

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**EFFECTS OF NISIN ON MOST ABUNDANT FOOD
PATHOGENS AND ITS COMBINATION WITH GILABURU
(*Viburnum Opulus* L.) TO CONTROL *STAPHYLOCOCCUS*
*AUREUS***

SEDA KİRAZLI
**A THESIS SUBMITTED FOR DEGREE OF
DOCTOR OF PHILOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS**

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**THESIS SUPERVISOR
PROF. DR. SEDEF TUNCA GEDİK**

GEBZE

2023

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

NİSİNİN SIK BULUNAN GIDA
PATOJENLERİ ÜZERİNE ETKİSİ VE
STAPHYLOCOCCUS AUREUS'UN
KONTROLÜNDE GİLABURU (*Viburnum*
Opulus L.) İLE BİRLİKTE KULLANIMI

SEDA KİRAZLI
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. SEDEF TUNCA GEDİK

GEBZE
2023



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SUMMARY

In this study, the antimicrobial effect of nisin was studied for gram positive and negative bacteria under different pH (4-9), salt concentrations (NaCl, 0.5-8.5 % w/v) and temperatures (8-45 °C). Nisin was more effective on gram positive bacteria than gram negatives. The best nisin activity was found to be against *S. aureus*. Then, its use with gilaburu (*Viburnum opulus* L.) was evaluated to control *S. aureus*. Nisin exhibited the best antibacterial activity at pH 5 with a minimum inhibitory concentration (MIC) of 25 µg/mL. The MIC value of gilaburu was found to be 50% in all conditions studied. According to checkboard assay results, a synergy was found between gilaburu and nisin (FIC=0.25) for 2.5% and 4.5% w/v salt concentrations at pH 5. The synergistic interaction between nisin and gilaburu was also determined by agar disk diffusion assay. The positive effect of gilaburu on nisin activity was further confirmed through the time-kill assay, flow cytometry, and measurement of TTC dehydrogenase activity, conductivity, DNA, and protein leakage. Briefly, in the presence of gilaburu, nisin was found to exert the same effect on *S. aureus* at a concentration approximately 8-fold lower than the MIC value (25 µg/mL). The synergistic effect of nisin and gilaburu was shown for the first time. Besides, nisin and gilaburu were successfully integrated with gelatine and chia mucilage-based edible films. FTIR and SEM analysis supported integration of antimicrobials into the film. This new combination is promising to protect food from *S. aureus* contamination and contribute to food safety.

Keywords: Antimicrobial activity, Antimicrobial packaging, Food safety, Gilaburu, Nisin, *Viburnum opulus* L.

ÖZET

Bu çalışmada, nisinin gram pozitif ve gram negatif bakteriler üzerinde antimikrobiyal etkisi farklı pH (4-9), tuz konsantrasyonu (NaCl, 0.5-8.5 % w/v) ve sıcaklıklarda (8-45 °C) çalışılmıştır. Nisin gram pozitif bakterilere gram negatiflerden daha etkili olmuştur. En iyi nisin aktivitesi *S. aureus* için elde edilmiştir. Daha sonra, *S. aureus*'un kontrolünde nisinin gilaburu (*Viburnum opulus* L.) ile birlikte kullanımı değerlendirilmiştir. Nisin minimum inhibitör konsantrasyonu (MIK) 25 µg/mL ile pH 5'te en iyi antibakteriyel aktiviteyi göstermiştir. Gilaburu'nun MIK değeri ise tüm koşullarda %50 bulunmuştur. Dama tahtası test sonuçlarına göre, pH 5'te %2,5 ve %4,5 w/v tuz konsantrasyonlarında gilaburu ve nisin arasında sinerjik etki olduğu bulunmuştur (FIC=0,25). Nisin ve gilaburunun sinerjistik etkileşimi, agar disk difüzyon testi ile de belirlenmiştir. Gilaburu'nun nisin aktivitesi üzerindeki olumlu etkisi, zaman öldürme testi, akış sitometrisi, TTC dehidrojenaz aktivitesi, iletkenlik, DNA ve protein sızıntısı ölçümü yoluyla da doğrulanmıştır. Kısaca, gilaburu varlığında nisin, MIK değerinden (25 µg/mL) yaklaşık 8 kat daha düşük bir konsantrasyonda *S. aureus* üzerinde aynı etkiyi gösterdiği bulunmuştur. Nisin ve gilaburu'nun *S. aureus*'a karşı sinerjik etkisi ilk kez gösterilmiştir. Bununla birlikte, nisin ve gilaburu jelatin-chia musilajı temelli yenilebilir filme başarıyla entegre edilmiştir. Antimikrobiyallerin filme entegrasyonu, FTIR ve SEM analizleri ile doğrulanmıştır. Bu yeni kombinasyon gıdaların *S. aureus* kontaminasyonundan korunmasında ve gıda güvenliğinin artırılmasında umut vericidir.

Anahtar Kelimeler: Antimikrobiyal aktivite, Antimikrobiyal ambalaj, Gıda güvenliği, Gilaburu, Nisin, *Viburnum opulus* L.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and</u> <u>Acronyms</u>	<u>Explanations</u>
ABA	: Absciscic acid
ABU	: Aminobutyric acid
Abu-S-Ala	: β -methyllanthionine
ADI	: Acceptable daily intake
ALA	: Alanine
Ala-S-Ala	: Lanthionine
BHI	: Brain Heart Infusion
BSA	: Bovine serum albumin
CFU	: Colony forming unit
CLSI	: Clinical and Laboratory Standards Institute
CM	: Chia mucilage
DHA	: Dehydroalanine
DHB	: Dehydrobutyrine
DNA	: Deoxyribonucleic acid
DW	: Distillated water
EDTA	: Etilendiamin tetraasetic acid
EFSA	: European Food Safety Authority
FDA	: Food and Drug Administration
FIC	: Fractional inhibitory concentration
FICI	: Fractional inhibitory concentration Index
G	: Gilaburu
GAA	: Glacial acetic acid
GE	: Gelatine
Gl	: Glycerol
GlcNAc	: N-acetylglucosamine
GRAS	: Generally Regarded as Safe

H	: Hour
HCl	: Hydrochloric acid
Kbp	: Kilo base pair
LPS	: Lipopolysaccharide
MBC	: Minimum bactericidal concentration
MIC	: Minimum inhibitory concentration
Mg	: Milligram
ml	: Milliliter
MRSA	: Methicillin resistant <i>Staphylococcus aureus</i>
MSNs	: Silica nanoparticles
MurNAc	: N-acetylmuramic acid
N	: Normality
NaCl	: Sodium chloride
NaOH	: Sodium hydroxide
Ni	: Nisin
OD	: Optical density
PBS	: Phosphate buffered saline
Ph	: Power of hydrogen
Pi	: Isoelectric point
PI	: Propidium iodide
PKa	: Dissociation constant
PLA	: Poly lactic acid
PVL	: Panton-Valentine Leucocidin
RDA	: Relative dehydrogenase activity
Rpm	: Revolutions per minute
S	: Sulphur
SEA	: Staphylococcal enterotoxin A
SEB	: Staphylococcal enterotoxin B
SEC	: Staphylococcal enterotoxin C
SED	: Staphylococcal enterotoxin D
TPF	: 1,3,5-triphenyl tetrazolium formazan
TSST 1	: Toxic shock syndrome toxin 1

TTC	:	2,3,5-triphenyl tetrazolium chloride
TVC	:	Total viable count
UDP	:	Uridine diphosphate
WHO	:	World health organization



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1. INTRODUCTION

Many foodborne illnesses result from the consumption of food contaminated with pathogenic bacteria. According to many food microbiological researches, a number of pathogens have raised significantly important, which are responsible for foodborne illness. These pathogens include *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa* [Hameed et al., 2020]. *Staphylococcus aureus*, a food pathogen, is a gram-positive microorganism that is highly resistant to adverse living conditions and is ubiquitously found in the environment. *S. aureus*, which can also be found in the skin and nasal mucosal flora of humans and animals, can contaminate foods hereby can cause foodborne diseases and even death with its toxins and enzymes [Sun et al., 2022]. *L. monocytogenes* is a gram positive psychrotrophic pathogen that causes listeriosis and it has a high mortality rate. It can be found in many types of food especially milk and milk products. *Bacillus cereus* is a gram positive widespread spore forming pathogen and highly resistant to environmental factors [Martinez et. al., 2016]. *Escherichia coli* is a gram negative bacterium naturally found in human and animal gut flora. Pathogenic *E. coli* strains like enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E.coli*/enterohemorrhagic *E.coli* (STEC/EHEC), *Shigella*/enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and enterotoxigenic *E. coli* (ETEC) are related with foodborne illnesses and indicates fecal contamination of food [Yang et. al., 2017]. *Pseudomonas aeruginosa* is a gram negative bacterium which can secrete exotoxin and enterotoxins and mostly found in water and meat [Liu et. al., 2020].

There are many food preservation methods and technologies to prevent foodborne diseases such as heat processing, freezing, refrigeration, antimicrobial agents, salts and chemical preservatives, etc. [Lee and Paik, 2016]. However, these applications can form undesirable effects such as a decrease in the nutritional value and organoleptic properties of foods and the formation of some allergic reactions and unwanted diseases in humans. For these reasons, studies have focused on natural antimicrobials [Lee and Paik, 2016], [Pisoschi et al., 2018]. One of these

antimicrobials is nisin (E 234) which is classified as Generally Recognized As Safe (GRAS) and currently an authorized food additive in the European Union (EU) for use in several food categories [Breukink and de Kruijff, 2006], [Khan and Oh, 2016], [Younes et al., 2017]. Nisin is a group of lanthionine bacteriocin, which is produced by *Lactococcus lactis* subsp. and contains unusual post-translationally modified amino acids. In general, it is highly effective against spore-forming and non-spore-forming gram-positive bacteria such as lactic acid bacteria, *Listeria*, *Mycobacterium*, *Staphylococcus*, *Clostridium*, and *Bacillus* [I. Khan & Oh, 2016]. Nisin binds to the lipid II precursor of bacteria and acts by disrupting the integrity of the peptidoglycan structure, which is the main component of the bacterial cell wall [Bauer & Dicks, 2005].

The antibacterial activity of nisin, however, is influenced by environmental factors such as pH, temperature, composition, and microbiota of the foods. Accordingly, to enhance its effectiveness as a food preservative, it is usually used in combination with other chemicals and antimicrobial agents [Alves et al., 2016b], such as EDTA [Fang and Tsai, 2003], lysozyme [Morsy et al., 2018], lauric arginate and ϵ -polylysine [Martínez-Ramos et al., 2020], reuterin [Arqués et al., 2008] and bovicin [Pimentel-Filho et al., 2014]. Furthermore, nisin has also been combined with different kinds of plant extracts or their components such as rosemary extract [Gao et al., 2014], grape seed extract [Zhao et al., 2020], thymol [Campion et al., 2017], [Hossain et al., 2022] and eugenol [Hossain et al., 2022], cinnamaldehyde, and citric acid [Campion et al., 2017], carvacrol [Campion et al., 2017], [Heckler et al., 2021] and oregano [Takundwa et al., 2021]. These plant extracts contain polyphenols which are responsible for damaging bacterial cell membranes [Mei et al., 2019].

Viburnum opulus L. is a fruiting plant generally known as gilaburu in Turkey. Since this plant contains a wide variety of biological substances such as phenolic compounds including polyphenolics (mostly chlorogenic acid) [Barak et al., 2019], flavonoids (catechin, epicatechin, quercetin), anthocyanins together with ascorbic acid, and L-malic acid [Kraujalytė et al., 2012], [Velioglu et al., 2006], it was found worthy of investigation in terms of its antimicrobial [Aksoy et al., 2006], [Cesonienė et al., 2012], [Česonienė et al., 2014], [Şapcı et al., 2017], anti-oxidative, anti-cancer, anti-inflammatory, anti-diabetic and anti-obesity properties [Kajszczak et al., 2020].

The ever-increasing modern consumer demand for safe food and nutrition is driving the industry to reduce the use of unnatural ingredients and develop healthier food products. For this reason, in recent years, studies have focused on the application of harmless substances or processes to foods. Active packaging systems with loading natural antimicrobial agents for controlling the growth of spoilage and pathogenic microorganisms have a growing interest in order to decrease the use of unnatural additives in food industry [Moradi et al., 2021].

This study aims to elucidate the inhibitory activity of nisin against different foodborne pathogens which are three gram positive (*Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*) and two-gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and to show the effect of gilaburu (*Viburnum opulus* L.) on the MIC value of nisin for a selected pathogen (*S. aureus*) under different conditions (pH, salt concentration and temperature). In addition, it was aimed to carry out preliminary studies to prepare a natural edible packaging material based on chia mucilage and gelatin complex coacervation system in which nisin and gilaburu integrated.

2. ANTIMICROBIAL AGENTS

There are different kind of food preservation techniques for preventing foodborne diseases and spoilage of food. Although heat treatment is the most basic food preservation method in foods, the use of food additives has also been a very effective food preservation method [Pisoschi et al., 2018].

The Codex Alimentarius defines food additive as

“any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities.” [Web 1, 2021].

Food additives basically divided into 6 groups which are preservatives, additives for nutritional purpose, flavoring agents, texturing agents, coloring agents and miscellaneous agents. Preservatives are also divided into antimicrobials, antioxidants and anti-browning agents [Carocho et al., 2015].

Antimicrobial agents refer to all substances that are effective against microbial organisms. Antimicrobial agent is a general term that refers to antibacterial, antifungal, antiviral, and antiprotozoal substances. Antimicrobial agents act by killing microbes (microbiocidal) or slowing the growth of microbes (microbiostatic) [Asif, 2018]. Antimicrobial agents can originate from unnatural or natural sources. Unnatural agents can cause allergic reactions and serious life threatening side effects. Interest in natural antimicrobials has increased in recent years as an alternative to unnatural chemicals and also to ensure food safety without affecting food quality standards like nutritional value, taste, color, texture and odor [Pisoschi et al., 2018].

Natural antimicrobials can be defined as substances produced by living organisms to defend themselves. The sources of these substances are plants (such as spices and herbs, essential oils), microorganisms (bacteriocins and organic acids) and animals (chitosan, lysozymes, lactoperoxidase, lactoferrin) [El-Saber Batiha et al., 2021], [Rodrigo and Palop, 2021]. They control microbial contamination in food and

contribute shelf-life extension technologies via delaying microbial spoilage. Besides, they have strengthening effects on immune cells in humans [Tajkarimi et al., 2010].

2.1. Nisin

The bacteriocin nisin discovered in England by Rogers and Whittier in 1928 [Paul Ross et al., 2002]. Nisin is an antimicrobial peptide which is used as a food preservative. It was isolated in the late 1930s and produced in 1950s. It is recognized as a food additive in the USA and used for food preservation all over the world since 1960s [Chen et al., 2020].

Bacteriocins are antibacterial proteins which are produced by bacteria to damage other bacteria. They are divided into class I (Ia and Ib), class II (Ia and Ib) and III. Class I bacteriocins have unusual amino acids, such as lanthionine, methyl-lanthionine, dehydroalanine and dehydrobutyrine. Class Ia bacteriocins have cationic and hydrophobic peptides which form pores in cell membranes. Class II bacteriocins has non-modified and heat stable peptides. Class III bacteriocins are large and heat labile proteins. These bacteriocins are often and erroneously confused with antibiotics. However, there are many differences such as synthesis pathway, mechanism of action, and toxicity [Cleveland et al., 2001]. These differences are summarized in Table 2.1.

Table 2.1: Different characteristics of bacteriocins and antibiotics.

Characteristics	Bacteriocins	Antibiotics
Synthesis	Ribosomal	Secondary metabolite
Mode of action	Membrane pore formation	Membrane or intracellular
Activity	Narrow spectrum	Wide spectrum
Immunity in host	Yes	No
Side effect/Toxicity	Not clear	Yes
Interaction	Non specific	Specific targets

Nisin is a type Ia bacteriocin which is produced by *Lactococcus* species especially *Lactococcus lactis subsp. lactis* and some *Streptococcus* species [Gedarawatte et al., 2021], [Shin et al., 2016]. Nisin is recognized as Generally Regarded As Safe (GRAS) status and approved in 1969 by the World Health

Organization (WHO) and in 1988 by the US Federal Food and Drug Administration (FDA) as a food preservative [Lewies et al., 2018]. Nisin (E 234) is currently an authorized food additive in the EU under Annex II of Regulation (EC) 1333/2008 for use in several food categories and its acceptable daily intake (ADI) is confirmed as 0.13 mg/kg body weight (bw) in EFSA panel, 2017. Besides, in the same regulation, the maximum level of nisin (E 234) allowed in foods including 10 mg/kg in creams and unripened cheese; 12.5 mg/kg in ripened cheese, processed cheese, and cheese products; 6.25 mg/kg-L in egg and egg products, and 3 mg/L or mg/kg in desserts. In 2017, nisin level for unripened cheese was discussed and accepted as a maximum level of 12 mg/kg and as a maximum level 25 mg/kg for heat treated meat products [Younes et. al., 2017].

Nisin is colorless, tasteless, odorless and has a good antimicrobial activity on gram positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus* so it is widely used in dairy products such as cheese [Cui et al., 2017], [Tong, et al., 2014a]. Besides, it is generally protected by food ingredients and keeps its antimicrobial activity after sterilization, pasteurization, and other food processing steps [Gharsallaoui et al., 2016].

2.1.1. Structure and Chemical Properties of Nisin

Nisin is ribosomally synthesized antimicrobial peptide with a 3,5 kDa molecular mass which is composed of 34 amino acid and post-translationally modified peptide with four methyl-lanthionine rings, one lanthionine and unusual amino acids such as dehydrobutyrine and dehydroalanine. Post translational modifications include dehydration of the threonine and serine in order to form 3,6-unsaturated amino acids, continued with the modification of these residues with the sulfhydryl group of the cysteines and these reactions compose the lanthionines [Kupke and Götz, 1996]. Because of these unusual amino acids, nisin is also called lantibiotic [De Vos et al., 1995], [Mitchell et al., 2018].

Nisin has five sulphide bridges which are called ring A, B, C, D and E (Figure 2.1). The ring A is formed by lanthionine. The ring B-E is formed by the four β -methyl lanthionine residues. Besides, nisin contains three $\alpha\beta$ unsaturated amino acids. These

are dehydrobutyrine (DHB) or β -methyl dehydroalanine at the position 2 and dehydroalanine (DHA) at the position 5 and 33. The first 1-19 amino acid residues, namely the first three lanthionine rings (A, B and C), form the N-terminus of Nisin. The other 23-28 residues (D and E rings) which is continued a flexible stretch of last 6 residues form C-terminus of nisin. Nisin has unusual thioether amino acids such as lanthionine (ALA-S-ALA) at the positions 3-7 and β -methyl lanthionine (ABA-S-ALA) at the positions 8-11, 13-19, 23-36 and 25-28 [Berridge et al., 1952],[Gross & Morell, 1971], [Hurst, 1981].

As shown in Figure 2.1, C and D rings in the C-terminal (amino acids 23-34) divided by a hinge region (amino acids 20-22) and this region contributes the flexibility of nisin. The N-terminal region of nisin is more hydrophobic than the C-terminal region this gives nisin an amphipathic property, besides, the hydrophobic parts are opposite the hydrophilic parts along the molecular length also contributes to this property [Breukink et al., 1997].

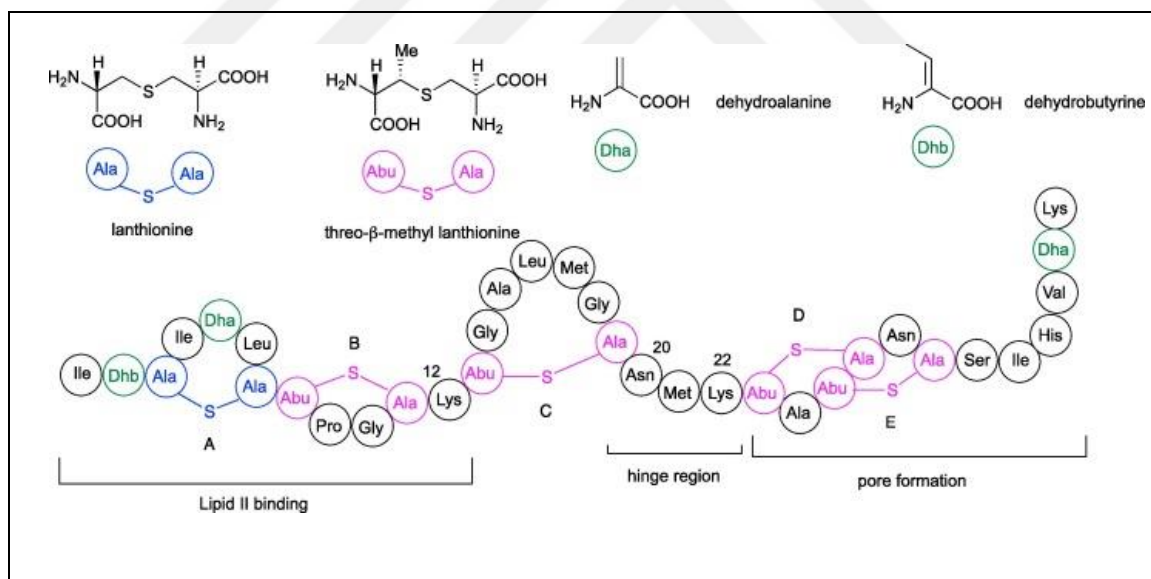


Figure 2.1: Structure of Nisin (Z) and the residues which have important functions in antimicrobial activity. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β -methyl lanthionine.

Nisin A, produced by *Lactococcus lactis*, is the natural form of nisin, and different variants have been described, differing from nisin A up to 10 amino acids. Nisin Z, F and Q are also produced by *Lactococcus lactis*, while nisin U and U2 are

produced by *Streptococcus* spp. [Piper et al., 2011]. Nisin Z differs from nisin A in only one amino acid which in position 27 is Asn in nisin Z while His in same position in nisin A [Mulders et al., 1991]. Structures of different types of nisin are shown in Figure 2.2.

Nisin has cationic property because C terminal has positively charged histidine and lysine residues. The side chain in lysine and histidine pKa values are 10 and 6.5, respectively. So, nisin's net charge is pH dependent [Abee and Delves-Broughton, 2003], [Tramer and Fowler, 1964]. According to Liu and Hansen, solubility and stability of nisin increase at lower pH levels and very stable at pH 2, however it is inactivated and unstable at high pH levels. Besides, nisin can be autoclaved and stored in refrigeration for months without any chemical and functional change [W. Liu and Hansen, 1990].

