T.R.
KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE

ISOLATION AND IDENTIFICATION OF POTENTIAL NISIN-PRODUCING *Lactococcus lactis* spp. *lactis*

Ayaz Mohamed Saleh MAMSIN

MASTER THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

KAHRAMANMARAŞ – TURKEY 2015

T.R.
KAHRAMANMARAŞ SÜTÇÜ İMAM UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCE

ISOLATION AND IDENTIFICATION OF POTENTIAL NISIN-PRODUCING *Lactococcus lactis* spp. *lactis*

Ayaz Mohamed Saleh MAMSIN

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Bioengineering and Sciences

KAHRAMANMARAŞ – TURKEY 2015

M.Sc. thesis entitled "Isolation and Identification of Potential Nisin-Producing *Lactococcus lactis* spp. *lactis*" and prepared by Ayaz Mohamed Saleh MAMSIN, who is a student at Department of Bioengineering and Sciences, Graduate School of Natural and Applied Science, Kahramanmaraş Sütçü İmam University, 22/4/2015 was certified by all the majority jury members, whose signatures are given below.

Prof. Dr. Özlem TURGAY (Supervisor) …….

Department of Food Engineering

Kahramanmaraş Sütçü İmam University

Assoc. Prof. Dr. Ismail AKYOL (Member) …….

Department of Agriculture Biotechnology

Kahramanmaraş Sütçü İmam University

Assist. Prof. Dr. Bahri ÖZSİSLİ (Member) …….

Department of Food Engineering

Kahramanmaraş Sütçü İmam University

I approve that the above signatures related to the members.

Assoc. Prof. Dr. Mustafa ŞEKKELİ …….

Director of Graduate School

Kahramanmaraş Sütçü İmam University

DECLARATION

I declare and guarantee that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. Based on these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Ayaz Mohamed Saleh MAMSIN

This study supported by: Kahramanmaraş Sütçü İmam University.

Project Number: 2014/1-8 YLS

POTANSİYEL NİSİN ÜRETEN *Lactococcus lactis* spp. *lactis'*in İZOLASYONU VE TANIMLANMASI

(YÜKSEK LİSANS TEZİ)

Ayaz Mohamed Saleh MAMSIN

ÖZET

Peynir üretiminde belirli starter kültürler kullanılmaktadır. Bunlar çoğunlukla laktik asit bakterileridir (LAB). *Lactococcus lactis* spp. *lactis* Lactik Asit Bakterilerinden olup starter kültür olarak kullanılmaktadır.

Bu çalışmada Kuzey Irak, Duhok'ta evlerde yapılan bir tür peynirden nisin üreten *Lactococcus lactis* spp. *lactis* izole ve identifiye edilmiştir. Peynir örnekleri koyun, keçi ve inek sütünden yapılmıştır. Toplam 20 peynir örneğinden GM17 ve MRS 'e yapılan ekimlerde 100 şüpheli koloni elde edilmiştir. Bunlardan 39 tanesi Gram pozitif, kok ve katalaz negatif olarak belirlenmiştir. Nisin *Lactococcus lactis* spp. *lactis* tanımlanması 16S rRNA ve *nisA* geni ile yapılmıştır. İzolatlardan 22 tanesininde 16S rRNA ve *nisA* geninin bulunduğu belirlenmiş ve nisin üreten *Lactococcus lactis* spp. *lactis* olduğu tespit edilmiştir.

Anahtar Kelimeler: Bacteriosin, *Lactococcus lactis*, Nisin, Laktik asit bakterileri.

Kahramanmaraş Sütçü İmam Üniversitesi

Fen Bilimleri Enstitüsü

Biyomühendislik ve Bilimleri, 2015

Danışman: Prof. Dr. Özlem TURGAY

Sayfa sayısı : 45

ISOLATION AND IDENTIFICATION OF POTENTIAL NISIN-PRODUCING *Lactococcus lactis* spp. *lactis*

(M.Sc. THESIS)

Ayaz Mohamed Saleh MAMSIN

ABSTRACT

Specially selected starter cultures are required to be used in cheese production industry. These starter cultures are mainly composed of Lactic Acid Bacteria (LAB), and it is important to identify novel LAB from traditional cheese. *Lactococcus lactis* spp. *lactis* is one of the important Lactic Acid Bacteria used as a starter culture.

In this study potential Nisin-producing *Lactococcus lactis* spp. *lactis* were isolated and identified from home-made cheeses taken from different towns in Duhok province of Northern Iraq. The cheeses were manufactured from milk of sheep, goat and cows. A total 100 potential isolated strains were from 20 samples of home-made cheese using GM17 and MRS culture media, after Gram staining and catalase test, Only 39 pure isolates were sharing (gram-positive, cocci and Catalase negative) characteristics with small, raised and circular colony morphology, after excluding the strains with bacilli and coccobacilli shapes. For identification of potential Nisin-producing *Lactococcus lactis* spp. *lactis* colony PCR conducted for the potential strains based on two primers 16S rRNA and *nisA* gene. 22 isolates of the suspected strains were confirmed as *Lactococcus lactis* spp. *lactis* by detecting 16S rRNA and confirmed as potential Nisin-producing strains by detecting Nisin structural gene, *nisA,* in all 22 isolates.

Key words: Bacteriocins, *Lactococcus lactis***,** Nisin, Lactic acid bacteria.

University of Kahramanmaraş Sütçü İmam
Graduate School of Natural and Applied Science
Department of Bioengineering and Sciences, 2015

Supervisor: Prof. Dr. Özlem TURGAY

Page number: 45

ACKNOWLEDGMENT

I would like to express my great appreciate to my supervisor Prof. Dr. Özlem TURGAY for her endless support, guidance, valuable information and patience during this research. I wish to acknowledge the help provided by Food Microbiology Laboratory and BIGEM staff.

I would like to thank Dr. Diyar Tayib the Head of the Directorate of Veterinary Medicine in Duhok for his unlimited support.

Finally, I offer sincere thanks to my family members especially my parents for their support and lofty love.

TABLE OF CONTENTS

Page No

LIST OF TABLE

LIST OF FIGURE

SYMBOLS AND ABBREVIATIONS

LAB **:** Lactic Acid Bacteria.

GRAS **:** Generally Recognized as Safe.

HACCP **:** Hazard Analysis and Critical Control Points

G+C **:** Guanine + Cytocine.

NAD^+ **:** Nicotinamide Adenine dinucleotide.

DNA **:** Deoxyribonucleic acid.

PCR **:** Polymerase Chain Reaction.

dNTPs **:** Deoxyribonucleotide triphosphates.

ml **:** Microlitter.

pH **:** Power of Hydrogen.

Mg **:** Microgram.

RNA **:** Ribonucleic Acid.

ATP **:** Adenosine triphosphate.

kDa **:** Kilodalton.

Da **:** Dalton.

GM17 **:** Glucose-Maltose 17.

MRS **:** De-man, Rogosa and Sharpe.

MRSA **:** Methicillin-resistant *Staphylococcus aureus*.

PMF **:** Proton Motive Force.

Bp **:** Base pair.

EtBr **:** Ethidium Bromide

1. INTRODUCTION

Foodborne diseases are a pervasive problem and the most serious and costly public health concerns worldwide, being a major cause of morbidity. Reported numbers of foodborne illnesses and intoxications still increased over the past decade in spite of modern technologies, good manufacturing practices, quality control, and hygiene and safety concepts such as risk assessment and HACCP. Several methods have been used to control and prevent the growth of pathogenic microorganisms.

Over the past decade, several health problems like recurrent outbreaks of diarrhea, assembled with the natural resistance of the causative agents, contributed to its status as hazard. The problem of selection of resistant bacteria to antibiotics and the increasing demand for safe foods, with less chemical additives, has increased the interest in replacing these compounds by natural products, which have no negative effects on the host or the environment (Parada *et al.,* 2007).

The scientists have become in challenge to grant a safer and healthier food. Especially after increasing the demand of packaged and processed food after 1960s due to breakdown of large families into nuclear families, growing urbanization and increasing the number of working women. Otherwise, before that a very few food items were processed and packaged. For inhibition of microbial growth, chemical preservatives and other traditional barriers have been used, which lead to serious health risks. Food preservation has become a major issue because food borne pathogens can cause havoc in both preserved and fresh food items at high temperature and even at low temperature. Consumers demand for faster, healthier and ready-to-eat products. Therefore, Consumers prefer the use of food preserved by natural preservatives instead of chemical preservatives (Guatam and Sharma, 2009).

Biopreservation is the use of controlled non-pathogenic microorganisms and/or their metabolites as preserving method for promoting microbiological safety and extending shelf-life of foods (Parada *et al.,* 2007). Among the biopreservatives, Bacteriocin-producing microorganisms have a significant deal of attention by microbiologists due to thier overthrown the problems that implement the requirements of food preservation (Guatam and Sharma, 2009), since bacteriocins are effective in a nanomolar range and have no toxicity (Parada *et al.,* 2007). Added to that, they have proteinaceous structures which make them safer for human consumption (Guatam and Sharma, 2009). Due to their

ability to improve human health, bacteriocins acquired the attention of academia and industry which lead to increase the number of published researches on production, purification, application and genetics of bacteriocins (Pingitore *et al.,* 2007).

Colocins were the first antimicrobial proteinaceous compounds discovered which are produced by, *Escherichia coli*, a gram-negative bacterium (Khan, 2013). Both gram-positive and gram-negative bacteria may produce Bacteriocins (Yang *et al.,* 2012). 99% of all bacteria could produce at least one type of bacteriocins and the only cause of that we haven't detected more is that few researchers have worked for isolating them (Riley and Wertz, 2002).

Recently, LAB that are bacteriocin-producing microorganisms have attracted more interest because their safety, their bacteriocins are considerd as generally recognized as safe GRAS product and have potential use as safe additives to improve the safety and shelf life of the food (Yang *et al.,* 2012). LAB found naturally in various raw food materials like milk, meat and flour used to produce foods (Savadogo *et al.,* 2006) , and it has a long history of use by man in food and feed fermentation and preservation either as the natural microflora or as starter cultures added under controlled conditions (Yang *et al.,* 2012). They have inhibitory effect against many microorganisms, including food spoilage organisms and pathogens (Savadogo *et al.,* 2004). The LAB are also used to improve nutriotional value, quality rather than for flavor and texture (Nagalakshmi *et al.,* 2013).

Bacteriocins and lantibiotics, metabolites produced by LAB, have a very important role in preventing the growth of spoilage and pathogenic bacteria, and have been extensively studied in recent years due to their potential use as novel, natural food preservative (Villani *et al.,* 2001).

The last two decades was the golden age of novel bacteriocins discovery that produced by LAB, because the bacteriocin-relate projects were supported financially by funding agencies such as European Union. To date the number of bacteriocin purified to homogeneity and characterized at the amino acid level is more than one hundred projects and the majority of them were discovered in the last twenty years (Khan, 2013). The lantibiotics are the most extinsivley studied group of bacteriocins (Ingham *et al.,* 2003), especially which are secreted by Lactococci. Nisin perhaps be the best best-stidied lantibiotic which is produced by speciefic strains of *Lactococcus lactis* subsp. *Lactis*, and it has been approved used as a food preservative. It has antimicrobial activity against several

gram-positive microorganisms and mostly similar type, including LAB, Clostridia and some other pathogens (Villani *et al.*, 2001).

The antimicrobial spectrum of Nisin shows that it's active towards clinical isolates of the Methicillin-resistant *Staphylococcus aureus* (MRSA), *Streptococcus pyogenes*, and several of the most severe human pathogens including the multi-resistant *Streptococcus pneumoniae* and vancomycin-resistant *E. faecium* or *E. faecalis*, against which new effective antibiotics are most necessoriely needed (Abts *et al.*, 2011).

Significant work has been done on the inhibitory effect of nisin on different spoilage bacteria and pathogens such as *Listeria monocytogenes* and its application in various food products. Fresh fruits and vegetables harbor various microorganisms, some of which are psychrotrophic. *Listeria monocytogenes* is one of the pathogenic bacteria that has the ability to grow at refrigerator temperature. Moreover, it's tolerant to acidic pH and salt concentration up to 10%. Therefore it's important to find bio-preservatives that control both pathogenic and spoilage microorganisms that are not easy to control and cause health issues including *Listeria monocytogenes* (Yang *et al.*, 2012).

Keeping in view the importance of LAB and their bacteriocins and the continuing interest of researchers in this area of research, the current study was planned with aim isolation and identification of potential Nisin-producing *Lactococcus lactis* subsp. *lactis* molecularly based on 16s rRNA and *nisA* genes, from home-made collected from Northern of Iraq. *Lactococcus lactis* subsp. *lactis* was selected among a number of LAB isolates because it produces Nisin which has a broad range of antimicrobial activity against many bacteria. The bacteriocin Nisin is non-toxic to humans, is produced by a bacterium not known to exert pathogenesis against humans, and not used for clinical therapies.

2. LITERAURE REVIEW

2.1. Biopreservation

The risk of microbiological contamination has been increased due to market globalization, introduction of the novel foods, new food-production processes, and raising consumers' awareness which leads to growing demand for minimally processed, ready-to-eat and fresh-cut products. To cover this risk, among the different choices of food preservatives and preservation techniques, special attention has been paid to biopreservation to improve hygienic quality, extend shelf-life and save nutritional and organoleptic properties of the food products (Garcia *et al.*, 2010).

