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

INVESTIGATION OF PLASMIDS ISOLATED FROM LACTIC ACID BACTERIA OF DOMESTIC DAIRY PRODUCTS AND THIER GENETIC CHARACTERIZATION

Saman Said Taha SAID AHMED

MASTER THESIS

DEPARTMENT OF BIOENGINEERING AND SCIENCES

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M.Sc thesis entitled “INVESTIGATION OF PLASMIDS ISOLATED FROM LACTIC ACID BACTERIA OF DOMESTIC DAIRY PRODUCTS AND THIER GENETIC CHARACTERIZATION” and prepared by *Saman Said Taha SAID AHMED*, who is a student at Bioengineering and Sciences Department, Graduate School of Natural and Applied Sciences, Kahramanmaraş Sütçü İmam University, was certified by all the majority of jury members, whose signatures are given below.

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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.

Saman Said Taha SAID AHMED

YERLİ SÜT ÜRÜNLERİNDEKİ LAKİK ASİT BAKTERİLERİNDEN PLAZMİT İZOLASONU VE GENETİK KARAKTERİZASYONUNUN İNCELENMESİ

(YÜKSEK LİSANS TEZİ)

Saman Said Taha SAID AHMED

ÖZ

Bu tezin amacı, Kuzey Iraktaki süt ürünlerinden izole edilen laktik asit bakterinin plazmit içerikleri ve onlara ait antibiyotik dirençlilikleri ve metabolitleri araştırıldı. Bu çalışma sırasında Kuzey Iraktan toplanan 52 adet yoğurt, peynir ve sarmısaklı peynir örneğinde plazmiti olup olmadığı analiz edildi. Bakterilerin izolasyonu ve sınıflandırmasında MRS ve GM17 besiyeri kullanılmış daha sonra plazmit izolasyonu yapılmıştır. GM17 besiyerinde 9 şuş plazmit içerirken MRS besiyerinde 10 suşda plazmit gözlenmiştir. Plazmit taşıyan laktik asit bakteri türleri koloni PZR kullanılarak tanımlandı, suşlar arasında *Lactobacillus plantarum'un* baskın olduğu belirlenirken S42 suşu *Lactobacillus spp.* olarak tanımlandı ve S49 suşunun tanımlanması yapılamadı. Plazmit barındıran şuşlara antibiyotik direnç testi yapıldı ve bu şuşlardan bazılarının vankomisin ve tetrasiklin antibiyotiklerine direnç gösterdiği belirlenmiştir. Aynı büyüklüğe sahip plazmitler arasındaki farklılığı belirlemek için plazmitler restriksiyon enzimleriyle kesilmiştir. Ayrıca plazmid taşıyan ve taşımayan bazı suşların metabolitleri incelenmiştir. Plazmitlerin tanımlanması ve genlerinin çalışılması süt ürünlerinin geliştirilmesinde önemlidir buna ek olarak tarım ve tıp alanlarında uygulanabilir.

Anahtar Kelimeler: Laktik asit bakterisi, plazmid, antibiyotik, restriksiyon enzimleri, metabolit.

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INVESTIGATION OF PLASMIDS ISOLATED FROM LACTIC ACID BACTERIA OF DOMESTIC DAIRY PRODUCTS AND THIER GENETIC CHARACTERIZATION

(M.Sc. THESIS)

Saman Said Taha SAID AHMED

ABSTRACT

The aim of this thesis is to investigate the plasmid contents of Lactic acid bacteria isolated from dairy products of Northern Iraq and study of their antibiotic resistantcy and metabolites. During this research 52 samples of yoghurt, cheese and cheese mixed with garlic were collected from Northern Iraq and analyzed to find out whether they have plasmid or not. The bacteria have been isolated and classified using MRS and GM17 media, and then plasmid isolation has been done. From MRS media, 10 strains contained plasmids while from GM17 media 9 strains were observed. To identify which lactic acid bacterial species that carry plasmids, colony PCR conducted for the strains which they were found plasmids, *Lactobacillus plantarum* was dominance on all strains except from strain S42 which identified as *Lactobacillus spp* and strain S49 has not been identified. Antibiotic resistance tests were also carried out on these strains that harbour plasmids and the results show the resistance of vancomycin and tetracycline antibiotic to some of these strains. Digestion of plasmid with restriction enzyme were conducted to differentiate between plasmids that have the same sizes. Metabolic activity of some strains that have plasmids and no contain plasmids were conducted to compare between them. Identification of plasmids and study of their genes aid to improvement of dairy products in addition to customized it in the field of medicine and agriculture.

Key Words: Lactic acid bacteria, Plasmid, Antibiotics, Restriction enzyme, end products.

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LIST OF ABBREVIATIONS

LAB:	Lactic acid bacteria.
GRAS:	Generally Recognized as Safe.
G+C:	Guanine + Cytocine.
IS:	Insertion elements.
DNA:	Deoxyribonucleic acid.
Hfr:	High frequency of recombination.
inc:	Incompatability.
ori:	Origin of replication.
Tet:	Tetracycline.
Sul:	Sulfonamide.
Cam:	Chloramphenicol.
Str:	Streptomycin.
RC:	Rolling circle mechanism.
ssDNA:	Single strand DNA.
OH:	Hydroxyl group.
dsDNA:	Double strand DNA.
sso:	Single strand origin.
dso:	Double strand origin.
EM:	Electron microscopy.
RNA:	Ribonucleic acid.
ATP:	Adenosine triphosphate.
GTP:	Guanosine triphosphate.
kDa:	Kilodalton.
bp:	Base pair.
FAO:	Food and Agriculture Organization.
WHO;	World Health Organization.
GIT:	Gastro intestinal tract.
LDL:	Low density Lipopolysaccharide.
EDTA:	Ethylenediaminetetraacetic acid.
UV:	Ultra violet.
mcg:	Micrograms.

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1. INTRODUCTION

In past centuries, the term "milk souring organisms" was given to lactic acid bacteria (LAB), as generally this bacteria return to the late nineteenth century, bacterium lactic (i.e. *Lactococcus lactis*) was the first pure culture of LAB obtained in 1873 by Lister, J., (Axelsson, 1998). This bacteria have great credit in the food industry (Nettles and Barefoot, 1993). Generally LAB known to be safe (Generally Recognized as Safe-GRAS), and under controlled condition this bacteria can be used as natural competitive microbiota or as specific starter cultures, in addition to its main role in the fermented products and preservation of foods (Cintas *et al*., 2001). Lactic acid bacteria encompass a heterogeneous group of microorganisms, which have as a common metabolic property the production of lactic acid from the fermentation of carbohydrates (Carr *et al*., 2002). LAB are gram-positive, non-spore forming fermentative bacteria, catalase negative, anaerobic, acid tolerant, and occur as cocci, coccobacilli or rods, and cells are usually non motile. They have a condition for complex growth factors such as vitamins and amino acids (Axelsson, 2004). LAB ferment carbohydrates and produce lactic acid as an end product, these are known as homofermentative LAB, and the homofermentative group of LAB includes *Streptococcus*, *Pediococcus*, *Lactococcus* and *Lactobacillus* species (Table 1.1), while heterofermentative LAB produce lactic acid and other compounds such as acetic acid, CO_2 and ethanol (Carr *et al*., 2002; Axelsson, 1998).

Homofermentation and heterofermentation are differentiated by the production of aldolase which is the main enzyme for glycolysis. The homofermenters have this key enzyme which ferments glucose to lactic acid, while heterofermenters do not possesses this enzyme and instead they follow pentose monophosphate pathway (Figure 1.1). They cannot breakdown fructose 1, 6 diphosphate to triose phosphates, and only oxidize glucose-6- phosphate to 6-gluconate by phosphoketolase (Bulut, 2003).

Table 1.1. Differentiation of the lactic acid bacteria according to the carbohydrate metabolism (Carr *et al*., 2002).

Genus	Gram stain Morphology	Type of lactic acid	CO2 production from glucose	Ribose Ferment – ation	Gluconate Fermen- tation	Arginine Hydro- lysis
Homofermenters						
Streptococcus	*Cocci*/chains	L+	-	-,+	ND	V
Pediococcus	*Cocci*/tetrad	DL, L+	-	V	-	V
Lactococcus	*Cocci*/chains	L+	-	-	-	V
Lactobacillus	*Bacilli*/pairs	D-, L+	-	+,-	+,-	-
Heterofermenters						
Betabacteria (*Lactobacillus)*	Pair/ Chains	DL	+	+	+	+
Leuconostoc	Pair/ Chains	D-	+	V	-,+	-

Abbreviations: +, - indicates most positive strains, an occasional negative strain; -, + indicates most negative strains, an occasional positive strain; + indicate positive strains; - indicate negative strains; V indicates variable; ND indicates no data is available.

Historically and till now LAB used in the fermentation of traditional foods as dairy products (yoghurt, butter, cheese, kefir, kumiss), meat (salami, sausages), bread and cereals (e.g., sourdough, ogi) vegetables (sauerkraut, pickles, olives), wine and silage (Geis, 2003).

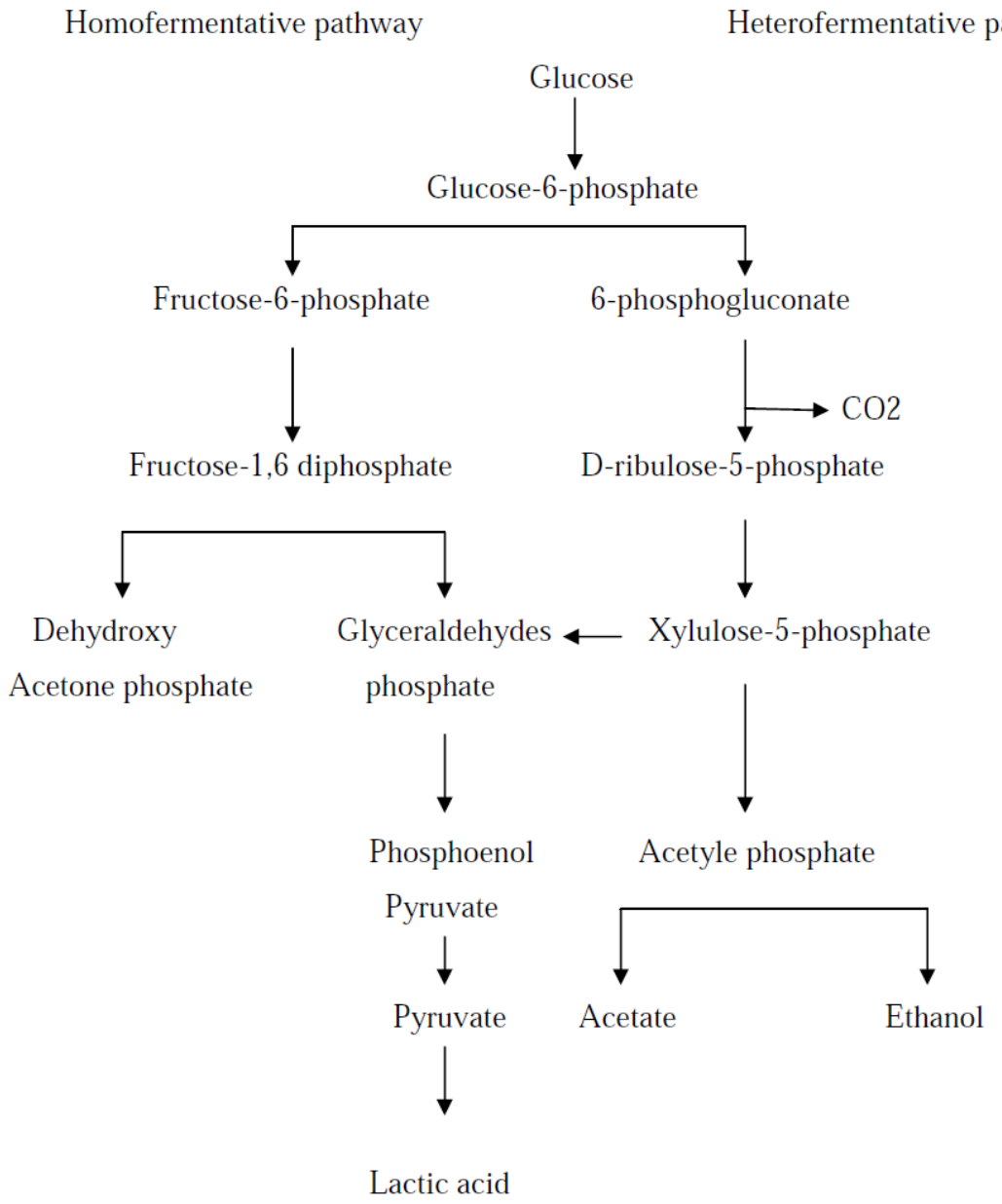

Figure 1.1. Two different pathways for glucose fermentation (Bulut, 2003).

Lactic acid bacteria excrete lactic acid as a main fermentation product into the medium. This biochemical definition associates lactic acid bacteria of different phylogenetic branches of bacterial evolution: the 'low G+C (guanine + cytosine) taxa (less than 55mol% G + C content in their DNA, e.g., *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Pediococcus* and *Streptococcus* and the'high G+C (more than 55mol% G + C content in their DNA' genus *Bifidobacterium* (Figure 1.2) (Schleifer and Ludwig, 1995).

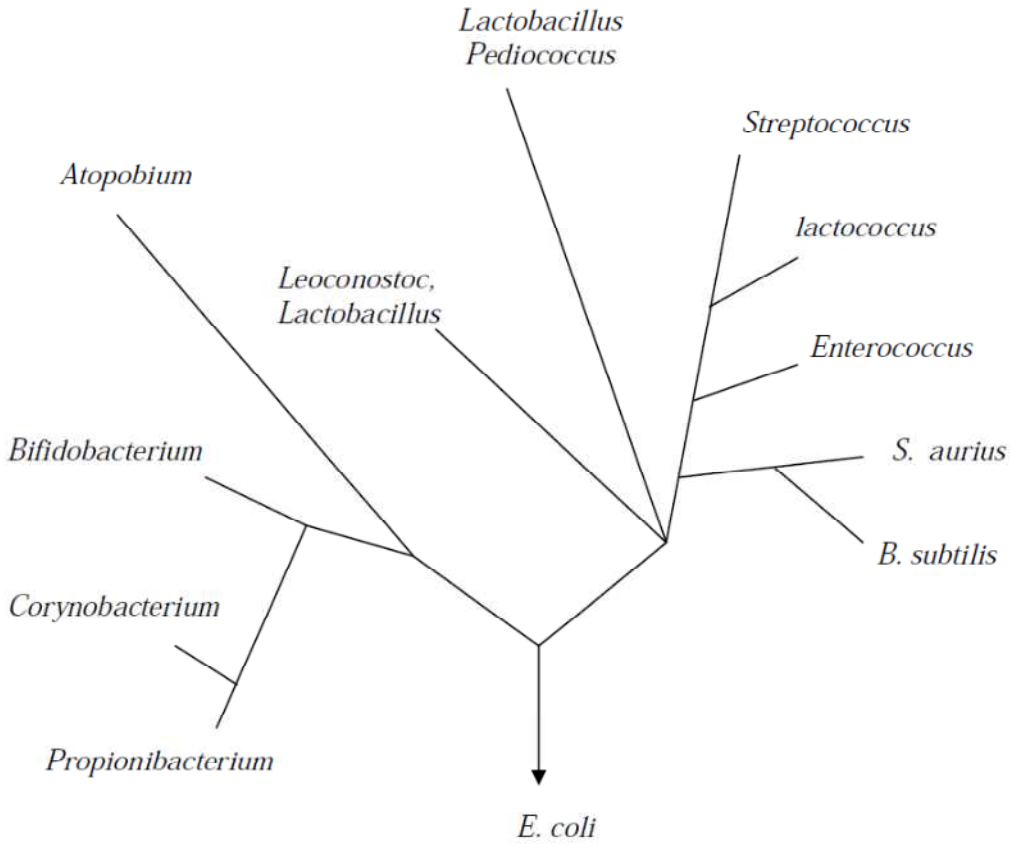

Figure 1.2. Phylogenetic tree of Gram-positive bacteria (Schlifer and Ludwig, 1995).