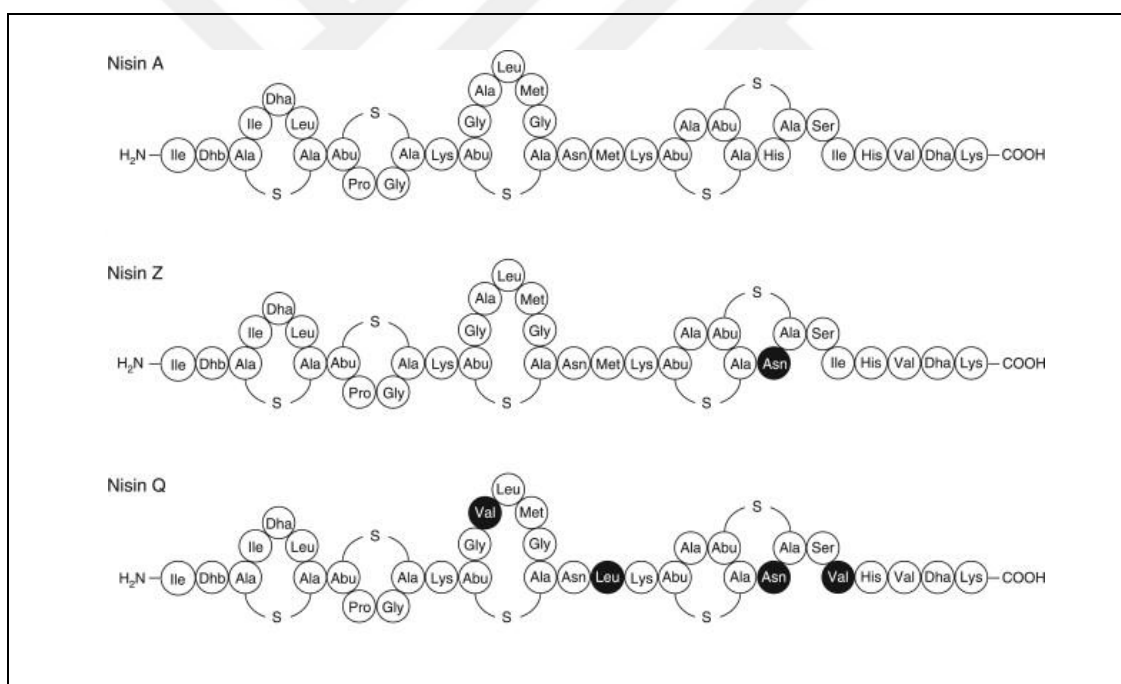


Figure 2.2: Structures of different types of nisin. Dha, dehydroalanine; Dhb, dehydrobutyrine; Ala-S-Ala, lanthionine; Abu-S-Ala, β -methyllanthionine. Filled circles are substituted amino acids from the corresponding residues of nisin A.

2.1.2. Biosynthesis of Nisin

Nisin is a precursor peptide which is synthesized ribosomally. This precursor peptide is enzymatically cleaved in order to form pro-nisin. Then, it undergoes a posttranslational modification process and forms mature lantibiotic. The primer transcript which is called prepronisin has a N terminal leader peptide with 23 aminoacids and it is followed by a C terminal peptide with 34 aminoacids [Kuipers et al., 1995]. Schematic outline of the biosynthesis of nisin A is shown in Figure 2.3.

Nisin biosynthesis genes are encoded by a 70 kbp conjugative transposon which is located on the chromosome. The nisin genes are located into an operon-like structure, the genes nis A, B, T, C, I, P, R, K, F, E, G of nisin and their functions are summarized in Figure 2.4 [Delves-Broughton, 2014].

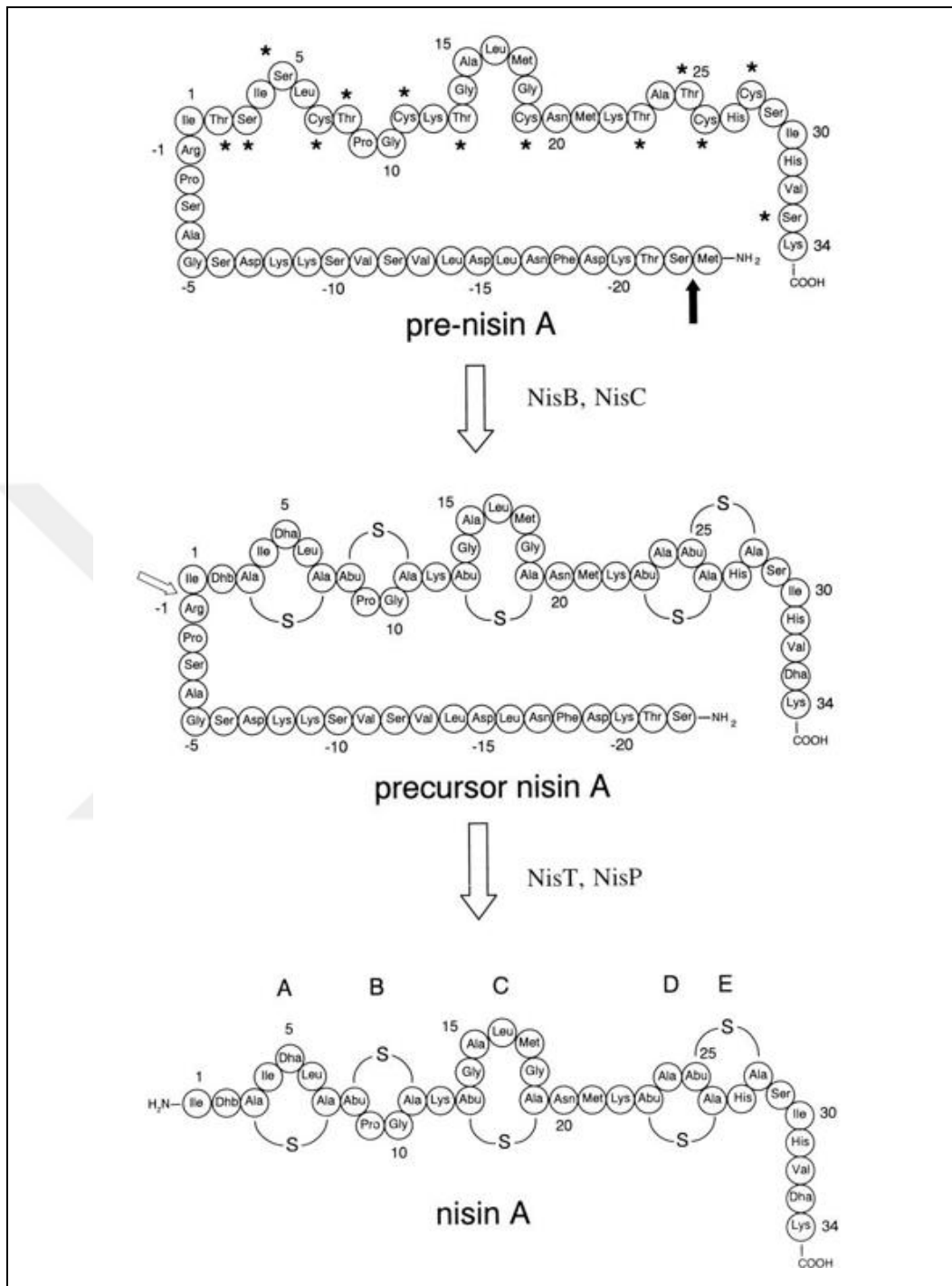


Figure 2.3: Schematic outline of the biosynthesis of nisin A. Rings are labeled A-E. Asterisks indicate residues that will be modified. The black arrow indicates processing of the N-terminal Met residue, while the small white arrow indicates processing of the leader peptide by the action of NisP. Dha, dehydroalanine; Dhb, dehydrobutyrine.

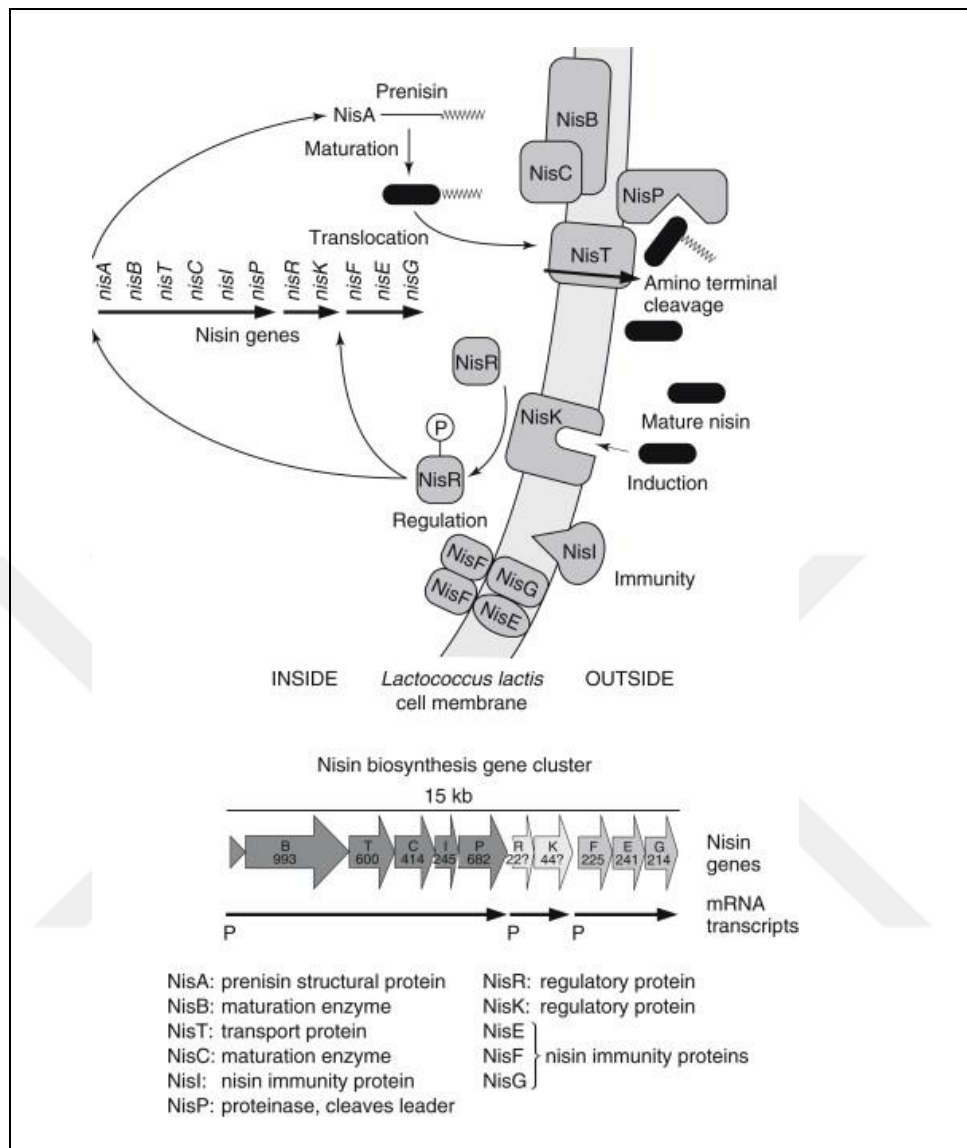


Figure 2.4: Nisin biosynthesis with corresponding genes.

2.1.3. Mechanism of Antibacterial Activity

Nisin inhibits the bacterial cells by creating pores on their cell wall. It binds to the lipid II which is the precursor molecule for synthesis of cell wall and causes an inhibition. This leads to the membrane permeabilization [Scherer et al., 2015].

Lipid II is a cell membrane anchored precursor molecule which has peptidoglycan monomer (N acetylglucosamine-N-acetylmuramic acid (pentapeptide)), pyrophosphate and 11 polyisoprene residues (Figure 2.5a)

[Punyauppa-path et al., 2015]. As shown in Figure 2.5b, bacterial cell wall synthesis is summarized in three stages. At stage I, UDP-GlcNAc and UDP-MurNAc-pentapeptide are produced from intermediate metabolites in cytoplasm. Lipid II is formed when MurNAc-pentapeptide binds to Lipid I which is embedded in the inner side of the bilayer and then GlcNAc binds to MurNAc sugar residue (stage II). At last stage (III) lipid II, is transferred onto periplasmic side and in here the monomer unit of peptidoglycan is integrated into the growing chain [Chugunov et al., 2013], [Panina et al., 2018].

The first stage of antibacterial activity of nisin starts by electrostatic interactions between positively charged section of nisin which has basic amino acid residues and negatively charged outer surface of the bacteria membrane because of phosphate groups of lipopolysaccharides and lipoteichoic acid structures of bacteria. These interactions do not need any specific receptors at the membrane of the bacteria [Jenssen et al., 2006].

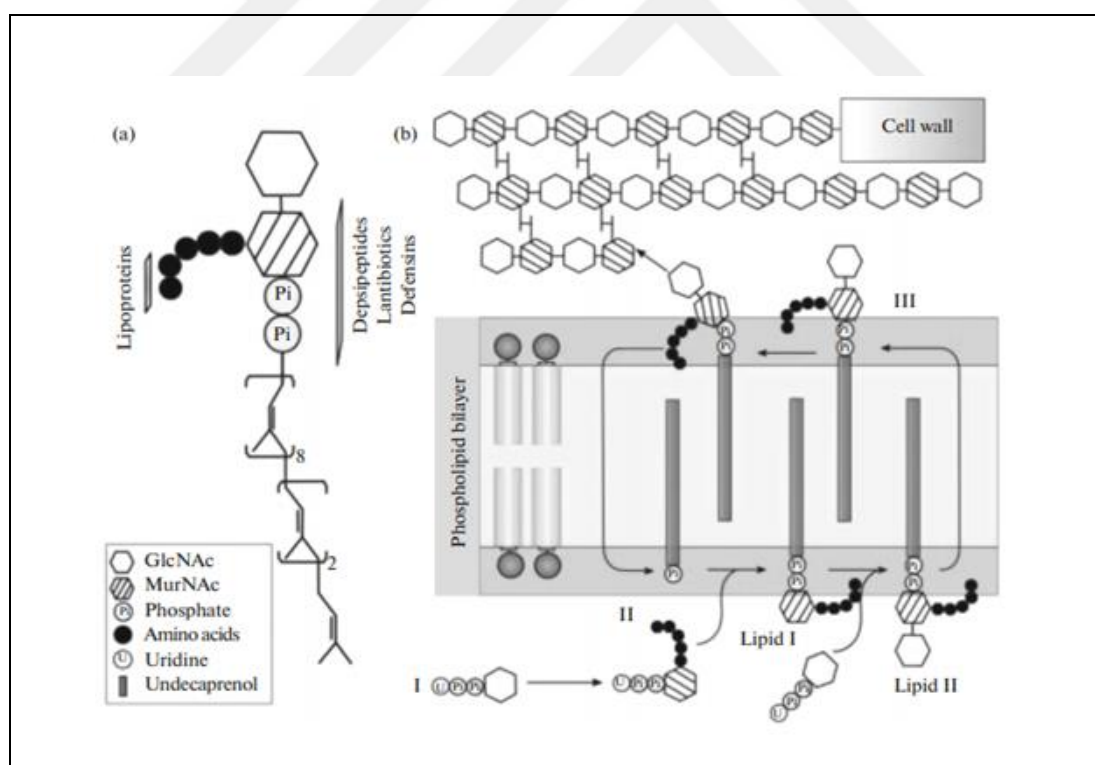


Figure 2.5: a) Lipid II structure. Lipid II has GlcNAc and MurNAc sugar residues, a pentapeptide (L-Ala-D- γ -Glu-L-Lys-D-Ala-D-Ala), a pyrophosphate linker and an undecaprenol tail b) Bacterial cell wall synthesis.

Nisin acts on cell membranes in two ways. First, nisin can bind lipid II and disrupt the integration of peptidoglycan monomer into the growing peptidoglycan chain (Figure 2.6). Second, nisin can bind carbohydrate-pyrophosphate part of lipid II via its N terminal part after that C terminal part of molecule can be inserted into the cell membrane and this creates pores (Figure 2.7). Pore formation can cause an increase in membrane permeability which cause an efflux of cytoplasmic contents from cell and cause cell death at the end [Punyaappa-path et al., 2015], [Shawali and Samy, 2015], [Wiedemann et al., 2004]. According to Van Kraaij et. al. pore formation mechanism of nisin occurs via translocation of the C-terminal region of the peptide across the membrane [Van Kraaij et al., 1998]. According to another study, the N-terminal of nisin interacts to the carbohydrate moiety of lipid II, firstly. After that, the C-terminal part of nisin undergoes a translocation across the cytoplasmic membrane of the bacteria. During the peptide interaction with the lipid II, nisin forms pores in cell membrane which have 2 to 2.5 nm pore diameter and are stable for seconds [Wiedemann et al., 2001, 2004].

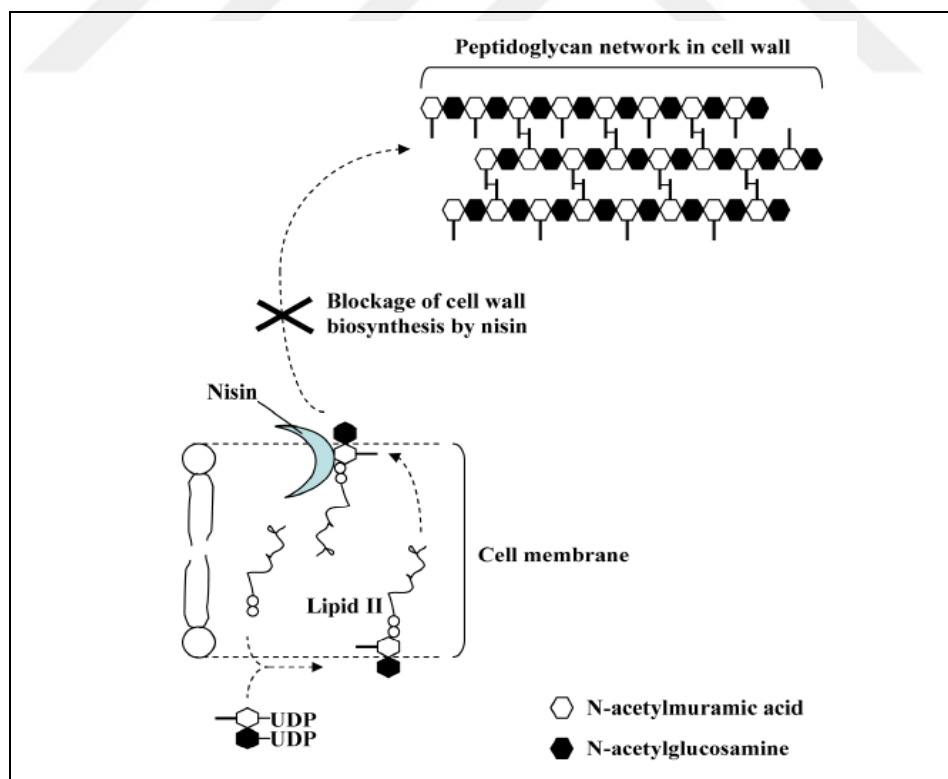


Figure 2.6: Inhibition of cell wall synthesis by nisin.

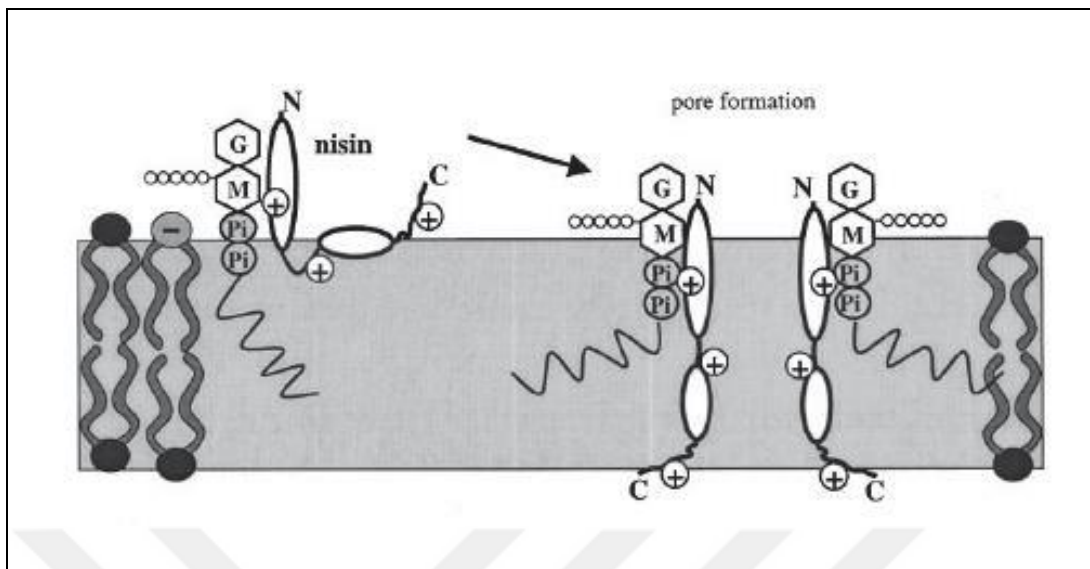


Figure 2.7: Pore formation of nisin in cell membrane.

Nisin is effective against most of Gram-positive and some gram negative foodborne pathogenic bacteria. Gram-negative cells are durable to nisin because of lipopolysaccharide (LPS) contents. LPS acts like a barrier for nisin on the cell wall. However, chelating agents restricts the divalent calcium and magnesium ions of the LPS which disrupt stabilization of the layer. Thereupon, nisin can be moved through the LPS layer and forms pores in the cytoplasmic membrane [Belfiore et al., 2007], [Millette et al., 2004]. Previously, nisin was reported as no inhibitory activity against viruses, yeast cells and filamentous fungi [Gänzle et al., 1999]. However, Le Lay et al. showed the inhibition of *Candida albicans* at a concentration of 500 g/mL of nisin Z. [Le Lay et al., 2008]. A paper published by Akarey et. al about inhibition of changing of *C. albicans* from blastopore to hyphal forms [Akarey et. al., 2009]. Abreu et al. were successfully created nisin loaded nanoparticles for inhibition of vaginal candidiasis [Abreu et. al., 2016]. Besides, Małaczewska et al. published that nisin at high concentrations and long incubation period was effective on the bovine viral diarrhea virus (BVDV) [Małaczewska et al, 2009].

According to the literature, nisin can show its antimicrobial effect even at room temperature. In a paper, Korean popular meal seasoned beef is packaged and kept in storage for 60 days at low (4 °C) and room temperature (25 °C). According to the results, in seasoned beef with nisin slight increase in the growth of mesophilic

microorganisms was seen, but in seasoned beef without nisin these microorganisms grow efficiently [Lee and Paik, 2016]. Nisin is known to be an FDA-approved compound, however recent studies has also proven that it does not show toxicity. Nisin integrated silica nanoparticles (MSNs) were studied against *S. aureus* and it was shown that free nisin and MSNs have any important toxic effects on the mouse fibroblast L929 cell line [Behzadi et al., 2018] In another study from Wang et al. showed that there was no cytotoxicity of nisin and perilla oil incorporated with chitosan-based films [Wang et al., 2021].

Along with being a direct antimicrobial agent, nisin has also immunomodulatory activity. The immunomodulatory effect of nisin can be listed as follows (1) regulation of cytokine production (e.g. interleukins, chemokines, TNF and TGF- β), and (2) regulation of adaptive (e.g. B and T lymphocytes) and innate (e.g. neutrophils, monocytes, macrophages) immune cells. So that, regulation of nisin by innate immune cells causes that bacterial infections can be removed by neutrophil catch, lysozyme, phagocytosis, and oxidative eruption. Nisin can stimulate immune cells in a similar way as natural host defence mechanisms by activating receptors and signal pathways (NF-B, p38, ERK MAPK, Akt/CREB , Src family kinases, PKC, and PKA) [Khan et al., 2022].

