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Subjecting Local *Lactobacillus* Isolates to Mutagensis for Improving Inhibitory Activity Against *Staphylococcus aureus*.

A Thesis

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By

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Dedication

Above all i would like to dedicate my thesis to my parents; you have given me so much, thanks for your faith in me, everlasting love, and for teaching me that I should never surrender.

To my supervisor Prof. Dr. Abdul Wahid B. Al-Shaibani, your unconditional support and guidance throughout my work was most helpful. It was honorable to be your student.

Is also dedicated to my sisters (Enas and Nibras) and my brothers (Sameer and Mustafa) thanks for your understanding, patience and love during this long journey.

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Summary

Sixteen isolates of *Lactobacillus* spp. were obtained from different sources including dairy products, vinegar, pickles and two locally vaginal isolates by primary culturing on De Mannes Rogoza Sharp (MRS) agar before subjection to cultural, microscopic and biochemical tests. To examine their inhibitory activity against methicillin resistant *Staphylococcus aureus* (MRSA), all isolates were cultured once on solid and other in liquid MRS media. Results revealed that high inhibitory effect was detected by the isolates that propagated in liquid medium than on the solid. Upon such result, the MRS broth was used to propagate *Lactobacillus* isolates in the next experiments.

After propagation, filtrates of isolates were obtained. No observable inhibitory effect was detected against the test bacteria when the unconcentrated *Lactobacillus* filtrates were used. While, after concentration, the one-fold concentrated filtrates gave positive results, especially after 24 hours (hrs.) of incubation period. Among the 16 isolates, *Lactobacillus acidophilus* (Lb.G2) and *Lactobacillus gasseri* (Lb.G1) from vagina origin gave the best inhibitory activity, therefore, they were chosen for the further experiments.

Inhibitory effect of the two-fold concentrated filtrates was increased to (17) millimeter for *L. acidophilus* and (18) millimeter for *L. gasseri* in comparison to (12) millimeter and (11) millimeter, respectively, for the one-fold filtrates after incubation at 37°C for 24 hrs. Moreover, the three-fold concentrated filtrates gave the highest inhibitory activity with inhibition zones of (21) millimeter for *L. acidophilus* and (23) millimeter for *L. gasseri*.

When the effective compounds produced by the two lactic isolates were evaluated, organic acids were found to be the most responsible factor for the inhibitory effect against methicillin resistant *Staphylococcus aureus*.

Susceptibility of the lactic isolates toward (13) commonly-used antibiotics was also determined. Results declared that they were resistant to streptomycin,

ciprofloxacin, gentamycin, ampicillin and nalidixic acid, but sensitive to chloramphinicol, cephotaxime and amoxicillin, while they were varied in their sensitivity towards the rest antibiotics.

After subjection of the two isolates to mutagenesis for obtaining mutants with improved inhibitory effect against test microorganism, physical mutagenesis by ultra violet led to increase their antagonistic effect against the methicillin resistant *S. aureus* when their inhibition zones increased to (16.5) millimeter for *L. acidophilus* and (15) millimeter for *L. gasseri* by comparison with their wild type (12 and 11 millimeter) respectively. On the other hand, results of chemical mutagenesis revealed that inhibitory activity of the two isolates increased after treatment with ethedium bromide when the inhibition zones against *S. aureus* raised to (15.5) millimeter for *L. acidophilus* and to (14) millimeter for *L. gasseri* compared with the controls (12 and 11 millimeter), respectively. In contrast, treating with the acridin orange resulted in no effect on the isolates inhibitory effect.

Upon isolation and visualization of the plasmid contents of wild and mutant types, plasmid profile was found to be identical when each native isolate and its mutant carried one plasmid band.

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List of Abbreviations

Abbreviation	***Meaning***
D.W.	Distilled Water
DNA	Deoxyribonucleic acid
EDTA	Ethylene dye tetra acetic acid
EMS	Ethyl methane sulfonate
Kb	Kilobase
LAB	Lactic Acid Bacteria
L.	*Lactobacillus*
mg/ml	Milligram per milliliter
MRS	De Man Rogosa Sharpe
MRSA	Methicillin resistant *Staphylococcus aureus*
MNNG	N-Methyl-N-nitro-N- nitrosoguanidine
Mb	Megabase
NCCLs	National committee of clinical laboratories
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulfate
Spp.	Species
TBE	Tris borate EDTA

Chapter One

Introduction

and

Literatures Review

1. Introduction and Literatures review

1.1. Introduction

In addition to their pathogenic and deteriorating effects, microorganisms are known also by their various beneficial uses. Within these organisms, lactic acid bacteria are considered to be one of the most common useful group of bacteria. This group contains several genera and species used in food processing, especially in dairy fermentation, as well as in production of industrial and healthy metabolites.

Lactic acid bacteria are Gram-positive cocci and bacilli, non-sporulating and non-motile bacteria which produce lactic acid as a major or sole product from sugar fermentation (Salminen and Arvilommi, 2001). Historically, Lister in 1873 was the first scientist to isolate lactic acid bacteria (LAB) and named it as *Bacterium lactis*; which is now known as *Lactococcus lactics*). Later at the beginning of the 20th century, precisely in 1910 Metchinkoff proposed the first scientific phrase which is known today as "Probiotics" when he reported about the healthy benefits of yogurt fermented by LAB (Wood, 1998).

Probiotic term is derived from Greek two words "for life", and defined as "mono or mixed cultures of live microorganisms which beneficially affect the host upon ingestion by improving properties of endogenous microflora" (Dhanasekaran *et al*., 2010). Lactic acid bacteria have the ability to inhibit various Gram-positive and Gram-negative bacteria, since they can produce a variety of antimicrobial substances including: organic acids, carbon dioxide, hydrogen peroxide and bacteriocins (Javed, 2009). These substances provide LAB with a protective effect against several food-borne and other pathogenic bacteria such as *Clostridium botulinum, Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* (*S. aureus*) (Alvarez-Olmos and Oberhelman, 2001).

Continuous increasing in the antibiotics resistancey among bacterial populations makes the scientific and commercial sectors be more interested in

using probiotics as alternatives in the treatment and prevention of microbial diseases (Petrova *et al*., 2009). Lactic acid bacteria exhibited a potential inhibitory effect against food-poisoning and infectious *S. aureus* and this inhibition affected by different factors like LAB species, media and type of produced antimicrobial substances (Charlier *et al*., 2009).

It was reported that production of H_2O_2 by some LAB has inhibitory effect towards *S. aureus* (Haines and Harmon, 1973). Also, bacteriocins of lactic acid bacteria were found to have effect in reducing level of *S. aureus* in the contaminated foods (Arqués *et al*., 2005). *Staphylococcus aureus* is a major opportunistic pathogen that can cause a variety of infections such as skin abscesses, bone and soft tissue surgical infections, sepsis and toxic shock syndrome (TSS) (Z´arate *et al*., 2007).

One of the most medically important *S. aureus* strains is Methicillin resistant *S. aureus* shortly (MRSA). The strains of this bacteria are found to be not only resistant to the methicillin, but also to certain other antibiotics including, oxacillin, penicillin and amoxicillin (Abd El-Moez *et al*., 2011). Due to the increasing incidents of the antibiotics resistancey, searching for alternative medical treatment has been practiced (Settanni and Corsetti, 2008). LAB is known as friendly and safe bacteria, so it might be suitable as alternative treatment against MRSA (Tinh *et al*., 2007).

Mutagenesis is considered to be as a beneficial genetic engineering technique that could be used to improve the inhibitory effect of various probiotic microorganisms, such as lactic acid bacteria (McKay and Baldwin, 1990).

Rodríguez-Quiñones *et al.* (1984) declared that existing strains of LAB may be subjected to the mutagenesis followed by selection of mutants with new traits.

Such LAB mutagenesis might be of interest for fermented dairy foods and probiotic therapy (Le Blanc *et al*., 2010). LAB characterized by low content of

G-C and small genome size ranging between (1.8 and 3.3) Mb. About 38% of species of the genus *Lactobacillus* were found to contain plasmids with different sizes (from 1.2 to 150 Kb) with varied copy numbers (1 or more) (Rodríguez and Vidal, 1990).

It was found that some of *Lactobacillus* plasmids were encoding to several cellular functions like metabolism of carbohydrates, production of bacteriocins, exopolysaccharides and antibiotic resistance (Wang and Lee, 1997). Ultimately, continuous search and investigations are required to overcome the problem of new arose MRSA strains, as well as for improving the inhibitory activity of LAB through mutagenesis.

Therefore, this study was aimed to isolation, identification and selection of an efficient isolates of lactic acid bacteria to be used as a probiotic against methicillin-resistant *Staphylococcus aureus,* then subjection of the selected isolates to chemical and physical mutagenesis in order to obtain more efficient mutants in this regard.

1.2 Literatures Review

1.2.1 Probiotics:

The fact of using harmless bacteria for inhibiting pathogens has been recognized for many years. Generally, probiotics have been used for as long as people have eaten fermented foods (Rolfe, 2000). Historically, it was Metchnikoff at 1907 who first suggested that ingested bacteria could have a positive influence on the normal microbial flora of the intestinal tract. He hypothesized that lactobacilli were important for human health, and considered yoghurt and other fermented foods as healthy (Mozzi *et al*., 2010).

The term "probiotic" which is derived from the Greek language, meaning “for life” was first used by Lilly and Stillwell in 1965 to describe “substances secreted by one microorganism which stimulates the growth of another”

(Schrezenmeir and De verse, 2001). An accurate definition of probiotics was then given by Fuller, (1989) who redefined it as "a live microbial feed supplement beneficial to the host (man or animal) by improving microbial balance within its body ".

Most probiotics fall into the group of microorganisms known as lactic acid-producing bacteria (Parvez *et al*., 2006); such as *Lactobacillus*, *Bifidobacterium* and the yeast *Saccharomyces boulardii*. Probiotics are normally consumed in the form of yoghurt, fermented milks or other fermented foods (Agarwal, 2008).

Probiotics have become widely accepted as a natural means to promote human and animal health which is taken as safe supplements in food and feeds. They can be used to replace the antibiotic therapy or chemical supplements (Kosin and Rakshit, 2006).