Biopreservation is using of non-pathogenic microorganisms and/or their metabolites to extend shelf-life of the foods and make them microbiologically safe. It refers to enhance safety and extend storage life of the foods using natural microflora and/or their antimicrobial products. Biopreservation can be defined as the using of natural and controlled microbiota and/or their antimicrobial compounds to extend the shelf-life of the foods. Arguably fermentation is one of the most used methods of biopreservation, it's based on the growth of microorganisms in foods, whether added or naturally present. Fermentation causes breakdown of complex compounds and produces many other compounds like acids and alcohols, improvement of organoleptic qualities such as production of aroma and flavor compounds, synthesize Vitamin-B12, riboflavin and vitamin C precursor, produce antifungal and antibacterial compounds such as bacteriocins, diacetyl and acetaldehyde. Enzymes, CO_2, hydrogen peroxide, etc. (Nath *et al*., 2014). Choosing a method for food preservation depends on the availability of the method, structure or components of the raw material that wanted to be preserved, cost, effect of the preservation method on the food and the degree of changing in flavor and nutritional feature of the food product. Fermentation is an example of biopreservation methods which is cheap, easy to apply and it meets today's increasing consumer's demand for minimally processed/preserved food products (Kongo, 2013).

Several requirements should be fulfilled by any biopreservative to be acceptable and used commercially, includes; it should not be toxic and accepted by a recognized authorities, it should be economical to the industry, it should not show any deleterious effect toward the organoleptic properties of the preserved materials, it should be efficient even if it is used in a relatively low concentrations, it should be sufficiently stable in

storing conditions and have no pharmaceutical uses, and bacteriocins fulfill all these requirement and hence gained popularity in the food industry day-by-day (Guatam and Sharma, 2009).

2.2. Lactic Acid Bacteria

The term Lactic Acid Bacteria (LAB) was first used at the beginning of the 20th century, other terms like "milk souring" and "lactic acid producing bacteria" had been used before for these bacteria making slight confusions (Khalid, 2011). A large group of bacteria, single species or single strains included belong "Lactic Acid Bacteria" (LAB) (Ikeda *et al.*, 2013). The first lactic acid bacterium pure culture (*Bacterium lactis*) was obtained in 1873 by J. Lister. The first time used as starter culture in cheese was in 1890, while it has been used by human in fermented foods for more than 5000 years (Halasz, 2009; König and Fröhlich, 2009).

LAB are beneficial probiotics in the human digestive tract and widely distributed in the nature and are generally regarded as safe (GRAS) for human consumption (Ikeda *et al.*, 2013). Biopreservation by LAB is a non-thermal method for food preservation which is one of the oldest and highly effective techniques (Kongo, 2013). LAB have nutritional benefits and extend shelf-life via producing organic acids, mainly lactic acid as a main catabolic end product which leads to acidification of the raw material. They also produce acetic acid, ethanol, aroma compounds, bacteriocins, exo-polysaccharides and several important enzymes. They are able to inhibit the growth of different microorganisms and play an important role in food preservation and safety (Vuyst and Leroy, 2007). Many LAB inhibit the growth of food borne and spoilage gram positive microorganisms including antibiotic resistant bacteria (Garcia *et al.*, 2010), gastrointestinal pathogenic microorganisms such as *Helicobacter pylori, Escherichia coli* and *Salmonella* species (Vuyst and Leroy, 2007). They show also antifungal activity (Masood *et al.*, 2011).

LAB are widely used traditionally as starter culture added under controlled condition to make beverages (Parada *et al.*, 2007), pickles, juices, cheese, yogurt, sausage (Masood *et al.*, 2011), fermented milk, meat, fish and vegetable products in which lead to decrease their pH by production of lactic acid which restrict the growth of pathogenic and spoilage microorganisms. LAB are normal flora of mucosal surfaces of human and animals (Aarnikunnas, 2006) in the mouth, intestine and vagina of mammals (Rattanachaikunsopon *et al.*, 2010).

Lactic acid bacteria LAB are group of gram-positive, rods or cocci, non-motile, catalase negative, non-spore forming bacteria (with the exception of genus sporolactobacillus), they can be range from aerophilic, aero-tolerant, to strict anaerobic (Tan, 2010). LAB produce lactic acid as a major metabolite of the fermentation of carbohydrate for gaining energy because they don't have the ability to synthesize cytochromes and porphyrins (component of respiratory chain), therefore cannot generate ATP and depend on fermentation of carbohydrates to gain energy. Lactic acid bacteria need carbohydrates, amino acids, peptides, nucleic acids and vitamins for their growth, they are nutritionally fastidious (Rattanachaikunsopon *et al.,* 2010).

Bacteriocins are from the primary metabolites released LAB, which are considered as generally regarded as GRAS (Zacharof *et al.,* 2013), and their production is related to the LAB growth in which are produced throughout the growth phase and cease at the end of exponential (or sometimes before the end of the growth) (Savadogo *et al,.* 2006).

Phenotypic technique has been used commonly for identification of LAB, but recently genetic techniques such as 16S rDNA sequencing have been used which are more consistent and have more accuracy for identification of individual strains (Khalid, 2011). Bacteriocins-producing strains can be readily identified in a deferred antagonism assay, in which colonies of the putative producer are overlaid with a bacterial lawn of a sensitive strain (Nagalakshmi *et al.,* 2013).

2.2.1. Classification and taxonomy of LAB

The classification of LAB was elaborated and published by 1919 by Orla-Jensen (Halasz, 2009). The classification of LAB into various genera is mostly based on morphology (cocci or rods, tetrad formation), method of glucose fermentation (homo- or heterofermentation), growth at different temperatures (e.g., 10°C and 45°C), structure of the lactic acid produced (D, L, or both), gram reaction, ability to grow at high salt concentrations, and acid or alkaline tolerance (Rattanachaikunsopon *et al.,* 2010; Khalid, 2011).

The LAB belong to phylum *Firmicutes*, class *Bacilli* and order *Lactobacillales* (Zacharof *et al.,* 2013). The LAB could be comprised of about 20 genera. The major members of the LAB are *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Leuconostoc*, *Pediococcus*, *Aerococcus*, C*arnobacterium*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weisella* Species. *Lactobacilli*, *Carnobacteria* and some *Wersella* are rods

while the remaining genera are cocci. *Lactobacillus* is largest of these genera, comprising around 80 known species (Aarnikunnas, 2006) and is one of the four main genera used for food fermentation with *Leuconostoc, Pediococcus* and *Streptococcus* (Mohammed *et al.,* 2013). The measurement of true phylogenetic relationship with rRNA sequencing have aided the classification of LAB and clarified the phylogeny of the group (Khalid, 2011).

LAB can be divided according to the end products formed as a result of glucose fermentation into two different groups (Figure 2.1.); Homofermentative lactic acid bacteria such as *Pediococcus*, *Lactococcus* and some lactobacilli produce lactic acid as the main or sole end-product of glucose fermentation (Rattanachaikunsopon *et al.,* 2010), LAB convert nearly all of sugars they use especially glucose into lactic acid (Khalid, 2011). Homofermentative lactic acid bacteria use the Embden-Meyerhof-Parnas pathway to produce two moles of lactate from each mole of glucose and derive approximately double as much energy from each mole of glucose as heterofermentative lactic acid bacteria. Heterofermentative lactic acid bacteria such as *Leuconostoc* and *Weissella* and some lactobacilli release equimolar amounts of CO_2, lactate and ethanol from glucose through hexose monophosphate or pentose pathway (Rattanachaikunsopon *et al.,* 2010).

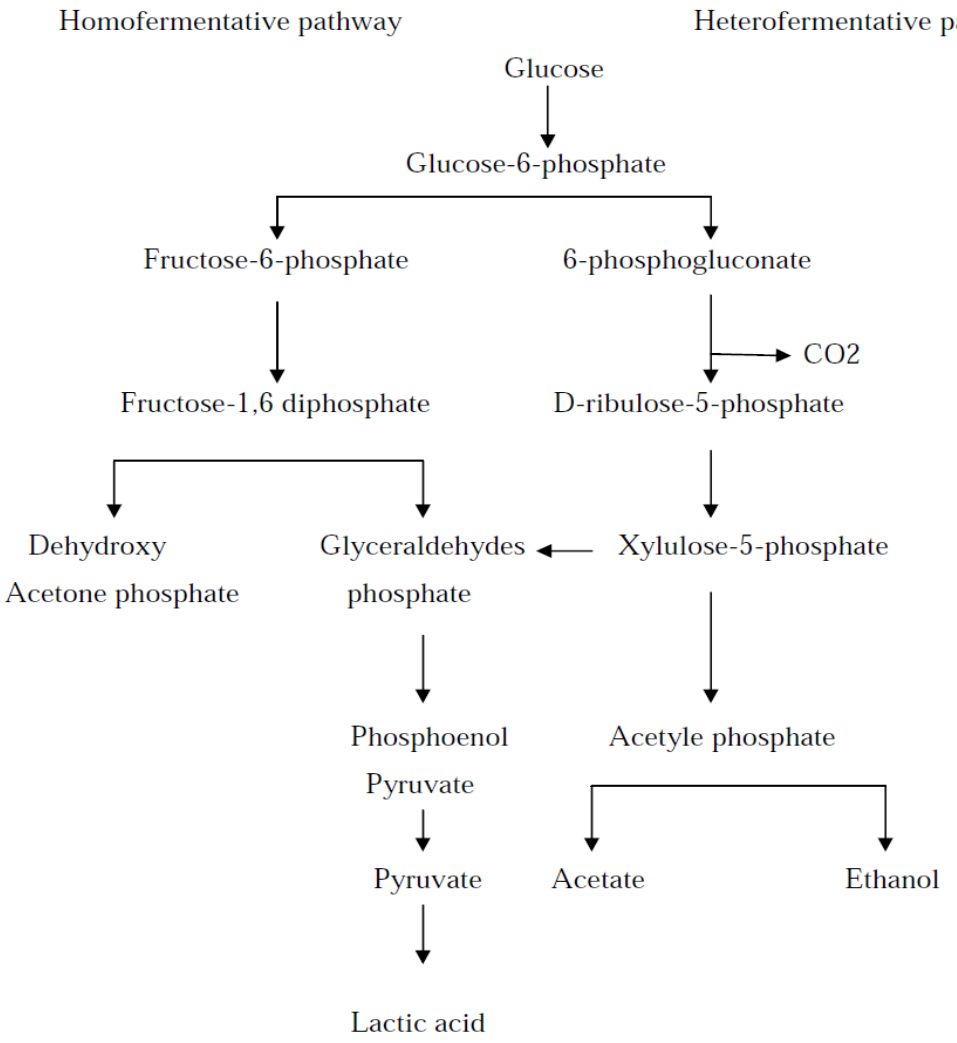

Figure 2.1. Two different pathways for glucose fermentation (Bulut, 2003).

In practice, to different the two groups, a test for gas production from glucose can be used. Differences in the growth rate in different temperature can be notified, pH of media, and sodium chloride tolerance. Growth is normally tested at 18°C and 45°C, sodium chloride concentration of 6.5% and 18%, and pH of 4.4 and 9.3. Finally for distinguishing between different genera, the formation of different isomeric forms of lactic acid (L-lactic acid or D-lactic acid) may be can be used (Halasz, 2009).

2.2.2. Genetically modified LAB

LAB have a great economic importance, for this reason, studies about culture improvement have been accelerated, and developments in the gene technology help this process. Introducing of new genes to make starters much better for the technological processes or improve organoleptic properties. There is expectation of more researches for understanding the physiology and genetic of LAB which will lead to improve the strains,

and better selections to be used in the future (Bulut, 2003). The first gene transformation was in 1928 when *Pneumococcus* was transformed *in vivo* in mice by Griffith. The first LAB that has been sequenced completely was *Lactococcus lactis* IL 1403 and this facilitate understanding and development in LAB genetic engineering (Renault, 2002).

Some bacteria strains have been engineered to aid autolysis which leads to lyse of the cells and elaborate some enzymes into the product matrix and aid in production of amino acids by degradation of peptides. In addition to that, there is possibility of transferring a gene from a LAB to other lactic-producer starter strains or its modification. For example, glutamate dehydrogenase (GDH) gene has been brought to *L. lactis* from *Peptostreptococcus asaccharolyticus* which aid in producing alpha-ketoglutarate from glutamate, an amino acid present in cheese, which convert amino acids to aroma compounds, and GDH-producing strains also produce carboxylic acids that are major aroma compounds. As a result of that, they don't need to add alpha-ketoglutarate as supplement (Bulut, 2003). To produce *Lactobacillus bulgaris* strains that do not ferment lactose efficiently and do not cause acidification of yogurt after completing of fermentation, chemicals modification has been applied, the result is products that can be preserved for long time without considerable drop in pH which lead to more desirable taste (Ahmed, 2003). For improvement of nisin Z production by *Lactococcus lactis* ssp. *lactis*, Genome shuffling has been applied to *Lactococcus lactis* ssp. *lactis* YF11 by ultraviolet irradiation and diethyl sulphate mutagenesis, after 4 rounds of genome shuffling, the best performing strain F44 produced which has obvious tolerance to glucose and nisin. Genome shuffling leads to increase the titter of nisin produced by F44 strain, which was 2.4 times that of YF11 strain, with the same structure of nisin from both strains (Zhang, 2014).