2.1.4. Application of Nisin in Food

Nisin has been studied for antimicrobial control in its free form without encapsulation, however, it exhibited loss of activity because of deactivation or degradation. Hence, nisin integrated films have a great interest in recent years. Films with encapsulated nisin could preserve it from proteases and environmental factors such as temperature, oxygen and pH [Ben Amara et al., 2017]. On the other hand nisin resistancy is a problem for many gram positive bacteria such as *L. monocytogenes*, *S. aureus*, and *Streptococcus* spp. The mostly known mechanisms of nisin resistance are variation of membrane phospholipid and fatty acid composition, enzymatic degradation of nisin by nisinase, thickening of cell wall, DNA mutations [Zhou et al., 2014]. Besides, nisin has a strong antimicrobial activity against gram-positive bacteria but less inhibitory activity for gram-negative bacteria, yeasts, and molds [Tiwari et al., 2009]. The outer membrane of gram-negative bacteria does not enable nisin to contact

cell membrane. Nisin with chelators can change the outer membrane permeability of gram-negative bacteria [Boziaris and Adams, 1999] for this reason and in order to avoid resistance, nisin has been combined with chelators and other antimicrobials [Fang and Tsai, 2003].

In the literature, nisin is mostly used in combination with other substances and implementations. In 2004, Arqués et. al, combined nisin with another natural antimicrobial name as reuterin and they researched the antimicrobial activity of reuterin alone or in combination with nisin against different gram-positive and gram-negative pathogens in milk. Reuterin (8 AU/ml) had bacteriostatic activity against *L. monocytogenes* and bactericidal activity against *S. aureus* and gram negatives. Reuterin combined with nisin (100 IU/ml) had synergistic effect on *L. monocytogenes* and additive effect on *S. aureus* were detected. However, combination of reuterin with nisin did not improve the antimicrobial effect of reuterin against gram-negatives [Arqués et al., 2004].

Sugandha Bhatia et al studied the antibacterial activities of nisin, EDTA and lysozyme in a starch film-based food packaging film. The results exhibited that nisin and EDTA have synergistic effect on antibacterial activities [Bhatia and Bharti, 2014].

Alves et. al. studied the antibacterial activity of nisin combined with phenolic compounds (carvacrol, thymol, eugenol, cinnamaldehyde) at proportions $\frac{1}{4}$ MIC of nisin and $\frac{1}{4}$ MIC of phenolic compounds against *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 15313 in cow milk. In all combinations bacteriostatic effect and synergism were detected [Alves et al., 2016].

Shi et. al. combined nisin with *p*-Anisaldehyde which is an extract from *Pimpinella anisum* seeds to prevent nisin resistance of *S. aureus*. They found synergism (FIC index ranging from 0.25 to 0.375) between nisin and *p*-Anisaldehyde against *S. aureus* and confirmed it with other tests such as agar diffusion and flow cytometry assays. They concluded that the combination of nisin and *p*-Anisaldehyde which exhibited antibacterial activity could not only decrease resistance but also be used as a natural food preservative [Shi et al., 2017b].

In another study, sophorolipids which are extracellular biosurfactants produced by yeasts are used as an antibacterial agent alone and in combination with nisin against foodborne pathogen *S. aureus*. In the study, sophorolipids and nisin showed good

antibacterial activities with minimum inhibitory concentrations of 32 and 0.5 $\mu\text{g mL}^{-1}$, respectively. In combination, fractional inhibitory concentration (FIC) index was 1.5 and this was interpreted as an additive effect [Chen et al., 2020].

In a different study, *Zataria multiflora* Bioss and *Mentha longifolia* essential oils alone and in combination with nisin and acid acetic, exhibited bacteriostatic and bactericidal effects against *Escherichia coli* O157: H7 and *S. aureus*. In the checkerboard test, FIC index was <0.5 and study showed that nisin had synergistic interaction with both types of oils [Jahani et al., 2021]. Zhang et al. studied the effect of nisin and oregano essential oil on the shelf life and microbial community of modified atmosphere packaged on fish fillets. Oregano essential oil and nisin alone or in combination caused a significant increase in shelf-life and inhibited the microbial growth of fish fillets [Zhang et al., 2021]. In another study by Chang et al., it was shown that EDTA–nisin combination increased the antimicrobial activity of PLA-chitosan films for fish fillets against gram negative bacteria (Coliforms, *Aeromonas*, *Pseudomonas* and *Vibrio* spp.) By this study, it was shown that EDTA binds metallic ions (Ca^{2+} and Mg^{2+}) in the outer membrane of gram-negative bacteria and so that nisin open pores on the cell wall [Chang et al., 2021].

Li. et al. searched for the interactions of nisin and carvacrol. For this purpose, they determine the MIC of nisin and carvacrol against *S. aureus* as 60 and 125 $\mu\text{g/mL}$, respectively. On the other hand, combination of nisin and carvacrol had synergic effect with FICI 0.28125. According to the results, the combination could completely kill *S. aureus* within 8 h [Li et al., 2022].

2.2. Gilaburu (*Viburnum Opulus* L.)

Viburnum opulus L. belongs to Caprifoliaceae family. This plant is known as American cranberry bush, European cranberry, rose elder, guelder rose, cherry-wood, whitten tree, and crampbark tree and originates from Asia, Europe, North Africa, and western Russia. It is known in Turkey with different local names such as gilaburu, gilaboru, gilgili, and gildar [Karaçelik et al., 2015], [Sagdic et al., 2014], [Eryilmaz et al., 2014].

Gilaburu is a branching shrub up to 4 m tall. The flowers of this species are white and fruits are 8–10 mm long, red and drupe (Figure 2.8) [Paşayeva et al., 2021].

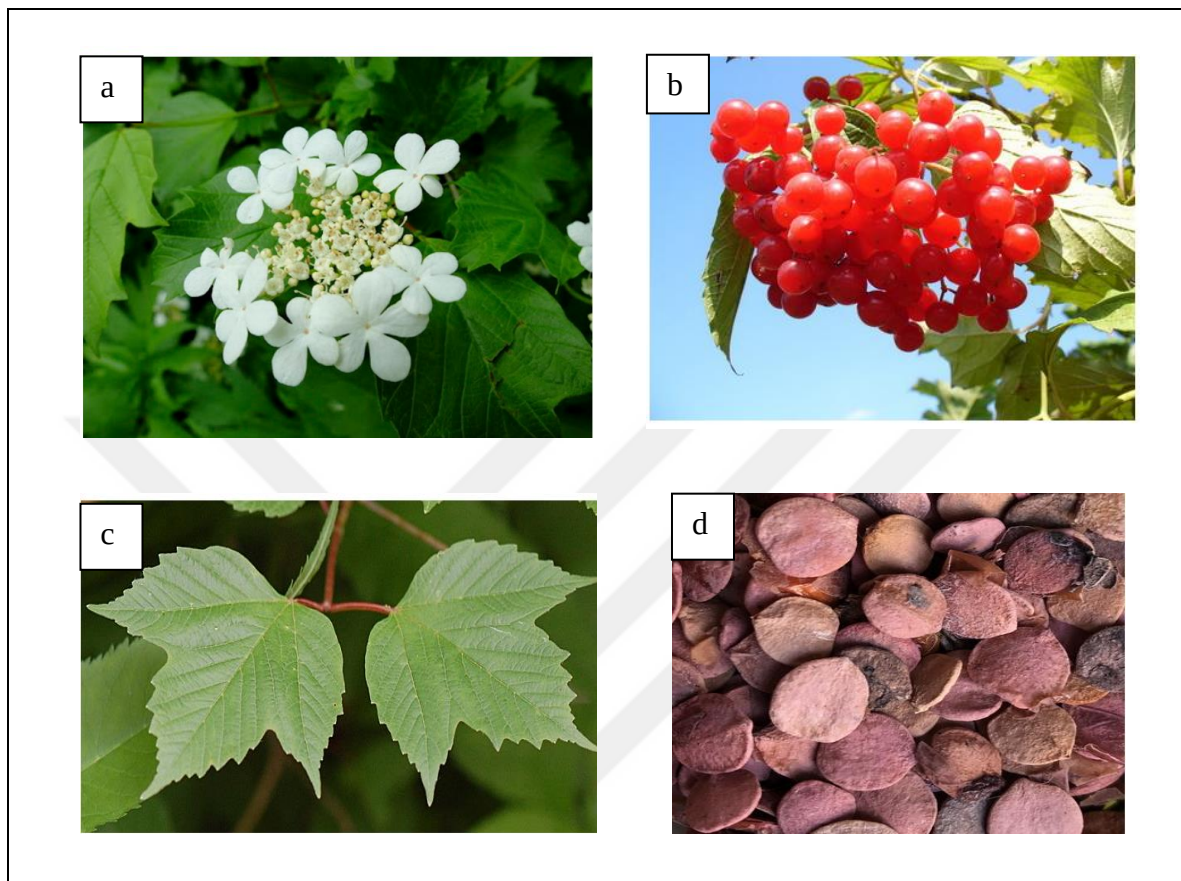


Figure 2.8: Different parts of *V. Opulus* L. plant. Flowers (a), Fruit (b), Leaves (c), Seed (d).

Gilaburu has red, edible bitter taste fruit which ripened in August–September. The juice from the fruit is the mostly used product. On the other hand, cooked fruits can be preserved and consumed like jams, marmalades or fermented product. *Caprifoliaceae* species has high level of polyphenolic compounds, especially anthocyanin. So, they are found remarkable and lately received attention because of their high antioxidant properties [Moldovan et al., 2012]. Besides anthocyanins, gilaburu has high level of polyphenols such as organic acids flavonoids, iridoids, phenolic acids and triterpenoids. *Caprifoliaceae* species has various biological activities including antioxidant, antibacterial, cytotoxic, hypoglysemic, anticancer, anti-inflammatory, antispasmodic and hepatoprotective [Paşayeva et al., 2021].

Because of these biological activities, gilaburu is used traditionally in order to treat colds, stomach and kidney diseases and high blood pressure [Moldovan et al., 2012].

Turker et. al. tested eight different wild fruits including *Viburnum opulus* L. by using disc diffusion assay (Kirby-Bauer Method). For each fruit, 8 different extracts (aqueous and ethanol extracts prepared from hot and cold treatments of fresh and dried fruits) were obtained. *Viburnum opulus* L. dry fruit hot water extraction exhibited antibacterial activity against *S. aureus* [Turker et al., 2012].

In another study, *Lonicera tatarica* and *Viburnum opulus* extracts were studied for their antibacterial activity against *S. aureus* and *S. epidermidis* which are biofilm producing strains. The antimicrobial activity was determined by broth microdilution method and the biofilm forming ability was quantified by the microtiter assay. Their results exhibited that the *Lonicera tatarica* mostly interfered with the bacterial growth exhibiting an inhibitory effect, while *Viburnum opulus* attenuated the ability of all tested strains to colonize the inert substratum and to form biofilms. So, they hypothesized that these two species could be used for the development of novel antimicrobial products for preventing the formation of biofilms [Bubulica et al., 2012].

Eryilmaz et. al. studied antimicrobial activities of aqueous and ethanolic extracts of *Viburnum opulus* L., *V. orientale* Pallas, *V. tinus* L. and *V. lantana* L. against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300 (MRSA), *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* RSKK 574 and *Candida albicans* ATCC 10231 with the disc diffusion and tube dilution techniques. The ethanolic extracts of *Viburnum* exhibited better antimicrobial activity than that of aqueous extracts [Eryilmaz et al., 2014].

Cesonienė et. al. studied antimicrobial activities of fruit juices and ethanol extracts of *Viburnum opulus*. A total of ten Gram-positive and Gram-negative bacteria and nine yeast strains were tested by using the agar well diffusion method. The most effective antibacterial activity was exhibited by the fruit juices against *Salmonella typhimurium*, *Salmonella agona*, and *L. monocytogenes*. Fruit juice of *Viburnum opulus* was also showed antibacterial activity on *S. aureus* [Česonienė et al., 2014]. Although different studies determined the importance of gilaburu for human health, research about this valuable plant is still very limited.

3. STAPHYLOCOCCUS AUREUS AS A FOOD PATHOGEN

Foodborne bacteria are an important public health concern worldwide. Most of people are affected by foodborne diseases such as diarrhea, vomiting, abdominal pain, headache and enteritis. Contaminated food with foodborne bacteria is a very serious problem for food industry such as meat processing, dairy, poultry and brewing [Zhu et al., 2022].

S. aureus is a spherical gram-positive foodborne pathogen (Bencardino et al., 2021) that can be reason for septicemia, pneumonia, endocarditis, meningitis and toxic shock syndrome [L. Lin et al., 2023]. *S. aureus* can grow in a broad range of temperatures (7° to 48.5° C; optimum 30 to 37°C), pH (4.2 to 9.3; optimum 7 to 7.5), up to 15 % sodium chloride concentration, and in dry and stressful environment [Kadariya et al., 2014].

S. aureus is a well-known pathogen that locates skin, the nasal cavity and oropharynx. *S. aureus* has an infectious mechanism via producing a variety of exoproteins during their growth in order to colonize and rise infections in mammalian hosts. Almost all *S. aureus* strains can secrete enzymes and cytotoxins which contains four haemolysins (alpha, beta, gamma, and delta), proteases, lipases, nucleases, hyaluronidase and collagenase. Some strains can secrete exoproteins which includes staphylococcal enterotoxins (basically SEA, SEB, SEC and SED), leukotoxins (such as Panton-Valentine Leucocidin (PVL)) and toxic shock syndrome toxin-1 (TSST-1) [Dinges et al., 2000], [X. Li et al., 2022], [Pereira et al., 2022].

In order to protect themselves from environmental stress, bacteria can form biofilms as a complex lifestyle. *Staphylococcus spp.* is one of the bacteria which can form biofilm [Zhu et al., 2022]. This biofilm composed of bacteria and extracellular polymeric substances which is used for attachment onto a surface. Biofilm former *S. aureus* is very resistant to hard stress conditions including acids, detergents, salts and antibiotics. Hence, the presence of *S. aureus* on food contact areas is a major problem for food industry because it can cause spoilage of product and be source of disease [Wen et al., 2021].

S. aureus strains can have methicillin-resistance because of acquisition of *mecA* gene. *S. aureus* evolves to MRSA with horizontal insertion of staphylococcal chromosome cassette *mec* elements which has resistance gene *mecA* that codes penicillin-binding protein. This protein has low affinity for β -lactam antibiotics. MRSA was reported in 1961 for the first time and rised by the time. In many countries, the high morbidity and mortality was in the 1980s. Antibiotics are basic treatment strategy for *S. aureus* but unconsciously and excessive antibiotic usage led to antibiotic resistancy [Gan et al., 2021]. In order to treat Methicillin-resistant *S. aureus* (MRSA), Vancomycin is used. However, vancomycin-resistant MRSA has also been isolated [Shahid et al., 2021].

The source of risky food in terms of *S. aureus* are meat and meat products, poultry and egg products, milk and dairy products, bakery products, salads, pastries, cakes and sandwich type foods [Kadariya et al., 2014]. *S. aureus* is one of the most frequently isolated agents from bovine mastitis. Itis the most common etiological agent of clinical and subclinical bovine mastitis, so milk is the most important source for this bacterium [Fluit, 2012].

4. ACTIVE PACKAGING

4.1. Antimicrobial Packaging and Nisin

Antimicrobial agents which are used sprayed or dripped on the food directly have not been found very effective because of the high diffusion rate of antimicrobials and the interactions of them with the food components. These substances can have negative effects on the taste of food. Hence, antimicrobial integrated packaging studies have gotten attention for a long time [Gharsallaoui et al., 2016].

Packaging is any kind of material which protects food from tampering or contamination from external factors such as physical (i.e. oxygen, water vapor, ultraviolet light), chemical and biological. This material protects food after food processing and enables it to travel safely to consumer. Active packaging described by Labuza and Breene (1989) is an approach in which the package, the product, and the environment interaction contributes shelf life, safety and sensory features of food, so that it aims at increasing the quality of the product. Intelligent packaging is another approach for monitoring the condition of packaged foods in order to get information about the quality of the packaged food during transport and storage [Prasad & Kochhar, 2014].

Biopolymer based films have excellent properties for developing edible and active antimicrobial packaging from biodegradable polymers such as polysaccharides, proteins and lipids. In vitro and in vivo studies have focused on various antimicrobial biodegradable films from different sources such as starch, cellulose, alginate, pectin, chitosan as polysaccharides, casein, whey protein, gelatine, zein, soy, gluten as proteins and oils, waxes, triglycerides as lipids [Chawla et al., 2021]. These natural biopolymers can be good barriers for food as well as extend the shelf life and safety of food due to their satisfactory mechanical properties [Prasad & Kochhar, 2014].

Antimicrobial biopolymer based films have many advantages for food. They can inhibit microbial growth on food surfaces where is the main part of onset of spoilage. Besides, the release of antimicrobials from packaging material gradually gives a longlife protection and prevention of antimicrobials from food components such as

lipids, enzymes etc. which reduces the effectiveness of them in food and this gradual release is the most advantageous method according to dipping and spraying antimicrobials to packaging material. In literature, it was shown that nisin release is pH and temperature dependent and low pH-high temperatures are the best conditions for nisin activity. Moreover, antimicrobial usage in the packaging material contributes decrease the time necessary for the sterilization process of food in food industry. Another advantage of antimicrobial polymers is reducing post-processing contamination via using them on equipment surfaces and personel equipments which has a direct contact with food [Santos et al., 2018].

In the literature, various methods have been used to introduce nisin into food packaging systems so far. These are mainly emulsions (such as ultrasonication, high-pressure homogenization, phase inversion, microfluidization), liposomes (micro and nano-liposomes), coacervated systems, films with spray drying and fibers [Eghbal et al., 2022], [Khan & Oh, 2016]. Pattanayaiying et al. have developed pullulan films containing lauric arginate and nisin Z, alone or in combination. They searched for effect of films on foodborne pathogens in muscle foods after long-term refrigerated storage. According to the results, the films incorporated with lauric arginate and nisin succesfully reduced *Salmonella typhimurium*, *Salmonella enteritidis*, *Staphylococcus aureus* and *Listeria monocytogenes* Scott A [Pattanayaiying et al., 2015]. Morsy et al produced nanoparticles with combinations of lysozyme 500 U/mL, nisin 1000 IU/mL, EDTA 5 mM, and ZnO 12 mM for minced beef. Lysozyme-Nisin-EDTA and Lysozyme-Nisin-EDTA-Zein combinations exhibited very strong antibacterial effect against *Escherichia coli* O157:H7, *Bacillus cereus*, and *Listeria monocytogenes*. They reduced the number of microorganisms about 2–4 log CFU/cm² in packaged minced beef during storage at 4 °C for 15 days [Morsy et al., 2018]. Liu et al. used a nanoemulsion based coating for Yao meat which contains nisin, star anise essential oil, and polylysine. The shelf life was extended from 8 days to 16 days because of inhibition of bacterial growth during storage. Besides, the coating had no any effect on moisture of product for 20 days without abnormal color and odor [Liu et al., 2020]. Martillanes et al. searched for the effect of high-pressure processing in combination with chitosan films incorporated with nisin and rice bran on dry-cured Iberian ham inoculated with *Listeria monocytogenes*. According to the results, the combination of

high pressure processing with the developed films reduced the population of *Listeria monocytogenes* by 6 log CFU/g [Martillanes et al., 2021].

Other materials also used in order to produce antimicrobial films. Pires et al. made antimicrobial films by incorporating nisin, natamycin, and a combination of both into a cellulose matrix. The antimicrobial effects were studied against *S. aureus* ATCC 6538, *L. monocytogenes* ATCC 15313, *Penicillium* sp., and *Geotrichum* sp. Nisin incorporated films showed an antimicrobial effect in vitro against *S. aureus* and *L. monocytogenes* and natamycin incorporated films were found efficient in vitro against *Penicillium* sp. and *Geotrichum* [Santos Pires et al., 2008]. Imran et al. studied antibacterial effects of nisin loaded films against *L. monocytogenes* CIP 82110 and *S. aureus* CIP 4.83 and the release rate of fluorescently labeled nisin from chitosan, sodium caseinate, hydroxypropyl methylcellulose, and polylactic acid (PLA) films in water-ethanol solution at the temperature of 4 °C and 40 °C. The study showed that hydroxypropyl methylcellulose has the higher nisin diffusion coefficient, while chitosan has the lowest diffusion coefficient. When temperature increased, nisin release from hydroxypropyl methylcellulose and sodium caseinate increased. A higher inhibitory effect against microorganisms was seen in hydroxypropyl methylcellulose and sodium caseinate based films with nisin [Imran et al., 2014]. Wang et al. formed nisin-loaded chitosan/PLA antimicrobial films by coating method. The structure, mechanical properties and antibacterial effect of the films were evaluated for *S. aureus* ATCC 6538. Besides, diffusion features of nisin were investigated. According to the results, the diffusion of nisin from film was found strongly dependent on pH and ionic strength. Moreover, films showed well-controlled release of nisin and better antimicrobial activity against *S. aureus* [Wang et al., 2015]. Chang et al. built up PLA films with nisin and used it as food contact material. They aimed to understand the migration properties of films. According to their results, the overall migration values were low according to the limit of European Commission, which is 10 g/dm². So that, they showed the safety of the tested films [Chang et al., 2021].

4.2. Coacervation as a Method for Packaging

Coacervation is a kind of oldest encapsulation technique which is mostly used. Its name comes from Latin word *acervus*, means aggregation, and the prefix *co* represents the fusion of colloid particles. This method is relatively simple according to emulsification technique [Devi et al., 2017]. There are two types of coacervation as simple and complex. In simple coacervation there is one polymer and involves elimination of the water from the dispersed colloid by substances which having greater affinity for water like salts and alcohols. The dehydrated molecules of polymer make an aggregate with surrounding molecules to occur the coacervate. [Gu et al., 2010]. The interaction of negatively charged polysaccharides with positively charged proteins below their isoelectric points enables to form phase separation in solutions which is known as complex coacervation in polymer technology. The underlying mechanism is the separation of the hydrocolloid from the primary solution pursued by agglomeration into a separate, liquid phase which is named “coacervate”. The coacervates are called the “continuous phase” and the second phase is called the “equilibrium solution”. The coacervation course can be classified into four stages: First, suspension of the material particles in the liquid phase. Second, production of a three-phase system, i.e., secretion of the second liquid phase (coacervate). Third, deposition of liquid polymer around the core. Lastly, gelling and solidification of the microcapsule wall (Figure 4.1) [Devi et al., 2017].

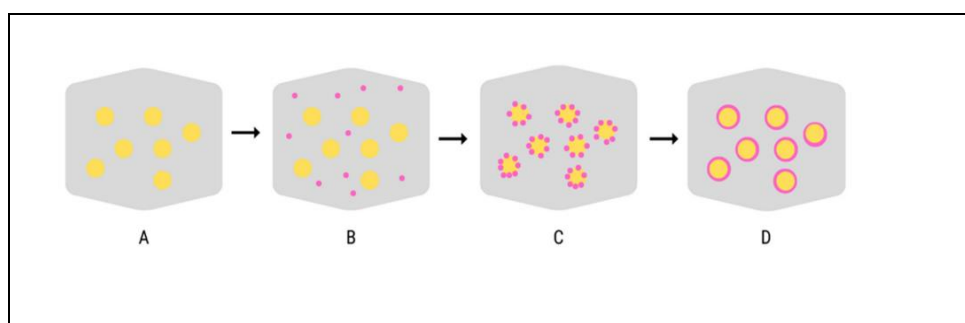


Figure 4.1: The mechanism of microcapsule formation in the coacervation method, liquid phase (A), suspending the core material in the liquid phase (B), deposition of liquid polymer around the core (C), gelling, and solidification of the microcapsule wall (D).