The belief in beneficial effects of probiotics is due to the knowledge that intestinal flora can protect humans against infection, and disturbance of this flora can increase susceptibility to infection (Rolfe, 2000).

Consumption of probiotics has several prophylactic and therapeutic effects which are approved clinically (Ouwehand *et al*., 2002; Kaur *et al*., 2002; Mercenier *et al*., 2003). These effects are:

- Balancing the intestinal flora.
- Increasing lactose tolerance and ingestion.
- Absorption of calcium.
- Modulating the immunological system.
- Increased nutritional value (better digestibility, increase bioavailability of minerals and vitamins).
- Promotion recovery from diarrhea (rotavirus, travelers).
- Prevention of intestinal tract infections (bacteria or virus induced, candida enteritis, *Helicobacter pylori* ulcers, neoplasia).
- Reduction of catabolic products eliminated by kidney and liver.

- Prevention of arteriosclerosis (reduction of serum cholesterol).
- Improved well-being.
- Synthesized nutrients (folic acid, niacin, riboflavin, vitamins B_6 and B_{12}).
- Decreasing prevalence of allergy in susceptible individuals.
- Reduction of blood pressure in hypertensives.

Production of antimicrobial substances is one of the selection criteria for probiotics, and many probiotic bacteria were found to produce them. In addition to the growth-inhibiting metabolites such as organic acids and hydrogen peroxide, other substances such as bacteriocins and adhesion inhibitors are also produced (Tuomola *et al*., 2001).

Several mechanisms may be used by the probiotic organisms to inhibit pathogens *invitro* and *invivo* such as: producing a variety of inhibiting substances, utilizing nutrients that may consumed by pathogenic microorganisms and blocking off adhesion sites on intestinal epithelial surfaces (Sherman *et al*., 2009). Because of their natural adaptation to the gut environment and their beneficial impact, lactic acid bacteria are considered to be the best choice for not only improving the microbial safety of food products but as a probiotic supplements (Mishra and Lambert, 1996).

So, during the last decades progressive attention has been focused on biological and molecular characterization and improvement of these bacteria (Ajmal and Ahmed, 2009).

1.2.2 Lactic acid bacteria (LAB):

1.2.2.1 History:

Lactic acid bacteria have been involved for thousands of years in food fermentations and considered as one of the most ancient preservation techniques. First signs of LAB utilization date back to the BC time, describing the fermentation of milk, meat (1.500 BC) and vegetable products (300 BC)

(Mansilla, 2008). Terms such as "milk souring" and "lactic acid producing" bacteria were used previously to name lactic acid bacteria (Magnusson, 2003).

According to Wee *et al*. (2006) lactic acid bacteria were first discovered in sour milk by Scheele in 1780 who initially considered it a milk component. In 1789 Lavoisier named this milk component "acide lactique" which became origin of the current terminology for lactic acid bacteria then, in 1857 Pasteur reported that it was not a milk component but certain microorganisms. Later, in 1878 lactic acid bacteria was isolated by Lister from rancid milk.

In 1900, it was also isolated from intestinal tract by Moro who named it *Bacillus acidophilus* due to its unusual acid tolerance (Azizpour *et al.,* 2009). In 1930, Dr. Minoru Shirota made the first stable culture of *Lactobacillus casei* strain Shirota which then in 1945 was incorporated in dairy products with promoted health benefits (Vasilievic and Shah, 2008). Products fermented and preserved by LAB proved to be safe for human consumption and have increased quality and functions (digestibility, taste and flavor), also these microorganisms possessed strong antagonistic effect against spoilage microorganisms and pathogens (Taniguchi *et al*., 1998).

1.2.2.2 Characterization and occurrence:

Lactic acid bacteria are Gram positive, rod or cocci in shape, heterotrophic usually non-motile, non-sporulating and produce a main product which is lactic acid (Gasson and De Vos, 1994). They also characterized as catalase-negative organisms that are devoid of cytochromes, strictly anaerobic or aerotolerant, fastidious, acid tolerant and strictly fermentive. LAB are considered to be mesophilic to slightly thermophilic bacteria with 5-45˚C as a range for their growth temperature (Buchanan and Gibbons, 1974).

These microorganisms also lack many biosynthetic capabilities; thus, they need complex nutritional requirements of amino acids and vitamins. Due to this complexity, LAB can be found in nutrient rich environments such as milk,

meat, decomposing plant materials, and fermented foods (yoghurt, cheese, olives, pickles), oral cavity, gastrointestinal tract and vagina of humans and other animals (Axelsson, 2004; Ljungh and Wadström, 2008).

Lactic acid bacteria are used as natural or selected starters in food fermentation such as dairy products, infant foods, meats and beverages in which they perform acidification through producing lactic and acetic acids flavor (Sybesma *et al*., 2006; Parada *et al*., 2007). Many species of LAB have significant role in improving the shelf life of foods and inhibiting pathogenic and spoilage microorganisms by producing antagonistic substances such as organic acids and bacteriocins (Cinats *et al*., 2001).

1.2.2.3 Classification of lactic acid bacteria:

Lactic acid bacteria consist of six genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Enterococococcus*, and *Streptococcus*. These divers genera are mainly grouped into either homofermenters or heterofermenters based on the end products of their sugar fermentation (Axelsson, 2004).

The homofermentatives produce lactic acid as the major end product of glucose fermentation, while the heterofermentative produce a number of products beside lactic acid, including, carbon dioxide, acetic acid and ethanol (Buchanan and Gibbons, 1974).

1.2.3 Genus *Lactobacillus*:

Lactobacilli belong to the lactic acid bacteria since their main end product of carbohydrate metabolism is lactic acid (Bešić, 2008). This genus comprises a large heterogeneous group of low G-C Gram positive bacteria. They are nutritionally fastidious and requiring rich media (carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives, and vitamins) for their growth (Ljungh and Wadström, 2008).

Lactobacilli are Gram positive rods or coccobacilli and non spore-forming microorganisms. They are catalase-negative, fermentative microaerophilic or aerotolerant and chemo-organotrophic which requiring rich media to grow (Narins, 2003; Felis and Dellaglio, 2008).

Cells occur single, but more than often in chains of varying lengths, growth temperature ranges from 2 to 53˚C with the optimum between 30 to 40˚C, all species are aciduric with 5.5 to 6.2 as optimal growth pH (Holt and Krieg, 1986).

Genus *Lactobacillus* contains the most dominant species of lactic acid bacteria which are widely distributed in the nature. They can be found as a component of normal intestinal flora of healthy humans such as *L. acidophilus*; other species such as *L. bulgaricus* and *L. casei* are mostly found in dairy products, fruits and vegetables (Silva *et al*., 1987). Others are found in meat or fish products, cereal products, and waste water as well as a part of microflora of the oral cavity (Hultberg, 2006; Samac *et al*., 2009). *Lactobacillus* species are generally regarded as safe (GRAS) and therefore, they are widely used in production of probiotics (Modzelewska-Kapituła1 *et al*., 2008).

1.2.3.1 Classification of *Lactobacillus*:

Orla and Jensen originally classified *Lactobacillus* into three groups (the thermobacteria, streptobacteria and betabacteria) based on their growth temperature and ability to produce CO_2 from glucose, and these three names are still in common use (Kandler and Weiss, 1986).

The thermobacteria are thermo-tolerant and homofermentative, not producing carbon dioxide from glucose fermentation and unable to grow at or below 15˚C but grow at 45˚C or greater, they also fail to hydrolyze arginine. While, streptobacteria are homofermentative, mesophilic and produce lactic acid from glucose fermentation. Betabacteria grow optimally at 15˚C, heterofermentative and hydrolyze arginine (Robinson, 1990).

Another classification divided lactobacilli into three main groups based on their metabolic pathways and fermentation end products (Hammes and Vogel, 1995). These are:

Group I: Homofermentative bacteria utilizing the Embden Meyerhof-Parnas (EMP) pathway to produce lactic acid, examples on some species in this group are *L. acidophilus* and *L. delbrueckii*.

Group II: Facultative heterofermenters producing lactic acid or a mixture of lactic acid, acetic acid, ethanol and formic acid. This group contains several species like, *L. plantarum, L. casei, L. coryniformis, L. curvatus* and *L. farciminis*. Group III: Obligate heterofermentative organisms producing lactic acid, acetic acid, carbon dioxide and possibly ethanol. They are capable of growth at a wide range of temperatures (2-53˚C) but with optimum growth at 30-40˚C. This group contains *L. brevis*, *L. buchneri*, *L. bifermentans*, *L. fermentum*, *L. cellobiosus* and *L. viridescens*.

1.2.4 Antimicrobial compounds produced by LAB:

Lactic acid bacteria are industrially important micoorganisms because of their fermentative ability as well as their health and nutritional benefits. LAB are provided with a competitive advantage over other microorganisms by producing metabolites and reductive products during their carbohydrate fermentation which can significantly affect growth of many spoilage bacteria and pathogens (Mishra and Lambert, 1996)

This antimicrobial activity is expressed through synthesized organic acids (lactic and acetic acids), hydrogen peroxide, carbon dioxide, diacetyl and bacteriocins (Samac *et al*., 2009).

1.2.4.1 Organic acids:

Sugars fermentation by lactic acid bacteria leads to reduction in pH due to the production of lactic and other organic acids such as acetic and propionic

acids which is an important factor against growth of undesired microorganisms (Parada *et al*., 2007). It is considered that direct antimicrobial effect of organic acids produced by most LAB species during fermentation process appears through their effect on cytoplasmic membrane by exchanging potentials and inhibiting active transport (De Vuyst and Vandamme, 1994; Cleveland *et al*., 2001).

Levels and types of organic acids depend on the species of microorganisms, culture composition and growth conditions (Ouwehand and Vesterlund, 2004). Lactic, acetic, and propionic acids and their mixture are approved for use in several industries as food preservatives and inhibitors against many spoilage and pathogenic microorganisms; also as acidulant, textile, and flavor as well as in pharmaceutical applications (Dumbrepatil *et al*., 2008; Ndaw *et al*., 2008).