2.2.3. Benefits of LAB in food products and health

Lactic acid bacteria are important microorganisms due to their nutritional and health benefits, and their fermentative property which has role in food fermentation (Rattanachaikunsopon *et al.*, 2010). Food fermentation is a cheap and effective method for food preservation that helps in improvement of texture, flavour and nutritional value rather than extending the shelf-life of many food products (Kongo, 2013), which is one of the oldest methods used traditionally for food production and preservation (Guatam and Sharma, 2009). Preservation of milk by fermentation was used early in human history. Sumerian writings about dairying go back to about 6000 B.C. Procedures for the

fermentation of meat was developed as early as the 15th century B.C. in Babylon and China. And fermentation of vegetables was known in China in the 3rd century B.C. (Halasz, 2009). Fermentation performed by adding LAB as starter culture to the food matrix (Vuyst and Leroy, 2007). Several species of LAB have been used as starter culture for food fermentation belong to the genera *Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus* and the newly recognized *Carnobacterium* which have been isolated from various natural sources such as grains, green plants, dairy and meat products, fermenting vegetables, and the mucosal surfaces of animals, and have been used to prevent the growth of microorganisms causing spoilage of foods and preserve food through natural fermentation, LAB are used commercially as starter cultures in dairy products, baking, meat, vegetables and alcoholic beverages industries (Rattanachaikunsopon *et al.*, 2010), involved in production of red, white and sparkling wine (Khalid, 2011). The isolation and screening of microorganisms from natural sources has always been the most powerful means for obtaining and genetically stable strains for industrially important products (Yang *et al.*, 2012).

In addition to food products benefits, LAB have many proposed health benefits, they have been used as probiotics to reduce the risk of intestinal disorders such as lactose intolerance, acute gastroenteritis, constipation (Halasz, 2009), and also used to treat lowered blood cholesterol and prevention of cancer (Savadogo *et al.*, 2006). LAB have role in managing of lactose mal-absorption, reducing of viral and drug induced diarrhea, post-operative pouchitis, irritable bowel syndrome, inflammatory bowel syndrome, antineoplastic effects on human cell line, keeping normal level of insulin in the blood and aid the absorption of fatty acids by intestine. They have role in treating ulcer, prevention of colon cancer (Masood *et al.*, 2011) and increase immune response (Soomro *et al.*, 2002).

2.2.4. Antimicrobial effects of LAB

LAB have been used as natural food preservatives due to their antimicrobial activity against various spoilage and pathogenic microorganisms, this activity results from several metabolites produced after lactic fermentation including; organic acids which result from sugar fermentation that are used as a nutrient source. Organic acids such as lactic acid cause dropping of pH of the food matrix, which make organic acids soluble in lipids, allowing them to break through the cell membrane and reach the cytoplasm of the pathogens. Hence, prevent the growth of undesirable microorganisms. In addition to

organic acids, other bioactive components like diacetyl, acetaldehyde, hydrogen peroxide, ethanol, CO_2, reuterin and reutericycline released. Some of these materials, rather than being microbial inhibitory compounds that extend shelf-life and make food microbiologically safe through specific mechanisms; they make a desirable taste, colour and texture of the food (Parada *et al.,* 2007; Guatam and Sharma, 2009; Rattanachaikunsopon *et al.,* 2010; Kruger *et al.,* 2013; Nagalakshmi *et al*., 2013)**.**

Many LAB strains also synthesize bacteriocins and bacteriocin-like molecules that display antimicrobial activity. Beside the production of bacteriocins, some LAB are able to release other antimicrobial peptides that may also contribute to food preservation and microbiological safety. For instance, strains of *Lactobacillus plantarum*, isolated from sourdough and grass silage, display antifungal activity, which release materials with low molecular mass metabolites and/or cyclic dipeptides (Savadogo *et al.,* 2006; Vuyst and Leroy, 2007). The competition for necessary nutrients, accumulation of D-amino acids and diminution of the oxirredutive potential also have role in their inhibitory activity (Parada *et al.,* 2007).

2.2.5. *Lactococcus lactis* spp. *lactis*

Lactococcus lactis spp. *lactis* belongs to the genus *Lactococcus*, which is shared in four other species: *L. garvieae, L. piscium, L. plantarum,* and *L. raffinolactis*. Among these species, only *Lactococcus lactis* spp. *lactis* and *Lactococcus lactis* spp. *cremoris* are used to produce starter culture. They are gram positive, homofermentative, microaerophilic, non-spore forming, non-motile and do not have flagella *Lactococcus lactis* appear ovoid or spehrical and are typically 0.5-1.5 µm in diameter. They appear in pairs or forming chains. They are well studied and a complete genomic sequence is available for the strain *Lactococcus lactis* spp *lactis* IL 1403. *Lactococcus lactis* is commonly used in diary industry to produce dairy products like yogurt, buttermilk, and certain types of cheese. In New Zealand, *Lactococcus lactis* is also used to make lactic casein. *Lactococcus lactis* are also used in other industries to produce pickled vegetables, beer, some bread kinds and other fermented foods (Robinson, 2005; Giridhar, 2013).

There are several lantibiotics and non-lantibiotics bacteriocins, antimicrobial peptides, synthesized by *Lactococcus lactis,* and are well studied and characterized. Nisin was the first bacteriocin isolated and identified among the bacteriocins produced by *Lactococcus lactis.* Other bacteriocins produced by *Lactococcus lactis* are lacticin; which

is from lantibiotics, and lactococcin which is non-lantibiotic pediocin-like bacteriocin (Alegría, 2010).

2.3. Bacteriocins

Bacteriocins are protein or protein complexes with antimicrobial activity. They are different in their biochemical properties, molecular weight, range of sensitive hosts and mode of action. They have bactericidal activity against the species that are closely related to the producer bacterium (narrow spectrum) (Soomro *et al*., 2002) or cross genera (broad spectrum) (Yang *et al.*, 2012). In all cases, the producer cell has specific immunity to avoid the action of its bacteriocins (Nagalakshmi *et al*., 2013).

Bacteriocins are peptides synthesized ribosomally, secreted by both gram positive and gram negative bacteria (Yang *et al.*, 2012). *E. coli*, gram negative bacteria, produce colicins, which are the first characterized bacteriocin group discovered in 1925 by Andre Gratia and his workgroup (Nath *et al.*, 2014). Colicins are large complex proteins, 29-90 kDa in size, with a structure involved in cell attachment, translocation and bactericidal effect. They act on the outer parts of the target cell by binding to specific receptors. The bacteriocins of gram positive bacteria are small peptides and have a size between 3-6 kDa (Soomro *et al.*, 2002) and they attracted attention in recent years, specially LAB bacteriocins due to their generally regarded as safe GRAS status and their use as safe additive for food preservation (Yang *et al.*, 2012). LAB have been traditionally associated to food and are regarded as safe so nowadays the term bacteriocin is mostly used for LAB bacteriocins, they are small, heat-stable cationic and display a wider spectrum of inhibition (Garcia *et al.*, 2010). Recently, Studies on bacteriocin-producing LAB have expanded, to apply the bacteriocins or bacteriocin-producing microorganisms as a natural additive for food preservation (Savadogo *et al.*, 2004). Most of new bacteriocins are belong Class II bacteriocins which are small (30-100 amino acids), heat-stable and commonly not post-transitionally modified, while most bacteriocin-producers secrete one bacteriocin, it has been shown that many LAB produce more than one bacteriocin (2-3 bacteriocins) (Nath *et al.*, 2014).

Bacteriocin-producers which have been used in natural fermentation will most likely have the best opportunities for application of their bacteriocins in near future, in opposite to those without GRAS status that will require premarket approval (Rattanachaikunsopon *et al.*, 2010). Bacteriocins are produced by bacteria and are

normally not named antibiotics in order to avoid confusion with pharmaceutical antibiotics, which may cause allergic reactions in human and lead to other medical issues (Nath *et al.*, 2014).

Most of bacteriocins are bactericidal with some exceptions, being bacteriostatic. They mostly inhibit gram positive bacteria. They are bactericidal to sensitive strains even at a very low concentration. The sensitivity of gram positive or gram negative bacteria to bacteriocins has been demonstrated on the bases of structure of the cell wall. Gram positive bacteria become sensitive to bacteriocins effect when it is destabilized by chemical or physical stresses. The outer layer of gram negative bacteria composed of proteins, phospholipids and lipopolysaccharides and this layer is impermeable to most molecules. The pores present in this layer allow the free diffusion of molecular mass bellow 600 Da while the smallest bacteriocin produced by LAB is approximately 3kDa and too large to reach their target cytoplasmic membrane (Guatam and Sharma, 2009). Using of bacteriocins as a biopreservative agent into processed food has been shown to be effective in the control of spoilage and pathogenic microorganisms. Nisin produced by *Lactococcus lactis* spp. *lactis* was the first approved bacteriocin to be used commercially as food preservative and date back to more than 50 years ago (Mohammed *et al.,* 2013). Bacteriocins like pediocin are also have potential use in food products, but they are not accepted as safe antimicrobial food additive yet (Yang *et al.,* 2012). Bacteriocin-producing microflora commonly isolated from food and food products. In a mixed natural population and under adequate circumstances the incidence of bacteriocin producer strains among fresh isolates of any gram positive species may reach 100% (Guatam and Sharma, 2009). Bacteriocin production is affected by type and level of carbon, nitrogen and phosphate sources, cation surfactant and inhibitors (Savadogo *et al.,* 2006).

In general, several features should be considered when selecting bacteriocins producers for food application including; they should be GRAS, have a broad spectrum effect or else high specific activity, thermostabilty, beneficial effect and improve safety, it should have no adverse effect on quality and flavour (Nath *et al.,* 2014). Many bacteriocins are active against foodborne pathogens. The important ones are Nisin, bulgarican, acidophilin, helveticins, diplococcin, lactacins and plantaricins (Savadogo *et al.,* 2006).

2.3.1. Classification of bacteriocins

On the basis of structure and mode of action, bacteriocins are divided into four major groups; Class I termed Lantibiotics, heat-stable peptides acting on membrane structure (Parada *et al.,* 2007) small (< 3 kDa), they are post transitionally modified (Abts *et al.,* 2011) lead to generation of unusual thioether amino acid (Guatam and Sharma, 2009). Characterized by distinctive thioether based intra-molecular rings of lanthionine and beta-methyl lanthionine (Garcia *et al.,* 2010). Example of this class is Nisin, lacticin 481 of *l. lactis*, citolysin of *E. faecalis* and lacticin 3147 of *L. lactis*, among others (Nath *et al.,* 2014). Class II, The bacteriocins of this class are usually small (<10 kDa), heat stable, membrane active peptides (Guatam and Sharma, 2009). They are non-modified peptides of 37-58 amino acids. In general, they are short cationic peptides with high iso-electronic points of particular relevance for food biopreservation is the potent anti-listeria activity display by the pediocin-like bacteriocins (Garcia *et al.,* 2010). In general, bacteriocins have amphiphilic helical structure, which help them to insert into membrane of target cell, leading to depolarization and death. They are divided into three subclasses on the bases of either a distinctive N-terminal sequence, the pediocin-like bacteriocins (class II1) (e.g. pediocin PA-1/AcH produced by *Pediococcus*), the lack of leader peptide (class II2) (e.g. enterocin EJ97 by *E. faecalis*), or neither of the above traits (class II.3) (e.g. enterocin L50A by *E. faecalis* (Nath *et al.,* 2014). Class III: Big peptides with molecular weight (>30 kDa) heat-labile protein (Guatam and Sharma, 2009) with modest prospects as food biopreservatives with exception of Colicin V and microcins, gram negative bacteriocins fall in this class (Garcia *et al.,* 2010). Members of this class formerly termed bacteriolysins, large (730 kDa), examples of bacteriocins belong to this class; such as helviticin J of *L. helveticus* and bacteriocin Bc. 48 of *E. faecalis*. It can function directly on the cell wall of gram positive targets, leading to the death and lysis of the target cell (Nath *et al.,* 2014). Class IV: The bacteriocins belong to this class are complex bacteriocins composed on not only proteins but also contain essential lipid, carbohydrate moieties (Guatam and Sharma, 2009).

Most LAB bacteriocins which have been applied in food preservation belong to the class I, II and III.

2.3.2. Mechanism of action of bacteriocins

Bacteriocins have various mode of action. Generally, the bacteriocins produced by gram-positive bacteria are more effective against other species of gram-positive bacteria. Mostly, the cell membrane is the target of bacteriocins action. Interfering with enzyme's function is another mechanism that shows by bacteriocins, bacteriocins inhibit important enzymes within the cell. Bacteriocins have hydrophobic nature which helps the compound to affect the lipid bilayer of the bacterial cell cytoplasm. The bacteriocins effect can be receptor-mediated when a specific strain or species is targeted; this happened in case of using narrow spectrum bacteriocins, while in case of using broad spectrum bacteriocins (e.g. nisin, which is active against various bacteria strains) the interaction is non-specific. Sometimes, the availability of proton motive force PMF is necessary for aiding the interaction between the bacteriocins and the targeted organelles. But in other cases, the interaction of bacteriocins with the membrane happened spontaneously. The result of this interaction is generating of pores in the cytoplasmic membrane that allow the ions, protons and amino acids to exit but not cytoplasmic proteins. This process affects negatively on membrane potential and destroys the cell system which is responsible for energy production. Both pore formation and opening need energy, which is produced by metabolizing bacterial cells. The studies about mechanism of pore formation have been done on artificial cytoplasmic membrane system (Oscáriz, 2000; McAuliffe *et al.*, 2001).