Generally LAB strains that are used in food fermentations are associated with habitats rich in nutrients such as various food products. Other LAB are important microorganisms as members of the normal flora in the human and animal body (intestine, vaginal mucosa and nasopharyngeal) and in environments (mainly plants) where there are spontaneous fermentations of carbohydrate containing substrates.

These sudden events developed into important food fermentations of plant material (sour dough, sauerkraut, etc.) and animal products (sausages, cheeses and sour milk from meat and milk, respectively) (Teuber, 1993). Other LAB excrete antimicrobial compounds that cause low molecular weight, cationic proteins, and bactericidal to other gram positive bacteria (Klaenhammer, 1988; Daeschel, 1990; Ray, 1992). These antimicrobial compounds have long been used in fermentations to preserve the nutritive qualities of different foods. Perhaps this bioactive compound may be proteinaceous bacteriocin (Tagg *et al.*, 1976), a lantibiotic (lanthionine containing proteins; Jung, 1991) or a bacteriocin like inhibitory substance.

Continuous isolation and identification the new strain of LAB is important for industrial application and for probiotic discovery. Also search of plasmid in some strain of LAB gives an incentive to study these bacteria. The plasmid of LAB offers new possibility for differentiating between strain that is isolated before (Sewaki *et al*., 2001), this plasmid after undergoing to genetic modification used as food grad cloning system and for construction of cloning and expression vectors (Shareck *et al*., 2004; Tarakanov *et al*., 2004; Yeng *et al*., 2009). Through the metabolic engineering in lactic acid bacteria plasmid are used to changing of pyruvate metabolism to production of desired flavour compounds by constructed mutant strains have no antibiotic resistant and foreign DNA gene (Akyol *et al.*, 2009). The remarkable development in the field of genetic engineering of LAB was made possible due to (i) technologically identification of important functions borne by plasmid (e.g. Bacteriocin production, lactose utilization, casein degredation) (Teuber and Geis, 2006), (ii) The adaptation of *Escherichia coli* derived technology for the isolation and manipulation of LAB borne plasmid. As a result, food and pharmaceutical industries could benefit from both LAB and its plasmid (Narayan *et al*., 2011). There is an important point in the growth of LAB in food; they act as antagonistic microorganisms because they are capable of inhibiting other food-borne bacteria by production of organic acids, hydrogen peroxide and/or bacteriocins (De Vuyst and Vandamme, 1994; Holzapfel *et al*., 1995). LABs are not considered to be pathogenic to human and animals with the exception of some *Streptococci*, there have been reports of the involvement of LAB in human clinical infections (Aguirre and Collins, 1993). In the majority of these clinical cases, patients had a history of underlying disease, should be considered as immune compromised and/or may have been treated with antibiotics. Therefore, some LAB may fall into the category of opportunistic pathogens. Nevertheless, there is no evidence to doubt the safety of ingesting large numbers of LAB in fermented foods, and because of this long history of safe use.

2. LITERATURE REVIEW

2.1. Plasmid as General and in Lactic Acid Bacteria

Plasmid, as in generally present in prokaryotes and in some lower eukaryotes, are non-essential extra-chromosomal genetic elements, usually its double-stranded molecules, that replicate independently of the host cell chromosome and are inherited stably. Like chromosomes, plasmids are also closed circular and supercoiled; although certain plasmids (Eg. *Borrelia*) are known to be linear. *Borrelia burgdorferi* has at least 11 plasmids, 2 are circular and 9 are linear molecules. A cell may have different plasmids existing together. Also plasmids may have a mosaic structure (i.e. composed of a collection of genetic sequences encoding for different functions (Figure 2.1)). In the term of size there are a variety of plasmids (1.5 kb to more than 600 kb), copy number (1 to several hundred per cell) and phenotypes conferred to their hosts (Osborn *et al.*, 2000). The most important requirement for a plasmids to display its function is the presence of the origin of replication (which gives the ability to replicate), therefore, the genes contained within plasmids, have a definition either an essential function (also called the plasmid backbone) or an accessory function which may have different phenotypic traits (Osborn *et al.*, 2000).

Plasmids, with exception of a few small plasmids, contain a large number of inserted and/or extra chromosomal mobile genetic elements such as Insertion elements, transposons, integrons, gene cassettes and conjugative transposons (Figure 2.1) (Toussaint and Merlin, 2002; Osborn *et al*., 2000). These accessory genes are effectively 'hitchhiking' on the plasmid backbone, giving the plasmid (and the bacterial host) a selective advantage in exchange for their maintenance, and possible transfer to other hosts (Osborn *et al*., 2000).

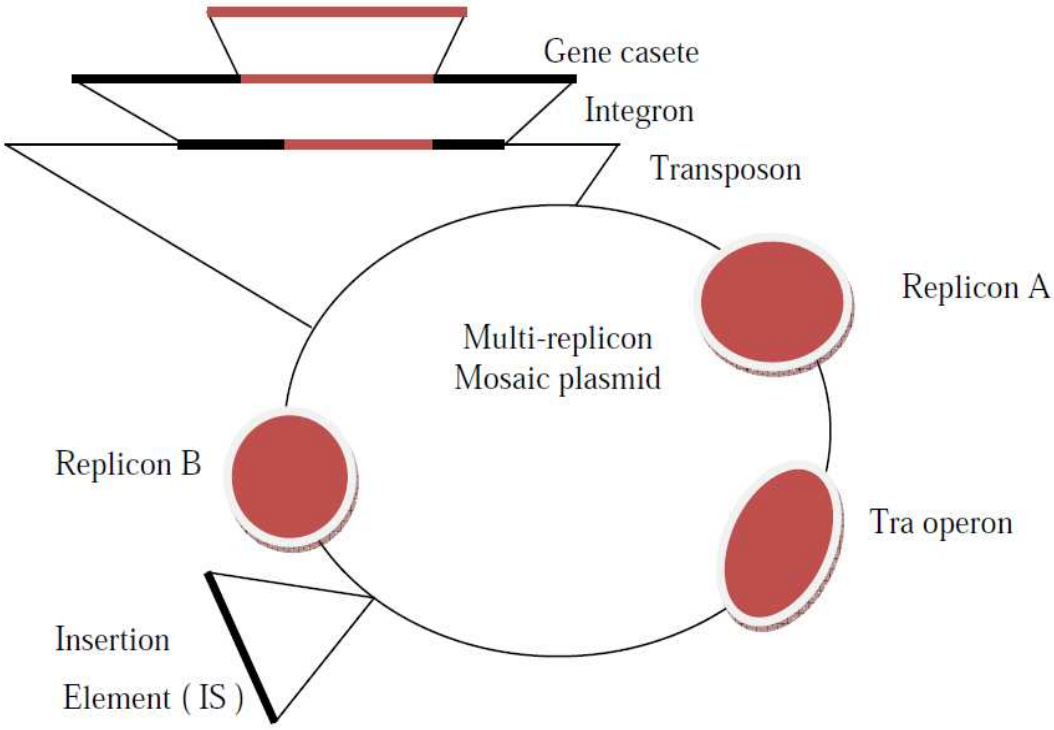

Figure 2.1. A simplified model to describe the mosaic structure of a circular plasmid (Osborn et al., 2000).

Plasmids differ from chromosomes in small size and genes encoded are non-essential for the bacterial survival. Absence of plasmids does not affect on growth of the bacterial cells, but their presence provides additional benefits to the bacteria such as hydrolysis of proteins, metabolism of carbohydrates, amino acids and citrate, production of bacteriocins, exo-polysaccharides, pigments and resistance to antibiotics, heavy metals and phages have been found to be plasmid-encoded (Wang and Lee, 1997). Nevertheless, many of the plasmids that occur in LAB are cryptic, usually small and plentiful, have no known effect on the host's phenotype, range in size from 1 to >100 kb, and have been reported in many groups of LAB (von Wright and Sibakov, 1998). Plasmids have two important traits, the ability to replicate and to partition themselves between the daughter cells after cell division. Plasmids does not freely float in the cell cytoplasm, instead are membrane bound (Kado and Liu, 1981).

Some plasmids usually the large one have ability to transfer themselves among cells (Figure 2.2). They contain genes (the *tra* genes) that encode all the functions they need for transfer. Transconjugation is most commonly mediated by such plasmids and it is called conjugative plasmid.

During conjugation between two bacterial strains, plasmids can be transferred along with the bacterial DNA and this activity is controlled by a set of transfer (*tra*) genes. The proteins produced by these transfer genes bind to the DNA at the ori site to form a DNA-protein complex known as a relaxosome. This complex makes a nick, or break, in one of the two strands of the double-stranded plasmid DNA molecule. The place where this break occurs is called the "nic" site, and the nicked DNA is said to be "relaxed" because the DNA unwinds as a result of the nick in one of the strands. The single-stranded DNA that is generated by the nick is thought to be unwound and transferred through the pilus, or mating bridge, that connects the two bacteria entering the recipient bacteria. The other strand is left in the donor bacteria. It acts as a template for the synthesis of a new complementary DNA strand forming a double-stranded plasmid DNA molecule (Snyder and Champness, 2007; Szpirer *et al.*, 1999; Noirot, 2004).

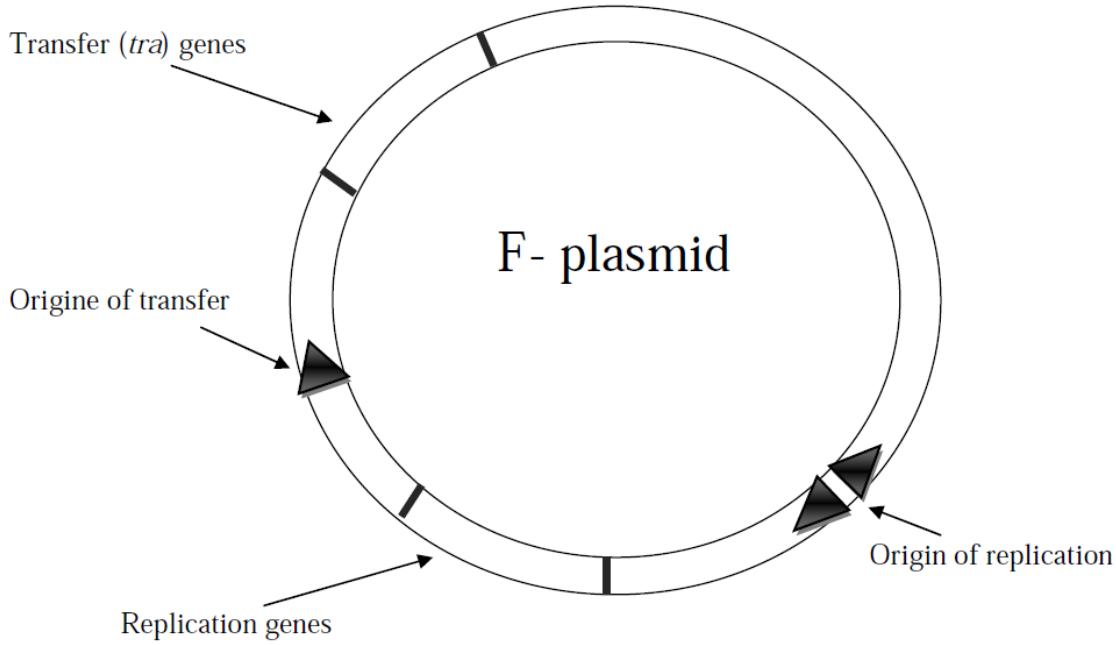

Figure 2.2. An F-plasmid, an example of a transferable plasmid (www.science.siu.edu/microbiology/micr302).

Conjugative plasmids take a larger size, this is due to the absorption of genes coding for self transfer. Conjugative plasmids in Gram positive bacteria tend to be smaller than those in Gram-negative bacteria (Dionisio, 2005).

Conjugative plasmids can exhibit broad or narrow host range. In case of narrow host range which we see most in nature, transfer is restricted generally to and between a small numbers of similar bacterial species. In case of broad host range, the plasmid is able to transfer between widely different bacterial species. This specialist characteristic of BHR plasmids can cause problems in human society such as the spread of multiple antibiotic resistance among pathogenic bacteria (Selimovic *et al*., 2007). Plasmids which have ability of bidirectional transfer between two bacterial strains are called shuttle vectors; they possess two different origins of replication (Mulligan, 2004). Whereas, non conjugative plasmids are those that cannot mediate conjugation. They are usually smaller than conjugative plasmids and they lack one or more genes to initiate self transfer but do encode the functions needed specifically for transfer of their own DNA. A non conjugative plasmids can be transferred by conjugation if the cells also harbours a second conjugative plasmids and this is called mobilization as long as they carry the necessary (*mob*) genes (Figure 2.3) (Snyder and Champness, 2007).

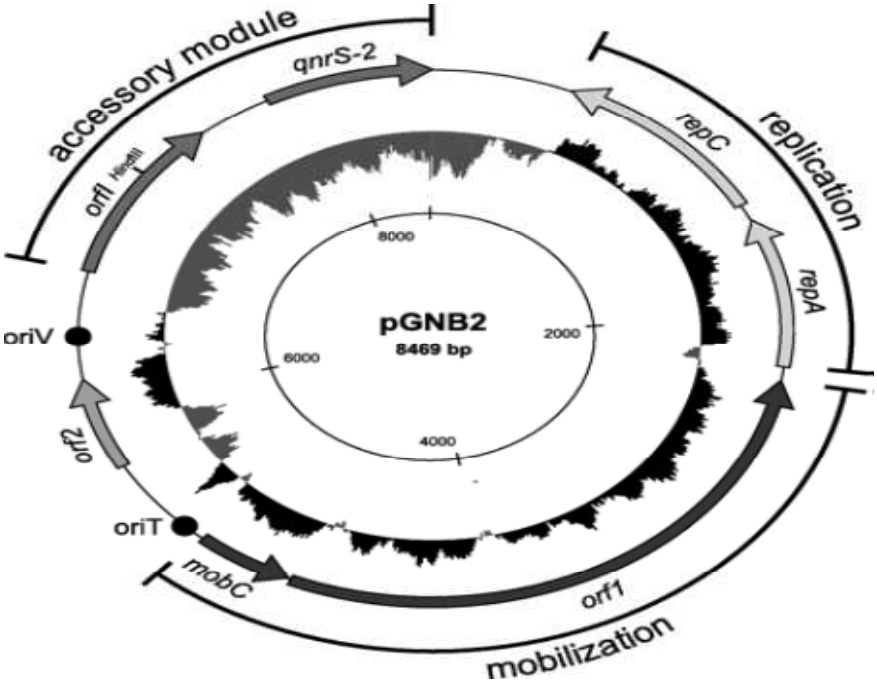

Figure 2.3. Plasmid pGNB2, an example of a mobilizable R-plasmid containing the qnrS2 gene that confers low-level quinolone resistance (Bönemann *et al*., 2006).