- **Lactic Acid:**

Lactic acid is the final product of homofermentive activity of lactic acid bacteria (Yang, 2000). The first antimicrobial effect of lactic acid is reduction of pH, which makes lactic acid lipossoluble allowing breaking through cell membrane and reaching the cytoplasm of pathogens and reduces the rate of macromolecule synthesis.

Lactic acid can create a harmful environment for pathogenic *Escherichia coli*, *Salmonella* spp. and methicillin resistant *Staphylococcus aureus* (MRSA) by lowering intestinal pH (Anuradha, 2005).

Therefore, an acidic pH and short chain fatty acids (SCFA), especially lactic acid have been used as food preservatives to prevent the growth of contaminating microorganisms (Ogawa *et al*., 2001)**.** There are several growth factors which stimulate growth of lactic acid bacteria therefore, increasing the produced lactic acid rate such as peptides, mixture of amino acids and phosphates (Wee *et al*., 2006).

1.2.4.3 Hydrogen peroxide:

Hydrogen peroxide is produced by many lactic acid bacteria in presence of oxygen and since LAB lack catalase enzyme, H_2O_2 accumulates in the environment (Ammor *et al*., 2006).

The cytotoxic effect of hydrogen peroxide is due to its ability to act as intermediate in oxygen reduction to generate more reactive and cytotoxic free radicals such as hydroxyl ($OH^{\cdot}$) and superoxide ($O^{\cdot}$), which are powerful oxidants and can initiate oxidation of biomolecules (Byczkowski and Gessner, 1988).

Several studies reported that H_2O_2 has inhibitory effect against both Gram negative and positive bacteria (Mishra and Lambert, 1996). Its production by *Lactobacillus* and *Lactococcus* strains inhibited *Staphylococcus aureus* (*S. aureus*), *Pseudomonas* spp. and various spoilage microorganisms in foods (Cords and Dychdala, 1993).

1.2.4.4 Carbon dioxide (CO_2):

Carbon dioxide is produced heterofermentatively and contributes to the appearance of anaerobic conditions by which it inhibits aerobic microorganisms of decay such as molds (Cleveland *et al*., 2001). Accumulation of CO_2 in the membrane lipid bilayer may cause a dysfunction in permeability (Eklund, 1984).

1.2.4.5 Bacteriocins:

- **Discovery and effect:**

Lactic acid bacteria are capable of producing a wide range of protein compounds with an important antimicrobial effect which are known as bacteriocins (Hilmi, 2000). Bacteriocins were defined by Klaenhammer, (1988) as "Proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer microorganisms".

Another definition by Cotter *et al.* (2005) as the "Ribosomally synthesized proteins and peptides which have antimicrobial activity to compete with other bacteria of the same species (narrow spectrum) or to counteract bacteria of other genera (broad spectrum)".

Most studies have been focused on bacteriocins produced by lactic acid bacteria especially those by dairy starter cultures due to their potential industrial application as natural food preservatives (Dimov *et al*., 2005). It has been reported that LAB bacteriocins have antagonistic activity against food pathogenic microorganism such as *Listeria monocytogens*, *S. aureus*, *Clostridium* spp., *Enterococcus* spp., and *Bacillus* spp. (Holzapfel *et al*., 1995).

Bacteriocins of LAB have the potential to cover a very broad field of application including both the food industry and the medical sector (De vuyst and Frederic, 2007). Several bacteriocins from lactic acid bacteria have been detected, purified and characterized such as acidocin 8912 from *L. acidophilus* TK8912 and gassericin A from *L. gasseri* LA39 (Mojgani *et al*., 2006). LAB bacteriocins have attracted increasing attention, especially in food fermentation due to their preservative action in food products. Moreover, they are active in a nanomolar rang and have no toxicity (Gautam and Sharma, 2008).

- **Classification of bacteriocins:**

Klaenhammer, (1993) divided bacteriocins into four classes according to size, mode of action and structural characteristics. Class I; bacteriocins called "lantibiotics" which contain post-translationally modified amino acids. They usually contain 19 to over 50 amino acids (Altena *et al*., 2000). Class II; bacteriocins contain small heat-stable, non-lantibiotics, and non-modified peptides (Ukeyima *et al*., 2010). Class III bacteriocins are large and heat stable while class IV is composed of an undefined mixture of proteins, lipids and carbohydrates (Chikindas *et al*., 2001).

Depending on their spectrum of activity, bacteriocins can also be classified into two classes, one includes those active against bacteria taxonomically close to the producer strain, and the other class is composed of bacteriocins with a relatively broad spectrum of activity against Gram positive and some Gram negative bacteria (Rogelj and Bogovič-Matijašič, 1994).

- **Mode of action:**

Bacteriocins mode of action on sensitive cells is a two step process; first is the adsorption of bacteriocins on specific or non specific receptors on cell envelopes of host bacteria. The second irreversible step involves pathological changes in the target cell which is specific to each bacteriocin (Tagg *et al*., 1976; Deegan *et al*., 2006). For instance, Mesentericin Y105 which produced by *Leuconostoc mesenteroides* Y105 is membrane active peptides which act to form pores in cell membrane of the antagonized cells (Fleury *et al*., 1996).

1.2.5 *Staphylococcus*:

Staphylococcus belongs to the family *Micrococcaceae*. This genus is characterized as Gram positive cocci that occur in grape-like clusters which varies from (0.5-1.5) μm in diameter. It is wide spread in the nature and many species belong to this genus found as normal flora on the skin and mucous membranes of the upper respiratory tract of mammals.

Staphylococcus aureus (*S. aureus*) is considered as the most medically important species of this genus (Anthoniraj *et al*., 2005). It is known as "the golden staph" which is the most common cause for several diseases such as wound infections, blood poisoning (Sepsis) and toxic shock syndrome (TSS) (Iqbal, 1998).

In addition to that, the nosocomial infections that are caused by a special strain of *S. aurous* named as methicilin resistant *S. aureus* shortly known as "MRSA" (Prescott *et al*., 2002).

Staphylococcus aureus is considered most virulent and clinically important among other *Staphylococcus* species due to its several potent virulence factors such as: surface proteins (promote colonization of host tissues), capsule (surface factor that inhibit phagocytic engulfment), biochemical properties (helped in their survival in the phagocytes like catalase production) and production of several types of toxins like hemolysins that lyses cell membranes (Talaro and Talaro, 2006).

1.2.6 Genetics of lactic acid bacteria:

Lactic acid bacteria are Gram-positive bacteria with low G-C content and characterized by small genomes ranging between (1800-3300) Kbp in size (Klaenhammer *et al*., 2005). For *Lactobacillus* genus it had a genome size ≈ 2000 Kbp and a number of genes spanning ≈ (1600-3000) depending on species (Makarova and Koonin, 2007). Most of *Lactobacillus* species carry one or more of plasmids varied in their size ranging from 1.2 to 150 Kbp, and these plasmids coding for several biological processes, like lactose metabolism, bacteriocin production and exopolysacharide (EPS) production (Alpert *et al*., 2003). Nowroozi and Mirzaii, (2004) found that *Lactobacillus plantarum* carries one plasmid with 4.5 Kbp in size.

Dimov *et al.* (2005) reported that most of bacteriocin genes are located on plasmids. Genome analysis of LAB had revealed important information about their metabolic processes and bioprocessing capabilities which reflect their nutrient diversity and the range of environments they inhabit in addition to their potential roles in health and well-being as probiotics (Klaenhammer, *et al*., 2005).

1.2.7 Mutagenesis of LAB:

Mutagenesis means the induction of inheritable changes "mutations" into the genetic material of any organism resulting in changing its genotype and can

be detected as modified phenotype of the organism which named as "mutant of the wild type" (Pühler, 1993). Mutagenesis process is important in many aspects and considered a basic requirement of evolution such as in microbial strains improvement and in plant cultivation for breeding new varieties (Lewin, 2004).

Occurrence rate of mutations can be increased by treatment with chemical compounds (such as acridin orange) or physical mutagens (ultra violate irradiation). Thus, mutagens can be defined as "factors that increase the rate of mutation above spontaneous rate (Dale and Park, 2004). Most mutagens act directly by modifying a particular base of DNA strand resulting in miss-pairing during replication process, or indirectly by incorporating into nucleic acid strands (intercalating agents) and cause structural changes that lead to miscopying of the template DNA during replication process (Campbell *et al.,* 2005).

Yoghurt made with a mutant strain of *Lactobacillus bulgaricus* which had altered lactose metabolism and limited fermentation capacity is characterized by little post-fermentation acidification during the shelf life period (Mollet and Delley, 1990).

The genetic alterations that occurs during exposing LAB to mutagenic conditions such as UV irradiation or chemicals like, MNNG or ethyl methyl sulphate may resulted in identification of new strains with improved traits like, flavors and nutritional value which may attract both food industry as well as consumers (Sybesma *et al*., 2006). In a study done by Ramnelsberg *et al*. (1990) it was observed that treating *Lactobacillus casei* with mutagen mitomycin C induced a 5 to 7 fold increase in caseicin synthesis.

1.2.7.1 Chemical mutagens:

- **Acridine orange:**

The acridins are chemicals widely used in many medical, industrial and scientific fields. Acridin orange and its derivatives have the ability to form

complexes with DNA by intercalation between adjacent nucleotides pairs with the help of its planner aromatic ring system which has dimensions similar to the Watson-Crick base pairs (3-10˚A) (Figure 2-1), and this results in multiple biological effects (Ladoulis and Gill, 1970; Hoffmann *et al*., 2003).

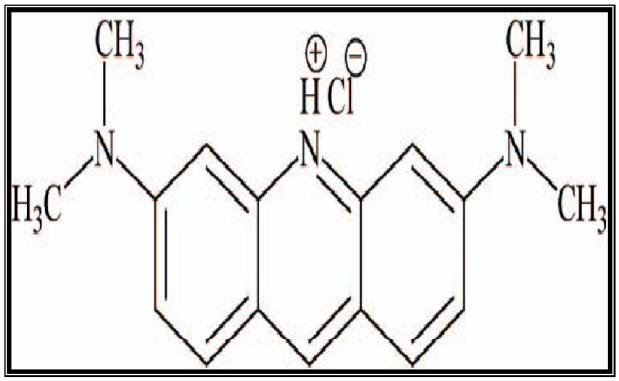


Figure 2-1: Chemical structure of acridine orange. (Hoffmann *et al*., 2003)

It has been reported that these acridine orange-DNA (A.O-DNA) complexes inhibit cell growth as well as synthesis of DNA, RNA and protein. Also it can induce morphological changes, inhibit DNA repair, cause curing of plasmids and induce mutations in a wide variety of organisms (Marcos *et al*., 1987). A study by Margino *et al*. (1998) on *Lactobacillus plantarum* that has been mutagenized by acridine orange showed increasing in antibacterial compounds in the mutants comparing with the wild-type.

