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

ISOLATION AND CHARACTERIZATION OF SOME LACTIC ACID BACTERIA FROM HOME-MADE DAIRY PRODUCTS AND INVESTIGATION OF SOME ANTIMICROBIAL AND ENZYMATIC PROPERTIES

Ahmed Karem Ramadan RAMADAN

MASTER THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

KAHRAMANMARAŞ- TURKEY 2014

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M.Sc thesis entitled “Isolation and characterization of some lactic acid bacteria from home-made dairy products and investigation of some antimicrobial and enzymatic properties” and prepared by Ahmed Karem Ramadan RAMADAN, who is a student at Bioengineering and Sciences Department, Graduate School of Natural and Applied Sciences, Kahramanmaraş Sütçü İmam University, 20/11/2014 was certified by all the majority of jury members, whose signatures are given below.

Prof. Dr. M. Sait EKİNCİ (Supervisor) ……...…….
Department of Bioengineering and Sciences
Kahramanmaraş Sütçü İmam University

Prof. Dr. Emin ÖZKÖSE .....
Department of Bioengineering and Sciences
Kahramanmaraş Sütçü İmam University

Assoc. Prof. Dr. Ismail AKYOL .....……
Department Agricultural Biotechnology
Kahramanmaraş Sütçü İmam University

I approve that the above signatures related to the members.

Prof. Dr. M. Hakkı ALMA …….
Director of Graduate School

THESIS STATEMENT

I hereby declare that all information in the thesis has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all the material and results that are not original to this work.

Ahmed Karem Ramadan RAMADAN

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BAZI LAKTİK ASİT BAKTERİLERİNİN EV YAPIMI SÜT ÜRÜNLERİNDEN İZOLASYONU, KARAKTERİZASYONU VE ANTİMİKROBİYAL VE ENZİMATİK ÖZELLİKLERİNİN ARAŞTIRILMASI

(YÜKSEK LİSANS TEZİ)

Ahmed Karem Ramadan RAMADAN

ÖZET

Bazı fermente ürünlerden laktik asit bakterilerinin izolasyonu ve tür düzeyinde tanımlamaları yapılmıştır. Bu amaçla geleneksel yöntemlerle Kuzey Irak'ta yapılmış 40 farklı fermente ürün (peynir, kaşar, yogurt, sarımsaklı peynir ve fermente sosis) izolasyon amaçlı kullanılmıştır. Morfolojik özelliklerine göre *Leuconostoc* sp. ve *Pediococcus* sp. olarak tanımlanmış izolelerin moleküler tanımlamalarında koloni PZR yöntemi kullanılmıştır. *Leuconostoc* sp. izolelerinin moleküler tanımlanmasında 23S rRNA kullanılırken, *Pediococcus* sp. izolelerinin tanımlanmalarında 16S rRNA kullanılmıştır. Toplam 38 izole *Pediococcus* ve *Leuconostoc* olarak moleküler yöntemlerle tanımlanmış ve bu izolelerin bazılarının *S. paratyphi* ve *E. faecali* türlerine karşı antimikrobiyal etki gösterdikleri tesbit edilirken hiçbir izole *P. mirabilis, P. aeruginosa, E. coli* ve *C. jejuni* türlerine karşı antimikrobiyal etki göstermemiştir. Kullanılan 11 farklı antibiyotik dayanıklılık testi sonuçları izolelerin çoğunluğunun ceprofloxacine, erythromycine ve penciline antibiyotiklerine karşı dayanıklı olduğunu ortaya koymuştur. Diğer taraftan hiç bir izole kanamycin, tetracycline, vancomycine ve streptomycine karşı dayanıklılık göstermemiştir. Tüm izoleler yüksek proteinaz aktivitesi gösterirken, 10 izolenin 1 den fazla plazmide (>2kb) sahip olduğu belirlenmiştir.

Anahtar Kelimeler: *Leuconostoc, Pediococcus,* Antibiotics test, Antimicrobial activity, Protienase activity, Plasmid

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ISOLATION AND CHARACTERIZATION OF SOME LACTIC ACID BACTERIA FROM HOME-MADE DAIRY PRODUCTS AND INVESTIGATION OF SOME ANTIMICROBIAL AND ENZYMATIC PROPERTIES

(M.Sc. THESIS)

Ahmed Karem Ramadan RAMADAN

ABSTRACT

Isolation and identification of lactic acid bacteria from some fermented products at the level of the species were carried out. For this aim, 40 traditionally made fermented produtcs (cheese, cottage cheese, yoghurt, cheese with garlic and sausage) in Northern Iraq were used for isolation of lactic acid bacteria. Colony PCR were conducted for molecular identification of isolates which they were preliminary identified as *Leuconostoc* sp. and *Pediococcus* sp. according to their morphological characters. For identification of *Pediococcus* sp. 23S rRNA and for Leuconostoc sp. 16S rRNA have been targeted. Total 38 isolates were identified as *Pediococcus* and *Leuconostoc* and more than one of these strains exhibited antimicrobial activity against to *S. paratyphi* and *E. faecali*. All tested strains showed no antimicrobial activity against *P. mirabilis*, *P. aeruginosa*, *E. coli* and *C. jejuni*. Eleven different antibiotic resistance tests were also carried out and the results revealed that majority of the isolates were resistant to Ceprofloxacine, Erythromycine and Penciline. None of the isolates showed resistance against Kanamycin, Tetracycline, Vancomycine and Streptomycin. All the isolated strain showed high proteolytic activity. Ten isolates showed the presence of multiple plasmids (1-4) corresponding to the molecular weights of 2.0, 2.6 and other plasmids >10 kb.

Key Words: *Leuconostoc*, *Pediococcus*, Antibiotics test, Antimicrobial activity, Protienase activity, Plasmid

Kahramanmaraş Sütçü İmam University

Graduate School of Natural and Applied Sciences
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Supervisor: Prof. Dr. M. Sait EKİNCİ

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LIST OF ABBREVIATIONS

LAB:	Lactic acid bacteria.
GRAS:	Generally recognized as safe.
G+C:	Guanine + Cytocine.
NAD^+:	Nicotinamide adenine dinucleotide.
DNA:	Deoxyribonucleic acid.
PCR:	Polymerase chain reaction.
dNTPs:	Deoxyribonucleotide triphosphates.
µl:	Microlitter.
pH:	Power of hydrogen.
µg:	Microgram.
RC:	Rolling circle mechanism.
dso:	Double strand origin.
RNA:	Ribonucleic acid.
ATP:	Adenosine triphosphate.
kDa:	Kilo dalton.
bp:	Base pair.
GM17:	Glucose-Maltose 17.
MRS:	De-man, Rogosa and Sharpe.
MSE:	Mayeux, Sandine and Elliker.

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1. INTRODUCTION

Lactic acid bacteria, commonly referred to as LABs, are a group of commercially important organisms that are classified by their ability to ferment hexose sugars into mainly lactic acid. LABs are best known for their use as starter culture in the manufacture of dairy products such as acidophilus milk, yogurts, buttermilk cottage cheese, hard cheese and soft cheese (Kok and Vos, 1994; Kunji *et al*., 1996; Mierau *et al*., 1997). It has a long history of use by man for food production and food preservation. There is 21 different commercial vegetable fermentation in Europe along with a large number of fermented vegetable juices and blends, the most economically relevant of these are the fermentations of olives, cucumbers, and cabbage (Tayeb idoui *et al*., 2009).

Lactic bacterium (i.e. *Lactococcus lactis*) was the first pure culture of LAB obtained in 1873 by Lister, J. (Axelsson, 1998). A number of strains of LABs have been proposed and safely used as probiotics, when ingested in certain numbers exert health benefits beyond inherent basic nutrition. Probiotics are widely used to aid digestion and help restore gut bacterial balance after antibiotics (Ganguli, 2013).

The heterogeneous group of LAB includes the rod-shaped bacteria like *lactobacilli*, and *cocci* have been used for manufacturing products like cheese, yoghurts, fermented milk products, beverages, sausages and olives. A variety of strains have been reported to be antagonistic to pathogens and spoilage organisms associated with those products (Chaillou *et al*., 2005; Chen and Hoover, 2003; Holzapfel *et al*., 1995). These food-grade bacteria can also improve the safety, shelf life, nutritional value, flavour and quality of the product. Moreover, it can be used for the production of food additives and aroma compounds (Sybesma *et al*., 2006).

There is increasing interest in the genetic manipulation of LAB to improve existing characteristics or introduce novel, industrially pertinent phenotypes (Julie, 2005). and development in the biotechnology of LAB: One area where genetic engineering would be of particular benefit to the dairy industry is in the genetic modification of LAB which are commonly used as starter cultures in the production of fermented dairy foods (Soomro *et al*., 2002). Therefore many scientists have been concentrated on the isolation of new LAB strain from natural resources for biotechnological application.

2. LITERATURE REVIEW

2.1. Lactic Acid Bacteria

2.1.1. Historical background of lactic acid bacteria

Lactic acid bacteria have a long history of use in the production and utilization of fermented foods and beverages. It is important to the food industry that lactic acid was produced as an end/by product of fermentation, which is a biological method for centuries to improve the storage qualities and nutritive value of perishable foods such as milk, vegetables, meat fish and cereals. In developed world, it mainly related with fermented dairy products such as cheese, buttermilk and yogurt. The conception of the name of 'lactic acid bacteria' was created for bacteria causing fermentation and coagulation of milk to producing lactic acid alone or acetic and lactic acids, alcohol and carbon dioxide (depending on metabolic pathway).

LABs are a cluster of Gram-positive, non-sporing, carbohydrate-fermenting lactic acid producers, acid tolerant of non-aerobic habitat and catalase negative bacteria. They are subdivided basically into four genera *Streptococcus, Leuconstoc, Pediococcus*, and *Lactobacillus*. The recent classification reviews suggest that lactic acid bacteria group could be includes of genera *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus,* and *Vagococcus* (Orla-Jensen, 1919).

2.1.2. Importance and general characteristics of LAB

LABs are importance to the food industry, since these bacteria have generally been regarded as safe (GRAS status). A number of LAB species are concerned in fermentation of a large range of foods and beverages, as dairy, meat, fish, vegetable, sourdough, wine, and cider (Liu, 2003). The LAB contains to group first cocci (*Lactococcus, Vagococcus, Leuconostoc, Pediococcus, Aerococcus ،Tetragenococcus, Streptococcus*) and second rods (*Lactobacillus, Carnobacterium ،Bifidobacterium*). In addation phylogenetically they are parts of the Clostridium-Bacillus subdivision of Gram-positive eubacteria LAB secretes antimicrobial compounds which were used in fermentations to preserve the nutritive qualities of various foods (Tagg *et al*., 1976; De Vuyst and Vandamme, 1994). Lactic acid bacteria used in food as a preservative and flavor enhancer (van Maris *et al*., 2004). In addition, LAB produces metabolites giving

aroma and flavor to the fermented producst And moreover they are used as texturizing agent and stabilizetor in food (Caplice and Fitzgerald, 1999). They also play an important role in processing of animal feeds like silages (Aukrust and Blom, 1992; Driehuis and Elferink, 2000; Holzer *et al*., 2003).

Lactic acid has two optical stereoisomer's, the (D-) and L (+) isomers. D-lactate was considered to be a non-physiological isomer, which cause unpleasant effects for infants and patients suffering from short-bowel disease and intestinal failure. However, L-lactate was chosen for pharmaceutical applications and food because this was a midway in mammalian metabolism (Lapierre *et al*., 1999).

The antimicrobial effect of LAB is mostly due to their lactic and organic acid production, causing the pH of the growth environment to decrease (Caplice and Fitzgerald, 1999; Kuipers *et al*., 2000). Low pH makes organic acids to become lipid soluble and diffuse through the cell membrane into the cytoplasm (Gottschalk, 1988). LABs also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodriguez *et al*., 2003).

Today, taxonomical methods are based on phenotypic characterization (cell-wall composition, protein fingerprinting, electrophoresis mobility, ect) and genotypic analysis (Chagnaud *et al*., 2001). A taxonomic perspective, the classification of lactic acid bacteria into different genera is mostly based on the characteristics used by (Orla-Jensen, 1919). This classification are divided into unlike genera are mainly based on morphology, mode of glucose fermentation, as *S. thermophilus*, the lactobacilli and the leuconostocs are grouped with the other lactic acid bacteria and *Bacillus* spp. within a supercluster of the clostridial sub-branch of the Gram-positive eubacteria, characterized by a low genomic G+C content (Stackebrandt *et al.,* 1983; Stackebrandt and Teuber, 1988).

In bacterial taxonomy, morphology is regarded as dubious as a key character (Woese, 1987), and is very significant in the existing descriptions of the lactic acid bacterial genera, based on morphology (*Lactobacillus* and *Carnobacterium*) and cocci (all other genera). The lactic acid bacteria, although consisting of a number of diverse genera, are grouped as (Figure 2.1) either homofermenters which convert glucose to lactic acid or heterofermenters which ferment glucose to lactic acid, ethanol/acetic acid,

and CO_2 (Sharpe, 1979; Axelsson, 1998), based on the end product of their fermentation. The method of glucose fermentation under standard conditions as an important way was used in the differentiation of the lactic acid bacteria genera, i.e., nonlimiting concentrations of glucose, growth factors (amino acids, vitamins and nucleic acid precursors) and limited oxygen availability. Leuconostocs and a subgroup of Lactobacillus are heterofermentative; all other lactic acid bacteria are homofermentative (Sharpe, 1979; Carr *et al*., 2002).

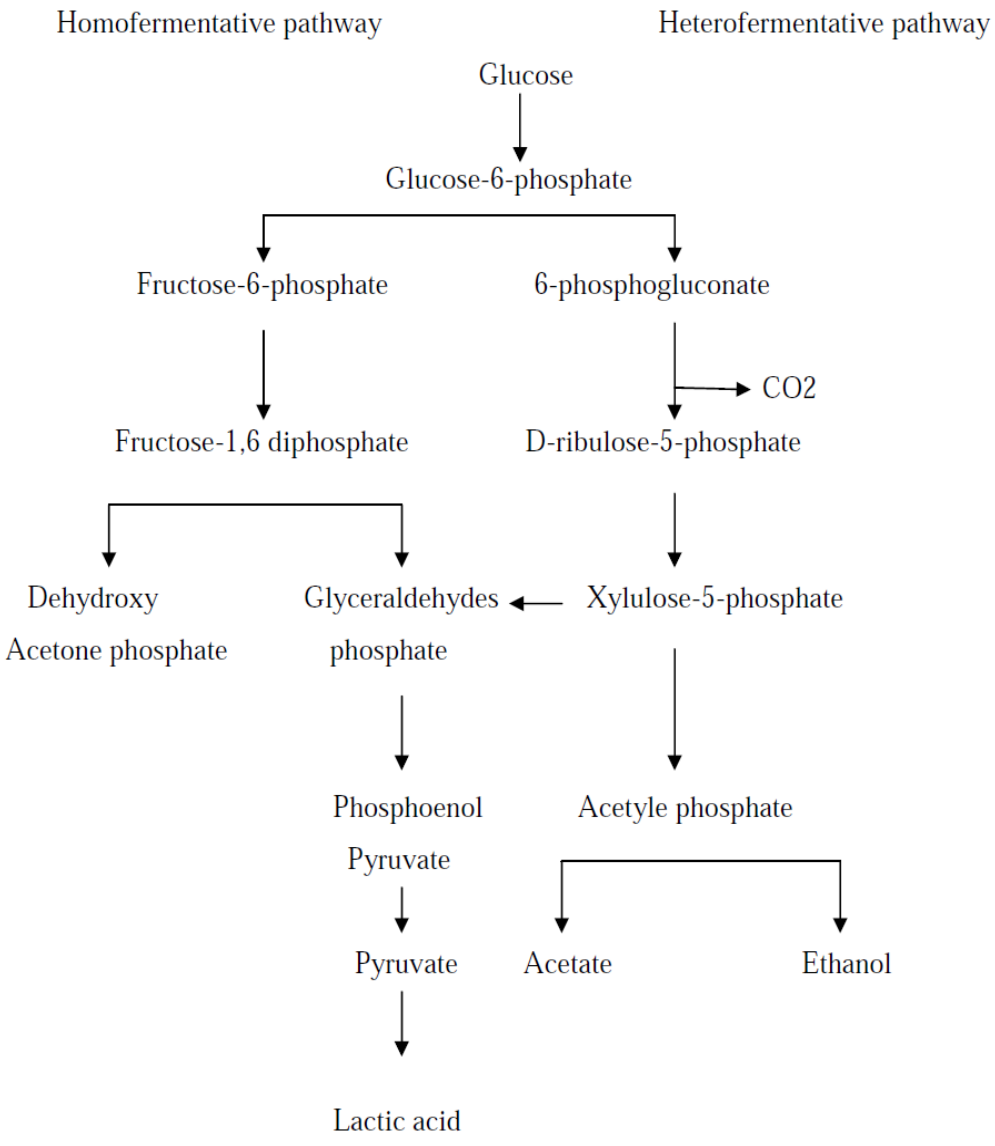

Figure 2.1. Two different pathways for glucose fermentation (Bulut, 2003).

