of storage and continued until 7th days of storage for the highest two concentrations tested. This rapid antimicrobial effect of nisin disappeared after the 7th day and the count increased gradually. However, synergistic inhibition of *L. innocua* by highest two concentration of LYS:NIS proceeded throughout the storage.

The statistically significant interaction between lysozyme and pH was reported by Johansen et al. (1994) who worked with relatively high concentrations of lysozyme at 5000 IU/mL and 10000 IU/mL at pH range of 5.5 to 7.2 and at 5 °C, and showed that lowering pH from 7.2 to 5.5 increased lag phases of *L. monocytogenes* cells. This finding of Johansen et al. (1994) was also in accordance with our results where increased lag phase in control and LYS groups were apparent at pH 4.5. Unlike the growth trend at pH

4.5, nisin containing sample groups showed a rapid inhibition within two days of storage, while it was followed by regrowth of *L. innocua* cells especially starting from the 7th day of storage at pH 6.0. This regrowth pattern was also monitored by Schillinger et al. (2001), Harris et al. (1991) and Gallo et al. (2007). In the former study, the researchers monitored the short-term inhibition of *L. monocytogenes* Scott A cell by nisin (3000 IU/mL) in tofu and claimed that initial inhibition may have been due to organic acid produced by the bacteriocin-producing strains in tofu. However, the latter two researchers reported that the survivor cells of *L. monocytogenes* have gain acquired resistance to nisin due to membraneous changes in their fatty acid and phospholipid compositions of the cell wall.

The results herein also proved that the antilisterial synergy is much stronger when the pH of the medium is close to neutrality (pH 6.0) instead of slightly acidic (pH 4.5) that caused suppression of lysozyme enzymatic activity. Besides, the antilisterial activity of nisin was much more effective in the earlier stages of storage than those of lysozyme. The activity loss of nisin is likely related to the acquired resistance of the bacterium. In contrast, lysozyme enables to exert antimicrobial activity at the later stages and delays regeneration of listerial growth.

4.3.2. Antilisterial Activity of LYS and/or NIS Based on the pH during Mild Heating

The growth profile of *L. innocua* at pH 4.5 (Figure 4.2A and Figure 4.3A) and pH 6.0 (Figure 4.2B and Figure 4.3B) conducted in the presence of LYS and/or NIS at both 50 °C and 60 °C are presented in Table 4.3.

According to the results at 50°C, a more rapid antimicrobial activity was observed at 7.5 min and pH 6.0 in NIS and LYS:NIS with 4.08 D and 5.91 D reductions, respectively, compared to NIS (2.32 D) and LYS:NIS (5.77 D) at pH 4.5. Moreover, these reductions were significantly higher ($P < 0.05$) compared to both control and LYS at both pH values, whereas LYS containing samples at both pH did not cause a remarkable ($P > 0.05$) antimicrobial activity compared to control group at 7.5 min. Similarly, the combined effect of heat and nisin against *L. monocytogenes* has been studied by Budu-Amoako et al. (1999) who demonstrated that 5 min heat processing of canned cold-pack lobster meat (≈ pH 8) at 60 °C or 65 °C with the addition of nisin at the concentration of 25 µg/g of can content yielded a 3D- to 5D reduction in different size of cans. On the

Table 4.1. Antilisterial activities of LYS and/or NIS against *L. innocua* at pH 4.5 & 4 °C

Antimicrobials	Concentration µg/mL	*L. innocua* (Log CFU/mL)						
		Day 0	Day 1	Day 2	Day 5	Day 7	Day 9	Day 12
Control	-	$3.65\pm0.10^{ab,A}$	$3.67\pm0.09^{a,AB}$	$3.68\pm0.09^{a,AB}$	$3.70\pm0.13^{a,A}$	$3.69\pm0.11^{a,A}$	$3.70\pm0.12^{a,A}$	$3.57\pm0.14^{b,A}$
LYS	0.976	$3.66\pm0.09^{ab,A}$	$3.61\pm0.17^{b,ABC}$	$3.72\pm0.05^{ab,A}$	$3.76\pm0.14^{a,A}$	$3.68\pm0.09^{ab,A}$	$3.67\pm0.08^{ab,A}$	$3.66\pm0.08^{ab,A}$
	1.953	$3.70\pm0.05^{a,A}$	$3.73\pm0.04^{a,A}$	$3.72\pm0.01^{a,A,AB}$	$3.74\pm0.05^{a,A}$	$3.71\pm0.07^{a,A}$	$3.70\pm0.04^{a,A}$	$3.67\pm0.04^{a,A}$
	3.906	$3.67\pm0.15^{a,A}$	$3.65\pm0.06^{a,ABC}$	$3.69\pm0.04^{a,ABC}$	$3.71\pm0.03^{a,A}$	$3.70\pm0.05^{a,A}$	$3.67\pm0.12^{a,A}$	$3.64\pm0.16^{a,A}$
	7.812	$3.58\pm0.06^{ab,AB}$	$3.60\pm0.05^{ab,ABC}$	$3.56\pm0.11^{ab,BCDE}$	$3.62\pm0.12^{a,AB}$	$3.64\pm0.08^{a,AB}$	$3.46\pm0.23^{b,BC}$	$3.49\pm0.08^{ab,AB}$
	15.625	$3.62\pm0.13^{a,A}$	$3.55\pm0.11^{a,ABC}$	$3.54\pm0.15^{a,CDE}$	$3.61\pm0.03^{a,ABC}$	$3.58\pm0.03^{a,ABC}$	$3.56\pm0.05^{a,AB}$	$3.53\pm0.10^{a,AB}$
NIS	0.976	$3.70\pm0.05^{a,A}$	$3.62\pm0.06^{ab,ABC}$	$3.51\pm0.08^{abc,DE}$	$3.36\pm0.15^{cd,DE}$	$3.46\pm0.15^{bc,CD}$	$3.26\pm0.20^{d,D}$	$3.21\pm0.25^{d,C}$
	1.953	$3.63\pm0.05^{a,A}$	$3.61\pm0.09^{ab,ABC}$	$3.47\pm0.02^{abc,EF}$	$3.43\pm0.02^{bcd,CDE}$	$3.37\pm0.14^{cd,DE}$	$3.30\pm0.20^{cd,CD}$	$3.28\pm0.18^{d,C}$
	3.906	$3.68\pm0.08^{a,A}$	$3.48\pm0.04^{b,C}$	$3.34\pm0.14^{bc,F}$	$3.26\pm0.11^{c,E}$	$3.23\pm0.11^{cd,E}$	$3.07\pm0.14^{de,E}$	$2.92\pm0.12^{e,D}$
	7.812	$3.47\pm0.10^{a,BC}$	$1.92\pm0.03^{b,E}$	$1.73\pm0.05^{bc,H}$	$1.69\pm0.12^{bc,G}$	$1.54\pm0.08^{cd,G}$	$1.39\pm0.17^{d,G}$	$1.09\pm0.22^{e,F}$
	15.625	$3.38\pm0.20^{a,C}$	$1.23\pm0.33^{b,F}$	$1.15\pm0.21^{b,I}$	$1.00\pm0.42^{b,HI}$	<0.69	<0.69	<0.69
LYS:NIS	0.97:0.97	$3.70\pm0.10^{a,A}$	$3.63\pm0.12^{abc,ABC}$	$3.66\pm0.06^{ab,ABCD}$	$3.61\pm0.07^{abc,ABC}$	$3.52\pm0.02^{c,BCD}$	$3.54\pm0.04^{c,AB}$	$3.56\pm0.05^{bc,AB}$
	1.95:1.95	$3.66\pm0.12^{a,A}$	$3.53\pm0.07^{b,BC}$	$3.49\pm0.04^{bc,DEF}$	$3.46\pm0.09^{bc,BCD}$	$3.45\pm0.12^{bc,CD}$	$3.42\pm0.01^{bc,BCD}$	$3.38\pm0.04^{c,BC}$
	3.90:3.90	$3.57\pm0.10^{a,AB}$	$2.44\pm0.30^{b,D}$	$2.35\pm0.10^{b,G}$	$2.09\pm0.13^{c,F}$	$2.02\pm0.05^{cd,F}$	$1.88\pm0.07^{cd,F}$	$1.76\pm0.27^{d,E}$
	7.81:7.81	$3.47\pm0.05^{a,BC}$	$2.13\pm0.15^{b,E}$	$1.63\pm0.35^{c,H}$	$1.08\pm0.12^{d,H}$	$1.00\pm0.20^{d,H}$	$0.92\pm0.21^{d,H}$	$0.80\pm0.28^{d,G}$
	15.625:15.625	$2.17\pm0.09^{a,D}$	$1.05\pm0.31^{b,F}$	$0.80\pm0.21^{b,J}$	$0.81\pm0.23^{b,I}$	<0.69	<0.69	<0.69

a-e and A-J values within each row and column followed by the same letter are not significantly different ($P > 0.05$), respectively.

Table 4.2. Antilisterial activities of LYS and/or NIS against *L. innocua* at pH 6.0 & 4 °C

Antimicrobials	Concentration µg/mL	*L. innocua* (Log CFU/mL)						
		Day 0	Day 1	Day 2	Day 5	Day 7	Day 9	Day 12
Control	-	$3.70\pm0.07^{d,A}$	$3.81\pm0.05^{d,A}$	$3.90\pm0.08^{d,A}$	$4.47\pm0.26^{c,A}$	$4.94\pm0.53^{c,A}$	$5.90\pm0.86^{b,A}$	$6.64\pm0.96^{a,A}$
LYS	0.976	$3.97\pm0.19^{a,A}$	$3.82\pm0.12^{ab,A}$	$3.69\pm0.09^{bc,B}$	$3.75\pm0.11^{b,B}$	$3.57\pm0.07^{c,C}$	$3.39\pm0.09^{d,C}$	$3.08\pm0.03^{e,DEF}$
	1.953	$3.85\pm0.09^{a,A}$	$3.69\pm0.12^{ab,AB}$	$3.69\pm0.10^{ab,B}$	$3.58\pm0.12^{ab,BC}$	$3.47\pm0.12^{bc,C}$	$3.28\pm0.21^{cd,C}$	$3.13\pm0.32^{d,DE}$
	3.906	$3.76\pm0.10^{a,A}$	$3.69\pm0.07^{a,AB}$	$3.71\pm0.12^{a,B}$	$3.23\pm0.08^{b,DE}$	$2.93\pm0.13^{c,D}$	$2.59\pm0.26^{d,DE}$	$2.38\pm0.24^{d,FGH}$
	7.812	$3.80\pm0.03^{a,A}$	$3.77\pm0.12^{a,AB}$	$3.63\pm0.07^{a,B}$	$2.94\pm0.18^{b,E}$	$2.35\pm0.06^{c,E}$	$2.16\pm0.23^{c,EF}$	$1.88\pm0.28^{d,GHI}$
	15.625	$3.67\pm0.18^{a,A}$	$3.66\pm0.13^{a,B}$	$3.74\pm0.12^{a,AB}$	$3.17\pm0.16^{b,DE}$	$2.65\pm0.14^{c,DE}$	$2.14\pm0.32^{d,EF}$	$1.77\pm0.18^{e,HI}$
NIS	0.976	$3.99\pm0.03^{c,A}$	$3.85\pm0.23^{c,A}$	$3.76\pm0.23^{c,AB}$	$4.45\pm0.32^{bc,A}$	$4.86\pm0.45^{b,A}$	$5.08\pm0.50^{b,B}$	$6.21\pm0.59^{a,AB}$
	1.953	$3.89\pm0.04^{c,A}$	$2.94\pm0.13^{de,D}$	$2.81\pm0.14^{e,C}$	$3.16\pm0.07^{d,DE}$	$3.75\pm0.26^{c,C}$	$4.39\pm0.13^{b,B}$	$5.04\pm0.14^{a,C}$
	3.906	$3.67\pm0.26^{d,A}$	$2.59\pm0.30^{e,E}$	$2.56\pm0.14^{e,D}$	$3.46\pm0.16^{d,BCD}$	$4.34\pm0.23^{c,B}$	$4.82\pm0.21^{b,B}$	$5.96\pm0.21^{a,B}$
	7.812	$1.65\pm0.46^{b,C}$	<0.69	<0.69	$0.77\pm0.0^{b,G}$	$0.99\pm0.47^{b,F}$	$1.64\pm0.51^{b,F}$	$2.64\pm0.48^{a,EFG}$
	15.625	$2.58\pm0.43^{ab,B}$	<0.69	<0.69	<0.69	$0.87\pm0.38^{c,F}$	$1.85\pm0.17^{b,EF}$	$3.20\pm0.08^{a,DE}$
LYS:NIS	0.97:0.97	$3.71\pm0.21^{a,A}$	$2.25\pm0.07^{de,F}$	$2.18\pm0.12^{e,E}$	$2.41\pm0.13^{d,F}$	$2.81\pm0.27^{c,D}$	$2.96\pm0.08^{c,CD}$	$3.42\pm0.07^{b,D}$
	1.95:1.95	$3.83\pm0.12^{a,A}$	$3.17\pm0.09^{cd,C}$	$2.99\pm0.23^{d,C}$	$3.35\pm0.29^{bc,CD}$	$3.49\pm0.22^{b,C}$	$3.49\pm0.09^{b,C}$	$3.54\pm0.09^{b,D}$
	3.90:3.90	$2.32\pm0.64^{a,B}$	$0.80\pm0.17^{b,G}$	<0.69	<0.69	<0.69	<0.69	$0.95\pm0.06^{b,I}$
	7.81:7.81	$2.54\pm0.29^{a,B}$	<0.69	<0.69	<0.69	<0.69	<0.69	<0.69
	15.625:15.625	$1.32\pm0.39^{a,C}$	<0.69	<0.69	<0.69	<0.69	<0.69	<0.69

a-e and A-I values within each row and column followed by the same letter are not significantly different ($P > 0.05$), respectively.

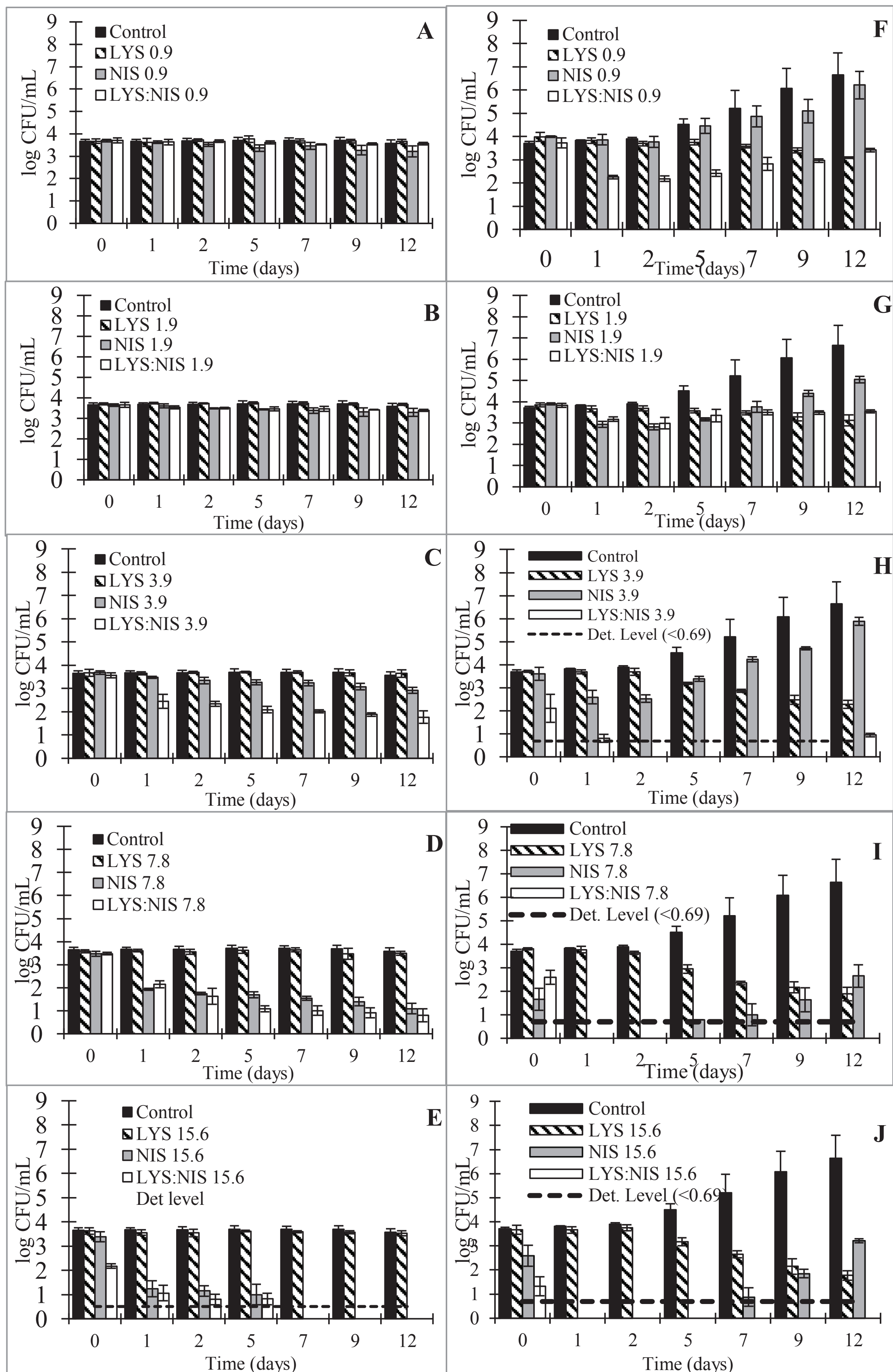

Figure 4. 1. Antimicrobial activities of LYS and/or NIS against *L. innocua* at pH 4.5 (A, B, C, D, E) and at pH 6.0 (F, G, H, I, J) & 4 °C

other hand, the synergistic effect of LYS:NIS was monitored in the early minutes of incubation, notably at 7.5 and 15 min of incubation at both pH values.

This synergy can be related to enhanced membrane damage, cell lysis, or destruction of energy-dependent processes that enable to repair the damage occurred in the cell. Furthermore, the heat applied in addition to lysozyme and nisin mixture may also affect the alteration in membrane permeability of the bacterial cell (Mangalassary et al., 2007). The combined application of mild heating at 50 °C, lysozyme, and nisin substantially reduced the count with a 6.86 D and 6.18 D at pH 4.5 and 6.0 at the end of 45-min incubation, respectively. On the other hand, lysozyme started to affect the bacteria from 15th min at which time LYS resulted in 2.5 D more reduction at pH 4.5 compared to those at pH 6.0. Even if lysozyme is more active close to neutral pH, the presence of both mild heating and slightly acidic medium (pH=4.5) created a hurdle effect. In addition, the enzymatic activity of lysozyme was monitored almost two-fold higher at pH 6.0 than those at pH 4.5 (Fig. 3.3), the conflicting situation observed at 50 °C might be due to the difference of substrate which is *L. innocua* in this case, whereas in the other case the substrate was *Micrococcus lysodeikticus* that is used as a substrate to determine standardized enzymatic activity of lysozyme obtained from different sources and it is notably sensitive microorganism to lysozyme (Hanušová et al., 2013). It was also reported that HEWL enabled to create a reduction greater than 5.2 D in the count of *M. lysodeikticus*, while this reduction remained at 2.6 D in the count of *L. innocua* at pH 7.0 (Nakimbugwe et al., 2006b).

The second part of this study was conducted at 60 °C using the same concentrations of antimicrobials at pH 4.5 (Fig. 4.3A) and pH 6.0 (Fig. 4.3B). According to the results obtained at 60 °C, dramatic decreases in the counts were monitored as of 2.5th min of incubation in all sample groups. This may be attributed to higher temperature incubation. Unlike the synergy observed at 50 °C, a sudden synergistic reduction occurred at pH 6.0 at which synergy is more apparent compared to those at pH 4.5. Moreover, nisin containing sample is significantly different from lysozyme at 2.5th min and it achieved to diminish the count below the detection limit (1 log CFU/mL) as of 5th min of incubation at pH 6.0. In addition to these results, lysozyme at pH 6.0 was found to be as efficient to inhibit the bacterium as nisin and lysozyme at pH 4.5.

As a result, mild heat treatment at 50 °C boosted the synergy between lysozyme and nisin against *L. innocua* and created a potential antilisterial hurdle.