- **Ethidium bromide:**

Ethidium Bromide (Et.Br) is the common name for 3, 8-diamino-5-ethyl-6-phenylphenanthridinium bromide (Figure 2-2), an intercalating agent usually used in molecular genetics and in the structural studies of DNA (Ouchi, 2007). This cationic dye which interacts strongly and specifically with double helical RNAs and DNAs, is widely used in spectrofluorimetric studies because of the striking fluorescence that displays upon binding. It is also used in molecular

studies as nucleic acid stain such as in agarose gel electrophoresis. Also it has been used to probe tRNA structure, chromatin structure and ribosomal RNA (Olmsted and Kearns, 1977).

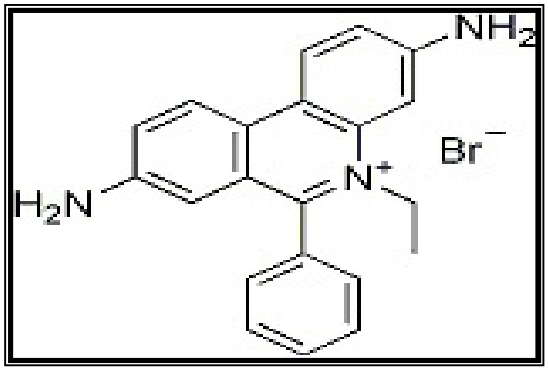


Figure 2-2: Ethidium bromide structure (Tomchick and Mandel, 1964)

The planner ring of Et.Br dye is completely intercalated between DNA base pairs by the formation of hydrogen bonds between amino groups of ethidium bromide ring and phosphate group of the polynucleotides (Luedtke *et al.,* 2003).

The resulting Et.Br-DNA complex may inhibit enzymes involved in the synthesis of nucleic acids such as DNA and RNA polymerases which leads finally to inhibit DNA replication and RNA transcription (Aktipis and Martz, 1974; Baranovsky *et al*., 2009).

The mutagenic effect of ethidium bromide sometimes could be a useful tool for improving industrially important microorganisms. Sudi *et al*. (2008) demonstrated that mutagenesis of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* by ethidium bromide leads to increasing in acid production and fructose utilization which give the opportunity for using these mutants as starter cultures in the dairy industry.

1.2.5.7.2 Physical mutagens:

- **Ultra violet radiation:**

Ultra violet radiation represents one of the most commonly used physical mutagen that may cause DNA damage and mutations. It is electromagnetic radiation with wavelengths range of 100-400 nm.

Generally, UV spectrum comprises of three wavelengths bands: UV A (320-400 nm), UV B (290-320 nm) and UV C (180-290 nm) (Yasbin, 2002). Such irradiation (ultra violate) exerts its negative effects in all living organisms ranging from prokaryotic bacteria to eukaryotic lower and higher plants, animals and even humans (Kodym and Afza, 2003).

These damaging effects targeting several cell components like DNA, proteins, pigments, and lipids. Ultra violet have direct or indirect mutagenic effects, directly by producing thymine dimmers and indirectly through generating free reactive radicals (Sommaruga and Buma, 2000; Sinha and Häder, 2002). The basic effect of ultra violate radiation is the production of pyrimidine dimmers and sometimes referred to as thymine dimmers; this reaction occurred when two thymine bases adjacent to each other in DNA molecule fuse covalently together under UV effect which resulting in deforming DNA double helix and increase the possibility of errors in DNA replication. It can act as a block to transcription and replication (Britt, 1995; Dale and Bark, 2004).

A study was done by Arihara and Itoh, (2000) in which UV irradiation was used to generate mutants of *Lactobacillus gasseri* resisting sodium chloride and sodium nitrite that could be utilized as a starter culture to develop probiotic meat products. Patel and Goyal, (2010) demonstrated that modification of *Lactobacillus delbrueckii* (NCIM 2365) by uv-mutagenesis and screened for novel mutants showing enhanced lactic acid productivity which may find commercial applications.

Ultra violet irradiation is more preferred than other types of radiations in the molecular researches due to its ease control (although eye and skin protection is necessary) and requires only comparatively inexpensive equipment (Dale and Bark, 2004).

Chapter Two

Materials and Methods

2. Materials and Methods:

2.1. Materials

2.1.1. Apparatus: The following equipment and apparatus were used throughout the study:

Apparatus and Equipment	Company (Origin)
Anaerobic Jar	Rod Well (England)
Autoclave	Express (Germany)
Balance (portable)	Ohans (France)
Balance (sensitive, digital)	Delta Range (Switzerland)
Compound Light Microscope	Olympus (Japan)
Cooled centrifuge	Harrier (UK)
Electric Oven	Gallen Kamp (England)
Eppendorf centrifuge	Sigma (USA)
Forceps	Olympus
Incubator	Memmert (Germany)
Laminar air flow hood	Heraeus (Germany)
Micropipette	Gilson (France)
PH-meter (Digital)	Radiometer (Denmark)
Sensitive Balance	Delta Rang (Switzerland)
Shaking Incubator	GLF (Germany)
UV- Transillaminator	Vilber Lourmat (France)
Vortex	Giffin (England)
Water Distillator	GLF

2.1.2 Chemicals and biological materials: The following chemicals and biological materials used in this study are listed with their manufacturing companies in the table below:

Material	Company (Origin)
Acridin Orange	Sigma (USA)
Agar-Agar	Himedia (India)
Agarose	Sigma
Ammonium acetate	Merck (Germany)
Arabinose	BDH (England)
Boric acid	BDH
Bromophenol Blue	Promiga (USA)
Calcium carbonate ($CaCO_3$)	BDH
Cellobiose	BDH
Chlorophenol red	BDH
Chlorophorm	BDH
Ethanol	Locally produced (Iraq)
Ethedium bromide	Sigma
Ethylen diamine tetra acetic acid (EDTA)	Sigma
Fructose	BDH
Galactose	BDH
Gelatine	Oxoid (England)
Glacial acetic acid	Fluka (Switzerland)
Glycerol	BDH
H_2O_2 (3%)	Locally produced (Iraq)

Hydrochloric acid (HCl)	BDH
K_2HPO_4	BDH
KH_2PO_4	BDH
L-Histidine	BDH
Lactose	BDH
Maltose	BDH
Mannitol	BDH
Mannose	BDH
Melibiose	BDH
$MgSO_4.7H_2O$	Merck
$MnSO_4.7H_2O$	Merck
Na_2HPO_4	Merck
NaOH	Merck
Peptone	BDH
Raffinose	BDH
Sodium acetate hydrate	Merck
Sodium dodecyl sulfate (SDS)	Sigma
Sorbitol	BDH
Sucrose	BDH
Tris-OH	Sigma
Trehalose	BDH
Trypsin	US Biological (USA)
Triammonium citrate	Fluka
Tween 80	Oxoid
Yeast extract	Himedia

2.1.3 Culture media:

2.1.3.1 Ready-to-use powdered media:

Medium	Company (Origin)
Brain Heart broth	Biolife (Italy)
Blood agar base	BDH (England)
Litmus Milk Media	Biolife
Mannitol salt agar	Himedia (India)
De Manns Rogoza Sharpe (MRS) broth	Oxoid (UK)
Nutrient agar	Oxoid
Nutrient broth	Biolife

2.1.3.2 Laboratory-prepared media:

- MRS-$CaCO_3$
- Gelatin agar.
- Carbohydrate fermentation broth
- Peptone broth.
- Blood agar.

2.1.4 Bacterial isolates: Bacterial isolates used in this study were obtained from the fallowing sources:

Isolate	Source	Supplied by
Lactobacillus acidophilus *Lactobacillus gasserii*	Vaginal Swap	Biotechnology Research Center/AL-Nahrain University.
Staphylococcus aureus	Nasal Swap	Biotechnology Department/ College of Science/ Baghdad University

2.1.5 Ready-to-use antibiotic discs: (Bioanalyse/ Turkey)

Antibiotic	Symbol	Concentration (µg/disc)
Ampicilline	AM	30
Amoxycillin	AX	25
Ciprofloxacin	Cip	5
Cephalothin	KF	30
Cephalexin	CL	30
Chloramphenicol	C	30
Cefotaxime	CTX	30
Gentamycin	CN	10
Methicilline	ME	5
Novobiocine	NV	30
Nalidixic acid	NA	30
Penicillin	P	10
Rifampin	RA	5
Streptomycin	S	10
Tetracycline	TE	30
Trimethoprim	TMP	5
Vancomycine	VA	30

2.2 Methods:

2.2.1 Buffers, Reagents and Solutions:

- **Physiological solution:**

It was prepared according to Atlas *et al*. (1995) by dissolving 0.85 g of NaCl in 1 liter of distilled water. pH was adjusted to 7 then, it was sterilized by autoclaving.

- **Phosphate buffer saline pH 7.0: (Cruishank *et al*., 1975)**

This solution was prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4 in 950 ml distilled water. pH was adjusted to 7 then, it was sterilized by autoclaving.

- **Chlorophenol red reagent (Cowan, 1974):**

This reagent was prepared by dissolving 0.2 g of chlorophenol red in 2 ml of 95% ethanol then the volume was completed to 100 ml with D.W.

- **Stock solutions of mutagens (Mims *et al*., 1998):**

Stock solutions of mutagen (ethidium bromide and acridin orange) were prepared by dissolving 0.01 gm of mutagen in 10 ml of D.W. (1mg/ml), then the mixture was stored in a dark bottle until use.

- **TE buffer:**

It was prepared according to Maniatis *et al*. (1982) as follows**:** 1 mM EDTA and 10 mM Tris-OH were dissolved in distilled water. pH was adjusted to 8.0 before it was autoclaved and stored at 4˚C.