Some plasmids have the ability to integrate into the host chromosome this is called episomes. Most of the plasmid DNA isolated from cells is in the supercoiled configuration, which is the most compact form within the cells. A single break (nick) in one of the two strands causes the supercoil to convert to an open circular form, and when breaks occur in both stands at the same place, a linear duplex structure is formed (Mdigan *et al.*, 1997). Many study have been done to use plasmid as a vector such as cloned polysaccharidase genes and transformed into ruminal and other Gram positive bacteria (Ekinci and Flint, 2002).

2.2. Plasmid Classification

F (fertility) plasmids: They have complete gene set to mediate self transfer by conjugation. The F plasmid is a circular DNA molecule its size about 100 kilobase pair, one region of the plasmid contains genes involved in regulating DNA replication such as incompatability, *inc*, and origin of replication *oriS* (Matson and Ragonese, 2005). It also contains a number of transposable elements involved in its ability to function as an episome. It has a large region of DNA, the *tra* region, containing genes that permit it to be transferred from one cell to another. Cells that possessing donor F plasmid are termed F^+ or male and those lacking it recipient are termed F^- or female. When F plasmid is integrated into the host chromosome, the cells are termed as Hfr cell (high frequency of recombination), because homologous recombination occur at a high frequency between the host bacterial chromosome and the introduced DNA (Masson and Ray, 1988).

R (resistance) plasmids: These plasmids are large conjugative plasmids that carry one or more antibiotic resistance genes and as well as genes resistance to mercury, cadmium, nickel, cobalt, zinc and arsenic in pseudomonas (Smit *et al*., 1998). R plasmid were discovered the first in Japan in dysentery causing *shigella* shown to be transferred to and from intestinal *E.coli*. sulfonamide (Sul) resistance was found first, followed rapidly by tetracycline (Tet), chloramphenicol (Cam), and streptomycin (Str) (Watanabe, 1963). genes for replication, partition and transfer in R plasmid are located on one part of the R factor and the resistance gene are located on another part which are often parts of transposons, many of R plasmid and F plasmid have related *tra* regions, and recombination can occur between F and R plasmids (Khan and Musharraf, 2004).

Col (colicin) plasmids: They are small plasmids which encode the genes to synthesize colicins (bacteriocines) (Riley *et al*., 2000). Colicins and bacteriocins are lethal proteins made by one bacterial strain to kill other bacteria. The structural gene for the bacteriocin and the genes encoding proteins involved in processing and transporting bacteriocin are often carried by a plasmid or a transposon (Jerman *et al.,* 2005). Colicins can be divided in to two groups, such as pore-former and nuclease colicine.

Virulence plasmids: Carry one or several genes that confer virulence properties on the bacterial cell so are related to the adhesion, colonization in the host organism and cause damage of the host by formation of substances such as toxin, enzymes and other molecules. The haemolysin and enterotoxin in enteropathogenic *E. coli* are encoded by plasmid (Clarke *et al*., 2003), moreover the exfoliative toxin in *S. aureus* is known to be plasmid linked (Yamaguchi *et al*., 2001). Furthermore two *Bacillus anthracis* toxins are composed of three proteins, protective antigen, lethal factor and the structural gene for these three component are located on the virulence plasmid pXol (Sirard *et al*., 1994). The circular Ti plasmid of agrobacterium tumefaceince enable the bacterium to infect plant cells and produce a tumor (crown gall tumor) (Otten and De Ruffray, 1994).

Metabolic plasmids: They carry some genes that help in cells metabolism, take part in various physiological functions, for example lactose and sucrose utilization (LeBlanc *et al*., 1979; Hardesty *et al*., 1987). The first reports of plasmid that carry genes coded for catabolic pathways were made in the early 1970; these were for catabolism of camphor, octane, naphthalene, salicylate, and toluene and were all found in strains of the Gram negative genus *Pseudomonus* (Williams, 2004). The *Rhizobium* species nodulate the legumes because of *nod*, *sym* and *fix* genes located on plasimds which are necessary for nitrogen fixation (Ahmad, 2001).

Integrative plasmids: These are plasmids that can occasionally integrate into chromosome (previously called episomes). In *Bacteroides uniformis*, certain sections of chromosome separate themselves from chromosome and become plasmids, which are capable of conjugation (Mazodier and Davies, 1991).

Cryptic plasmids: are those that no phenotype can be observed on the host cell. They may be present for possible exclusion of plasmids that are incompatible with the resident plasmid; the properties of these cryptic plasmids are presumably of some use to the host cell but are still unknown. As their functions and products of their genes are unknown thus far.

The presence of cryptic plasmids can help to identify certain medical important bacterial strains, especially for identification of some strains in genus *Enterobacteriacea* (Tompkins, 1985) However, some plasmid-encoded functions have been discovered in cryptic plasmids originating from *Lactobacillus, Streptococcus thermophilus* and *Pediococcus spp*. and can be used as selective marker systems in vector construction (Shareck *et al.,* 2004).

2.3. Plasmid Replication Mechanism

The set of sequences need for autonomous replication of the plasmid (or chromosome) is known as replicon. Several genes and sequences for replication can be present within the plasmid the first is the origin (s) of replication (generically termed *ori*), which is characteristic of each replicon, many plasmids encode a protein involved in the initiation of replication, usually termed trans-acting replicator protein. The third is the plasmid-borne genes involved in the control of replication. The requirement of a plasmid-encoded initiator is reflected by the presence of DNA cognate sites in the origin of replication, where protein-DNA interactions take place. These specific sites are the hallmark of a class of replicons that are different from replicons that do not require specific initiators (Del Solar *et al.,* 1998).

The mode of replication of plasmids has an important impact on some characteristics of plasmid-derived vectors, namely host range, stability and copy number. In LAB, the most common replication mechanisms are the *sigma* and *theta* modes of replication (Shareck *et al.,* 2004).

2.3.1. Sigma-replicating plasmids (Rolling-circle mechanism)

This Replication mechanism has to be unidirectional and it is considered to be an asymmetric process because synthesis of the leading strand and synthesis of the lagging strand are uncoupled (Del Solar *et al*., 1998).

A lot of small plasmid replication (smaller than 10 kb) affiliated to this model of replication but all small plasmids do not necessarily replicate by the RC mode. For example, small plasmids like pRJF1 (2.6 kb) and pWV02 (3.8 kb), isolated from gram-positive bacteria, replicate by the theta mode (Hefford *et al*., 1993; Kiewiet *et al.*, 1993). Genetic elements that are involved in RC replication are the *rep* gene that encodes the replication initiation protein controlled by a repressor and its target site the plus origin of replication or double-stranded origin.

In addition most RC plasmids have a minus origin of replication or single strand origin, a specific sequence that enables the conversion of ssDNA intermediates into double-stranded DNA molecules (Gruss and Ehrlich, 1989).

The Rep protein is a site-specific nuclease which produces a single-stranded nick at the plus strand, initiating positive strand replication and terminating it when a leading strand is synthesized (Figure 2.4) (Gruss and Ehrlich, 1989). The nick leaves a 3'-OH end that is used as a primer for leading-strand synthesis, which most probably involves host replication proteins. Elongation from the 3'-OH end, accompanied by the displacement of the parental plus strand, continues until the replisome reaches the reconstituted *dso*, where a DNA strand transfer reaction (s) takes place to terminate leading strand replication (Del Solar *et al*., 1998). The leading strand replication generates: a dsDNA molecule constituted by the parental minus strand and the newly synthesized positive strand and a ssDNA intermediate that corresponds to the parental positive strand. Generation of ssDNA is the trademark characteristic of RC replication. Finally, lagging strand synthesis occurs and parental plus strand (ssDNA intermediates) are converted to dsDNA forms by host proteins initiating at the single-strand origin (*sso*), which is physically distant from the *dso*. The last step would be the supercoiling of the replicated DNAs by the host DNA gyrase (Espinosa *et al.*, 2000).

Cloning vectors based on RC replication usually have low segregational stability due to accumulation of ssDNA intermediates (Posno *et al*., 1991; Vujcic and Topisirovic, 1993) and insertion of foreign DNA may further reduce their stability (Gruss and Ehrlich, 1989). Accumulation of ssDNA is generally due to the absence of an sso, consequently hindering the conversion of ssDNA to dsDNA molecules (Del Solar *et al.*, 1987).

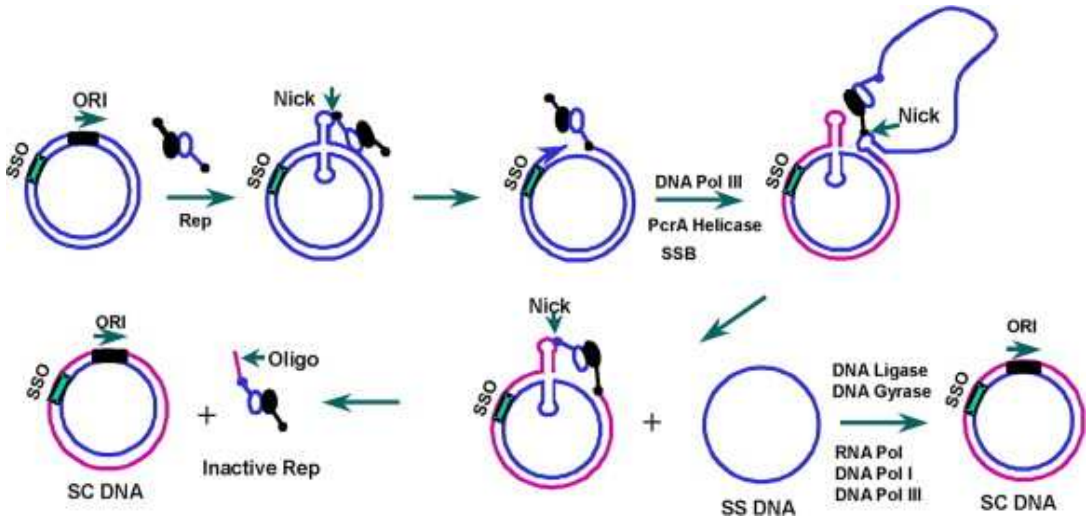

Figure 2.4. A model for Rolling circle replication plasmid.

Replicates the original double-stranded plasmid, plus the single-stranded plasmid DNA with the single-stranded origin sso. However, the single-stranded DNA containing the sso is then replicated to create a second double-stranded plasmid. Thus the overall process produce two plasmid dsDNA to complete the replication (http://www.emergentcomputation.com/mtDNA.html).

2.3.2. Theta replicating plasmids

Theta type mechanism has been studied most extensively among the circular plasmids of gram-negative bacteria, and has also been described for plasmids isolated from gram-positive bacteria (Bruand *et al*., 1991), some lactococcal replicons (Kiewiet *et al.*, 1993), and at least one *Bacillus subtilis* plasmid (Meijer *et al.*, 1995). Theta-replicating plasmids usually occur in medium and large-size plasmids that encode important metabolic functions, enzymes such as lactase (Horng *et al*., 1991), citrate permease (Jahns *et al.*, 1991) and lactase-protease (Frere *et al.*, 1993), bacteriophage resistance (Lucey *et al.*, 1993), pediocin production (Motlagh *et al.*, 1994; Kantor *et al.*, 1997) and exo-polysaccharide production (Van Kranenburg and De Vos, 1998). Also theta replication has been observed in some cryptic plasmids such as: pVS40 (Von Wright and Raty, 1993), pCI305 (Hayes *et al*., 1991), and pWV02 (Kiewiet *et al.*, 1993).

Theta replication mode is not limited to large plasmids only, but has included some very small plasmids , namely p4028 (Zuniga *et al*., 1996), pTXL1 (Biet *et al.*, 2002), and pMB1 (Matteuzzi *et al*., 1990; Corneau *et al.*, 2004). When there is comparison between sigma and theta replication the last one to be the best because theta-replicating plasmids do not produce ssDNA intermediates. This results in greater structural and segregational stability, making theta-replicating plasmids better candidates for vector construction as they can stably maintain large heterologous DNA inserts (Kiewiet *et al*., 1993; De Vos and Simons, 1994).

DNA replication through the theta mechanism involves melting of the parental strands, synthesis of a primer RNA (pRNA), and initiation of DNA synthesis by covalent extension of the pRNA (Kornberg and Baker, 1992). DNA synthesis is continuous on one of the strands (leading strand) and discontinuous on the other, although synthesis of the two strands seems to be coupled (Figure 2.5) (Kelman and O'Donnell, 1995; Zavitz and Marians, 1991). With some exceptions, plasmids using the theta mechanism of replication require a: an initiator protein (Rep) necessary for strand opening, an origin of replication (*ori*) with specific DNA structural organization for strand opening and initiator-protein binding and a host-encoded polymerase I (DNA Pol I) during the early stages of leading-strand synthesis (Del Solar *et al.*, 1998; Alpert *et al*., 2003). Theta-type DNA synthesis can start from one or from several origins, and replication can be either uni- or bidirectional. Under electron microscopy (EM), the replication intermediates are seen as typical Ô ("theta")-shaped molecules that, when digested with enzymes that cleave within the replicated region, yield Y-shaped molecules (Del Solar *et al*., 1998). In the theta mode, sites for priming of leading and lagging-strand synthesis are located close to one another within the replication origin. During replication, both DNA strands remain covalently closed except during the resolution of daughter molecules (De Vos and Simons, 1994).

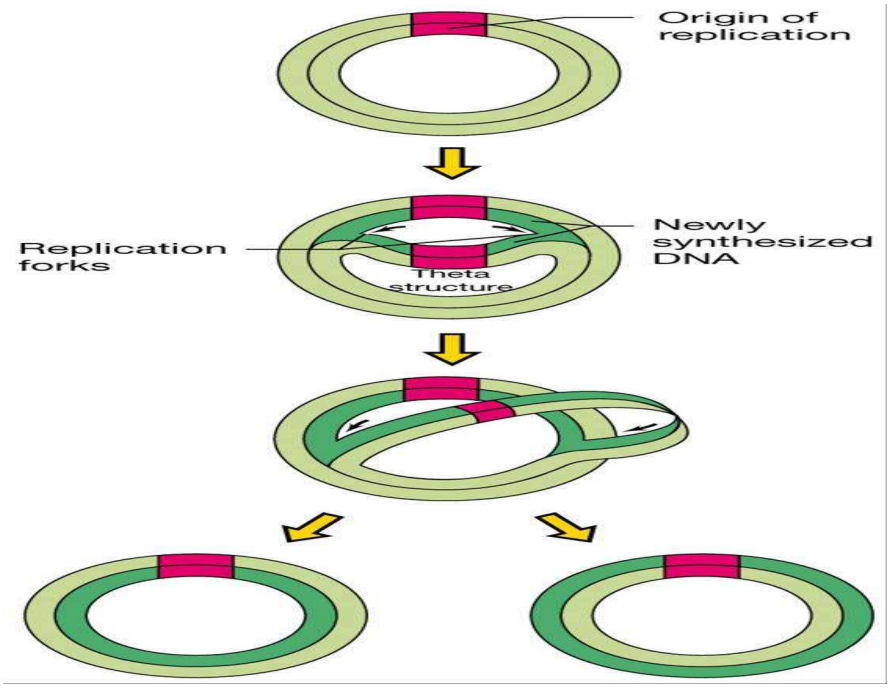

Figure 2.5. Theta replication plasmids
http://202.204.115.67/jpkch/jpkch/2008/wswx/chapter%209.htm.