The optimum pH ratio of polymers and ionic strength strongly effects coacervation success [Hernández-Nava et al., 2019], [Timilsena et al., 2016]. In many studies have been investigated and suggested using complex coacervation system for development of films and coatings as food package [Timilsena et al., 2016].

According to the literature, various wall materials for coacervation have been used up to now. Gum arabic which is a complex anionic polysaccharide and gelatine are the most commonly used materials. However, chemical induction by formaldehyde, glutaraldehyde, glyoxal, or epichlorohydrin, which are known as toxic for health is mostly used in order to enhance strength of gum arabic-gelatine coacervation. Besides arabic gum, chitosan, carrageenan and alginates are mostly used polysaccharides for complex coacervation. These polysaccharides were studied with different protein types. For example, amphipathic milk proteins used in coacervation, however, it exerted limitations such as conformational changes or denaturation because of techniques such as high pressure, ultrasound, temperature during preparation of coacervates. However, proteins from animal origin can cause allergic reactions. Instead of animal-based proteins, plant-based proteins from peas, rice, lentils, or wheat are becoming interest in recent years. Mucilage as a plant-based polysaccharide is very attractive in last years because they are environmentally friendly, low cost, available, and have interesting functional properties like other plant-based proteins [Napiórkowska and Kurek, 2022].

4.3. Gelatine as a Component of Coacervation Systems

Gelatine is obtained with thermal process from collagen which can originate from cattle bone, hides, pig or cattle skins, and fish. The gelatine can be produced either by the acid process (type A gelatine), or by the alkaline process (type B gelatine). The isoelectric point (pI) of gelatine A produced differs from pH 6–9, whereas gelatine B has a pI of 4.8–5 [Muhoza et al., 2022]. Gelatine is a kind of digestible protein which contains all the essential amino acids except tryptophan. The amino acid composition can differ from species to species particularly with respect to proline and hydroxyproline [Marriott and Adam, 2013]. Gelatine has an excellent film forming, gas and oil barrier, low price and non-toxicity but low mechanical and thermal

properties. Gelatine is widely studied together with chitosan in order to form a complex coacervate because of its hydrophilic nature which has low water resistancy leads to restrict the usage of gelatine alone [Abdollahzadeh et al., 2021], [Ge et al., 2017].

4.4. Chia Mucilage as a Natural Source for Active Packaging

Recently, plant-based polysaccharides including mucilage had a great interest. The examples of plant-based mucilage sources are chia seeds (*Salvia hispanica*), cress seeds (*Cardamine*), flax seeds (*Linum usitatissimum*), marshmallow (*Althaea officinalis*), aloe vera, prickly pear (*Opuntia ficus-indica*) [Napiórkowska and Kurek, 2022].

Chia (*Salvia hispanica* L.) is an annual herbaceous plant from the mint family (*Lamiaceae*) [Capitani et al., 2013]. Chia seed which has a water retention of 27 times its weight has complex slightly sticky and transparent polysaccharide mucilage when introduced with water [Muñoz et al., 2012]. This mucilage structure has been reported as complex anionic heteropolysaccharide to be created by α -D-glucose, 4-O-methyl- α -D-glucuronic acid and D-xylose on a ratio of 1:1:2, respectively [Lin et al., 1994]. Chia seeds has been commonly used in healthy diet programs and as an additive in food because of its functional properties as prebiotic [Kassem et al., 2021] and high omega-3 fatty acid, protein and antioxidant content [Capitani et al., 2012]. Mucilage of chia seed has been applied in yoghurt in order to increase unsaturated fatty content of fermented milk products [Derewiaka et al., 2019]. Besides, possible use of chia seed mucilage as stabilizer and emulsifier for food has been reported due to their water holding capacity and it was applied in ice cream successfully [Campos et al., 2016], [Segura-Campos et al., 2014]. In addition, it could be used successfully as coating agent and packaging film in food. In order to extend the shelf life of seafood products, chia mucilage was studied in combination with both propolis and gojiberry extract separately as a coating agent and satisfactory results were obtained [Coban and Coban, 2020], [Coban and Ergur, 2021], [Salazar Vega et al., 2020]. Chia mucilage and protein concentrate coacervated film has been developed and film has had best properties when they used in a ratio of 2:1, respectively [Salazar Vega et al., 2020].

According to another research the best conditions to form films via complex coacervation of gelatine and chia mucilage (GE-CM) were with a mass ratio of 2:1 and a pH value of 3.6 [Hernández-Nava et al., 2019]. Besides, Oregano essential oil encapsulation was achieved in a complex coacervation system introduced by GE-CM [Hernández-Nava et al., 2020], [Luo et al., 2019] and compared with gelatine-arabic gum encapsulation system [Luo et al., 2019].



5.MATERIALS AND METHODS

5.1. Materials

Table 5.1: Devices.

Product	Company	Catalog Number
Autoclave	Tomy	SX-700
Scale	And	GR-200
Water Bath	Memmert	WNB 45
Hotplate Stirrer	Stuart	SB 162-3
Colony Counter	Rocker	Galaxy 330
pH Meter	Mettler Toledo	34S210-U
Micropipet 100-1000 μ L	Eppendorf	Research plus 100-1000 μ L
Micropipet 10-100 μ L	Eppendorf	Research® plus 100-1000 μ L
Flow Cytometry	Bd	Accuri C6
Vortex	Fisher Brand	15786959
Class II Cabinet	Metisafe	MSC IIA C150
Incubator	Memmert	IN110
Centrifuge	Hermle	Z306
Spectrophotometer	Perkin Elmer	Lambda 35
Elisa Reader	Biotek	EQOLE1495
Shaker Incubator	Ika	KS3000i
McFarland Spectrophotometer	bioMérieux	3335
Multimeter	WTW Inolab	7320
Lyophilizer	SP Scientific	VirTis Ultra 25
Ultrasonic Homogenizator	Branson	250
SEM	Philips	XL SFEG
Sputter	Quortech	Quorum SC 7620,
FTIR	Perkin Elmer	Spectrum 100

Table 5.2: Consumables.

Product	Company	Catalog Number
Tips 2-200 µL	Eppendorf	022492039
Tips 100-1000 µL	LP Italiana	L151546
Parafilm M	Bemis	PM996
Eppendorf Tubes	Axygen	MCT-060-C,MCT-150-C
Falcon 15 / 50 ml	Capp	510015 / 510050
96 well Microplate	Citotest	36010096D
Cryovial	Nest	607001
Petri Dishes 100 mm	Corning, Gosselin	SH91-101
Syringe Filter (0.22 µm)	Milex,Millipore	MILFSLGS033SS
Syringe 10 ml	Beybi	24.09001
Erlenmayer	Superior	C411210
Loop 10 ul	LP Italiana	L.X8677F
Paper Disks	Whatman no: 1	-

Table 5.3: Chemicals, Media and Kit.

Product	Company	Catalog Number
Brain Heart Infusion Broth	Merck	1.10493.0500
Brain Heart Agar	Merck	1.13825.0500
BSA	Sigma Aldrich	9048-46-8
Glycerol	Merck	104092
HCl	Merck	109058
NaOH	Merck	106462
NaCl	Merck	106462
PI Solution	BD	556463
PBS	Merck	524650
TTC	Merck	108380
Tris buffer	Merck	648314
Glucose	Merck	108337
Methylbenzene	Merck	108325
GAA	Merck	100063
Bradford Assay Kit	Thermo scientific	23238

5.4: Bacterial strain.

Product	Company	Catalog Number
<i>Staphylococcus aureus</i> NCTC 10788	bioMérieux	56045
<i>Listeria monocytogenes</i> ATCC 19111	Microbiologics	0277E3
<i>Bacillus cereus</i> ATCC 10876	Microbiologics	0998E3
<i>Escherichia coli</i> NCTC 12923	bioMérieux	56034
<i>Pseudomonas aeruginosa</i> NCTC 12924	bioMérieux	56040

Table 5.5: Antimicrobials and biopolymers.

Product	Company	Catalog
Nisin A	Sigma-Aldrich	1414-45-5
Gilaburu	Bio bitkisel A.Ş.	-
Chia	Yayla Agro Gıda San. ve Tic. A.Ş.	-
Gelatin (Type B)	Halavet Gıda A.Ş.	-

5.2. Methods

5.2.1. Preparation of Bacterial Cultures

S. aureus (NCTC 10788, bioMérieux, France), *Listeria monocytogenes* (ATCC 19111, Microbiologics, USA), *Bacillus cereus* (ATCC 10876, USA), *Escherichia coli* (NCTC 12923, bioMérieux, France), *Pseudomonas aeruginosa* (NCTC 12924, bioMérieux, France) maintained as -80°C frozen stock culture in Brain Heart Infusion (BHI) broth (Merck, Germany) with 20% (v/v) glycerol (Merck, Germany) and they were used as test microorganisms. Bacteria were inoculated into 50 ml BHI broth (Merck, Germany) and incubated with 150 rpm agitation overnight at 30 °C. Cell density was adjusted to 0.5 McFarland (10^8 CFU/ml) with a spectrophotometer (DensiCHEK, bioMérieux, France) and then diluted with BHI broth to obtain an inoculum with a concentration of 10^6 CFU/ml.

5.2.2. Preparation of Antimicrobial Agents

Nisin A from *Lactococcus lactis* (2.5% in balance sodium chloride and denatured milk solids) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nisin stock solution was prepared by dissolving 0.2 g powder in 10 ml 0.02 N HCl (pH 2.5) and centrifuged at 4500 rpm for 15 min. The supernatant was sterilized by filtration through 0.22 µm filters (Millex, Millipore) and stored at -20°C.

Gilaburu extract, which contains only pulp and juice of the fruit without any preservatives and additives was purchased from a local market as an organic food supplement (Bio Bitkisel Inc., Kayseri, Turkey). This commercial extract was considered as 100% concentration and used as stock gilaburu solution. Gilaburu extracts were centrifuged at 10,000 rpm for 5 min and supernatant sterilized by filtration through 0.22 µm filters (Millex, Millipore) and stored at -20°C. Nisin and gilaburu stock solutions were diluted with Brain Heart Infusion (BHI) broth and they were stored at dark at 4°C for a maximum of 1 week.

5.2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) in BHI

MIC and MBC of nisin and gilaburu were determined at different pH (4,5,6 and 7,8 and 9) and salt concentrations (0.5, 2.5, 4.5 and 6.5%) at 30 °C (Memmert, Germany). The pHs (4,5,6,7,8 and 9) were adjusted with 1N NaOH (Merck, Germany) and 1N HCl (Merck, Germany) with a pH meter (Mettler Toledo, USA). Different salt concentrations adjusted with NaCl (Merck, Germany) at concentrations of 0.5, 2.5, 4.5 and 6.5% w/v. Since BHI broth already contains 0.5% salt, other salt concentrations were calculated accordingly. The working temperature was chosen as 30 °C since there was no significant difference in nisin activity on bacteria at mesophilic temperatures.

The MIC of antimicrobials was determined by a slightly modified broth dilution reference method according to CLSI document M07-A11 [Web 2, 2021]. The initial concentration of nisin and gilaburu was maintained to 2000 µg/ml and 50%, respectively. 100 µl of BHI broth was dispersed with a micropipette (Eppendorf, Germany) to a 96-well microtiter plate. Then, 100 µl nisin or gilaburu was supplemented to the wells of the first column in aseptic conditions and mixed. 100 µl of solution from the first column was taken and added to wells of the second column. The transfer of solution to the other wells was repeated in the same way and finally, wells of the column had half of the substance concentration of the previous column. Eventually, 10 µl of the bacterial culture (10^6 CFU/well) were added into the wells. The plates were incubated for 24 h at 30 °C with 150 rpm agitation. The MIC was defined as the concentration of antimicrobial agent which completely inhibits the growth of the organism, as detected with the naked eye.

For determination of MBC, 10 µl of the solution from the MIC microtiter plate was transferred to BHI agar plates by using the drop plate method and incubated for 24 h at 30 °C. The MBC was defined as the concentration at which no bacterial colony formed. All experiments were performed in triplicate.

5.2.4. Checkboard Assay

The synergistic antimicrobial effect of nisin and gilaburu was investigated and evaluated using a checkboard assay. The assay was conducted for *S. aureus* in different salt concentrations (0.5%, 2.5% and 4.5% w/v), at pH 5 and 30 °C. The rows of a microplate included the same concentrations of nisin, diluted two-fold from 50 to 3.125 µg/ml along the Y-axis. Similarly, the column included the same concentration of gilaburu, diluted two-fold from 50% to 6.25% along the X-axis. *S. aureus* was inoculated into each well (10^6 CFU/mL) and the plate was incubated at 30 °C for 24 h with 150 rpm agitation [Shi et al., 2017], [Tong et al., 2014]. Checkboard assay was performed three times on different days. The combinational effects of nisin and gilaburu were evaluated by calculating the fractional inhibitory concentration (FIC) index. The following equation was used to calculate the FIC index. FIC index: FIC A + FIC B, where FIC A means MIC of antimicrobial A in combination/MIC of A alone and MIC B means MIC of antimicrobial B in combination/MIC of B alone. The interpretation of the FIC index was as follows: a FIC index value of 0.5 represented synergy, a FIC index value of 0.5-4 represented additive effect, and a FIC index value > 4 represented antagonism [Schelz et al., 2006].

5.2.5. Agar Disk Diffusion Assay

S. aureus culture (10^6 CFU/100 µl) was inoculated uniformly on agar plates. Paper disks (6 mm diameter) impregnated with nisin and gilaburu (5 µl from different concentrations) alone or in combination were placed onto the agar surface. The samples were incubated at 30 °C for 24 h and the diameter of clear inhibition zones were measured in millimetre [Zhao et al., 2016]. The assays were performed in triplicate. The antimicrobial activity was expressed with the following cut-off values: <1 mm/µg means low, 1 to <10 mm/µg means intermediate, and 10 mm/µg means high antimicrobial activity.

5.2.6. Time Kill Assay

Time-kill assays were performed by incubating *S. aureus* (10^6 CFU/ml) with different concentrations of nisin and gilaburu alone or in combination, in different salt concentrations at 30 °C and pH 5. Total viable count (TVC) was determined at certain time intervals (0, 4, 8, 12, 24 h) by spread plating on BHI agar. After incubation at 30 °C for 24 h, the average bacterial cell number was calculated for each time point. The assays were done in triplicate. Interactions between antimicrobial agents were interpreted as synergistic when the mixture caused a ≥ 2 -log decrease in TVC compared to the antimicrobial agents alone [Chi and Holo, 2018].

5.2.7. Flow Cytometry

Propidium Iodide (PI) dye is used in flow cytometry to detect dead cells. Dead cells with a damaged cell membrane are permeable to PI dye [Emerim Lemos et al., 2021]. Since the lowest number of viable cells in the time-kill assay belonged to the 12th hour samples, these samples were used in the flow cytometry analysis. 1 ml sample was taken after 12-hour incubation and centrifugated at 4°C for 20 min at 3500 rpm. Then, the pellet was washed three times with sterile phosphate-buffered saline (PBS; pH 7.4) and dissolved in 1 ml PBS. 100 µl of the bacterial solution was mixed with 5 µl of PI solution (BD Biosciences, San Diego, CA, USA) and incubated in dark for 10 min. Then the samples with three biological replicates were analysed by BD ACCURI C6 (BD Biosciences, San Diego, CA, USA) flow cytometer. PI fluorescence was determined by using FL2 bandpass filter (585/20). Data for the unstained control sample was obtained as the gate of live bacterial cells and this gate was used for detection of live or dead cells in stained control and antimicrobial treated samples. The percentage of PI-positive cells in the samples were used as the dead cell ratio.

5.2.8. Measurement of Relative TTC Dehydrogenase Activity

2,3,5-triphenyl tetrazolium chloride (TTC) is a white crystalline water-soluble powder that gives an irreversible reduction reaction through the dehydrogenase

enzymes present in metabolically active cells. In this reaction, the heterocyclic ring of TTC forms 1,3,5-triphenyl tetrazolium formazan (TPF) which is a reddish-colored insoluble complex. This chemical transformation is interpreted as an indicator of vitality in cells. The tetrazolium-formazan system has been shown by many researchers as an indicator of microbial growth in liquid and solid culture media [Saviano and Lourenco, 2018]. In the TTC dehydrogenase activity experiment, 2 ml of 1.5 mol/L Tris-HCl buffer (pH 8.8) and 2 ml of TTC-glucose solution (mixture of 4% TTC solution and 0.1 mol/L of glucose solution in 1:1 ratio) were added into 6 ml of *S. aureus* culture grown for 12 h in the presence of antimicrobials. Then, the mixture was incubated at 37°C for 2 h. After incubation, 7.5 ml of methylbenzene (Merck, Germany) and 7.5 ml glacial acetic acid (GAA) (Merck, Germany) were added to the samples and they were kept at room temperature for 1 h. The upper phase of samples was used for the measurement of absorbance at 485 nm using a UV spectrophotometer (Lambda25, Perkin Elmer (USA)). Methylbenzene was used as a blank solution during relative dehydrogenase activity (RDA).

$$\text{RDA (\%)} = (A_X/A_0) \times 100 \quad (5.1)$$

where, A_X indicates the absorbance of treated samples, and A_0 indicates the absorbance of the control sample [Ding et al. 2016], [Ersoy et al. 2019].

5.2.9. Measurement of Conductivity

10 ml *S. aureus* culture grown for 12 h in the presence of antimicrobials was centrifuged at 3500 rpm, 4°C for 20 min. The ion concentration of the culture supernatants was measured by a multimeter (WTW Inolab, Germany) to detect cellular membrane disruption.

5.2.10. Measurement of DNA and Protein Leakage

The leakage of DNA from the cells was determined by measuring the absorbance of culture supernatants at 260 nm by a spectrophotometer (Lambda25, Perkin Elmer, USA). An absorbance of 1 unit at 260 nm corresponds to 50 µg DNA per ml [Yang et al. 2016].

Bradford assay was used to determine the amount of released protein in the supernatant of the samples as described in the kit manual (Thermo Fisher Scientific, Waltham, USA). 1 ml of supernatant was mixed with 1 ml of Coomassie Brilliant Blue G-250 solution and incubated for 10 min at room temperature in dark. Then, the absorbance of the samples was measured at 595 nm by using a spectrophotometer (Lambda25, Perkin Elmer (USA)). A calibration curve was prepared with BSA (bovine serum albumin) and the concentration of the proteins in the samples was calculated according to the equation of this curve. Spectrophotometric readings resulting from the added nisin and BHI were subtracted from the last readings when calculating the protein leakage because, unlike gilaburu, nisin affects the total protein amount; and the BHI medium contains protein.

5.2.11. Extraction of Chia Mucilage

The modified method described by Hernández-Nava was used. Chia seeds were dispersed in distilled water in a ratio of 1:20 (w/v). In order to enable water absorption of mucilage, it was kept in stirrer for 4 hr at 35 ± 1.0 °C, so that the seeds became swollen. Chia seeds were mechanically separated from mucilage by using a piece of cloth. Chia mucilage was condensed in 50°C during 24 hours. Later it was freeze-dried (VirTis Ultra 25, SP Scientific, USA) [Hernández-Nava et. al., 2019]. After the system was placed under vacuum at 50 kPa pressure, the freezing process was started. The freezing process was terminated after the shelf temperature was reduced to -30°C and the samples were ensured to be frozen. Condenser temperature lowered to -40°C. The system pressure was reduced to 5 Pa and the drying process was started under vacuum. The shelf temperature was kept constant at 15°C during the drying process. After 48 hours of freeze-drying, the product was taken from the drying unit.

5.2.12. Coacervation of Gelatine and Chia

The method described by Hernández-Nava was used with small modifications. Gelatine (GE) and chia mucilage (CM) were mixed at mass ratios of 2:1. pH is adjusted to 3.5 by adding HCl 0.1 N. GE and CM solutions were prepared in distilled water with constant stirring (350 rpm) at 40 ± 1.0 °C until total dissolution was obtained. Then, solution is homogenized by ultrasound sonicator (for 20 min applying an amplitude of 10%). The system was cooled down to 25 °C [Hernández-Nava et. al., 2019].

5.2.13. Preparation of Antimicrobial Films

Antimicrobial films were prepared with coacervated gelatine (GE) and chia mucilage (CM) solution. Glycerol (Gl) added into the solution as a plasticizer. Nisin (Ni) and gilaburu (G) were integrated into the solution according to their MIC levels for *S. aureus*. All films were prepared at a final 10 ml solution with constant stirring (350 rpm) at 40 ± 1.0 °C. They poured on wax paper and kept at room temperature for drying during 24 h. The film ingredients, amount of substances and film properties was shown in Table 5.6.

Table 5.6: The amount of film components (GE: Gelatine, CM: Chia mucilage, Gl: Glycerol, G: Gilaburu, Ni: Nisin, DW: Distilled water).

Components	Amount of components
GE + CM	0.8 g + 0.4 g / in 10 ml DW
GE + CM + Gl	0.8 g + 0.4 g / in 0.8 ml Gl + 9.2 ml DW
GE + CM+ GI + G	0.8 g + 0.4 g / in 0.8 ml Gl + 5 ml G + 4.2 ml DW
GE + CM + GI + Ni	0.8 g + 0.4 g / in 0.8 ml Gl + 1 ml (250 µg) Ni + 8.2 ml DW
GE + CM+ GI + G + Ni	0.8 g + 0.4 g / in 0.8 ml Gl + 1 ml (31,25 µg) Ni + 2.5 ml G + 5.7 ml DW

5.2.14. FTIR and SEM Analyses

Chemical interactions data were obtained in the range of wave number from 4000 to 600 cm^{-1} from a FTIR instrument (Spectrum 100, Perkin Elmer, ABD).

Morphological assessment of films was performed using a scanning electron microscope (SEM) (XL 30S FEG, Philips, Holland). SEM applied with an accelerating voltage of 5 kV. Surfaces of the film samples were coated with gold using sputter (Quorum SC 7620, Quorum tech, UK) at 3 kV for 60 s. SEM images were acquired with different magnifications under high vacuum conditions.

5.2.15. Evaluation of antimicrobial property of films on *S. aureus*

Antimicrobial films were prepared as defined in Table 5.6. After drying and UV sterilization in class II cabinet at room temperature for 24 h, 1 cm square films (0.04 g of each) were placed on BHI agar plates. BHI agar plates prepared as two layers: first layer (bottom) had 1.5% agar and the second layer (upper) had 0.5% agar. *S. aureus* (10^6 CFU/100 μl) cells were mixed with melted soft agar, mixed and then poured onto the first layer BHI agar. The plates were incubated at 30 °C for 24 h and the inhibition zones were evaluated.