2.2.2 Pure yield plasmid DNA miniprep system: (Promega-USA)

It was used for plasmid DNA extraction from *Lactobacillus* isolates. Miniprep kit includes the following:

- Cell lysis buffer (CLC).
- Neutralization solution (NSC).
- Endotoxin removal wash (ERB).
- Column wash solution (CEC).
- Elution buffer (EBB).
- Pure yield minicolumns.
- Pure yield collection tubes.

2.2.3 Electrophoresis solutions:

These solutions were prepared according to Maniatis *et al*., (1982) as follows:

- **Loading dye:**

This solution was prepared by mixing 30% sucrose, 50% TBE (1X), 20% distilled water and 0.25% bromophenol blue then, stored at 4˚C.

- **Ethidium bromide solution:**

It was prepared by dissolving 0.05 g of ethidium bromide in 10 ml of distilled water. The solution was then, kept in a dark container.

- **Tris borate-EDTA buffer solution TBE (5X)**

This solution was prepared by mixing 54 g of Tris-base and 27.5 g boric acid in 20 ml of 0.5 M EDTA solution, pH was adjusted to 8.0 with 1 M NaOH, volume completed to 1 Liter by D. W. then sterilized by autoclaving and kept in 4 ºC.

- **Agarose gel (0.7%):**

It was prepared by dissolving 0.35 g of agarose in 50 ml of Tris borate (1X). Crystals of agarose was completely dissolved under heating.

2.2.4 Media preparation:

2.2.4.1 Ready-to-use powdered media:

The media listed in (2.1.3.1) were prepared according to the instructions by their manufacturer fixed on the containers. After adjustment of pH, they were sterilized by autoclaving at 121ºC for 15 min. unless otherwise stated.

2.2.4.2 Laboratory prepared media:

- **MRS-$CaCO_3$ agar:**

This medium was prepared according to Harrigen and MacCane, (1976) by mixing powdered MRS broth media with 1.5% (w/v) agar, then addition of 1% (w/v) $CaCO_3$ to this mixture, and sterilized by the autoclave. This medium was used for the LAB isolation.

- **Gelatin medium:**

This medium was prepared according to Atlas *et al*. (1995) by dissolving 12 g of gelatine in 90 ml MRS broth. Volume was completed with MRS broth to get a final concentration of 12% (w/v) then sterilized by autoclaving for 20 min.

- **Carbohydrate fermentation broth:**

It was used for identification of *Lactobacillus* spp. and was prepared according to Cowan, (1974) by dissolving the following ingredients in 950 ml of distilled water:

Ingredient	Weight (g/L)
Peptone	10
yeast extract	5
Sodium acetate hydrate	5
K_2HPO_4	2
Triammonium citrate	2
$MgSO_4.7H_2O$	0.2
$MnSO_4.7H_2O$	0.05
Tween 80	1ml

Then, 2% of Chlorophenol red was added as indicator and the pH was adjusted to (6.2-6.4). After that, the volume was completed to 1000 ml with D.W, and then sterilized by autoclaving. Carbohydrates solutions were prepared then, added aseptically and separately to the sterilized medium at a final concentration of 2%.

- **Blood agar (Atlas *et al*., 1995):**

This medium was prepared by dissolving 37 g of blood base agar in 950 ml of D.W. and autoclaved after pH was adjusted to 7.0. After cooling to 50˚C, the

blood was added as a final concentration 5%, mixed well and distributed into Petri dishes under aseptic conditions.

- **Peptone broth (Mackie and MacCartney, 1996):**

Quantity of 5 g peptone was dissolved in 100 ml of D.W., and sterilized by autoclaving. Then 5 ml aliquots were dispensed in sterile test tubes and stored at 4˚C until use.

2.2.5 Sterilization methods (Baily *et al.*, 1990):

Three methods of sterilization were used:

- **Wet-heat sterilization:** All bacterial cultural media and solutions were sterilized by autoclaving at 121 ˚C (15 Ib/inch2) for 15 min, unless otherwise stated.
- **Dry-heat sterilization:**

  Electric oven was used to sterilize Glassware at 180˚C for 2hr.
- **Membrane sterilization (Filtration):**

Bacterial filtrates and sugar solutions were sterilized throughout 0.45 and 0.22 μm in diameter millipore filter units.

2.2.6 Samples collection:

A total of 53 samples of locally made dairy products (cheeses, crude milk and yoghurts), pickles and vinegar were collected from different regions of Baghdad governorate in sterile containers and transported to the laboratory under aseptic cooled conditions.

2.2.7 Isolation of *Lactobacillus* isolates:

Lactobacillus spp. was isolated according to Buck and Gilliland, (1995) as following:

One ml of each sample was transferred to a test tube containing 9 ml of sterilized MRS broth and incubated anaerobically (by using anaerobic jar) overnight at 37˚C. Serial dilutions were made using peptone water, and 1 ml of

the appropriate dilution (which was chosen according to previous experiment) was cultured on MRS-$CaCO_3$ agar and incubated for 24 hr. at 37˚C under anaerobic conditions. After incubation, only acid producing bacterial colonies were selected. This was observed by the clear zones formed around colonies as an indicator of $CaCO_3$ dissolving by acid. These colonies were picked up and purified by restreaking on the same agar medium. Morphological, microscopical examinations and biochemical tests were then performed.

2.2.8 Identification of *Lactobacillus* isolates:

2.2.8.1 Morphological characteristics:

Phenotyping and cultural characteristics of the suspected *Lactobacillus* isolates were first identified according to their colony shape, color, size, edges and height on MRS agar plates (Harely and Prescott, 1996).

2.2.8.2 Microscopical characteristics:

A touch from single colony of each suspected *Lactobacillus* isolates was fixed on a microscopic slide and stained by Gram staining, then examined under compound light microscope. Cells shape, Gram reaction, grouping and spore forming were detected (Harely and Prescott, 1996).

2.2.8.3 Biochemical tests:

- **Catalase test (Atlas *et al*., 1995):**

One drop of hydrogen peroxide (3%) was added to part of the grown isolated colonies which was picked up from the agar by sterile wooden stick applicator and placed on the microscopic slide. Production of gaseous bubbles within 20-30 sec. indicates a positive result.

- **Oxidase test (Atlas *et al*., 1995):**

A clump of colonies from bacterial growth was picked with a sterile wooden stick applicator and placed on a filter paper that moistened with a few drops of a freshly prepared oxidase reagent. A positive reaction is indicated by intense deep purple color appearing within 5-10 sec.

- **Gelatin liquification test (Baron *et al*., 1994):**

This test was performed by stabbing each of the test tubes containing gelatin medium with the bacterial isolates. After incubating in anaerobic jar at 37˚C for 48 hr., the test tubes were then reincubated at 4˚C for 30 min. Liquifiaction of gelatin represents a positive result.

- **Acid production and clot formation test (Kandler and Wiess, 1986):**

Test tubes, each containing 10 ml of Litmus milk medium, were inoculated with 100 µl of fresh culture of *Lactobacillus* spp. then incubated under anaerobic conditions at 37˚C for 48 hr. Presence of pink color, curd production and reduction of medium pH indicate a positive result.

- **Carbohydrate fermentation Test (Atlas *et al*., 1995):**

Tubes containing fermentation media were inoculated with 1% of LAB isolates and incubated at 37ºC for 5 days (including the positive control consisted of MRS broth without glucose and meat extract), and the negative control (MRS broth only). Changing the medium color from red to yellow indicates positive result.

- **Antibiotics susceptibility test of *Lactobacillus* isolates (Atlas *et al*., 1995):**

Disk diffusion test was used for testing antibiotic sensitivity of *Lactobacillus* isolates. Ten ml of MRS broth medium inoculated with the bacterial isolate then, cultures were incubated anaerobically at 37ºC for 18hrs. then, 0.1ml of culture was transferred to MRS agar plates. A sterile cotton swab was used in three different planes to obtain an even distribution of the inocula. The selected antibiotic disks were placed on the inoculated plates using sterile

forceps and incubated at 37ºC for 24 hrs. in an inverted position, then diameters of inhibition zone were measured in millimeter.

2.2.9 Identification of test microorganism (*Staphylococcus aureus*):

2.2.9.1 Microscopical and morphological examinations:

A touch of fresh culture of suspected *S. aureus* was fixed on a microscopic slide then examined microscopically as mentioned in (2.2.8.2).

The isolate was also cultured on three media (Nutrient agar, Mannitol salt agar and blood agar).

2.2.9.2 Biochemical tests:

- **Catalase and oxidase tests**:

They were done as mentioned in item (2. 2. 8. 3.).

- **Coagulase test (Atlas *et al*., 1995):**

Several colonies of *Staphylococcus aureus* are transferred using a sterile loop to a tube containing 0.5 ml of human plasma. The tube was covered to prevent evaporation before incubating at 37˚C for 4 hrs.

Result was reading by tilting the tube and observing clot formation in the plasma, which indicates positive result. If the plasma remains free-flowing with no evidence of clot, the test considered to be negative.

- **Hemolysin production test (Cruickshank *et al*., 1975):**

This test was achieved by streaking freshly prepared blood agar plate with fresh culture of *S. aureus*. Appearance of clear zone around the colonies after 18 hrs. of incubation at 37˚C indicates a positive result.

- **Mannitol fermentation test (Benson, 2002):**

This test was done by streaking the *S. aureus* isolate on mannitol salt agar plate and incubated at 37˚C for 18 hr. changing medium color from red to yellow indicates a positive result.

2.2.9.3 Antibiotic sensitivity test of *S. aureus*:

Susceptibility of *S. aureus* isolate to different antibiotics (especially methicillin) was studied by the standard disc diffusion method (NCCLS, 2002). Five ml of sterile brain heart infusion broth was inoculated with *S. aureus* isolate and incubated at 37 ˚C for 18 hr. Then serial dilutions were made and 0.1 ml of the 10^{-7} dilution (adjusted by McFarland solution) was spread on nutrient agar plates in different three planes by rotating the plate approximately 60º after each streaking to obtain an even distribution of the inoculums.