2.4. Control of Plasmid Replication and Copy-Number

Accumulation of plasmid copies cause the metabolic burden of the host. This leads to slower cell growth and causes cell death. Plasmid-free cells can accumulate rapidly if the plasmid mutates to higher copy numbers (Matsunaga *et al*., 1997). The copy-number of a plasmid in the cell is determined by regulating the initiation of plasmid replication. The initiation of plasmid replication may be controlled by regulating the amount of available primer for the initiation of DNA replication regulating the amount of essential replication proteins, or regulating the function of essential replication proteins (Friechs, 2004).

The Rep proteins bind and stimulate the iterons which are the binding sites of Rep protein in origin of replication. The Rep protein not only stimulates replication but also act as negative regulators of own synthesis (repressor of transcription).

Once the Rep proteins bind the repeated iterons, additional proteins are recruited to the site resulting in bending of DNA and strand separation. This facilitates binding of DNA helicase and primase, which then permit the activity of DNA polymerase. Thus, a plasmid gets replicated (Del Solar *et al*., 1998).

Two major mechanisms are used to control the initiation of plasmid replication:

1. Regulation by antisense RNA:

Antisense RNAs are the main elements to control on the plasmids copy number (Wagner and Simons, 1994) that occurring naturally, its untranslated transcripts that pair to target RNAs at specific regions of complementarity to control their biological function (Spiegelman *et al*., 1972). This means that plasmids concentrations (i.e. copy numbers) can be measured and that the replication frequency can be adjusted accordingly to correct deviations from the normal steady-state copy number (Nordstrom, 1990; Novick, 1989). These RNAs are constitutively synthesized and rapidly turned over, so that their concentration inside the cell, at any time during the cell cycle, correlates with the plasmid concentration (measuring function). The mechanism by which RNA/RNA interaction inhibits replication can, however, differ: inhibition of primer formation (Tomizawa, 1986), inhibition of Rep protein synthesis through prevention of leader peptide translation (Blomberg *et al*., 1992; Wu *et al*., 1992), inhibition of pseudoknot formation which is tertiary RNA elements containing at least two stem-loop structures that is required for Rep synthesis (Asano *et al.*, 1991; Wilson *et al.*, 1993) or induction of premature termination of a rep mRNA (Novick *et al*., 1989; Brantl *et al*., 1993).

2. Regulation by binding of replication proteins to repeated 18-22 bp sites called iterons**:**

Replication origins of a family of bacterial plasmids have multiple sites that contains several repeats of a certain set of DNA bases, called iterons sequences, for binding a plasmid-specific replication initiator protein. The iteron-initiator interactions are essential for plasmid replication as well as for inhibition of plasmid over replication. The inhibition increases with plasmid copy number and eventually shuts plasmid replication off completely. There are two mechanisms for regulating plasmid replication by iterons:

1- RepA protein repress its own synthesis by binding to its own promoter.
2- RepA protein can link two plasmid by binding to their iteron sequences and thereby preventing them from initiating replication (Chattoraj, 2000).

2.5. Resolution of Plasmid Multimers

Sometimes, a plasmid is said to be “lost” when the progeny cells dose not receive the plasmid. The loss of plasmids in a population is sometimes referred to as plasmid segregation. The process of obtaining plasmid-free isolates is termed 'curing'(Ghosh *et al.*, 2000). While multimer is refers to individual copies of the plasmid molecules linked to each other, so during replication if the plasmid form multimer cured of plasmid will increased. Recombination between sister plasmids during or after replication can readily give rise to plasmid dimers or higher multimers. This decreases the number of plasmid copies available for segregation to daughter cells. Since many large plasmids are present as only two or three copies per cell, such events would lead to frequent plasmid loss (Austin *et al*., 1981; Nordstrom and Austin, 1989).

In addition, many plasmid that use special type for its replication control makes them vulnerable to a cumulative effect of dimer formation termed “dimer catastrophe” (Summers *et al*., 1993) further decreasing plasmid stability. For accurate segregation, it is important that multimers are resolved to monomers before cell division occur. This is accomplished by enzyme-mediated, site-specific recombination systems. Large plasmids encode their own recombinase systems consisting of genes for a specific recombinase and a recombination site at which they act. Dimers contain two such sites that are cut, exchanged, and rejoined by the protein to yield two separate circular monomers. Two families of recombinases are represented in various plasmid species: active-site tyrosine recombinases and active-site serine recombinases (Stark *et al.*, 1992).

2.6. Partitioning System

Plasmids avoid being lost from dividing cells by carrying partitioning system, which ensure at least one copy of the plasmid segregates into each daughter cell during replication (Abeles, 1985). As copy number of large plasmid is low (one to three copies per cell), therefore distribution of these copies randomly to daughter cells may lead to increase the proportion of the loss of plasmid (Nordstrom and Austin, 1989).

Plasmids overcome this problem by having plasmid partition systems that ensure that each daughter cell receives at least one copy of the plasmid DNA (Gordon and Wright, 2000). Partition systems direct the active segregation of the plasmids to either side of the cell center prior to cell division, minimizing the chances of plasmid loss.

All low-copy number plasmids appear to encode a partition (par) system. They consist of an ATPase or a GTPase motor protein which is a specific DNA binding adaptor protein and a cis-acting centromere-like site at which they act. The proteins are generally produced from an operon that is tightly auto regulated by one of the products (Gerdes, 2000). Plasmid par systems can be classified into three types. Type I par systems encode a deviant Walker-type P-loop ATPase. Type II encodes an actin/hsp70 type of ATPase, and the recently described type III parsystem encodes a GTPase. Type I partition systems can be further classified into Ia and Ib (Gerdes, 2000). Type Ia systems have large ATPase motor proteins that serve as the operon autorepressor. Type Ib systems have smaller motor proteins and use the DNA binding protein as an autorepressor (Sengupta and Austin, 2011).

2.7. Bacteriocins of LAB

Some antimicrobial substances can be produced by Lactic acid bacteria such as bacteriocins, which is mostly small proteins that affecting unrelated or closely related microorganisms. These antibacterial peptides have an important role in food industry as natural preservatives and possible substitutes for chemical preservation (Abee *et al*., 1995; Cleveland *et al.*, 2001). Bacteriocins differ from most therapeutic antibiotics in being proteinaceous agents that are digested rapidly by proteases in the human digestive tract. They are ribosomally synthesized peptides, and this fact reflects the possibility of improving their characteristics to enhance their activity and spectra of action (Saavedra *et al*., 2004). Production of bacteriocin could be considered as an advantage for food and feed producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources (Deegan *et al.*, 2006). Considering the antimicrobial spectrum, producing species, molecular weight, stability, physical chemical properties and mode of action of bacteriocins, they form a heterogeneous group.

There is the classic type, which has a spectrum of activity only against homologous species, while a second type, less common, which shows action against a wide range of gram-positive microorganisms. One example of this second type is nisin, which is produced by certain strains of *Lactococcus lactis* subsp. *lactis* (De Vuyst, 1994; Rodriguez, 1996; Moreno *et al*, 2000). Nisin, produced by *L. lactis* subsp. *lactis*, is active against Gram-negative bacteria, but only when used at high concentrations or when the target cells have been pre-treated with EDTA (Stevens *et al*., 1991). Bacteriocins in some times are not active against Gram-negative bacteria. The outer membrane of this bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents and dyes from reaching the cytoplasmic membrane (Stevens *et al*., 1991). However, some studies have already reported bacteriocin activity against this group of bacteria. Examples are plantaricin 35d, produced by *Lactobacillus plantarum* and active against *Aeromonas hydrophila* (Messi *et al.*, 2001); bacteriocin ST151BR, produced by *Lactobacillus pentosus* ST151BR (Torodov and Dicks, 2004) and a bacteriocin produced by *Lactobacillus paracasei* Spp. *paracasei* active against *E. coli* (Caridi, 2002); thermophylin, produced by *Streptococcus thermophilus* actives against *E. coli*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* among the gram-negative species and actives against several *Bacillus* species, *Listeria monocytogenes* and *Salmonella typhimurium* among the Gram-positives (Ivanova *et al.*, 1998). Bacteriocins ST28MS and ST26MS, produced by *Lactobacillus plantarum* isolated from molasses (Torodov and Dicks, 2005) inhibited the growth of *E. coli* and *Acinetobacter baumanii*.

2.8. Classification of Bacteriocins

LAB produce a wide number of bacteriocins (Table 2.1) and according to their biochemical and genetic characteristics they can be classified (Klaenhammer *et al*., 1994; Gonzalez-Martinez *et al*., 2003).

Class I. – Lantibiotics: it is small about (< 5 kDa) heat-stable peptides acting on membrane structures; after translation they are extensively modified, resulting in the formation of characteristic thioether, aminoacids, lanthionine and methyllanthionine.

These arise via a two-step process, originated from posttranslational modifications: firstly, gene-encoded serine and threonine are subjected to enzymatic dehydration to give rise to dehydroalanine and dehydrobutyrine, respectively (Sahl and Bierbaum, 1998). A well known example of this group is nisin (Broadbent *et al*., 1989). Based on structural similarities the lantibiotic bacteriocins were initially divided into two subclasses. *Subclass Ia* included relatively elongated, flexible and positively charged peptides; they generally act by forming pores in the cytoplasmatic membranes of sensitive target species. The prototypic lantibiotic nisin is a member of this group. *Subclass Ib* peptides are characteristically globular, more rigid in structure and are either negatively charged or have no net charge. They exert their action by interfering with essential enzymatic reactions of sensitive bacteria (Deegan *et al*., 2006).

Class II. – Non-Lantibiotics: contain small heat-stable, nonmodified peptide (<10 kDa), containing regular amino-acids. This group are subdivided into:

Class IIa: peptides active against *Listeria*, that contain the recently described lactococcin MMFII (Ferchichi *et al.,* 2001) and sakacin G (Simon *et al*., 2002).

Class IIb: consisting in the association of two different peptides for full activity, such as lactococcin G or lacticin F (Nissen-Meyer *et al*., 1992).

Class III. – Big peptides, with molecular weight over 30 kDa. this group is not well documented (Muriana and Klaenhammer, 1991).

Lantibiotics are the most studied and explored industrially. Nisin, a lantibiotic is the first characterized bacteriocin of lactic acid bacteria. Also, it is the most extensively described one usually produced by *Lactococcus lactis* ssp. *lactis* (Harris *et al.,* 1992) is used as an additive in foods. All of the variants of nisin are active against Gram-positive bacteria, like *Listeria* sp, *Micrococcus* sp and also on sporulating bacteria, like *Bacillus* sp and *Clostridium* sp (Gonzalez-Martinez *et al*., 2003). This bacteriocin breaks down the electrochemical potential of bacterial membranes (Teuber, 1995). Lactococcin is a small heat stable non-lanthonine bacteriocin produced mainly by *Lactococcus lactis* ssp. *cremoris*. Lactococcins have three different types, A, B, and M. All types of lactococcins are coded by plasmid DNA. Their host range is rather narrow affecting only lactococcal strains. Their functions are on the cytoplasmic membranes of susceptible organisms (Teuber, 1995).

Table 2.1. Bacteriocins of lactic acid bacteria and their main characteristics (Klaenhammer *et al*., 1994; Gonzalez-Martinez *et al.,* 2003).

Producing species	Bacteriocin	Spectrum of action	Characteristics
Lactococcus lactis ssp. lactis	Nisin	Gram-positive bacteria	Class I lantibiotic, 3,5 kDa, 34 amino-acids, commercially available
	Lacticin 3147	*Clostridium sp* *Listeria monocytogenes* *Staphylococcus aureus* *Streptococcus dysgalactiae* *Enterococcus faecalis* *Propionibacterium acne* *Streptococcus mutans*	Class I two-component lantibiotic, 4,2 kDa, heatstable, active under acid and physiological pH
Lactococcus lactis ssp. cremoris	Lactococcin B	*Lactobacillus*	Class II bacteriocin, approx. 5 kDa, narrow spectrum of action
Lactobacillus acidophilus	Acidocin CH5	Gram-positive bacteria *Lactobacillus*	Class II bacteriocin, forms high molecular weight Aggregates
	Lactacin F	*Lactobacillus fermentum* *Enterococcus faecalis* *Lactobacillus delbrueckii* *Lactobacillus helveticus*	Class II bacteriocin, 6,3 kDa, 57 amino-acids, heatstable at 121° C for 15 minutes
	Lactacin B	*Lactobacillus debrweckii* *Lactobacillus helveticus* *Lactobacillus.bulgaricus.* *Lactococcus lactis.*	Class III bacteriocin, 6,3 kDa, heat-stable, detected only in cultures maintained between pH 5.0 to 6.0

Lactobacillus amylovorus	Lactobin A	*Lactobacillus acidophilus* *Lactobacillus delbrueckii*	Class II bacteriocin, 4,8 kDa, 50 amino-acids, narrow spectrum of activity
Lactobacillus casei	Lactocin 705	*Listeria monocytogenes* *Lactobacillus plantarum*	Class II two-component bacteriocin (33 amino-acids each component), 3,4 kDa,
Leuconostoc gelidum	Leucocin A	*Lactobacillus* *Enterococcus faecalis* *Listeria monocytogenes*	Class II bacteriocin, 3,9 kDa, 37 amino-acids, stable at low pH values, even after heating (100°C for 20 min)
Leuconostoc mesenteroides	Mesentericin Y105	*Enterococcus faecalis* *Listeria monocytogenes*	Class II bacteriocin, 3,8 kDa, 37 amino-acid residues, heat stable (60°C for 120 min at pH 4.5)
Pediococcus acidilactici	Pediocin F	Gram-positive bacteria	Class II bacteriocin, 4,5 kDa, sensitive to proteolytic enzymes, resistant to heat and organic solvents, active under a wide range of pH
	Pediocin PA-1	Listeria monocytogenes	Class II bacteriocin, 4,6 kDa, 44 amino-acids,
	Pediocin AcH	Gram-positive and Gramnegative bacteria under stressing situations	Class II bacteriocin, 4,6 kDa, 44 amino-acids, broad spectrum of action

Pediococus pentosaceous	Pediocin A	*Lactobacillus* *Lactococcus* *Leuconostoc* *Pediococcus* *Staphylococcus* *Enterococcus* *Listeria* *Clostridium*	Class II bacteriocin, 2,7 kDa, sensitive to proteolytic enzymes and heat stable (10 min 100°C)
Enterococcus faecium	Enterocin A	*Listeria monocytogenes* *Pediococcus*	Class II bacteriocin, 4,8 kDa, 47 amino-acid residues, heat-stable
Lactobacillus sake	Lactocin S	*Lactobacillus* *Leuconostoc* *Pediococcus*	Class I bacteriocin, 3,7 kDa, active between pH of 4,5 and 7,5
	Sakacin P	*Listeria monocytogenes*	Class II bacteriocin, 4,4 kDa, heat-stable
Lactobacillus curvatus	Curvacin A	*Listeria monocytogenes* *Enterococcus faecalis*	Class II bacteriocin, 4,3 kDa
Lactobacillus helveticus	Helveticin J	*Lactobacillus bulgaricus* *Lactococcus lactis*	Class III bacteriocin, 37 kDa, narrow spectrum of action, sensitive to proteolytic enzymes, reduction of activity after 100° C for 30 min

2.9. Antibiotic Resistance Profiles of LAB

For several decades, studies on the selection and spread of antibiotic resistance have focused mainly on clinically relevant species. However, several researchers have suggested recently that commensal bacteria such as lactic acid bacteria may act as reservoirs of genes resistance to antibiotics similar to those found in human pathogens (Levy *et al*., 2002). The main concerns associated with these bacteria is that these resistance genes can be transferred to pathogenic bacteria (Perreten *et al.*, 1997a).