Table 4.3. Change in *L. innocua* count (log CFU/mL) at different pH and temperature conditions

Antimicrobial concentrations µg/mL				***L. innocua* count**				
LYS	**NIS**	**pH**	**T (°C)**	**Time (min)**				
				0	**7.5**	**15**	**30**	**45**
-	-	4.5	50	7.86 ± 0.09 $^{a,A,A'}$	7.71 ± 0.12 $^{a,A,A'}$	7.62 ± 0.14 $^{a,A,A'}$	7.24 ± 0.30 $^{b,A,A'}$	6.21 ± 0.55 $^{c,A,B'}$
500	-	4.5	50	7.86 ± 0.09 $^{a,A,A'}$	7.33 ± 0.43 $^{b,A,A'}$	5.36 ± 0.88 $^{c,B,C'}$	3.52 ± 0.29 $^{d,B,C'}$	3.52 ± 0.25 $^{d,B,CD'}$
-	15.625	4.5	50	7.86 ± 0.09 $^{a,A,A'}$	5.54 ± 0.48 $^{b,B,B'}$	5.01 ± 0.53 $^{b,B,C'}$	3.33 ± 1.28 $^{c,B,C'}$	2.88 ± 0.70 $^{c,B,D'}$
500	15.625	4.5	50	7.86 ± 0.09 $^{a,A,A'}$	2.09 ± 0.59 $^{b,C,D'}$	1.23 ± 0.25 $^{c,C,E'}$	1.15 ± 0.16 $^{bc,C,D'}$	1.00 ± 0.00 $^{c,C,E'}$
-	-	6.0	50	7.74 ± 0.09 $^{a,A,B'}$	7.72 ± 0.14$^{a,A,A'}$	7.75 ± 0.13$^{a,A,A'}$	7.73 ± 0.13 $^{a,A,A'}$	7.69 ± 0.17 $^{a,A,A'}$
500	-	6.0	50	7.74 ± 0.09 $^{a,A,B'}$	7.47 ± 0.39 $^{ab,A,A'}$	6.46 ± 1.01 $^{b,B,B'}$	4.65 ± 1.40 $^{c,B,B'}$	3.65 ± 1.36 $^{c,B,C'}$
-	15.625	6.0	50	7.74 ± 0.09 $^{a,A,B'}$	3.66 ± 1.33 $^{b,B,C'}$	3.57 ± 0.98 $^{b,C,D'}$	3.55 ± 1.18 $^{b,C,C'}$	3.83 ± 0.71 $^{b,B,C'}$
500	15.625	6.0	50	7.74 ± 0.09 $^{a,A,B'}$	1.83 ± 1.22 $^{b,C,D'}$	1.84 ± 1.23 $^{b,D,E'}$	1.59 ± 1.00 $^{b,D,D'}$	1.56 ± 0.96 $^{b,C,E'}$
				0	**2.5**	**5**	**10**	**15**
-	-	4.5	60	7.69 ± 0.12 $^{a,A,A'}$	4.52 ± 0.62 $^{b,A,A'}$	4.38 ± 0.44 $^{b,A,A'}$	3.74 ± 0.55 $^{c,A,A'}$	2.17 ± 0.37$^{d,A,B'}$
500	-	4.5	60	7.69 ± 0.12 $^{a,A,A'}$	3.97± 0.49$^{b,AB,AB'}$	2.84 ± 0.26 $^{c,B,B'}$	2.35 ± 0.62 $^{d,B,BC'}$	1.68 ±0.54$^{a,AB,B'}$
-	15.625	4.5	60	7.69 ± 0.12 $^{a,A,A'}$	3.33 ± 0.22 $^{b,B,B'}$	2.72 ± 0.06 $^{c,B,B'}$	1.52 ± 0.73 $^{d,B,C'}$	1.15 ± 0.21 $^{d,B,B'}$
500	15.625	4.5	60	7.69 ± 0.12 $^{a,A,A'}$	2.03 ± 0.39 $^{b,C,C'}$	1.00 ± 0.00 $^{c,C,C'}$	<1	<1
-	-	6.0	60	7.55 ± 0.14 $^{a,A,B'}$	4.08 ± 1.04 $^{b,A,AB'}$	3.71 ± 0.88 $^{b,A,A'}$	3.28 ± 1.02 $^{b,A,AB'}$	3.44 ± 0.93$^{b,A,A'}$
500	-	6.0	60	7.55 ± 0.14 $^{a,A,B'}$	3.47 ± 0.70 $^{b,A,B'}$	2.44 ± 1.10 $^{cd,B,B'}$	2.81 ± 1.23 $^{bc,A,ABC'}$	1.67 ± 0.70 $^{d,B,B'}$
-	15.625	6.0	60	7.55 ± 0.14 $^{a,A,B'}$	2.15 ± 0.78 $^{b,B,C'}$	< 1	<1	< 1
500	15.625	6.0	60	7.55 ± 0.14 $^{a,A,B'}$	< 1	< 1	< 1	< 1

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

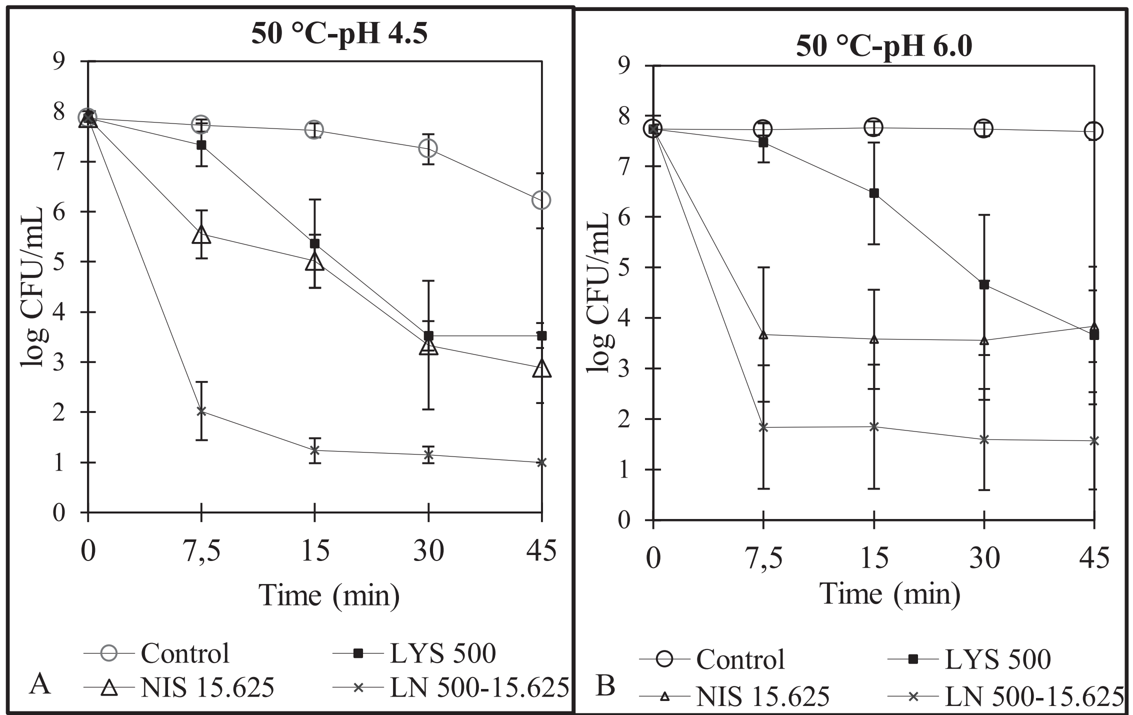

Figure 4. 2. Growth profile of *L. innocua* at 50 °C- pH 4.5 (A) and 50 °C pH 6.0 (B)

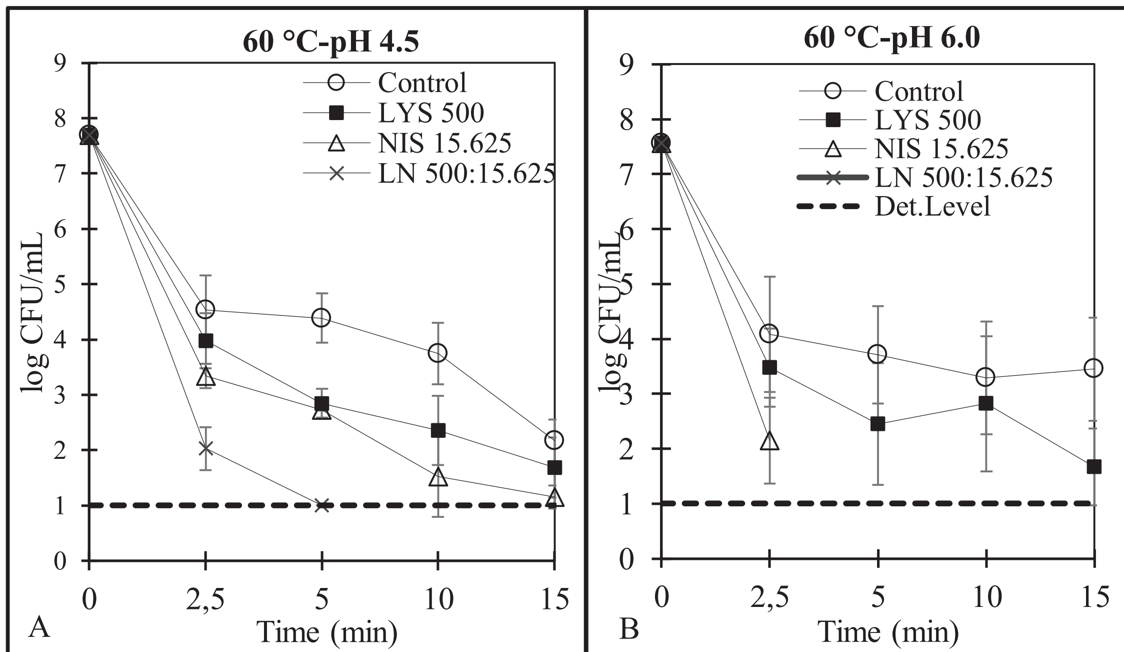

Figure 4. 3. Growth profile of *L. innocua* at 60 °C-pH 4.5 (A) and 60 °C-pH 6.0 (B).

4.3.3. Antilisterial Activity of LYS and/or NIS in Heat-Treated Raw Milk

The antilisterial behavior of lysozyme and/or nisin directly added in raw milk containing 3.3 - 3.4% milk fat was monitored at both 50 °C (Figure 4.4 and Table 4.4) and 60 °C (Figure 4.5 and Table 4.5).

Lysozyme did not show any antimicrobial activity compared to the control group ($P > 0.05$). This finding may be related to the gain of resistance of the bacterium against lysozyme by milk minerals (potassium, calcium, zinc, magnesium, etc.) that could stabilize the cell of the bacteria (Kihm et al., 1994). But, NIS and LYS:NIS sample groups had substantial inhibitory effects on *L. innocua* in milk. Their suppressing effects progressively increased as the incubation period was prolonged to 45 min. However, LYS:NIS group had remarkable inhibitory activity with 3.16 D, 4.74 D and 5.45 D reductions in the count at the 15th, 30th and 45th min of incubation, respectively. Whereas, the antimicrobial activity of NIS remained at a lower level compared to those in LYS:NIS ($P < 0.05$). Similarly, the inhibitory action of nisin against *L. monocytogenes* in milk stored both at 4 °C and 8 °C during 12 days was also reported by Arqués et al. (2004). Moreover, the antimicrobial activity of nisin is enhanced by moderate heat treatment against *L. monocytogenes* in milk (Maisnier-Patin et al., 1995). According to this research, 3 D reductions in the count of *L. monocytogenes* in milk were accomplished with heat treatment at 54 °C for 16 min and in the presence of 25 IU/mL nisin. This antimicrobial activity of nisin with a mild heat heating can be attributed to both rapid efflux of cytoplasmic constituents like ATP, amino acids or potassium due to membrane damage and the change in cell wall structure by a heat treatment that results in the augmentation of nisin adsorption (Maisnier-Patin et al., 1995).

According to the results obtained at 60 °C, the initial bacterial population (7.81 log CFU/mL) dramatically decreased to the range of 2.71 and 3.42 log CFU/mL in all sample groups by the effect of temperature in 5 min. Thus, no significant difference was observed between the counts of the samples at 5th min ($P > 0.05$). However, the count of LYS:NIS sample varied considerably from the control group whose count was 0.87 log units more than those of LYS:NIS group at 10th min of incubation. Moreover, NIS and LYS:NIS counts decreased below to the detection limit at 15th min, while the count of LYS sample was significantly lower compared to the control group.

Contrary to results monitored in broth media, nisin exhibited its antimicrobial effect on the bacterium later in raw milk. This can be attributed to the fat content of milk. Because, the interference of nisin on target cells can be hindered by adsorption of nisin on fat globules in milk (Zapico et al., 1999). Therefore, the composition of food is substantially effective in the activity of nisin (Bhatti et al., 2004). Besides, the presence of high-fat content and divalent cations like Ca^{2+} or Mg^{2+} in milk have been pointed out to harm nisin activity (Zapico et al., 1998). Moreover, homogenization is another factor that adversely affects nisin activity in milk. It results to decrease in the size of fat globules while increasing their number and surface area (Bhatti et al. 2004).

It can be concluded from the results that the developed hurdle effect of lysozyme-nisin-mild heating has been proved as an efficient preservation method of raw milk from the risk of listeriosis, particularly when the initial count of the bacterium is at a lower level than those studied here.

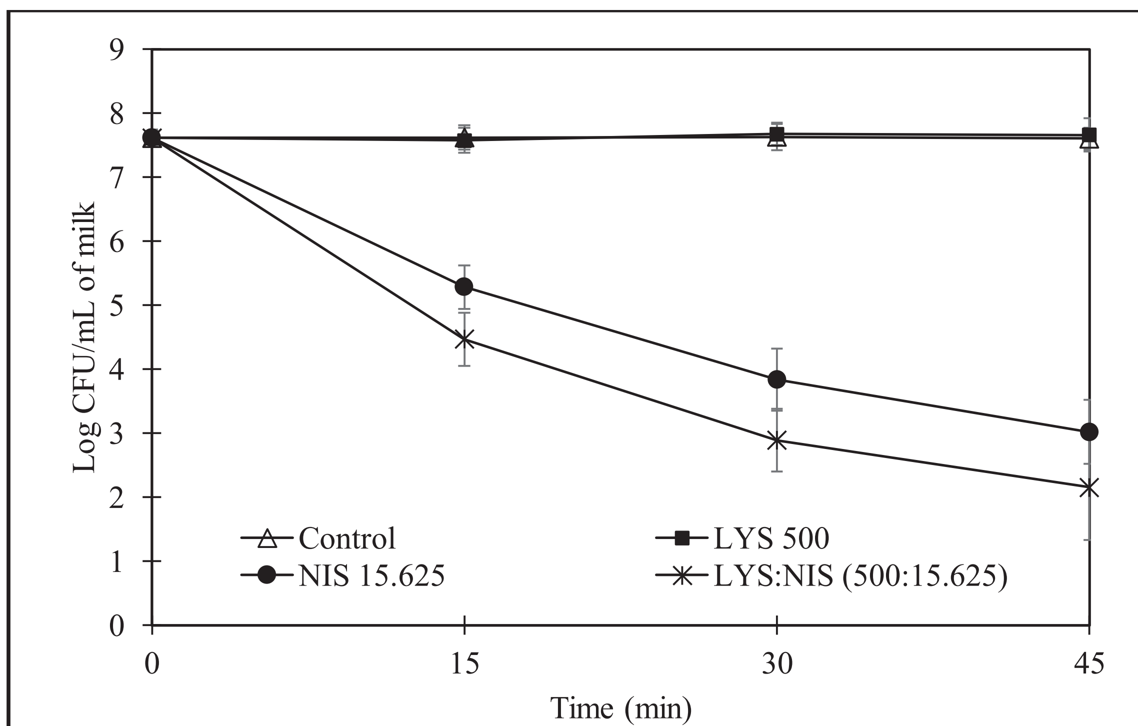

Figure 4.4. The antilisterial activity of LYS and/or NIS against *L. innocua* in raw milk exposed to heating at 50 °C

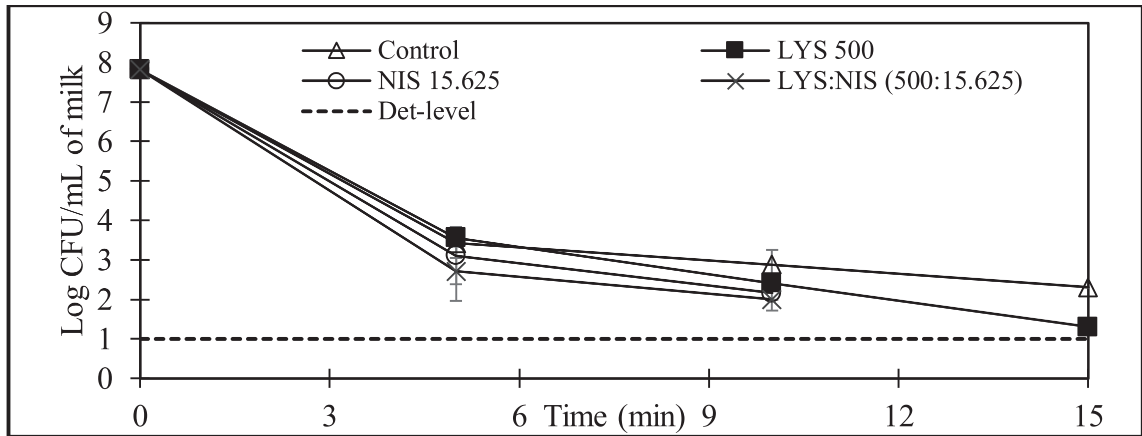

Figure 4.5. The antilisterial activity of LYS and/or NIS against *L. innocua* in raw milk exposed to heating at 60 °C

Table 4.4. Antilisterial effect of LYS and/or NIS in raw milk heat treated at 50 °C

Antimicrobial concentrations (µg/mL)			***L. innocua* count (log CFU/mL)**			
LYS	**NIS**	**T (℃)**	**Time (min)**			
			0	**15**	**30**	**45**
-	-	50	7.61 ± 0.13 ^{a,A}	7.62 ± 0.18 ^{a,A}	7.63 ± 0.20 ^{a,A}	7.60 ± 0.17 ^{a,A}
500	-	50	7.61 ± 0.13 ^{a,A}	7.57 ± 0.19 ^{a,A}	7.67 ± 0.17 ^{a,A}	7.66 ± 0.26 ^{a,A}
-	15.625	50	7.61 ± 0.13 ^{a,A}	5.28 ± 0.34 ^{b,B}	3.83 ± 0.48 ^{c,B}	3.02 ± 0.49 ^{d,B}
500	15.625	50	7.61 ± 0.13 ^{a,A}	4.46 ± 0.41 ^{b,C}	2.89 ± 0.49 ^{c,C}	2.15 ±0.82 ^{d,C}

a-c values within each row followed by the same letter are not significantly different (*P* >0.05). A-C values within each column for each replicate followed by the same letter are not significantly different (*P* > 0.05).

Table 4.5. Antilisterial effect of LYS and/or NIS in raw milk heat treated at 60 °C

Antimicrobial concentrations (µg/mL)			***L. innocua* count (log CFU/mL)**			
		T	**Time (min)**			
LYS	**NIS**	**(℃)**	**0**	**5**	**10**	**15**
-	-	60	7.81 ± 0.06 ^{a,A}	3.42 ± 0.36 ^{b,A}	2.87 ± 0.37 ^{c,A}	2.30 ± 0.00 ^{c,A}
500	-	60	7.81 ± 0.06 ^{a,A}	3.56 ± 0.21 ^{b,A}	2.40 ± 0.09 ^{c,AB}	1.30 ±0.00 ^{d,B}
-	15.625	60	7.81 ± 0.06 ^{a,A}	3.10 ± 0.72 ^{b,A}	2.16 ± 0.44 ^{b,AB}	< 1
500	15.625	60	7.81 ± 0.06 ^{a,A}	2.71 ± 0.72 ^{b,A}	2.00 ± 0.00 ^{b,B}	< 1

a-c values within each row followed by the same letter are not significantly different (*P* >0.05). A-C values within each column for each replicate followed by the same letter are not significantly different (*P* > 0.05).