Although the phenotypic techniques are one method, it has proven to be useful; there is a general awareness that strains with similar phenotypes do not necessarily have closely related genotypes. Wild type strains isolated from natural habitats show phenotypic variability and are repeatedly classified (Milliere *et al*., 1996).

Genotypic techniques have diverse levels of discrimination, from species level to individual strain level. Most of these techniques are based on Polymerase Chain Reaction (PCR), which are based on DNA marker system. That technique enables the amplification of targeted DNA fragments by the use of designed primers under controlled reaction conditions. The most powerful and most extensively used phylogenetic marker is 16S ribosomal RNA and the genes code for it (Morata *et al*. 1999). The 16S or 23S rDNA sequencing are other useful methods which were particularly used for systematic studies. Obtained sequences are compared with the sequences previously deposited in a database. Stackobrandt and Goobel (1994) reported that strains that are more than 3% divergent in 16S rRNA are nearly always members of different species, as determined by DNA-DNA hybridization studies. While the strains with less than 3% divergency are generally members of the same species (Cohan, 2002).

2.2. Leuconostoc spp.

Leuconostocs were traditionally originated in association with plant matter, fermenting vegetables, milk, dairy products, and wines and meats. The name Leuconostoc means “colourless nostoc” where “nostoc” pertains to an algal genus (Euzeby, 2009). Garvie described the characteristics of the genus *Leuconostoc* in the previous edition (9th) of Bergey’s manual of systematic bacteriology (Garvie, 1986). The cells are non-motile and do not form spores. *Leuconostoc spp*. is considered psychrotrophic mesophiles with optimal growth at 14-30°C. The temperature limits for growth vary among species and strains ranging from 1-10°C to 30-40°C. The ability to grow at chilled temperatures (4°C or below) is particularly characteristic for strains of *L. carnosum, L. gasicomitatum, L. gelidum* and *L. inhae* (Holzapfel *et al*., 2009). Other characteristics are resistance to vancomycin and a lack of larginine dihydrolase and catalase activities. *Leuconostoc* strains, including *Leuconostoc mesenteroides*, occupy a variety of niches including plants and fermented food products (Hemme and Foucaud-Scheunemann, 2004). The *Leuconostoc* was also originally described in 1878, *Leuconostoc* are Gram positive cocci, occurring in pairs and chains, and form D (-)

lactic acid and carbon dioxide from the fermentation of glucose. These microorganisms from small, grey, flat colonies on agar media, the genus is commonly found on the surface of and inside of fruits and vegetables, in dairy products. The genus plays an important role in the fermentation of numerous dairy products, the production of sauerkraut, and various meats. The *Leuconostoc* are heterofermentative, unlike the similar Betabacteria, produce D (-) lactate and are unable to hydrolyze arginine to form ammonia. They have complex nutritional requirements and are considered vancomycin resistant (Carr *et al*., 2002).

Phylogenetically the genus of *Leuconostoc* belonging to the family of *Leuconostocaceae*, phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales. Leuconostocs* (Ln) are associated to *Fructobacillus, Oenococcus* and *Weissella*, and both they are generally known as the "*Leuconostoc* group" of LAB. This genus includes catalase negative, Gram-positive cocci or ovoid cells occurring singly, in pairs or in chains and this genus was found generally in milk, dairy products and other fresh fruits and find commercial application in production of wine cheese and sugar. It is non-pathogenic, and however, a few species of these was measured pathogenic in humans (Martinez-Murcia and Collins, 1990). The group *Leuconostoc* is divided into three distinct lineages: the genus *Leuconostoc sensu stricto*, the *Leuconostoc paramesenteroides* group, and *Leuconostoc oenos* (Martinez-Murcia and Collins, 1990). Many of the recent studies were reported that both genetic and phenotypic tests were lead to several taxonomic of the group Leuconostoc (Collins *et al*., 1993; Dicks *et al*., 1995; Endo and Okada, 2008).

In the dairy industry, *Leuconostoc dextranicum* and *Leuconostoc cremoris* are of vital importance as starter culture. They produced a flavor compound diacetyl in the manufacture of such dairy products as butter, buttermilk, and cheese products (Dessart and Steenson, 1995; Thunell, 1995). *Leuconostoc mesenteroides* has been used in the manufacture of dextrans, which have been used as blood plasma extenders. While in meats, the *Leuconostoc* may cause spoilage, which may be accompanied with the production of dextran slime. Most slime-producing *Leuconostoc* may be differentiated from the meat contaminant, *Lactobacillus viridescens*, by *Leuconostoc* failure to ferment trehalose orhydrolyze arginine. The *Leuconostocs* have caused serious problems in the sugar can industry by the formation of dextran slime. These dextrans

affect not only the clarity of the refined sugar but may affect the appearance, quality, and quantity of manufactured product (Carr *et al*., 2002).

At present, *Leuconostocs* are major and important group of microorganisms in the manufacturing of fermented dairy, vegetable and cereal foods and they contribute to the taste, texture, nutritional value and safety of fermented foods (Hemme and Foucaud-Scheunemann, 2004; Vedamuthu, 1994). When used as dairy starters, *Leuconostocs* are often combined with acid-producing *Lactococcus lactis* strains whereas *Leuconostoc* strains are used due to their role in the formation of the buttery aroma (Vedamuthu, 1994).

2.2.1. Physiology and energy metabolism

Leuconostocs are referred to as nutritionally, revealing their limited biosynthetic capacity and their requirements for multiple growth factors, preformed amino acids, purine and pyrimidine bases and many other nutrients. Sugars are primary energy and carbon sources for *Leuconostoc* spp., and most species are able to utilise a wide variety of mono- and disaccharides (Bjorkroth and Holzapfel, 2006). In contrast to many other LABs, *Leuconostocs* lack Embden-Meyerhof-Parnas pathway, the most common type of glycolysis. Instead, leuconostocs are obligate heterofermentative organisms, which ferment glucose via the phosphoketolase pathway and produce equimolar amounts of lactic acid, CO_2 and ethanol. Under anaerobic conditions, the reduction of acetyl-phosphate (acetyl-P) to ethanol is essential and serves to oxidise the NADH generated during the conversion of hexoses to pentoses (Cogan and Jordan, 1994). When *Leuconostocs* grow on pentoses, the NADH is not produced and acetyl-P is directed to the acetate branch of phosphoketolase pathway yielding acetate and extra ATP. In addition, leuconostocs may utilise alternative routes for NADH reoxidation (Axelsson, 2003; Zaunmuller *et al*., 2006). Many *Leuconostoc* strains also possess NADH oxidases which take over NAD+ regeneration in the presence16 of oxygen (Lucey and Condon, 1986). Furthermore, *Leuconostoc* use pyruvate, fructose or citrate as electron acceptors and subsequently generate acetate instead of ethanol (Erten, 1998; Zaunmuller *et al*., 2006). This so-called cofermentation or co-metabolism of multiple carbon sources and growth in the presence of oxygen generates more ATP from glucose and leads to an increase in growth yield and specific growth rates (Borch and Molin, 1989; Cogan and Jordan, 1994; Lucey and Condon, 1986; Zaunmuller *et al*., 2006).

Other characteristic metabolic features of *Leuconostoc* species include production of D (-) lactate enantiomer, fermentation of fructose to mannitol and acetate, and co-metabolism of citrate and carbohydrate under reducing conditions to diacetyl (2,3-butanedione), CO2 and acetoin (3-hydroxy-2- butanone) (Cogan and Jordan, 1994).

2.2.2. Importance of *Leuconostoc*

Members of *Leuconstoc spp*. are frequently used in production of fermented foods because of their ability to produce lactic acid and diacetyl, and in generally that is considered as spoilage bacteria. Because they are heterofermentative abilities, they produce carbon dioxide which effects and changes the texture of fermented foods. However, certain strains produce diacetyl and acetoin from citrate and contribute to the typical flavour and taste of many food and especially dairy products (Sandine and Elliker, 1970; Collins and Speckman, 1974; Garvie, 1984; Sozzi and Pirovano, 1993).

The leuconostocs characterize a small percentage of the mesophilic microflora, which is mostly composed of homofermentative *Lactobacilli, LLactococci* and *Pediococci*. However, in a fermentation broth including antibiotics, antibacterial substances, or bacteriophages, the leuconostocs to be the main lactic acid produce bacteria. Exclusion of the leuconostocs often causes a reduction in flavour and aroma, and a common lowering in the typical characteristics of the product. It is therefore important to study the development contacts between *Leuconostoc* and the other lactic acid bacteria, the volatile compounds they produce, and their resistance to bacteriophages (Sozzi and Pirovano, 1993).

At present most of the research programmers on *Leuconostoc* spp. are concerted on the organism's antagonistic action and ability to advance the organoleptic properties in the following main field's fermented dairy products, in other foods, vegetables, and wine production.

2.2.3. *Leuconostoc* in fermented other products

Leuconostoc is considered to be useful microorganisms to society, are involved in the manufacture of thousands of fermented foods. The positive effects of leuconostocs on dairy products were recognized already in the early 20^{th} century. *Leuconostoc mesenteroides* subsp. *cremoris* and *Leuc. lactis* change citrate to diacetyl and acetoin at

low pH by using an inducible citrate lyase (Cogan *et al.,* 1981). The active metabolism of citrate and lactose, resistance to antibiotics, and growth interactions with other microorganisms are important criteria in the selection of a strain when incorporated in a dairy starter culture. The organoleptic quality, consistency, texture, and eye formation of the following cheeses improved when selected strains of *Leuc. mesenteroides* and/or *Leuc. lactis* were included in the starter cultures: Manchego (Barneto and Ordonez, 1979; Ramos *et al.*, 1981), Danbo (Birkkjaer and Thompsen, 1981). Minas (Bonassi *et al.,* 1983), Sovetskii (Gudkov *et al.,* 1980), Fynbo (Barraquio *et al.,* 1983), Gouda (Toyoda and Kikuchi, 1983), Kefalotyri (Litopolou-Tzenetaki, 1990). water-buffalo Mozzarella (Coppola *et al.,* 1990), and Aracena (Garrido-Gomez *et al.,* 1991).

As with all the other *leuconostocs,* which play an important role in a large number of various traditional food fermentations, the starter cultures used in milk fermentations can be attacked by bacteriophages, causing a loss of specific aromatic properties. The acidification time of the fermented milk is, however, not necessarily influenced (Sozzi and Pirovano, 1993). *Leuconostoc* phages were isolated from Hungarian semi-hard cheese (Babella and Mike, 1977). The phages isolated from cheese and butter dairy factories in Columbia had isometric heads and long non-contractile flexible tails and were identified as members of the *Siphoviridae* group of viruses (Sozzi and Pirovano, 1993).

Leuconostoc spp connected with *lactobacilli* is considered very important in the fermentation of dough. Azar *et al.* (1977) reported that the *Leuconostocs* produce the desirable flavour compounds and sour taste in sangak dough. However, in additional study is needed to determine the correct composition of these leuconostocs bacteria in the complex mixture of microorganisms. Several papers have been published on the spoilage of meat by lactic acid bacteria. *Leuconostoc* spp. was isolated from spoiled space packed beef (Roxbeth *et al.,* 1991), dry sausages and raw hams (Hechelmann, 1986), and raw ripened sausage (Schillinger *et al*., 1989).

2.3. Pediococcuss spp.

The genus *Pediococcus* was first described by wochnschre F. Balcke in 1884. *Pediococci* are coccoidal or ovoid, Gram-positive, homofermentative non-motile, catalase-negative, facultative anaerobes of the family *Streptococcaceae* (Facklam *et al.,* 1989; Facklam and Elliot, 1995; Ruoff, 1995; Fugelsang and Edwards, 2007). The cells

are usually found in pairs or tetrads though single cells are also common. Chain formation has not been found. DL-Lactic acid is formed from the metabolism of glucose via the Embden-Meyerhof pathway while carbon dioxide and ethanol are not produced. The genus contains eight species though only a few have been observed in wine (Dicks and Endo, 2009).

The *Pediococci* are found commonly associated with various plants and their products such as cabbage and saukeraut, cucumbers and pickles, grapes and wine and wort and grain mashes. *Pediococcus damnosus* and *Pediococcus acidilactici* are useful in the manufacture of fermented sausage, the fermentation of Lebanon bologna (summer sausage), bacon, as well as pickles and olives. The Pediococci also play a prominent role in marinated products, particularly in marinated herring. This genus is of economic importance as a contaminant in beer, where it may be responsible for a cloudy, acid-tasting beer due to the production of diacety (sarcinasick) beer. It may also be a problem in the pickle industry where they can cause swelling of cucumbers. The hop- tolerant species (resistance to humulone and colupulone) formerly referred as *Pediococcus cerevisiae* has been reclassified as *Pediococcus damnosus*. The *Pediococcus*, unlike the other lactics, have a tetrad arrangement. The *Pediococcus* growth on MRS media and growth may be enhanced, as with the *Leuconosto*c, by culturing them under reduced atmospheric condition. They are particularly useful as starter culture for various types of sausages; however, in the pickle industry and in the wine and beer industry they are unwanted contaminants when they produce off flavors (Carr *et al*., 2002).

The *pediococci* are saprophytes often found in fermenting vegetable material, many of these organisms that are commonly used in the industrial fermentation of vegetables and meat that they are involved in a variety of food fermentations, i.e., (Franklin *et al.,* 1963) and sausage products (Smith *et al*., 1981). Processes involving the use of *Pediococci* as starter cultures have been developed for some of these products. The ecologic niche of *pediococci* in humans appears to be the enteral tract (Ruoff, 1995).

Genus *Pediococcus*, out of seven species only two of these species as *Pediococcus acidilactici* and *Pediococcus pentosaceus* (Golledge *et al*., 1990; Facklam and Elliot, 1995) are mostly studied. *Pediococci* appear on Gram's stains to

be arranged in tetrads (Facklam *et al*., 1989; Facklam and Elliot, 1995; Ruoff, 1995) and group and are commonly resistant to vancomycin and teicoplanin (Facklam *et al.*, 1989; Riebel and Washinton, 1990; Facklam and Elliot, 1995; Ruoff, 1995). Although this genus was described as harmless bacteria, it was rarely recovered from human respiratory region and spit and also from other clinical specimens (Facklam *et al*., 1989; Golledge *et al*., 1990; Facklam and Elliot, 1995; Ruoff, 1995).

In addition they also have an ability to produce bacteriocin. Bacteriocins produced by *Pediococcus spp.*, designated as pediocins, are useful against several gram positive pathogenic and food spoilage bacteria including *Listeria monocytogenes* (Tagg *et al*., 1976; Daeschel and Klaenhammer, 1985; Bhunia *et al*., 1988; Pucci *et al.*, 1988).