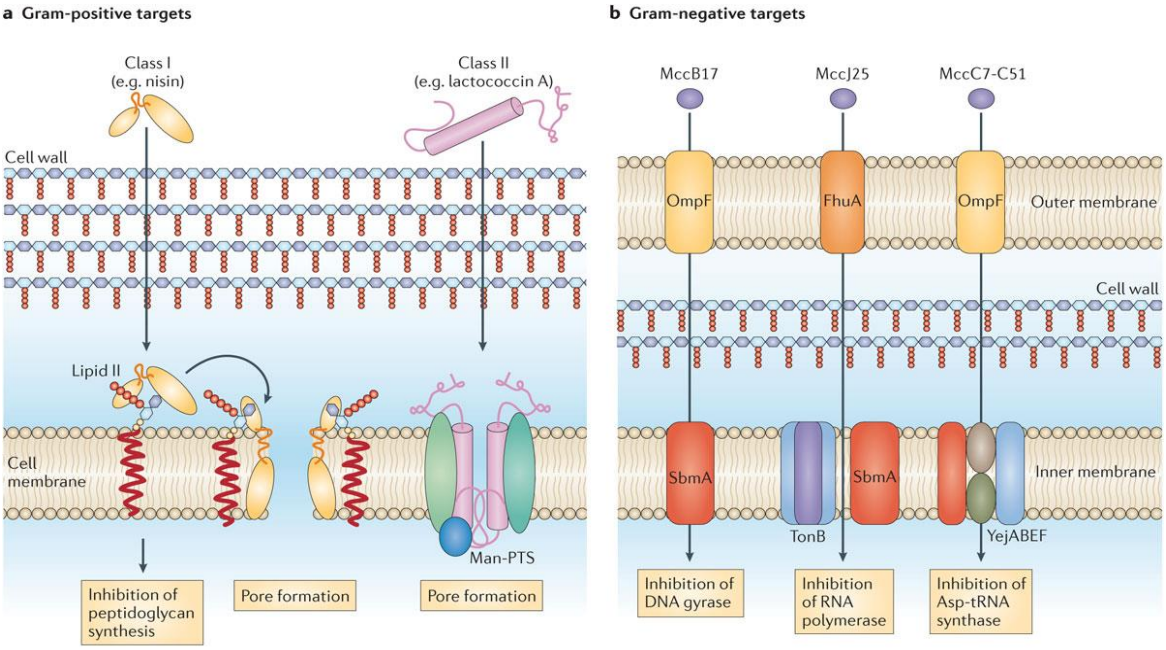

Figure 2.2. (a) | Some bacteriocins, and in particular many of those that inhibit Gram-positive bacteria, function by targeting the cell envelope. Some class I bacteriocins inhibit lipid II on the cell membrane, thereby abrogating peptidoglycan synthesis. Other bacteriocins form pores to inhibit or kill their target bacterium. For example, class II bacteriocins such as lactococcin A bind to the pore-forming receptor mannose phosphotransferase system (Man-PTS). Nisin and some other class I bacteriocins both inhibit peptidoglycan synthesis and form pores. Other class I peptides, such as the thiopeptides and bottromycins, control Gram-positive bacteria by targeting translation (not shown). (b) | Many bacteriocins that inhibit Gram-negative bacteria (and thus need to be transported through the outer and, in many cases, inner membranes before functioning) control their target bacteria by interfering with DNA, RNA and protein metabolism. For example, microcin B17 (MccB17) inhibits DNA gyrase, MccJ25 inhibits RNA polymerase, and MccC7-C51 inhibits aspartyl-tRNA synthatase. There are also exceptions, such as MccE492, that function through pore formation (Updated from Cotter *et al.*, 2013).

2.3.3. Bacteriocins of gram-positive bacteria

Many bacteriocins have been isolated and documented from various gram positive non-LAB bacteria including *Staphylococcus*, *Clostridium* and *Bacillus* spp. but using of these bacteriocins are excluded from food products because they are may be produced by pathogens, but bacteriocins produced by gram positive LAB attract special attention because of several factors including their proteinaceous nature which make them easily inactivated by gastrointestinal proteolytic enzymes, non-toxicity and non-immunogenic nature when tested to laboratory animals, inactivity against eukaryotic cells, generally thermo-resistant and broad spectrum activity affecting most of gram positive bacteria and some damaged gram negative bacteria including various pathogens such as *Listeria*

monocytogenes, *Salmonella*, *Staphylococcus aureus* and *Bacillus cereus* (Nath *et al.*, 2014). Most of bacteriocin-producing gram positive bacteria are membrane active compounds that affect the permeability of the cytoplasmic membrane. They often show a much wider bactericidal spectrum activity than colicins (Gram negative bacteriocins produced by *E. coli*) (Savadogo *et al.*, 2006) and require many more genes for their production than gram negative bacteriocins (Riley and Wertz, 2002). Recent developments in genetic engineering techniques have made the transfer of gene encoding for bacteriocin production from both gram positive and gram negative bacteria to food grade microorganism possible.

2.3.4. Bacteriocins of gram-negative bacteria

A wide variety of bacteriocins produced by gram negative bacteria, which are named after the genius of producing bacteria e.g., (klebicins of *Klebsiella pneumoniae*), or after species like (colicins of *Escherichia coli*), (marcescins of *Serratia marcescens*), (alveicins of *Hafnia alvei*), (cloacins of *Enterobacter cloacae*) and pyocins are bacteriocins produced by *Pseudomonads*. the bacteriocins produced by gram negative bacteria according to their size can be divided into three groups, first; large colicin-like bacteriocins which are (25-80 kDa) in size, the second; microcins which are much smaller (<10 kDa) and are similar to bacteriocins of gram positive bacteria (Chavan and Riley, 2007) which divided into two classes according to size and mode of action, class I microcins with less than 5 kDa size with post-transitional modifications which effect on intracellular targets, while class II microcins which range in size between 7 to 10 kDa and possess no modifications and act on bacterial membrane , both classes are similar in amino acid compositions and hydrophobicity (Dirix *et al.*, 2004), and the third group; phage tail-like bacteriocins, which are multimetric peptides assemblies, nuclease and protease resistant rod-like particles and kill sensitive cells by depolarization of the cell membrane, the example of this group is pyocins produced by *Pseudomonads* (Chavan and Riley, 2007).

2.3.5. Bacteriocins as food preservatives and health care products

Bacteriocins have been used to preserve foods due to its inhibitory effect against both spoilage and pathogenic bacteria, they are safe for human consumption because of their proteinaceous nature which are degradable by protease of gastrointestinal tract and deactivated and do not affect the microflora of intestinal tract (Guatam and Sharma, 2009).

They have been used in prevention and treatment of infectious diseases in both medical and veterinary sectors (Chaimanee *et al.,* 2009).

Purified, crude bacteriocins or bacteriocin-producing strains (starter culture) have been used in food products processing (Zacharof *et al.,* 2013). Bacteriocins are applied to the food products with several techniques, including; using bacteriocin solution by direct soaking of food items into it, using polyethylene based edible cellulosic films and plastic films, Adsorption of bacteriocin on different surfaces such as polyethylene, ethylene vinyle acetate, polypropylene, polyanile, acrylic, poly vinyle chloride and salinized silica etc. Bacteriocins can be used in hurdle technology which utilizes synergies of combined treatment to more effectively preserve food, can be used with chemical or physical methods for better results; one of the best-studied examples is the use of nisin in meat systems. Nitrates are commonly used in meat to prevent clostridial growth; however, nitrate is not so desirable and there is seeking for alternatives in food preservation. Nisin or combined with lower levels of nitrate can prevent the growth of *Clostridium* (Cleveland *et al.,* 2001), pediocin has been used with low dose of irradiation and hade better inhibiting effect on the growth of *L. menestroids* or can be used with another bacteriocin, like nisin, they have been used together to increase shelf-life of fish, meat and dairy products (Guatam and Sharma, 2009).

Bacteriocins have been used in preservation of meat and fish products, fruit juices, beverages, fresh vegetables (Zacharof *et al.,* 2013), seafood and dairy products (Khan, 2013). Health related application of purified bacteriocins could be their inclusion in pharmaceutical and cosmetic products since nisin has been successfully included in dental-care products, ulcers and colon infection treatment products and potential birth control (Zacharof *et al.,* 2013). Bacteriocins produced by *Lactobacillus acidophilus* may aid in the treatment of chronic or persistent diarrhea and bacteria over growth related diarrhea in children (Mohammed *et al.,* 2013)**.**

Several factors affect the activity of bacteriocins in food preservation negatively including; development of bacterial resistance to the bacteriocins, improper environmental conditions for biological activity, retention of bacteriocin molecules by food system components like fat, effect of other additives like salts, slower diffusion and solubility and/or uneven distribution of bacteriocin molecules in foods (Nath *et al.,* 2014).

2.3.6. Lantibiotics

Lantibiotics are a group of bacteriocins which is one of the most extensively studied group, consisting of peptide (<5kDa) in size, containing unusual amino acids such as lanthionine and methyllanthionine in their primary structure, produced by extensive post-transitional modifications. The lantibiotic name is originated from L (anthionine) containing antibiotics. Until now, there are about 60 lantibiotics of gram positive bacteria which are characterized and about 20 of them are LAB-produced bacteriocins (Khan, 2013). Lantibiotics consist of between 19 to 38 amino acids, and are categorized according to their structure and functional aspects into type A and type B, the type A are elongated flexible amphiphiles and act on the cell membrane of the sensitive cell by forming pores in bacterial membranes, while type B are globular and are peptides that inhibits enzyme function (Tan, 2010). According to classifications, the lantibiotics are Class I bacteriocins, an example of lantibiotics; Nisin which is a bacteriocin produced by *Lactococcus lactis* spp. *lactis* consists of a group of small peptides and characterized by the presence of several unusual amino acids in its structure (Savadogo *et al.,* 2006).

2.3.7. Nisin

Nisin is the most prominent antimicrobial peptide and firstly discovered in England in 1928 (Guatam and Sharma, 2009), used to control the outgrowth of *Clostridium botulinum* spores in cheese spreads in England (Riley and Wertz, 2002). Nisin-producing bacteria were firstly isolated and identified in fermented milk products, then from different dairy products, fermented meat products, traditional fermented vegetables, river water and human milk (Nagalakshmi *et al.,* 2013). Nisaplin® was the first commercial name of extracted Nisin produced by Aplin and Barrett, Ltd in 1957 (Schimidt, 2009). It is the most commercially used bacteriocin (Cleveland *et al.,* 2001) because it is the only bacteriocin that has been approved as a food preservative (Khan, 2013). Nisin has been approved as a safe food preservative by Food and Agriculture Organization/World Health Organization (FAO/WHO) Committee on Food Additives in 1969 and considered as generally regarded as safe product by the Food and Drug Administration (FDA) in the USA in 1988 (Schimidt, 2009). To date it is approved in more than 45 countries as a safe food preservative (Riley and Wertz, 2002).

Nisin is secreted by *Lactoccocus lactis* spp. *lactis* and it is the most extensively studied bacteriocin produced by LAB (Nagalakshmi *et al.,* 2013) and has a broad spectrum

against gram positive bacteria including many pathogens and it is effective against Bacillus and Clostridium spores and can prevent their outgrowth which helps in reduction in thermal processing of the food products, it has been used as a food preservative in processed and fresh cheese, canned foods, processed vegetables and baby foods (Rattanachaikunsopon *et al.,* 2010)**,** milk, cured meat (Guatam and Sharma, 2009), pasteurized processed cheese, salad dressing (Murdock *et al.,* 2007), and also in dental care products (Jozala *et al.,* 2005). It has been used for more than 50 years as food preservative and no significant resistance has been reported against it (Abts *et al.,* 2011).

Table 2.1. World-wide use of nisin (Cleveland *et al.,* 2001)

Country	**Food in which nisin is permitted**	**Maximum level (IU/g)**
Argentina	Processed cheese	500
Australia	Cheese, processed cheese, canned tomatoes	No limit
Belgium	Cheese	100
Cyprus	Cheese, clotted cheese, canned vegetables	No limit
EU	E234, may also labeled as "natural preservative"	Varies according to product and number state
France	Processed cheese	No limit
Italy	Cheese	500
Mexico	Nisin is a permitted additive	500
Netherland	Factory cheese, processed cheese, cheese powder	800
Peru	Nisin is permitted additive	No limit
Russia	Dietetic processed cheese, canned vegetables	8000
UK	Cheese, canned foods, clotted cream	No limit
US	Pasteurized processed cheese spread	10000

2.3.7.1. Chemical and physical properties of Nisin

Nisin is antimicrobial peptide produced by *Lactococcus lactis* spp. *lactis*; it kills or inhibits the growth of other bacteria. According to its structure, it is belong to the Class I bacteriocins which are lantibiotics. Nisin is formed by 34 amino acids with a small molecular weight 3510 Daltons (Schimidt, 2009). Its synthesis is complex, involving processes of transcription, secretion, processing and signs of transduction. There is two types of Nisin; Nisin A and Nisin Z, the difference between the two types is only in the amino acid 27 which is in Nisin A is Histidine while in Nisin Z is replaced by asparagine (Parada *et al.*, 2007). The mature nisin molecule is post-transitionally modified, in which serine and threonine residues are dehydrated and several thioether bridges are produced, as a result of this five ring structures formed (Soomro *et al.*, 2002). Nisin stability and solubility depend on the pH, its activity decrease with the increasing of pH to neutral or alkaline pH, its stability is optimal at pH from 3 to 3.5, when pH reach 5 it loss about 40% of its activity and loss about 90% when the pH become 6.8 (Schimidt, 2009) with complete inactivation after 30 min at 63 C at pH 11 (Jozala *et al.*, 2005).