4.3.4. Amount of Soluble and Bound Lysozyme and Nisin in Raw Milk

The amounts of soluble and bound (by milk proteins and/or milk fat) lysozyme and nisin in unheated and heated milk samples were presented in Figure 4.6. In nisin added milk samples, 67.26% and 72.97% of nisin were retained soluble in whey of unheated and heated milk, respectively. Therefore, it seemed that 32.73% and 27.02% of nisin remained in the fat or milk protein fraction of the curd of unheated and heated milk, respectively. On the other hand, the soluble lysozyme contents in unheated and heated milk were determined as 64.76% and 62.67%, respectively. These results showed that the majority of lysozyme and nisin exist in soluble form. The mild heating applied to increase listerial inactivation caused a slight increase in soluble nisin content while heating had almost no effect on the amount of soluble lysozyme in the milk.

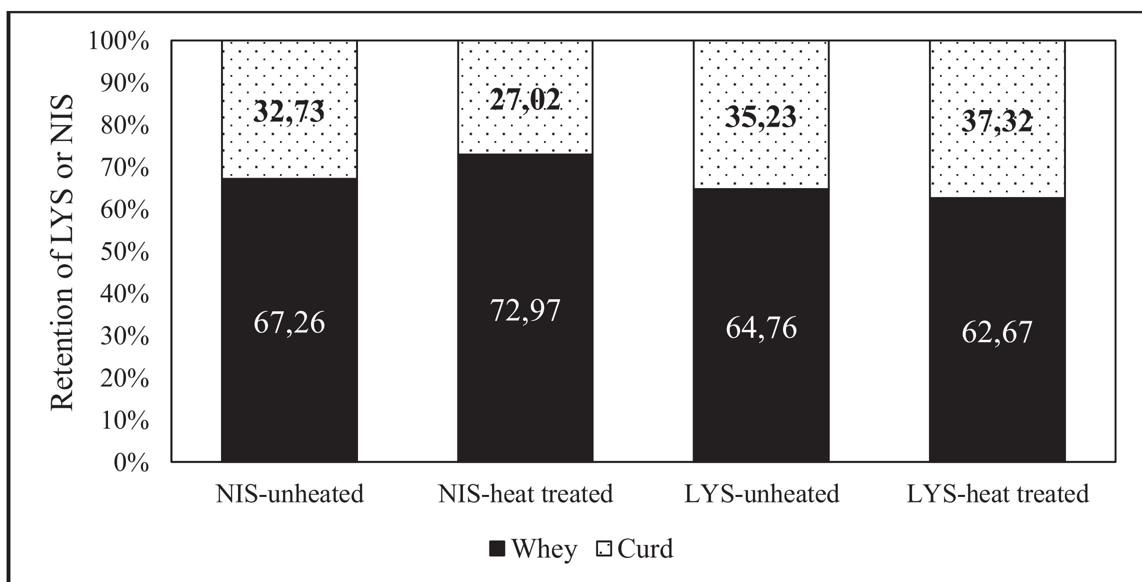

Figure 4.6. Percentages of soluble (in whey) and bound (in curd) LYS or NIS in heated and unheated milk samples

Nisin can interact with food depending on the ingredient of the food and thus, its efficiency may decrease with regard to interaction rate (da Silva Malheiros et al., 2010). However, concerning the milk, the adsorption of nisin on the fat globules in milk has been demonstrated (Jung et al., 1992; Zapico et al., 1998; Zapico et al., 1999). On the other hand, lysozyme is positively charged at pH values below pI that is 11.4 (Boyacı et al., 2016). Thus, ionic binding occurs between positively charged lysozyme and negatively charged casein (pI=4.6) in the milk that has a pH of almost 6.5 (Fagan et al., 2017;

Schneider et al., 2010). The electrostatic interactions between positively charged lysozyme and negatively charged whey proteins, namely β-lactoglobulin and α-lactalbumin might also be observed depending on the pH and the concentration of these proteins (Howell et al., 1995). Howell and Li-Chan (1996) also showed the possibility of hydrophobic interactions formed between lysozyme and whey proteins. However, the results of this work clearly showed that the enzyme maintained its solubility in the whey. On the other hand, the retention of part of the nisin and lysozyme in the curd suggested that the lysozyme-nisin synergy might show an antilisterial activity also in the cheese. Moreover, the synergy between these natural antimicrobials might also be exploited against *Clostridium tyrobutyricum* which causes gaseous type spoilage (late blowing) in maturated semi-hard or hard cheeses (D'Amato et al., 2010). Further studies are needed to show antimicrobial benefits of lysozyme-nisin synergy in cheese.

4.4. Conclusion

The test of different lysozyme-nisin combinations at refrigeration conditions (+4 °C) clearly showed the presence of a strong synergy of these biopreservatives against *L. innocua*. The results also proved that the antilisterial synergy is much stronger when the pH of the medium is close to neutrality (pH 6.0) instead of slightly acidic (pH 4.5) that caused suppression of lysozyme enzymatic activity. The detailed incubation tests under refrigerated conditions also showed that the initial antilisterial capacity of nisin is much higher than that of lysozyme. However, the nisin lost its antilisterial capacity by cold storage due to neutralization effects or acquired resistance of the bacterium, while lysozyme maintains its antilisterial potential at the later stages and delays regeneration of listerial growth.

The combination of lysozyme-nisin synergy with mild heating at 50 °C boosted the antilisterial potential obtained against *L.* innocua and created an effective hurdle. The test of developed hurdle effect (lysozyme-nisin-mild heating) on inoculated raw milk proved the effectiveness of developed preservation mechanism to prevent the risk of listeriosis from raw milk and its whey. Moreover, the retention of a significant portion of lysozyme and nisin in the curd suggested that the preservation mechanism could also provide an antilisterial potential for dairy products obtained. However, further studies are

needed to prove the effectiveness of developed antilisterial mechanism in dairy products obtained from the curds of lysozyme-nisin containing milk.

CHAPTER 5

LYSOZYME-NISIN SYNERGY IN COMBINATION WITH CHITOSAN COATING AGAINST *LISTERIA INNOCUA*: TEST OF FINDINGS ON COATED INOCULATED SPROUTED SEEDS

5.1. Introduction

The main consideration of food packaging is to ensure preservation and protection of foods from any spoilage factors such as microbiological or oxidative. For this purpose, plastic-based materials -particularly polyethylene- have been extensively using in the food packaging industry due to their good oxygen and aroma barrier properties, flexibilities and also their low costs. However, increasing use of these non-biodegradable materials results in environmental pollution due to the accumulation of huge amount of plastic waste (Tharanathan, 2003). Therefore, the increasing demand of consumers for natural, eco-friendly and bio-degradable packaging materials to keep their foods fresher and safer has induced many researchers and food processors to substitute biodegradable packaging materials for non-biodegradable polymers (Cutter, 2006). These biodegradable materials could be obtained from agricultural feedstock or by-products, wastes of fish industry and animal or microbial sources (Marsh and Bugusu, 2007). Polysaccharides such as starch, pectin, chitosan, alginates, cellulose, xanthan or pullulan, plant-based proteins from corn, soy, wheat, animal-based proteins like casein, whey and gelatin are the examples of these biodegradable polymers. (Cutter, 2006). These mentioned biodegradable materials can be used in the production of edible films as a film forming matrix. Edible films can be defined as a thin layer of continuous matrices that are used to prolong the shelf life of the food on which is coated or covered with the thin layer of edible film or coating that can be separated just before eating or consumed together with the food (Dehghani et al., 2018). The coating of edible film on the food surface is a more practical application method compared to the application of precast self-standing films. It can be applied in several ways. Food can be directly dipped into a film-forming solution

or the film-forming solution can be sprayed or brushed on the food surface (Yemenicioğlu, 2017). The main aim of using edible films and coatings is not only to keep food undamaged but also to protect food from any kind of chemical, physical and microbiological deterioration (Dehghani et al., 2018).

The main aim of this chapter was to develop an edible film incorporated with lysozyme and nisin. Because, there is only one study focused on the development of a coating solution incorporated with these two antimicrobial agents in the literature (Datta et al., 2008). In this study, *Salmonella anatum* or *Listeria monocytogenes* contaminated smoked salmon samples were coated with calcium alginate coating solution incorporated with nisin and hen egg white or oyster lysozyme. This study suggests that combinational use of these antimicrobials in calcium alginate edible film is more efficient to control microbial growth in smoked salmon during 35-days storage than those of calcium alginate edible film incorporated with lysozyme or nisin alone. On the other hand, few researchers have been endeavored to incorporate these two antimicrobial agents into edible films in the presence of EDTA (Cha et al., 2002; Bhatia and Bharti, 2015). Bhatia and Bharti (2015) reported the antimicrobial activity of a starch-based film incorporated with lysozyme, nisin, and EDTA against the microorganisms contaminated from environmental sources to food sample including cooked rice with pulses. Whereas, Cha et al., (2002) incorporated the combination of lysozyme, nisin, and EDTA into Na-alginate films or K-carrageenan films to monitor their effectiveness against various bacteria that were *Micrococcus luteus, L. innocua, Salmonella enteritidis, E. coli, and S. aureus.* Na-alginate films incorporated with these three agents were determined as more efficient in terms of antimicrobial activity.

On the other hand, the main objective of this chapter was to develop an antimicrobial chitosan film incorporated with lysozyme and nisin and to determine its efficacy against *L. innocua.* For this purpose, firstly, chitosan films were prepared using different molecular weight chitosan and the most potent chitosan in terms of antimicrobial activity was selected for further analyses. After that, the release test for both lysozyme and nisin was conducted at 4 °C. Then, chitosan film solution incorporated with the combination of lysozyme and nisin was applied as a coating material on legume seeds contaminated with *L. innocua* to reduce the initial load of the bacteria at room temperature.

5.2. Importance of Microbial Load in Seeds and Sprouts

Sprouted legumes as a raw ready-to-eat food are considered healthy and become popular among western society in the last few decades. Alfalfa, broccoli, lentils, mung beans, onions, soybeans, peanuts, radish, sunflower, and wheat are the mostly sprouted seeds. Sprouts are rich in nutrients such as minerals, vitamins, phytochemicals, fiber and phenolic compounds (Trząskowska et al., 2018; Iacumin and Comi, 2019). However, sprouts produced by the germination of seeds are associated with many recalls and outbreaks originated from *L. monocytogenes* (CDC, 2014c), *E. coli* O104:H4 (EFSA, 2011a), *Salmonella* (EFSA, 2011b; CDC, 2016c), *Yersinia enterocolitica* and *Bacillus cereus* (EFSA, 2011b). These outbreaks are inevitable since they are generally consumed as raw. Moreover, the presence of enteric pathogens on seed has been reported and they can also withstand the favorable conditions of sprouting (Trząskowska et al., 2018). The rapid increase in microbial count after 48 h sprouting has been demonstrated by Piernas and Guiraud (1997) who inoculated rice seeds at 3.5 log CFU/g and 2.7 log CFU/g concentrations of *L. innocua* and *B. cereus* and enumerated the counts as 7.3 log CFU/g and 8.2 log CFU/g, respectively. This increase in the counts has arisen from sprouting conditions. These conditions include high humidity and warm temperatures for a couple of days with the presence of abundant nutrients in germinating seeds that can be contaminated by high microbial loads and may harbor pseudomonads, enterobacteria, lactic acid bacteria, and yeasts as well. Besides, pathogen contamination in sprouts often originates from their seeds which may be contaminated during growing and harvesting from soil and irrigation water or during processing steps like washing, soaking, packaging (Iacumin and Comi, 2019). Therefore, to eliminate the risk of infection due to consumption of sprouts, many decontamination methods like dry heat, hot water, the use of organic acids, antagonistic microorganisms and their metabolites, ozonized water, chlorine, pulsed ultraviolet light, irradiation, supercritical carbon dioxide, high hydrostatic pressure and ultrasound can be applied on the seed (Millan-Sango et al., 2017; Trząskowska et al., 2018). Moreover, non-carcinogenic chlorine dioxide is also considered as an alternative way to decontamination of seeds. The maximum permitted concentration of aqueous chlorine dioxide for the contact of intact products was determined as 3 ppm by FDA (Millan-Sango et al., 2017) that also permitted the use of 20000 ppm calcium hypochlorite solution for steeping of the seed before sprouting to

reduce microbial load (Studer et al., 2013). In addition, antimicrobial treatment for accomplishing minimum 3 D reductions in the microbial pathogen load of seeds before sprouting was recommended by the Canadian Food Inspection Agency (CFIA, 2018).

In this context, it was aimed to achieve at least 3 log reductions in *L. innocua* count in intentionally contaminated seeds of mung bean, lentil, and wheat by coating with chitosan-based antimicrobial film solution incorporated with lysozyme and nisin as a food application of chitosan film. This will be the first application of the antimicrobial coatings on seeds that are processed into sprouts.

5.3. Chitosan

Chitosan was used as a film-forming material in this study. It is a deacetylated form of chitin that is the second most plentiful polysaccharide in nature after cellulose and is the major constituent of the shells of crustaceans like crab, shrimp, lobster and crawfish (No et al., 2007) and in the cell walls of fungi. Chitosan is a linear polysaccharide that is composed of the D-glucosamine and N-acetyl-D-glucosamine subunits linked to each other through the β (1-4) linkage (Di Martino et al., 2005). Chitosan is derived from chitin by alkaline deacetylation as depicted in Figure 5.1 in the presence of concentrated alkaline solution and high temperatures. The conditions of this deacetylation process such as the concentration of the alkaline solution, the temperatures used and the duration determine the degree of acetylation and molecular weight of chitosan obtained. These characteristics of chitosan are also effective on the solubility, viscosity and antimicrobial activity of chitosan as well (Vargas and González-Martínez, 2010).

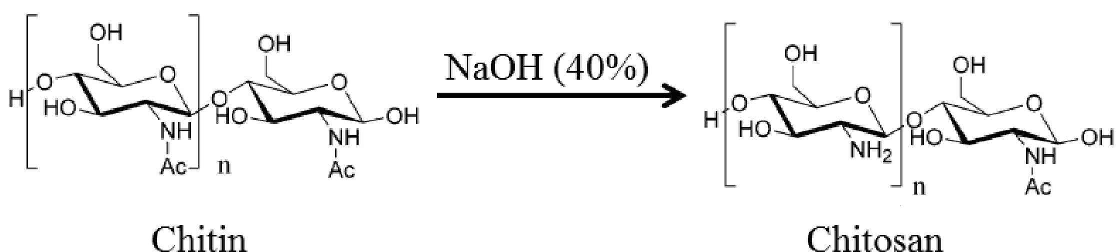

Figure 5.1. Alkaline deacetylation of chitin into chitosan (Source: Rabea et. al., 2003)

The use of chitosan has attracted significant attention of many industries such as food, medicine, agriculture, textile, environment, and bioengineering for many different

reasons some of which are its antimicrobial activity, biodegradability, biocompatibility, non-toxic and non-antigenic nature and chelating ability (Wang et al., 2018). Although the main antimicrobial action of chitosan is still unclear (Rabea et al., 2003; Kong et al., 2010; Alqahtani et al., 2019), the generally accepted hypothesis attributes its antimicrobial activity to its polycationic nature. Thereby, electrostatic interactions between positively charged chitosan molecule and negatively charged cell membrane take place and cause the change in permeability of the cell wall from where intracellular constituents are leaked to the outside of the cell (Rabea et al., 2013; Vargas and González-Martínez, 2010). Therefore, it is expected to monitor the higher antimicrobial activity of chitosan with a higher deacetylation degree that means a higher number of positively charged groups (Vargas and González-Martínez, 2010). One of the other hypothesis proposed for the antimicrobial activity of chitosan emphasize the interaction between diffused hydrolysis products and microbial DNA, that results in the inhibition of protein and mRNA synthesis, whereas another hypothesis claims that chitosan exerts its antimicrobial activity by chelating the metals in the cell membrane or essential nutrients (No et al., 2007). Antimicrobial activity possessed by chitosan is effective on Gram-positive and Gram-negative bacteria, filamentous fungi, and yeasts. However, mammalian cells are more resistant to its activity (Kong et al., 2010).

During the last few decades, chitosan has gained attention due to its properties as mentioned earlier in the food industry as well (Vargas and González-Martínez, 2010). Its use in food as a food additive was approved in Japan and Korea since, 1983 and 1995, respectively (No et al., 2007). Chitosan can be added into food as an additive for the following purposes which include texture controlling, emulsifying, color stabilization, clarification and deacidification of fruits and beverages. In addition, it can also be added to improve the nutritional quality of the food to increase dietary fiber, reduce the lipid absorption, produce a hypocholesterolemic effect. Besides, it can be used in the purification of water by recovering metal ions, pesticides, phenols and removing of dyes (Shahidi et al., 1999). On the other hand, chitosan has the film-forming ability, thanks to its solubility in dilute acids like acetic or lactic acid solution (Wang et al., 2018). Therefore, several chitosan films have been fabricated by many researchers to extend the shelf life of different fresh fruits like kiwifruit, pear and peach (Du et al., 1997) and strawberries (Zhang and Quantick, 1998; Han et al., 2004; Park et al., 2005; Vargas et al., 2006), minimum processed fruits such as fresh cut carrots (Vargas et al., 2009) and sliced mango fruits (Chien et al., 2007), meat products like ground beef (Ouattara et al., 2002),

chilled pork (Sagoo et al., 2002), fish products like fish patties (Lopez-Caballero et al., 2005), lingcod fillets (Duan et al., 2010) and herring and codfish (Jeon et al., 2002) and chicken eggs (Yuceer and Caner, 2014).

To the best of our knowledge, the combination of lysozyme and nisin, as antimicrobial agents used in this Ph.D. thesis, has not incorporated into a chitosan film before. However, each of them was separately incorporated into a chitosan film or coating. Food applications of nisin incorporated chitosan films have been performed to extend the shelf life of fresh strawberries (Duran et al., 2016), to monitor antimicrobial efficiency against *L. monocytogenes* in ham steaks (Ye et al., 2008a) and cold-smoked salmon (Ye et al., 2008b) and to control microbial growth in minimally processed pears (Cé et al., 2012). Moreover, antimicrobial activities of chitosan films incorporated with nisin were also evaluated in agar media (Cha et al., 2003; Pranoto et al., 2005; Imran et al., 2014). On the other hand, Duan et al., (2008) showed the antimicrobial efficiency of lysozyme incorporated chitosan film on both *E. coli* and *L. monocytogenes*, while Park et al. (2004) evaluated the antimicrobial activity of the lysozyme-chitosan films against *E. coli* and *Streptococcus faecalis.* In addition, the effect of chitosan coating incorporated with lysozyme on the shelf life extension and the enhancement of microbial quality and organoleptic properties were studied in both brined and salt reduced Halloumi cheeses (Mehyar et al., 2018). Nevertheless, lysozyme incorporated chitosan films were applied on eggs in order to extend shelf life and improve functional properties of them (Yuceer and Caner, 2014), on tomatoes stored at ambient temperatures to prolong shelf life without undesirable change in their quality (Thumula, 2006) and on mozzarella cheeses to enhance microbial safety (Duan et al., 2007).

5.4. Experimental Study

In the following sections, both materials and methods performed for chitosan coating applied on seeds to be sprouted were given in detail.

5.4.1. Materials

Hen egg white lysozyme (L6876), nisin from *Lactococcus lactis* (N5764), *Micrococcus lysodeikticus* as a substrate of lysozyme in enzyme activity determination,

Tween 20, low molecular weight (LMW) chitosan (448869), medium molecular weight (MMW) chitosan (448877) and high molecular weight (HMW) chitosan (419419) were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA). The details about the chitosan used in this study were also presented in Table 6.1. MRS broth and glycerol were obtained from Merck (Darmstadt, Germany). The strain of *Lactobacillus plantarum* (NRRL-B4496) obtained from ARS Culture Collection (NRRL) was kindly provided by Dr. Burcu Öztürk, from the Department of Food Engineering, Izmir Institute of Technology, İzmir.

Mung bean, lentil, and wheat used in food application experiments were purchased from a local market in İzmir.

Table 5. 1. Molecular weights and degree of acetylation of chitosan

Chitosan	**Degree of acetylation (%)**	**Molecular weight (Da)**
LMW	75-85	50000 - 190000
MMW	75-85	190000-300000
HMW	>75	310000-375000

5.4.2. Methods

The methods given below were performed to test chitosan coating on sprouted seeds.