The inoculated plates were placed at room temperature for at least 30 min (to allow absorption of excess moisture), then, antibiotic discs were placed on the inoculated plate and incubated at 37˚C for 18 hrs. Diameters of the inhibition zones were measured and compared with that of standards of the National Committee for Clinical Laboratory Standards.

2.2.10 Maintenance of bacterial isolates:

2.2.10.1 Maintenance of *Lactobacillus* isolates: (Conteras *et al*., 1997).

- **Daily working culture:**

Broth media were inoculated with each of the isolates and then incubated at 37˚C for 24 hr. under anaerobic conditions then, kept in the refrigerator at 4˚C and reactivated weekly.

- **Long term storage:**

Lactobacillus isolate was cultured in MRS broth medium for 24 hrs. at 37˚C under anaerobic conditions and then, 1.5 ml of the freshly preparation of bacterial growth was added to bottles containing 8.5 ml of 20% Glycerol, and stored at (-20)˚C.

2.2.10.2 Maintenance of *Staphylococcus aureus* (Maniatis *et al*., 1982):

- **Short–term storage:**

Bacterial isolates were maintained for a period of few weeks on nutrient agar plates then, wrapped tightly with parafilm before storing at 4˚C.

- **Medium–term storage:**

Bacterial isolate was maintained as slab culture for months. Such cultures were prepared in small screw capped bottles containing 5-7 ml of nutrient agar as slant and stored at 4˚C.

- **Long–term storage:**

Test tubes contained 10 ml of sterile brain heart infusion broth were inoculated with a single colony of *S. aureus* and incubated at 37˚C for 24 hrs., then 1.5 ml of the culture was mixed with 8.5 ml of glycerol (20%), and stored at -20 ˚C without loose in viability.

2.2.11 Testing the inhibitory effect of *Lactobacillus* isolates:

- **On solid medium:**

A culture of the *Lactobacillus* isolate, which was previously inoculated in MRS broth and incubated anaerobically (using anaerobic jar) at 37˚C for 24 hrs. was recultured by streaking on MRS agar plates and incubated at 37˚C for 24 hrs. After incubation, a disc of the growth was obtained with the aid of a sterile cork borer (5 mm in diameter). The disc was fixed on the surface of nutrient agar plate that is previously spreaded with test microorganism (*Staphylococcus aureus*). After incubation at 37˚C for 24 hr. then, Diameter of inhibition zone around the disc was measured after subtracting the diameter of the well (5 mm). (Silva *et al*., 1987).

- **In liquid media:**

In a test tube, a quantity of 5 ml of MRS broth medium was inoculated with 1% of fresh culture of *Lactobacillus* spp., and incubated anaerobically at 37˚C

for 24 hr. Then, it was centrifuged at 6000 rpm for 15 min. The supernatant was taken and sterilized by filtration (ErdoĞrul and Erbilir, 2006).

Inhibitory effect of *Lactobacillus* crude filtrate was examined against the test microorganism (*Staphylococcus aureus*) using well diffusion method (by making wells on nutrient agar surface previously inoculated with *S. aureus* and filling them with crude filtrates of *Lactobacillus* spp.). After incubation at 37˚C for 24 hr., the diameters of inhibition zones around wells were measured (in millimeter) after subtracting the diameter of the well (5 mm) and compared with the control which contained MRS broth only (Ryan *et al*., 1996).

2.2.12 Detection of inhibitory substances:

In order to characterize the inhibitory compounds produced by the *Lactobacillus* isolates, their culture supernatants were assayed for organic acids and bacteriocins presence. The well diffusion assay method was used.

For organic acids, the culture supernatants were neutralized to pH 7 with the addition of 1 M NaOH, for bacteriocin the supernatants were treated with trypsin (final concentration 1mg/ml) for 12 hr. at 37˚C. From each treated supernatant 0.1 ml was transferred into the wells of the agar that previously inoculated by test microorganism and 0.1 ml from the untreated culture supernatant was also transferred into the wells. The presence or absence of inhibitory zones around the wells was determined after incubation for 24 hrs. at 37˚C. This method was mentioned by Jin *et al*. (2000).

2.2.13 Mutagenesis of *Lactobacillus*:

2.2.13.1 Physical mutagenesis:

- **Ultra violet mutagenesis:** (Sudi *et al*., 2008)

Cultures of *L. gasseri* and *L. acidophilus* grown in MRS broth at 37˚C for 18 hr. anearobically. were centrifuged at 5000 rpm for 15 min. then, the pellets were taken, washed twice in 5 ml of phosphate buffer (pH 7) and resuspended

in same volume of this buffer. Then, 5 ml portions of this bacterial suspension were transferred to sterile Petri dishes and irradiated with ultra violet light source at 254 nm wavelength for five different periods (5, 10, 15, 20 and 25 sec.). Each irradiated sample was then diluted serially using 0.9% NaCl solution and 0.1 ml of the appropriate dilution were plated onto MRS agar by spreading using L-shape glass rod and incubated at 37˚C for 18hr. After that, the total viable count was examined for both isolates and the suspected mutants were picked up and further identified according to their inhibitory effect against *S. aureus*.

2.2.13.3 Chemical mutagenesis:

- **Ethedium bromide mutagenesis:** (Sudi *et al.,* 2008)

The two isolates (*Lactobacillus acidophilus* and *Lactobacillus gasserii*) were separately grown in MRS broth under anaerobic conditions at 37˚C for 18 hr. The cells were harvested and washed twice and resuspended with sterile phosphate buffer solution then ethidium bromide was added to 5 ml of each cell suspension at final concentrations (0.5, 0.1, 1.5, 2 and 2.5) g/L.

The mixture was aerated in a shaking incubator at 37˚C for 30 min. The treated cells were cultured and incubated in 10 ml MRS broth then, washed twice before resuspending in phosphate buffer solution. Serial dilutions were made and spreaded on MRS agar plates before incubation anearobically at 37˚C for 24hr. The total viable count was examined for both isolates after treatment and then, colonies that might be mutants were isolated according to their antagonistic effect against *S. aureus*.

- **Acridine orange mutagenesis:** (Margino *et al*., 1998)

To each one of the twelve tubes containing 5 ml of MRS broth, (0, 25, 50, 75, 100 and 125) µL of a 1 mg/ml acridine orange solution were added. All tubes were inoculated with 50 µL of a primary culture of *L. acidophilus* and *L.*

gasseri isolates. The incubation was performed at 37˚C in an orbital shaker at 150 rpm for 30 min. Then, samples were serially diluted and 0.1 ml from each appropriate dilution was spreaded on MRS agar plates, before incubation at 37˚C for 24 hrs. and the total viable count was examined for both isolates. Mutants were then picked up depending on their inhibitory effect against test microorganism.

2.2.14 Extraction of plasmid DNA of *Lactobacillus* isolates:

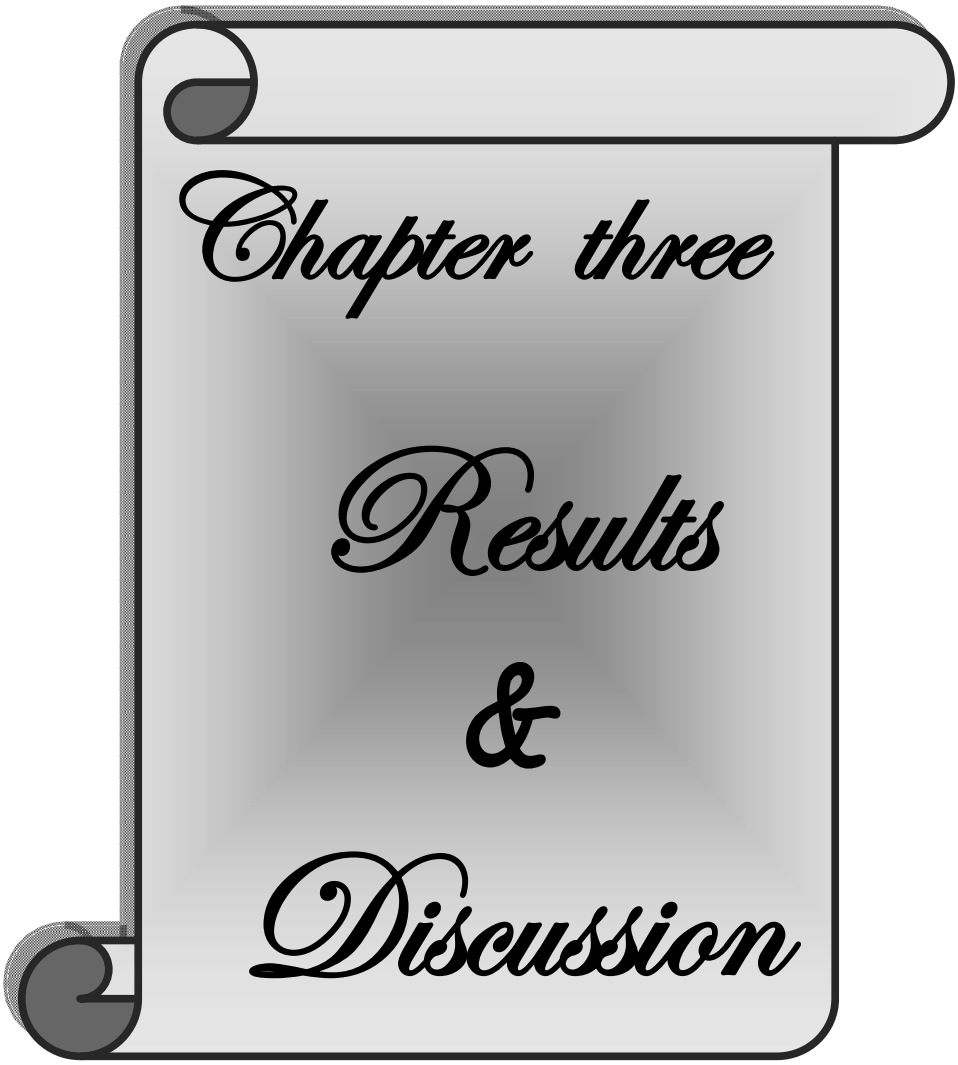
Plasmid DNA extraction was performed by using plasmid miniprep system and according to the instructions by the manufacturer as follows:

1. A quantity of 5-10 ml of MRS broth was inoculated with 0.1 ml of the selected bacterial isolates then, cultures were incubated overnight at 37˚C in shaker incubator (180 rpm).
2. Bacterial cultures were transferred into a 10 ml tubes and cells recovered by centrifugation at 4000 rpm for 10 mins. at 4˚C.
3. Supernatants were discarded by gentle aspiration, leaving the bacterial pellet as dry as possible.
4. The bacterial pellet was resuspended in 600 µl of TE buffer completely.
5. A volume of 100 µl of cell lysis buffer was added to each bacterial suspension, and the contents were mixed immediately by inverting for six times (not more two mins.). Complete lysis indicated by changing the solution color from opaque to clear blue.
6. A quantity of 350 µl of ice-cold neutralization solution was added then, tubes were mixed by inverting several times (forming of yellow precipitate insured complete neutralization) and they were centrifuged at maximum speed (12000 rpm) for 3 min. at 4˚C.
7. The resulted supernatant (~ 900 µL) was transferred using piptte to a pure yield minicolumn then, the minicolumn placed into a pure yield collection tube, and centrifuged at maximum speed for 15 sec.