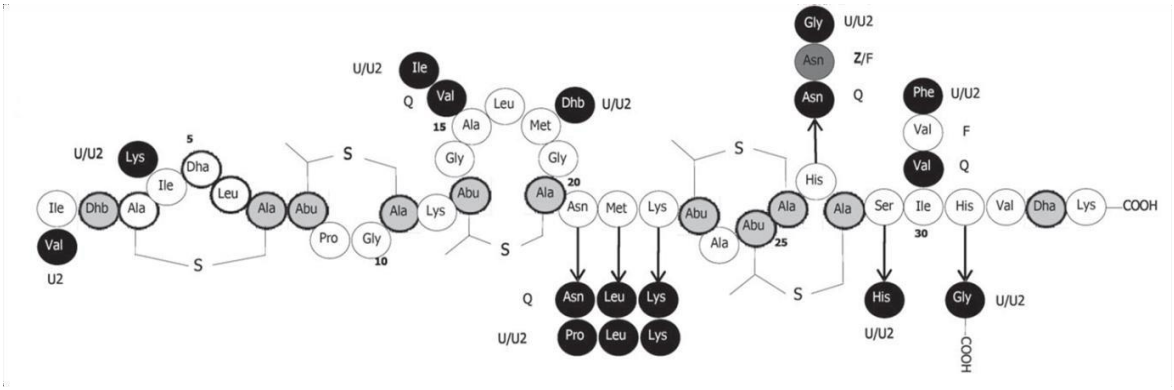

Figure 2.3. Comparison of structures of A (illustrated as basis), Z, Q, F, U and U2 after post-translational modification (adapted from Piper *et al.*, 2010).

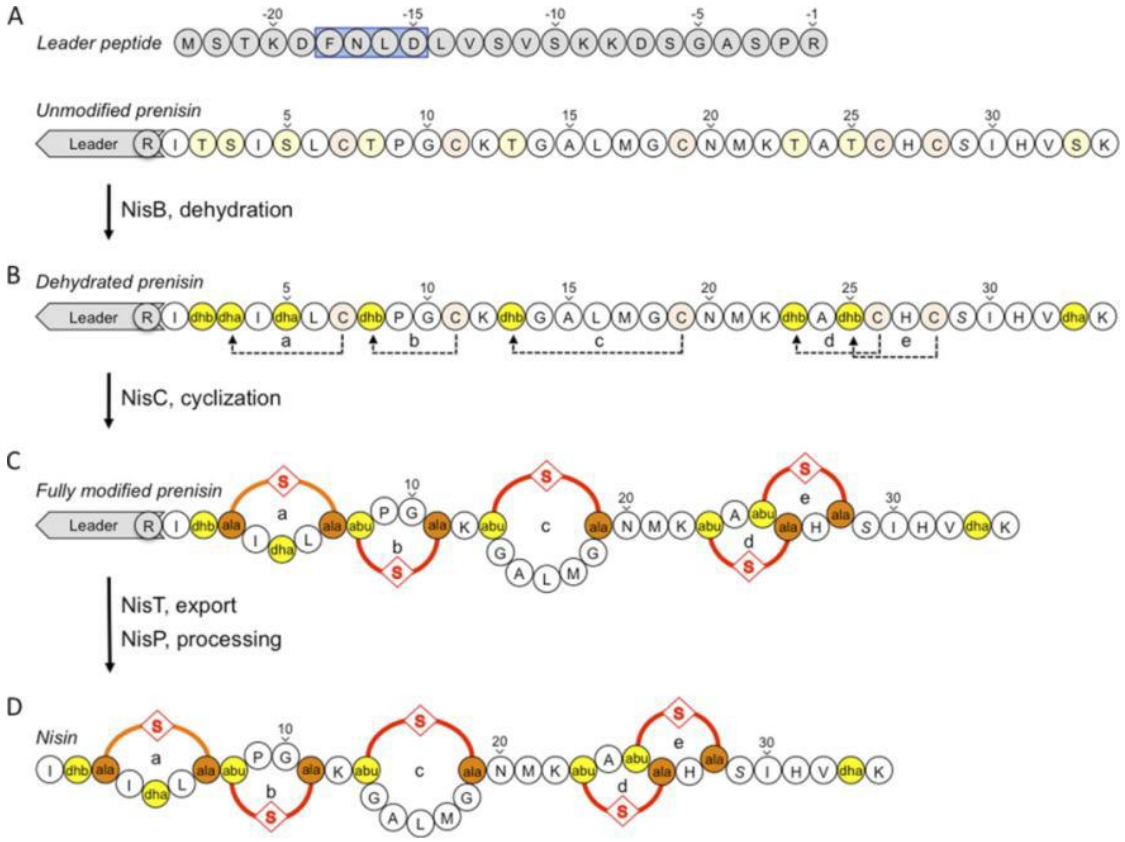

Figure 2.4. Posttranslational modification of amino acids of nisin A (updated from Mavaro *et al*., 2011).

2.3.7.2. Antimicrobial spectrum

Nisin has a broad spectrum activity against gram positive bacteria, and also can prevent germination of many spores of different pathogenic bacteria (Rattanachaikunsopon *et al.*, 2010). Nisin has antimicrobial activity against *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* when used *in vitro,* it can prevent the growth of *Salmonella* spp. (Ingham *et al.*, 2003). It acts on the gram positive bacteria which cause mastitis (Parada *et al.*, 2007)**,** it has been used to prevent Clostridial spoilage of natural and processed cheese, and prevent the growth of spoilage Lactobacilli in wine and beer fermentations and provides additional protection against Bacillus and Clostridial spores in canned foods, it is active against *Listeria* species (Savadogo *et al.*, 2006) for this reason, it is widely used as a natural biopreservative.

Nisin has little activity or no activity against some gram negative organism due to their wall structure, nisin cannot penetrate their wall. However, when used in hurdle technology or combined with other bacteriocins which help Nisin to penetrate the wall and reach the inner structures of the cell, it becomes more effective. It has been used with

lactoferrin to control the growth of gram negative pathogens such as *E. coli* O157:H7 (Murdock *et al.,* 2007) and has been used with chemicals; nisin (25 µg/m) with 1% H_2O_2, 1% sodium lactate and 0.5 citric acid has been used to decontaminate melon surfaces from *L. monocytogenes* and *E. coli* and keep the safety and quality of fresh cut melon. (Nagalakshmi *et al*., 2013) the typical amount of nisin used in food range between 2.5 to 100 ppm (Rattanachaikunsopon *et al.,* 2010).

Table 2.2. The activity of nisin increase in hurdle technology (Cleveland *et al.,* 2001)

Bacteriocins	**Other factors**	**Antibiotics**
Nisin A	N_2; CO_2; low temperature	Effect on *L. monocytogenes*: increase in the lag phase 400 IU/ml.; inhibition of growth 1250 IU/ml.
Nisin A	Milk lactoperoxidase (LP) and low temperature	Nisin-producing *L. lactis* acts synergistically with LP in reduction of *L. monocytogenes*
Nisin A	Calcium alginate gel	Gel-immobilized nisin is delivered more effectively than pure nisin and suppresses growth of Bro. thermosphacta on beed carcasses
Nisin	Sucrose fatty acid esters	Synergy against *L. monocytogenes*, *B. cereus*, *L. plantarum* and *S. aureus*
Nisin	CO_2	Synergistic when used against wild-type and nisin-resistant *L. monocytogenes*
Nisin	Pulsed electric field	Synergistic activity against *B. cereus* (06 µg/ml nisin and 16.7 kV/cm, 100 µs duration PEF)
Nisin	Modified atmosphere packaging (MAP)	Combination was more effective than either treatment alone at preventing growth of *L. monocytogenes*

2.3.7.3. Mode of action

It has been proposed that Nisin has various mechanisms to kill gram positive microorganisms; Nisin binds to lipid II, an essential membrane-anchored cell wall precursor, and lead to inhibition of cell wall synthesis, and also Nisin integrate with the membrane through ionic interactions of the C-terminus of the nisin (Abts *et al.*, 2011) which is positively-charged to the negatively-charged membrane (Khan, 2013), which composed of eight nisin and four lipid II molecules, and cause pore formation, These actions result in collapse of membrane potential and ultimately cause death of the bacterial cell (Abts *et al.*, 2011). The effectiveness of nisin against gram negative cells is low due to inability of nisin to penetrate cell wall but when the microorganisms treated with chelators such as ethylenediaminetetraacetic acid (EDTA) it shows better effect (Parada *et al.*,2007).

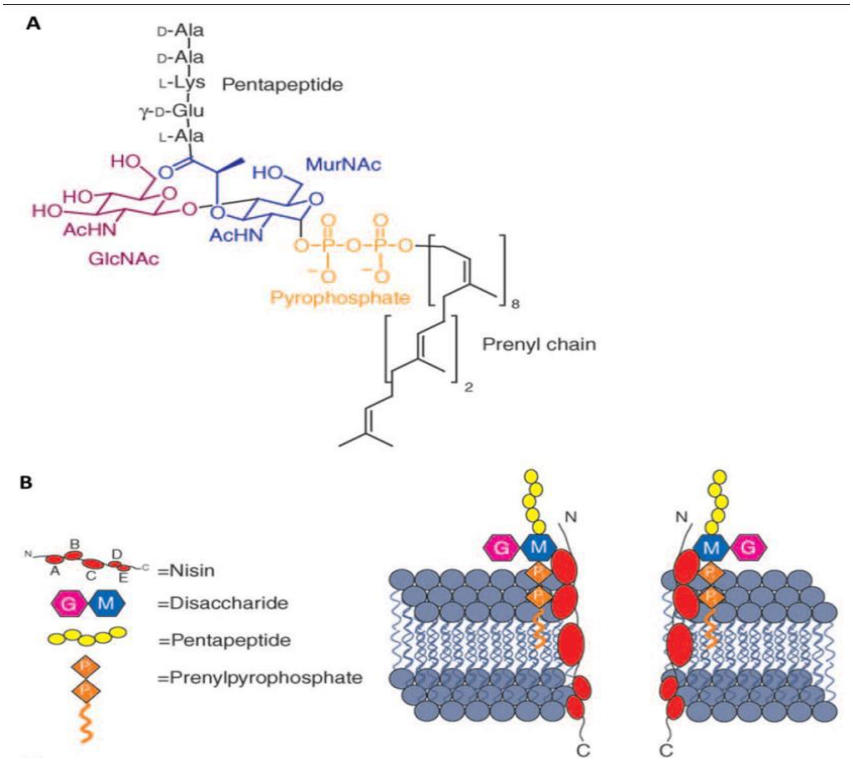

Figure 2.5. The chemical structure of lipid II (A) and the binding mechanism of Nisin to lipid II (B) (updated from Patton and Vab der Donk, 2005)

2.3.7.4. Nisin resistance

Some gram positive bacteria develop Nisin resistance when expose to high concentrations of nisin through some physiological and molecular mechanisms, including changes in the cell structures such as increasing the thickness of the cell, raising in cell wall hydrophobicity, alteration of concentration of membrane phospholipid and production of nisinase and nisin-resistant protein by some bacteria species. Some environmental conditions also can affect the activity of nisin like pH, food composition and structure, temperature and food microbiota (Zhou *et al.,* 2014).

2.4. Bacteriocins vs. Antibiotics

Bacteriocins synthesis have a role in competing with other bacteria which are closely related or present in the same ecological niche while antibiotic is a microbial metabolite or its derivative that has bactericidal or bacteriostatic activity on sensitive microorganisms (Guatam and Sharma, 2009). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous compounds that are rapidly digested by proteases in the human digestive tract. They are peptides produced ribosomally, and this fact makes the chance of improving their characteristics to aid their activity and spectrum. Antibiotics are generally considered to be secondary metabolic products that have inhibitory effect even in low concentration, excepting the inhibition caused by metabolites such as ammonia, organic acids and hydrogen peroxide. It is likely that most of not all bacteria have the ability to produce a heterogonous array of molecules in the period of their growth *in vitro* (and perhaps also in their natural habitats) that may be inhibit the growth of either themselves or other microorganism (Parada *et al.,* 2007).

Table 2.3. Bacteriocins vs. antibiotics (Cleveland *et al.*, 2001)

Characteristics	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell	Usually adaptation affecting cell membrane composition	Usually a genetically transferable determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly pore formation, butin a few cases possibly cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity/side effects	None known	Yes

2.5. Objective of Thesis

In this study, it has been aimed that to isolate and identify *Lactococcus lactis* spp. *lactis*, which is an important LAB commonly used for industrial application, from Home-made cheese. Especially, isolation and identification of potential Nisin-producing strains of *Lactococcus lactis* spp. *lactis* by molecular analysis.