5.4.2.1. Preparation of Chitosan Film

Chitosan films were prepared by a slight modification of the method of Ünalan et al. (2011b). Briefly, 1.5 % (w/w) LMW chitosan, MMW chitosan or HMW chitosan was dissolved in a 0.5% acetic acid solution by stirring overnight at 300 rpm. Then, 100% glycerol (w/ w chitosan) was added into the solution which was stirred for 30 min at 300 rpm. The film solution was filtered by using cheesecloth to remove impurities and antimicrobial agents (nisin at 0.5 mg/cm^2, lysozyme at 0.5 mg/cm^2 or 3.5 mg/cm$^{2)}$ were added into film forming solution right before homogenization which takes place using a Heidolph Silent Crusher M equipped with a 12F shearing tool (Heidolph Instruments

GmbH, Schwabach, Germany) at 10000 rpm for 3 min. Then, the film making solutions were centrifuged for degassing air bubbles at 5000 g for 5 min. After degassing, 20 g of film solution was poured into a disposable Petri dish (8.5 cm in diameter) and dried for 22±2 h at 45 °C.

5.4.2.2. Determination of Antilisterial Activities of Different MW Chitosan Films Incorporated with LYS and/or NIS

Antilisterial activities of different MW chitosan films incorporated with lysozyme and/or nisin were determined in broth medium. Initially, chitosan films incorporated with lysozyme and/or nisin were prepared as described in section 5.4.2.1 and then, films were cut into disk-shaped specimens by a sterile 13 mm-diameter cork-borer under aseptic conditions. After that, disk-shaped films containing lysozyme (0.5 mg/cm^2 for different MW chitosan or 3.5 mg/cm^2 for LMW chitosan) and/or nisin (0.5 mg/cm^2) were immersed into 2.389 mL of nutrient broth (at pH 6.0) to adjust the final concentration of the antimicrobials as 250 µg/mL and 0.265 µL of inoculum of *L. innocua* at 10^7 CFU/mL was added into the sterile capped tubes. The enumeration was performed on the 0th, 1st, 2nd, 6th and 12th day of the storage at 4 °C. No film added tubes were also prepared to monitor the growth of the bacteria and no antimicrobial added chitosan film was considered as a control film. The enumeration was conducted by the spread plate method on nutrient agar. The colonies were enumerated after 24 h incubation at 37 °C. Two tubes were prepared for each day of sampling. The analysis was carried out in duplicate and the enumeration was performed in triplicate.

5.4.2.3. Determination of Release Profiles of Lysozyme

The release test of lysozyme from LMW chitosan films was carried out in Na-phosphate buffer at pH 6.0 and 4 °C by shaking during the release study. Briefly, chitosan films were cut into 16 cm^2 square-shaped films and placed into an Erlenmeyer flask containing 15 mL of Na-phosphate buffer at pH 6.0. The flasks were stored at 4 °C in a cooled incubator and shaken with an orbital shaker working at 160 rpm. The release test was terminated when the equilibrium was reached for the release of lysozyme or an insignificant increase was observed in lysozyme activity. Lysozyme activity was

measured using a spectrophotometer by taking 0.2 mL (3 repeats) aliquots from the release test buffer at different time intervals. The activities were expressed as Units (0.001 absorbance change in 1 min per mL of enzyme) released per cm^2 of films tested.

The LYS recoveries from the films were calculated by the following formula; % LYS recovered = (maximum U LYS released form film/ total U LYS incorporated into film) x 100.

5.4.2.4. Determination of Release Profiles of Nisin

The release test of nisin from LMW chitosan films was carried out in Na-phosphate buffer at pH 6.0 and 4 °C by shaking during the release study. Briefly, chitosan films were cut into 16 cm^2 square-shaped films and placed into an Erlenmeyer flask containing 15 mL of Na-phosphate buffer at pH 6.0. The flasks were stored at 4 °C in a cooled incubator and shaken with an orbital shaker working at 160 rpm. The release test was terminated when the equilibrium was reached for the release of lysozyme or an insignificant increase was observed in nisin activity. Releasing concentration of nisin was determined by agar diffusion method as described in section 4.2.2.3 by taking 50 μL (3 repeats) aliquots from the release test buffer at different time intervals.

The concentration of nisin was estimated using the following equation which was obtained from the standard curve of nisin:

$$D = 5.24 \log C + 0.8473$$

where D is the diameter of the inhibition zone and C is the concentration of nisin. The nisin concentration was expressed as International Units (IU) released per cm^2 of films tested.

The NIS recoveries from the films were calculated by the following formula; % NIS recovered = (IU NIS released form film/ total IU NIS incorporated into film) x 100

5.4.2.5. Determination Morphology and Mechanical Properties of Films

The cross-sectional morphology of chitosan films incorporated with lysozyme (3.5 mg/cm^2) and/or nisin (0.5 mg/cm^2) was determined by using scanning electron microscopy (SEM) (Philips XL 30S FEG, FEI Company, Netherlands) under high vacuum mode at an operating voltage varying between 2 and 3 kV. The films were placed into liquid nitrogen for fast freezing and crashed for SEM examination. After that, the samples were gold coated with a sputter coater (Emitech K550X, Quorum Technologies Inc., UK) under 15mA for 1 min. The thickness of the films was measured from SEM cross-sectional views of films from 500X magnified images.

Tensile strength, elongation at break and Young's modulus of the chitosan films were determined by using a Texture Analyser TA-XT2 (Stable Microsystems, Godalming, UK) according to ASTM Standard Method D 882-02 (ASTM, 1999). Conditioning of the films was performed at 25 °C and 50% RH for 24 h in an environmental chamber. Then, the conditioned films were cut into 8 mm × 80 cm strips. The initial grip distance and crosshead speed were set to 50 mm and 50 mm/min, respectively. The mentioned mechanical parameters were calculated by using stress to strain plot. At least seven replicates of each film were tested.

5.4.2.6. Inoculation of Legume Seeds

For the antimicrobial test, seeds were firstly soaked in 5% (v/v) sodium hypochlorite solution for 15 min and washed with sterilized distilled water for 20 min. After washing, they were dried in a laminar flow cabin.

Meanwhile, *L. innocua* was activated by transferring one loop of frozen culture to 9 mL nutrient broth and incubating at 37 °C for 24 h. Then, the culture was diluted ten-fold into the nutrient broth to adjust to a final concentration of 10^7 CFU/mL. After that, each type of seed in 10 g of portions was immersed into a culture solution and stirred with a sterile glass rod for 15 min to distribute the inoculum evenly. The culture solution was then removed and the inoculated seeds were placed into sterile Petri dishes and kept for drying in a laminar flow cabin for 2 h.

5.4.2.7. Monitoring of Antilisterial Activities of Coating on Legume Seeds

Chitosan solutions added with lysozyme (3.5 mg/cm^2) and/or nisin (0.5 mg/cm^2) were prepared as described in section 5.4.2.1. Each type of inoculated seed in 10 g of portions was dipped into the flasks including the film solutions and stirred with a glass rod to distribute the solution evenly. The excess amount of the film solution was then removed. Seeds coated with the film solutions were placed into sterile Petri dishes which were kept at 25 °C for 4 h. Throughout the drying of the coating solution, seeds were stirred with a glass rod at 1 h intervals and kept at 25±2 °C under aseptic conditions. Immediately after drying of the coating solution, each sample was diluted ten-fold with 0.1% peptone water and the samples were stirred vigorously in an Erlenmeyer flask for 60 s. The serial decimal dilutions were prepared from each ten-fold diluted sample and appropriate dilutions were prepared were spread plate onto Oxford Listeria Selective Agar supplemented with Oxford Listeria Selective Supplement. The enumeration of small black colonies with a halo on the plate was performed after 48 h incubation at 37 °C. The enumeration was conducted in triplicate plates. Two separate samples from each replicate for each coating treatment were used in the microbiological analysis. Uncoated seeds were considered as the control group.

5.4.2.8. Determination of Seed Germination Rate

The germination rate of seeds was determined by a slight modification of the method given by Pierre and Ryser (2006). Briefly, 120 coated or uncoated seeds were placed on moistened cotton in petri dishes (40 seeds per Petri plate X 3 plates per replicates) and stored in an environmental chamber at 22 °C and 50% RH for 5 days in the dark. Seeds were moistened daily with 5 mL of water. Seed germination was monitored daily and a seed was considered to be germinated when the radicle was 2 mm long. The germination rate was determined according to the following formula for each treatment and seed type at days of 3, 4 and 5.

$$Germination\ rate\ (\%) = \frac{\text{No. of sprouts per plate}}{\text{No. of seeds per plate}} \times 100$$

5.4.2.9. Statistical Analyses

The results presented are averages and standard deviations that were calculated from these replicate measurements (Microsoft Excel, Microsoft Corporation, Redmond, WA). The Analysis of Variances (ANOVA) and Fisher test were applied to determine whether the treatments were different from each other and which treatment significantly differed from the others, respectively, with significance assigned at $P < 0.05$ by using a statistical software of Minitab release 16 (Minitab Inc., State College, Pa., U.S.A.)

5.5. Results and Discussion

The detailed results and discussion for this chapter will be given in the following sections.

5.5.1. Effects of Different MW Chitosan Films Incorporated with LYS and/or NIS against *L. innocua*

Different molecular weight chitosan films incorporated with lysozyme and/or nisin at equal concentrations (0.5 mg/cm^2) were prepared to compare their antilisterial activities at two different inoculum concentrations (10^4 CFU/mL or 10^6 CFU/mL) at pH 6.0. The results from the initial load of 10^4 CFU/mL and 10^6 CFU/mL were given in Table 5.2 and Table 5.3, respectively.

The results presented in Table 5.2 showed that LYS alone in LMW chitosan film was effective on *L. innocua* during 12-day incubation. Besides, it resulted in a significantly lower count of the bacteria than those in MMW or HMW chitosan films incorporated with LYS ($P<0.05$). In addition, a significant reduction in respect of final *L. innocua* counts of LMW chitosan film alone (4.84 D) was also monitored compared to those of MMW (3.62D) or HMW (1.66 D) chitosan film alone ($P<0.05$). LYS:NIS incorporated chitosan films caused a synergistic reduction in both LMW and MMW chitosan films on the 2nd day of incubation. On the other hand, the antilisterial effect of the films incorporated with nisin was not permanent especially after the 6th days of the incubation due to short-term inhibition effect of nisin on *L. monocytogenes* reported as Harris et al. (1991) and Gallo et al. (2007). This finding was particularly obvious in

LYS:NIS incorporated different MW chitosan film whose the count of the bacteria increased after 6th days of storage.

The results presented in Table 5.3 clearly showed a gradual decrease in the reduction of *L. innocua* occurred in all types of control films. But, control film produced from LMW chitosan was the most efficient one with 5.25 D reduction in the count of the bacterium (P<0.05) and it was followed by MMW and HMW chitosan films with 3.52 D and 2.83 D reductions at the 12th day of incubation, respectively. Similarly, 5.70 D reduction in the count was significantly different in LYS incorporated-LMW chitosan film from LYS incorporated MMW (4.20 D) or HMW chitosan film (4.42D) at the end of the incubation period as well. On the other hand, nisin lead to gradual decrease starting from the 1st and 2nd day of the incubation in LMW and HMW chitosan films respectively, whereas the antilisterial activity of nisin obtained at the 1st day of incubation remained stable during the further incubation period in MMW chitosan film. However, the synergistic effect of LYS and NIS on the bacteria has been monitored between the 1st and 6th days of incubation in both LMW and MMW chitosan films, while in HMW chitosan films synergy was only observed at the 6th day of the incubation. In addition, the highest and significant reduction with a 6.3 D decrease in the count was also observed in LMW chitosan films incorporated with LYS and NIS. But, the synergistic effect disappeared in all chitosan films on the 12th day of incubation. This could be related to the acquired resistance of nisin by bacteria.

These results suggest that the combinational incorporation of lysozyme and nisin into LMW or MMW chitosan films has the potential to inhibit *L. innocua* almost a week of storage at refrigerated conditions, particularly at the lower concentration of the bacteria. But also, the synergistic use of these antimicrobials can suppress the growth of the bacteria at the higher concentration of the bacteria. Therefore, initial inoculum concentration is considerably important on the efficiency of antimicrobials which have a more inhibitory effect when the initial inoculum concentration is lower (Francis and Beirne, 1997; Janes et al., 2002; Balciunas et al., 2013). On the other hand, chitosan alone has also an antimicrobial activity against a wide range of microorganisms (Coma et al., 2002; No et al., 2007; Joerger, 2007).

The present result of chitosan film yet again confirms the antimicrobial activities of chitosan alone. Besides, different antimicrobial mechanisms of chitosan have been proposed to explain the effect of chitosan on Gram-positive and Gram-negative bacteria by Zheng and Zhu (2003) who suggested that chitosan forms a cell surface-located

polymer membrane to prevent nutrient intake into the cell of Gram-positive bacteria, whereas chitosan enters into the cell of Gram-negative bacteria via pervasion. However, this finding is contradictive. Because Kim et al. (2007) studied the antimicrobial effect of different MW chitosan on *Salmonella* Enteritidis which was determined as the most sensitive to the lowest MW chitosan tested. Contrarily, *S. enterica* serovar Enteritidis was more efficiently inhibited by HMW chitosan with MW of 310-375 kDa as same as used in our study (Leleu et al., 2011). Besides, in that study, HMW chitosan was also tested against different Gram-positive bacteria such as *L. monocytogenes, Carnobacterium* sp, *Staphylococcus warneri* and Gram-negative bacteria like *Acinetobacter baumanii, Alcaligenes* sp., *E. coli* (O157:H7), *Pseudomonas* sp. *Salmonella* Typhimurium and *Serratia marcescens*. The most affected bacterium was demonstrated as *Serratia marcescens* with 1.59 D reduction. Therefore, antimicrobial activity of chitosan is influenced by its molecular weight (Shin et al., 2001; Rabea et al., 2003; Zheng and Zhu, 2003; Qin et al., 2006; No et al., 2007), the type of bacterium tested (Kim et al., 2007) and degree of acetylation (DA) (Qin et al., 2006; Dutta et at., 2009; Goy et al., 2009). However, no specific standard defines the discrimination of chitosan with regard to their molecular weight (Goy et al., 2009). But, HMW chitosan with high DA has been reported as having higher antimicrobial activity compared to those of LMW chitosan with lower DA (Shin et al., 2001; Vargas and González-Martínez, 2010). Contrarily, the greater antimicrobial activity of LMW chitosan on *S. aureus* has been reported compared to those of higher MW chitosan (Zheng and Zhu, 2003). This conflicting data can also arise from the undefined classification of chitosan in terms of their MW. In the results given in this Ph.D. thesis, antimicrobial activity of LMW chitosan film incorporated with lysozyme and/or nisin is more effective to control the growth of *L. innocua.* Therefore, LMW chitosan was selected for further studies.

The synergistic effect of the combined use of lysozyme and nisin in chitosan films or coatings has not been researched yet. Therefore, in this study, it was focused on to develop an antimicrobial chitosan film incorporated with lysozyme and nisin. On the other hand, the antimicrobial studies of chitosan films incorporated with lysozyme (Park et al., 2004; Duan et al., 2007; Duan et al., 2008) or nisin (Pranoto et al., 2005; Guo et al., 2014; Imran et al., 2014; Duran et al., 2016) are available in the literature.

For instance, nisin incorporated MMW chitosan film revealed antimicrobial activity on *S. aureus, L. monocytogenes* and *B. cereus* (Pranoto et al., 2005). In addition, chitosan films incorporated with 486 IU/cm^2 of nisin resulted in around 2 D reductions in

turkey meat slices, regardless of chitosan concentration used (Guo et al., 2014). Besides, Duan et al. (2007) coated 60% of lysozyme incorporated chitosan films on mozzarella cheese to monitor the antimicrobial effect of the coating on the growth of *E. coli, P. fluorescens* or *L. monocytogenes* throughout 14 day-storage at 10 °C. *L. monocytogenes* was the most sensitive at the end of the storage period with almost 1.5 D reduction and followed by *E. coli* (1.25 D) and *P. fluorescens* (1D). Similarly, Mehyar et al. (2018) coated Halloumi cheeses with chitosan solution incorporated with 60% of lysozyme to prolong its shelf life by controlling the growth of anaerobes, psychrotrophs, LAB, yeast, and mold. Chitosan-lysozyme coating has been reported to achieve at least a 5-day extension of shelf life in 5% or 10% cheeses, while the reductions in the count of the mentioned microorganisms have been found between 1.8 D and 2.2 D in 15% brined cheeses with hurdle effect of salt as well.

Table 5.2. Effect of chitosan films on *L. innocua* in broth media (initial microbial load at 4 log CFU/mL)

	***L. innoua counts* (log CFU/mL)**				
LMW Chitosan	**Day 0**	**Day 1**	**Day 2**	**Day 6**	**Day 12**
Ctrl w/no film	4.53±0.22 ^{c,A,A}	4.62±0.12 ^{c,A,A}	4.80±0.23 ^{c,A,A}	5.31±0.31 ^{b,A,A}	6.57± 0.79 ^{a,A,A}
Ctrl film	4.45±0.21^{a,A,B}	3.72±0.46 ^{b,B,B}	2.52±0.68 ^{c,B,B}	1.44±0.48 ^{d,B,B}	1.73±0.48 ^{d,B,B}
LYS	4.67±0.08 ^{a,A,A}	3.26±0.22 ^{b,C,A}	1.43±0.28 ^{c,C,B}	1.10±0.17 ^{c,B,B}	< 0.69
NIS	3.90±0.53^{a,B,AB}	2.14±0.37^{b,D,A}	1.75±0.50^{bc,C,A}	1.00±0.30 ^{d,B,B}	1.44±0.68 ^{cd,B,B}
LYS:NIS	3.55±0.42 ^{a,C,B}	2.21±0.36 ^{b,D,A}	0.93±0.33 ^{c,D,A}	< 0.69	1.43±0.36 ^{bc,B,AB}
MMW Chitosan	**Day 0**	**Day 1**	**Day 2**	**Day 6**	**Day 12**
Ctrl w/no film	4.53±0.22 ^{c,A,A}	4.62±0.12 ^{c,A,A}	4.80±0.23^{c,A,A}	5.31±0.31^{b,A,A}	6.57± 0.79^{a,A,A}
Ctrl film	4.67±0.15^{a,A,AB}	3.79±0.64^{b,B,B}	2.53±0.60^{c,B,B}	2.78±0.33^{c,B,A}	2.95±0.45^{c,B,A}
LYS	4.45±0.56 ^{a,A,A}	2.52±0.02^{b,C,B}	2.57±0.45^{b,B,A}	2.00±0.53^{b,C,A}	2.47±0.72^{b,B,A}
NIS	4.38±0.29 ^{a,A,A}	2.25±0.24^{b,CD,A}	2.06±0.36^{b,B,A}	2.64±0.29^{b,BC,A}	2.29±0.83^{b,B,A}
LYS:NIS	4.70±0.11 ^{a,A,A}	1.73±0.61^{b,D,A}	1.04±0.26^{c,C,A}	< 0.69	1.05±0.10^{c,C,B}
HMW Chitosan	**Day 0**	**Day 1**	**Day 2**	**Day 6**	**Day 12**
Ctrl w/no film	4.53±0.22^{c,A,A}	4.62±0.12^{c,A,A}	4.80±0.23 ^{c,A,A}	5.31±0.31 ^{b,A,A}	6.57± 0.79 ^{a,A,A}
Ctrl film	4.93±0.19 ^{a,AB,A}	4.84±0.13^{a,A,A}	4.81±0.06^{a,A,A}	3.19±0.15 ^{b,B,A}	3.18±0.64 ^{b,B,A}
LYS	4.98±0.22^{a,A,A}	3.36±0.26^{b,B,A}	1.63±0.33^{c,BC,B}	1.32±0.21^{cd,D,AB}	0.69±0.00^{d,D,A}
NIS	3.57±0.36^{a,C,B}	2.38±0.31^{bc,C,A}	1.93±0.19^{b,B,A}	2.36±0.34^{cd,C,A}	2.84±0.43^{b,BC,A}
LYS:NIS	3.82±0.75^{a,C,B}	1.34±0.49^{b,D,A}	1.30 ±0.00^{b,C,A}	1.29±0.11^{b,D}	1.98±0.65 ^{b,CD,A}

a-d Values at each row followed by different lowercase letters indicate statistically significant differences. A-D Values at each column followed by different capital letters indicate statistically significant differences in each chitosan film. *A-B* Values at each column followed by different capital letters indicate statistically significant differences between each treatment in different MW chitosan films ($P < 0.05$).