2.3.1. Morphology of *Pediococci*

In a single culture, the cells of *Pediococci* are spherical and of uniform size, 0.36-1.43 ~m in diameter (Gunther and White, 1961). They are never elongated. Division occurs alternately in two planes at right angles, the type of division of *Pediococci* were the subject of dispute stated that the cells divided in two perpendicular directions in a single plane is a distinctive characteristic of *Pediococci*, and in recognition of this, derived the name *Pediococcus* from two Greek nouns: *pedium*, meaning a plane surface, and *coccus*, meaning a berry (Balcke, 1884). Later descriptions of tetrad-forming cocci described them as dividing in two planes, rather than in one, as Balcke had suggested (Gunther, 1959; Herrmann, 1965; Shimwell, 1949) reported that tetrads were more noticeable than chains of cells, and this may have explained the preoccupation of bacteriologists with such morphological features. *Pediococci* are sphere-shaped, so it is not possible for them to divide in more than one plane while undergoing only two cell divisions (Simpson, 1994).

The genus *Pediococcus* is genetically heterogeneous. Mol% G+C values of *Pediococcus* spp., determined by various methods, lie in the range 34-44 (Sakaguchi and Mori, 1969; Kocur *et al*., 1971; Solberg and Clausen, 1973; Back, 1978). In *P. pentosaceus*, the ability to ferment raffinose, melibiose and sucrose is associated with three different plasmids. Sucrose hydrolase and a-galactosidase activities are connected with plasmid encoded raffinose utilization (Gonzalez and Kunka, 1986).

Lactose fermentation in some strains of *P. pentosaceus,* and sucrose fermentation in some strains of *P. acidilactici,* may be plasmid-linked (Hoover *et al*., 1988; Kayahara *et al*., 1989) found that 92 of 160 strains of *P. halophilus,* isolated from miso and soya sauce factories, harboured plasmids. In *P. acidilactici,* erythromycin resistance is coded for by a 40 MDa plasmid (Torriani *et al*., 1987). *Pediococci* can be transformed by electroporation or conjugation (Kimet *et al*., 1992). Plasmids can be transferred from genera such as *Enterococcus, Streptococcus* and *Lactococcus to Pediococcus* spp. and vice versa (Gonzalez and Kunka, 1983).

2.3.2. Pediococci in dairy products and foods

Dacre, (1958a) was the first in identifying *Pediococci* in cheese. Starter cultures of *Pediococcus* spp. have since been employed in cheese production (Dacre, 1958b; Bhowmik and Marth, 1990). *Pediococci* characterize only a small amount of the total lactic acid bacteria in cheese (Litopolou-Tzanetaki *et al.,* 1989), their precise influence on cheese quality (Bhowmik and Marth, 1990; Fox *et al.,* 1990; Olson, 1990).

Pediococci are basic starter cultures with widespread use in dairy industry, consisting of a selected strain of *P. acidilactici,* were used in the preparation of semi-dry sausages. *Pediococcus pentosaceus* can be used instead of *P. acidilactic,* and it was better suited to dry sausage fermentation as it has a lower optimal growth temperature and a lower minimum temperature for fermentation (Raccach, 1987). *Pediococci* was used to care for other types of sausage from pathogens, such as *Listeria monocytogenes,* since they produce bacteriocins (Berry *et al.,* 1991; Yousef *et al.,* 1991; Foegeding *et aI.,* 1992; Luchansky *et al.,* 1992). Commercial starter cultures of *P. acidilactici* have been used to prevent meat spoilage (Gibbs, 1987). *Pediococci* were offered in different fermented foods counting buroung dalag marinated herrings (Blood, 1975) fermented shrimps and a wide range of Thai fermented foods (Tanasupawat and Daengsubha, 1983).

Some *Pediococci* produce off-flavours in wine, caused by diacetyl and acetoin (Pilone and Kunkee, 1965). Others spoil wine by producing rope, a D-glucan consisting of a trisaccharide repeating unit of D-glucose, which increases the viscosity of the wine to such an extent as to make it unpalatable (Liauberes *et aI*., 1990). Wines with higher than average pH values are more susceptible to growth of *Pediococci* (Edwards and Jensen, 1992).

2.4. Antibiotic resistance in lactic acid bacteria

An antibiotic is a substance produced by bacteria, fungi and other organisms that can destroy or inhibit the growth of other microorganisms (Levy, 1992). This resulting was extensively used in the prevention and handling of infectious diseases. The term antibiotic is a term that refers to a drug which is cures disease caused by bacteria, while term an antimicrobial agent is a term that refers to a group of drugs that contains antibiotics, antifungals, antiprotozoals, and antivirals. By (Fleming, 1929) reported that, the first antibiotic, penicillin, was discovered when he observed that a common mold (Penicillum) produced a substance that lysed colonies of Staphylococcus spp. After this discovering, in the following decades, many new antibiotics were discovered, including streptomycin, chloramphenicol, and tetracycline (Levy, 1992).

Lactobacilli, Pediococci and *Leuconostoc spp* was reported to have a maximum natural resistance to vancomycin, a property that is positive to separate them from other Gram-positive bacteria (Hamilton- Miller and Shah, 1998; Simpson *et al*., 1988). Some lactobacilli also have a maximum natural resistance to bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, gentamicin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin trimethoprim/ sulphamethoxazole, and vancomycin (Danielsen and Wind, 2002).

There have been few systematic studies to investigate acquired antibiotic resistance in LAB from different food (Jett *et al*., 1994; Moellering, 1998). The antibiotic resistances reported that in the other food-associated LAB which can be summarized by stating that only a incomplete number of papers reported the occurrence of antibiotic resistance in mostly *Lactobacillus* spp. isolated from raw meat and fermented food products. In another hand few studies was reported that generally susceptibility to antimicrobial in strains used as meat starter cultures (Raccach *et al*., 1985; Holley and Blaszyk, 1997) or dairy starter cultures (Katla *et al*., 2001; Reinbold and Reddy, 1974).

2.5. Antimicrobial activity and agents

The antibacterial activity of lactic acid bacteria (LAB) isolated from dairy products, these antimicrobial compounds were used in fermentations to preserve the nutritive qualities of various foods (Tagg *et al*., 1976). The antimicrobial action of starter cultures bacteria was attributed to the production of metabolites such as organic

acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl and acetaldehyde (Vanderbergh, 1993).

Antimicrobial agents characterize a major therapeutic tool to manage, control and care for a diversity of bacterial infectious diseases. The first antimicrobial compounds have been used in modern medicine were created and isolated from living organisms such the penicillins from fungi of the genus Penicillium, or streptomycin produced by bacteria of the genus Streptomyces. Antimicrobial agents can be divided as either bactericidal or bacteriostatic. Bactericidals kill bacteria directly while bacteriostatics prevent them from dividing. However, both are able of ending a bacterial infection (Hancock, 2005).

Anti microbial resistance develops through a number of different mechanisms are first changes in the permeability of the bacterial cell wall, second, active efflux of the anti microbial out the cell; third, mutation in the target site; fourth, modification of the anti microbial agent; and final acquisition of alternative metabolic pathways to those inhibited by the antimicrobial agent (Table 2.1).

Table 2.1. Mechanisms of antimicrobial resistance (McDermott *et al*., 2003).

	Mechanisms of resistance	**Antimicrobial (s) affected**
1	Modification of the antimicrobial agent	Aminoglycosides, chloramphenicol, and ß-lactams.
2	Alteration or protection of the target site	Aminoglycosides, ß-lactams, macrolides, quinolones, rifampicin, trimethoprim, and tetracycline.
3	Decreased antibiotic accumulation • Decreased uptake • Increased efflux	Many antibiotics. Tetracycline, macrolides, quinolones,and chloramphenicol.
4	Alteration of the metabolic pathway	Sulfonamides, trimethoprim.

Intrinsic resistance was describing as a natural phenomenon; it was depend on a function of the physiological or biochemical structure of all members of a species (Harbottle *et al.,* 2006). Acquired resistance is not present in the whole species but within only certain pedigree of bacteria derived from a susceptible parent.

As *Pediococci* are used as useful microbes in food microbiology and animal husbandry (e.g., wine, cheese, yogurt and for the production of silage), *Pediococcus* isolates in the ferment industry. These isolates frequently harbour one or more ABC MDR genes, suggesting that resistance to hop-compounds may also confer resistance to other antimicrobial compounds（Sami *et al*., 1997). The enriched media that are commonly used to allow increase of *Pediococci* might be inhibitory to some of the antimicrobial compounds. The usage of antimicrobial compounds by some industries to combat *Pediococcus* contaminants (e.g., hop-compounds, Penicillin, Virginiamycin) is longstanding, yet knowledge about the resistance of pediococci to antimicrobial agents is minimal (Ammor *et al*., 2007).

2.6. Bacteriocins

Bacteriocins were first discovered by Gratia in 1925. He was searched the process for ways to kill bacteria, which also resulted in the development of antibiotics and the discovery of bacteriophage. He called his first discovery a colicin because it killed *E. coli.* Bacteriocins are family of antimicrobial peptides. These substances have increasing interest. Their proteinaceous nature implies their putative degradation in the gastro-intestinal tract of man and animals (Atrih *et al*., 2001).

Bacteriocins are of interest in medicine because they are made by non pathogenic bacteria that normally colonize the human body. Most bacteriocins act on responsive cells by destabilization and permeabilization of the cytoplasmic membrane during the formation of passing poration complexes or dissipation of the proton motive force (Cintas *et al*., 1998; Herranz *et al*., 1999; Luders *et al*., 2003). All bacteriocin producing strains require themselves to contain protection mechanisms in order to avoid cell death. The protein is generally encoded in the bacteriocin operon (Cintas *et al*., 2001; McAuliffe *et al*., 2000).

Bacteriocins are ribosomally synthesized anitimicrobial compounds that are produced by different bacterial species, including member of the LAB (Garneau *et al*., 2002). A number of bacteriocins shaped by LAB inhibit not only closely related species such as Gram-positive spoilage microorganisms including Bacillus sp. and *Enterococcus faecalis* but also effective against food-borne pathogens such as *Listeria*

monocytogenes, Clostridium botulinum, Staphylococcus aureus (Delves-Broughton, 1990).

Bacteriocins of LAB are a heterogenous group of bacterial antagonist ranging in molecular size from a few thousand Daltons to complex protein structures that possibly contain carbohydrate or lipid moieties was reported by (Van Belkum and Stiles, 2000).

It was defined that the major peptides of bacteriocins usually consisting of 20-60 amino acids (Eijsink *et al*., 2002). There is huge difference among the peptides, e.g. in terms of length, amino acid sequence and composition, secretion and processing machinery, post-translational modifications, and antimicrobial activity. Almost all bacteriocins have a positive charge at neutral or slightly acidic pH and they usually contain stretches of sequence that are hydrophobic and/or amphiphilic.

A number of LABs produce bacteriocins, antibacterial proteinaceous substances with bactericidal activity against connected species or across genera (Rogelj, 1994; Cotter, 2005). In fermented foods, lactic acid bacteria present many antimicrobial activities and a wide variety of antimicrobial substances including organic acids, hydrogen peroxide and diacetyl. Bacteriocins of LAB have extensive interest since they have potential marketable value of these antimicrobials in food preservation and other biological applications (Devuyst and vandamme, 1994, Ross *et al*., 1999). Bacteriocin biosynthesis has characteristic for strain selection as it provides an important mechanism of pathogen keeping out in fermented foods as well as in the gastrointestinal environment (Klaenhammer, 1993)

2.6.1. Classification of bacteriocins

Bacteriocins are commonly divided into four groups (Ennahar *et al*., 2000; Oscáriz and Pisabarro, 2001). They are I) Lantibiotics; II) small hydrophobic heat-stable peptides (< 13,000 Da); III) large heat-labile proteins and IV) carbohydrate (Ouwehand, 1998).

Class I bacteriocins termed lantibiotics, are small, consist of post-translationally modified peptides (Van Belkum and Stiles, 2000; O'Sullivan *et al*., 2002) and peptides is containing unusual amino acids as lanthionine, β-methyllanthine (MeLan) and a number of dehydrated amino acids (McAuliffe *et al*., 2001) as shown in Table 2.2

Table 2.2. Antibiotics produced by LAB (Ouwehand, 1998; McAuliffe *et al*., 2001)

Bacteriocin	**Producer strain**
Nisin A.	*L. lactis* NIZOR5, 6F3,NCFB894, ATCC11454
Nisin Z.	*L. lactis* N8, NIZO22186
Lactocin S	*Lb. sake* 145
Lactococcin	*Lb. lactis* ADRI85L030
Lacticin 481	L. lactis CNRZ481, ADRIA85LO30
Cytolysin	*E. faecalis* DS16
Lacticin3147	*L. lactis* DPC3147
Salvaricin A	*St. salvarius* 20P3
Streptococcin A-FF2	*St. pyrogens* FF22
Carnocin U149	*Carnobacterium pisicola*
Variacin 8	*Micrococcus varians* MCV8

Class II bacteriocins of lactic acid bacteria (LAB) was appeared in recent years as the mainly promising bacteriocin candidate for food protection, since they was shown to display overall better act, bacteriocins are ribosomally synthesized as in active prepeptides that are revised by post translational division of the N-terminal leader peptide commonly at a double glycine (-2, -1) (Van Belkum and Stiles, 2000).

Class II bacteriocins include a huge group of heat-stable original peptide bacteriocins (O'Sullivan *et al*., 2002) with molecular masses lesser than 10 kDa (Oscáriz and Pisabarro, 2001). This class which is subdivided; first, group IIa are consists listeria-active peptides that conserved N-terminal sequence Try-Gly-Asn-Gly-Val and two cysteines forming an S-S (disulfide bridges) in their N-terminal half of the peptide. In addation, second group are includes of pore-forming complexes requiring two petides for their activity. And third group are consists thiol-activated peptides, which involve reduced cysteine residues for activity (Carolissen-Mackay *et al*., 1997; Cleveland *et al*., 2001; Oscáriz and Pisabarro, 2001; O'Sullivan *et al*., 2002).

Class III includes of bacteriocins that are heat-labile proteins with have a large molecular mass (>30kDa), (Van Belkum and Stiles, 2000; Oscáriz and Pisabarro, 2001). They are usually inactivated within 30 minutes by temperature of 100 °C or less (Dodd and Gasson, 1994). Most of them are shaped by bacteria of the genus Lactobacillus (Ouwehand, 1998; Oscáriz and Pisabarro, 2001).

Class IV bacteriocins includes compound of bacteriocins (Ouwehand, 1998). Lipoid or carbohydrate moieties show to be essential for action (Van Belkum and Stiles, 2000). This group produced by lactic acid bacteria as Lactocin 27; *Lb helveticus*, *Leuconocin* S; *Leuconostoc paramesenteroides* Pediocin, SJ-1; *Pediococcus acidilactici* (Ouwehand, 1998).

2.6.2. Characterization of bacteriocins

LAB produced the most of the bacteriocins that are consists cationic, hydrophobic, or amphiphilic molecules composed of 20 to 60 amino acid residues (Chen and Hoover, 2003). Bacteriocins that have small-size are active more than a wide pH range (3.0-9.0), and while resistance to great pH values of 1.0 (acidocin B) and 11.0 (bavaricin A) was observed, most of these bacteriocins are cationic at pH 7.0, lactocin, with a net charge of -1 at neutral pH, being the exception. Their high isoelectric point allows them to interact at physiological pH values with the anionic surface of bacterial membranes (Eguchi *et al*., 2001).