3. MATERIALS AND METHODS

3.1. Tools and Equipment

Incubator (Nüve), Analytical balance (Vibra), Pipette (AXYPET), Microscope (Olympus), Microscope slides (Sailing Boat), Beaker (Iso Lab); Fume hood (Nüve), Vortex (Velp); Centrifuge (JP Selecta), Autoclave (Nüve), Microwave oven (Gosonic), Heating magnetic stirrer (Velp), Thermoblock TMR (Bunsen), Gel electrophoresis apparatus (Cole-parmer), Transilluminator (UVP), Digital Camera (Canon); Microtiter plate (Italy), Thermocycler (AB applied biosystem), Bunsen burner.

3.2. Chemicals and Reagents

All chemicals and reagents used were from Sigma-Aldrich (Germany), Merck (England), Favorgen (Taiwan) unless otherwise stated.

3.3. Sample Collection

For this study 20 different samples of Home-made cheese were collected from Duhok Province of Northern Iraq, within sterile packaging materials. Then all samples were stored in 4 ºC until delivery to laboratory and stored in refrigerator (4 ºC).

Table 3.1. List and details of samples

Sample no.	Origin of milk	Age of the sample	Location
1	Goat	1 month	Sarsing
2	Sheep	23 days	Sarsing
3	Sheep	2 months	Amedi
4	Sheep	2 months	Center
5	Cattle	1 week	Sarsing
6	Cattle	20 days	Zawite
7	Sheep	2 weeks	Sarsing

Sample no.	Origin of milk	Age of the sample	Location
8	Cow	2 weeks	Akre
9	Cow	3 months	Sarsing
10	Cow	1 month	Amedi
11	Cow	1 month	Akre
12	Sheep	5 days	Sarsing
13	Sheep	5 days	Amedi
14	Goat	10 days	Bamerne
15	Sheep	2 months	Bamerne
16	Cow	3 weeks	Sarsing
17	Cow	1 month	Center
18	Sheep	5 days	Sarsing
19	Sheep	5 days	Akre
20	Sheep	5 days	Akre

3.4. Culture Media

3.4.1. GM17 media

GM17 media was made by mixing M17 broth with distilled water making concentration 42.5 g/l for making GM17 broth, and M17 agar with concentration 55 g/l for GM17 agar, then autoclaving at 121 °C for 15 minutes. Then, Glucose was added, as a source of sugar, to the final concentration of 0.5% after autoclavation separately.

Table 3.2. Ingredients of GM17

Ingredients	**g/L**
Tryptone	2.5
Meat peptone (peptic)	2.5
Soya peptone (pepainic)	5
Yeast extract	2.5
Meat extract	5
Sodium glycerophosphate	19
Magnesium sulphate	0.25
Ascorbic acid	0.5
Lactose	5

3.4.2. MRS media

Man, Rogosa and Sharpe (MRS) agar is used for the growth and enumeration of cultures of Lactobacillus in dairy and other food products and in animal feeding stuffs. MRS media was made by mixing MRS broth with distilled water making concentration 52.2 g/l MRS broth, and MRS agar with distilled water making concentration 68.2 g/l MRS agar, then autoclaving at 121 °C for 15 minutes.

Table 3.3. Ingredients of MRS

Ingredients	**g/L**
Peptone from casein	10
Meat extract	8
Yeast extract	4
D(+) Glucose	20
di-potassium hydrogen phosphate	2
Tween 80	1
di-Ammonium hydrogenocitrate	2
Sodium acetate	5
Magnesium sulphate	0.2
Manganese sulphate	0.04

3.4.3. Mueller-Hinton agar

This media was used in antimicrobial susceptibility testing by the disc diffusion method. This formula conforms to Clinical and Laboratory Standard Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS).

Table 3.4. Ingredients of Mueller-Hinton Agar

Ingredients	**g/L**
Beef infusion solids	2 g
Acid Hydrolysed casein	17.5 g
Agar	17 g
Starch	1.5 g

3.4.4. Sodium chloride Peptone Solution

It is made to dissolving, suspending and diluting test samples, this fluid provides osmotic stability, a stable pH value and maintains the viability of microorganisms during preparation of samples for testing, with a pH 7.0 .

Table 3.5. Ingredients of Peptone Broth

Ingredients	**g/L**
Proteose Peptone	1
Potassium Dihydrogen Phosphate	3.6
Sodium Chloride	4.3
Disodium Hydrogen Phosphate	7.2

3.5. Isolation of Nisin-producing *Lactococcus lactis* spp. *lactis*

Samples were analyzed by weighing 10 grams of each sample and homogenizing it in 90 ml Buffered Sodium Chloride-Peptone Solution. A loopful from each streaked on MRS and GM17 agars and incubated in 37 °C for 24-48 hrs. To obtain pure culture, randomly a single colony from the petri dish inoculated to 5 ml MRS and GM17 broth and incubated for overnight then 500 µl of the overnight culture added to 500 µl of 30% glycerol, gently mixed and stored at -20 °C as a stock culture.

3.5.1. Morphological examination

Morphological characteristic examination of colony on the base of (colour, size, shape, margin and surface) has been done by eye after streaking repeatedly on MRS and GM17 agar plates.

3.5.2. Gram staining and catalase Test

The isolated strains were investigated and biochemically characterized on the basis of catalase were also carried out by pouring a drop of hydrogen peroxide (H_2O_2) on the colony and observed the reaction in order to identify suspected LAB catalase negative, and gram positive were isolated. Lactic acid bacteria tend to be blue-purple color microscopically which is indicate to gram-positive bacteria. Collected samples underwent to the gram status by light microscopy after staining. From the fresh pure culture broth a drop spread on a slide to form a thin smear, by exposure to flam smear fixation be done by quickly passing it two to three times through a flame, then immerse the smear with crystal violet for 1 minute and gently washed with tap water then immersed again with iodine for 1 minute and again washed with tap water, after that decolorized by adding alcohol 95% by

holding the slide at an angle to allow the decoloriser to drain for about 6 seconds then gently rinse off excess decoloriser with tap water. Flood the smear with safranin for 30 seconds and washed with tap water. Finally drain slide and allow it to air dry followed by examination of slides under light microscope.

3.5.3. Polymerase Chain Reaction (PCR)

Identification of potential *Lactococcus lactis* spp. *lactis* were done by detection of specific genes by PCR amplification using two primers Table 3.6. The process were conducted by pick up one colony from agar plate with sterile pipette tip attached to the pipetter and suspended in a PCR eppendorf containing 10 µl of sterile distilled water and pipetted up and down to mix and then 1 µl were used as DNA template in the reaction volume with the substrates mentioned in Table 3.7.

Table 3.1. Primers sequences and the expected fragment size of PCR reaction

Primers	**5'——————› 3'**	**Position[b]**	**Reference**
16S rRNA	F: GCGGCGTGCCTAATACATGC R: ATCTACGCATTTCACCGCTAC	700	(Perin and Nero, 2014)
nisA	F: GGATAGTATCCATGTCTG R: CAATGATTTCGTTCGAAG	250–269	(Li and O'Sullivan, 2002)

Table 3.2. Substrates added to PCR eppendorf to perform colony PCR

Chemical	**Amount**
DNA sample	1 µl
Distilled water	32 µl
Taq DNA polymerase buffer	4 µl
Deoxyribonucleotide triphosphates (dNTP)	1 µl
Forward primers	1 µl
Reverse primers	1 µl
Taq DNA polymerase enzyme	1 µl

Then mixtures were putted in thermal cycle under condition given in Table 3.8. to amplify 16S rRNA gene, in Table 3.9. to amplify *nisA* gene.

Table 3.3. Condition of PCR used to amplify *nisA* gene

First denaturation		**Denaturation**	**Annealing**	**Extension**	**Last extension**
Temperature	95 °C	95 °C	48 °C	72 °C	72 °C
Time	3 min	30 sec	30 sec	40 sec	10 min
Cycle	1	35			1

Table 3.4. Condition of PCR used to amplify 16S rRNA gene

First denaturation		**Denaturation**	**Annealing**	**Extension**	**Last extension**
Temperature	95 °C	95 °C	55 °C	72 °C	72 °C
Time	3 min	30 sec	30 sec	40 sec	10 min
Cycle	1	35			1

3.5.4. Gel electrophoresis

DNA fragments were separated via 1% (w/v) agarose gel electrophoresis. 1 g of standard agarose was dissolved in 100 ml of 1X Tris Borate EDTA buffer (TBE) by boiling in microwave oven until all particles were dissolved. After cooling, the agarose solution was poured into the gel casting stand and combs were placed. After the gel was cooled, the combs were removed gently. The casting tray carrying the agarose gel was placed into the electrophoresis tank and 1X Tris Borate EDTA (TBE) buffer was added until the buffer cover the gel. 5 µl of DNA were taken and mixed with 1 µl of gel loading buffer. After that the samples and DNA ladder were loaded into the wells. The electrophoresis conditions used were voltage of 110 volts electric current of 5 mA, and the gel was run for 75 min. When the electrophoresis was complete, the gel was removed from the electrophoresis tank with care and immersed in ethidium bromide (200 ml dH_2O containing 200 µl of EtBr) for 10-15 min which is a DNA intercalating agent, which fluoresces orange when exposed to ultraviolet (312 nm).

4. RESULTS AND DISCUSSION

4.1. Biochemical and Morphological Identification of LAB Isolates

Out of 20 home-made cheese samples collected from Duhok province, 100 potential colonies were isolated. Only 39 pure isolates were sharing (gram-positive, cocci and Catalase negative) characteristics with small, raised and circular colony morphology, after excluding the strains with bacilli and coccobacilli shapes. Under light microscope, the suspected strains' cells shapes were cocci and their arrangements were (pairs and short chains) after simple staining as shown in Figure 4. 1.

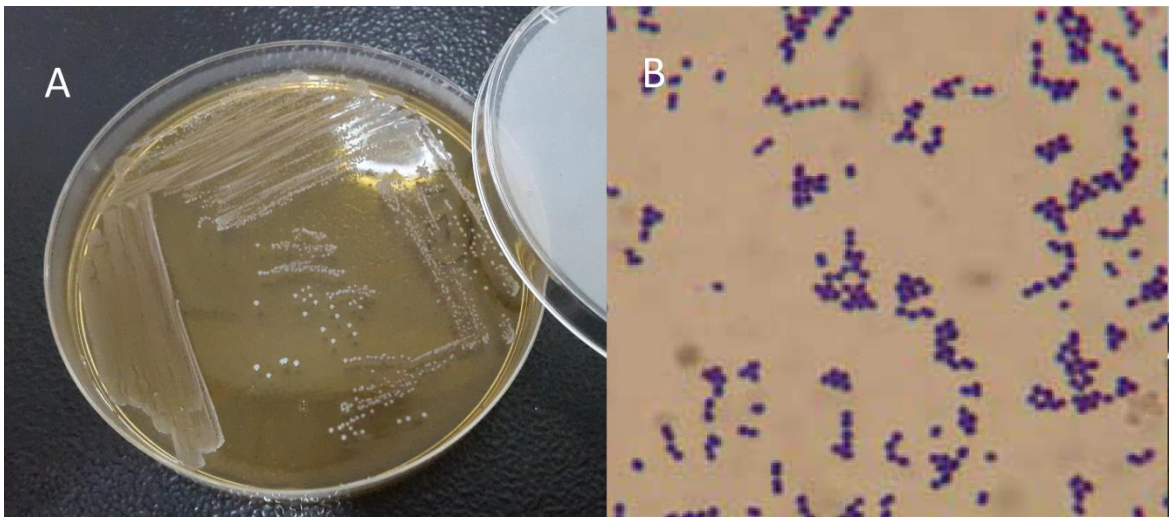

Figure 4.1. (A) Colony morphology on GM17 agar (B) Gram staining of *Lactococcus lactis* spp. *Lactis*

Table 4.1. Microbiological and Biochemical properties of suspected pure cultured isolates after elimination of strains which have various cell shape (not cocci) and gram positive and catalase negative

Suspected strains	Morphology	Gram	Bacteria
1 A	Cocci	+	*Lactococcus lactis spp. lactis*
1 B	Cocci	+	Not Defined
1 D	Cocci	+	Not Defined
2 B	Cocci	+	Not Defined
3 C	Cocci	+	*Lactococcus lactis spp. lactis*
3 D	Cocci	+	*Lactococcus lactis spp. lactis*

Suspected strains	Morphology	Gram	Bacteria
3 E	Cocci	+	*Lactococcus lactis spp. lactis*
4 B	Cocci	+	*Lactococcus lactis spp. lactis*
5 B	Cocci	+	*Lactococcus lactis spp. lactis*
5 D	Cocci	+	Not Defined
5 E	Cocci	+	Not Defined
6 B	Cocci	+	Not Defined
6 D	Cocci	+	*Lactococcus lactis spp. lactis*
6 E	Cocci	+	*Lactococcus lactis spp. lactis*
7 D	Cocci	+	Not Defined
8 B	Cocci	+	*Lactococcus lactis spp. lactis*
9 A	Cocci	+	Not Defined
9 B	Cocci	+	Not Defined
9 C	Cocci	+	Not Defined
9 D	Cocci	+	*Lactococcus lactis spp. lactis*
9 E	Cocci	+	*Lactococcus lactis spp. lactis*
10 A	Cocci	+	*Lactococcus lactis spp. lactis*
10 B	Cocci	+	*Lactococcus lactis spp. lactis*
10 C	Cocci	+	*Lactococcus lactis spp. lactis*
10 D	Cocci	+	Not Defined
10 E	Cocci	+	Not Defined
11 A	Cocci	+	Not Defined
11 B	Cocci	+	Not Defined
13 C	Cocci	+	Not Defined
14 D	Cocci	+	Not Defined