Table 5.3. Effect of chitosan films on *L. innocua* in broth media (initial microbial load at 6 log CFU/mL)

	L. innoua counts (log CFU/mL)				
LMW Chitosan	**Day 0**	**Day 1**	**Day 2**	**Day 6**	**Day 12**
Ctrl w/no film	6.53±0.23 ^{d,A,A}	6.77±0.20 ^{c,A,A}	6.89±0.13 ^{c,A,A}	7.70±0.31^{b,A,A}	8.23±0.20^{a,A,A}
Ctrl film	6.48±0.28^{a,AB,B}	5.51±0.52 ^{b,B,B}	5.22±0.59 ^{b,B,B}	3.90±0.70^{c,B,B}	2.98 ±0.56^{d,B,C}
LYS	6.71±0.13^{a,A,A}	5.50±0.26 ^{b,B,A}	4.54±0.12 ^{c,C,A}	2.40±0.42^{d,C,B}	2.53±0.13^{d,B,B}
NIS	6.27±0.30^{a,B,B}	3.86±0.51 ^{b,C,A}	3.14±0.81 ^{bc,D,B}	2.80±0.60^{c,C,B}	2.47±0.59^{c,B,B}
LYS:NIS	5.68±0.32^{a,C,A}	3.06±0.56 ^{b,D,B}	2.52±0.22^{c,E,B}	1.40±0.19^{d,D,B}	2.58±0.24^{bc,B,B}
MMW Chitosan	**Day 0**	**Day 1**	**Day 2**	**Day 6**	**Day 12**
Ctrl w/no film	6.53±0.23^{d,A,A}	6.77±0.20^{c,A,A}	6.89±0.13^{c,A,A}	7.70±0.31 ^{b,A,A}	8.23±0.20 ^{a,A,A}
Ctrl film	6.72±0.15^{a,A,AB}	6.42±0.13^{a,B,A}	6.35±0.18^{a,B,A}	4.91±0.57^{b,B,A}	4.71±0.24^{b,B,B}
LYS	6.73±0.09^{a,A,A}	4.25±0.19^{b,C,B}	3.15±0.48^{c,C,B}	4.04±0.70^{b,C,A}	4.03±0.27^{b,CD,A}
NIS	6.70±0.13^{a,A,A}	4.27 ±0.04 ^{b,C,A}	4.10±0.10^{b,D,AB}	4.02 ±0.37^{b,C,A}	4.37±1.10^{b,BC,A}
LYS:NIS	5.68±0.82^{a,B,A}	3.23 ±0.20 ^{b,D,B}	2.11±0.18^{c,E,B}	3.22±0.30^{b,D,A}	3.59±0.40 ^{b,D,A}
HMW Chitosan	**Day 0**	**Day 1**	**Day 2**	**Day 6**	**Day 12**
Ctrl w/no film	6.53±0.23 ^{d,A,A}	6.77±0.20^{c,A,A}	6.89±0.13^{c,A,A}	7.70±0.31^{b,A,A}	8.23±0.20^{a,A,A}
Ctrl film	6.88±0.27^{a,A,A}	6.85±0.14^{a,A,A}	6.88±0.20^{a,A,A}	5.43± 0.18^{b,B,A}	5.40 ±0.05^{b,B,A}
LYS	6.68±0.40^{a,A,A}	4.59±0.49^{b,B,B}	3.67±0.45^{c,C,B}	3.82±0.46^{c,C,A}	3.80 ±0.53^{c,C,A}
NIS	6.58±0.31^{a,A,AB}	4.30±0.10^{bc,BC,A}	4.57±0.16^{b,B,A}	3.99±0.25^{cd,C,A}	3.73±0.44^{d,CD,AB}
LYS:NIS	6.50±0.14^{a,A,A}	4.15±0.12 ^{b,C,A}	3.49±0.41^{c,C,A}	3.28±0.18^{c,D,A}	3.29±0.27^{c,D,AB}

a-d Values at each row followed by lowercase capital letters indicate statistically significant differences. A-C Values at each column followed by different capital letters indicate statistically significant differences in each chitosan film. *A-C* Values at each column followed by different capital letters indicate statistically significant differences between each treatment in different MW chitosan films ($P < 0.05$).

5.5.2. Release Profiles of Lysozyme and Nisin in Chitosan Film

The release profiles of lysozyme at pH 6.0 were determined for LMW chitosan that was selected as the most potent antilisterial chitosan form (Table 5.2 and Table 5.3). The lack of any enzyme activity release form LMW chitosan films with LYS at 1.25 mg/cm^2, and only a slight release of the enzyme from films with LYS at 2.5 mg/cm^2 clearly showed binding of lysozyme by chitosan film matrix (Fig. 5.2A.). The retention of lysozyme by positively charged chitosan film should not be due to charge-charge attractions since lysozyme is also positively charged at pH 6.0. Thus, it seemed that lysozyme binds on chitosan matrix with H-bonds as proposed by Yao & Li (1994). However, a significant and concentration-dependent lysozyme release started from LMW

chitosan films as enzyme concentration increased ≥ 3.5 mg/cm^2. The recoveries of lysozyme from films with 3.5, 4.0 and 5 mg LYS/cm^2 were 46, 59 and 74%, respectively (Fig. 5.3A). It appears that chitosan matrix started to release excessive lysozyme after all H-bonding groups were filled with the bound enzyme. These results were in line with recent findings of Park et al. (2004) who also observed a concentration-dependent increase in lysozyme release from chitosan films.

Nisin at 0.5 mg/cm^2 was also bound by LMW chitosan matrix effectively (Fig. 5.2B.), but unlike LYS, NIS at this concentration released slowly from the films with a recovery of almost 10% (Fig. 5.3B).

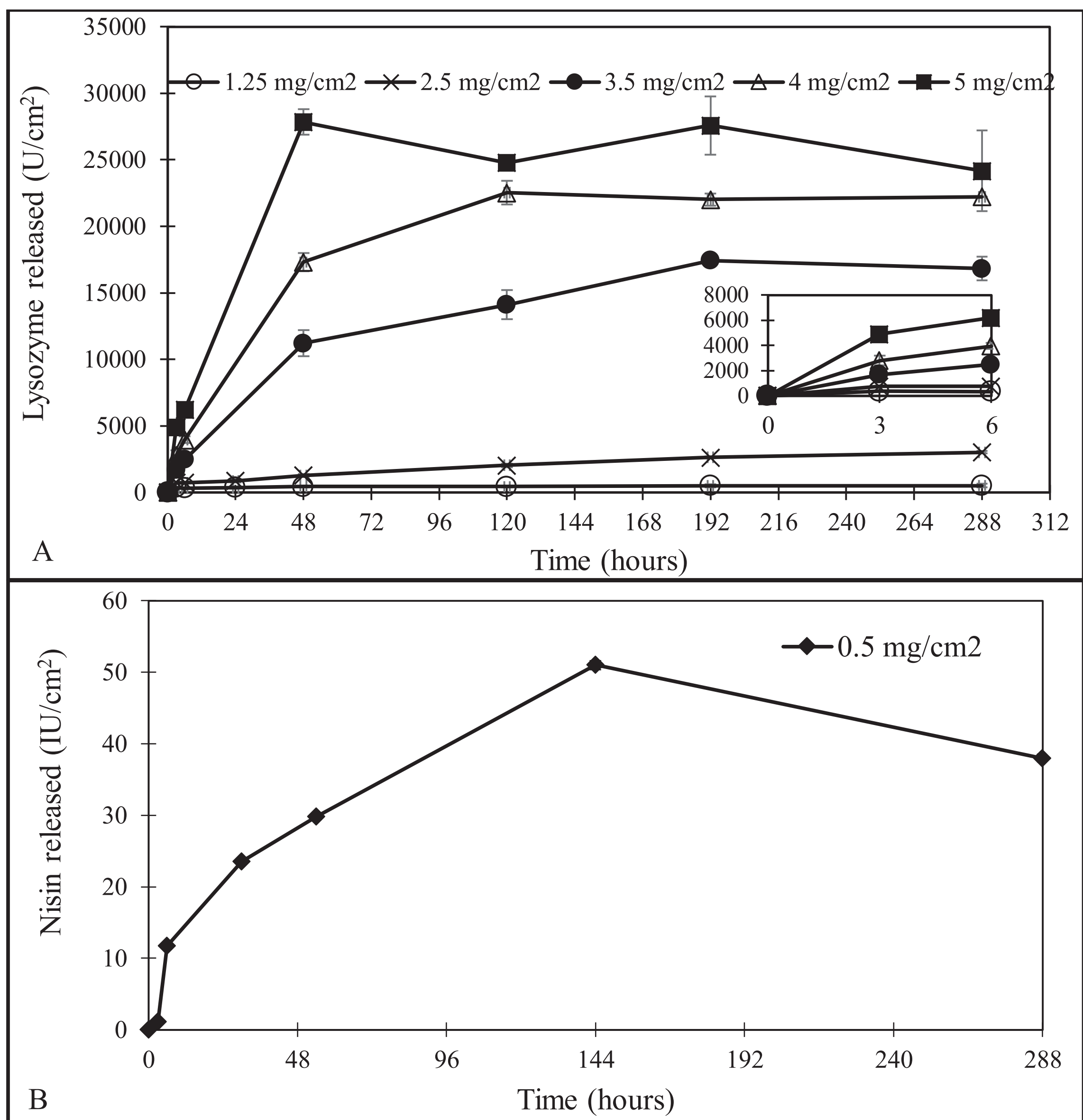

Figure 5.2. Lysozyme (A) or nisin (B) release profile of chitosan films containing lysozyme or nisin (release profiles of lysozyme in the first 6 h is given separately).

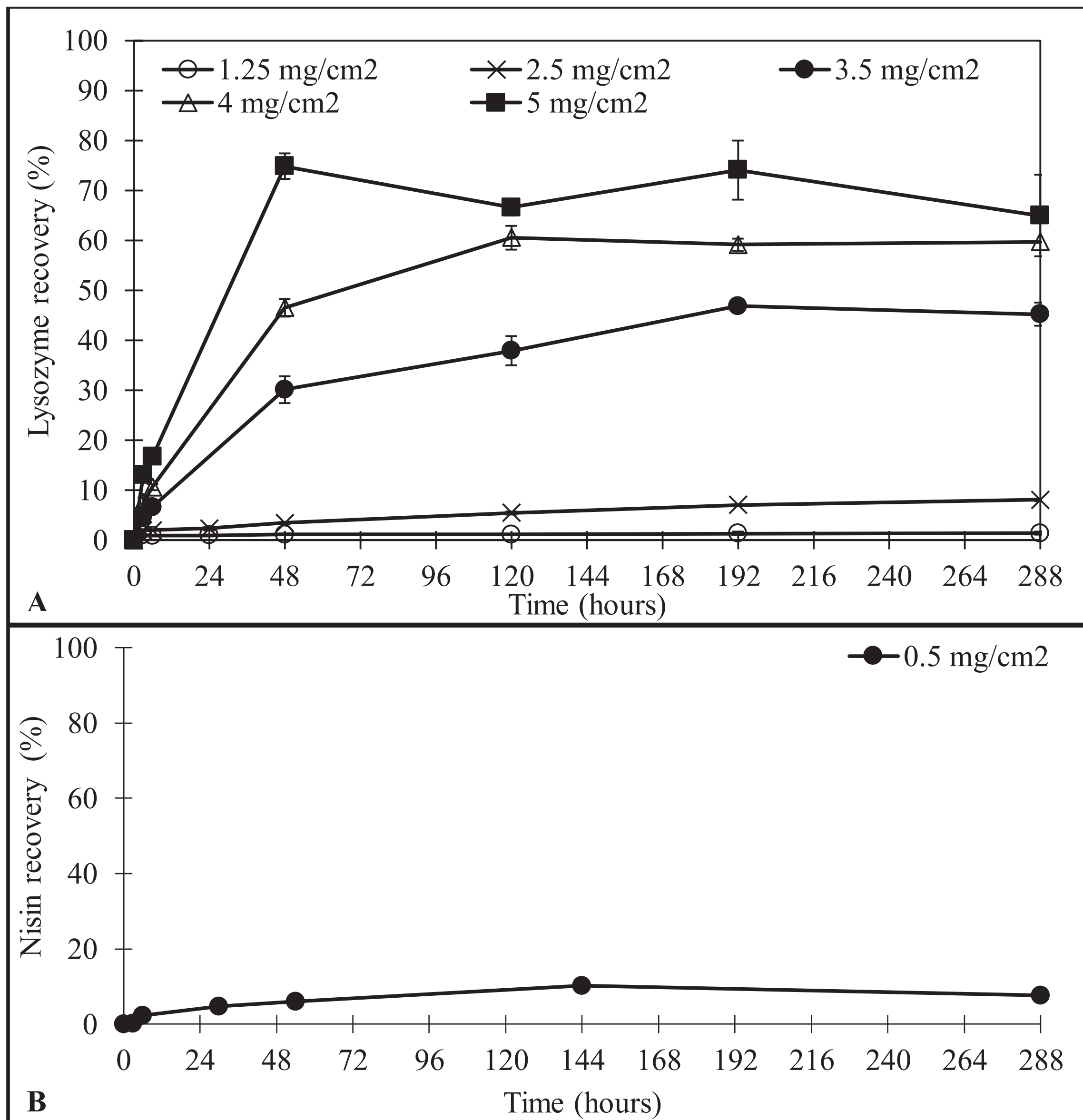

Figure 5.3. Recovery levels of lysozyme (A) and nisin (B) from different LMW chitosan films

5.5.3. Morphology of Chitosan Films

The morphological structures of the developed films were investigated through SEM cross-sectional images which were given in Figure 5.4. The incorporation of NIS or LYS alone did not cause a dramatic change in the dense structure of LMW CHI films observed with SEM (Fig 5.4A), but this resulted in the formation of some limited number of visible protein aggregates within the film matrix (Fig. 5.4B and 4C). In contrast, the addition of LYS-NIS mixture caused the formation of extensive protein aggregates within the films (Fig. 5.4D). It appeared that the combination of LYS and NIS caused

aggregation of these protein-based agents. It is also evident that the aggregates formed contain both LYS and NIS that could not be bound by the film matrix and solubilize slowly by swelling of films.

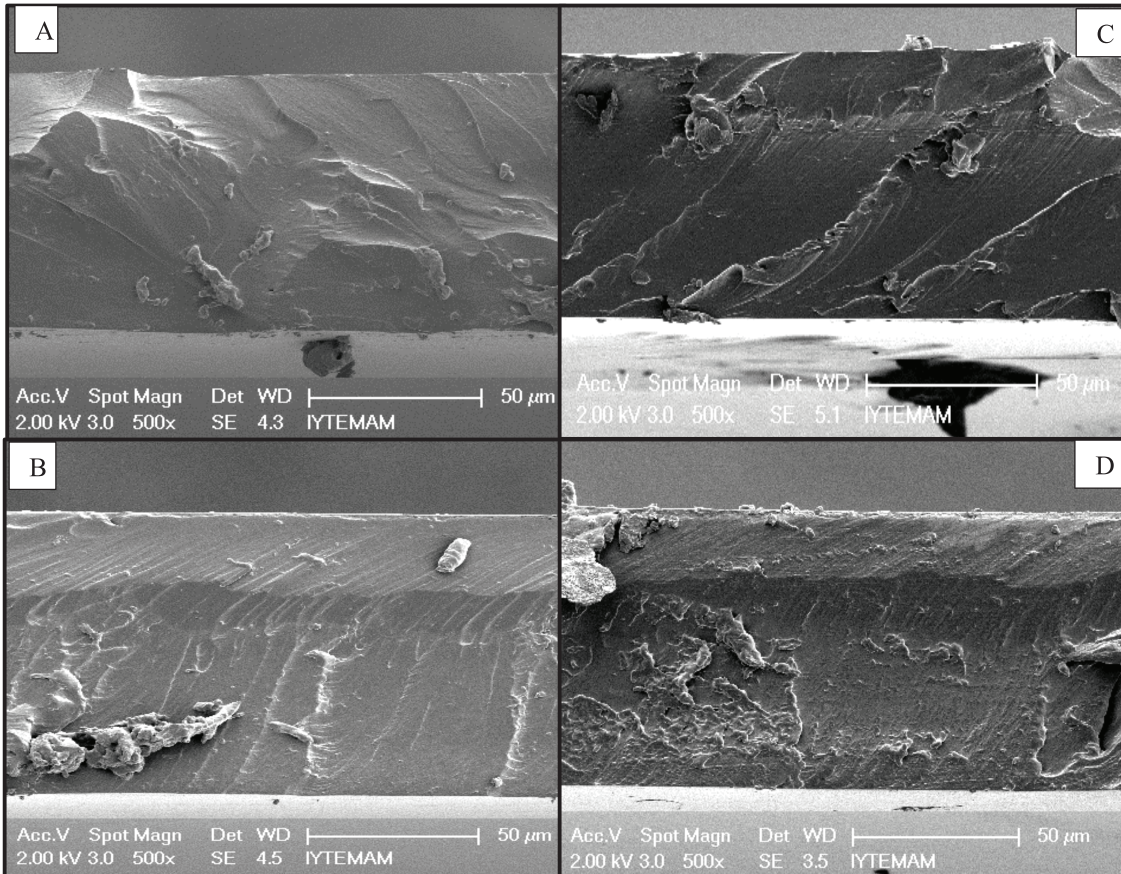

Figure 5.4. SEM photographs of chitosan films (Magnification x500; A: Control; B: 3.5 mg/cm^2 lysozyme; C: 0.5 mg/cm^2 nisin; D: 3.5 mg/cm^2 lysozyme and 0.5 mg/cm^2 nisin

5.5.4. Mechanical Properties of Chitosan Films

Mechanical properties of developed films comprising of tensile strength, elongation and Young's modulus are presented in Table 5.4. No significant changes occurred in tensile strength and elongation of chitosan films by incorporation of nisin and lysozyme alone. The incorporation of LYS-NIS into films did not also cause significant changes in elongation of films, but it caused a slight reduction in film's tensile strength that could be related to interruptions in the film matrix caused by LYS-NIS aggregates (see SEM micrographs). On the other hand, the Young's modulus of films showed a greater variation than their other parameters. In particular, the significant increase in

Young's modulus of films by incorporation of lysozyme alone indicated increased film networking possibly due to the binding of lysozyme by the LMW chitosan matrix.

Table 5.4. Mechanical properties of chitosan films

Incorporated concentrations (mg/cm2)		**Tensile Strength (MPa)**	**Elongation (%)**	**Young's modulus (MPa)**
LYS	**NIS**			
-	-	2.98 ± 0.86^{A}	30.67 ± 7.19^{AB}	0.09 ± 0.01^{D}
3.5	-	2.26 ± 0.46^{AB}	36.99 ± 6.69^{A}	1.78 ± 0.20^{A}
-	0.5	2.79 ± 1.04^{AB}	34.50 ± 7.49^{AB}	0.72 ± 0.16^{B}
3.5	0.5	1.79 ± 0.18^{B}	28.15 ± 7.42^{B}	0.43 ± 0.18^{C}

A-D Values at each column followed by different capital letters indicate statistically significant differences ($P < 0.05$).

5.5.5. Antilisterial Activites of Chitosan Coatings

The antimicrobial effect of chitosan coating incorporated with LYS (3.5mg/cm^2) and NIS (0.5mg/cm^2) on *L. innocua* in inoculated seeds of mung bean, wheat and lentil was shown in Figure 5.5 and the statistical differences between coated and uncoated seeds were also given in Table 5.5. The initial load of the seeds was adjusted to 10^5 CFU/g of seed that was decreased by the help of both control coating and LYS:NIS chitosan coating. 2.5 D, 3.6 D and 2.8 D reductions were monitored in the counts of mung bean, wheat and lentil seed coated with control chitosan film, respectively. The initial load of mung bean was significantly lower compared to the other seeds. This can be attributed to its surface structure. Because, mung bean surface has no cracks or pores to allow the water absorption (Miano et al., 2016), whereas wheat has rough surface structure (Cromey et al., 1998) and thus the bacterium may easily cling to its surface. On the other hand, the reductions were more efficient and significant in seeds coated with LYS:NIS chitosan film compared to seeds coated with control film. The highest inhibition resulted from LYS:NIS chitosan coating against *L. innocua* was obtained in wheat seeds with the more than 4.06 D and it was followed by lentil (3.42 D) and mung bean (3.3 D). The crucial point in these reductions is that, LYS:NIS chitosan coating in all type of seeds tested achieved more than 3 D reductions in the bacterium count as recommended by CFIA

(2018) which stated a requirement of antimicrobial treatment for accomplishing minimum 3 D reductions in microbial pathogen load of seeds before sprouting. In addition, a decontamination method to employ for seeds to be sprouted is also recommended by the FDA (Peñas et al., 2010). Therefore, this method can be considered as an innovative treatment for eliminating pathogens from seeds to be sprouted. Because, contaminated seeds are the main reason of pathogen contaminated sprouts whose consumption is associated with many outbreaks originated from *L. monocytogenes* (CDC, 2014c), *E. coli* O104:H4 (EFSA, 2011a), *Salmonella* (EFSA, 2011b; CDC, 2016c), *Yersinia enterocolitica* and *Bacillus cereus* (EFSA, 2011b). In order to eliminate these pathogens from the seed, many methods can be applied. However, some of the decontamination methods may adversely affect the germination rate and thus, sprout yield, or result in chemical residuals that have negative health effects on consumers. Moreover, some of the methods such as irradiation, high-pressure processing has been reported as insufficient to decontaminate the seeds, whereas hot water treatment of seed was stated as efficient, but leads to undesirable changes in organoleptic properties of mung bean seeds (Mohammad et al., 2019).