A sensitive detection method for bacteriocins can be functional to track purification procedures, to identify bacteriocin production in test involving genetic manipulation, and detect bacteriocins in food (Rose *et al*., 1999). MALDI-TOF MS is effective for peptides and proteins with molecular masses ranging from 0.5-30 kDa and it was used to determine the masses of purified class I and II bacteriocins (Hindre *et al*., 2003). In addition, molecular weight of SA-FF22 bacteriocin-like substance was analyzed by ESI-MS (Jack *et al*., 1994). The last most important data required for complete characterization are their amino acid sequence determined by Edman degradation using automated protein sequencer (Jack *et al*., 1996; Meyer *et al*., 1994). However, (Walk *et al*., 1999) was reported that a number of problems might occur during automated protein sequencing of compounds containing nonproteinogenic amino acids. In some cases, the unusual amino acids effectively ‘block’ the further determination of sequential information.

2.6.3. Mechanism of action of bacteriocins

Bacteriocins have an effect on diverse important purposes of the living cell (transcription, translation, replication, and cell wall biosynthesis), although a large amount of them act by pores that destroy the energy potential of sensitive cells this due

to the large variety of their chemical structures (Oscáriz and Pisabarro, 2001). It was established that the primary target for many of these small, cationic peptides was the cytoplasmic membrane of sensitive cells, where they act to dissipate the proton motive force (PMF) through the formation of discrete pores in the cytoplasmic membrane, and thus deprive cells of an essential energy source (Montville and Bruno, 1994; McAuliffe *et al*., 2001). The PMF is composed of a chemical component (the pH gradient: ΔpH) and an electrical component, drive ATP synthesis and the accumulation of ions and other metabolites through PMF-driven transport systems in the membrane. Collapse of the PMF, induced by bacteriocin action, leads to cell death through cessation of energy requiring reactions (Ennahar *et al*., 2000; McAuliffe *et al*., 2001; Chen and Hoover, 2003).This mechanism is described in Figure 2.2.

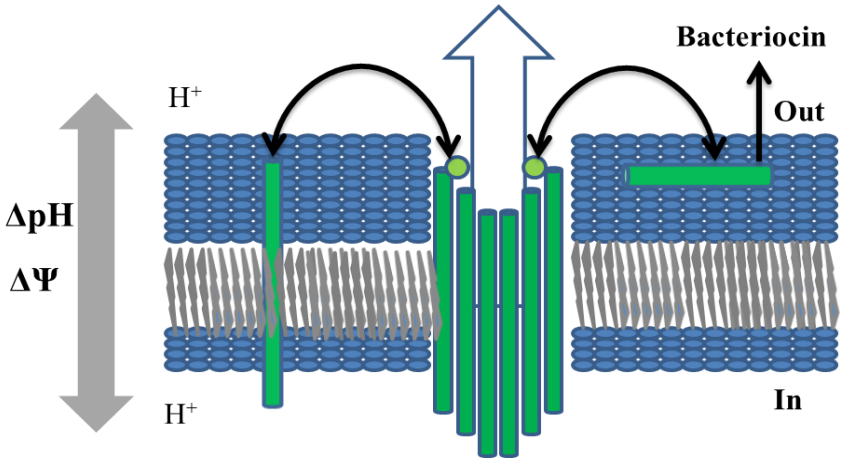

Figure 2.2. General models for mechanism of pore formation by bacteriocins Source: (McAullife *et al*., 2001).

2.7. Proteinases of Lactic acid bacteria

The Proteolytic activity exerted by most LABs is not only a prerequisite for growth, but also affects product texture and flavour, especially in dairy products. Proteolytic activity encompasses proteinases that degrade proteins, such as caseins, into relatively large number of different oligopeptides and peptidases that break down protein fragments into small peptides and free amino acids (Schmidt, 1982). Cell wall-associated LAB proteinases are responsible for this main proteolytic action and function with transport systems (Doeven *et al*., 2005). Casein includes all of the amino acids required for cell growth and certain required portions of casein proteins are hydrolyzed by PrtP into oligopeptides to meet the nutritional requirements of LAB (Kunji *et al*.,

1996). The extracellular location of PrtP was established. The proteinase can be liberated from the cell-wall with Ca2+-free buffers (Tsakalidou *et al*., 1999) or lysozyme (Fernandez-Espla *et al*., 2000).

Extracellular proteinases are secreted as "free" enzymes outside the cell. The same organism also produces an intracellular proteinase with some properties in common with the supposed extracellular enzyme. Actively growing or metabolizing cells release a constant amount of proteinase into the medium, irrespective of its Ca^{+2} content. Only the enzyme in the cell wall is affected by the Ca^{+2} (Abhijit, 2013). Intracellular proteinases are also equally important in the turnover of denatured or defective proteins, the activation of zymogens, and the termination of newly synthesized proteins *Lactobacilli* generally produce neutral proteinases active on α-, β- and κ-casein but the intensity of their activity is extremely variable from strain to strain. β-casein is the most preferred substrate (Abhijit, 2013). Many studies were shown that degradation of caseins starts with a single cell envelops proteinases (CEP) (Smid *et al*., 1991; Tan *et al*., 1993, Pritchard and Coolbear, 1993; Kok and Vos, 1994). In general, the proteinase is a monomeric serine proteinase with a molecular mass between 180-190 kDa (Laan and Konings, 1989).

In general, LAB proteinases are classified into two groups based on their specificity of the casein degradation (Pritchard and Coolbear, 1993). The first type is PI tproteinase degrades β-casein (β-CN) readily with slight tendency to act upon on αs1-CN and κ-CN. The second type is PIII proteinase also cleaves β-casein, but at diverse sites from the first type and shows a greater tendency towards κ-CN and αs1-CN (Law and Haandrikman, 1997; Stepaniak, 2004). The second is the amino acid residues at 747-748 positions. The β-CN cleavage site of PI-type is characterized by glutamine and serine residues and this region was having low charge, high hydrophobicity, and high proline content, PIII-type PrtP cleaves β-CN at GL-X-X or X-GL-X peptide bonds, where X is in the main a hydrophobic residue such as methionine, phenylanine, leucine or tyrocine (Tan *et al*., 1993). They have two regions in the PrtP contribute to the differentiation in substrate specificity of these two types PrtP, the first region is around the centre of the active site and is homologous with substrate-binding site of subtilising (Kunji *et al*., 1996).

Nutritionally LAB require a complex and rich medium (Axelsson, 1998). They form lactic acid as a most important end product of their carbohydrate metabolism; prevented the increase of other microbes that have lower pH in the external environment. Proteolytically generated oligopeptides are transported into the cell and undergo further hydrolysis to provide the building blocks and nitrogen (Axelsson, 1998; Lehninger, 1982). The proteolytic system of LAB is contained of three distinctive parts: first, extracellular serine-proteinases, second transport systems, and finally intracellular peptidases. They are systematically prepared to make protein utilization through peptide formation by proteinases, delivery by the transport systems, and hydrolysis by peptidases.

Proteinases from dairy LAB are main factor in determining the nature of the degradation products generated from milk proteins. These products conclude the increase of lactic acid bacteria in milk and in fermented milk products. In addition, this specificity was developed in to a functional tool to classify lactococcal proteinases. Therefore, extensive attention was focused in recent years on the substrate specificity of the *L. lactis* proteinases. While only a limited number of substrates are degraded by these proteinases and only newly synthetic chromogenic substrates was created that are hydrolyzed (Exterkate, 1990). For classification of proteinases, a various attempts was made to classify proteinases. The Polyclonal and monoclonal antibodies were used to distinguish lactococcal proteinases and provided a classification that was based on caseinolytic specificity (Hugenholtz *et al.*, 1984; Laan *et al.*, 1988). Therefore this classification was based on substrate specificity provides a reliable way to differentiate the diverse lactococcal proteinases (Visser *et al.*, 1983). These ways have been extended that is based on PCR-amplification of regions determining substrate specificity followed by sequence analysis of relevant regions (Bruinenberg and de Vos, 1992).

2.8. Plasmids

Lederberg was the first one described plasmids in 1952, who used the term to describe extra chromosomal hereditary determinants (Lederberg, 1952). They were reported in an extensive range of host, counting prokaryotes, archaea and eukaryotic fungi (Erauso *et al*., 1996; Griffiths, 1995).

Plasmids are extra-chromosomal DNA molecules that replicate independently of the chromosome (Baron *et al*., 1996). Typically they are circular molecules of double stranded DNA, although some bacteria have linear plasmids (Casjens *et al*., 2000). There may be one copy, generally for large plasmids to hundreds of copies of the plasmid in a single cell, and cells can harbor more than one type of plasmids. Plasmids vary in size from about five to more than several hundred kilobase pairs and they generally do not encode functions essential to bacterial growth, such as RNA polymerase, ribosomal subunits, or enzymes of the tricarboxylic acid cycle (Baron *et al*., 1996). Plasmids are inherited independently from the bacterial chromosome, but some do rely on proteins encoded by the host for their replication and transcription. While plasmids are not normally essential for the growth of bacteria, specific phenotypes such as (i) hydrolysis of proteins, (ii) metabolism of carbohydrates, amino acids and citrate, (iii) production of bacteriocins, exopolysaccharides, pigments and (iv) resistance to antibiotics, heavy metals and phages have been found to be plasmid-encoded (Wang and Lee, 1997).

The plasmid of LAB offers new possibility for differentiating between strains that is isolated before (Sewaki *et al*., 2001). Many cryptic plasmids from lactic acid bacteria have been isolated, characterized and used to construct cloning vectors (Shareck *et al*., 2004). Plasmids may present a selective benefit under certain conditions, as enzymes for the utilization of unusual carbon sources resistance to substances such as heavy metals and antibiotics (Williams and Murray, 1974; Zhang *et al*., 2011; Silver and Misra, 1988; Schwarz *et al*., 2001). and synthesis of toxins and other proteins that allow the successful infections of higher organisms (Sengupta and Austin, 2011). Plasmids depend on having an origin of DNA synthesis of its own (oriV). In addition, mainly plasmids encode for specific replication initiator proteins that bind to this oriV (Scott, 1984; dei Solar *et al*., 1998; Gerdes *et al*., 2000).

Plasmids play significant role in biotechnology of *Enterococci* today most recent study associate plasmids with cloning vectors and tools for genetic manipulation. They represent an immense reservoir of genetic variability and contribute to genetic exchange between bacteria (Norman *et al*., 2009) The ability to discover and classify plasmids based on their phylogenetic relationship was provide an essential tool for investigating their distribution among bacteria and their importance in the host cell, such as their role in dissemination of antimicrobial resistance. The new PCR based plasmid classification

system for Gram positive bacteria, targeting replication initiation genes, can be of great value in the detection and identification of enterococcal plasmids (Lynch *et al*., 1997). Isolated plasmids were classified depend on physical and phenotypic characteristics, or by incompatibility testing, replicon typing and complete plasmid sequencing (Smalla *et al*., 2000).

In addition, the classification system is based on DNA and protein homology of replication initiation genes. In addition to multiple rep genes in one plasmid, for detecting fewer plasmids with S1 nuclease assays compared to rep typing. When the classification system has been developed, the number of plasmids analyzed in each rep family was limited and primer sequences were in general targeting the supposed most conserved region. Recently detected plasmids that were not analyzed in the classification scheme may in theory be detected by the PCR primers but differ so much in the region between the primers that the similarity threshold is below hybridization stringency.

Plasmids can be physically present in almost all bacterial species, and plasmids are naturally present in LAB, and many was isolated and characterized (Shareck *et al*., 2004). Two modes of replication are commonly found among LAB plasmids, rolling-circle (RC) and theta. RC plasmids encode a site-specific nuclease that nicks double-stranded DNA at its target site (DSO), initiating the synthesis of the leading strand of DNA (dei Solar *et al*., 1998).

Size, function and distribution that found on plasmids are generally different, (Davidson *et al*., 1996; Wang and Lee, 1997). The functions found on plasmids contain hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins and exopolysaccharides, and resistance to antibiotics, important metals and phages. At least 25 species of lactobacilli contain native plasmids (Wang and Lee, 1997). and frequently appear to include multiple (from 1 to 16) diverse plasmids in a single strain. R-plasmids encoding tetracycline, erythromycin, chloramphenicol, or macrolide-lincomycin-streptogramin resistance have been reported in *Lb. reuteri* (Vescovo *et al*., 1982; Axelsson *et al*., 1988; Lin *et al*., 1996; Tannock *et al*., 1994). *Lb. fermentum* (Ishiwa and Iwata, 1980; Fons *et al*., 1997), *Lb. acidophilus* (Vescovo *et al*., 1982), and *Lb. plantarum* (Ahn *et al*., 1992; Danielsen, 2002) isolated from raw meat, silage and faeces. Most of these R-plasmids had a size smaller than 10 kb (5.7 – 18 kb).

The reported prevalence of antibiotic resistance genes such as erythromycin, vancomycin, tetracycline, chloramphenicol, and gentamicin resistance genes, on transferable genetic elements in enterococci is more extensive, both on plasmids (Christie *et al*., 1987; Rice *et al*., 1998; West and Warner, 1985; Clewell *et al*., 1974; Murray *et al*., 1988) and transposons (Perreten *et al*., 1997; Clewell *et al*., 1995).

2.9. Objective of thesis

In this study, isolation and identification of new strains of LAB which are important for industrial application and for probiotic discovery from home-made chesses and sausage were aimed. Especialally isolation and identification of some types of bacteria like *Leuconostoc* and *Pediococci* from natural fermented milk samples and meat by molecular analysis were targeted. Searching plasmid conted and plasmid related resistancy to some antibiotics, proteinase activity and antimicrobial properties were also studied.

3. MATERIALS AND METHODS

3.1. Tools and Equipment

Analytical balance (Vibra); incubator (Nüve); Pipette (Biohit); Microscope (Olympus); Microscope slides (Sailing Boat); Beaker (Iso Lab); Fume hood (Nüve); Vortex (Velp); Centrifuge (JP Selecta); Micro-centrifuge (Hettich); autoclave (Nüve); Microwave oven (Vestal); Ph meter (JP Selecta); Heating magnetic stirrer (Velp); Thermoblock TMR (Bunsen); Gel electrophoresis apparatus (Cole-parmer); Transilluminator (UVP); Digital Camera (Canon); Microtiter plate (Italy); Thermal cycle Thermal cycle (Favorgen).

3.2. Chemicals and Reagents

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

3.3. Culture Media

3.3.1. M17 broth

M17 broth was developed for the growth and enumeration of *Lactic streptococci* (lactococci) in milk and dairy products. It favours the growth of mutants unable to ferment lactose. It is well adapted to the culture of *Lactococcus lactis* (a particularly fastidious species) and *Streptococcus* at 37ºC, pH 7.0 ± 0.2 (Terzaghi and Sandine, 1975) (Table 3.1).

Table 3.1. Typical ingredients of M17 for 1 liter of medium.

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

3.3.2. MRS broth.

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. The medium can be used to culture slowly-growing lactobacilli such as *Lactobacillus brevis* and *Lactobacillus fermentum* at 30ºC and pH 5.7 +/- 0.2 (Table 3.2).

Table 3.2. Typical ingredients of MRS for 1 liter of medium.

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

3.3.3. MSE broth

This medium, developed by Mayeux, Sandine and Elliker in 1962, is an elective medium for the detection and enumeration of *Leuconostoc* in millk, dairy products and sweet foods.

Table 3.3. Typical ingredients of MSE for 1 liter of medium.

Ingredients	g/l
Tryptone	10.0 g
Gelatin	2.5 g
Yeast extract	5.0 g
Sucrose	100.0 g
Glucose	5.0 g
Sodium citrate	1.0 g
Sodium azide	75.0 mg
Bacteriological agar	15.0 g

3.3.4. Acetate broth

Acetate broth is used for the isolation and cultivation of *Leuconostoc* and *Pediococcus* species (Table 3.4).

Table 3.4. Typical ingredients of Acetate broth for 1 liter of medium.