The films prepared as described above were also evaluated in liquid culture of *S. aureus* (10^6 CFU/ml). 0.5 g film were put into 100 ml *S. aureus* culture in BHI broth and incubated at 30 °C. Optical density in 600 nm was measured in spectrophotometer (Lambda25, Perkin Elmer (USA)) at certain time intervals (0 h, 6 h, 24 h and 48 h).

6. RESULTS

6.1. Determination of Antimicrobial Effects of Nisin on Different Bacteria

The antimicrobial effects of nisin on *S. aureus* (NCTC 10788, bioMérieux, France), *Listeria monocytogenes* (ATCC 19111, Microbiologics, USA), *Bacillus cereus* (ATCC 10876, USA), *Escherichia coli* (NCTC 12923, bioMérieux, France) and *Pseudomonas aeruginosa* (NCTC 12924, bioMérieux, France) were evaluated separately under different pHs (4, 5, 6, 7, 8 and 9) and salt concentrations (NaCl, 0.5, 2.5, 4.5 and 6.5% w/v) at 30 °C in BHI broth. Besides, the effect of temperature on nisin activity was evaluated. In the experiments with different salt concentrations and temperatures, pH was kept constant at 7. BHI broth contains 0.5% salt in its original formulation, other salt concentrations were calculated according to this value. The effect of different pHs, salt concentrations and temperatures on MIC and MBC values were summarized in Table 6.1, Table 6.2 and Table 6.3, respectively.

Table 6.1. Minimum inhibitory concentrations (µg/ml; MIC) and minimum bactericidal concentrations (µg/ml; MBC) of nisin in different pHs.

Type of bacteria		pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
<i>S. aureus</i>	MIC	12.5	25	50	50	100	100
	MBC	50	100	100	100	100	100
<i>L. monocytogenes</i>	MIC	ND	31.25	62.5	62.5	250	250
	MBC	ND	125	125	125	500	500
<i>B. cereus</i>	MIC	ND	31.25	31.25	125	125	62.5
	MBC	ND	125	125	125	125	62.5
<i>E. coli</i>	MIC	<15.625	250	1000	1000	1000	1000
	MBC	<15.625	500	1000	2000	2000	2000
<i>P. aeruginosa</i>	MIC	ND	1000	2000	2000	2000	2000
	MBC	ND	>2000	>2000	>2000	>2000	>2000

ND: Growth not detected

Table 6.2. Minimum inhibitory concentrations ($\mu\text{g/ml}$; MIC) and minimum bactericidal concentrations ($\mu\text{g/ml}$; MBC) of nisin in different salt concentrations.

Type of bacteria		0.50%	2.50%	4.50%	6.50%	8.50%
<i>S. aureus</i>	MIC	50	50	50	12,5	12,5
	MBC	100	100	100	100	100
<i>L. monocytogenes</i>	MIC	62.5	31.25	7.81	<3.9	<3.9
	MBC	125	62.5	62.5	31.25	31.25
<i>B. cereus</i>	MIC	125	62.5	15.62	<3.9	ND
	MBC	125	62.5	31.25	<3.9	ND
<i>E. coli</i>	MIC	1000	500	250	62.5	<15.625
	MBC	2000	500	250	250	125
<i>P. aeruginosa</i>	MIC	2000	1000	250	62.25	<15.625
	MBC	>2000	2000	2000	2000	2000

ND: Growth not detected

Table 6.3. Minimum inhibitory concentrations ($\mu\text{g/ml}$; MIC) and minimum bactericidal concentrations ($\mu\text{g/ml}$; MBC) of nisin in different temperatures.

Type of bacteria		8°C	15°C	20°C	25°C	30°C	35°C	45°C
<i>S. aureus</i>	MIC	6.25	25	25	50	50	50	<3.125
	MBC	100	100	100	100	100	100	<3.125
<i>L. monocytogenes</i>	MIC	15.62	31.125	125	125	62.5	62.5	ND
	MBC	125	125	125	125	125	125	ND
<i>B. cereus</i>	MIC	<7.81	125	125	125	125	125	125
	MBC	62.5	250	250	250	125	125	125
<i>E. coli</i>	MIC	1000	1000	1000	1000	1000	1000	ND
	MBC	2000	2000	2000	2000	2000	2000	ND
<i>P. aeruginosa</i>	MIC	2000	2000	2000	2000	2000	2000	ND
	MBC	>2000	>2000	>2000	>2000	>2000	>2000	ND

ND: Growth not detected

The lowest MIC of nisin against *S. aureus* grown in BHI (0.5% salt) was obtained at pH 4 as 12.5 $\mu\text{g/ml}$ (Table 6.1). When the pH level was increased to 5, the MIC level was determined as 25 $\mu\text{g/ml}$. Increase in pH was found directionally proportional with the increase in MIC values. At pH 8 and 9, MIC level was detected

as 100 µg/ml. The MBC level in different pHs were same as 100 µg/ml except pH 4 where MBC was detected as 50 µg/ml. The MIC of nisin was found 50 µg/ml at 0.5%, 2.5%, and 4.5% salt concentrations when the pH was kept at 7. However, if salt concentrations were changed to 6.5% and 8.5%, the MIC of nisin had a dramatic decrease as 12.5 µg/ml. The MBC of nisin was 100 µg/ml in all salt concentrations and it was not affected by the changes in salt concentrations (Table 6.2). According the results in Table 6.3, when temperatures were decreased, the MIC of nisin was also decreased. The MIC level was same as 50 µg/ml at 25°C, 30°C and 35°C and 25 µg/ml at 15°C and 20°C. But in 8°C and 45 °C, the MIC level of nisin was very low as 6.125 µg/ml and <3.125 µg/ml, respectively.

The effects of nisin on *L. monocytogenes* were investigated under different conditions. It was observed that as the pH value decreased, the MIC and MBC values of nisin also decreased (Table 6.1). *L. monocytogenes* did not grow at pH 4, so the MIC and MBC values could not be determined. The lowest MIC and MBC values was observed at pH 5 (31.25 µg/ml). In studies conducted with different salt concentrations for *L. monocytogenes* as shown in Table 6.2, as the salt concentration increased, a decrease was observed in both MIC and MBC values and the lowest MIC and MBC values were determined at 6.5% and 8.5 % salt ratios as <3.9 µg/ml and 31.25 µg/ml, respectively. The effect of nisin on *L. monocytogenes* was analyzed at different temperatures (Table 6.3). There was a significant decrease of MIC in lower temperatures. The lowest MIC value was 15.62 µg/ml at 8 °C. MIC values were same for 30 °C and 35 °C (62.5 µg/ml) and 20 °C and 25 °C (125 µg/ml). There were no significant difference in MBC values (125 µg/ml) at all temperatures. *L. monocytogenes* did not grow at 45 °C.

As can be seen in Table 6.1, *B. cereus* did not grow at pH 4 and as the pH value decreased, the MIC values of nisin also decreased. However the MIC and MBC values of nisin were 62.5 µg/ml at pH 9. There was no difference in MIC and MBC values at pH 7 and pH 8. There was a significant decrease in MIC and MBC values of nisin at pH 5 and 6 (31.25 µg/ml). In studies conducted with different salt concentrations, as the salt concentration increased, a decrease was observed in the MIC and MBC values and the lowest MIC value was determined at 6.5 % salt ratio as <3.9 µg/ml. *B. cereus* did not grow at 8,5 % salt concentration. The effect of nisin on *B. cereus* was analyzed

at different temperatures and no significant difference was found in MIC values (125 µg/ml) at all temperatures (Table 6.3). MBC values for 30 °C, 35°C and 45°C were same as MIC values but they were two times higher than MIC values for 15 °C, 20 °C and 25 °C. Besides, the MIC value of nisin was <7.81 µg/ml and MBC was 62.5 µg/ml for 8 °C.

In the experiments we performed at different salt concentrations, pHs and temperatures, we saw that nisin had no efficient antimicrobial effect on gram negative *E. coli* and *P. aeruginosa* (Table 6.1, Table 6.2, Table 6.3).

When all the results are evaluated together, it can be concluded that nisin was more effective on bacteria at low pH, the increase in salt concentration could have a positive effect on nisin activity, and mesophilic temperatures did not have an important effect on the activity of nisin. The effect of nisin on gram-negative bacteria (*E. coli* and *P. aeruginosa*) could only be demonstrated at very high nisin concentrations. It is safe to say that *P. aeruginosa* was the most resistant and *S. aureus* was the most susceptible bacteria to nisin.

Nisin can not be synthesized chemically, its production is expensive and complex downstream processing are required. To reduce the amount of nisin used as a food preservative, its use with gilaburu (*Viburnum opulus* L.) was tested on *S. aureus*.

6.2. Determination of Antimicrobial Effects of Gilaburu on *S. aureus*

The antimicrobial effect of gilaburu on *S. aureus* was evaluated separately under different pHs (5, 6, and 7) and salt concentrations (NaCl, 0.5, 2.5, and 4.5% w/v) at 30 °C in BHI broth. BHI broth contains 0.5% salt in its original formulation, other salt concentrations were calculated according to this value. The MIC and MBC values of gilaburu did not change in response to different pH or salt concentrations and were found as 50% and >50%, respectively.

Table 6.4: Minimum inhibitory concentrations ($\mu\text{g/ml}$; MIC) and minimum bactericidal concentrations ($\mu\text{g/ml}$; MBC) of gilaburu on *S. aureus* in different pHs and salt concentrations at 30°C in BHI broth.

Antimicrobial		pH			Salt concentrations		
		pH 5	pH 6	pH 7	0.5 %	2.5 %	4.5 %
Gilaburu	MIC	50%	50%	50%	50 %	50 %	50 %
	MBC	>50%	>50%	>50%	>50 %	>50 %	>50 %

6.3. Determination of the Effect of Gilaburu on Nisin Activity

6.3.1. Checkboard Assay Results

Checkboard assays were conducted in different salt concentrations (0.5%, 2.5% and 4.5% w/v), at pH 5 and 30 °C to determine the existence of synergy between nisin and gilaburu (Table 6.5). The FIC value was 0.625 for 0.5% w/v salt concentration showing and additive effect between nisin and gilaburu when they were used together. Also, a synergy was found between nisin and gilaburu (FIC=0.25) for both 2.5% and 4.5% w/v salt concentrations.

Table 6.5: MIC values of nisin and gilaburu alone or in combinations and the FIC values of combinations against *S. aureus* at pH 5 and 30°C.

MIC ^a		MIC (FIC) in combination		
Nisin	Gilaburu	Nisin+Gilaburu	Nisin+Gilaburu	Nisin+Gilaburu
(0.5% Salt)		(0.5% Salt)	(2.5% Salt)	(4.5% Salt)
25	50	3.125/25 (0.625)	3.125/6.25 (0.25)	3.125/6.25 (0.25)

^a Concentrations are given in $\mu\text{g/mL}$ for Nisin and % for Gilaburu

6.3.2. Agar Disk Diffusion Assay Results

Agar disk diffusion test was carried out to further investigate the synergistic interaction between nisin and gilaburu (Figure 6.1, Table 6.6). Nisin at 3.125 $\mu\text{g/ml}$ concentration was mixed with different concentrations of gilaburu at different salt concentrations (0.5 and 2.5% w/v). Combination of nisin (3.125 $\mu\text{g/mL}$, 8 fold less

than the MIC value at 0.5% salt) with a 100% concentration of Gilaburu at both salt concentrations showed high antimicrobial activity. At 0.5% salt concentration, growth of *S. aureus* was inhibited by nisin (3.125 $\mu\text{g}/\text{mL}$) with different concentrations of gilaburu which ranged from 100% to 12.5%. Nisin (3.125 $\mu\text{g}/\text{mL}$) combined with 50% and 25% gilaburu also showed a high antimicrobial activity at 2.5% salt concentration, while combination with 12.5% gilaburu showed an intermediate activity.

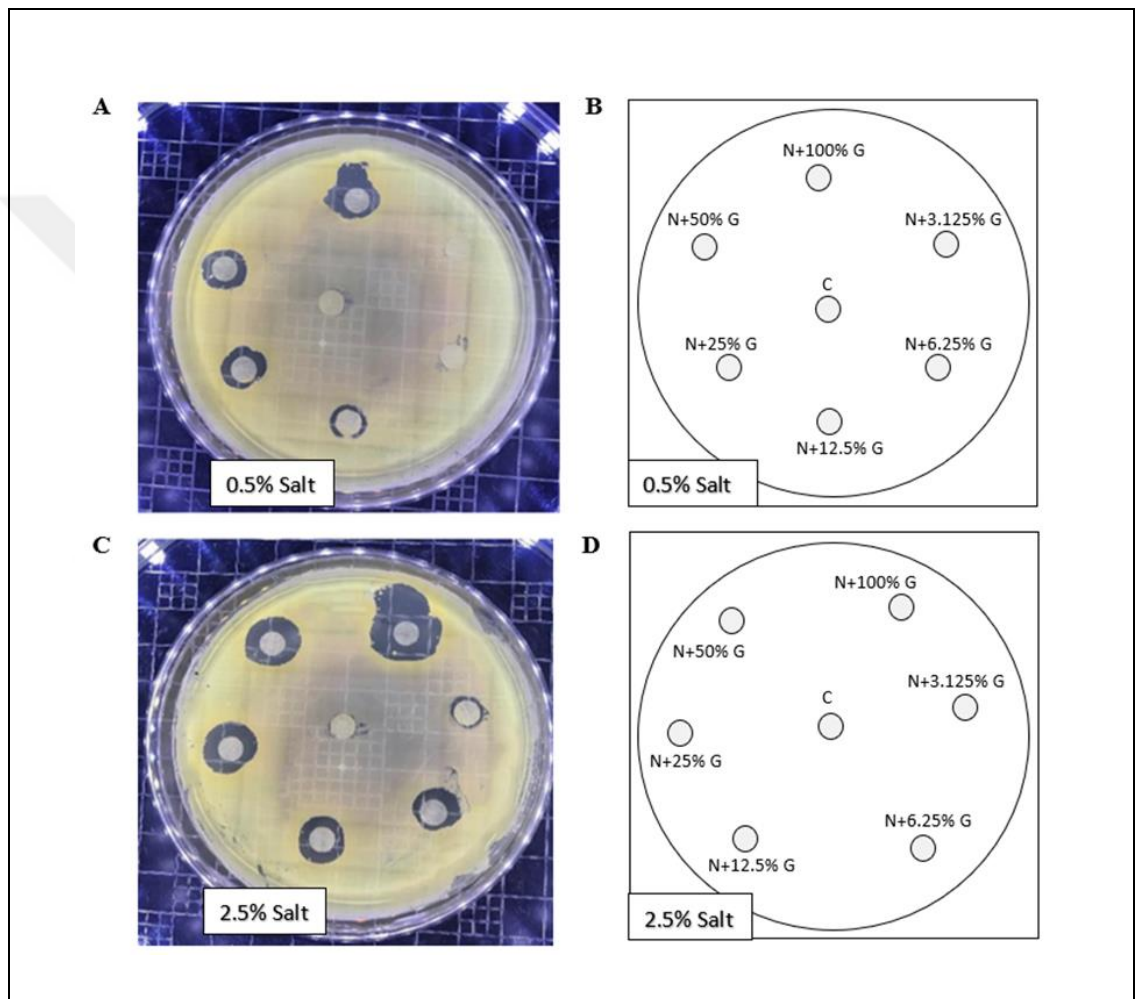


Figure 6.1: Agar disk diffusion assay for nisin combined with different concentrations of gilaburu against *S. aureus*. The concentration values of antimicrobials for A (0.5% salt) and C (2.5% salt) were given in B and D, respectively. (Ni: 3.125 $\mu\text{g}/\text{mL}$ Nisin; G: Gilaburu %; C: Control, 3.125 $\mu\text{g}/\text{mL}$ Nisin).

Table 6.6: Inhibition zone diameters of nisin and gilaburu combinations against *S. aureus* in different salt concentrations at pH 5 and 30°C. Inhibition zone diameters are given as mean $\pm$ standard deviation. Statistics are given in Appendix B.

Concentrations ^a (Nisin+Gilaburu)	Inhibition zone diameters (mm)	
	0.5% Salt	2.5% Salt
0/0 (Control)	0 $\pm$ 0	0 $\pm$ 0
3.125/100	10 $\pm$ 0.2	15 $\pm$ 0.15
3.125/50	8 $\pm$ 0.1	10 $\pm$ 0.1
3.125/25	7 $\pm$ 0.11	10 $\pm$ 0.15
3.125/12.5	6.7 $\pm$ 0.2	8 $\pm$ 0.15
3.125/6.25	0 $\pm$ 0	7.5 $\pm$ 0.2
3.125/3.125	0 $\pm$ 0	6.5 $\pm$ 0.2

^a Concentrations are given in $\mu\text{g/mL}$ for nisin and % for gilaburu.

6.4. Effect of Nisin and Gilaburu on *S. aureus*

6.4.1. Time Kill Assay Results

The effect of nisin and gilaburu alone or in combination on *S. aureus* was also studied in a time-kill assay at different time intervals. In the experiments, nisin and gilaburu concentrations were used at their MIC levels, 25 $\mu\text{g/mL}$ and 50%, respectively. Regarding checkboard assay results, nisin and gilaburu combinational treatments were conducted as 3.125 $\mu\text{g/mL}$ nisin + 25% gilaburu and 3.125 $\mu\text{g/mL}$ nisin + 6.25% gilaburu at 0.5% and 2.5% salt concentrations, respectively.

Nisin and gilaburu affected the growth of *S. aureus* negatively and a continuous decrease in cell number was seen until the 12th hour of growth in the presence of antimicrobials (Figure 6.2). Nisin and gilaburu combinations both in 0.5% and 2.5% salt concentrations had caused a ≥ 2 -log reduction in *S. aureus* cell number at the 12th

hour. This result also showed that antimicrobial agents resulted in a synergistic effect when they are used together.

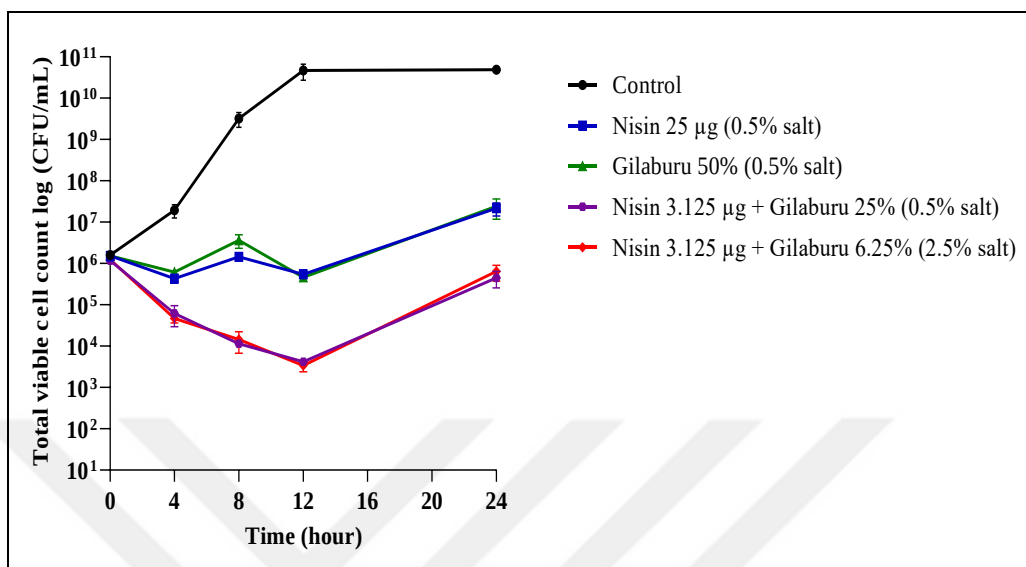


Figure. 6.2: Time-kill assay results of nisin and gilaburu (alone or in combination) against *S. aureus* grown at pH 5 and 30°C. Statistics are given in Appendix B.

6.4.2. Flow Cytometry

Since the lowest number of viable cells in all combinations in the time-kill assay belonged to the 12th hour of bacterial growth for all combinations, flow cytometry analysis was carried out using the samples collected at this time point. The dead cell ratio was taken as the percentage of PI-positive cells in the samples. According to the mean of triplet results, nisin (25 µg/ml) and gilaburu (50%) alone caused a 60.7% and 54.9% dead cell ratio, respectively (Figure 6.3A). When nisin (3.125 µg/ml) and gilaburu (25%) were used together, the dead cell ratio was found to be 54.9 % in the same conditions. In the presence of 2.5% salt in the medium, a dead cell ratio of 51.9% was obtained with 3.125 µg/ml nisin and 6.25% gilaburu. The flow cytometry result of one of the biological replicates was shown in Figure 6.3B.

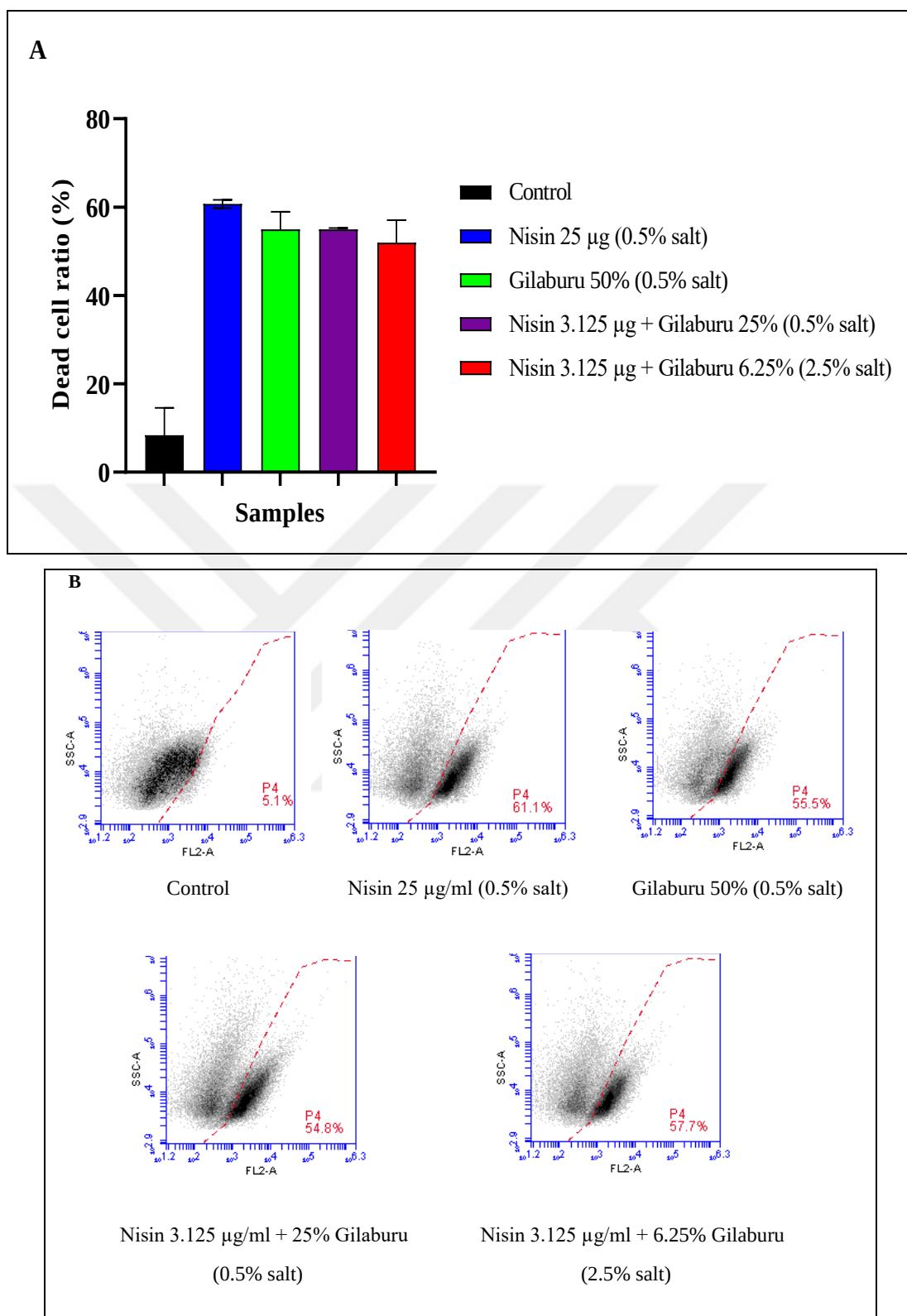


Figure 6.3: (A) The dead cell ratios of *S. aureus* at 12th hour of growth in the presence of nisin and gilaburu (alone or in combination) by Flow cytometry. (B) The original flow cytometry results of one of the biological replicates. Statistics are given in Appendix B.