Genes that give resistance property to tetracycline, erythromycin and vancomycin have been detected and characterized in *Lactococcus lactis, Enterococci* and in *Lactobacilli* isolated from fermented meat and milk products (Mathur and Singh, 2005). One example of the this resistance is the presented by *Lactobacilli, Pediococci* and *Leuconostoc spp.* which have been reported to have a high natural resistance to vancomycin, a property that is useful to separate them from other Gram-positive bacteria (Hamilton and Shah, 1998). LAB often contain plasmid of different size and some antibiotic resistant determinants located on this plasmids have been reported to occur in *L. lactis* and various *Lactobacillus* and *Enterococcus* species (Gevers *et al.*, 2003a).

The resistance range of *Bifidobacterium* was lately described by (Charteris *et al.*, 1998a). The Examination of (probiotic) bifidobacteria showed us a sensitivity towards ampicillin, penicillin G, cephalosporin, bacitracin, chloramphenicol, erythromycin, clindamycin, nitrofurantoin and tetracycline. And resistances towards vancomycin, gentamicin, kanamycin, streptomycin, fusidic acid, trimethoprim, norfloxacin, nalidixic acid, metronidazol, polymyxin B and colistin. The mechanisms of resistances are unknown (Charteris *et al.*, 1998b).

Lactococcus lactis strains when investigated were susceptible to ampicillin, amikacin, chloramphenicol, first generation cephalosporine, erythromycin, imipenem, gentamicin, oxacillin, penicillin, trimethoprim/ sulfomethoxazol, piperacillin, sulfonamide, tetracycline, and vancomycin (De Fabrizio *et al*., 1994). There was little sensitive towards carbenicillin, ciprofloxacin, dicloxacillin and norfloxacin.

Resistances were towards colistin, fosfomycin, pipemidic acid and rifamycin. In *L. lactis* subsp. *lactis* MG1363 multiple drug efflux proteins were discovered (Van Veen and Konings, 1998), one of these is an ABC transporter (*lmrA*), the other a proton motive force dependent drug transporter (*lmrP*). Both are responsible for a resistance to high concentrations of ethidium bromide.

A similar transporter (*horA*) described in *Lactobacillus brevis* ABBC45 and encoded on a high copy number plasmid is obviously responsible for a resistance to the bitter-tasting hop compounds humulone (acylphloroglucine substituted with two isoprenoid side chains) and isohumulone, and a low level resistance towards ethidium bromide (Sami *et al.*, 1997). The membrane permeability disturbing hop compounds (Teuber and Schmalreck, 1973) have antimicrobial activities against Gram-positive bacteria (Schmalreck *et al.*, 1975).

A resistance to them induces a serious beer spoilage potential of lactobacilli (Sami *et al*., 1997). Indeed, it should be investigated whether the detected efflux proteins have a natural function in providing resistance to phenolic compounds in bacteria which live in and on plant tissues like many LAB including *Lactococcus lactis* (Teuber, 1995).

Twenty-six strains of *L. lactis* ssp. *cremoris* and ssp. *lactis* were all resistant to trimethoprim and almost all to sulfathiazole. Resistances to gentamicin, kanamycin, lincomycin, nafcillin, neomycin, nisin, rifampin and streptomycin varied (Orberg and Sandine, 1985). *Lb. delbrueckii* ssp. *bulgaricus* as components of yoghurt cultures showed intrinsic resistance towards mycostatin, nalidixic acid, neomycin, polymyxin B, trimethoprim, colimycin, sulfamethoxazol and sulfonamides. Susceptibilities to cloxacillin, doxycyclindihydrostreptomycin, furadantin, novobiocin, oleandomycin, oxacillin, and streptomycin were prominent, but kanamycin and streptomycin susceptibilities varied (Sozzi and Smiley, 1980). Many strains of *Lb. plantarum, Lb. casei, Lb. salivarius, Lb. leichmannii, Lb. acidophilus* carry intrinsic resistance genes towards vancomycin which is due to the presence of D-alanine: D-alanine ligase-related enzymes (Elisha and Courvalin, 1995). Intrinsic vancomycin resistance of *Lactobacillus, Leuconostoc* and *Pediococcus* species has been used to separate them from other Gram-positive bacteria on vancomycin supplemented selective media (Simpson *et al.*, 1988).

Fifteen strains of *Streptococcus thermophilus* from yoghurt cultures showed varying levels of resistance to colimycin, gentamicin, kanamycin, mycostatin, nalidixic acid, neomycin, streptomycin and sulfonamides (Sozzi and Smiley, 1980).

In order to obtained of LAB the resistance genes from other bacteria naturally without the intervention of the laboratory must have the possibility of active communication with these bacteria with the aid of conjugative plasmids and transposons. Indeed, plasmids are common in *Enterococci, Lactococci, Leuconostoc, Pediococci*, and present in some strains of *S. thermophilus, lactobacilli* and *bifidobacteria* (Janzen *et al.*, 1992; Mercenier *et al*., 1994; Dellaglio *et al*., 1995; Devriese and Pot, 1995; Sgorbati *et al.*, 1995; Simpson and Taguchi, 1995; Teuber, 1995). Conjugative transposons (broad and narrow host range) have been described in *Enterococci, Lactococci* and *Streptococci* (Clewell, 1994).

To be able to bacteria live in the middle of antibiotics must acquire antibiotic resistance genes from other bacteria, and this was observed in lactobacilli in habitats such as the bovine udder surface, the intestinal, vaginal and nasopharyngeal mucosae of man and animals (Teuber *et al.*, 1999) (Table 2.2). Strains of *Lactobacilli* resistant to vancomycin (intrinsic), cefazolin, penicillin, tetracyclin, trimethoprim-sulfamethoxazole and ciprofloxacin occasionally have been identified in cases of human *Lactobacillus-bacteremia* (Antony *et al*., 1996). In the term of food borne pathogens, lactic acid bacteria contaminate milk and meat obtained from animals treated with antibiotics and carrying populations of resistant bacteria (Teuber *et al.*, 1999) (Table 2.3). Antibiotic Resistances in the Food-associated LAB: An overview of antibiotic resistances reported in the food-associated LAB is compiled in (Table 2.4).

Table 2.2. Antibiotic resistances and resistance plasmids in enteric *Lactobacillus* species from animals.

Species	Source	Plasmid name and Size	Observed resistances or resistance genes	Reference
Lactobacillus acidophilus Strain PA3	Pig		Tetracycline, erythromycin Oleandomycin Kanamycin Neomycin Gentamicin	Klaenhammer and Sutherland 1980
Lactobacillus acidophilus (20 strains)	Pig & calf feces	Several	penicillin (1 strain) ampicillin (1) cloxacillin (7) aminoglycosides (20) tetracycline (15) erythromycin (10) chloramphenicol (1) bacitracin (6)	Vescovo *et al.*, 1982

Lactobacillus reuteri (16 strains)	Pig & calf feces	Several	cloxacillin (16 strains) aminglycosides (16) tetracycline (16) erythromycin (13) chloramphenicol (3) rifamycin (3) bacitracin (10)	Vescovo *et al.* 1982
Lactobacillus reuteri	Poultry	pET 633 9.8 kb	*ermGT* (similar to *ermC* of pE 194)	Tannock, 1987
Lactobacillus reuteri G4	Chicken	pTC82	*catTC* (81% identity to *catPC* 194)	Lin *et al*., 1996
Lactobacillus fermentum	Pig	pLEM3 5.7 kb	*Erm* (98.2% identity to Tn*1545 erm*)	Fons *et al*., 1997
Lactobacillus plantarum	raw pork	pCAT 8.5 kb	*Cat*	Ahn *et al*., 1992

Table 2.3. Intrinsic antibiotic resistance profile of LAB and *Bifidobacterium spp*. (modified from Teuber *et al*., 1999).

Type of bacteria	Intrinsic Antibiotic Susceptibility	Intrinsic Antibiotic Resistance
Bifidobacterium	Ampicillin, penicillin G, bacitracin, cephalosporin, chloramphenicol, erythromycin, clindamycin, nitrofurantoin, tetracycline.	Vancomycin, gentamycin, fusidic acid, streptomycin, polymyxin B, trimethoprim, aminoglycosides, colistin, metronidazol
Enterococci	erythromycin, streptomycin, gentamycin, penicillin G, tetracycline, chloramphenicol	Kanamycin

Lactococcus lactis	Amikacin, ampicillin, 1st generation cephalosporine, chloramphenicol,erythromycin, gentamicin, penicillin, imipenem, oxacillin, sulfonamide, tetracycline, vancomycin	Colistin, fosfomycin, pipemidic acid and rifamycin.
Lactobacilli	Chloramphenicol, streptomycin, gentamycin, penicillin G, tetracycline and Erythromycin	Aminoglycosides, fluoroquinolones, glycopeptides and vancomycin

Table 2.4. Overview of antibiotic resistances in the food-associated LAB.

Foods	Species	Resistance	Detection and location of gene	References
Chinese yoghurts	*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*	Ampicillin, kanamycin, chloramphenicol, chlortetracycline, tetracyclines, neomycin and gentamycin	*tet* M, *ant* 6, aph 3'-IIIa	Zhou *et al.*, 2012
Chinese fermented foods-pickles, sausages	*L. plantarum, L. fermentum, L. helveticus, Ent. faecium*	Tetracycline, erythromycin, chloramphenicol, kanamycin	*tet* M and *erm* B, - plasmid and chromosome; gene *aph* A3, - plasmid, gene *mef* A, -chromosome	Pan *et al.*, 2011

Chinese fermented foods	*L. fermentum* NWL24 and *L. salivarius* NWL33; *L. plantarum* NWL22 and *L. brevis* NWL59 *L. brevis* and *L. kefiri*	Erythromycin 11%., tetracycline 17%., gentamycin 65%., ciprofloxacin 85%.	*erm* B, *tet* M, *tet* S	Nawaz *et al.*, 2010
Italian fermented products	*L. paracasei* 197 strains.	Tetracycline 22%., erythromycin 6%.,		Comunian *et al.*, 2010
Italian Sola cheese made from raw milk	*L. sakei* Rits 9	Tetracycline, erythromycin	*tet* M, - transposon; *tet* L, - plasmid	Ammor *et al.*, 2008
Italian dairy product	*Lc. lactis, Stre. bovis, Ent. faecalis,*	Tetracycline, erythromycin		Devirgiliis *et al.*, 2010
Raw milk, starter-free cheese	Lc. Lactis	Tetracycline	*tet* M, on plasmid	Florez *et al.*, 2008
Turkish yoghurt	*S. thermophilus*	Vancomycin 65%.		Aslim and Beyatli, 2004
Fermented dry sausages	*Lactobacillus* species	Tetracycline gentamicin 79%. penicillin g 64%. kanamycin 79%.		Gevers *et al.*, 2003b

Indian vegetables and fermented foods	*L. plantarum, L. fermentum, Weissella* spp. *P. parvulus*	Gentamicin, vancomycin, norfloxacin, kanamycin		Patel *et al.*, 2013
European probiotic products	*L. acidophilus, L. rhamnosus, L. casei, L. reuteri, L. johnsonnii,L. plantarum, L. delbreukii spp. Bulgaricus*	Tetracycline 26%. Penicillin g 23% Erythromycin 16%. chloramphenicol 11%.		Temmerman *et al.*, 2002
Raw milk soft cheese	*Lc. lactis* K214	Streptomycin, tetracycline, chloramphenicol	*Str-tet* S. *-cat*	Perreten *et al.*, 1997b
Greek cheese	*L. acidophilus* ACA-DC 243	Penicillin		Charteris *et al.*, 1998b
Yoghurt starter cultures	*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*	Neomycin, polymyxin B		Sozzi and Smiley, 1980
Nigerian fermented foods and beverages	*L. pentosus, L. acidophilus, L. casei, L. brevis, L. plantarum, L. jensenii*	Tetracycline 42.5%. Erythromycin 17.5%. Ampicillin 47.5%. cloxacillin 80%. ; penicillin 77.5%. ;		Olukoya *et al.*,1993

2.10. Probiotics

Probiotics are defined as “live microorganisms which exhibit a beneficial effect on the health of the host when administered in adequate amounts by improving its intestinal microbial Balance” (FAO/WHO 2001). Most probiotic microorganisms belong to Lactic Acid Bacteria, such as *Lactobacillus* sp, *Bifidobacterium* sp and *Enterococcus* sp (Klein *et al*., 1998).

Regardless of their source most of the LAB strains harbor at least one indigenous plasmid and often more (Ghosh *et al.*, 2000; Posno *et al.*, 1991; Pouwels and Leer, 1993). In which some LAB may carry potentially transmissible plasmid encoded antibiotic resistance genes (Ahn *et al.*, 1992; Ishiwa and Iwata, 1980; Lin *et al*., 1996) and any strains containing antibiotic resistance plasmids are considered unsuitable for use as human or animal probiotics (Kalavathy *et al*., 2003; Ghosh *et al*., 2000). Probiotic bacteria, which are commensals of the human gut, have been reported to inhibit the growth of harmful microorganisms and food poisoning bacteria, such as *Salmonella*, that can be encountered in the gastrointestinal tract (Hughes and Hoover, 1991; Lim *et al*., 1993). In order to survive passage through the GIT, Probiotic must have the ability to acid and bile tolerance and it must have antimicrobial activity against intestinal pathogens, and ability to adhere and colonize the intestinal tract. Beneficial effects of probiotic include alleviation of lactose intolerance, control of diarrhea, inhibition of intestinal pathogens, enhanced immune response and anticarcinogenic activity (Mishra and Prasad, 2005). Probiotics that used in food industry have good technical properties for large scale cultivation, acceptable shelf life and contribution to good taste is essential (Ouwehand *et al*., 2002). Probiotic bacteria have been suggested to promote human health by the inhibition of pathogens, improvement of the epithelial barrier function, and modulation of host immune responses (Lebeer *et al.*, 2008).

Generally the desirable attribute in probiotic *Lactobacilli* is good adherence capacity, as it may promote the gut residence time, exclude pathogens, and interact with host cells for the protection of epithelial cells or initiate immune modulation (Servin 2004).

Beneficial effects of probiotics bacteria

Managing lactose intolerance

Since the lactic acid bacteria convert lactose into lactic acid, their ingestion may help lactose intolerant individuals tolerate more lactose than what they would have otherwise (Sanders, 2000).