Ingredients	g/l
Meat extract	50.0 g
Glucose	10.0 g
Peptone	5.0 g
Yeast extract	5.0 g
Sodium acetate buffer	100.0 ml
Tween 80	0.5 ml

3.3.5. Mueller-Hinton agar

This media was used in antimicrobial susceptibility testing by the disk diffusion method. This formula conforms to Clinical and Laboratory Standard Institute (CLSI), formerly National Committee for Clinical Laboratory Standards (NCCLS) (Table 3.5).

Table 3.5. Typical ingredients of Mueller-Hinton Agar for 1 liter of medium.

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

3.4. Sample Collection and Isolation of Lactic Acid Bacteria

During this research 40 samples of cheese, cottage cheese, yoghurt, cheese with garlic and sausage were collected in disinfectant test tube, aseptically from different parts of Northern Iraq (Table 3.6). Then all samples were stored in 4 ºC until delivery to laboratory and stored in refrigerator (4-5 ºC).

Table 3.6. The samples and their origin used in this study.

Sample No.	Sample type	Location		Age of sample	Origin of milk
		City	Village		
1	Cottage cheese	Dohuk	Akre	55 days	Sheep
2	Cottage cheese	Dohuk	Akre	2 months	Cow
3	Cheese	Dohuk	Amadia	3 months	Cow
4	Cheese	Musel	Center	2 months	Buff
5	Cheese	Dohuk	Sersink	20 days	Goat
6	Cheese	Dohuk	Zawite	3 months	Sheep
7	Cheese	Arbil	Kalate	4 days	Sheep
8	Cheese	Dohuk	Akre	2.5 months	Goat
9	Cheese	Dohuk	Sersink	20 days	Goat
10	Cheese	Dohuk	Amadia	15 days	Goat
11	Cheese with Garlic	Dohuk	Akre	2 month	Sheep
12	Cheese with Garlic	Arbil	Diana	2.5 months	Sheep
13	Cheese	Dohuk	Amadia	10 days	Cow
14	Cheese	Dohuk	Bamerne	3 months	Sheep
15	Cheese	Dohuk	Bamerne	1 months	Sheep
16	Cheese	Dohuk	Center	5 days	Goat
17	Cheese	Dohuk	Center	20 days	Sheep
18	Cheese	Dohuk	Akre	1.5 month	Sheep
19	Cheese	Dohuk	Akre	2 months	Goat
20	Cheese	Dohuk	Akre	2 days	Goat
21	Cheese	Dohuk	Zakho	3 days	Goat
22	Cheese	Musel	Center	1 months	Goat
23	Cottage cheese	Dohuk	Akre	2 months	Sheep
24	Cheese	Dohuk	Akre	40 days	Sheep
25	Cheese with Garlic	Dohuk	Akre	1.5 months	Sheep
26	Cottage cheese	Arbil	Diana	1months	Cow
27	Sausage	Dohuk	Akre	1 days	Cow
28	Yoghurt	Musel	Center	1 days	Water buffalo
29	Yoghurt	Dohuk	Akre	1 days	Goat
30	Yoghurt	Dohuk	Akre	3 days	Cow
31	Yoghurt	Dohuk	Akre	2 days	Sheep
32	Cheese	Dohuk	Akre	3 days	Sheep
33	Cheese	Dohuk	Akre	3 days	Sheep
34	Cheese	Dohuk	Akre	2 days	Goat
35	Cheese	Dohuk	Akre	4 days	Goat
36	Cheese	Dohuk	Akre	4 days	Sheep
37	Cheese	Dohuk	Akre	5 days	Sheep
38	Cheese	Dohuk	Akre	2 days	Sheep
39	Cheese	Dohuk	Akre	3 day	Goat
40	Cheese	Dohuk	Akre	4 days	Goat

3.5. Isolation of Lactic Acid Bacteria

2-3 gr of each sample inoculated in 5 ml of Acetate, MSE, MRS and GM17 broth and incubated at 30°C and 37°C for MRS, GM17 and Acetate while incubated just at 25- 37°C for MSE media for overnight. A 100 µl of culture broth inoculated again in another 5 ml of mentioned media to obtain pure culture and treated with glycerol 30 % and preserved as stock culture at -20°C.

3.6. Morphological Examination

Morphological characteristics of both colony and bacterial cell on the base of color, shape, margin and surface, arrangement, and Gram reaction were investigated. Biochemical characterizations 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 identified suspected LAB catalase negative or positive.

3.7. Gram Staining

Lactic acid bacteria tend to be blue-purple colour microscopically wich is indicate to Gram positive bacteria. Collected samples underwent to the gram status by light microscopy after staining. From surface agar one colony treated with one drop of water on microscope slide and spread it 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 30 seconds and gently washed with tap water then immersed again with iodine for half minutes 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 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.

3.8. Polymerase chain reactions

Lactic acid bacteria were identified at species level by PCR amplification according to the two primers used during this thesis (Table 3.7). Screening for the presence of *Leuconostoc* and *pediococcus* were conducted by pick up one colony from agar plate with sterile wooden applicator and suspended in a PCR eppendorf containing

10 µl of sterile distilled water then mixed well and follows substrate in Table 3.8. were added in a PCR eppendorf.

Table 3.7. Primers used in this study

Primers	5'----------› 3'	**Region**	**Resource**
PEDİO_R	R: GCGTCCCTCCATTGTTCAAACAAG	*23s rRNA*	Pfannebecker and Fröhlich 2008.
PEDİO_F	F: GAACTCGTGTACGTTGAAAAGTGCTGA	*~700 bp.*	
Leu-R	R: TTTGTCTCCGAAGAGAACA	*16s rRNA ~973 bp*	Jang *et al*.
Leu-F	F: CGAAAGGTGCTTGCACCTTTCAAG		2003.

Table 3.8. Substrate that added to PCR eppendorf to performed colony PCR.

Chemical	**Amount**
DNA Sample	1 µl
Distilled water	32 µl
Taq DNA polymerase buffer	4 µl
deoxyribonucleotide triphosphates (*dNTPs*)	1 µl
Forward primers	1 µl
Reverse primers	1 µl
Taq DNA polymerase enzyme	1 µl

Then mixtures are putted in thermal cycle under condition given in Table 3.9.

Table 3.9. Condition of PCR used to amplify 16S rRNA gene.

First denaturation		**Denaturation**	**Annealing**	**Extension**	**Last extension**
Temperature	94 °C	94 °C	55 °C	72 °C	72 °C
Time	3 min	1 min	1 min	1 min	4 min
Cycle	1	35			1

3.9. Electrophoresis of PCR product and plasmid DNA

To determine the amplified DNA size agarose gel electrophoresis was conducted, DNA tends to be negative charge and through a highly cross-linked agarose matrix migrate to the positive pole (Robyt *et al.*, 1990).

In this study DNA sample electrophoresed in 0.8 % agarose gel prepared as follow; 800 mg of agarose was dissolved in 100 ml of 1X Tris Borate EDTA buffer (TBE) then boiled in microwave oven until all particles were dissolved and then allowed to cool (approx. 20 min) at room temperature and poured into the gel casting stand and combs were placed. When gels become solid the combs were removed gently, the agarose gel was placed into the electrophoresis tank and 1X Tris Borate EDTA buffer (TBE) was added until the buffer cover the gel then 8 µl of DNA sample mixed with 2 µl loading dye (bromophenol blueand xylene cyanol FF) for visual tracking of DNA migration during electrophoresis. The electrophoresis conditions were used as; voltage of 130 V, electric current of 5 mA, and the gel was run for 1-1.30 hours. For plasmid electrophoresis one gram of agarose was (1% w/v) dissolved in 100 ml of 1X TBE buffer and the electrophoresis conditions were used like; 80 volts, electric current of 5 mA and the electrophoresis was performed for 3-4 hours.

3.10. Protienase Test

Special agar for protienase were conducted by addition of 1 gr of skim milk and 1.5 gr of agarose in 100 ml of distilled water. After treated with microwave oven and poured on Petri dish each plate were divided to 8 parts to investigated 8 samples then 10 µl of sample added to the determined part of Petri dish and incubated for 5 hours at 37 °C, then the diameters of the inhibition zones were measured using electronic digital caliper.

3.11. Antimicrobial Test

Food borne pathogens and spoilage microorganisms (*Salmonella paratyphi, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli, Campylobacter jejuni, Klebsiella pneumoniae, Yersinia P1*) for testing antimicrobial activity were received from the culture collection of Department of Bioengineering and Sciences (KSU Turkey). Test micro-organisms were inoculated to Mueller-Hinton agar and poured on Petri dish and each one divided to 8 parts for testing 8 samples of

Pediococus and *Leuconostoc*, wells of 5 mm in diameter were performed on each parts. The wells were filled with 10 µl of samples after that all plates were incubated in refrigerator (4°C) for one hour then incubated for 20-24 hours at 37°C. After the incubation, inhibition zones appearing around the wells were measured in millimeter (mm).

3.12. Antibiotic Test

Studies of antibiotic resistance of LAB have not been extensively investigated until recently, in contrast to the situation with pathogenic species and their antibiotic resistance. However, interest in LAB and their antibiotic resistances has recently gained strength since the resistant determinants are known to be able to be transferred between bacterial species, also from beneficial bacteria to pathogens.

After stock culture reactivated, bacterial isolate inoculated uniformly by the sterilized swab onto the surface of Acetate agar plate then antibiotic disc of Penicillin 10 µg, Tetracycline 30 µg, Chloromphenicol 30 µg, Ampicillin 10 µg, Erythromycin 15 µg, Amoxicillin 25 µg, Streptomycin 10 µg, Rifompin 5 µg, Kanamycin 30 µg, Vancomycine 30 µg, Gentamycin 10 µg were applied on the agar surface plate. The whole process has been done under aseptic condition. Thereafter agar plates with antibiotic discs were incubated for 24 h at 37 ºC, the diameters of the inhibition zones were measured using electronic digital caliper. The results were expressed as sensitive (S), marginally susceptible (I) and resistant (R).

3.13. Plasmid screening

The plasmid DNA of LAB offers new possibilities for differentiating the isolates at the strain level (Sewaki *et al*., 2001). Plasmids of these bacteria have been used, after genetic modification as food grade cloning system and for the construction of cloning and expression vectors (Shareck *et al*., 2004; Tarakanov *et al*., 2004; Yeng, *et al*., 2009).

Plasmid DNA from LAB were isolated by GeneJET plasmid Miniprep Kit according to manufacturer's protocol as described as follows:

LAB stock cultures were reactivated in 5 ml of acetate broth during 24 hours at 37 ºC. Then pellet cells were resuspended in 250 µl of the resuspension solution and then transferred cell suspension to a microcentrifuge tube and vortex well until no cells clumps remain. A 250 µl of the lysis solution was added and tubes were shaken gently 4-6 times until the solution become viscous and slightly clear, then 350 µl of the neutralization solution added and again shaken gently 4-6 times after that centrifuged for 5 minutes and the supernatant transferred to the supplied GeneJET spin column with the taking into consideration transferring the white precipitate. Centrifuge the GeneJET spin column for 1 minutes and discard the flow-through and place the column back into the same collection tube. Then 500 µl of the wash solution added to the GeneJET spin column and centrifuged for 30-60seconds and again discard the flow-through and place the column back into the same collection tube. The washing procedure was repeated another time then discard the flow-through and centrifuge for an additional 1 minute to remove residual wash solution to avoid residual ethanol in plasmid preps. Then the GeneJET spin column transferred into a fresh microcentrifuge tube and 500 µl of the elution buffer added to the center of GeneJET spin column membrane to elute the plasmid DNA then incubate at room temprature for 2 minute and centrifuged for 2 minutes then column discarded and purified plasmid DNA stored at -20 ºC.

4. RESULTS AND DISCUSSION

4.1. Biochemical test and gram staining of LAB

Within 40 samples of cheeses, cottage cheese, yoghurt, cheese with garlic and sausage collected from Northern Iraq. From that samples to isolate different bacteria they were grown in different media such as acetate media, MRS media, GM17 media and MSE media. After growing them in different media, 34 strain from acetate media, 38 strains from MRS media, 36 strains from GM17 media and 34 strains from MSE media were detected. According to biochemical and morphological tests, it has been found that all isolates were catalase negative and gram positively stained and categorized as lactic acid bacteria (Table 4.1., 4.2., 4.3. and 4.4.). On the Figure 4.1 Gram staining of representative cocci shaped and bacilli shaped isolates were demonstrated. Our result are similar to the (Fatma and Benmechernene, 2013). They isolate 83 strains of *Leuconostoc mesenteroides* from 12 samples of raw camel milk collected from different Algerian zones based on morphological, biochemical and physiological characters tests.

Table 4.1. Microbiological and biochemical properties of isolated LAB strains from grown in Acetate media at 37 ºC.

Strains in Acetate	Cell form	Gram	Catalase	Bacteria
Ac A1	*Coc*	+	-	*Pediococcus*
Ac A2	*Bacilli*	+	-	ND
Ac A3	*Coc*	+	-	*Pediococcus*
Ac A4	*Coccobacilli*	+	-	ND
Ac A5	*Coccobacilli*	+	-	*Pediococcus*
Ac A6	*Coccobacilli*	+	-	ND
Ac A7	*Coccobacilli*	+	-	*Pediococcus*
Ac A8	*Bacilli*	+	-	ND
Ac A9	*Coc*	+	-	*Pediococcus*
Ac A10	*Coccobacilli*	+	-	ND
Ac A11	*Coccobacilli*	+	-	*Pediococcus*
Ac A12	*Bacilli*	+	-	ND
Ac A13	/	/	+	/
Ac A14	*Coccobacilli*	+	-	*Pediococcus*
Ac A15	*Coc*	+	-	ND
Ac A16	*Coc*	+	-	*Pediococcus*
Ac A17	*Coccobacilli*	+	-	*Pediococcus*
Ac A18	*Coc*	+	-	*Pediococcus*
Ac A19	*Coc*	+	-	ND

Ac A20	*Coccobacilli*	+	-	*Pediococcus*
Ac A21	*Coccobacilli*	+	-	*Pediococcus*
Ac A22	*Coc*	+	-	*Pediococcus*
Ac A23	*Coc*	+	-	*Pediococcus*
Ac A24	*Coccobacilli*	+	-	*Pediococcus*
Ac A25	*Bacilli*	+	-	ND
Ac A26	/	/	+	/
Ac A27	*Coccobacilli*	+	-	ND
Ac A28	/	/	+	/
Ac A29	/	/	+	/
Ac A30	/	/	+	/
Ac A31	/	/	+	/
Ac A32	*Coccobacilli*	+	-	ND
Ac A33	*Coccobacilli*	+	-	*Leuconostoc*
Ac A34	*Coc*	+	-	*Leuconostoc*
Ac A35	*Bacilli*	+	-	ND
Ac A36	*Coccobacilli*	+	-	ND
Ac A37	*Coc*	+	-	ND
Ac A38	*Coc*	+	-	*Leuconostoc*
Ac A39	*Coccobacilli*	+	-	*Leuconostoc*
Ac A40	*Coccobacilli*	+	-	ND

Table 4.2. Microbiological and biochemical properties of isolated LAB strains from grown in MRS media at 30 ºC.