6.4.3. Measurement of Relative TTC Dehydrogenase Activity

2,3,5-triphenyl tetrazolium chloride (TTC) is reduced in living cells by dehydrogenase enzyme to produce 1,3,5-triphenyl tetrazolium formazan which is an insoluble red complex. TTC-dehydrogenase activities of 12 h grown *S. aureus* were measured. The results were 51.01%, 51.18%, 52.87% and 50.36% after treatments with 25 µg/ml nisin, 50% gilaburu, 3.125 µg/ml nisin + 25% gilaburu (0.5% salt) and 3.125 µg/ml nisin + 6.25% gilaburu (2.5% salt), respectively (Figure 6.4).

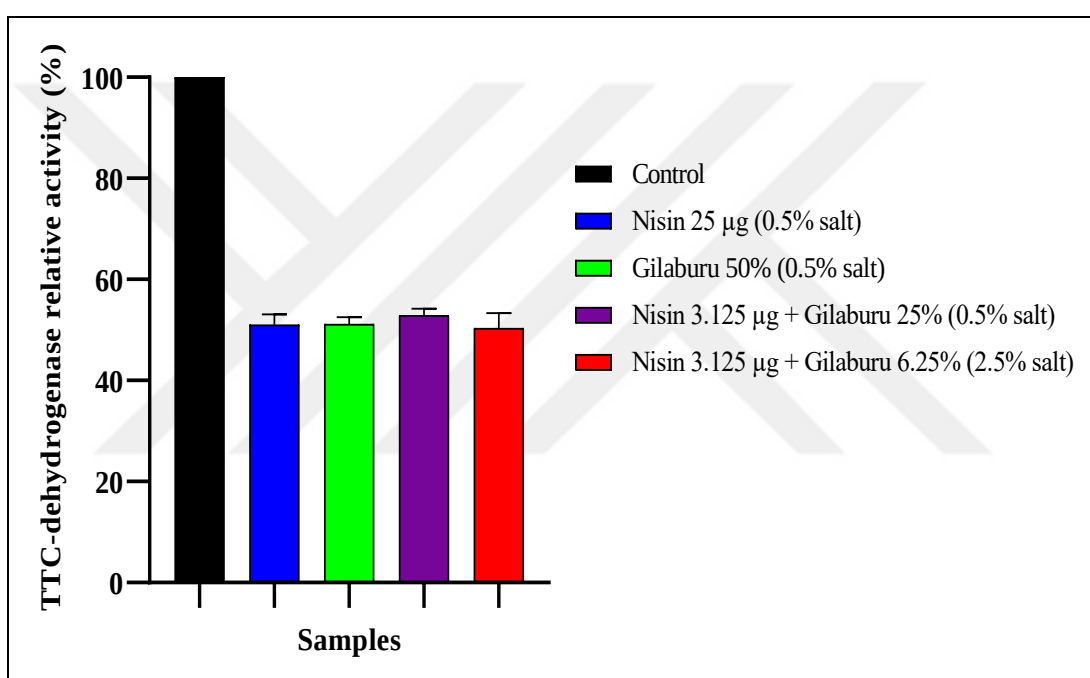


Figure 6.4: Relative TTC-dehydrogenase activity of *S. aureus* at 12th hour of growth in the presence of nisin and gilaburu (alone or in combination). Statistics are given in Appendix B.

6.4.4. Measurement of Conductivity

The changes in the conductivity of supernatants of *S. aureus* after treatment with nisin, gilaburu, and their combinations are shown in Figure 6.5. According to the results, it was seen that the conductivity increased in all samples by comparison with the control sample. This was interpreted as an indication of membrane damage.

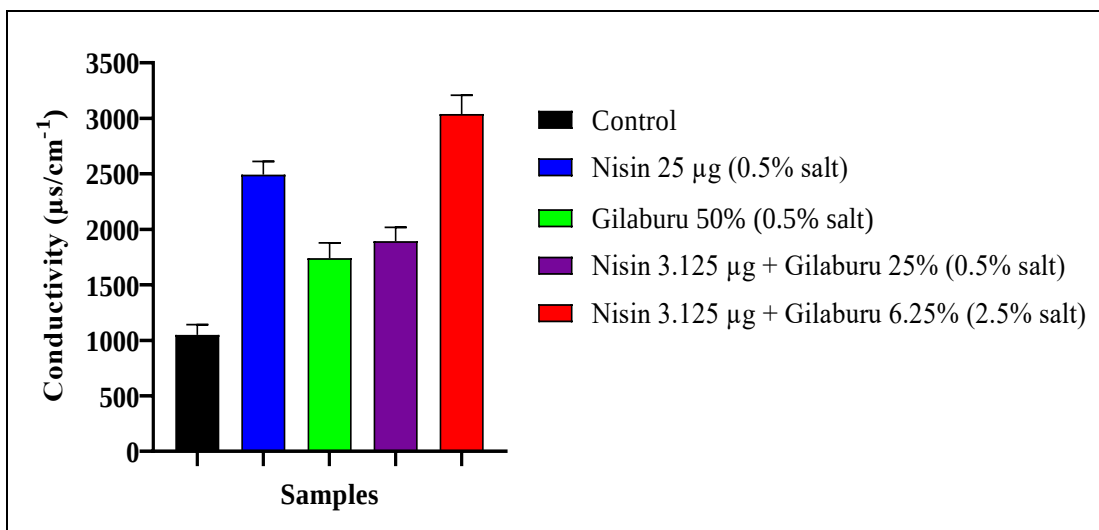


Figure 6.5: Conductivity of *S. aureus* supernatants at 12th hour of growth in the presence of nisin and gilaburu (alone or in combination). Statistics are given in Appendix B.

6.4.5. Measurement of DNA and Protein Leakage

The results of DNA leakage at OD₂₆₀ are shown in Figure 6.6. The highest level of DNA leakage was 26.82 µg/ml in the nisin treated-sample. This DNA leakage value was followed by nisin + gilaburu combination in 2.5% salt concentration as 20.58 µg/ml, nisin + gilaburu combination in 0.5% salt concentration as 15.74 µg/ml, and gilaburu alone as 12.03 µg/ml.

Protein concentrations in the supernatants of *S. aureus*. However, the protein leakage levels were very similar to each other and were 10.3 µg/ml for the nisin + gilaburu combination in 0.5% salt and 10.96 µg/ml for nisin + gilaburu combination in 2.5% salt (Figure 6.7).

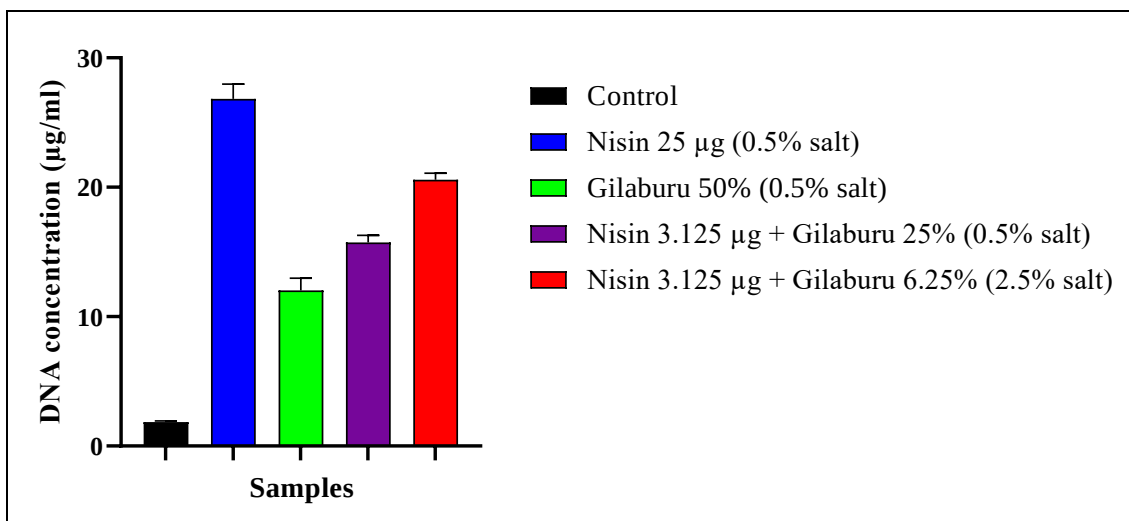


Figure 6.6: DNA concentrations of *S. aureus* supernatants at 12th hour of growth in the presence of nisin and gilaburu (alone or in combination). Statistics are given in Appendix B.

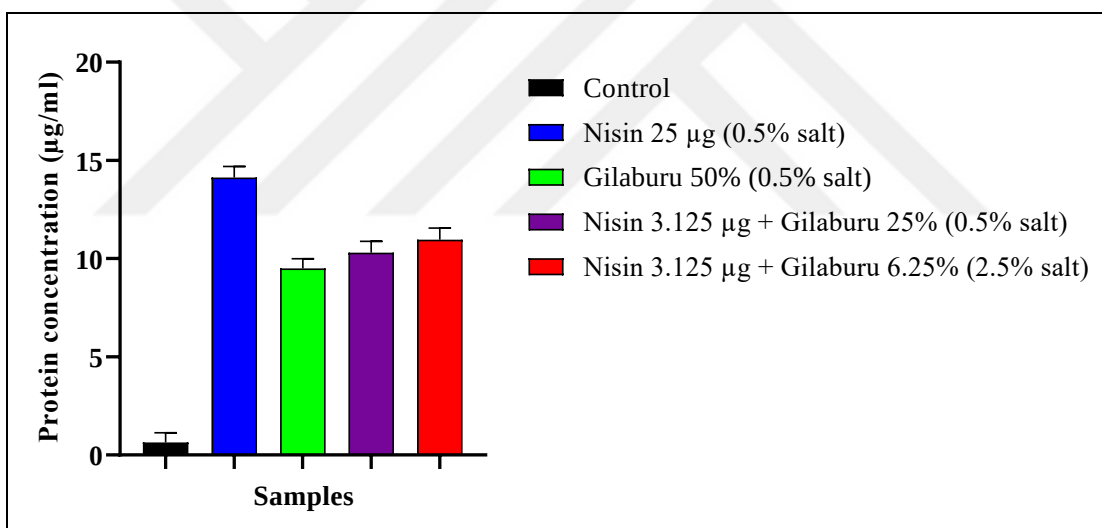


Figure 6.7: Protein concentrations of *S. aureus* supernatants at 12th hour of growth in the presence of nisin and gilaburu (alone or in combination). Statistics are given in Appendix B.

6.4.6. Extraction of Chia Mucilage

Swollen chia seeds and chia mucilage after separation from seeds were shown in Figure 6.8. Mucilage secretion can be seen around the seeds (Figure 6.8a). Extracted chia mucilage was yellowish in colour, tasteless with a slight odor (Figure 6.8b).

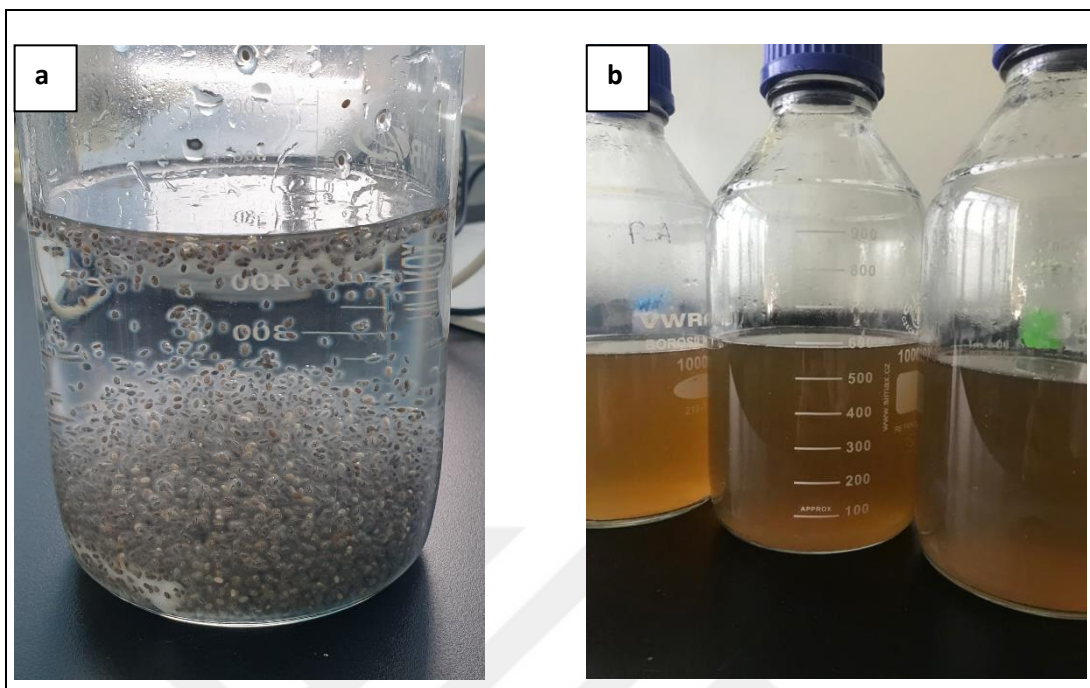


Figure 6.8: Swollen chia seeds (a) and chia mucilage after separation from seeds (b).

Lyophilized chia mucilage was obtained as cotton candy like texture and very light yellowish colour as seen in Figure 6.9. Since it was observed that it shrinks and becomes sticky when affected by the humidity of the air, it was stored at room temperature in a closed glass bottle containing moisture-retaining beads until used as packaging material.



Figure 6.9: Lyophilized chia mucilage; Right after lyophilization (a), recovered and shred mucilage (b).

6.4.7. Antimicrobial Agent Incorporated Films

Gelatine (GE) and chia mucilage (CM) was used as the main components of films. They are together had a rigid and fragile form. Addition of glyserol (GI) enabled flexibility of the film. Films with gilaburu or without gilaburu only had difference in colour because of gilaburu's original pinkish red colour. That is gilaburu (G) added films showed weak reddish colour than yellowish. Nisin (Ni) addition neither affected colour nor flexibility of the films. The components, properties and images of different films prepared in this study were shown below in Table 6.3 and Figure 6.10. Glycerol (GI) effect on the structure of film can be seen in Figure 6.11.

Table 6.7: Components and properties of films (GE: Gelatine, CM: Chia mucilage, GI: Glycerol, G: Gilaburu, Ni: Nisin).

Components	Film properties
GE + CM	Fragile, pale yellowish
GE + CM + GI	Flexible, pale yellowish
GE + CM+ GI + G	Flexible, pale reddish
GE + CM + GI + Ni	Flexible, pale yellowish
GE + CM+ GI + G + Ni	Flexible, pale reddish

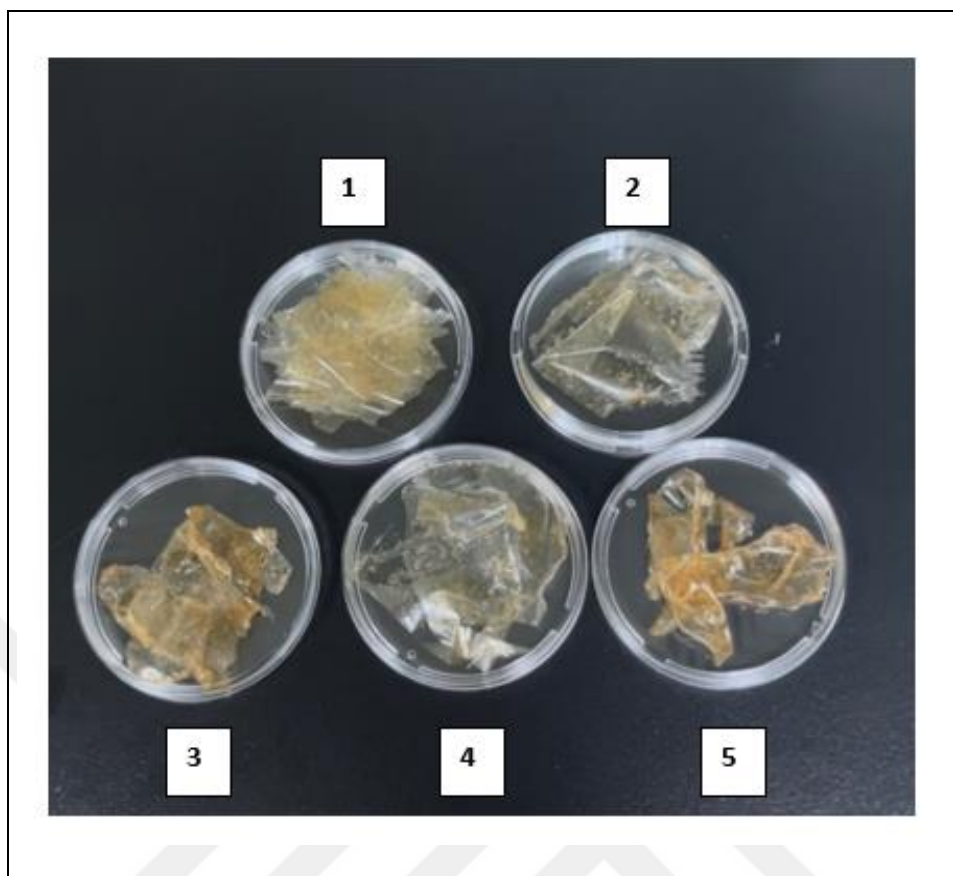


Figure 6.10: Films and antimicrobial agent incorporated films (GE: Gelatine, CM: Chia mucilage, Gl: Glycerol, G: Gilaburu, Ni: Nisin, 1: GE+CM, 2: GE+CM+Gli, 3: GE+CM+Gli+G, 4: GE+CM+Gli+Ni, 5: GE+CM+Gli+G+Ni).

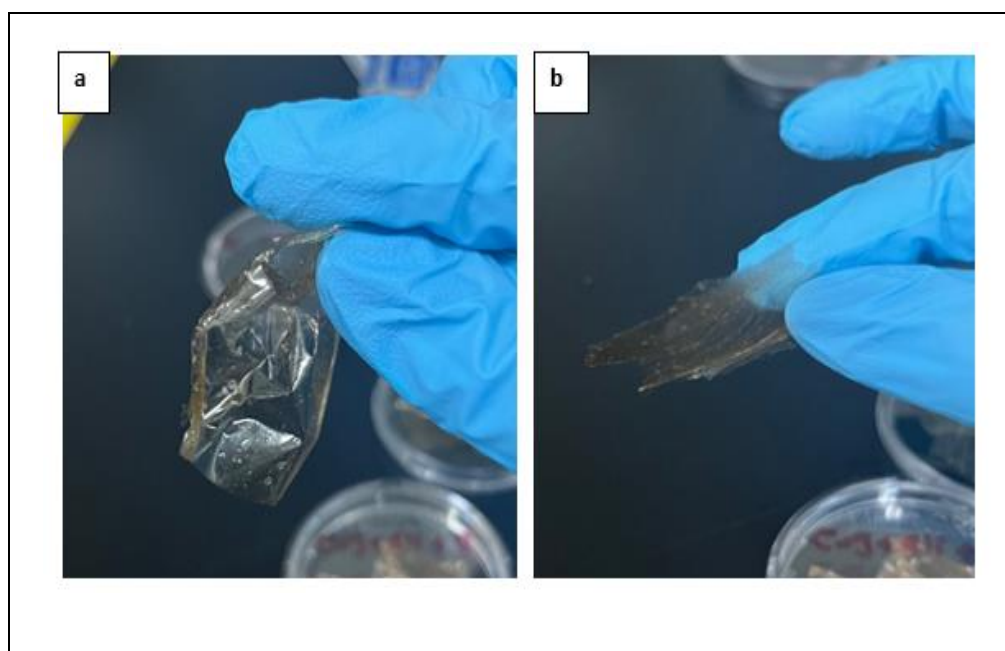


Figure 6.11: The effect of glycerol on coacervated film, GE+CM film with glycerol (a) and GE+CM film without glycerol (b) (GE: Gelatin, CM: Chia mucilage).

6.4.8. FTIR Results of Films

In FTIR spectrum, nisin (Ni) showed peaks at 3267.8 cm^{-1} , 2975.4 cm^{-1} . The peaks at 1628.3 cm^{-1} , 1577.3 cm^{-1} , 1259.4 cm^{-1} were detected for amide I, II and III region of Nisin. The peaks which are characterized by hydroxyl and carbonyl functional groups at 3291 , 2881.3 , 1720.2 and 1271.6 cm^{-1} were seen for gilaburu (G). According the results from this study, glycerol had a band at 3290 cm^{-1} for OH stretching, another bands at 2881 and 2936 cm^{-1} for C-H stretching and band at 1661 cm^{-1} for C=O stretching. The typical sharp bands were also seen at 1108.7 , 1033.9 and 993.5 cm^{-1} . For gelatine (GE), characteristic bands of amide I at 1630.8 cm^{-1} , amide II at 1528.5 cm^{-1} and amide III at 1237.4 cm^{-1} were detected. Chia musilage (CM), uronic acid peaks were at 1595 and 1409.4 cm^{-1} and the C-O-C stretching of 1→4 glycosidic bond and C-O-H bending exhibited a peak at 1034.2 cm^{-1} . All results are shown in Figure 6.12.