Prevention of colon cancer

Lactic acid bacteria have demonstrated antimutagenic effects thought to be due to their ability to bind with (and therefore detoxify) hetrocylic amines; carcinogenic substances formed in cooked meat (Wollowski *et al*., 2001).

Animal studies have demonstrated that LAB can protect against colon cancer in rodents, though human data is conflicting and limited. Most human trials have shown that lactic acid bacteria may act as anti-carcinogenic through decreasing the activity of an enzyme called β-glucuronidase (Brady *et al*., 2000).

Cholesterol lowering

Animal studies have demonstrated the impact of a range of lactic acid bacteria to decrease serum cholesterol levels in animals, presumably by breaking down bile in the gut, thus inhibiting its reabsorption which enters the blood as cholesterol. Some, but not all human trials have found that dairy foods fermented with lactic acid bacteria can affect with the limited rate on reducing in total and LDL cholesterol levels (Sanders, 2000).

Lowering blood pressure

According to the small clinical trials, consumption of milk that fermented with different strains of lactic acid bacteria can result in modest reductions in blood pressure. That is may be due to the Angiotensin Converting Enzyme inhibitor like peptides produced during fermentation (Sanders, 2000).

Improving immune function and preventing infections

There is a belief that the Lactic acid bacteria have several beneficial effects on immune function. They may protect against pathogens by competitive inhibition and there is evidence to suggest that they may improve immune function by increasing the number of IgA-producing plasma cells, increasing or improving phagocytosis as well as increasing the proportion of T-lymphocytes and Natural Killer cells (Reid *et al.*, 2003; Ouwehand *et al*., 2002). According to the clinical trials, probiotics may decrease the incidence of respiratory tract infections and dental caries in children (Nase *et al.*, 2001) and have role in the treatment of *Helicobacter pylori* infections which cause peptic ulcers in adults after mixed with standard medical treatments (Hamilton-Miller, 2003). Lactic acid bacteria foods and supplements have demonstrated to be effective in the treatment and prevention of acute diarrhea; decreasing the severity and duration of rotavirus infections in children as well as antibiotic-associated and travelers diarrhea in adults (Reid *et al.*, 2003; Ouwehand *et al.*, 2002).

Reducing inflammation

Dietary supplements that containing Lactic acid bacteria have been found to modulate inflammatory and hypersensitivity responses due to the impact of these bacteria to the regulation of cytokine function. Clinical studies declare that they can prevent reoccurrences of Inflammatory Bowel Disease in adults (Reid *et al*., 2003) as well as improve milk allergies (Kirjavainen *et al.*, 2003) and decrease the risk of atopic eczema in children (Kalliomaki *et al*., 2003).

As shown in the (Table 2.5), there are different groups of microorganisms have been used as probiotic supplement, the usual probiotic bacteria used include strains from the genera *Lactobacillus* and *bifidobacterium*, less commonly, species of other lactic acid bacteria, *Saccharomyces*, *E. coli* and *Bacillus* have been suggested for probiotic effects.

Table 2.5. Microbes used as probiotics (modified from Shah, 2000, Young, 1998).

Lactobacillus	*Bifidobacterium*	other lactic acid bacteria	Other species
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus spp.*
L. brevis	*B.animalis*	*Enterococcus faecium*	*E. coli*
L. casei	*B.bifidum*	*Leuconostoc lactis*	*Propionibacterium Freudenreichii*
L. cellobiosus	*B.breve*	*Leuconostoc mesenteroides*	*Saccharomyces cerevisiae*
L. cripatus	*B.infantis*	*Pediococcus acidilactici*	
L. delbueck subsp. Bulgaricus	*B.lactentis*	*Sporolactobacillus Inulinus*	
L. fermentum	*B.longum*	*Streptococcus cremoris*	
L. gallinarum	*B.thermophilum*	*Streptococcus intermedius*	

L. gasseri		*Streptococcus lactis*	
L. rhamnosus GG		*Streptococcus thermophilus*	
L .lactis			
L. plantarum			
L. reuteri			
L. rhamnosus			

2.11. Thesis Objective

Plasmids are extremely valuable tools in the fields of molecular biology and genetics, specifically in the area of genetic engineering. It occurs in many bacterial strains. Because of that this thesis focused on searching the traditional fermented milk product for LABs and their features, investigation their plasmid content, possible function of the plasmids and compares plasmid containing and not containing bacteria according to their antibiotic resistance and produced metabolits.

3. MATERIALS AND METHODS

3.1. Tools and Equipment

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

3.2. Chemicals and Reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Germany), Merck (England) and Favorgen (Taiwan) 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 MRS 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 sulfate	0.2
Manganese sulfate	0.04

3.4. Sample Collection and Isolation of Lactic Acid Bacteria

The 52 samples of cheese and yoghurt and cheese mixed with garlic were aseptically collected from different region of Northern Iraq in disinfectant test tube (Table 3.3). The isolation of LAB was performed in Biotechnology and Genetic Engineering laboratory of KSU, Animal Science Department.

Table 3.3. The samples and their origin used in this study.

Sample No.	Milk products	Location	Type of milk
1	Cheese	Arbil	Cow
2	Cheese	Arbil	Cow
3	Cheese	Duhok	Ewe
4	Cheese	Arbil	Ewe
5	Cheese	Duhok	Cow
6	Cheese	Duhok	Ewe
7	Cheese	Duhok	Ewe
8	Cheese	Duhok	Ewe
9	Cheese	Duhok	Cow
10	Cheese	Duhok	Ewe
11	Cheese	Duhok	Cow
12	Cheese	Duhok	Ewe
13	Cheese	Duhok	Ewe
14	Cheese	Duhok	Ewe
15	Cheese	Duhok	Cow
16	Cheese	Duhok	Ewe
17	Cheese	Duhok	Cow
18	Cheese	Duhok	Cow
19	Cheese	Duhok	Cow
20	Cheese	Duhok	Cow
21	Cheese	Duhok	Cow
22	Cheese with Garlic	Duhok	Ewe
23	Cheese with Garlic	Duhok	Cow
24	Cheese with Garlic	Duhok	Cow
25	Cheese with Garlic	Musel	Cow
26	Cheese with Garlic	Musel	Cow
27	Cheese with Garlic	Musel	Cow
28	Cheese with Garlic	Duhok	Ewe
29	Cheese with Garlic	Duhok	Ewe

30	Cheese with Garlic	Duhok	Cow
31	Cheese with Garlic	Duhok	Cow
32	Cheese with Garlic	Duhok	Cow
33	Cheese with Garlic	Duhok	Ewe
34	Cheese with Garlic	Duhok	Ewe
35	Cheese with Garlic	Duhok	Cow
36	Cheese with Garlic	Duhok	Cow
37	Cheese with Garlic	Duhok	Cow
38	Cheese with Garlic	Duhok	Ewe
39	Cheese with Garlic	Duhok	Cow
40	Cheese with Garlic	Duhok	Cow
41	Cheese with Garlic	Duhok	Cow
42	Cheese with Garlic	Duhok	Cow
43	Cheese with Garlic	Duhok	Ewe
44	Cheese with Garlic	Duhok	Cow
45	Yoghurt	Musel	Ewe
46	Yoghurt	Duhok	Ewe
47	Yoghurt	Duhok	Goat
48	Yoghurt	Duhok	Ewe
49	Yoghurt	Duhok	Goat
50	Yoghurt	Duhok	Cow
51	Yoghurt	Duhok	Cow
52	Yoghurt	Duhok	Cow

All samples were brought to the laboratory under refrigeration conditions and stored at 4 °C until their analysis. 2-3 grams were taken from each sample and inoculated in 5 ml in MRS and GM17 broth and incubated for 24 hours at 30 ^{o}C for MRS and 37 ^{o}C for GM17 for the culture to grow up. After that 100 µl were taken from each culture broth and inoculated again in another 5 ml of MRS and GM17 broth and on the same temperature to get a pure culture, after incubation 500 µl of each sample stored at -20 ^{o}C in the 30 % glycerol.

3.5. Culture Conditions

Strains were grown in the both MRS at 30 ^{o}C and in M17 (Terzaghi and Sandine, 1975) medium supplemented with 0.5% (w/v) glucose (GM17 medium) at 37 ^{o}C. Agar plates were made by adding 1.5% (w/v) bacto agar to broth media.

For M17 (42 g) while for MRS (52.2 g) are suspended in 1 litter of distilled water and let them soak. Boiled and dispensed in to suitable container and then medium were sterilized by autoclaving at 121°C for 15 min.

3.6. Morphological and Biochemical Characterization of Isolates

In order to get the lactic acid bacteria, morphological characters of the positive isolates like colony morphology (color, shape, margin and surface) and cell morphology (shape, arrangement, and Gram reaction) were studied and biochemical characterization 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. According to the catalase negative and gram positive suspected lactic acid bacteria isolated and become ready to plasmid isolations.

3.7. Plasmid Isolation

Plasmid DNA was isolated by using the following procedure (Klaenhammer, 1984) with some slight modifications as described follows.

Bacterial stock cultures were inoculated into 50 ml MRS and GM17 broth and then incubated for 24 hours at 30 ºC for MRS and 37 ºC for GM17. Cells were then harvested by centrifugation at 3500 *g* for 10 min and resuspended in 1 ml of cold ice (25% sucrose, 0.05 M Tris, pH 7.5, 5 mM EDTA). After holding the cell suspension for 10 min in ice bath 75 µl of lysozyme buffer (1 mg/ml in 0.05 M Tris, pH 7.5, 5 mM EDTA) was added. The sample were held in ice bath for 1 hours. Then cells were plated by centrifugation at 3500 *g* for 10 min and the supernatant discarded. The remaining pellet was resuspended in 500 µl of the following lysis solution and mixed well: 0.05 M Tris, 5 mM EDTA, 0.05 M glucose, 3% Sodium dodecyl sulfate (SDS). Immediately before used 1 ml of this solution mixed with10 µl of 10 N (Normality) NaOH, then mixture was heated at 62°C for 1 hour, then allowed to cool (approx.15 min) at room temperature. The 50 µl of 2 M Tris buffer (pH 7.0) was added and mixed gently, followed by addition of 70 µl of 5 M NaCl.

The sample was transferred into 2 ml eppendorf tube, the appearance of lysates at this step were often viscous, but remained turbid. Deprotienization of the sample was conducted using 500 µl of 3% NaCl-saturated phenol. After addition of phenol the tube was shaken vigorously for 3 seconds and held at room temperature for 5 min, to facilitate phase separation end 300 µl chloroform was added and centrifuged for 5 min at 14,000 *g* at room temperature. After centrifugation the upper phase transferred into the new eppendorf tubes and 600 µl of chloroform: isoamylalcohol mixture (24:1) was added.

After 5 min at room temperature the sample was again centrifuged at 14000 *g* for 5 min, then aqueous phase was harvested into the new eppendorf tubes.

The sample then treated using (10% v/v) 3 M Na-acetate buffer (pH 5.2) gently mixed then added double of the total volume of pure ethanol (cooled at -20 ºC), mixed and left for 30 min in crushed ice then centrifuged for at least 30 min at room temperature.

The plasmid DNA was pelleted and dried under vacuum for 10 min then plasmid DNA pellet were dissolved in 20 µl of sterile distal water and stored at -20 ºC.

3.7.1. Electrophoresis of plasmid DNA, PCR product and digested plasmids

Plasmid DNA was run in 0.8% (w/v) agarose gel. 800 mg of standard agarose was dissolved in 100 ml of 1X Tris Borate EDTA buffer (TBE) by boiling. After cooling, the agarose solution was poured into the gel casting stand and combs were placed. After the gel was cooled, the combs were removed gently. The casting tray carrying the agarose gel was placed into the electrophoresis tank and 1X TBE buffer was added until the buffer cover the gel. 10 µl of plasmid DNA sample were taken and mixed with 2 µl of gel loading buffer. After that the samples and DNA ladder were loaded into the wells. The electrophoresis conditions used were voltage of 80 volts electric current of 5 mA, and the gel was run for 3-4 hours.

For PCR product electrophoresis concentration of agarose were one gram (1%) dissolved in 100 ml of 1X TBE buffer and the electrophoresis conditions used were voltage of 130 volts, electric current of 5 mA and the electrophoresis was performed for 1-1.30 hours.

For digested plasmids electrophoresis 5 µl of isolated plasmids as control and 10 µl of restriction digestion reactions were loaded to 1.0 % (w/v) horizontal agarose gel in 100 ml 1X TBE buffer containing 100 µl ethidium bromide. The gel was run at 50 volts, 5 mA for 3-4 hours in TBE buffer, the gel was observed under the UV light.

3.7.2. Staining the gels

When the electrophoresis was complete, the gel was removed from the electrophoresis tank with care and immersed in ethidium bromide (200 ml dH_2O containing 200 µl of EtBr) for 15-20 min which is a DNA intercalating agent, which fluoresces orange when exposed to ultraviolet (312 nm).

3.8. Identification of LAB that have a Plasmid by PCR

Plasmid harboring lactic acid bacteria were further identified at species level by PCR amplification. Screening for the presence of *Lactobacillus bulgaricus, Lactococcus lactis ssp. lactis, Lactobacillus Streptococcus thermophilus, Lactobacillus plantarum, Lactobacillus spp., Leuconostoc spp.* and *Pediococcus spp.* were done by PCR protocol. The amplification was carried out by picking up one colony from culture agar surface with the aid of a sterile wooden applicator and the cells were suspended in 10 µl of sterile distilled water in a PCR eppendorf then from this 1 µl were taken to a new PCR eppendorf and added 1 µl deoxyribonucleotide triphosphates (dNTPs), 1 µl Taq DNA polymerase and 32 µl of distal water, 4 µl Taq DNA polymerase buffer and using 1 µl from both forward and reverse primers mentioned in section 4.3. primer for *L. bulgaricus* to give a 232 bp product for *Lactococcus lactis ssp. lactis* to give a 157 bp product for *Streptococcus thermophilus* to give a 157 bp product for *L. plantarum* to give a 300 bp product for *Lactobacillus spp* to give a 250 bp product for *Leuconostoc spp* to give a 976 bp product and for *pediococcus spp* to give a 701 bp product. The PCR conditions were as in Table 3.4**.**

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

First denaturation		Denaturation	Annealing	Extension	Final 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. Antibiotic Susceptibility Test

Resistance to antibiotics were tested using disc diffusion methods. The assay was carried out according to Khali (2009), in the disc diffusion test, the bacterial isolate is inoculated uniformly onto the surface of an agar plate and a filter disc impregnated with a standard amount of an antibiotic is applied to the surface of the plate, resulting in a gradient of the antibiotic surrounding the disk (Mayer, 2007). Following incubation a bacterial lawn appears on the plate and zones of inhibition of bacterial growth would be present around the antibiotic disc. The test is performed under standardized conditions hence the size of the inhibition zone is dependent on the degree of sensitivity of the microorganism to the antibiotic (Mayer, 2007).