Strains in MRS	**Cell form**	**Gram**	**Catalase**	**Bacteria**
MR A1	*Coc*	+	-	*Pediococcus*
MR A2	*Coc*	+	-	ND
MR A3	*Coc*	+	-	ND
MR A4	*Coccobacilli*	+	-	ND
MR A5	*Coc*	+	-	ND
MR A6	*Bacilli*	+	-	ND
MR A7	*Coc*	+	-	*Pediococcus*
MR A8	*Coc*	+	-	*Pediococcus*
MR A9a	*Coc*	+	-	ND
MR A9b	*Coccobacilli*	+	-	*Pediococcus*
MR A10	*Coc*	+	-	ND
MR A11	*Coccobacilli*	+	-	ND
MR A12	*Bacilli*	+	-	ND
MR A13	*Coccobacilli*	+	-	ND
MR A14	*Coc*	+	-	*Pediococcus*
MR A15	*Bacilli*	+	-	ND
MR A16	*Coccobacilli*	+	-	ND
MR A17	*Coccobacilli*	+	-	ND
MR A18	*Bacilli*	+	-	ND
MR A19	*Coc*	+	-	*Pediococcus*
MR A20a	*Bacilli*	+	-	ND

MR A20b	*Coccobacilli*	+	-	ND
MR A21	*Coc*	+	-	*Pediococcus*
MR A22	*Coc*	+	-	*Pediococcus*
MR A23	*Coccobacilli*	+	-	ND
MR A24	*Coccobacilli*	+	-	ND
MR A25	*Coccobacilli*	+	-	*Pediococcus*
MR A26	*Coc*	+	-	*ND*
MR A27a	*Coc*	+	-	*Pediococcus*
MR A27b	*Coccobacilli*	+	-	*Pediococcus*
MR A28	*Coc*	+	-	ND
MR A29	/	/	+	/
MR A30	/	/	+	/
MR A31	*Bacilli*	+	-	ND
MR A32	*Coc*	+	-	ND
MR A33	*Coc*	+	-	ND
MR A34	*Coccobacilli*	+	-	ND
MR A35	*Coccobacilli*	+	-	ND
MR A36	*Coc*	+	-	ND
MR A37	*Bacilli*	+	-	ND
MR A38	*Coc*	+	-	ND
MR A39	*Coccobacilli*	+	-	ND
MR A40	*Coccobacilli*	+	-	ND

Table 4.3. Microbiological and biochemical properties of isolated LAB strains from grown in GM17 media at 37 ºC.

Strain in GM17	**Cell form**	**Gram**	**Catalase**	**Bacteria**
G A1	*Coccobacilli*	+	-	ND
G A2	/	/	+	/
G A3	*Coc*	+	-	ND
G A4	*Coc*	+	-	*Pediococcus*
G A5	*Bacilli*	+	-	ND
G A6	*Coc*	+	-	ND
G A7	*Coc*	+	-	*Pediococcus*
G A8	*Coccobacilli*	+	-	ND
G A9	*Coc*	+	-	*Pediococcus*
G A10	*Coc*	+	-	ND
G A11	*Coccobacilli*	+	-	ND
G A12a	*Coccobacilli*	+	-	ND
G A12b	*Coccobacilli*	+	-	*Pediococcus*
G A13	*Coccobacilli*	+	-	ND
G A14	*Coc*	+	-	ND
G A15	*Coc*	+	-	ND
G A16	*Coccobacilli*	+	-	*Pediococcus*
G A17	*Bacilli*	+	-	ND
G A18	*Coc*	+	-	ND
G A19	*Coc*	+	-	*Pediococcus*

G A20	*Coccobacilli*	+	-	ND
G A21	*Coccobacilli*	+	-	ND
G A22	*Coccobacilli*	+	-	ND
G A23	*Coc*	+	-	*Pediococcus*
G A24	*Coc*	+	-	ND
G A25	*Coccobacilli*	+	-	ND
G A26	*Coccobacilli*	+	-	ND
G A27	*Coc*	+	-	ND
G A28a	*Bacilli*	+	-	ND
G A28b	*Coccobacilli*	+	-	*Pediococcus*
G A29	/	/	+	/
G A30	/	/	+	/
G A31	/	/	+	/
G A32	*Coc*	+	-	ND
G A33	*Coc*	+	-	ND
G A34	*Coccobacilli*	+	-	ND
G A35	*Coccobacilli*	+	-	ND
G A36	*Coccobacilli*	+	-	ND
G A37	*Coc*	+	-	ND
G A38	*Coc*	+	-	ND
G A39	*Coccobacilli*	+	-	ND
G A40	*Coccobacilli*	+	-	ND

Table 4.4. Microbiological and biochemical properties of isolated LAB strains from grown in MSE media at 25 ºC and 37 ºC.

Strain in MSE	**Cell form**	**Gram**	**Catalase**	**Bacteria**
MS A1	*Coc*	+	-	ND
MS A2a	*Coc*	+	-	ND
MS A2b	*Coc*	+	-	ND
MS A3	*Coccobacilli*	+	-	ND
MS A4	*Coc*	+	-	ND
MS A5	*Coc*	+	-	ND
MS A6	*Coccobacilli*	+	-	ND
MS A7	*Coccobacilli*	+	-	ND
MS A8	*Coccobacilli*	+	-	ND
MS A9	*Coc*	+	-	ND
MS A10	*Coccobacilli*	+	-	ND
MS A11	*Coc*	+	-	ND
MS A12	*Coc*	+	-	ND
MS A13	*Bacilli*	+	-	ND
MS A14	*Coccobacilli*	+	-	ND
MS A15	*Coc*	+	-	ND
MS A16	*Coc*	+	-	ND
MS A17	*Bacilli*	+	-	ND
MS A18	/	/	+	/
MS A19	*Coccobacilli*	+	-	ND

MS A20	*Coccobacilli*	+	-	ND
MS A21	*Coc*	+	-	ND
MS A22	*Bacilli*	+	-	ND
MS A23	*Coc*	+	-	ND
MS A24	*Coccobacilli*	+	-	ND
MS A25	*Bacilli*	+	-	ND
MS A26	/	/	+	/
MS A27	*Coc*	+	-	ND
MS A28	/	/	+	/
MS A29	/	/	+	/
MS A30	/	/	+	/
MS A31	*Coccobacilli*	+	-	ND
MS A32	*Coccobacilli*	+	-	ND
MS A33	*Coccobacilli*	+	-	ND
MS A34	*Coccobacilli*	+	-	ND
MS A35	*Coccobacilli*	+	-	ND
MS A36	*Coc*	+	-	ND
MS A37	*Coccobacilli*	+	-	ND
MS A38	/	/	+	/
MS A39	*Coccobacilli*	+	-	ND
MS A40	*Coccobacilli*	+	-	ND

Abbreviations: + indicates catalase positive strains; - indicates negative catalase strains; / indicates no data is available; ND not defined

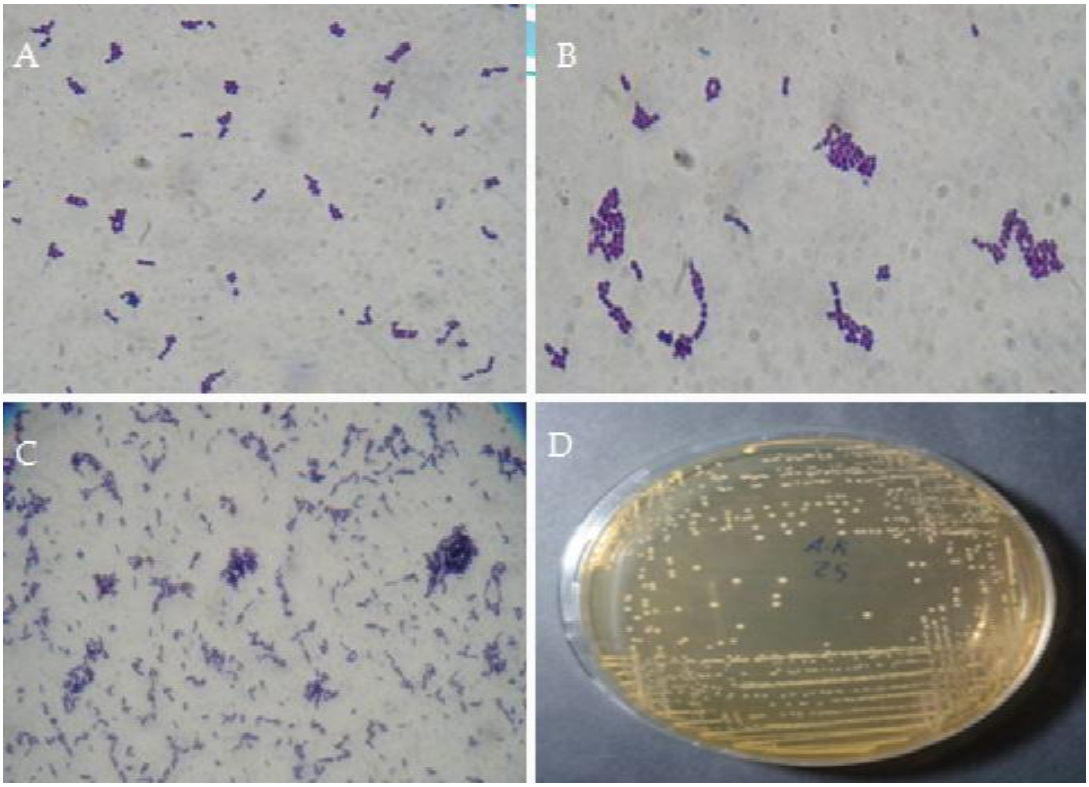

Figure 4.1. A. Gram staining of strain (G A34) showing coccobacilli shaped, B. Gram staining of strain (MS A4) showing cocci shaped, C. Gram staining of strain (MR A6) showing bacilli shaped, D. Colony morphology of typical LAB.

From all four media isolation of *Pediococcus* and *leuconostoc* were conducted as its clear in Table 4.5.

Table 4.5. Number of strains that identified from all four media.

Bacteria	MRS	GM17	Acetate	MSE
Pediococcus	11	8	15	-
Leuconostoc	-	-	4	-

4.2. Identification of LAB genetically

Isolated and microbiologically and biochemically defined as LABs were subjected to molecular analysis by PCR technique. PCR reaction was set up using two primers as mentioned earlier (Table 3.7) according to PCR amplifications it has been expected to get *ca* 700 bp for *Pediococcus* and *ca* 973 bp for *Leuconostoc* DNA fragments. The results showed that, 4 *Leuconostoc* from acetate agar and 11 *Pediococcus* from MRS agar, 15 from acetate agar and 8 from GM17 agar were detected as seen in Figures 4.2., 4.3., 4.4, and 4.5.

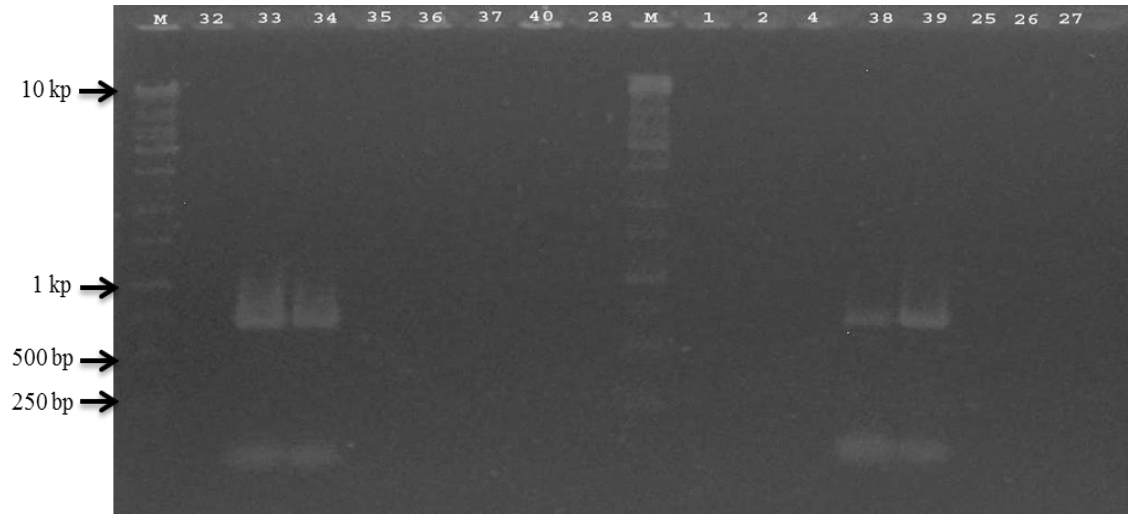

Figure 4.2. PCR products of amplified 16S rRNA gene spacer regions different LAB isolated by Acetate media. Lanes: M, 100 bp DNA marker. Lane 33, 34, 38 and 39 detected *Leuconostoc* as (~973 bp).

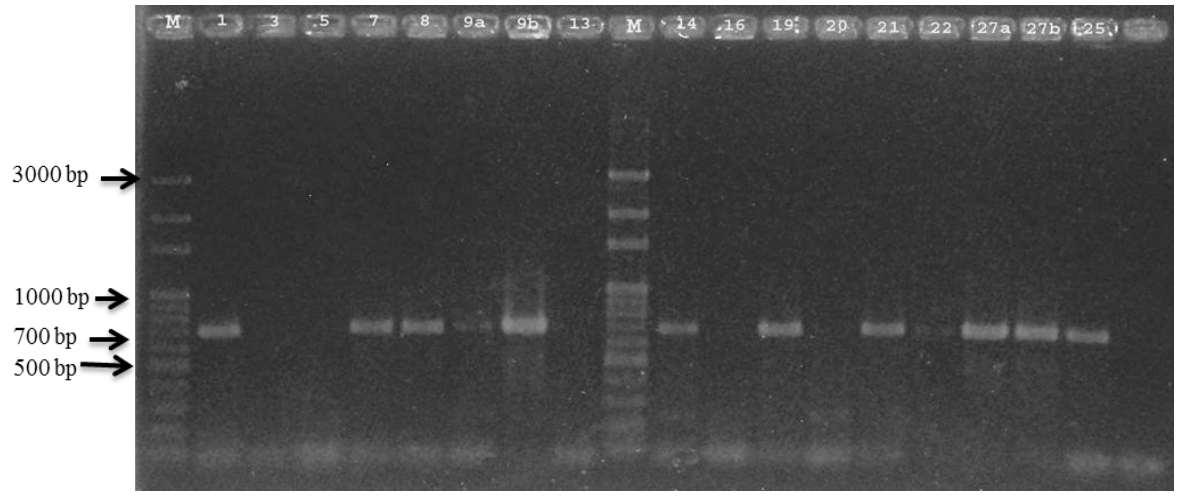

Figure 4.3. PCR products of amplified 23S rRNA gene spacer regions different LAB isolated by MRS media. Lanes: M, 100 bp DNA marker. Lane 1, 7, 8, 9b, 14, 19, 21, 22, 27a, 27b and 25 detected *Pediococcus* as (~700 bp).

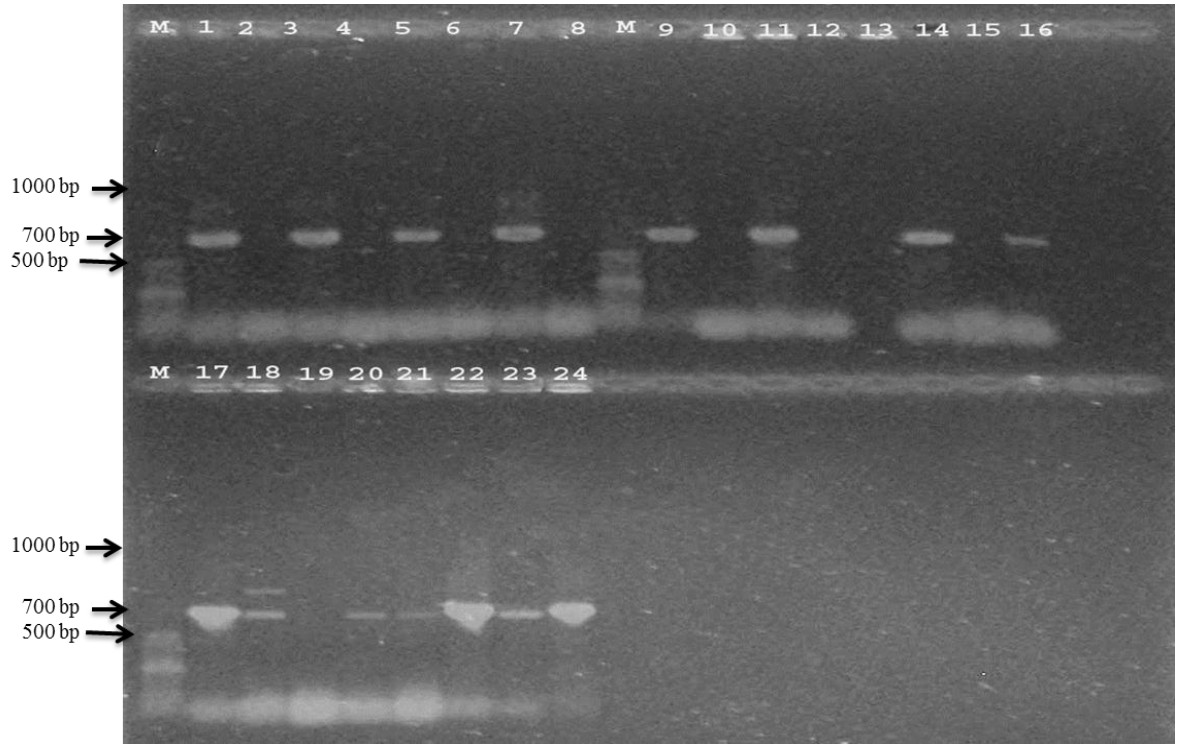

Figure 4.4. PCR products of amplified 23S rRNA gene spacer regions different LAB isolated by Acetate media. Lanes: M, 100 bp DNA marker. Lane 1, 3, 5, 7, 9, 11, 14, 16, 17, 18, 20, 21, 22, 23 and 24 detected *Pediococcus* as (~700 bp).