8. All supernatants were removed. Minicolumns were then, placed in the same collection tubes
9. A volume of 200 µL of endotoxin removal wash was added to the minicolumns and centrifuged at maximum speed for 15 sec.
10. Column wash solution was then added (400µL) to the minicolumns and centrifuged at maximum speed for 15 sec.
11. These minicolumns were then transferred to a clean 1.5 ml eppendorf tubes, then 30 µL of elution buffer was added directly to the minicolumns and let stand for 1 min. at room temperature.
12. Minicolumns were centrifuged for 15 sec. at maximum speed to elute the plasmid DNA. Eppendorf tubes were caped and stored at -20˚C until use.

2.2.15 Agarose gel electrophoresis:

Samples of extracted DNA were mixed with loading dye in 1-10 ratio and added to the wells on the agarose gel (0.7%), and then run horizontally in Tris Borate-EDTA buffer (TBE 1X). Generally, gel was run for 2-3 hrs. at 5 $v.cm^{-1}$ then, the agarose gel was stained with ethidium bromide by immersing it in ethidium bromide solution (0.5µg/ml) for 30-45 min. DNA bands were visualized by U.V trasilluminator cabinet, and photographed.


Chapter three
Results
&
Discussion

3. Results and Discussion

3.1Isolation of *Lactobacillus* spp.:

Fourteen *Lactobacillus* isolates were isolated from 53 samples of different food sources. These isolates were primarily identified by producing clear zones around their colonies (due to the production of acid) after growth on MRS agar containing $CaCO_3$. Results indicated in figure (3-1) showed that nine (56%)of the total isolates (16) were isolated from yogurt, two (12.5%) from crud milk and one (6.5%) from each of (vinegar, pickles, and cheeses), also two(12.5%) *Lactobacillus* isolates (from vagina)were obtained and reidentified.

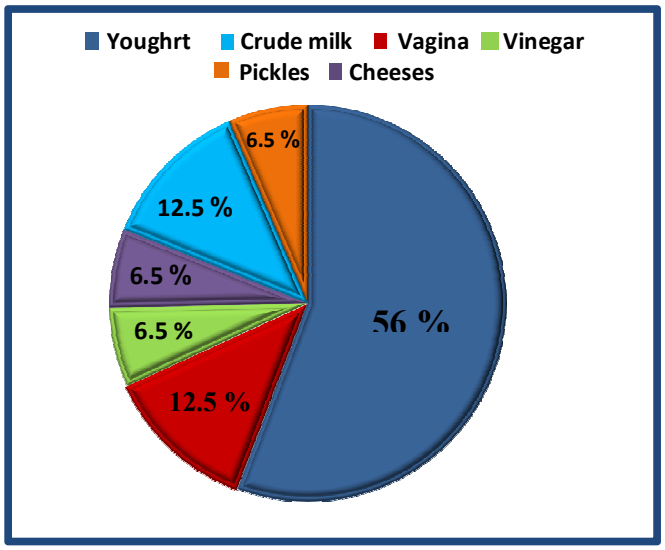


Figure 3-1: Percentages of *Lactobacillus* spp. from different food and clinical samples.

These isolates were identified according to their cultural, microscopical characteristics and biochemical tests, and as follows:

3.2 Identification of bacterial isolates:

3.2.1 Cultural characteristics:

Colonies of the LAB isolates appeared after culturing on MRS agar as; white to yellow in color, round shaped, (2-3) mm in diameter, some convex and

othersare relatively flat, soft, mucoid and having smooth edges. Such results are in accordance with those described by Hammes and Vogel, (1995).

3.2.2. Microscopical characteristics:

Microscopical examinations of the 16 isolates declared that the cells gave Gram positive reaction, short and long bacilli, mainly organized in chains containing (3-8) cells, but some found as singles or in pairs. They were non-spore formers. Similar characterization was given by Kandler and Wess, (1986) to the *Lactobacilli*.

Depending on the above findings, the sixteen isolates were suspected to be related to the genus *Lactobacillus*.

3.2.3 Biochemical tests:

After studying the cultural and microscopical characteristics of the bacterial isolates which may be belong to *Lactobacillus* spp., these isolates were subjected to biochemical tests.Results indicated in table (3-1) showed that all LAB isolates were negative to the indol, catalase, oxidase, gelatinase tests, while they were positive to the litmus milk test because of lowering the pH of litmus milk medium causing clots. After propagation of the 16*Lactobacillus* isolates in MRS broth and incubation at 45˚C for 24hr. under anaerobic conditions, results in table (3-1) declared that 11 of these isolates were able to grow at 45˚C while, the rest 5 were unable to do so.

According to the results of biochemical tests the 16 isolates were belonging to the genus *Lactobacillus* (Kandler and Weiss, 1986; Hammes and Vogel, 1995).

Ability of these isolates to ferment various carbon sources was used for further identification of *Lactobacillus* isolates. Results indicated in table (3-1) showed that all isolates were similar in their ability to ferment fructose, galactose, lactose, cellobiose and maltose carbohydrates with exception

ofLb.Cwhich was unable to ferment cellobiose, galactose, and maltose sugars, and Lb. M1 isolate was unable to ferment cellobiose

On the other hand, most of the LAB isolates were unable to ferment xylose sugarwith exception of Lb.M1isolate. While, sucrose sugar was fermented by all *Lactobacillus* isolates except one which was Lb. C.

Regarding the rest of carbon sources *Lactobacillus* isolates were varied in theirfermentation pattern.

Table 3-1: Biochemical tests for identification of locally isolated *Lactobacillus* spp.

Isolate Symb.	Biochemical test																		
	Catalase	Oxidase	Gelatenase	Litmus milk	Growth at 45°C	Indol test	Ara.	Cel.	Fru.	Gal.	Lac.	Mal.	Mann.	Mani.	Mel.	Raf.	Sor.	Suc.	Tre.	Xyl.
Lb. P	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Lb. Y1	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-
Lb.Y2	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-
Lb. Y3	-	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-
Lb. G1	-	-	-	+	+	-	-	+	+	+	+	+	+	-	+	+	-	+	+	-
Lb.M1	-	-	-	+	-	-	+	-	+	+	+	+	-	-	+	+	-	+	-	+
Lb. Y4	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-
Lb. Y5	-	-	-	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	-
Lb. C	-	-	-	+	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-
Lb. G2	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-
Lb. V	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-
Lb.M2	-	-	-	+	-	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-
Lb. Y6	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-
Lb. Y7	-	-	-	+	+	-	-	+	+	+	+	+	+	+	-	-	+	+	+	-
Lb. Y8	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-
Lb. Y9	-	-	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-

Gal: Galactose, Fru: Fructose, Suc: Sucrose, Sor: Sorbitol, Lac: Lactose, Mal: Maltose, Mann: Mannose, Mani: Mannitol, Mel: Mellibiose Xyl: Xylose, Tre: Trehalose, Raf: Raffinose, Ara: Arabinose, Cel: cellobiose. (+) positive result, (-) negative result.

After identifying *Lactobacillus* isolates by the cultural, microscobial and biochemical tests. Results mentioned in table (3-2) showed that seven of the total isolates (16) were identified as *L. acidophilus*, two from each of *L. plantarum*, *L. fermentum* and *L. casei* also one from each of *L. gasseri*, *L. bulgaricus*, and *L. brevis* (Holt and Kriege, 1986).

Table 3-2: Full identification of *Lactobacillus* isolates

Isolate Symbol	Source	Isolate sp.
Lb. P	Pickles	***L. plantarum***
Lb. Y1	Yoghurt	***L. acidophilus***
Lb.Y2	Yoghurt	***L.acidophilus***
Lb. Y3	Yoghurt	***L. fermentum***
Lb. G1	Vagina	***L. gasseri***
Lb.M1	Crude milk	***L. brevis***
Lb. Y4	Yoghurt	***L. acidophilus***
Lb. Y5	Yoghurt	***L. fermentum***
Lb. C	Cheese	***L. bulgaricus***
Lb. G2	Vagina	***L. acidophilus***
Lb. V	Vinegar	***L. plantarum***
Lb.M2	Crude milk	***L. casei***
Lb. Y6	Yoghurt	***L. acidophilus***
Lb. Y7	Yoghurt	***L. casei***
Lb. Y8	Yoghurt	***L. acidophilus***
Lb. Y9	Yoghurt	***L. acidophilus***

3.3 Reidentification of test microorganism:

Test microorganism (*S. aureus*) used in this study was isolated and identified in a previous study, but it was reidentified through some of its cultural and biochemical characteristics before use. The antibiotic sensitivity pattern was

also examined to confirm that the test isolate was methicillin-resistant *S. aureus* (MRSA).

Staining with Gram's stain showed that cells of *S. aureus* isolate were Gram positive cocci (0.5-1.5µm) appeared as grape-like clusters when viewed under the oil immersion lens of the light microscope. Additionally, colonies of this isolate were round, relatively large, yellow, raised when cultured on the enrichment medium (BHI). On the other hand, results of biochemical tests declared that the isolate gave positive results for catalase, mannitol fermentation, coagulase tests while it was negative to the oxidase.