10 ml of each of MRS and GM17 agar were prepared poured in test tube then underwent to the autoclave and 50 µl of stock culture were taken, added to 5 ml of MRS and GM17 broth that autoclaved before then incubated for 24 hours at 30 ºC for MRS and at 37 ºC for GM17. After that the MRS, GM17 agar tube were boiled until it became liquid then left at room temperature for 2-3 minute. After that 100 µl from culture broth added to both agar and has been spilled on Petri dish and left for 20-30 min to became solid and then antibiotic disc of Vancomycin 30 mcg , Penicillin 10 µg, Tetracycline 30 µg, Ciprofloxacin 5 µg, Trimethoprim 2.5 µg, Ampicillin 10 mcg, Erythromycin 15 µg, Amoxicillin 25 mcg, Gentamycin 10 µg, Amoxicillin 25 mcg were applied on the solidified agar surface plate. The whole process it has been done under aseptic condition. Thereafter agar plates with antibiotic discs incubated for 24 h at 30 ºC or 37 ºC depending on the agar media, 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).

3.10. Plasmid DNA Digestion

Sal I (Bio Labs), *Bgl II* (Sigma), *EcoR I* (Fermentas) and *Mbol II* (Fermentas) was used as restriction enzyme for digestion of Plasmid DNA. the digestion reaction was prepared as follows: 1 µl of restriction enzyme buffer mixed with 1 µl bovine serum albumin (Fermentas) and 2 µl sterile distal water with 5 µl plasmid DNA and 1 µl restriction enzyme were added in a PCR eppendorf and incubated at 37 ºC overnight. The mixtures were then incubated at 65 ºC for 20 min to inactivate the restriction enzyme.

3.11. Metabolic End Products Analysis by HPLC

To analyze metabolic end products from the growth medium, 1 mL of an overnight culture was centrifuged at 14,800 *g* for 5 min. The supernatant was placed in a fresh eppendorf tube, then samples diluted with 1% of meta phosphoric acid and centrifuged again at 14800 *g* for 5 min. the supernatant harvested in to the new eppendorf. The sample was then analyzed by HPLC or was stored at -20°C until further analysis. The eluant was monitored at 210, 277 and 190 nm. For HPLC analysis, a Shimadzu Prominence HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a PD-M20A diode array detector and two binary gradient pumps (Shimadzu LC-10AT), autosampler (SIL 20AC), column oven (CTO-20AC), and a communication bus module (CBM-20A) with valve unit FCV-11AL was used. All metabolite standards were purchased from Sigma-Aldrich (Munich, Germany). The HPLC system was calibrated using standards for sodium pyruvate (10 mg/mL), sodium lactate (10 mg/mL), sodium formate (10 mg/mL), sodium acetate (10 mg/mL), acetoldehyde (10 mg/mL), ethanol (10 mg/mL), 2-3 butanediol (10 mg/mL) and 3 hydroxy (10 mg/mL). Quantification of the peak area was performed using a PC1000 chromatographic data analysis system (Thermo Separation Products, San Jose, CA).

4. RESULTS AND DISCUSION

4.1. Medium and Growth Conditions

Lactic acid bacteria at the level of species were obtained by two different media MRS and GM17 also by two different temperatures in order to cultivate lactobacillus spp. at 30 ºC in the case of MRS and as described in section 3.3.2 all ingredients supply the nutritive elements required for the growth of *lactobacilli* and essential for the growth of these bacteria.

Ammonium citrate and sodium acetate inhibit the development of most contaminants, including *streptococci* and molds. In the case of GM17 media incubation temperature increased to 37 ºC for cultivate *Streptococcus thermophilus* and to enumerate of *lactococci*.

4.2. Catalase Test and Morphological Characteristics of LAB Strains

57 strains from MRS media and 61 strains from GM17 media isolated as subculture within 52 collected samples of cheese, yogurt and cheese mixed with garlic were detected as catalase negative, gram positive cocci, bacilli and coccobacilli bacteria and categorized as LAB according to the Bergey's manua (1923), and especially those that have rod shape, gram positive, catalase negative related to the genus *Lactobacillus* (Kandier and Weiss, 1996).

4.3. Screening of LAB for Plasmids

A total of 118 strain isolated from dairy product of Northern Iraq by two different media MRS and GM17 were screened for presence of plasmid. Only 19 strains are found to be harbouring plasmids. Nine out of 19 strains were isolated from the culture grown on GM17 medium and these results were given in Figure 4.1 and 4.2. Rest of the strains we obtained from the MRS medium and gel photograph of these results were documented in Figure 4.3. Since lactic acid bacteria are gram positive they have thick cell-wall and distraction of that wall is difficult. For current study several protocols were used to harvest plasmid DNA, however all of them failed without knowing why? Apart from alkaline lysis protocol that mentioned in the Materials and methods, which gave clear and detectable plasmid DNA bands on agarose gel electrophoresis.

Results obtained through plasmids isolation indicate the presence of a considerable number of large plasmids in cheese and only one plasmid from yoghurt.

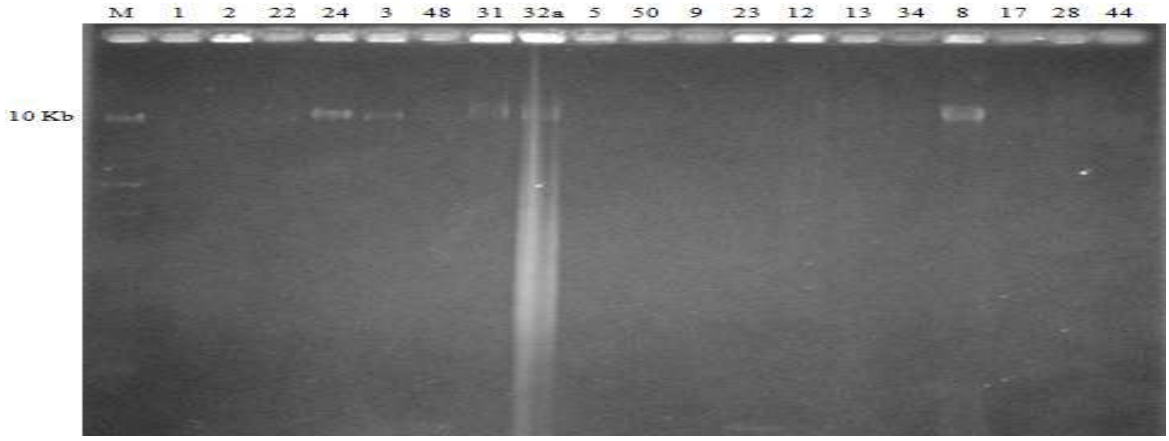

Figure 4.1. Electrophoresis of plasmid DNA extracted from LAB isolated on GM17 media. M: 1 Kb marker; 22: plasmid (10 Kb); 24: plasmid (>10 Kb); 3: plasmid (>10 Kb); 31: plasmid (>10 KB); 32a: plasmid (>10 KB); 8: plasmid (>10 Kb).

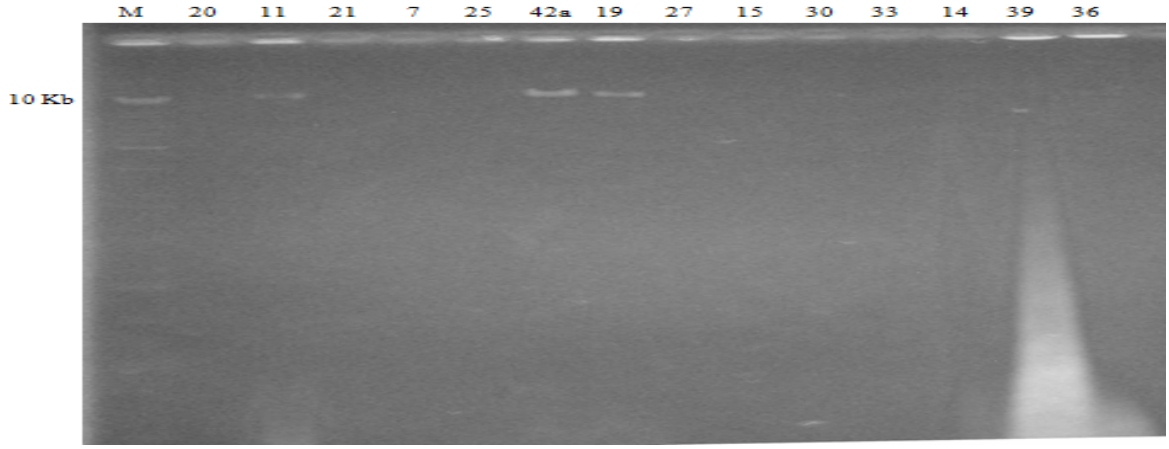

Figure 4.2. Electrophoresis of plasmid DNA extracted from LAB isolated on GM17 media. M: 1 Kb marker; 11: plasmid (10 Kb); 42a: plasmid (10Kb); 19: plasmid (10Kb).

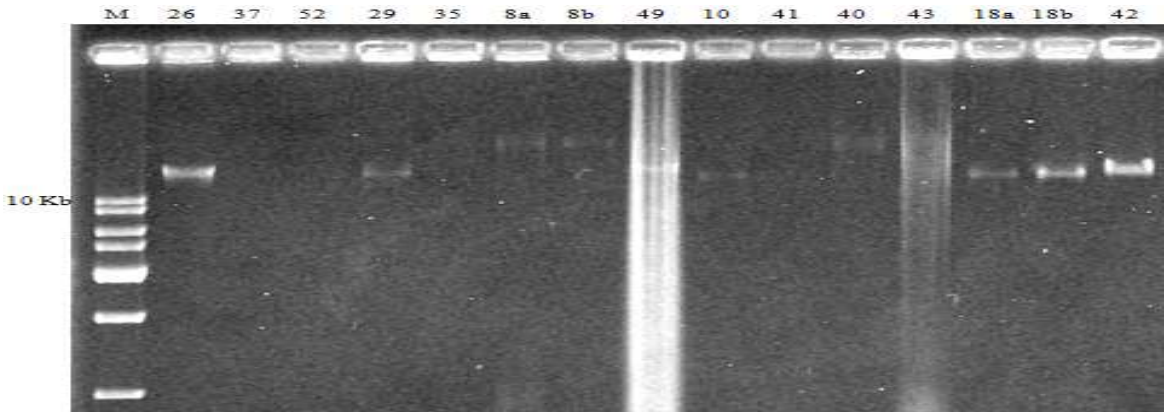

Figure 4.3. Electrophoresis of plasmid DNA extracted from LAB isolated on MRS media. M: 1 Kb marker; 26: plasmid (>10 Kb); 29: plasmid (>10 Kb); 8a: plasmid (>10 Kb); 8b: plasmid (>10 Kb); 49: plasmid (>10 Kb); 10: plasmid (>10 Kb); 40: plasmid (>10 Kb); 18a: plasmid (>10 Kb); 18b: plasmid (>10 Kb); 42: plasmid (>10 Kb).

4.4. Identification of Lactic Acid Bacteria that Contain Plasmids

All strains of LAB that contain plasmids underwent to PCR reaction and all of them identified as *Lactobacillus pluntaram* except strain 42 were identified as *Lactobacillus spp* and strain 49 which remain unidentified as is clear in Figure 4.4 according to a specific primers for LAB listed in Table 4.1.

Table 4.1. Species specific primers for lactic acid bacterial strains used in this thesis.

Species	Primers	Sequence 5'→ 3'
Lactobacillus spp.	LAB-F- LAB-R-	CTC AAA ACT AAA CAA AGT TTC CTT GTA CAC ACC GCC CGT CA
Pediococcus spp.	Pedio -F Pedio –R	GAA CTC GTG TAC GTT GAA AAG TGC TGA GCG TCC CTC CAT TGT TCA AAC AAG
Leuconostoc spp.	Leu F Leu R	TTT GTC TCC GAA GAG AAC A CGA AAG GTG CTT GCA CCT TTC AAG
Lactobacillus plantarum (recA)	PlanF PlanR	CCG TTT ATG CGG AAC ACC TA TCG GGA TTA CCA AAC ATC AC

Lactobacillus bulgaricus	YGLbF YGLbR	TCA AAG ATT CCT TCG GGA TG TAC GCA TCA TTG CCT TGG TA
Lactococcus lactis subsp. Lactis	LlacF LlacR	GTA CTT GTA CCG ACT GGT A GGG ATC ATC TTT GAG TGA T
Streptococcus thermophilus	YGStF YGStR	ACG CTG AAG AGA GGA GCT TG GCA ATT GCC CCT TTC AAA TA

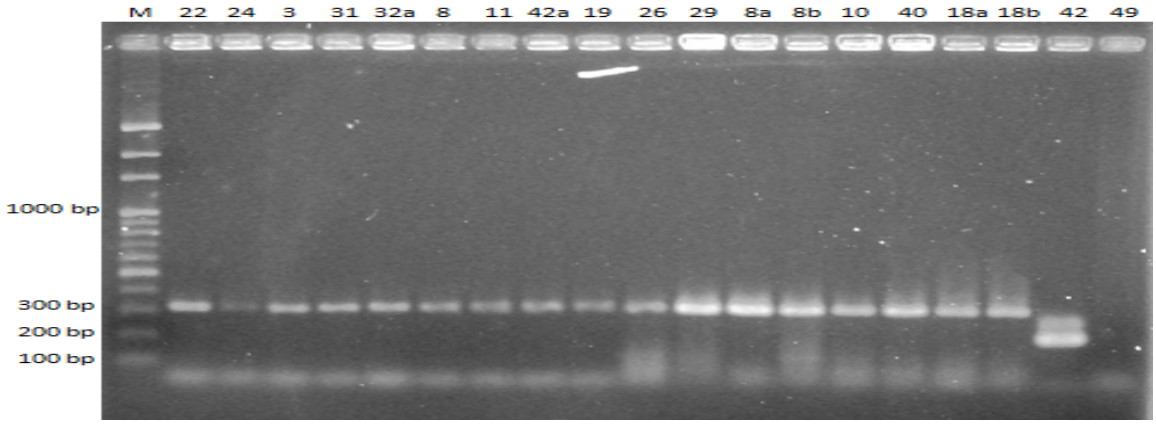

Figure 4.4. PCR products of amplified 16S rRNA gene spacer regions from different LAB. Lanes: M, molecular weight marker (100 bp); Lane 22 to Lane 18b have the same size (300bp) *Lb. plantarum*; Lane 42 (> 200 bp) *Lactobacillus Spp*.

Obtained results within lactic acid bacterial strain that harbour plasmids have been suggested that *Lactobacillus* genus especially *Lactobacillus plantarum* species were dominant according to the primers (PlanF, PlanR; Table 4.1) which were used to amplify the 16S region of rRNA gene. This primer pair was successful in amplifying a 300 bp fragment in 17 LAB strains out of 19 strains. Also primer (LABF, LABR) was successful in amplifying a > 200 bp fragment in one strain. In contrast no such fragment was amplified when DNA from *Pediococcus spp*. (701 bp), *Leuconostoc spp*. (976 bp), *Lactobacillus bulgaricus* (232 bp), *Lactococcus lactis ssp. lactis* (157 bp) and *Streptococcus thermopholus* (157 bp) were used as templates in the PCR assay.

4.5. Antibiotic Resistant Test

The 19 strain that harbor plasmid were assayed for susceptibility to 9 antibiotics, namely Vancomycin, Penicillin, Tetracycline, Ciprofloxacin, Trimethoprim, Ampicillin, Erythromycin, Amoxicillin and Gentamycin. Results showed in (Table 4.2) susceptibility of small number of strains to antibiotics. All strains were resistant to Gentamycin, Amoxicillin, Erythromycin, Ciprofloxacin, Trimethoprim, Penicillin, while the strains, 42a, 19, 8, 32a, 31 and 3 were observed with susceptibility to two antibiotics, Vancomycin and Tetracycline, with addition of strain 19 is also showed susceptibility to Ampicillin (Figure 4.5).