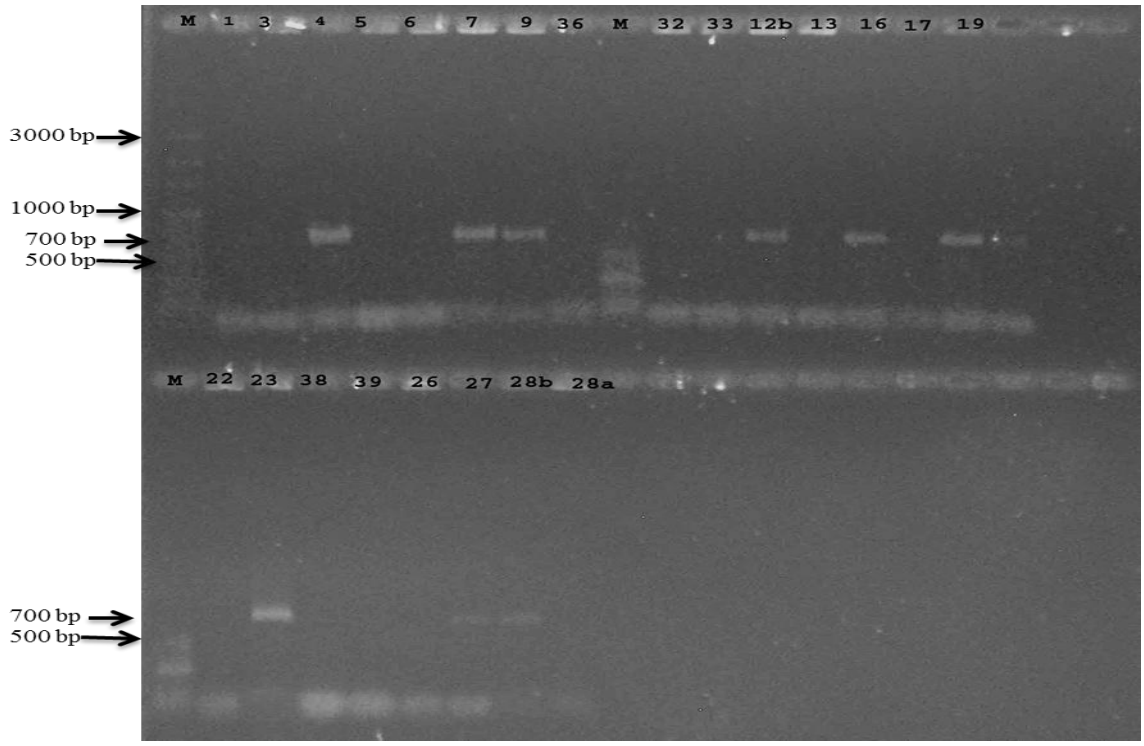

Figure 4.5. PCR products of amplified 23S rRNA gene spacer regions different LAB isolated by GM17 media. Lanes: M, 100 bp DNA marker. Lane 4, 7, 9, 12b, 16, 19, 23, and 28b detected *Pediococcus* (~700 bp).

4.3. Antimicrobial inhibitory effect of *Pediococcus* and *Leuconostoc*

The antimicrobial activity of the isolated LAB was determined by the agar well diffusion method (Tagg *et al*., 1976) using cell-free culture supernatants. A total of 38 *Pediococcus* and *Leuconostoc* strains were tested for their antimicrobial activity against food borne pathogens and spoilage bacteria such as *Salmonella paratyphi, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli, Campylobacter jejuni, Klebsiella pneumoniae, Yersinia P1.* The antimicrobial activity of *Pediococcus* and *Leuconostoc* strains are given in Table 4.6. More than one strains exhibited antimicrobial activity against to *Salmonella paratyphi* and *Enterococcus faecalis* (Figure 4.6). Also, all these 38 strains were found to show no antimicrobial effect against *Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli* and *Campylobacter jejuni* as the result that obtained by Herreros *et al.,* (2005) who screened thirty-one lactic acid bacteria isolated from Armada cheese for antimicrobial activity. Their results revealed that none of the strains showed antimicrobial activity against several pathogenic and spoilage reference strains.

Table 4.6. Inhibition of some food-borne pathogens and spoliage bacteria by *Pediococcus* and *Leuconostoc* strains.

Strains	*S. paratyphi (I)*	*E. faecalis (II)*	*P. mirabilis (III)*	*P. aeruginosa (IV)*	*E. coli (V)*	*C. jejuni (VI)*	*K. pneumoniae (VII)*	*Yersinia P1 (VIII)*
AK1	10.33	13.00	5.05	0	7.15	8.00	5.00	0
AK2	5.00	8.00	0	0	0	0	0	9.10
AK3	13.00	13.33	0	0	0	7.25	10.00	7.85
AK4	5.00	8.05	0	0	0	7.32	8.25	11.20
AK5	7.00	9.33	0	0	0	0	0	7.25
AK6	11.5	5.52	0	0	5.50	0	8.15	5.00
AK7	6.5	12.00	8.00	0	0	0	0	6.00
AK8	0	14.00	0	0	9.00	8.55	0	5.20
AK9	0	9.25	0	0	0	0	5.05	0
AK10	7.45	6.66	0	0	6.55	0	0	0
AK11	11.03	10.33	7.25	0	8.20	0	6.00	7.33
AK12	6.05	8.00	0	0	0	6.02	6.25	0
AK13	7.33	10.55	5.15	0	0	0	0	6.12
AK14	10.66	11.22	0	0	0	6.03	0	8.10
AK15	6.00	9.25	0	0	0	0	0	0
AK16	11.20	13.55	0	0	0	5.00	0	9.05
AK17	11.00	8.00	0	0	0	0	0	0
AK18	8.33	8.55	8.22	0	6.15	0	8.05	0
AK19	11.33	12.32	0	0	7.00	5.15	5.15	7.22
AK20	13.32	9.00	5.25	0	0	0	0	10.35
AK21	9.05	0	5.33	0	8.95	6.00	0	0
AK22	9.33	13.00	7.00	0	6.03	0	6.73	8.55
AK23	14.00	10.00	6.10	0	0	0,75	10.45	11.11
AK24	10.02	10.04	0	0	5.02	9.00	0	11.00
AK25	10.05	9.00	7.55	0	0	6.15	0	10.34
AK26	10.32	8.67	0	0	0	8.20	10.00	9.66
AK27	14.02	0	0	0	0	8.35	0	11.65
AK28	12.00	11.22	0	0	8.40	0	0	6.25
AK29	13.05	11.15	7.00	0	0	0	6.65	0
AK30	13.00	6.00	0	0	0	0	8.10	10.45
AK31	9.01	13.65	0	0	6.00	0	0	0
AK32	9.66	13.00	5.00	0	7.00	0	0	0
AK33	0	10.33	0	0	0	0	0	5.00
AK34	13.00	10.52	0	0	8.10	6.04	11.22	7.15
AK35	5.00	11.00	5.45	0	0	0	0	0
AK36	5.02	12.65	6.15	0	6.00	0	6.15	6.32
AK37	0	9.00	0	0	0	0	6.00	9.22
AK38	12.55	14.00	0	0	0	7.02	12.33	0

Bacteriocins are highly specific antibacterial proteins produced by the strains of bacteria active mainly against some other strains of the same or related species (Gaur *et al*. 2004). The bacteriocins produced by the LAB are potent biopreservative agents and the applications of these in food are currently subject of extensive research. The search for new bacteriocins with wider spectrum of activity and compatibility with different food system is being studied by some investigators. On the other hand, bacteriocins produced by *Pediococcus spp*. Appear to have a relatively broad spectrum of activity, that include non-lactobacillaceae gram positive bacterial species (Daeschel and Klaenhammer, 1985; Hoover *et al*., 1988).

For the agents tested in common, our results are similar to those of (Dat Nghe and Tu Nguyen, 2014) who tested bacteriocins produced by *Pediococus pentosaceus* (VTCC-B-601) which inhibited effectively the growth of *Salmonella typhimurium (*ATCC 19430), *Pseudomonas aeruginosa (*ATCC 27853), *Staphylococus aureus (*ATCC 25923), and *Micrococcus luteus (*ATCC 10240).

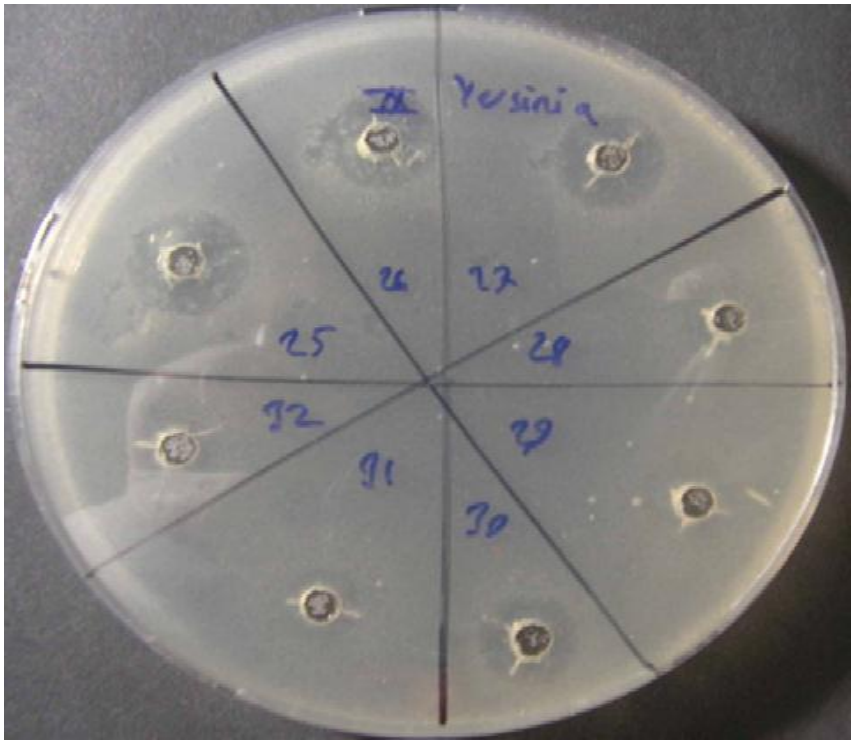

Figure 4.6. Antimicrobial activity of *Pediococcus* and *Leuconostoc* strains against to *Yersiria.*

4.4. Proteinase activity of isolated strains

Proteolysis is considered one of the most important biochemical processes involved in manufacturing many fermented dairy products (Fox, 1989). The proteolytic activities of all strains were assayed using skim milk agar and exhibited as diameter of clear zone. As showed in Figure (4.7) all the 38 strain of LAB showed highly proteolytic activity. The proteolytic systems of LAB play the key role because it enables these bacteria to grow in milk, thereby ensuring successful fermentation. (Rollan *et al*., 1985) performed isolation of bacterial strains from environmental samples and screened their capability of protease production using skim agar and reported that the *Leuconostoc oenos* isolated from red wine was the maximum producer of protease. On the other hand *Pediococcus acidilactici* (ATCC 8042) isolates were screened by (Bousquets *et al.,* 2008) for their abilities to produce extracellular protease by means of formation of clearing zones around the bacterial growth in skim milk agar plates and they found normal producer of protease. The proteinase activity of the isolates used in current study are presented in Table 4.7.

Table 4.7. Proteinase activity of isolated strains by measuring clear zone (mm)

Strains	**Proteinase**			**Mean**
AK1	14.56	11.18	12.96	12.9
AK2	11.26	11.77	12.66	11.89
AK3	14.13	10.11	9.81	11.35
AK4	12	11.76	9.8	11.18
AK5	11.09	10.02	12.26	11.12
AK6	11.26	12.7	11.35	11.77
AK7	11.81	11.52	12.65	11.99
AK8	13.09	10.46	12.52	12.02
AK9	14.98	10.45	10.27	11.9
AK1	11.8	10.58	10.97	11.11
AK11	9.5	11.2	12.05	10.91
AK12	12.34	10.7	9.78	10.94
AK13	9.07	12.7	10.72	10.83
AK14	10.45	10.58	13.02	11.35
AK15	11.83	10.23	10.22	10.76
AK16	10.49	12.33	10.2	11
AK17	14.7	12.91	11.33	12.98
AK18	13.46	13.03	12.55	13.01
AK19	11.83	12.88	11.87	12.19
AK20	11.23	13.18	12.9	12.43
AK21	10.92	10.73	11.03	10.89

AK22	11.55	12.34	10.23	11.37
AK23	14.18	13.5	11.42	13.03
AK24	12.8	13.24	11.05	12.36
AK25	12.91	14.1	11.76	12.92
AK26	12.15	11.04	11.7	11.63
AK27	12.1	12.05	12.42	12.19
AK28	13.06	11.69	13.87	12.87
AK29	12.36	11.96	13.76	12.69
AK3	14.14	12.57	14.34	13.68
AK31	11.46	12.85	9.34	11.21
AK32	11.06	13.05	10.66	11.59
AK33	9.55	12.29	12.5	11.44
AK34	10.56	13.3	12.63	12.16
AK35	9.1	12.76	11	10.95
AK36	9.4	10.5	13.2	11.03
AK37	9.66	9.82	12.47	10.65
AK38	10.07	13.9	14.76	12.91

An exemplified sample of proteolytic activities of some isolates in agar plates are shown in Figure 4.7. The celear zone represents the activity of the proteins enzyme against casein.

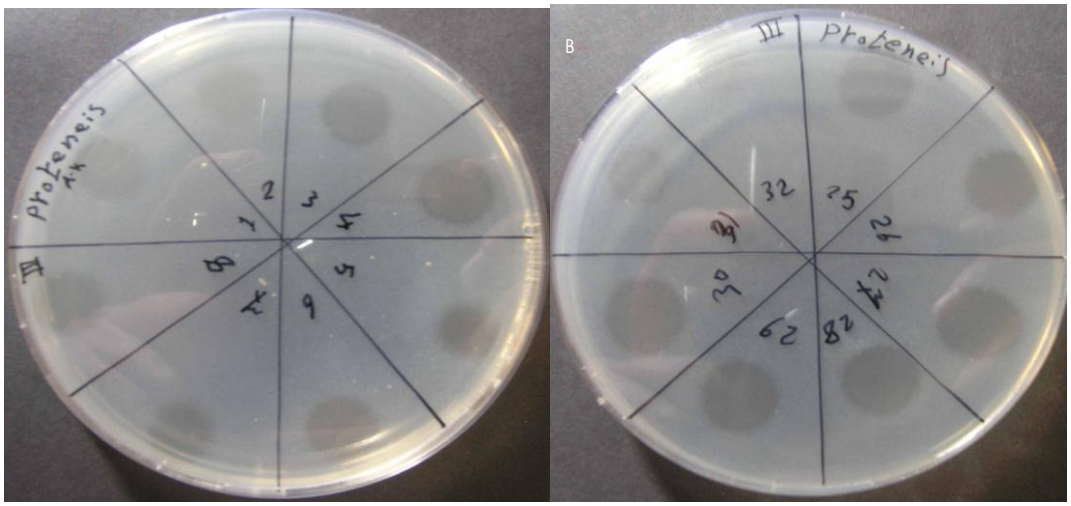

Figure 4.7. The zone of proteolytic enzyme activity of some strain isolated on skim milk agar plates.