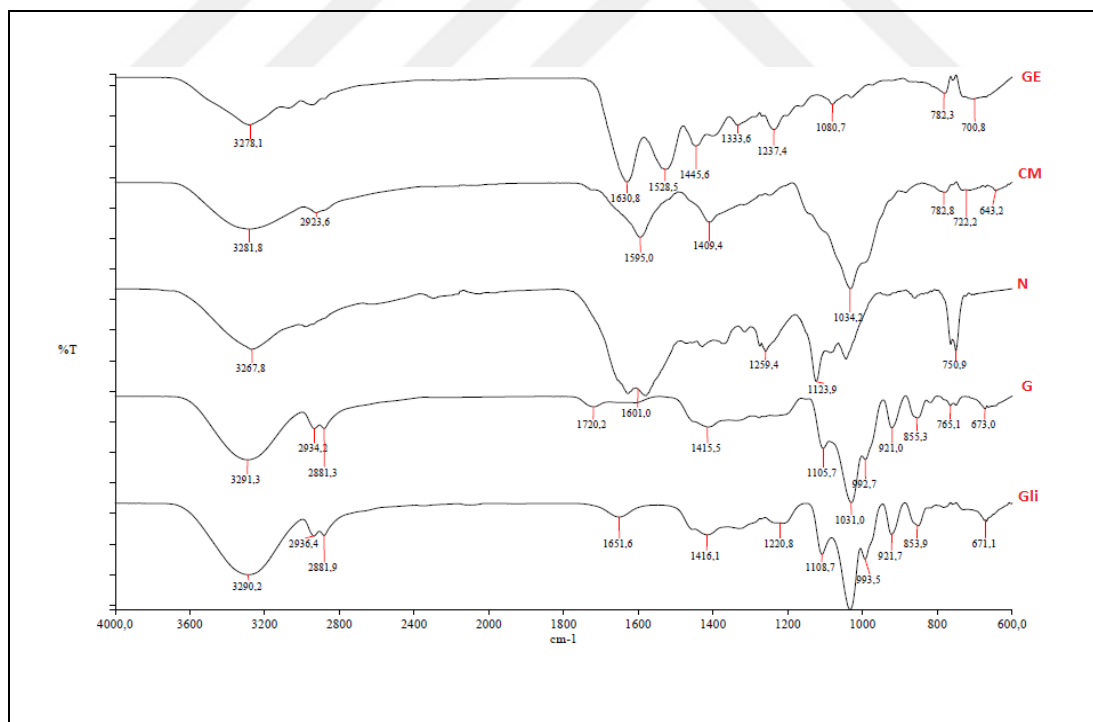


Figure 6.12: FTIR results of film components (GE: Gelatine, CM: Chia mucilage, Ni: Nisin, G: Gilaburu, Gli: Glycerol).

FTIR spectra of gelatine and chia mucilage film showed the combination of bands from gelatine and chia mucilage, however, dominated by gelatine. There is no new peaks in spectra, so it suggests an electrostatic interactions rather than chemical interactions between gelatine and chia mucilage. The interpretation of the peaks of different combinations showed that nisin and gilaburu did not react chemically with each other and also with gelatine and chia mucilage in the coacervate (Figure 6.13).

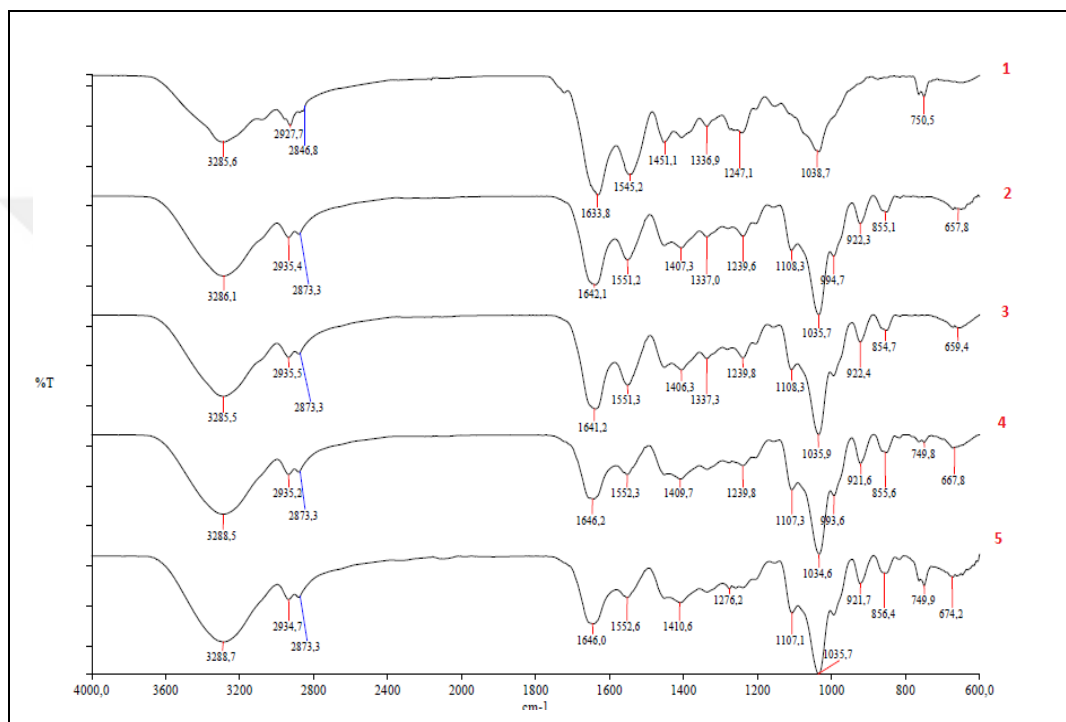


Figure 6.13: FTIR results of films (GE: Gelatine, CM: Chia mucilage, Ni: Nisin, G: Gilaburu, Gli: Glycerol, 1: GE+CM, 2: GE+CM+Gli, 3: GE+CM+Gli+G, 4: GE+CM+Gli+Ni, 5: GE+CM+Gli+G+Ni).

6.4.9. SEM Results of Films

Surface morphology of the gelatine and chia mucilage coacervate had a grape grain like appearance with sharp lined extensions as shown in Figure 6.14. After adding glycerol as a plasticizer, coacervate had a flatter and regular with grape agglomerate like ridges as shown in Figure 6.15. As can be seen in Figure 6.16 and Figure 6.17 pure nisin was seen as regular spherical clusters and gilaburu had a morphology like honeycomb structures, respectively. When nisin was integrated into

the glycerol added coacervate, regular spherical nisin particles was found to disperse in all area homogenously (Figure 6.18). Figure 6.19 shows SEM image of glycerol added gelatine and chia mucilage films integrated with both nisin and gilaburu.

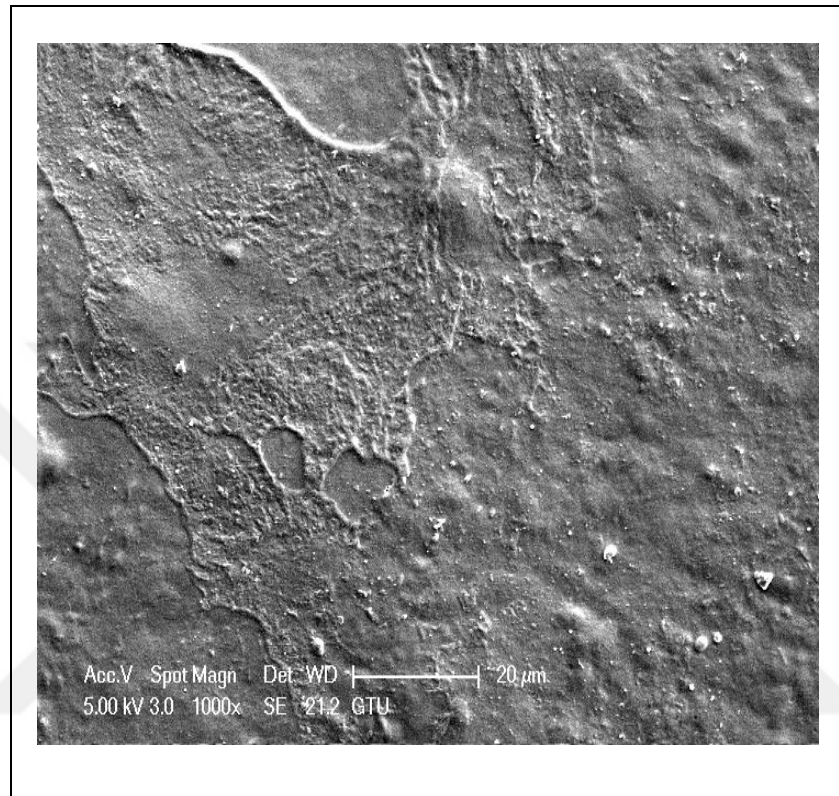


Figure 6.14: SEM image of gelatine and chia mucilage film (1000x).

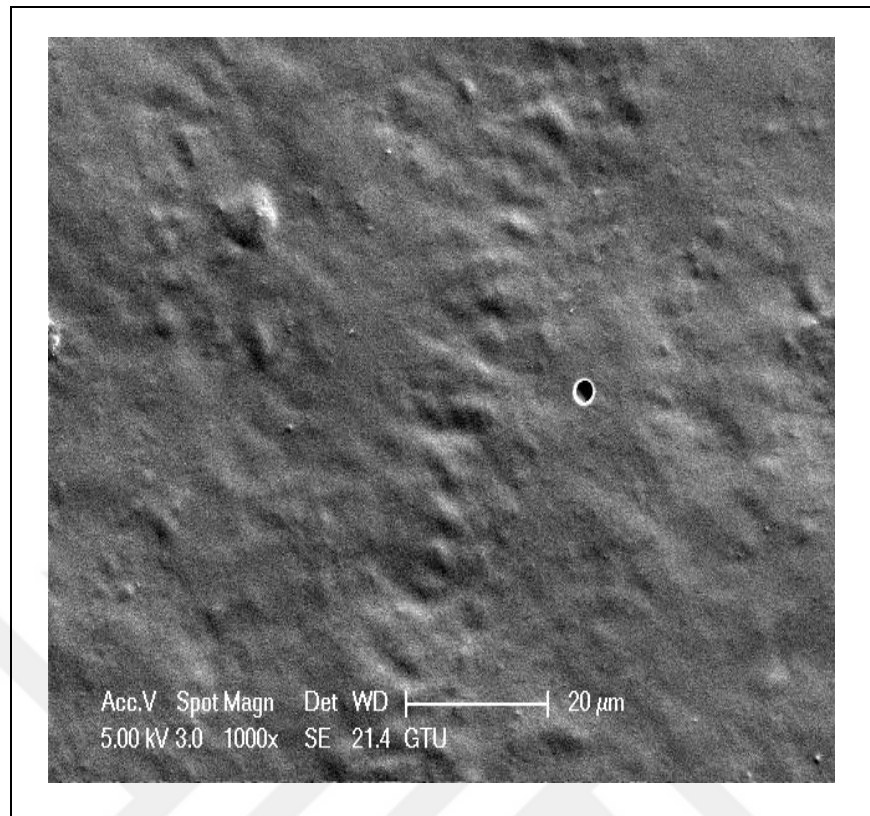


Figure 6.15: SEM image of gelatine and chia mucilage film with glycerol (1000x).

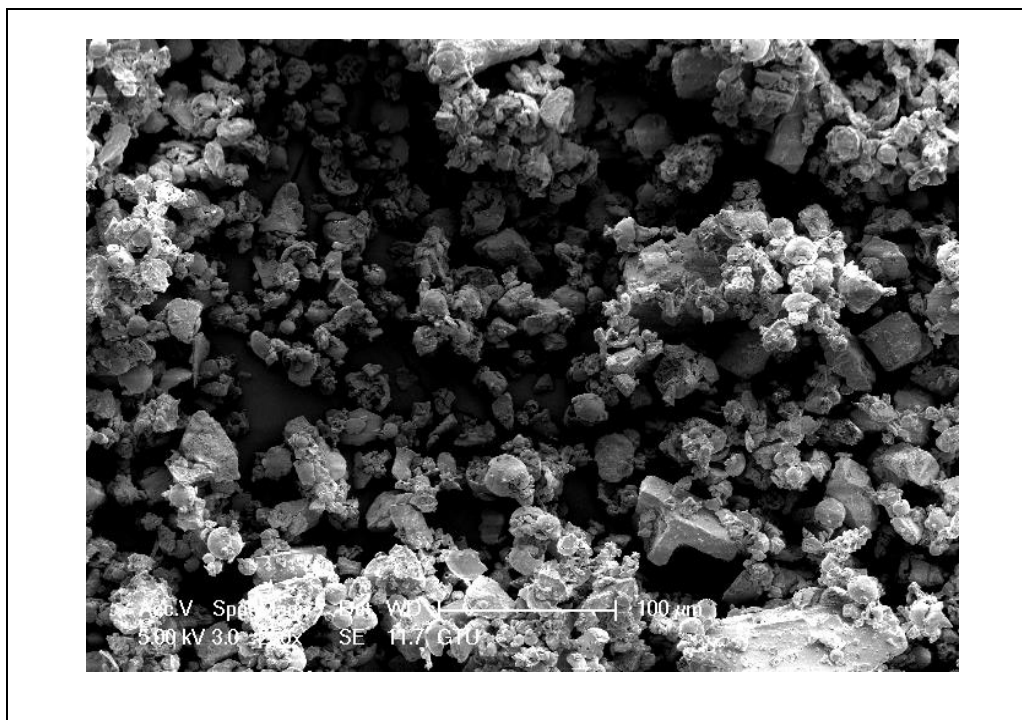


Figure 6.16: SEM image of nisin (250x).

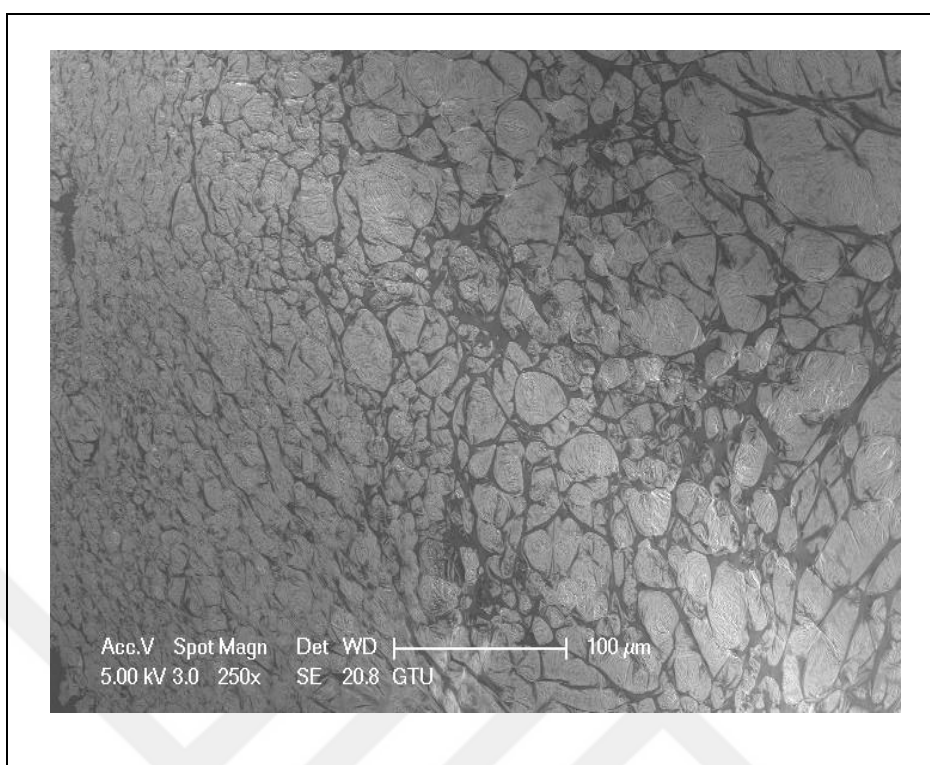


Figure 6.17: SEM image of gilaburu (250x).

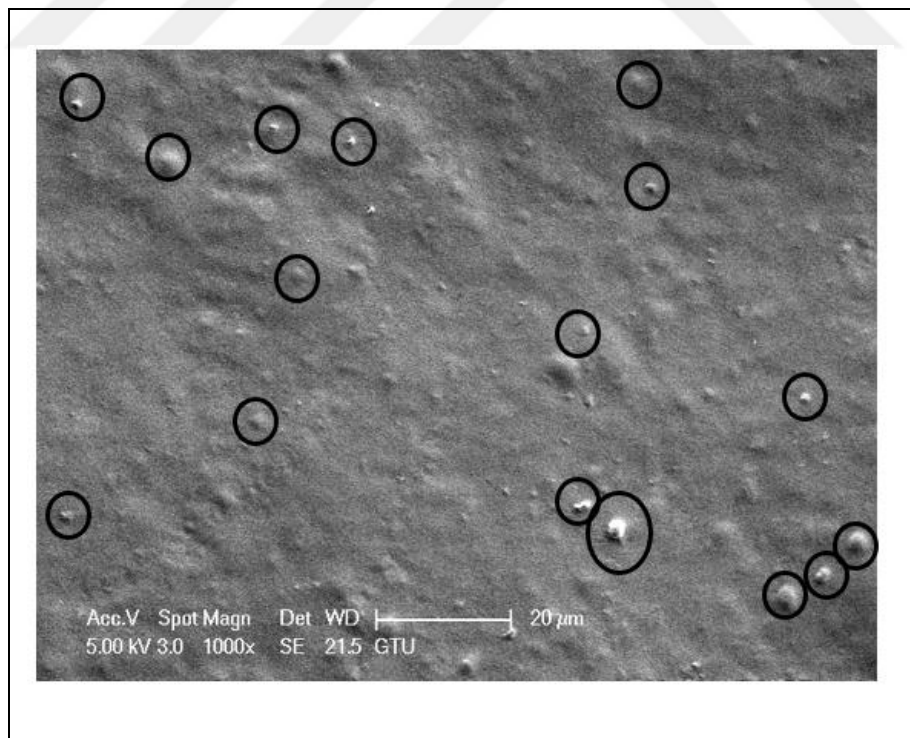


Figure 6.18: SEM image of glycerol added gelatine and chia mucilage films integrated with nisin (Some of nisin particles were marked with black circles) (1000x).

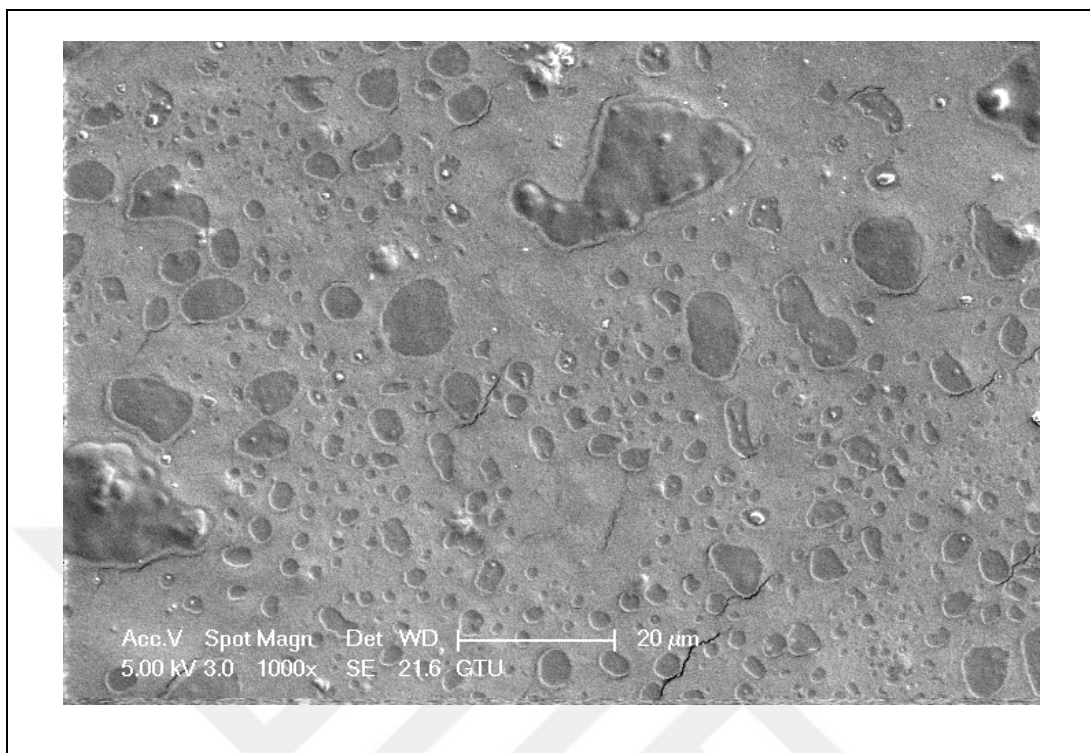


Figure 6.19: SEM image of glycerol added gelatine and chia mucilage films integrated with nisin and gilaburu (1000x).

6.4.10. Antimicrobial properties of the films

Antimicrobial properties of the films were evaluated in solid (Figure 6.20) and liquid media (Figure 6.21). According to agar disk diffusion assay, no clear inhibition zones around the antimicrobial incorporated films were seen up to 48 h. Zone appeared at the 60th h of incubation around the film incorporated with nisin (Figure 6.20). However, no inhibition zones were seen around other films at this hour.

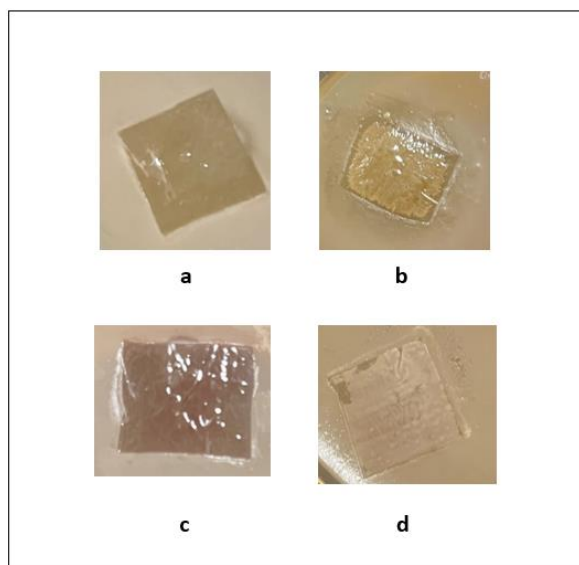


Figure 6.20: Agar disk diffusion assay results of films against *S. aureus*. Film without antimicrobials (a); Nisin (25 µg/ml) integrated film (b); Gilaburu (50%) integrated film (c); Nisin (3.125 µg/ml) and Gilaburu (25%) integrated film (d).

According to the results of OD measurements (Figure 6.21), films without antimicrobials in BHI broth did not show any effect on *S. aureus* growth. The films, integrated with nisin and gilaburu separately or in combination, inhibited the growth of the bacteria until 48 h of fermentation.