In the light of this knowledge, the newly developed non-chemical chitosan coating incorporated with antimicrobials having GRAS status meets the criteria of CFIA and the recommends of FDA can be considered as a novel method to eliminate *Listeria monocytogenes* in seeds to be sprouted.

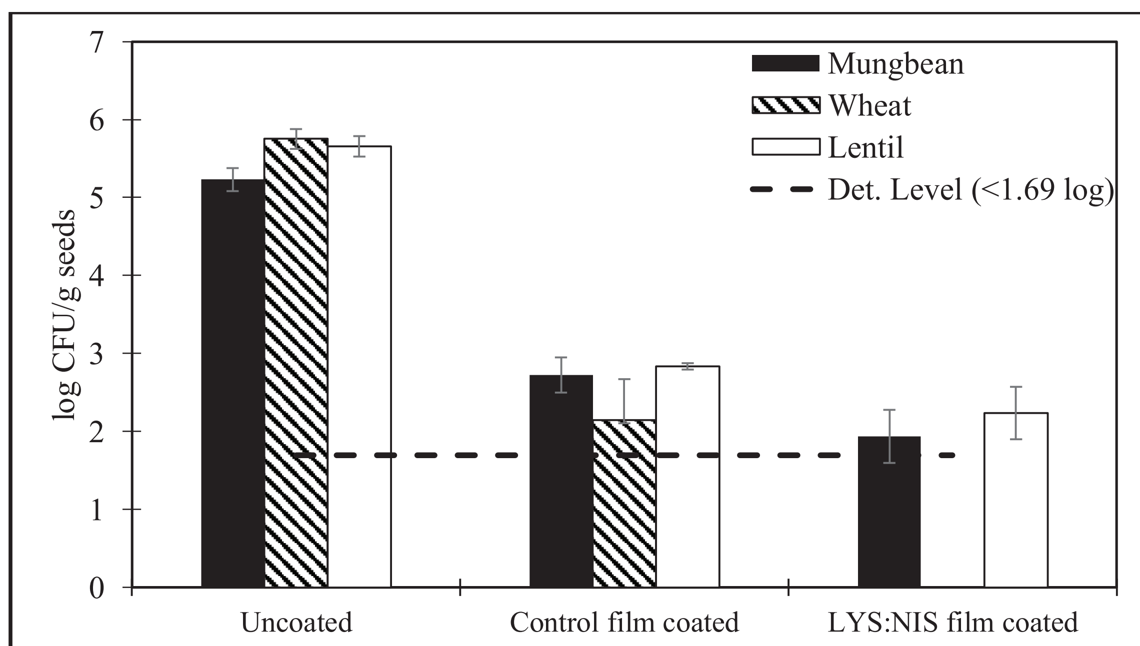

Figure 5.5. Effect of coating on *L. innocua* on seeds

Table 5.5. Effect of coating on *L. innocua* on seeds

Seeds	Uncoated	Control film coated	LYS:NIS film coated
Mung bean	5.23 ± 0.14 b,A	2.72 ± 0.22 a,B	1.93 ± 0.33 a,C
Wheat	5.75 ± 0.12 a,A	2.14 ± 0.52 b,B	< 1.69
Lentil	5.65 ± 0.13 a,A	2.83 ± 0.04 a, B	2.23 ± 0.33 a,C

a-b Values at each column followed by different lowercase letters indicate statistically significant differences ($P < 0.05$). A-C Values at each row followed by different capital letters indicate statistically significant differences ($P < 0.05$).

5.5.6. Effect of Coating on Germination Rate of the Seeds

Not only the antimicrobial activity of a decontamination method in seeds is important, but also germination rate of the seeds exposed to that decontamination method is worth to evaluate. The germination rates of mung bean, lentil and wheat were depicted in Figure 5.6 and the statistical differences during the germination period for each seed type and between treatments for each seed were given in Table 5.6. The germination rates in both coated and uncoated mung seeds did not differ from each other ($P > 0.05$). High-level germination rates ranging from 94% to 98% and from 95% to 98% among mung bean and wheat were monitored during the 3 days, respectively. Therefore, the coating of seeds did not affect on the germination rate of mung bean and wheat. However, the germination percentage of coated lentil was significantly lower compared to uncoated lentils during monitoring ($P < 0.05$). But, the rate reached 93% in coated lentils on the last day of monitoring.

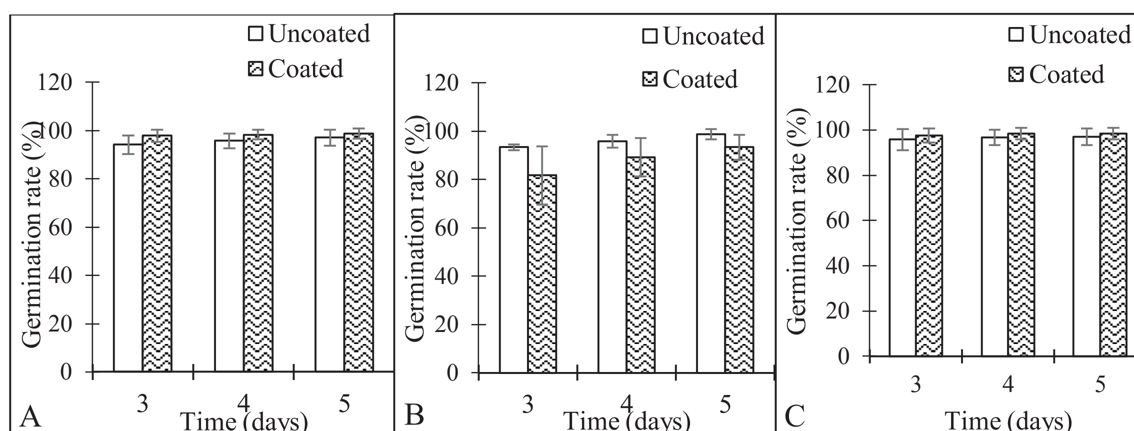

Figure 5.6. Germination rates of coated and uncoated mung bean (A), lentil (B) and wheat (C)

Table 5.6. Germination rates of coated and uncoated mung bean, lentil and wheat

Seed type	Germination rate (%)		
Mung bean	**Day 3**	**Day 4**	**Day 5**
Uncoated	94.17 ± 3.76 ^{a,A}	95.83 ± 3.03 ^{a,A}	97.08 ± 3.32 ^{a,A}
Coated	97.92 ± 2.46 ^{a,A}	98.33 ± 2.04 ^{a,A}	98.75 ± 2.09 ^{a,A}
Lentil	**Day 3**	**Day 4**	**Day 5**
Uncoated	93.33 ± 1.29 ^{b,A}	95.83 ± 2.58 ^{b,A}	98.75 ± 2.09 ^{a,A}
Coated	81.67 ± 12.11 ^{b,B}	89.17 ± 7.85 ^{ab,A}	93.33 ± 5.16 ^{a,B}
Wheat	**Day 3**	**Day 4**	**Day 5**
Uncoated	95.83 ± 4.65 ^{a,A}	96.67 ± 3.42 ^{a,A}	97.08 ± 3.68 ^{a,A}
Coated	97.50 ± 3.16 ^{a,A}	98.33 ± 2.58 ^{a,A}	98.33 ± 2.58 ^{a,A}

a-b Values at each row followed by different lowercase letters indicate statistically significant differences ($P < 0.05$). A-B Values at each column followed by different capital letters indicate statistically significant differences between treatments of each seed type ($P < 0.05$).

5.6. Conclusion

The results obtained at this chapter of the thesis clearly showed that the incorporation of synergetic lysozyme-nisin mixtures into inherently antimicrobial chitosan films created a highly potent antimicrobial mechanism against *L. innocua.* The antimicrobial tests conducted with high, low and medium molecular weight chitosan proved that the use of low molecular weight chitosan films employing synergetic lysozyme-nisin mixture is the most potent combination against *L. innocua.* Moreover, it is also determined that the potency of the developed lysozyme-nisin-chitosan films is inversely proportional to the initial bacterial load during refrigerated storage. The coating studies conducted for the first time in this thesis with different seeds such as lentil, wheat, and mung bean showed that the low molecular weight chitosan coatings with synergetic lysozyme-nisin mixtures could be effectively used to prevent listeriosis from sprouted seeds. The almost 3 decimal reduction of the listerial load of seeds met the Canadian Food Inspection Agency's disinfection requirements necessary to apply for seeds destined for sprouting.

CHAPTER 6

LYSOZYME-NISIN SYNERGY IN COMBINATION WITH REFRIGERATION AGAINST LACTIC ACID BACTERIA: TEST OF FINDINGS IN BOZA

6.1. Introduction

Boza is a traditional fermented beverage that is a colloidal suspension of hydrocolloids with a sweet and slightly sour taste. It is consumed in a large geographical area including the majority of countries in the Balkan Peninsula, in some parts of Caucasus and in Turkey where it is served together with cinnamon and unsalted roasted chickpeas during winter months (Gotcheva *et al.*, 2001, Yeğin and Üren, 2008). It is produced from a slurry containing a mixture of different pulses and cereals such as bulgur, maize, chickpea, millet, wheat or rice by fermentation of a microbial flora dominated heavily by lactic acid bacteria (LAB). Depending on its cereal composition, and fermentation and storage conditions total dry matter, protein, total sugar and ash content of boza could vary between 5.57% to 29.82%, 0.27 % to 2.75%, 10.62% to 22.59%, and 0.02% to 0.17%, respectively (Altay *et al.*, 2013). However, the characteristic pleasant sour taste of boza is originated from its lactic acid content that varies between 0.3% and 0.6% (w/v) (Akpinar-Bayizit *et al.*, 2010; Arici and Daglioglu, 2002; Petrova and Petrov, 2017). Some yeasts are also involved in boza fermentation (Akpinar-Bayizit *et al.*, 2010; Arici and Daglioglu, 2002), but the conditions during fermentation allow the formation of only 0.5% to 2% alcohol by volume in this beverage (Petrova and Petrov, 2017).

The global interest in fermented functional beverages including boza has been increasing continuously since they show different health benefits and they could be produced without the use of thermal processing and chemical additives (Altay et al., 2013; Marsh et al., 2014). The health benefits of boza are related mainly to its positive effects on the human gastrointestinal system and attributed mainly to its probiotic LAB and prebiotic content originated from exopolysaccharides produced by LAB (Heperkan et al., 2014) and cereal dietary fiber (Prado et al., 2008; Vasudha and Mishra, 2013; Petrova and

Petrov, 2017). Moreover, Kancabaş and Karakaya (2012) reported that boza is a good source for antihypertensive peptides. However, the content and profile of probiotic, prebiotic and bioactive peptide of boza in different countries could be variable due to differences in the mixture of cereals and microbiota used in fermentation. For example, it was reported that the Bulgarian boza is formed 70% by LAB, while microbiota of Turkish boza is formed 98% by LAB (Petrova and Petrov, 2017). *Lactobacillus plantarum* is of the most frequently isolated lactic acid bacteria in Turkish boza (Kivanc et al., 2011), but other lactic acid bacteria such as *Lactobacillus fermentum* (Hancioğlu and Karapinar, 1997; Kivanc et al., 2011), *Lactobacillus sanfrancisco*, *Lactobacillus coryniformis*, *Lactobacillus confusus, Leuconostoc paramesenteroides, Leuconostoc mesenteroides* subsp. *mesenteroides, Leuconostoc mesenteroides* subsp. *dextranicum, Leuconostoc oenos* (Hancioğlu and Karapinar, 1997), *Lactobacillus brevis* (Kivanc et al., 2011; Dogan and Ozpinar, 2017), *Lactobacillus paracasei subsp. paracasei, Lactobacillus acidophilus, Lactobacillus paraplantarum, Lactobacillus graminis, Lactococcus lactis subsp. lactis, Leuconostoc citreum, Enterococcus faecium* and *Pediococcus species* (Kivanc et al., 2011) had also been isolated from Turkish boza samples.

The shelf-life of boza varies from 3 to 15 days, depending on the amount of fermentable carbohydrates, the profile of microbiota and storage conditions (Gotcheva et al., 2001; Tangüler, 2014). The shelf-life highly correlates to the amount of organic acids (mainly lactic acid) produced in the beverage by fermentation, and a boza having a pH below 3.5 is generally considered unsuitable for consumption (Blandino et al., 2003; Altay et al., 2013). The temperature abuse during transportation and storage causes undesirable changes in composition (fermentable sugars, lactic acid and other organic acids, and alcohol) and sensory properties of boza due to the uncontrolled growth of microbiota (Akpinar-Bayizit et al., 2010). The pasteurization and chemical additives are not preferred in boza preservation since they destroy probiotic LAB and show negative effects on the characteristic aroma and flavor of this traditional beverage. Thus, the use of biopreservatives in boza at slightly inhibitory to bacteriostatic concentrations could be an attractive option to suppress LAB mediated spoilage without significant destruction of probiotic LAB and impairing original attributes of boza.

In this part of the Ph.D. thesis, it was aimed to use natural and Generally Recognised as Safe (GRAS) biopreservatives lysozyme (LYS) and nisin (NIS) alone or in combination in order to control LAB in Turkish boza and to delay its acidic spoilage

without causing considerable destruction in LAB. The preservation of boza with natural antimicrobials without significant destruction on LAB opens a new perspective to standardize/increase shelf-life of this probiotic beverage and increase its worldwide consumption as a functional food. Besides, all results presented in this chapter were already published by Sozbilen et al. (2018).

6.2. Experimental Study

In the following sections, both materials and methods performed for antimicrobial application in boza were given in detail.

6.2.1. Materials

Hen egg white lysozyme (L6876 with a minimum activity of 40000 unit/mg protein), nisin from *Lactococcus lactis* (N5764), *Micrococcus lysodeikticus* as a substrate of lysozyme in enzyme activity determination and Tween 20 were purchased from Sigma-Aldrich Chem. Co. (St. Louis, MO, USA). D-/L-lactic acid kit was obtained from NYZTech (Cat. No. AK00141, Lisbon, Portugal). MRS broth, peptone water and agar used in the enumeration of LAB were obtained from Merck (Darmstadt, Germany). The strain of *Lactobacillus plantarum* (NRRL-B4496) obtained from ARS Culture Collection (NRRL) was kindly provided by Dr. Burcu Öztürk, from the Department of Food Engineering, Izmir Institute of Technology, İzmir. Four batches of freshly prepared boza (obtained from the mixture of corn, wheat, and millet) were purchased from Şemikler Bozacısı in Karşıyaka, İzmir (Turkey) at different periods.

6.2.2. Methods

The methods given below were performed to test the effect of lysozyme and nisin effects in boza.

6.2.2.1. Effects of NIS and/or LYS on *L. plantarum* in Broth Medium

Variable amounts (250 or 500 µg/mL) of NIS and/or LYS were added into 45 mL of MRS broth adjusted to pH 4.0 with 0.1 N HCl and it was inoculated with 5 mL of *L. plantarum* culture (10^7 CFU/mL) that was used as model LAB to estimate the potential antimicrobial effects of NIS and LYS. The inoculated broth was then cold stored at 4°C for 21 days and the survivors of *L. plantarum* were enumerated periodically at 0th, 3rd, 7th, 14th and 21st days. For this purpose, broth from each group was serially diluted with 0.1% sterile peptone water and enumerated by using the pour plate method in MRS agar after 2-days incubation at 30 °C. The enumeration was performed in duplicate at each dilution.

6.2.2.2. Preparation of NIS and/or LYS Containing Boza for Cold-Storage

The experiments conducted during the cold storage of boza were carried out by using four different batches of boza samples. Batch#1 was used in the determination of LYS and NIS stability in cold-stored boza; Batch#2 was used in the determination of LYS and/or NIS effect on LAB, pH, titratable acidity, and D- and L- lactic acid content in boza during cold storage. Batch#3 and Batch#4 were used in sensory analysis and e-nose analysis, respectively. Each batch was separated into four different groups by weighting into sterile bottles under aseptic conditions. These treatment groups were as follows: (1) Control group; (2) NIS (250 µg/g) containing group; (3) LYS (500 µg/g) containing group; (4) LYS:NIS (500:250 µg/g) containing group. The applied NIS and LYS concentrations of boza were the maximum allowed concentrations in food products according to the US Food and Drug Administration (FDA) (FDA, 1988) and European Union Regulation (European Commission (EC) Regulation No. 2066/2001 (EC, 2016), respectively. After the addition of NIS and/or LYS samples stirred extensively with a sterile glass rod were cold-stored for 28 days at 4 °C. Samples taken periodically under aseptic conditions were used in different tests given below. Two bottles were prepared for each treatment group.

6.2.2.3. Stability of LYS in Cold-Stored Boza

The stability of LYS was determined by monitoring change of its activity in cold-stored boza samples at 0th, 3rd, 7th, 14th, 21st and 28th days. The measurements were conducted at pH 3.5 and pH 6.5 to determine activity changes at a minimal pH observed in boza and at pH close to the enzyme's optimum pH, respectively. Briefly, 0.5 mL of LYS containing boza was transferred into an Eppendorf tube and it was diluted 1:3 with 0.05 M citrate-phosphate or 0.05 M Na-phosphate buffers at pH 3.5 and 6.5, respectively. The samples were then centrifuged at 15000 g at 4 ºC for 15 min. After that 0.1 mL of the supernatant was then brought to 30 °C and mixed with 2.4 mL of *Micrococcus lysodeikticus* (0.26 mg/mL) solution prepared in 0.05 M citrate-phosphate buffer at pH 3.5 or 0.05 M Na-phosphate buffer pH 6.5 at 30 °C. The measurement of residual activity of LYS was conducted at 660 nm by a spectrophotometer (Shimadzu Model 2450, Japan) equipped with a constant temperature cell holder working at 30 °C. The absorbance measurements were recorded for 2 min and activity calculated from the slope of the initial linear portion was given in Units per g of boza (1 Unit corresponds to 0.001 changes in absorbance per min).

6.2.2.4. Stability of NIS in Cold-Stored Boza

The stability of NIS in boza was determined by monitoring change of its concentration in cold-stored samples at 0th, 3rd, 7th, 14th, 21st and 28th days. The NIS concentration was determined by the agar diffusion method (Teerakarn *et al.*, 2002) using *L. plantarum* NRRL-B4496 as test microorganism. Serial dilutions of NIS (500 IU/mL) prepared in sterile 0.05 M citrate-phosphate buffer at pH 4.0 was used to prepare the standard calibration curve. Briefly, the bacteria culture was inoculated into MRS broth and incubated for 24 h at 30 °C. Then, the culture of freshly grown cells was adjusted to 0.5 Mac Farland unit with 0.1% of peptone water, and the diluted culture was seeded into MRS test agar which was prepared by adding 0.75% of agar and 20 mL/L of 50% of Tween 20 into MRS broth. Twenty mL of the inoculated agar was then poured into Petri dishes and solidified for almost 3 h at room temperature. Three wells were then opened on the surface of each agar by using a sterile 6mm-diameter cork-borer, and 50µL of NIS containing boza sample (or NIS solution for preparation of standard curve) was added

into the wells. The boza samples were prepared by 1:3 dilution of 0.5 mL of the sample with sterile 0.05 M citrate-phosphate buffer at pH 4.0, and clarification of mixture by 15 min centrifugation at 4 °C and 15000 g. The Petri dishes were incubated in an anaerobic jar with Aerocult C (Merck, Darmstadt, Germany) at 37 °C for 16-18 h to grow the test bacteria and observe the formation of visible clear zones formed around wells by NIS. The diameter of each well and clear zone were measured from three different points using a digital caliper (Mitutoyo IP67, Japan). The experiment was conducted in duplicate with three replications. The standard curve was obtained by plotting the logarithm of NIS concentration *vs.* diameter of the clear zones. The concentration of NIS in boza was expressed as IU per g of boza.