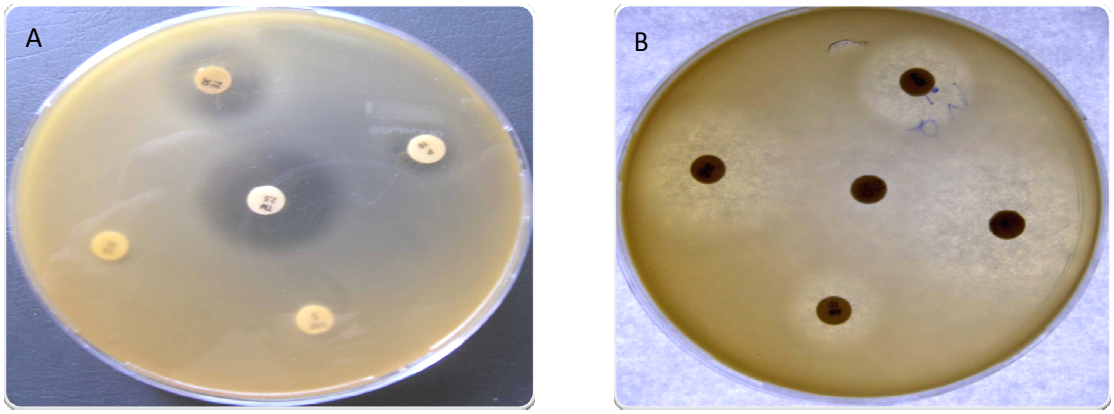

Figure 4.5. Antibiotic test to strains that harbour plasmids. (A) Strain 40 shows resistance to some antibiotics. (B) Strain 19 shows susceptibility to Vancomycin and Tetracycline.

Table 4.2. Antibiotic susceptibility tests to strains that contain plasmids. (++) Susceptible, (+) Intermediate Susceptible and (-) Resistant.

Strains	VA	P	TE	CIP	TM	AM	E	GN	AMX
MRS media									
18a	-	-	-	-	-	-	-	-	-
18b	-	-	-	-	+	-	-	-	-
8a	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-
8b	-	-	-	-	+	-	-	-	-
29	-	-	-	-	-	-	-	-	-
49	-	-	-	-	+	-	-	-	-
26	-	-	-	-	-	-	-	-	-
40	-	-	-	-	+	-	-	-	-
42	-	-	-	-	-	-	-	-	-
GM17 media									
11	-	-	-	-	-	-	+	-	-
42a	++	-	++	-	-	-	-	-	-
19	++	-	++	-	-	++	+	-	-
8	++	-	++	+	-	-	+	-	+
32a	++	-	++	-	-	+	+	-	-
31	++	-	++	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-
22	-	-	+	-	-	-	-	-	-
3	++	-	++	-	-	-	+	-	-

4.6. Digestion of Plasmids with Restriction Enzyme

The plasmids underwent to the digest with some restriction enzyme and their recognition sites were listed in the Table 4.3 to obtain different fragments. However plasmids have not recognition sites for the restriction endonucleases used (Figure 4.6).

Table 4.3. Restriction enzymes used in this project.

Restriction Enzyme	Recognition sequence
Sal I	5' G↓TCGAC 3' 3' CAGCT↑G 5'
Bg1 II	5' A↓GATCT 3' 3' TCTAG↑A 5'
EcoR I	5' G↓AATTC 3' 3' CTTAA↑G 5'
Mbol II	5' GAAGA (N) 8↓….3' 3' CT T CT (N) 7↑….5'

↑ ↓ indicate sites where the sugar-phosphate backbone is cut by the enzyme.

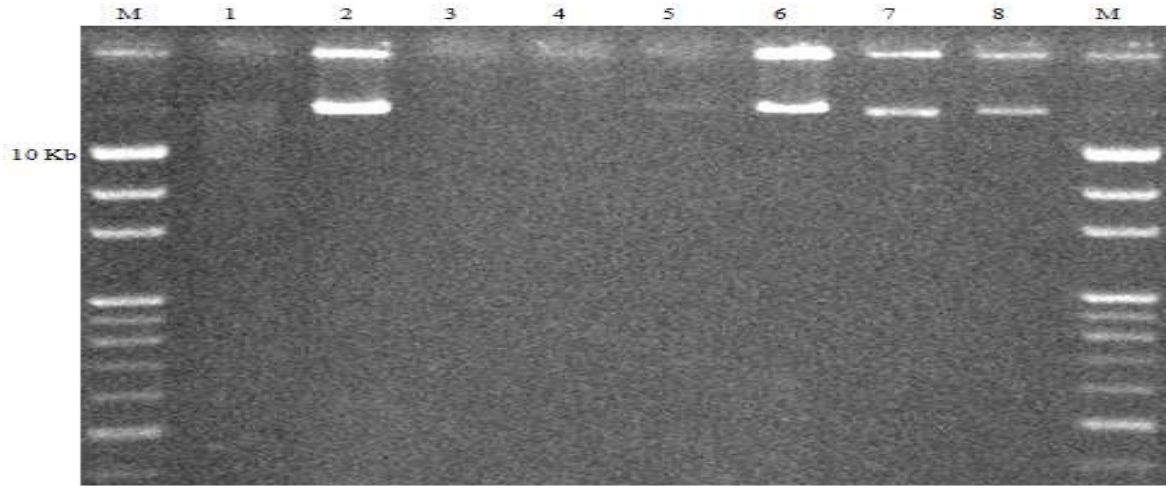

Figure 4.6. Plasmid digested with restriction enzyme: M: 1Kb marker, Lane (1) 24 digested with Sal I, Lane (2) 24 undigested (as control), Lane (3) 11 digested with Bgl II, Lane (4) 11 undigested, Lane (5) 42 digested with EcoR I, Lane (6) 42 undigested, Lane (7) 19 digested with Mbol II, Lane (8) 19 undigested.

4.7. Metabolic End-Product Analysis

The activity of some *L.plantarum* strains that contain plasmids and others not contain plasmids was studied by analyzing the metabolic end products to compare between them. The supernatants after overnight growth were analyzed by HPLC to measure the metabolic activity. When end-product formation from these strains was examined by HPLC, different level of metabolite values were observed between all strains under aerobic conditions (Table 4.4). According to the results of HPLC analysis, strains that contain and not contain plasmids showed a heterofermentative character under aerobic conditions, and it produced a mixture of Acetic acid, formate, pyruvate, lactate, 3 hydroxy, acetaldehydrate, ethanol and 2-3 butanediol as the end product.

Table 4.4. Main end products (mg/ml) formation from the metabolism of strains that contain and non contain plasmids under aerobic conditions.

Strains	Acetic acid	Formate	Pyruvate	Lactate	3 hydroxy	Acetaldehydrate	Ethanol	2-3 butanediol
24	61.30±26.59	49.16±15.63	0.54±0.13	50.82±12.69	16.40±4.24	77.12±17.09	111.65±22.52	9.51±0.76
3	61.19±6.53	35.13±11.46	6.89±1.15	37.80±5.09	7.17±0.97	26.62±11.23	138.24±60.23	5.37±0.42
31	71.24±4.78	2.03±0.12	3.85±0.27	35.17±3.89	6.44±1.87	5.05±0.34	122.20±10.73	12.57±1.20
32a	68.11±8.06	2.22±0.52	3.97±2.04	32.02±2.96	6.11±1.42	4.51±0.31	105.30±4.50	12.11±1.32
8	68.54±2.30	4.89±0.09	5.52±0.39	31.74±0.61	6.47±1.31	37.57±0.98	205.79±17.12	7.95±0.44
42	69.38±7.44	7.40±4.80	4.78±2.83	32.81±0.38	4.37±0.29	5.10±0.80	155.50±8.75	11.46±0.48
42a	67.83±4.56	4.12±3.20	5.20±0.83	33.52±2.54	5.78±1.12	48.06±2.80	196.24±38.15	9.08±0.09
19	61.96±1.07	5.71±0.58	4.23±0.21	32.35±0.57	7.16±2.38	25.07±7.03	171.06±50.76	8.83±0.72
12*	48.43±7.66	19.18±3.40	2.43±0.71	32.10±3.92	3.92±0.69	7.52±1.13	93.29±26.17	13.31±1.94
13*	55.37±18.33	1.33±0.47	2.70	33.98±11.85	6.56±3.23	109.02±46.84	90.18±27.76	11.06±3.80
14*	51.79±3.46	1.29±0.32	2.77±1.61	34.77±3.11	6.48±0.93	5.22±1.03	90.46±6.74	13.78±0.07
29*	35.24±4.32	1.78±0.10	2.87±1.51	47.17±1.44	16.55±11.03	178.36±31.58	75.25±28.37	11.33±1.52

* Strains have not contain plasmid

DISCUSSION

Unlike gram-negative bacteria, which are easily lysed using standard protocols, Gram-positive species are relatively more resistant to cellular lysis resulting from the extensive concentration of peptidoglycan within the cell wall. Therefore alkaline-detergent lysis methods (Klaenhammer, 1984) was used in present study. Klaenhammer during his study discovered that mutanolysin was more effective than lysozyme because it acted quickly in generating osmotically fragile cells. *Lactobacillus plantarum* speices were particularly insusceptible to lysozyme these data are consistent with previous reports noting the resistance of *lactobacilli* to lysozyme (Chassy and Giuffrida, 1980; Neujahr *et al*., 1973) and sensitive of gram positive bacteria to mutanolysin (Kondo and McKay, 1982; Monsen *et al.,* 1983; Yokogawa *et al*., 1975). The results obtained throughout this research compared to other research conducted in the field of isolating plasmids from different samples were fairly good. The experimental condition of current study was most directly comparable to those of O'Sullivan and Klaenhammer (1993) they found large plasmids were isolated from both *lactococci* and *lactobacilli,* including a 70-kb plasmid from *lactobacillus acidophilus* strain C7. A published methods suggested for the isolation of plasmid DNA from lactic streptococci (Anderson and McKay, 1983) was suitable for the extraction of plasmid DNA larger than 30 megadaltons, Through this protocol can be isolated amounts of plasmid DNA that required in molecular cloning as he has conducted by Richard *et al.,* (1985) within 23 strains of *Streptococcus thermophilus* examined, 5 were found to contain a single small cryptic plasmid. Another procedure reported for isolating plasmid of lactic streptococci yielded highly pure plasmid DNA but the protocol required rigidly controlled conditions including low-temprature incubations steps (Orberg and Sandine, 1984) and method was applicable to plasmids of at least 40 megadaltons. Methods that mentioned in present research was preferable over either of these two protocol since it yields highly purified plasmid DNA and may be carried out under ordinary laboratory conditions, 19 Plasmid (> 10 Kb) were detected in all strains of lactic acid bacteria and in the term of genus most of them are identified as *L. plantarum*.

The results indicate that some strains that harbor plasmids possesses antibiotic resistant gene against Vancomysin, Tetracycline and only one strain against Ampicillin, the present findings are in accordance with those of Elisha and Courvalin (1995) who recorded that many strains of *Lb. plantarum, Lb. casei, Lb. salivarius, Lb. leichmannii, Lb. acidophilus* carry intrinsic resistance towards Vancomycin which is due to the presence of D-alanine: D-alanine ligase-related enzymes. Intrinsic vancomycin resistance of *Lactobacillus, Leuconostoc* and *Pediococcus* species have been used to separate them from other Gram-positive bacteria on Vancomycin supplemented selective media (Simpson *et al.* 1988), While *tet(M*) has been found on a plasmid with a size of ca. 10 kb (Danielsen, 2002; Gevers *et al*., 2003b), which was also the case in the present study. On the other hand there is published research that recorded some *Streptococcus thermophilus* strains were isolated from yogurt samples carrying only one or no plasmid were observed to be susceptible to most antibiotics that used in his research (Aslim and Beyatli, 2004).

The results in this research indicate all strains whether contain plasmids or not have heterofermentative characteristic and that not comparable with those of Lindgren *et al* (1989) they found *lactobacillus plantarum* strains used as silage inoculante have homofermentative when glucose was absent from the medium and incubated anaerobically for (7-30 days), while when citrate was present in the medium together with the glucose during the initial fermentation the lactic acid produced was degraded.

5. CONCLUSION AND FUTURE PERSPECTIVE

Lactic acid bacteria (LAB) are common to the dairy industry, that produce lactic acid as the principle by-product of sugar fermentations. LAB are common in nature and are often associated with plant materials. They can also be found as part of the resident microflora of human and other mammals (e.g., oral cavity, gastro-intestinal track, etc.). The cultures of bacteria used in the manufacture of cheese and other fermented milk products are known as starters. They play a vital role in the production of these dairy products; they produce the lactic acid that influences important quality characteristics such as texture, moisture content and taste.

The objective of this research was to derive plasmids of the total 118 bacterial strain, results obtained were 19 plasmids in all strains and no plasmid DNA was detected for the other 99 isolated strains according to the protocol that mentioned before in material and methods, and that the isolates that they contain plasmids have been identified as *lactobacillus plantarum* and one was *lactobacillus spp*. and only one strain has not been identified. Therefore the difficulty in isolating plasmid DNA from *lactobacilli* may be associated with the lysozyme insensitivity of this genus (Neujahr *et al.,* 1973). Most of *Lactobacillu*s species, regardless of their source, harbour at least one indigenous plasmid (Pouwels and Leer, 1993). The functions of these plasmids have classically been correlated with phenotypical properties, including drug resistance, carbohydrate metabolism, bacteriocin production and amino acid metabolism. The discovery of plasmid DNA in the lactic acid bacteria is generally attributed to Cords *et al.* (1974) and has since been correlated with a number of commercially associated phenotypes in lactic acid bacteria, including lactose metabolism, proteinase activity, citrate fermentation, bacteriocin production, drug resistance, sugar transport and metabolism and the phage resistance mechanisms of restriction/ modification, adsorption resistance and abortive infection so on (Carr *et al.,* 2002; Cebra, 1999; Fernandes *et al.*, 1987; Zhou *et al.*, 2000).

In the other hand, results of current study shows that certain strains which would be used in manufacture of home-made yoghurt, cheese using natural starter have various antibiotic resistant activity, vancomycin and tetracycline among the antibiotic disc that used in this study were more susceptible to strains that have plasmids.

According to these results, in some strains the resistance to some antibiotics 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 b-lactamases (penicillinases) produced by many Gram-positive and Gram-negative bacteria. The enzyme is coded by chromosomal or plasmid genes (Hardy, 1981).

In the light of these results, it will be useful to determine other technological characteristics such as bacteriophage resistance, proteolytic and lipolytic activities, aroma and flavor compound production, antimicrobial activities and exopolysaccharide production as well as bioamines. Genes residing within the plasmids related to such functional properties could also be investigated. Function and expression of these genes should be carried out to provide an improved understanding. Technological methods could be developed to characterize these strains in order to provide the use of them in the dairy industry. Finally, strains that were genotypically characterized in this study could be selected by technological methods and used as starter cultures for the fermentation of foods in dairy industry.

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