4.5. Susceptibility of Antibiotic on isolated strains

Lactic acid bacteria are found in many natural environments however, antibiotic resistance in these bacteria is a growing concern (Korhonen *et al*., 2010). Thus, sensitivity to antibiotics must be determined before LAB strains can be used in food production (Jansson, 2005). Antibiotic-resistant strains can be detrimental to the health

of humans and animals (Korhonen *et al*., 2010), because they are capable of transferring antibiotic resistance genes to pathogenic bacteria (Herreros *et al*., 2005), which can contaminate raw food products such as meat or milk. Data on the antibiotic susceptibility of *Pediococcus spp*. isolated from food are limited. Penicillin, imipenem, gentamicin, netilmicin, erythromycin, clindamycin, rifampin, chloramphenicol, daptomycin, and ramoplanin are generally active against *Pediococcus* species (Zarazaga *et al.*, 1999; Danielsen *et al*., 2007)

However, susceptibility is thought to be species dependent. Table 4.8. shows the susceptibility test for LAB originating from the fermented sausages and raw cheese. We found that more isolated bacteria were susceptible to ceprofloxacine, erythromycine and penciline (Figure 4.8). The growth of all LABs were not influenced by kanamycin, tetracycline, vancomycine and streptomycin antibiotics. While gentamycin inhibited the growth of the strain AK21, rifompin inhibited the growth of the strain AK8, ampicillin inhibited the growth of the strain AK18 and amoxicillin inhibited the growth of the strains AK15 and AK38.

In contrast, studies have reported that LAB are often resistant to commonly used antibiotics such as β-lactams, cephalosporins, aminoglycosides, quinolone, imidazole, nitrofurantoin, and fluoroquinolones (Herreros *et al*., 2005 and Halami *et al*., 2000). ß-lactams, which are bactericidal, are the most widely used class of antimicrobial agent because of their broad spectrum of action and excellent safety profile.

Table 4.8. Results of evaluation of LAB sensitivity to Antibiotics

Strains	**C30**	**TE30**	**E15**	**CN10**	**S10**	**RA5**	**AM10**	**P10**	**K30**	**VA30**	**AX25**
AK1	12.42	10.65	11.75	-	11.22	12.60	10.28	12.07	10.55	-	14.88
AK2	20.12	10.87	17.75	10.10	-	19.80	18.39	16.81	-	-	17.49
AK3	15.31	-	14.80	-	-	14.65	9.75	13.52	-	-	18.10
AK5	18.60	8.52	20.20	9.77	12.10	16.00	13.82	20.02	-	-	17.32
AK8	25.10	10.55	14.36	13.43	-	21.75	16.25	17.95	8.02	-	19.35
AK11	23.64	14.32	19.93	-	14.46	19.72	19.05	20.77	13.20	-	-
AK15	14.02	16.28	18.60	9.95	-	19.60	15.20	15.91	-	-	23.10
AK16	21.02	9.42	23.25	-	12.30	14.45	15.02	13.64	-	-	13.33
AK18	23.17	14.00	11.03	15.05	-	18.60	22.87	18.75	11.62	-	19.31

AK20	25.60	-	21.75	-	-	16.74	16.48	21.60	10.69	-	17.69
AK21	17.95	12.06	16.85	21.10	-	18.57	16.72	15.93	9.37	-	18.12
AK22	16.50	12.90	22.75	-	-	17.95	16.46	-	-	-	15.85
AK23	14.82	-	26.33	-	-	16.39	11.40	9.63	-	-	-
AK24	12.95	-	19.06	-	-	18.70	17.39	17.80	14.60	-	18.75
AK25	13.81	-	-	-	-	19.64	-	-	-	-	-
AK27	21.34	-	20.32	8.35	-	17.63	18.42	13.20	-	-	16.09
AK28	27.65	-	21.00	9.82	-	18.02	18.98	15.85	12.10	-	9.95
AK31	18.41	14.07	17.10	-	-	18.70	-	14.45	11.03	-	-
AK37	23.02	11.90	15.61	11.48	-	17.50	19.37	27.60	8.30	-	19.28
AK38	21.84	12.82	19.22	-	-	19.20	15.17	24.46	-	-	26.66

Abbrevations: Chloromphenicol C30; tetracycline TE30; Erythromycin E15; Gentamicin CN10; Streptomycin S10; Rifompin RA5; Ampicilin AM10; Penicillin P10; Konamycin K30; Vancomycin VA30; Amoxicillin AX25

ß-lactams inhibit bacteria cell wall synthesis and have a lethal effect on gram-positive bacteria. Erythromycin is a macrolide antibiotic with a range of action and efficacy similar to that of penicillin. Macrolides, which are bacteriostatic, bind to ribosomes to block protein synthesis and are effective against gram-positive microorganisms (Liasi, 2009).

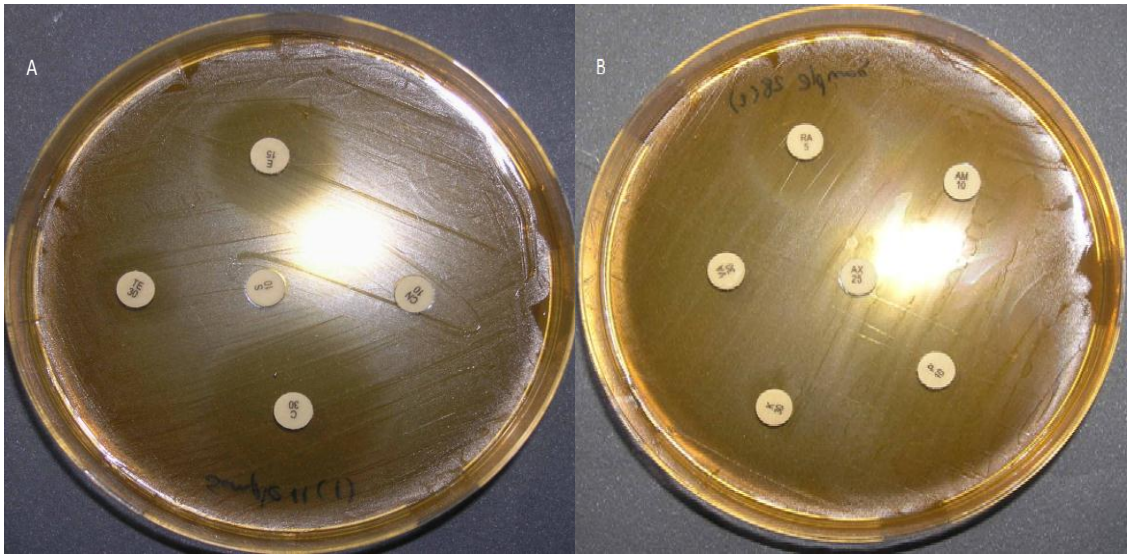

Figure 4.8. Antibiotic test of strains that isolated before. (A) Strain 11 shows susceptibility to Ciprofloxacin and Erythromycin, (B) Strain 38 shows susceptibility to Penicillin and Rifompin.

4.6. Investigation of isolated LAB plasmids:

A total of 38 strains isolated from dairy product of Northern Iraq by more then one media were screened for presence of plasmids. Out of these isolates screened, only ten strains were found to harbour plasmids according to the Gene JET plasmid Miniprep Kit methods. The plasmid profiles of 10 strains are shown blow (Figure 4.9.) Plasmids ranging in size from 7 to 20 kb were detected in all the examined strains and the number of plasmids observed in each samples are represented in Table 4.9. In which AK2, AK5, AK15, AK22, AK31 AK37 and AK38 strains were observed with maximum number of plasmids and AK3 strains had observed with only one plasmid.

Table 4.9: Detected plasmids from isolated LAB strains.

LAB strains	**Number of plasmids**
AK1	3
AK2	4
AK3	1
AK5	4
AK15	4
AK21	2
AK22	4
AK31	4
AK37	4
AK38	4

E.coli based plasmids for cloning and expression vector construction is predominant scientific approaches for a number of reasons. There is comparatively less work on LAB based plasmids (Shareck *et al*., 2004). Therefore, finding novel plasmids in LAB could be a significant importance for biotechnologists and genetic engineers. (Kumar *et al*., 2011), isolated 21 lactic acid bacteria from milk, water, soil and plant samples, however only 6 of them harbour the plasmids.

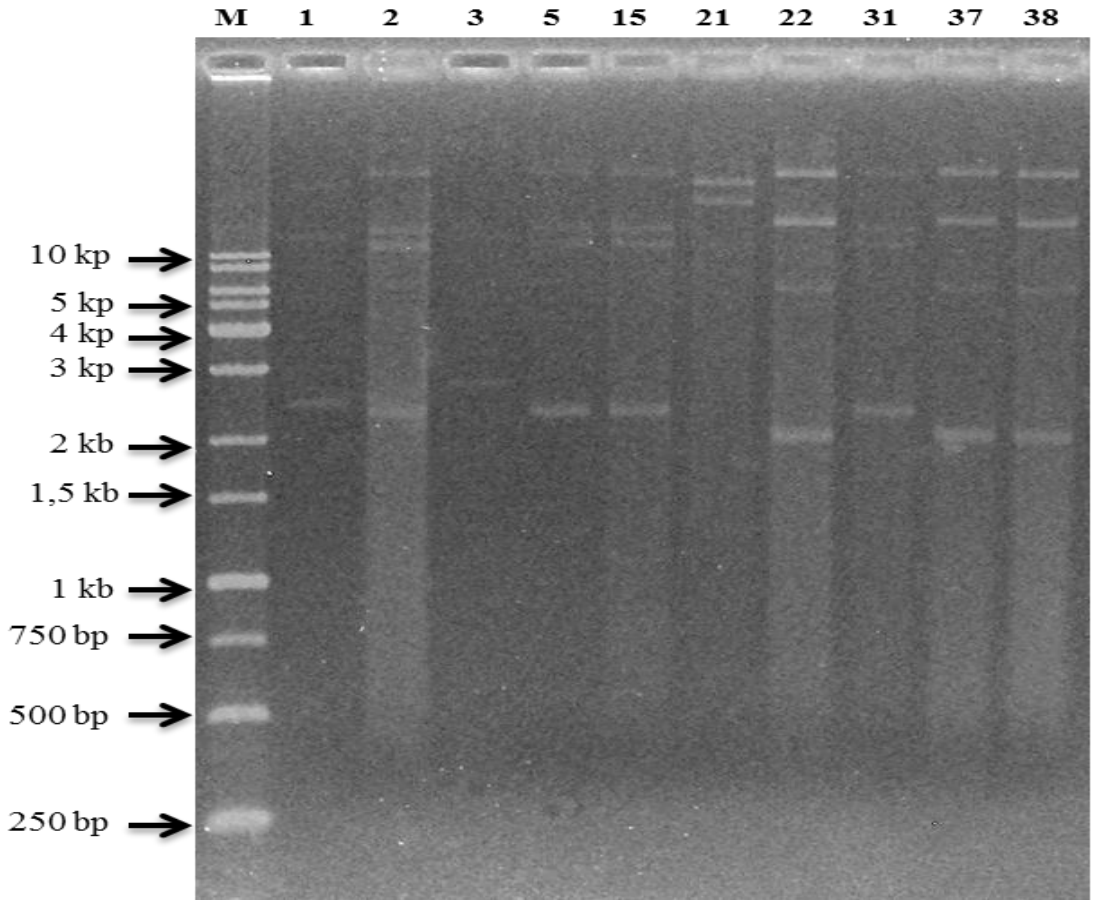

Figure 4.9. Electrophoresis of plasmid DNA extracted from LAB isolated. M: 1 Kb marker; 1: plasmid (10 Kb); 2: plasmid (>10 Kb); 3: plasmid (>10 Kb); 5: plasmid (>10Kb); 15: plasmid (>10 Kb); 21: plasmid (>10 Kb); 22: plasmid (>10 Kb); 31: plasmid (>10 Kb); 37: plasmid (>10 Kb); 38: plasmid (>10 Kb).

5. CONCLUSION AND FUTURE PERSPECTIVE

The lactic acid bacteria (LAB) have an important role in the formation of the specific organoleptic characteristics of dairy fermented products. They might also be found as part of the resident microflora of human and other mammals (e.g., oral cavity, gastro-intestinal track, etc.). Lactic acid bacteria are functionally related group of non-pathogenic, phylogenetically diverse bacteria that produce lactic acid as a primary metabolic end product from glucose and are often associated with food/feed fermentation. They play a vital role in the production of dairy products. They produce the lactic acid that influences important quality characteristics such as texture, moisture content and taste.

Isolation, molecular characterization and determination of probiotic properties of lactic acid bacteria isolated from dairy products were the main target of this study. Lactic acid bacteria were isolated from dairy products. Microbiologically 142 strains isolated as LAB from 40 samples of cheeses, cottage cheese, yoghurt, cheese with garlic and sausage collected from Northern Iraq. For molecular identification based on 16S rRNA and 23S rRNA gene only 38 strains were identified as *Leuconostoc spp* and *pediococcus spp*. (34 strains as *pediococcus spp* and 4 as *leuconostoc spp*,). Investigation of plasmids among the isolated LAB was the our target as their important in molecular biology, The use of these plasmids in genetic engineering techniques for the dairy industry will go a long way towards constructing a strain with all desirable characters. Results obtained were 10 plasmids in all strains and no plasmid DNA was detected for the other 28 isolated strains. On the other hand probiotic properties were conducted to check out their potential for food preservation and other applications such as antimicrobial activity tests against some pathogenic microorganism. *S. paratyphi* and *E. faecalis* are more sensitive to isolated strains than the other pathogenic organisms mentioned earlier in results and discussion. Also antibiotic test that applied on the isolated strains showed the susceptible of ceprofloxacine, erythromycine and penciline to more than one strains and resistant of kanamycin, tetracycline, vancomycine and streptomycin to all the isolated ones. According to these results, in some strains the resistance to some antibiotics and microorganisms may be under the control of plasmid DNAs; however, the resistance to some antibiotics may be coded by chromosomal genes, and different plasmids caused resistance to different antibiotics. Penicillins are inactivated by β-lactamases (penicillinases) produced by many Gram-positive and

Gram-negative bacteria. The enzyme is coded by chromosomal or plasmid genes (Hardy, 1981). Also proteinase activity would be conducted using skim milk agar and showed high proteolytic activity of all strains.

In the light of these result, it will be useful to determine other technological characteristics such as lipolytic activities, aroma and flavor compound production. Finally, strains that were genotypically characterized in this study could be selected by technological methods and used as starter cultured for the fermentation of foods in dairy industry.

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CURRICULUM VITAE

Personal information

Name : Ahmed Karem Ramadan RAMADAN

Country : Iraq

Date of birth : 27/06/1987 Akre

Marital Status : Single

Telephone : +964 (0) 7504072026 - +905346369218

E-mail : ahmed_akre97@yahoo.com

Education

Degree	Subject	Department/ University	Date
B.Sc.	Agriculture	College of Agriculture, Animal production Dohuk University, Iraq	2011