Suspected strains	Morphology	Gram	Bacteria
15 B	Cocci	+	Not Defined
16 B	Cocci	+	*Lactococcus lactis spp. lactis*
16 C	Cocci	+	*Lactococcus lactis spp. lactis*
16 D	Cocci	+	*Lactococcus lactis spp. lactis*
18 D	Cocci	+	*Lactococcus lactis spp. lactis*
20 B	Cocci	+	*Lactococcus lactis spp. lactis*
20 C	Cocci	+	*Lactococcus lactis spp. lactis*
20 D	Cocci	+	*Lactococcus lactis spp. lactis*
20 E	Cocci	+	*Lactococcus lactis spp. lactis*

4.2. Molecular Identification of the Isolates

Most natural isolates of lactococci belong to the lactis subspecies, but it has been reported that coccal-shaped lactic acid bacteria decrease towards the 28-days period of cheese ripening and lactobacillus, thought to be due to that lactobacillus bacteria have the ability to grow in low pH (Terzić-Vidojević *et al.,* 2009). Isolated and microbiologically and biochemically defined as LABs were subjected to molecular analysis by PCR technique to isolate strains of *Lactococcus lactis* spp. *lactis* from Home-Made cheese which has been previously isolated and molecularly identified from rocket salad (Kruger *et al.,* 2013), bean-sprouts (Cai *et al.,* 1997), different dairy products (Nagalakshmi *et al.,* 2013), sausages (Noonpakdee *et al.,* 2003) and others. PCR reaction was based on 16S primer as mentioned in Table 3.6., according to PCR amplifications. It has been expected to get about ~700 bp for 16S rRNA, DNA fragment. The amplicons from the PCR assays were analyzed using the agarose gel electrophoresis. The results showed that, 22 isolates were carrying both 16S rRNA gene which is used to provide genus and species identification specially for isolates that do not fit to any recognized biochemical profiles and highly useful in classification of bacteria (Janda and Abbott, 2007) as shown in Figure 4. 2. and Figure 4. 3.

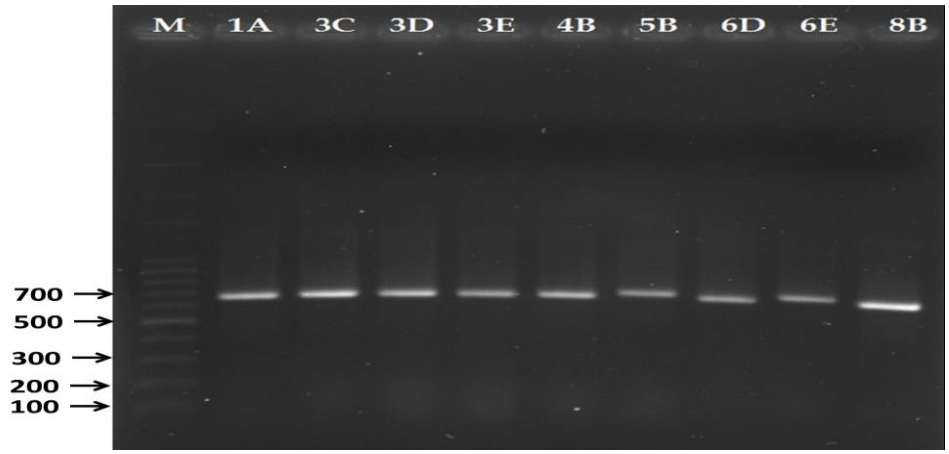

Figure 4. 2. PCR products of amplified 16S rRNA gene spacer regions different LAB isolated BY MRS and GM17 agars, Lanes: M, 100 bp DNA marker. Lane 1A, 3C, 3D, 3E, 4B, 5B, 6D, 6E, 8B isolates were *Lactococcus lactis* spp. *lactis* detected as (~700 bp).

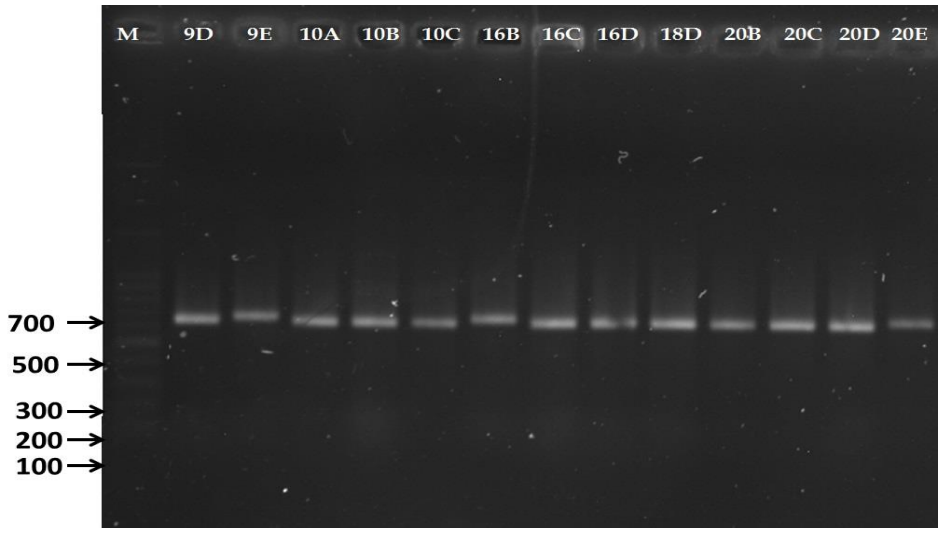

Figure 4. 3. PCR products of amplified 16S rRNA gene spacer regions different LAB isolated BY MRS and GM17 agars, Lanes: M, 100 bp DNA marker. Lane 9D, 9E, 10A, 10B, 10C, 16B, 16C, 16D, 18D, 20B, 20C, 20D and 20E isolates were *Lactococcus lactis* spp. *lactis* detected as (~700 bp).

4.3. Estimation of Nisin Production Capacity of Isolated *Lactococcus lactis* spp. *lactis*

To estimate the capacity of Nisin production by the isolated *Lactococcus lactis* spp. *lactis*, we characterized the Nisin structural gene, *nisA*, which is a gene involved in complex biosynthesis of Nisin (Kuipers *et al*., 1995). All 22 *Lactococcus lactis* spp. *lactis* isolates were positive for *nisA* gene, with a primer designed specifically to detect the presence of the Nisin structural gene, *nisA*, as mentioned in Table 3.6., and ~250 bp for *nisA* gene were expected, the results were as shown in Figure 4.4. and Figure 4.5., which indicate that the isolated *Lactococcus lactis* spp. *lactis* from home-made cheese were potential Nisin-producing strains, the results were similar to (Perin and Nero, 2014) in isolates from goat milk , and (Biscola *et al.,* 2013) in isolates from charqui, a Brazilian fermented, salted and dried meat product, and (Perin *et al.*, 2012) isolates from raw milk and cheese.

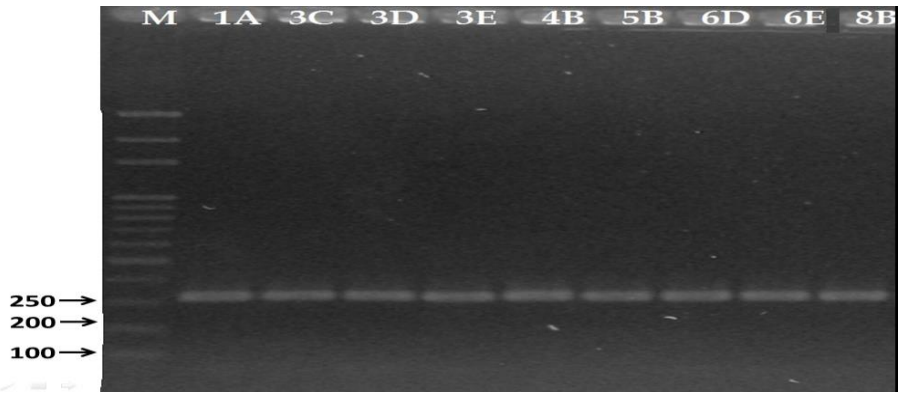

Figure 4.4. PCR products of amplified *nisA* gene spacer regions different LAB isolated BY MRS and GM17 agars, Lanes: M, 100 bp DNA marker. Lane 1A, 3C, 3D, 3E, 4B, 5B, 6D, 6E, 8B, 9D, 9E, 10A, 10B isolates were detected as (~250 bp).

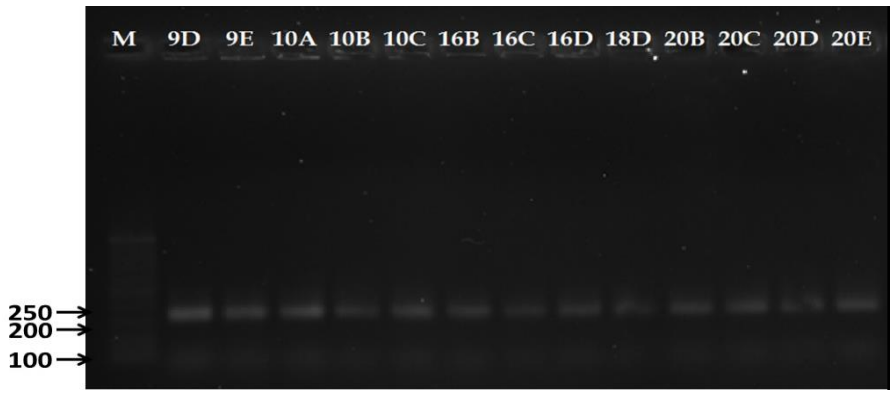

Figure 4.5. PCR products of amplified *nisA* gene spacer regions different LAB isolated BY MRS and GM17 agars, Lanes: M, 100 bp DNA marker. Lane 9D, 9E, 10A, 10B, 10C, 16B, 16C, 16D, 18D, 20B, 20C, 20D and 20E isolates were detected as (~250 bp)

5. CONCLUSION

LAB have a significant role in production of organoleptic properties of food products make by fermentation. They are related to group of non-pathogenic bacteria, phylogenetically diverse bacteria synthesize lactic acid as a primary metabolite from fermentation of glucose, and produce bacteriocins which are proteinaceous compounds have role in inhibition of many bacteria that are responsible for important food borne outbreaks or spoilage related bacteria. LAB are widely distributed in the nature, and play a vital role in dairy products manufacturing. They can be also found as a normal flora of human and other mammals (e.g. gastrointestinal tract, oral cavity, etc.). *Lactococcus lactis* spp. *lactis* belongs to the genus *Lactococcus*, is a LAB commonly used in diary industry to produce dairy products like yogurt, buttermilk, and certain types of cheese. *Lactococcus lactis* spp. *lactis* synthesize several lantibiotics and non-lantibiotics bacteriocins, antimicrobial peptides, and the most important one is Nisin. Isolation and molecular identification of potential Nisin-producing strains *Lactococcus lactis* spp. *lactis* from home-made cheese was the aim of this study.

The results of this study show that from total 100 potential strains isolated and genetically identified from 20 different samples of home-made cheese, 22 were potential Nisin-producing strains of *Lactococcus lactis* spp. *lactis*, which were confirmed using identification method based on PCR-amplification using 16S rRNA for identification of the targeted LAB and *nisA* for determination of the capacity of Nisin-production by the isolated strains. The detection of potential Nisin-producing strains of *Lactococcus lactis* spp. *lactis* in home-made cheese indicate that traditional dairy products can obtain promising strains that can produce Nisin and be used as bio-control agent to avoid food borne diseases, caused by several important food borne pathogenic bacteria that are according to researches sensitive to Nisin such as *L. monocytogenes, E. coli, S. aureus* and others.

Finally, the importance of bacteriocins producing strains of probiotics is in that after ingestion, if they are not affected by inadequate conditions such as the low pH in the stomach or by the enzymatic inactivation by gastric proteases, by bile salt stress, then they have the ability to pass through stomach and enter to gastro-intestinal tract, and produce active compounds that are effective in controlling pathogenic bacteria, especially which can cause diarrhea and gastro intestinal infections, and has role in correction of imbalance happened in microbiota resulted from antibiotic treatment.

REFERENCES

Aarnikunnas, J., 2006. Metabolic engineering of Lactic Acid Bacteria and characterization of novel enzymes for the production of industrially important compounds. Academic dissertation at Faculty of Veterinary Medicine of the University of Helsinki, Finland.

Abts, A., Mavaro, A., Stindt, J., Bakkes, P. J., Metzger, S., Driessen, A. J., Smits, S. H. J. Schmitt L., 2011. Easy and rapid purification of highly active nisin. *International Journal of Peptides*, (2011): 1-9.

Ahmed, F. E., 2003. Genetically modified probiotics in foods. *Trends in Biotechnology*, 21(11): 491-497.

Alegría, Á., Delgado, S., Roces, C., López, B., Mayo, B., 2010. Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk. *International Journal of Food Microbiology*, 143.1-2: 61-66.

Biscola, V., Todorov, S. D., Capuano, V. S. C., Abriouel, H., Gálvez, A., Franco, B. D. G. M., 2013. Isolation and characterization of a nisin-like bacteriocin produced by a *Lactococcus lactis* strain isolated from charqui, a Brazilian fermented, salted and dried meat product. *Meat Science*, 93(3): 607-613.