6.2.2.5. Effects of NIS and/or LYS on LAB in Cold-Stored Boza

The change in LAB counts of control, NIS (250 µg/g), LYS (500 µg/g) and LYS:NIS (250:500 µg/g) containing boza were monitored at 0th, 3rd, 7th, 14th, 21st and 28th days. For this purpose, 10 mL of boza sample from each group was serially diluted with 0.1% sterile peptone water. The LAB were enumerated by using double layer pour plate method in MRS agar incubated for two days at 30 °C. The enumeration was performed in duplicate for each sample and LAB counts were expressed as log CFU/mL of boza.

6.2.2.6. Monitoring of D- and L- Lactic Acid Concentrations in Cold-Stored Boza

The concentration of D-lactic acid and L-lactic acid in boza was determined spectrophotometrically at 340 nm for 0th, 14th, 21th and 28th days of cold-storage using D-/L-Lactic acid kit (NZYTech) according to manufacturer's instructions. The measurements were performed in duplicate and results were expressed as g per L of boza.

6.2.2.7. Monitoring of pH and Titratable Acidity in Cold-Stored Boza

10 mL of boza was diluted 10 fold with deionized water, mixed by vortex for 5 s, and measured with a pH meter (WTW, Inolab, Multilevel-3, Germany) (AOAC, 2006). The measurements were performed twice.

The titratable acidity of boza samples was determined by the titration method described by Cemeroğlu (2007). The samples were diluted 10 fold with deionized water and titrated with 0.1 N NaOH until the endpoint identified by phenolphthalein indicator. The acidity was expressed as a percent of lactic acid equivalents. The measurements were performed twice.

6.2.2.8. Sensory Analyses of Cold Stored Boza

Sensory analyses of cold stored boza samples were conducted at the 0th, 7th and 14th days of cold-storage as described by Akpinar-Bayizit *et al.* (2010). The analysis was performed by 10 semi-trained panelists who rated six attributes (color, odor, texture, taste, mouthfeel and overall acceptability) for each sample on a five-point hedonic scale where 1 and 5 corresponded to dislike extremely and like extremely, respectively. Panelists were chosen among the graduate students of Food Engineering Department at İzmir Institute of Technology who were willing to taste boza. Since the panelists did not consume boza frequently, a briefly 30-min training session was conducted to the panelists to remind desired attributes of boza using fresh boza at optimal quality and to inform them about possible negative changes in the sensory attributes of boza during storage. Panelists assessed the samples in individual booths at a sensory analysis laboratory. Samples were served at 10±1 °C in glasses coded with three-digit numbers. The sensory evaluation was performed twice in different sessions.

6.2.2.9. E-Nose Analysis of Cold-Stored Boza

The effects of NIS and/or LYS on aroma profiles of cold-stored boza were determined at 0th, 7th and 14th days by using an e-nose (zNose^{TM} 7100 vapor analysis system, EST, Newbury Park, CA, USA) containing a 1-m DB-5 column and a surface acoustic wave (SAW) detector with a parts per billion sensitivities. The analysis method was modified from Kadiroğlu *et al.* (2011). Briefly, 10 g of boza sample was put into a 20 mL septa-sealed screw cap vial and kept at 40 °C for 40 min in an incubator to equilibrate the headspace volatile components. The vapor of the sample was introduced into an e-nose device through an injection 5-cm-long needle. The operating conditions of e-nose were programmed as follows: injection time of 10 s, inlet temperature of 200 °C,

valve temperature of 165 °C, SAW detector temperature of 20 °C, column ramp temperature from 40 to 180 °C at 6 °C/s, helium flow rate of 4 cm^3/min and data acquisition time of 10 s. Data were collected in every 0.02 s using Microsense software (Newbury Park, CA, USA). Two vials for each treatment group were prepared and e-nose measurement for each vial was run in triplicate.

The data of e-nose analysis were analyzed by using partial component analysis (PCA) that is a multivariate method. This analysis was performed with SIMCA 13.0.3 software (Umetrics, Sweden). PCA is an unsupervised technique that reduces the dimensionality of the data matrix to convert a set of a large uncorrelated variable into a few new linearly correlated variables called as principal components (Uncu and Ozen, 2016). The first principal component (PC1) covers a maximum variation of data and it is orthogonal to the second principal component (PC2) which is also orthogonal to the PC3 and covers as much of the remaining variation in the data as possible compared to PC3 and so on (Kara, 2009). The scatter plot obtained from the results of PCA shows that how the different observations distributed to differentiate from each other by forming a cluster.

6.2.2.10. Statistical Analyses

The statistical comparisons of the mean values for microbiological, analytical and sensory analyses were performed by using analysis of variance (ANOVA) with Fisher's multiple comparison test (Minitab, State College, PA). Differences at $P<0.05$ were considered as statistically significant.

6.3. Results and Discussions

The detailed results and discussion for this chapter will be given in the following sections.

6.3.1. Effects of NIS and/or LYS on *L. plantarum* in Broth Media

This work aimed to delay the acidic spoilage of boza by LAB without causing a substantial inhibition in these potentially probiotic bacteria. *L. plantarum* is one of the

most frequently isolated lactic acid bacteria in boza not only in Turkey (Kivanc *et al.*, 2011), but also worldwide (Heperkan et al., 2014). Thus, different concentrations of NIS and/or LYS were first tested on this bacterium in MRS broth at pH 4.0, a pH close to that of freshly prepared boza. The results presented in Table 6.1. showed that LYS alone was not effective on *L. plantarum* during 21-day incubation. The samples with LYS:NIS combinations at 1:1 (250:250 µg/mL or 500:500 µg/mL) ratio showed significantly (P<0.05) lower *L. plantarum* counts than control at 0th day. In contrast, LAB counts of all other samples were quite similar to that of control at the 0th day. A significant reduction in respect of initial *L. plantarum* counts of each sample occurred within 3 days in presence of NIS alone at 250 µg/mL, and LYS:NIS combination at 250:500 µg/mL. It took 7 days for LYS:NIS combination at 500:500 µg/mL to cause a significant reduction (P<0.05) in respect of initial *L. plantarum* count of samples while this took 14 days for samples containing NIS alone at 500 µg/mL and LYS:NIS at 250:250 µg/mL. At the end of 14 days, a period equivalent to the expected shelf-life of boza, NIS alone at 250 µg/mL, NIS alone at 500 µg/mL, and LYS:NIS combination at 250:250 gave similar bacterial counts (P>0.05) that were 1.6 to 1.8 D lower than that of control. On the other hand, the combination of LYS:NIS at 500:500, 250:500, and 500:250 µg/mL gave the highest inhibition levels within 14 days with 3.4, 3.1 and 2.4 D lower *L. plantarum* counts than control, respectively. Further incubation of broths containing NIS alone at 250 or 500 µg/mL, and LYS:NIS combinations at 500:500 or 250:500 µg/mL for 21 days did not considerably change decimal differences between *L. plantarum* counts of these samples and control. However, decimal differences between broths containing LYS:NIS at 250:250 or 500:250 µg/mL and control reached to 2.4 and 2.9 D at the end of 21 days, respectively. Thus, at the studied storage conditions the ranking of the effectiveness on *L. plantarum* was as follows: LYS:NIS at 500:500, LYS:NIS at 250:500, LYS:NIS at 500:250, LYS:NIS at 250:250, NIS at 500, and NIS at 250 µg/mL. These results clearly showed the concentration-dependent inhibitory activity of NIS on *L. plantarum* during cold storage. Moreover, it is also clear that NIS was more effective on *L. plantarum* when it was combined with LYS. In the literature, the occasional synergy of LYS and NIS combinations against some LAB strains such as *L. sake* and *L. curvatus* had been reported by Chung and Hancock (2000). In the current work, the synergy between LYS and NIS was observed when test results at some specific concentrations were evaluated carefully. For example, it is important to report that LYS:NIS combination at 250:250 µg/mL

caused significantly lower (minimum 0.7 D) *L. plantarum* counts than LYS alone at 500 µg/mL or NIS alone at 500 µg/mL at the end of 21 days.

Table 6.1. Effects of different concentrations of NIS and/or LYS on *L. plantarum* in broth media at 4 °C

Concentrations (µg/mL)		*L. plantarum counts* (log CFU/mL)				
LYS	**NIS**	**Day 0**	**Day 3**	**Day 7**	**Day 14**	**Day 21**
-	-	6.62 ±0.15 ^{b,A}	6.99 ±0.20 ^{a,A}	6.88 ±0.10 ^{ab,A}	7.10 ±0.36 ^{a,A}	6.94 ±0.12 ^{ab,A}
250	-	6.66 ±0.12 ^{c,A}	6.74 ±0.13 ^{bc,B}	6.94 ±0.02 ^{a,A}	6.88 ±0.10 ^{ab,A}	6.99 ±0.12 ^{a,A}
500	-	6.68 ±0.10 ^{a,A}	6.74 ±0.12 ^{a,B}	6.80 ±0.12 ^{a,A}	6.82 ±0.10 ^{a,A}	6.80 ±0.05 ^{a,A}
-	250	6.62 ±0.02 ^{a,A}	5.52 ±0.02 ^{c,E}	5.74 ±0.02 ^{b,CD}	5.53 ±0.06 ^{c,B}	5.34 ±0.04 ^{d,B}
-	500	6.47 ±0.13 ^{a,AB}	6.37 ±0.06 ^{a,C}	6.14 ±0.41 ^{a,B}	5.29 ±0.05 ^{b,B}	5.22 ±0.43 ^{b,B}
250	250	5.95 ±0.48 ^{ab,C}	6.25 ±0.08 ^{a,C}	5.83 ±0.31 ^{ab,C}	5.30 ±0.38 ^{b,B}	4.53 ±0.89 ^{c,C}
500	500	6.26 ±0.36 ^{a,BC}	5.92 ±0.03 ^{ab,D}	5.50 ±0.16 ^{b,D}	3.73 ±0.51 ^{c,D}	3.51 ±0.05 ^{c,D}
250	500	6.39 ±0.08 ^{a,AB}	5.79 ±0.12 ^{b,D}	4.39 ±0.14 ^{c,E}	4.05 ±0.38 ^{cd,D}	3.76 ±0.41 ^{d,D}
500	250	6.59 ±0.13 ^{a,A}	5.52 ±0.09 ^{b,E}	5.65 ±0.11 ^{b,CD}	4.71 ±0.13 ^{c,C}	4.00 ±0.31 ^{d,CD}

Different lower-case and capital letters indicated statistically significant differences at rows and columns ($P < 0.05$), respectively.

6.3.2. Stability of LYS and NIS in Boza

The residual activities of LYS (at 500 µg/g) in cold-stored boza with or without the presence of NIS (at 250 µg/g) were determined at different pH values. Activity measurements at pH 3.5 were determined to understand the levels of LYS activity ($LYS_{3.5}$) at a minimal pH observed for boza (Fig. 6.1A). According to Smolelis and Hartsell (1952), LYS showed its optimal antimicrobial activity at pH 6.6. Thus, activities at pH 6.5 ($LYS_{6.5}$) were measured to determine changes in enzyme activity close to its optimal pH without acidic stress on the enzyme (Fig. 6.1B). The $LYS_{3.5}$ activities in boza changed between 2000 and 4000 U/g during 28-day cold storage. The activity at acidic conditions showed some fluctuations in the presence of NIS, but it did not drop below 2500 U/g during cold storage. The $LYS_{3.5}$ activity in boza without NIS remained quite stable within 14 days. However, $LYS_{3.5}$ showed a considerable loss (almost 50%) in activity at 21th and 28th days without the presence of NIS. In contrast, the $LYS_{6.5}$ activities of boza showed significantly lower fluctuation than $LYS_{3.5}$ activities, and they varied

between 47000 and 59000 U/g during 28 days of cold storage. Thus, it is clear that the LYS activity levels determined at pH 3.5 for boza accounted for only 3 to 8% of its activity determined at pH 6.5. It is also important to note that a minimum 80% of $LYS_{6.5}$ activities were maintained with or without the presence of NIS in boza during 28 days of cold storage. These results clearly showed that the factors that caused instability and activity loss of LYS in boza were reversible and appeared due to acidic conditions.

The stability of NIS (at 250 µg/g) in boza was also determined during 28 days of cold-storage (Fig. 6.2). The results clearly showed that almost 57% of initial antimicrobial activity for NIS was destabilized within 3 days. However, the destabilization of NIS in boza slowed down after 3 days. It should be reported that the NIS maintained almost 30% and 20% of its initial antimicrobial potential after 14 and 28 days of cold-storage, respectively.

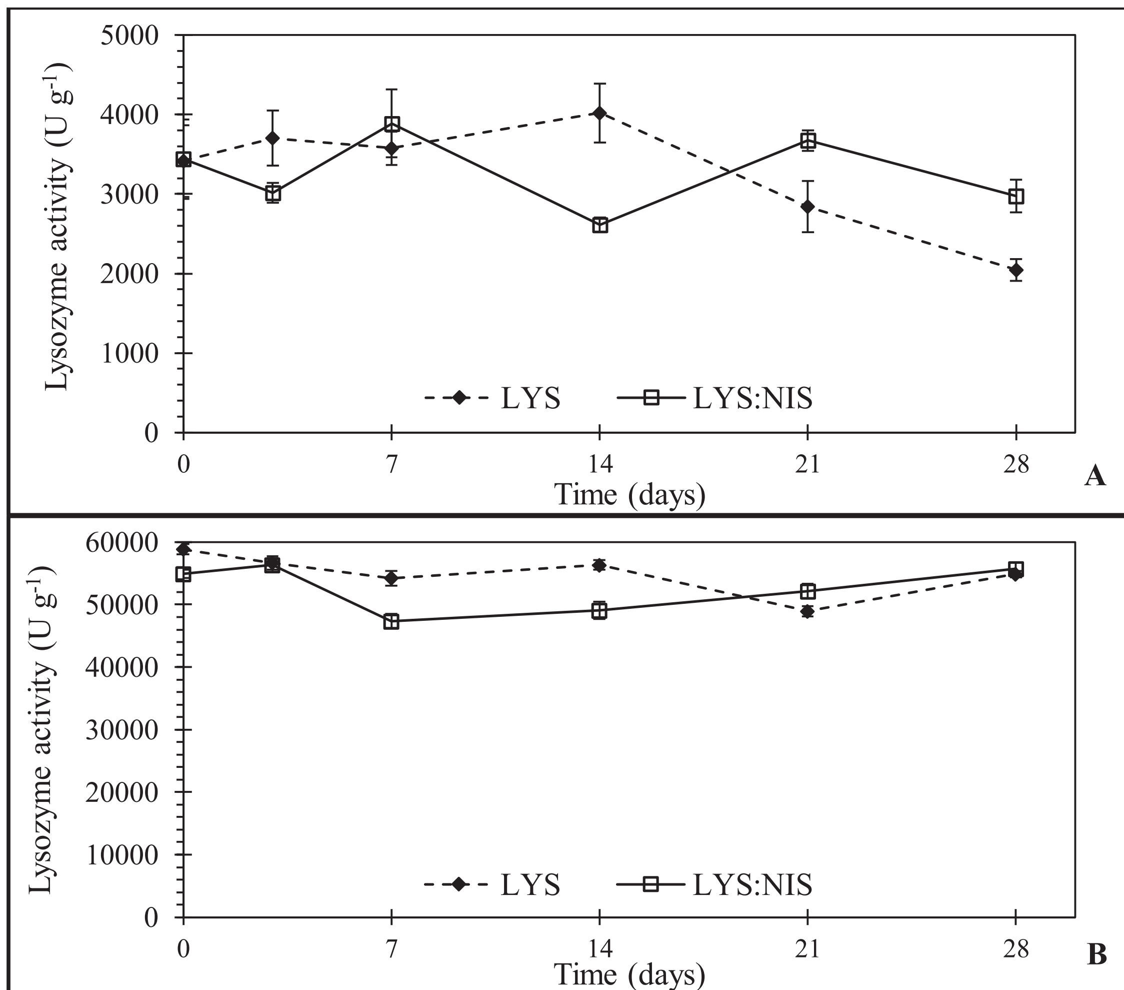

Figure 6.1. Stability of LYS in boza (Batch# 1) during cold storage at 4 °C (activity determination at pH 3.5 (A) and pH 6.5 (B)).

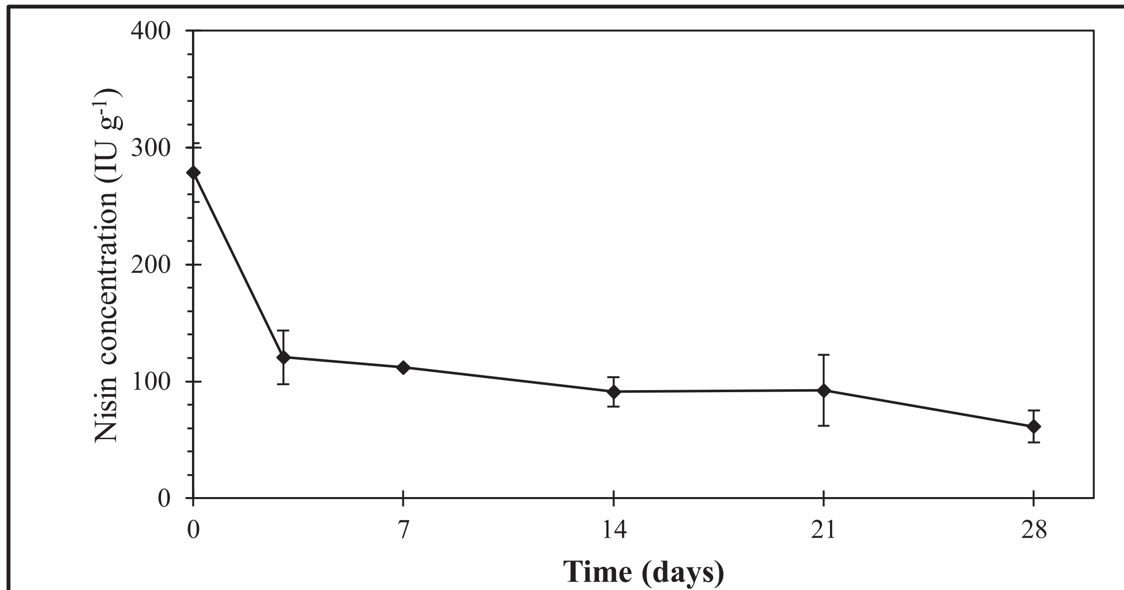

Figure 6.2. Stability of NIS in boza (Batch# 1) during cold storage at 4 °C.

6.3.3. Effects of NIS and/or LYS on LAB in Boza

The effect of NIS at 250 µg/g, LYS at 500 µg/g and LYS:NIS combination at 500:250 µg/g on LAB of cold-stored boza was presented in Table 6.2. The initial LAB counts of control, NIS, LYS or LYS:NIS containing boza samples did not show a statistically significant difference ($P > 0.05$). Thus, it is clear that NIS and/or LYS did not show a potent LAB inhibition at the applied concentrations. It is also important to report that the initial LAB count of control boza sample (6.9 log CFU mL^{-1}) was within the range of LAB counts determined for this fermented beverage by different researchers (between 5.9 and 7.9 log CFU mL^{-1}) (Gotcheva *et al.*, 2000; Morea, 2008; Osimani *et al.*,2015). The cold-storage of control and LYS containing boza samples for 28 days did not cause a significant change ($P>0.05$) in their LAB counts. This finding was expected since LYS alone did not also show a considerable antimicrobial activity on *L. plantarum* even in the broth media. In contrast, boza samples containing NIS alone and LYS:NIS combination showed significantly lower ($P<0.05$) LAB counts than control and LYS containing boza samples starting from the 3rd day of cold-storage. Samples containing NIS and LYS:NIS did not show a significant difference ($P>0.05$) in their LAB counts within the first 14 days of cold storage. However, boza samples containing LYS:NIS showed significantly lower ($P<0.05$) LAB counts than NIS containing boza samples at 21th and 28th days. The overall reduction in the initial LAB counts of boza samples at 14th and 28th days of cold-storage

reached to 0.54 and 0.67 D for NIS, and 0.7 and 0.87 D for LYS:NIS containing boza samples, respectively. The LAB counts of NIS containing boza samples were ≥ 6.0 log CFU/mL during 28 days of cold-storage. However, LAB counts of LYS:NIS containing samples after the 14th day of cold-storage drop below 6.0 log CFU/mL that is essential for a food to be accepted as a probiotic (Espitia *et al.*, 2016). These results suggested that the NIS is the main antimicrobial compound effective on LAB. The LYS showed the antimicrobial effect on LAB at the later stages of cold-storage only when NIS presented in the boza. This finding once more suggested the effectiveness of LYS and NIS combination against LAB.