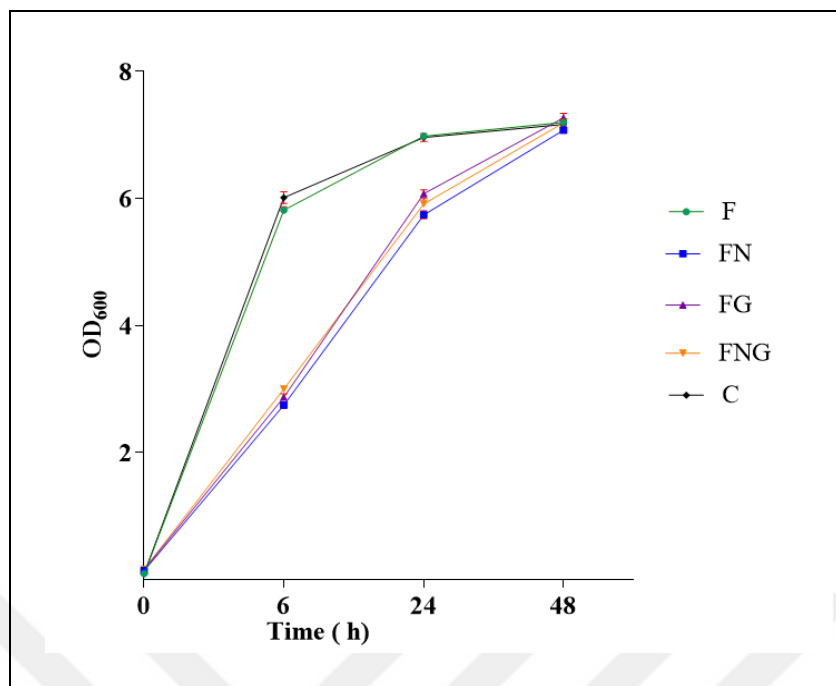


Figure 6.21: OD measurements of *S. aureus* culture growing in the presence of films. F: Films without antimicrobials; FN: Nisin (25 µg/ml) integrated films; FG: Gilaburu (50%) integrated films; FNG: Nisin (3.125 µg/ml) and Gilaburu (25%) integrated films; C: Control. Statistics are given in Appendix B.

7. DISCUSSION

It is well known that foodborne diseases are the most prevalent concern worldwide [Fetsch & Johler, 2018]. During the last decades, investigations have focused on natural substances and their different combinational usage to prevent such foodborne diseases and to enhance food safety. Nisin is one of the good alternatives because of its naturalness, harmlessness, and effectiveness [Gee Yap et al., 2022]. Moreover, when it is combined with other natural substances, it may be used in lower amounts so that more cost-effective, stable and strong antimicrobial combinations could be achieved [Ning et al., 2022]. Nisin combination with another antimicrobial can also lead to a decrease in bacterial resistance [O'Connor et al., 2020]. This type of combination can also diminish synthetic chemical preservative usages such as nitrates/nitrites, sulphites, sodium benzoate which are related to cancers and allergic reactions [Lee & Paik, 2016], [Pisoschi et al., 2018]. In the present study, the antimicrobial activity of nisin was determined on different pathogenic bacteria at different pHs, salt concentrations and temperatures. Besides, the effect of gilaburu on nisin activity against *S. aureus* was evaluated and preliminary studies were carried out to prepare a natural edible packaging material based on chia mucilage and gelatin complex in which nisin and gilaburu were integrated.

Experiments carried out under different pH, salt concentrations and temperatures showed that nisin was more effective on gram positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*) than gram negative ones (*Escherichia coli* and *Pseudomonas aeruginosa*) and the best nisin activity was found to be against *S. aureus*.

It is known that the solubility, stability, and biological activity of nisin are pH-dependent and it has high activity at acid pHs [Davies et al., 1998], [Deegan et al., 2006]. Supporting this information, in our study, nisin showed the best antimicrobial activity on *S. aureus* at pH 5 and MIC value was determined as 25 µg/mL. Similarly, Yu et al reported an increase in the MIC of nisin against *S. aureus* from 10 to 48 µg/ml when the pH of the LB medium was changed from 6.0 to 7.2 [Yu et al., 2020]. It has been reported that many different MIC values of nisin have been determined for *S. aureus* in various studies carried out to date. According to Jensen et. al., Nisin's MIC

values for methicillin-sensitive and resistant *S. aureus* isolates come from different kinds of food products, including pork and chicken, pasta, salad, and sushi were ranged from 6.4 to 12.8 µg/ml [Jensen et al. 2020]. In another study, the MIC range of nisin on *S. aureus* was determined to be 1.5 to 83.6 µg/mL [Mota-Meira M et al., 2000].

In order to decrease the MIC value of nisin on *S. aureus*, gilaburu was selected to be used in combination with nisin. Water extract of gilaburu fruit in 50% concentration was effectively inhibited the growth of *S. aureus* in all pH and salt conditions studied. To the best of our knowledge, literature on the antimicrobial activity of gilaburu (*Viburnum opulus*) is limited. Eryilmaz et al. determined that ethanolic extracts of *Viburnum* sp. showed antimicrobial activity against *S. aureus* ATCC 25923, *S. aureus* ATCC 43300 (MRSA), *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Candida albicans*, however water extracts of gilaburu showed weak antimicrobial effect only on *K. pneumonia* [Eryilmaz et al. 2014]. According to Turker et. al. hot water extracts of dry *Viburnum opulus* L. fruits showed strong antibacterial activity against *S. aureus*, *S. epidermidis*, and *S. pyogenes*. [Turker et. al., 2012]. In another study, ethanol extracts of gilaburu were found to suppress the growth of human pathogenic gram-negative and positive microorganisms including *S. aureus* [Česonienė et al., 2014]. Aksoy et al. reported that methanol extracts of *Viburnum opulus* L. dried fruits at 10% concentration successfully suppressed the growth of *S. aureus* Cowan 1 [Aksoy et al. 2006].

The synergistic interaction between nisin and gilaburu was determined by both checkboard assay and agar disk diffusion assay. Time kill assay results were interpreted as nisin and gilaburu combination at 0.5% and 2.5% salt concentrations had caused ≥ 2 -log reduction in the *S. aureus* at 12 h. In nisin and gilaburu combinations results were very close to each other although gilaburu concentration decreased 50% in one of the combination. It has been interpreted that this situation is related to the increase in salt concentration and that increase in salt concentration complements the effect of decreased concentration of gilaburu.

According to flow cytometry analysis, it is safe to say that gilaburu decreased the MIC value of nisin about eight-fold in the BHI medium and nisin presence decreased the MIC value of gilaburu two-fold. Salt addition to the BHI medium decreased only the MIC value of gilaburu four-fold in the presence of nisin.

The positive effect of gilaburu on nisin activity against *S. aureus* was further confirmed by TTC dehydrogenase assay, conductivity, and finally by DNA and protein leakage. The relative TTC-dehydrogenase activities of samples from *S. aureus* exposed to nisin and gilaburu alone or in combinations decreased compared to the control group, which supports the results obtained from time-kill assay and flow cytometry.

Leakage of cytoplasmic contents is a well-known indicator of bacterial membrane damage. Small ions such as phosphate and potassium first leak out of the cell, while other large molecules such as DNA, RNA, and proteins leak out afterward [Shen et al., 2015]. Ions leak out from the cytoplasm lead to an increase in the conductivity, and our results showed that conductivity of *S. aureus* culture supernatants increased after the treatment with nisin and gilaburu alone or in combinations. Similarly, it was found that DNA and protein leakage levels also increased in nisin and gilaburu treated cells according to the control samples. According to conductivity results, nisin and gilaburu combinations in 2.5% salt concentration had higher level of conductivity than others. Increase in conductivity may have been caused by increase in salt concentration. Leakage of ions, DNA, RNA, and proteins after nisin treatments alone or in combination with other chemicals were shown by also other studies [Schelegueda et al. 2016], [Yang et al. 2017], [Ning et al. 2022].

In the present study, it was also understood that gilaburu also contributes to membrane damage of *S. aureus*. As a precious plant with a variety of biological effects as folk medicine and a food ingredient, the mechanisms underlying the mode of action of *Viburnum opulus* L. on microorganisms are worth more research.

According to the literature, nisin has the characteristic absorption bands around 3288 cm^{-1} and 2960 cm^{-1} in response of attribution to axial O–H and N–H stretching. The peak at 1652 cm^{-1} can be attributed to amide band and the peak at 1449 cm^{-1} evidenced the presence of COO[–] symmetric stretching vibrations [Zheng et al., 2019]. According Gruskiene et. al., the peak at 3268 cm^{-1} means the stretching vibration of hydrogen bonded -OH and -NH. Besides, three characteristic peaks at 1635, 1515 and 1230 cm^{-1} are responsible for amide I, II and III region, respectively, which are related with secondary structure of nisin [Gruskiene et al., 2021]. Results from this study were

compatible with literature. Chlorogenic acid is the most abundant phenolic substance in the gilaburu extract and it is characterized by hydroxyl and carbonyl functional groups. The FTIR spectrum of the gilaburu represents bands at 3341, 2880, 1706, 1578, and 1260 cm^{-1} . Presence of phenolic compounds is defined by the peak at 3341 cm^{-1} which means the stretching vibration of the OH in H-bonded alcohols and phenols. The peak at 2880 cm^{-1} means C-H stretching vibration and the peak at 1706 cm^{-1} can be interpreted as the C=O stretching vibration. The peak at 1260 cm^{-1} can be interpreted as the C-O stretching vibration in the phenolic structure. The peaks between 1200–1300 cm^{-1} can also be interpreted as the hydroxyl groups attached to the benzene ring [Avachat et al., 2013], [Özdemir et al., 2022]. In this study, as seen in Figure 6.12, the peaks at 2881.3, 1720.2 and 1271.6 cm^{-1} were compatible with each other. Although other peaks were close to the peaks found in the literature, they were still slightly different. For example; the peak at 3341 cm^{-1} in the literature was obtained at 3291 cm^{-1} . It was concluded that the reason for these differences was due to the water that was not completely evaporated from the sample.

Glycerol can be defined with two bands appeared at 2890 and 2950 cm^{-1} which are coming from C-H (saturated) stretching and FTIR spectrum of glycerol exhibits sharp bands at 1105, 1035, and 920 cm^{-1} [Pérez et al., 2009]. According to another study, FTIR spectrum of the glycerol showed O-H stretching at 3265 cm^{-1} while C-H stretching was showed by the peaks in the region of 2810-2950 cm^{-1} . The C=O stretching band was found at 1655 cm^{-1} . Bending of the C-O-H group was seen in the region of 1400 to 1420 cm^{-1} of the C-O stretching of the primary alcohol [Danish et al., 2015]. According to the literature, the band 1626.9 cm^{-1} represents the C-N stretching and in-plane N-H bending of the amide I and this is known as characteristic for gelatin. C-N stretching with attending of N-H bending forms the amide II band, which is placed at the wavenumber 1525.1 cm^{-1} . The amide III bands which basically means C-N stretching, were found in the region 1232.7 cm^{-1} .

In CM spectra, the symmetric stretching of carboxyl group of uronic acid exhibits the peaks at 1597.0 cm^{-1} and 1418.4 cm^{-1} and this is a characteristic feature for polysaccharides from seed gums. The bending vibration of C-O-C present in the pyranose ring indicates a peak at 1150.3 cm^{-1} . Another characteristic of polysaccharides is C-O-C stretching of 1→4 glycosidic bond and C-O-H bending, gives a band at

1037.2 cm^{-1} . The band at 886 cm^{-1} presents the β -anomeric C-H deformation and glycosidic linkages assignable to glucopyranose and xylopyranose units [Hernández-Nava et al., 2019]. In short, the FTIR spectrum of glycerol, gelatine and chia mucilage which are detected in this study were compatible with the literature. Moreover, there were no new peaks in all components and combinations, so it was interpreted that they were only bound to each other by physical interactions.

In SEM images of films as seen in Figure 16.9, circular formations are seen. They are interpreted that may differ from the honeycomb structures observed in the gilaburu and nisin encapsulated with gilaburu. These circular formations are examined carefully, nisin particles are usually noticeable in their centres. This has been interpreted as positively charged nisin molecules interacting in some way with negatively charged gilaburu. These circular molecules are homogeneously dispersed in this matrix can be interpreted as physical interactions of nisin and gilaburu with gelatin (+ charged) and chia mucilage (- charged) coacervation system.

Antimicrobial activity of the films was evaluated according to agar disk diffusion assay. While nisin integrated film exhibited clear inhibition zone after 60 h of incubation, other types of films did not cause any inhibition in the growth of *S. aureus*. Antimicrobial films were effectively inhibited the growth of bacteria in liquid culture compared to the control. Although these methods can be optimized by trying different antimicrobial concentrations, incubation times, release states etc., trials in real solid food systems will give us reliable results. Further research is definitely needed to prepare effective antimicrobial edible films with nisin and gilaburu.

In summary, the antimicrobial effect of the nisin and gilaburu combination was evaluated for the first time in this study. It was determined that the similar effects of nisin and gilaburu at their MIC values on *S. aureus* were reached at much lower doses when they were used in combination. These in vitro findings suggest that gilaburu may significantly improve the antibacterial activity of nisin and further, gilaburu in combination with nisin has considerable potential to control *S. aureus* contaminations in foods. Although further research is needed to show the antimicrobial efficiency, successful integration of nisin and gilaburu to gelatine-chia mucilage coacervation system was shown for the first time in this study.

8. CONCLUSION AND FUTURE PERSPECTIVES

Nisin is the only WHO and FDA-approved GRAS (generally considered as safe) status bacteriocin mostly used in foods, especially in milk and other dairy products. In different studies conducted in recent years, it was aimed to increase both the antimicrobial activity and stability of nisin by combining it with other chemicals and to integrate nisin into packaging systems.

We have shown in this study that the antimicrobial activity of nisin can be effectively increased with another natural compound, gilaburu. According to our results, gilaburu and nisin alone have antimicrobial activity on *S. aureus*, they do not lose their antimicrobial activity when used together and even they show a synergistic antimicrobial effect in combination. This synergistic effect was achieved by combining both nisin and gilaburu below their MIC values. In the use of nisin as a food additive, gilaburu can contribute to a cost-effective approach because it reduces the dose of nisin to be used in foods. The fact that both are natural compounds makes them ideal for use in combination as food additives. In addition to the contribution of gilaburu to nisin's antimicrobial effect, it will also be possible to benefit from its antioxidant effect by using it in foods. Furthermore, the synergistic approach could help to minimize the problem of bacterial resistance. Hence, because of the literature on gilaburu is limited, more studies are needed on it.

Food packaging is very important to protect foods from deterioration in response to environmental factors and microorganisms. Therefore, it is important to develop safer and cheaper food packaging to meet the growing demand of the food industry. Polymer food packaging can be classified as biodegradable and non-biodegradable. Today, non-biodegradable food packaging is mostly used materials, however, their usage is being decreased for the reason of environmental pollution. In order to prevent pollution, in the long run, it is very important to enhance the usage of biodegradable food packaging, because it is healthy, green, environmentally friendly and renewable. This study demonstrated that GE and CM can be successfully used to form complex coacervates for being a candidate as a good edible and biodegradable packaging alternative. Furthermore, complex coacervates formed by GE-CM may be an alternative as encapsulating agents for antimicrobials. According to our knowledge,

there is no research about investigation on evaluation of nisin and gilaburu alone or together in GE-CM coacervation system. It was demonstrated that these two natural antimicrobials can be integrated successfully in GE-CM films which are also natural. These edible, green and safe films are promising to be used in food industry. These antimicrobial integrated coacervated films also did not have any unpleasant taste, colour and odor. However, further research is required to study the features of films such as physical properties (i.e. tensile strength, viscosity, turbidity, wapor and oxigen permeability, colour etc.), release kinetics of antimicrobials and most importantly applicability in real food systems.



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BIOGRAPHY

Seda Kirazlı graduated with B. Sc. Degree from Veterinary of Medicine in Uludağ University in 2011. Later, she worked as a PhD research assistant between 2012-2013 in Food Hygiene Department of Ondokuz Mayıs University. She has been working as Microbiologist and Specialist in Biogenetics and Food Laboratory of Turkish Standardization Institute since 2013. She has been a part of PhD programme of Molecular Biology and Genetics at Gebze Technical University since 2018 under the supervision of Prof. Dr. Sedef Tunca Gedik.



APPENDICES

Appendix A: Publications Within the Scope of Thesis Study

Kirazli S., Tunca S., (2022), “Nisin and gilaburu (*Viburnum opulus* L.) combination is a cost-effective way to control foodborne *Staphylococcus aureus*”, Food Control, 142, 109213.

Appendix B: Statistics

Table B1.1: One way ANOVA Analysis of data obtained in Agar Disk Diffusion Assay.

0.5 % Salt Concentration				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. 3.125 µg/mL Nisin + 100 % Gilaburu	-10.07	<0.0001	****	-10.36 to -9.773
Control vs. 3.125 µg/mL Nisin + 50 % Gilaburu	-8.000	<0.0001	****	-8.294 to -7.706
Control vs. 3.125 µg/mL Nisin + 25 % Gilaburu	-7.067	<0.0001	****	-7.360 to -6.773
Control vs. 3.125 µg/mL Nisin + 12.5 % Gilaburu	-6.700	<0.0001	****	-6.994 to -6.406
Control vs. 3.125 µg/mL Nisin + 6.25 % Gilaburu	0.000	>0.9999	ns	-0.2936 to 0.2936
Control vs. 3.125 µg/mL Nisin + 3.125 % Gilaburu	0.000	>0.9999	ns	-0.2936 to 0.2936
2.5 % Salt Concentration				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. 3.125 µg/mL Nisin + 100 % Gilaburu	-15.07	<0.0001	****	-15.43 to -14.71
Control vs. 3.125 µg/mL Nisin + 50 % Gilaburu	-10.00	<0.0001	****	-10.36 to -9.640
Control vs. 3.125 µg/mL Nisin + 25 % Gilaburu	-10.13	<0.0001	****	-10.49 to -9.774
Control vs. 3.125 µg/mL Nisin + 12.5 % Gilaburu	-8.033	<0.0001	****	-8.393 to -7.674
Control vs. 3.125 µg/mL Nisin + 6.25 % Gilaburu	-7.500	<0.0001	****	-7.860 to -7.140
Control vs. 3.125 µg/mL Nisin + 3.125 % Gilaburu	-6.500	<0.0001	****	-6.860 to -6.140

Table B1.2: One-way ANOVA analysis of data obtained in time kill assay.

0 hour				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	66667	0.9909	Ns	-512726 to 646059
Control vs. Gilaburu 50 %	76667	0.9849	Ns	-502726 to 656059
Control vs. Nisin 3.125 µg + Gilaburu 25 %	433333	0.1607	Ns	-146059 to 1012726
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	353333	0.2907	Ns	-226059 to 932726
4 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	19068000	<0,0001	****	11734043 to 26401957
Control vs. Gilaburu 50 %	18878667	<0,0001	****	11544710 to 26212623
Control vs. Nisin 3.125 µg + Gilaburu 25 %	19437833	<0,0001	****	12103877 to 26771790
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	19453067	<0,0001	****	12119110 to 26787023
8 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	3215210000	0,0001	***	1898933133 to 4531486867
Control vs. Gilaburu 50 %	3213043333	0,0001	***	1896766466 to 4529320200
Control vs. Nisin 3.125 µg + Gilaburu 25 %	3216655200	0,0001	***	1900378333 to 4532932067
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	3216652133	0,0001	***	1900375266 to 4532929000
12 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	46666120000	0,0003	***	26027991789 to 67304248211
Control vs. Gilaburu 50 %	46666203333	0,0003	***	26028075122 to 67304331544
Control vs. Nisin 3.125 µg + Gilaburu 25 %	46666662513	0,0003	***	26028534302 to 67304790724
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	46666663293	0,0003	***	26028535082 to 67304791504
24 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	48844966667	<0,0001	****	41697770986 to 55992162347
Control vs. Gilaburu 50 %	48842500000	<0,0001	****	41695304319 to 55989695681
Control vs. Nisin 3.125 µg + Gilaburu 25 %	48866218333	<0,0001	****	41719022653 to 56013414014
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	48866026667	<0,0001	****	41718830986 to 56013222347

Table B1.3: One-way ANOVA analysis of data obtained in flow cytometry measurements.

12 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	-52.33	<0.0001	****	-63.16 to -41.51
Control vs. Gilaburu 50 %	-46.57	<0.0001	****	-57.39 to -35.74
Control vs. Nisin 3.125 µg + Gilaburu 25 %	-46.57	<0.0001	****	-57.39 to -35.74
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	-43.57	<0.0001	****	-54.39 to -32.74

Table B1.4: One-way ANOVA analysis of data obtained in TTC dehydrogenase activity measurements.

12 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	48.99	<0.0001	****	44.09 to 53.89
Control vs. Gilaburu 50 %	48.82	<0.0001	****	43.92 to 53.72
Control vs. Nisin 3.125 µg + Gilaburu 25 %	47.13	<0.0001	****	42.23 to 52.03
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	49.64	<0.0001	****	44.74 to 54.54

Table B1.5: One-way ANOVA analysis of data obtained in conductivity measurements.

12 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	-1443	<0.0001	****	-1794 to -1092
Control vs. Gilaburu 50 %	-690.0	0.0005	***	-1041 to -339.1
Control vs. Nisin 3.125 µg + Gilaburu 25 %	-843.3	<0.0001	****	-1194 to -492.4
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	-1990	<0.0001	****	-2341 to -1639

Table B1.6: One-way ANOVA analysis of data obtained in DNA concentration measurements.

12 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	-24.98	<0.0001	****	-26.97 to -22.98
Control vs. Gilaburu 50 %	-10.19	<0.0001	****	-12.19 to -8.196
Control vs. Nisin 3.125 µg + Gilaburu 25 %	-13.89	<0.0001	****	-15.89 to -11.90
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	-18.73	<0.0001	****	-20.73 to -16.74

Table B1.7: One-way ANOVA analysis of data obtained in protein concentration measurements.

12 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control vs. Nisin 25 µg	-13.48	<0.0001	****	-14.94 to -12.02
Control vs. Gilaburu 50 %	-8.864	<0.0001	****	-10.32 to -7.405
Control vs. Nisin 3.125 µg + Gilaburu 25 %	-9.667	<0.0001	****	-11.13 to -8.208
Control vs. Nisin 3.125 µg + Gilaburu 6.25 % (2.5 % salt)	-10.32	<0.0001	****	-11.78 to -8.862

Table B1.7: One-way ANOVA analysis of data obtained in antimicrobial test of films in BHI broth.

0 hour				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control film vs. Nisin 25 µg integrated film	-0,04983	<0,0001	****	-0,05496 to -0,04470
Control film vs. Gilaburu 50 % integrated film	-0,06730	0,0059	**	-0,09239 to -0,04221
Control film vs. Nisin 3.125 µg + Gilaburu 25 % integrated film	-0,07347	0,0036	**	-0,09605 to -0,05089
6 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control film vs. Nisin 25 µg integrated film	3,072	<0,0001	****	2,914 to 3,231
Control film vs. Gilaburu 50 % integrated film	2,948	<0,0001	****	2,790 to 3,106
Control film vs. Nisin 3.125 µg + Gilaburu 25 % integrated film	2,817	<0,0001	****	2,668 to 2,966
24 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control film vs. Nisin 25 µg integrated film	1,235	0,0001	***	1,060 to 1,411
Control film vs. Gilaburu 50 % integrated film	0,9061	0,0003	***	0,7285 to 1,084
Control film vs. Nisin 3.125 µg + Gilaburu 25 % integrated film	1,067	0,0009	***	0,8256 to 1,307
48 hours				
Row Factor	Difference	P value	Summary	95% CI of diff.
Control film vs. Nisin 25 µg integrated film	0,1207	0,1046	ns	-0,03453 to 0,2760
Control film vs. Gilaburu 50 % integrated film	-0,07033	0,4825	ns	-0,2664 to 0,1257
Control film vs. Nisin 3.125 µg + Gilaburu 25 % integrated film	0,007667	0,9927	ns	-0,1410 to 0,1563

ns: not significant