Bulut, Ç., 2003. Isolation and molecular characterization of Lactic Acid Bacteria from cheese. Doctoral dissertation, 102 pages, İzmir Institute of Technology, Turkey.

Cai, Y., Ng, L. K., Farber, J. M., 1997. Isolation and characterization of nisin-producing *Lactococcus lactis* spp. *lactis* from bean-sprouts. *Journal of Applied Microbiology*, 83(4), 499-507.

Chaimanee, V., Sakulsingharoj, C., Deejing, S., Seetakoses, P., Niamsup, P., 2009. Screening and characterization of bacteriocin-producing bacteria capable of inhibiting the growth of bovine mastitis. *Maejo International Journal of Science and Technology*, 3(1): 43-52.

Chavan, M. A., Riley, M. A., 2007. Molecular evolution of bacteriocins in gram-negative bacteria. *In Bacteriocins: Springer Berlin Heidelberg*, pp. 19-43.

Cleveland, J., Montville, T. J., Nes, I. F., Chikindas, M. L., 2001. Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology*, 71(1): 1-20.

Cotter, P. D., Ross, R. P., Hill, C., 2013. Bacteriocins - a viable alternative to antibiotics? *Nature Reviews Microbiology*, 11(2): 95-105.

Dirix, G., Monsieurs, P., Dombrecht, B., Daniels, R., Marchal, K., Vanderleyden, J., Michiels, J., 2004. Peptide signal molecules and bacteriocins in gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. *Peptides*, 25(9): 1425-1440.

Garcia, P., Rodriguez, L., Rodriguez, A., Martinez, B., 2010. Food biopreservation: promising strategies using bacteriocins, bacteriophages and endolysins. *Trends in Food Science and Technology*, 21(8): 373-382.

Giridhar, A., 2013. Increasing the *Lactococcus lactis* Biomass Through Aerobic Growth. A thesis presented in partial fulfillment of the requirement for the degree of master of Engineering in Biotechnology at Massey University, Palmerston North, New Zealand.

Guatam, N., Sharma, N., 2009. Bacteriocin: safest approach to preserve food products. *Indian Journal for Microbiology*, 49(3): 204-211

Halasz, A., 2009. Lactic Acid Bacteria. *Food Quality and Standards*, 3: 70-82.

Ikeda, D. M., Weinert Jr, E., Chang, K. C., McGinn, J. M., Miller, S. A., Keliihoomalu, C., DuPonte, M. W., 2013. Natural farming: Lactic Acid Bacteria. Published by the College of Tropical Agriculture and Human Resources University of Hawai‘i.

Ingham, A., Ford, M., Moore, R. J. Tizard, M., 2003. The bacteriocin piscicolin 126 retains Antilisterial Activity *in vivo*. *Journal of Antimicrobial Chemotherapy*, 51 (6): 1365-1371.

Janda, J. M., Abbott, S. L., 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology*, 45(9): 2761-2764.

Jozala, A. F., Novaes, L. C. D. L., Cholewa, O., Moraes, D., Penna, T. C. V., 2005. Increase of Nisin production by *Lactococcus lactis* in different media. *African Journal of Biotechnology*, 4 (3): 262-265.

Khalid, K., 2011. An overview of Lactic Acid Bacteria. *Journal of Biosciences*, 1 (3): 1-13.

Khan, H., 2013. Production, characterization and utilization of the bacteriocin produced by *Enterococcus faecalis* B9510. A thesis presented in partial fulfillment of the requirement for the degree Doctor of Philosophy in Biotechnology at Massey University, Palmerston North, New Zealand.

Klijn, N., Weerkamp, A. H., De Vos, W. M., 1995. Genetic marking of *Lactococcus lactis* shows its survival in the human gastrointestinal tract. *Applied and Environmental Microbiology*, 61 (7): 2771-2774.

Kongo, J. M., 2013. Lactic Acid Bacteria as starter-cultures for cheese processing: past, present and future developments. *InTech: Open Science| Open mind*s, 1-20.

König, H., Fröhlich, J., 2009. Lactic Acid Bacteria. In Biology of Microorganisms on Grapes, in Must and in Wine. *Springer Berlin Heidelberg*, pp. 3-29.

Kruger, M. F., Barbosa, M. D. S., Miranda, A., Landgraf, M., Destro, M. T., Todorov, S. D., Franco B. D. G. M., 2013. Isolation of bacteriocinogenic strain of *Lactococcus lactis* subsp. *lactis* from Rocket Salad (*Eruca sativa Mill.*) and

evidence of production of a variant of Nisin with modification in the leader-peptide. *Food Control*, 33 (2): 467-476.

Kuipers, O. P., Rollema, H. S., Beerthuyzen, M. M., Siezen, R. J., De Vos, W. M., 1995. Protein engineering and biosynthesis of nisin and regulation of transcription of the structural *nisA* gene. *International Dairy Journal*, 5(8): 785-795.

Lepeuple, A. S., Van Gemert, E., Chapot-Chartier, M. P., 1998. Analysis of the bacteriolytic enzymes of the autolytic *Lactococcus lactis* subsp. *cremoris* strain am2 by renaturing polyacrylamide gel electrophoresis: identification of a prophage-encoded enzyme. *Applied and Environmental Microbiology*, 64(11): 4142-4148.

Li, H., O'Sullivan, D. J., 2002. Heterologous expression of the *Lactococcus lactis* bacteriocin, nisin, in a dairy *Enterococcus* strain. *Applied and Environmental Microbiology*, 68(7): 3392-3400.

Masood, M., I., Qadir, M., I., Shirazi, J., H., Khan, I., U., 2011. Beneficial effects of Lactic Acid Bacteria on human beings. *Critical Reviews in Microbiology*, 37(1): 91–98

Mavaro, A., Abts, A., Bakkes, P. J., Moll, G. N., Driessen, A. M. J., Smits, S. H. J., Schmitt, L., 2011. Substrate recognition and specificity of *NisB*, the lantibiotic dehydratase involved in nisin biosynthesis. *Journal Biological Chemstry*, 286(35): 30552-30560.

McAuliffe, O., Ross, R. P., Hill, C., 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews*, 25(3): 285-308.

Mohammed, S. S. D., Balogu, T. V., Yunusa, A. Aliya, H., 2013. Antimicrobial Efficiency of Purified and Characterized Bacteriocins Produced by *Lactobacillus bulgaricus* Y34 and *Lactococcus lactis* N22 Isolated from Fermented Milk Products. *International Journal of Advanced Research*, 1 (6): 63-70.

Murdock, C. A., Cleveland, J., Mathews, K. R. Chikindas, M. L., 2006. The synergistic Effect of Nisin and Lactoferrin on the Inhibition of *Listeria monocytogenes* and *Escherichia coli* O157:H7. *Letters in Applied Microbiology*, 44 (3): 255-261.

Nagalakshmi, P. K., Sumathi, R., Kanimozhi, K., Sivakumar, T., 2013. Isolation of Bacteriocin Nisin Producing *Lactococcus lactis* from Dairy Products. *Journal of Scientific and Industrial Research*, 1(10):1626-630.

Nath, S., Chowdhury, S., Dora, K.C., Sarkar, S., 2014. Role of biopreservation in improving food safety and storage. *International Journal of Engineering Research and Applications*, 4 (1): 26-32.

Noonpakdee, W., Santivarangkna, C., Jumriangrit, P., Sonomoto, K., Panyim, S., 2003. Isolation of nisin-producing *Lactococcus lactis* WNC 20 strain from nham, a traditional Thai fermented sausage. *International Journal of Food Microbiology*, 81(2): 137-145.

Oscáriz, J. C., Pisabarro, A. G., 2000. Characterization and mechanism of action of cerein 7, a bacteriocin produced by *Bacillus cereus* Bc7. *Journal of Applied Microbiology*, 89(2): 361-369.

Parada, J. L., Caron, C. R., Medeiros, A. B. P. Soccol, C. R., 2007. Bacteriocins from Lactic Acid Bacteria: purification, properties, and use as biopreservatives. *Brazilian Archives of Biology and Technology*, 50 (3): 521-542.

Patton, G. C., Van der Donk, W. A., 2005. New developments in lantibiotic biosynthesis and mode of action. *Current Opinion in Microbiology*, 8(5): 543-551.

Perin, L. M., Nero, L. A., 2014. Antagonistic Lactic Acid Bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*. *BMC Microbiology*, 14(1): 36.

Perin, L. M., Moraes, P. M., Viçosa, G. N., Júnior, A. S., Nero, L. A., 2012. Identification of bacteriocinogenic *Lactococcus* isolates from raw milk and cheese capable of producing nisin A and nisin Z. *International Dairy Journal*, 25(1): 46-51.

Pingitore, E. V., Salvucci, E., Sesma, F., Nader-Macías, M. E., 2007. Different strategies for Purification of Antimicrobial Peptides from Lactic Acid Bacteria (LAB). *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, 1: 557-568.

Piper, C., Hill, C., Cotter, P. D., Ross, R. P., 2010. Bioengineering of a Nisin A-producing *Lactococcus lactis* to create isogenic strains producing the natural variants Nisin F, Q and Z. *Microbial Biotechnology*, 4(3): 375-382.

Rattanachaikunsopon, P., Phumkhachorn, P., 2010. Lactic Acid Bacteria: their antimicrobial compounds and their uses in food production. *Annuals of Biological Research*, 1(4): 218-228.

Renault, P., 2002. Genetically modified Lactic Acid Bacteria: applications to food or health and risk assessment. *Biochimie*, 84(11): 1073-1087.

Riley, M. A., Wertz, J. E., 2002. Bacteriocins: evolution, ecology and application. *Annual Review of Microbiology*, 56 (1):117-137.

Robinson, R. K., 2005. Dairy microbiology handbook: the microbiology of milk and milk products. *John Wiley and Sons*, 784p.

Savadogo, A., Ourrara, C. A. T, Bassole, I. H. N, Traore, S. A., 2004. Antimicrobial activities of Lactic Acid Bacteria strains isolated from Burkina Faso fermented milk. *Pakistan Journal of Nutrition*, 3 (3): 174-179.

Savadogo, A., Outtara, C. A. T., Bassole, I. H. N, Traore, S. A., 2006. Bacteriocins and Lactic Acid Bacteria – a mini review. *African Journal of Biotechnology*, 5 (9): 678-683.

Schmidt, S. E., 2009. Antimicrobial efficacy of liposome encapsulated Nisin and Nisin's inhibition against *Listeria monocytogenes* in fluid milk at different storage temperatures. Master's thesis, Texas A&M University, USA.

Soomro, A., H., Masud, T., Awar, K., 2002. Role of Lactic Acid Bacteria (LAB) in food preservation and human health - a review. *Pakistan Journal of Nutrition*, 1(1): 20-24.

Tan, Y.,T., 2010. The development of *Lactococcus lactis* as an antimicrobial agent. A thesis submitted for the degree of Master of Applied Science at Queensland University of Technology, Australia.

Terzić-Vidojević, A., Nikolić, M., Veljović, K., Tolinački, M., Busarčević, M., Topisirović, L., 2009. Analysis of the Lactic Acid Bacteria microflora in traditional Caucasus cow's milk cheeses. *Archives of Biological Sciences*, 61(3): 395-406.

Villani, F., Aponte, M., Blaiotta, G., Pepe, O. Moschetti, G., 2001. Detection and Characterization of a Bacteriocin, graviecun L1-5, Produced by *Lactococcus gravieae* Isolated from Raw Cow's Milk. *Journal of Applied Microbiology*, 90 (3): 430-439.

Vuyst, L., D., Leroy, F., 2007. Bacteriocins from Lactic Acid Bacteria: production, purification, and food applications. *Journal of Molecular Microbiology and Biotechnology*, 13 (4): 194–199.

Yang, E., Fan, L., Jiang, Y., Doucette, C. and Fillmore, S., 2012. Antimicrobial activity of bacteriocin producing Lactic Acid Bacteria from cheese and yogurts. *AMB Express*, 2 (1): 1-12.

Zacharof, M. P., Coss, G. Mandale, M., S. J., Lovitt, R. W., 2013. Separation of Lactobacilli bacteriocins from fermented broths using membranes. *Process Biochemistry*, 48 (8): 1252-1261.

Zhang, Y. F., Liu, S. Y., Du, Y. H., Feng, W. J., Liu, J. H., Qiao, J. J., 2014. Genome shuffling of *Lactococcus lactis* subspecies *lactis* YF11 for improving nisin Z production and comparative analysis. *Journal of Dairy Science,* 97(5): 2528-2541.

Zhou, H., Fang, J., Tian, Y., Lu, X. Y., 2014. Mechanisms of nisin resistance in gram-positive bacteria. *Annals of Microbiology*, 64(2): 413-420.

CURRICULUM VITAE

Personal Information

Name, Surname : Ayaz MAMSIN

Nationality : Kurdish

Date of birth : June 20, 1986

Place of birth : Duhok, Iraq

Marital Status : Single

Telephone : (+90) 5346369249 – (+964)7504017749

E-mail : ayaz_salih86@yahoo.com

Education

Date	Faculty	Place of Education	Degree
2009	Veterinary Medicine	University of Duhok	B.Sc.
2004	---	Sarsing High School	High School Diploma

Work Experience

Date	Job Title	Institution
2009-2013	Veterinarian	Ministry of Agriculture and Water Resources

Language Skills

Kurdish, English and Arabic.

Computer Skills

Windows XP/7/8, Microsoft Office, Internet.