6.3.4. Effects of NIS and/or LYS on pH, Titratable Acidity and Lactic Acid Concentration of Boza

The pH and titratable acidity of samples containing NIS at 250 µg/g, LYS at 500 µg/g and LYS:NIS combination at 500:250 µg/g were presented in Fig. 6.3A and Fig. 6.3B, respectively. The drop of pH, but the increase of titratable acidity in control samples and samples containing LYS alone were observed very clearly during cold storage. The pH and titratable acidity of control and LYS containing boza samples did not differ significantly from each other during cold storage, but they differentiated significantly ($P<0.05$) from boza samples containing NIS and LYS:NIS combination after the 7th day of cold storage. These data supported the ineffectiveness of LYS alone on LAB to control their total acid production capacity. In contrast, the almost unchanged pH and titratable acidity of boza samples containing NIS and LYS:NIS combination clearly showed the successful control of acid production capacity of LAB in the presence of NIS. The measurements of D- and L-lactic acid in boza (Table 6.3) showed that LAB in boza lacked to form sufficient amounts of L-lactic acid (≤0.14 g/L). This result is expected since D-lactic acid was already reported as the main lactic acid isomer produced by LAB during boza fermentation (Gotcheva *et al.*, 2000). In the current study, the initial D-lactic acid contents in different boza samples were similar, and they changed between 0.8 and 1.0 g/L ($P>0.05$). D-lactic acid in different NIS containing (NIS or LYS:NIS) boza samples showed almost no change during 28 days of cold-storage. Thus, it is clear that NIS alone showed the most effective inhibition of D-lactic acid production capacity of LAB in boza.

Table 6.2. Effects of different concentrations of LYS and/or NIS on lactic acid bacterial counts in boza (Batch 2) at 4 °C

Concentrations (µg/g)		**LAB counts (log CFU/mL)**					
LYS	**NIS**	**Day 0**	**Day 3**	**Day 7**	**Day 14**	**Day 21**	**Day 28**
-	-	$6.84 \pm 0.23^{a,A}$	$6.72 \pm 0.24^{a,A}$	$6.71 \pm 0.12^{a,A}$	$6.71 \pm 0.18^{a,A}$	$6.66 \pm 0.18^{a,A}$	$6.80 \pm 0.07^{a,A}$
500	-	$6.53 \pm 0.36^{a,B}$	$6.80 \pm 0.19^{a,A}$	$6.68 \pm 0.36^{a,A}$	$6.74 \pm 0.25^{a,A}$	$6.65 \pm 0.10^{a,A}$	$6.78 \pm 0.08^{a,A}$
-	250	$6.80 \pm 0.14^{a,AB}$	$6.35 \pm 0.22^{b,B}$	$6.30 \pm 0.21^{bc,B}$	$6.26 \pm 0.19^{bc,B}$	$6.02 \pm 0.08^{d,B}$	$6.13 \pm 0.08^{cd,B}$
500	250	$6.70 \pm 0.04^{a,AB}$	$6.39 \pm 0.32^{b,B}$	$6.21 \pm 0.27^{bc,B}$	$6.00 \pm 0.29^{cd,B}$	$5.78 \pm 0.15^{d,C}$	$5.83 \pm 0.09^{d,C}$

Different lower-case and capital letters indicated statistically significant differences at rows and columns ($P < 0.05$), respectively

All other samples showed a moderate increase in their D-lactic acid content on the 14th day of cold storage. Samples containing LYS:NIS combination showed a decline in their D-lactic acid content at the 21st day of cold storage, and no more D-lactic acid formation was determined in these samples on the 28th day. In contrast, controls and samples containing LYS alone continued to form D-lactic acid at 21st and 28th days of cold storage, respectively. It is important to note that the D-lactic acid content of control samples increased much more rapidly than LYS containing samples, and it was almost doubled at the end of the 21st day. These results suggested that the LYS alone also delayed D-lactic acid formation in boza, but this finding did not show sufficient parallelism with respect to pH and titratable acidity measurements in boza. On the other hand, higher effectiveness of NIS alone than LYS:NIS combination to reduce D-lactic acid formation showed the different responses of D-lactic acid formation mechanism in LAB in the presence of different antimicrobials. Further studies are needed to determine the detailed organic acid profile of boza in presence of different preservatives. However, this work clearly showed the possibility of using NIS alone and LYS:NIS combinations to control acidic spoilage of boza by LAB.

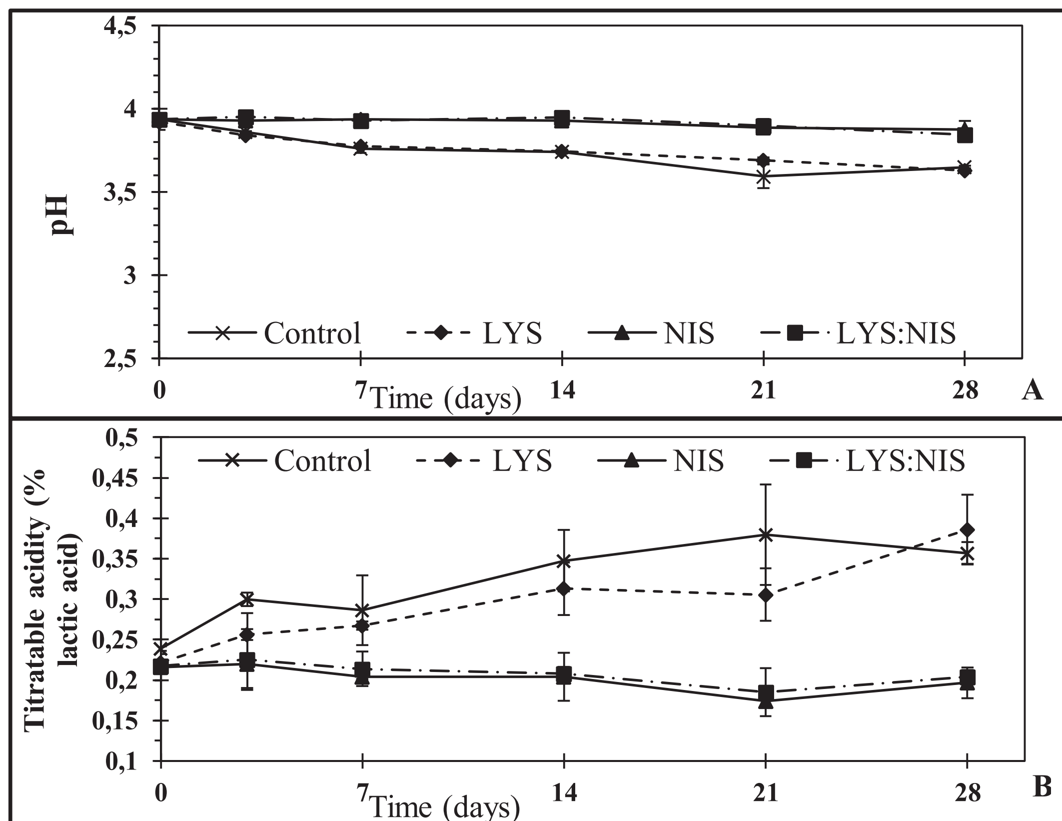

Figure 6.3. Changes in pH (A) and titratable acidity (B) of boza (Batch# 2) samples during cold storage at 4 °C.

Table 6.3. D-/L-Lactic acid concentrations of boza (Batch # 2) samples during cold-storage at 4°C

Concentrations (μg/g)		**D-Lactic acid concentrations (g/L)**			
LYS	**NIS**	**Day 0**	**Day 14**	**Day 21**	**Day 28**
-	-	0.84 ± 0.02 ^{d,A}	1.10 ± 0.04 ^{c,B}	1.61 ± 0.01 ^{a,A}	1.35 ± 0.01 ^{d,B}
500	-	0.89 ± 0.04 ^{c,A}	1.29 ± 0.04 ^{b,A}	1.20 ± 0.03 ^{b,B}	1.49 ± 0.01 ^{a,A}
-	250	0.92 ± 0.02 ^{b,A}	0.91 ± 0.00 ^{b,C}	0.99 ± 0.00 ^{a,C}	1.01 ± 0.00 ^{a,D}
500	250	0.83 ± 0.04 ^{c,A}	1.16 ± 0.01 ^{a,B}	1.03 ± 0.01 ^{b,C}	1.09 ± 0.01 ^{ab,C}
		L-Lactic acid concentrations (g/L)			
-	-	0.01 ± 0.01 ^{b,B}	0.08 ± 0.02 ^{a,A}	0.04 ± 0.00 ^{ab,A}	0.05 ± 0.03 ^{ab,B}
500	-	0.00 ± 0.00 ^{b,B}	0.14 ± 0.08 ^{a,A}	0.03 ± 0.00 ^{ab,A}	0.05 ± 0.01 ^{ab,B}
-	250	0.05 ± 0.00 ^{bc,A}	0.10 ± 0.02 ^{ab,A}	0.02 ± 0.01 ^{c,A}	0.12 ± 0.01 ^{a,A}
500	250	0.00 ± 0.00 ^{c,B}	0.06 ± 0.01 ^{a,A}	0.03 ± 0.00 ^{b,A}	0.05 ± 0.00 ^{ab,B}

Different lower-case and capital letters indicated statistically significant differences at rows and columns ($P < 0.05$), respectively.

6.3.5. Sensory Analysis

The average scores of sensory attributes during cold-storage of boza samples containing NIS at 250 μg/g, LYS at 500 μg/g and LYS:NIS combination at 500:250 μg/g were given in Figure 6.4A to 6.4C. The samples were evaluated for 14 days since their declared shelf-life was expired within 15 days. It should be reported that the panelists did not detect any significant differences among color, odor, and texture of different boza samples during 14 days of cold-storage (P>0.05). At the end of 7 and 14 days, NIS and LYS:NIS containing samples got the highest taste, mouthfeel and overall acceptability scores that are all significantly higher than those of the control (P<0.05). In contrast, taste, mouthfeel and overall acceptability scores of LYS containing samples were not significantly different from those of controls at the end of 7 days (P>0.05). The mouthfeel of LYS containing samples and controls did not also differ significantly after 14 days. However, LYS containing samples had significantly higher scores for taste and overall acceptability than controls at the end of 14 days (P<0.05). Moreover, no significant differences were determined among mouthfeel and overall acceptability scores of LYS, NIS, and LYS:NIS containing samples (P>0.05) at 7th and 14th days of cold storage.

However, taste scores of LYS:NIS and NIS containing samples were significantly higher than those of LYS containing samples at 7th and 14th days, respectively (P<0.05).

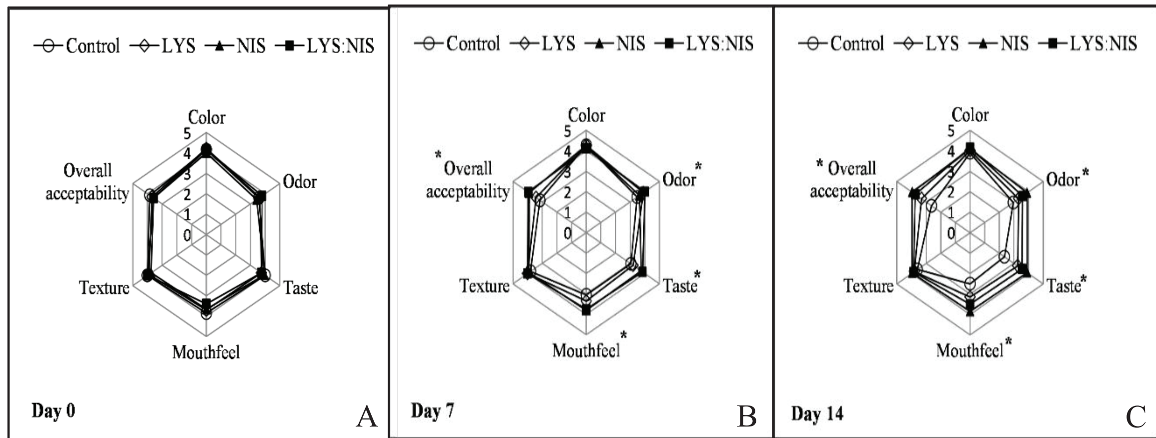

Figure 6. 4. Sensory attributes of different boza (Batch# 3) samples during cold storage at 4 °C (at 0th (A), 7th (B) and 14th (C) days); * indicates statistically differed attributes (P < 0.05)).

6.3.6. E-Nose Analysis

E-nose that mimics human olfactory system (Wilson and Baietto, 2009) has been used to detect the changes in food aroma originated form food spoilage, adulteration and loss of quality parameters (Casalinuovo et al., 2006; Hai and Wang, 2006; Kim et al., 2015; Marina et al., 2010). Besides, the combination of e-nose with chemometric methods showed a great potential to distinguish the aroma fingerprint of food products such as extra virgin olive oils obtained from olives of different cultivars, geographical origins and harvest years (Kadiroğlu et al., 2011). Wasnin et al. (2014) employed e-nose to detect aromatic changes in durian fruit pulp during fermentation. Rajamäki et al. (2006) used e-nose to detect aromatic changes in refrigerated modified atmosphere packaged broiler chicken carcasses. On the other hand, Santos et al. (2010) employed the device to detect early signs of deterioration in red wines.

In the current study, the e-nose analysis was conducted to differentiate the aroma fingerprints of boza samples treated with different antimicrobials on the 0th, 7th and 14th days of cold storage (Fig. 6.5A to 6.5C). The PCA results showed that the total variation (R^2) and prediction ability (Q^2) of e-nose data obtained at 0, 7 and 14th days were 73.8% and 21.4%, 92.4% and 52.1%, and 89.3% and 55.7%, respectively.

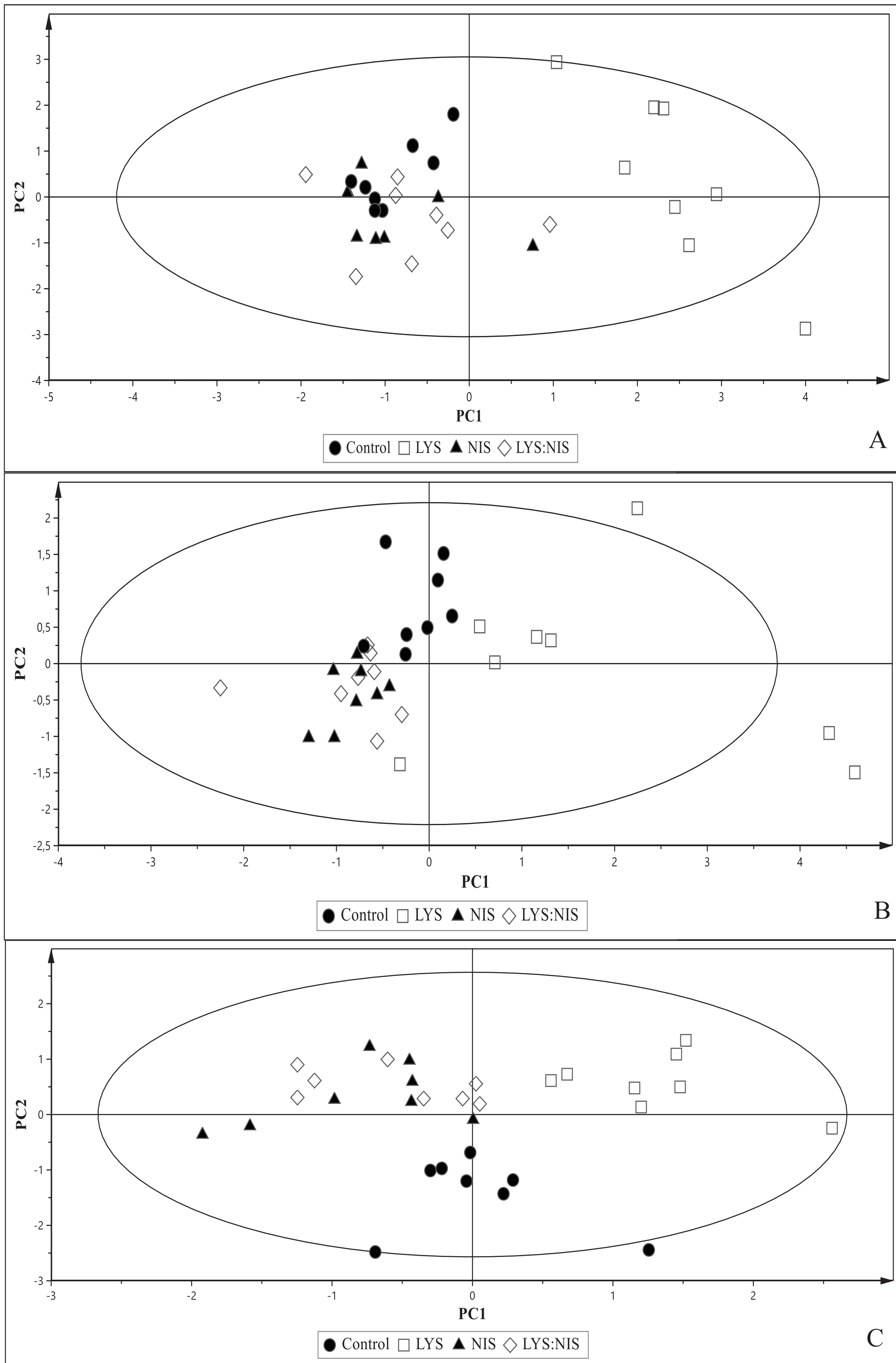

Figure 6. 5. PCA score plots of different boza (Batch# 4) samples during cold storage at 4 °C (at 0th (A), 7th (B) and 14th (C) days).

The results obtained at 0th day of cold storage showed that LYS containing group separated from control, NIS and LYS:NIS containing groups (Fig. 6.5A). The control group started to separate from NIS and LYS:NIS containing groups at the 7th day of storage (Fig. 6.5B). On the other hand, control and LYS containing groups clustered and got separated from NIS and LYS:NIS containing groups at the 14th day (Fig. 6.5C). In contrast, data for NIS and LYS:NIS containing groups did not show apparent discrimination at the 7th and 14th days. These clustering profiles showed parallelism with the sensory analysis that distinguished better taste of NIS and LYS:NIS containing samples than those of control and LYS containing samples.

6.4. Conclusion

For the first time in the literature, the results of this study clearly showed that the natural antimicrobial agents such as lysozyme and nisin could be employed to prevent acidic spoilage, to improve quality attributes and to extend the shelf-life of a fermented beverage like boza without causing considerable destruction of LAB. Using nisin alone or synergetic lysozyme-nisin mixture in boza yielded the most positive sensory effects during the storage of boza. The LAB counts of both nisin and lysozyme-nisin containing boza were almost at 6.0 log CFU/mL for 14-days cold storage. However, nisin alone gave significantly higher final LAB counts than synergetic lysozyme-nisin mixtures at storage periods exceeding 2 weeks. Thus, a slightly less destructive concentration of the synergetic lysozyme-nisin mixture is needed for long term preservation of boza. In contrast, due to the highly acidic nature of boza, LYS activity alone is insufficient to control LAB in this fermented beverage. The control of acidic spoilage in fermented probiotic beverages without causing microbial destruction is a great challenge. In fact, this is a major global problem in the commercialization of traditional fermented beverages. The results of this work are quite promising to obtain shelf-stable traditional probiotic beverages. However, further studies are needed at pilot scale with boza having different cereal composition and LAB profile. Moreover, further studies are also needed with alternative more popular LAB fermented beverages such as ayran, yogurt drinks, and acidophilus milk. Such easily scalable and economically feasible bio-based methods could help to make a great contribution to local economies and increase global consumers benefited from fermented functional foods.

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PUBLICATIONS

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