Also the MRSA isolate caused a blood hemolysis when cultured on blood agar medium. Results of morphological and biochemical characterization came in accordance with those mentioned by Morse, (1981) and Anthoniraj *et al*. (2005).

3.3.1 Antibiotic susceptibility of *Staphylococcus aureus*:

Antibiotic sensitivity towards methicillin was performed to insure that *S. aureus* isolate is a methicillin resistant (MRSA) one. Results insured that the isolate was completely resistant to this antibiotic. In this regard, Saravanan and Nanda, (2009) reported that a clinical isolate of *S. aureus* which caused septicemia was found to be resistant to methicillin when no inhibition zone was produced. Also, Murugan *et al*. (2008) found that more than half of the *S. aureus* isolates collected from foot ulcers exhibited resistance toward the methicillin antibiotic.

In addition to methicillin antibiotic, *S. aurues* isolate was screened for its sensitivity towards other eleven antibiotic types. Results indicated in table (3-3) showed that *S. aureus* isolate was resistant to streptomycin, amoxicillin, vancomycin, ampicillin and penicillin, but sensitive to rifampin, ciprofloxacin, novobiocin, cephalothin, tetracycline and trimethoprim (NCCLS, 2002).

Almost similar results were obtained by Onwubiko and Sadiq, (2011) when they found that collected clinical isolates of methicillin resistant *S. aureus* were also resistant to streptomycin, tetracycline, penicillin and amoxicillin, while some of them were sensitive to the ciprofloxacin. Maree, (2007) stated that *S. aureus* has become resistant to many commonly used chemotherapeutic agents.

Table 3-3: Antibiotics susceptibility of *Staphylococcus aureus*.

Antibiotic	Sym.	Conc.(µg/ml)	Result
Rifampin	RA	5	S
Streptomycin	S	10	R
Ciprofloxacin	CIP	5	S
Methicillin	ME	5	R
Novobiocin	NV	30	S
Cephalothin	KF	30	S
Tetracycline	TE	30	S
Amoxicillin	Amx	25	R
Vancomycine	VA	30	R
Ampicillin	AM	10	R
Penicillin	P	10	R
Trimethoprime	TMP	5	S

R: resist, S: sensitive.

3.4 Inhibitory effect of *Lactobacillus* isolates against *S. aureus*:

Inhibitory effect of *Lactobacillus* isolates (as probiotics) was evaluated against the methicillin resistant *S. aureus* isolate by two methods for propagating probiotics before use. First, on the solid medium (MRS agar), and the second in the liquid one (MRS broth). Results obtained were best to be explained and discussed as follows:

3.4.1 On solid medium:

Ability of *Lactobacillus* isolates to exhibit probiotic effect and produce inhibitory metabolites against *S. aureus* was evaluated by incubating isolates on the MRS agar under anaerobic conditions for 24 hr. at 37˚C.

Results mentioned in table (3-4) declared that the inhibitory effects of LAB isolates against the methicillin resistant *S. aureus* were varied. The inhibition zones ranged between (5-8) mm for *L. plantarum* (Lb.P), *L. acidophilus* (Lb.Y1), *L. bulgaricus* (Lb.C), *L. acidophilus* (Lb.Y8) and *L. casei* (Lb.M2), *L. acidophilus* (Lb.Y2) isolates. While, better effect was recorded by *L. fermentum* (Lb.Y3), *L. acidophilus* (Lb.Y9), *L. gasseri* (Lb.G1), *L. acidophilus* (Lb.G2) and *L. plantarum* (Lb.V) isolates when diameters of their inhibition zones raised to be between (9-10) mm. Adversely, *L. acidophillus* (Lb.Y4), *L. fermentum* (Lb.Y5), *L. acidophillus* (Lb.Y6), *L. casei* (Lb.Y7) and *L. brevis* (Lb.M1) isolates had no any inhibitory effect against the test microorganism.

In this regard, Schillinger and Lucke, (1989) found that not all *Lactobacillus* isolates exhibited antagonistic effect against test bacteria, and their effect was pH independent. According to Egorov, (1985) such variability in the inhibition ability of LAB against pathogenic bacteria may be related to the type of both LAB and pathogenic bacteria, also to the type and quality of the inhibitory substances, and their ability to be distributed in the medium. While, Vignolo *et al*. (1993) attributed that to the test bacteria only.

Tadesse *et al*. (2005) found that lactic acid bacteria isolated from fermented beverages have well documented antimicrobial activity against several food borne pathogens with a range of inhibition diameters between (16-17.5) mm against *S. aureus*. Metabolites produced by *Lactobacillus* spp. (those with antimicrobial activity) are usually accumulated in their environment at levels and proportions that depend on the species of LAB and chemical composition of the growth medium (Šuškovic *et al.,* 2010).

Some studies concluded that composition of the MRS medium (selective for lactobacilli) is very inducible to produce secondary metabolites possessing inhibitory effect. Also, Champagne *et al*. (1999) found that growth rate, maximum biomass levels and probiotic effect production were increased by raising yeast extract concentration from (0.5) to (5) g/L which is same concentration that used in the formula of MRS medium.

Table 3-4: Inhibitory effect of *Lactobacillus* isolates previously cultured on MRS agar against methicillin resistant *S. aureus* isolate after 24 hr. of incubation.

Isolate	Inhibition zone* (mm)
***L. plantarum* (Lb.V)**	**10**
L. acidophilus (Lb.Y1)	7
L. acidophilus (Lb.Y2)	5
***L. fermentum* (Lb.Y3)**	**9**
***L. gasseri* (Lb.G1)**	**9**
L. casei (Lb.Y7)	0
L. fermentum (Lb.Y5)	0
L. acidophilus (Lb.Y6)	0
L. bulgaricus (Lb.C)	8
***L. acidophilus*(Lb.G2)**	**10**
***L. casei* (Lb.M2)**	**8**
L. acidophilus (Lb.Y4)	0
L. plantarum (Lb.P)	8
L. brevis (Lb.M1)	0
L. acidophilus (Lb.Y8)	7
***L. acidophilus*(Lb.Y9)**	**9**

*** Diameter was calculated after substracting the diameter of the well (5 mm).**

(0): no inhibition zone.

As mentioned by Todorov and Dicks, (2005) production of the inhibitory materials by LAB was affected by the medium used for growth, when they found that inclusion of Tween 80 into the growth medium induced the production of proteins (bacteriocins) by 50%. Inhibition of methicillin resistant *S. aureus* by *Lactobacillus* species isolated from commercial food products was also reported by Karska-Wysocki *et al*. (2010).

Depending on the previous results, the most efficient six isolates that gave highest inhibitory effect among all other *Lactobacillus* isolates have been chosen for further studies. These are absently; *L. acidophilus* (Lb.Y9), *L. fermentum* (Lb. Y3), *L. gasseri* (Lb. G1), *L. casei* (Lb.M2), *L. plantarum* (Lb. V) and *L. acidophilus* (Lb.G2).

3.4.2 In liquid media:

To determine the inhibitory effect of the six chosen LAB isolates (item 3.4.1) against *S. aureus*, well diffusion method was used. The wells were made in nutrient agar plates that previously inoculated with *S. aureus* then, filled with the filtrate of each of the *Lactobacillus* isolates which previously incubated at 37˚C for different periods (24, 48, 72 hr.).

By comparison with the results of solid medium, the non-concentrated filtrate of LAB showed no effect on the growth of *S. aureus* when no inhibition zones were formed. Such findings were closed to those obtained by Elkins *et al*. (2008) who found that the non-concentrated filtrate gave no inhibitory effect in comparison with the concentrated one.

Anas *et al.* (2008) referred that the inhibitory effect of *Lactobacillus plantarum* is either to the production of organic acids (lactic or acetic acids), or to that of bacteriocins. Also, this could be explained in the way that neither the isolates have an inhibitory effect, nor the concentration of the secreted metabolite is far from that of the minimum inhibitory concentration (Barefoot,

1983). In order to improve the inhibitory activity of LAB filtrate, they were concentrated to one-fold. Results indicated in table (3-5) showed that concentrated filtrate resulted in a slight increase in the inhibition effect against test microorganism with an inhibition zone diameters ranged between (7-12) mm after 24 hr. of incubation.

Gupta *et al*. (1998) mentioned that supernatant of LAB bacteria which was previously grown in MRS broth have antagonistic activity against both Gram positive and negative bacteria like *B. subtilis* and *S. aureus* and with inhibition zones ranged between (13-19) mm.

Table 3-5: Inhibitory effect of *Lactobacillus* isolates against *Staphylococcus aureus* incubated in MRS broth medium for different incubation periods.

Incubation period (hr.)	Inhibition zone (mm)					
	L. casei **(Lb. M2)**	***L. acidophilus*** **(Lb.G2)**	***L. plantarum*** **(Lb.V)**	***L. gasseri*** **(Lb. G1)**	***L. acidophilus*** **(Lb.Y9)**	***L.fermentum*** **(Lb.Y3)**
24	7	12	6	11	7	8
48	7	14	6	13.5	8	11
72	4	10	5	11	6	9

Zalan *et al*. (2005) confirmed that best inhibitory effect was obtained by the hydrogen peroxide and bacteriocins produced by *Lactobacilli* after grown in MRS medium, and this might be explained by the fact that this medium is rich in organic compounds, peptides and salts that can be utilized by the bacteria.

Jagadeewari *et al*. (2010) declared that *Lactobacillus* spp. which was identified as (*L. plantarum, Lactococcus lactis* and *L. acidophilus*) had antagonistic activity against some indicator bacteria including *S. aureus*. They also reported that the well diffusion method gave more positive results

compared to the other methods used. Adversely, Spillmann *et al*. (1978) and Oyetayo, (2004) found that *Lactobacillus* isolates were incapable to produce inhibitory effect against *S. aureus*.

On the other hand, it was found there was an increase in the inhibitory effect was recorded with the increase of the incubation period to 48 hr. as mentioned in table (3-5) when zone diameters raised to (14, 13.5, 8 and 11) mm for *L. acidophilus* (Lb.G2), *L. gasseri* (Lb.G1), *L. acidophilus* (Lb.Y9) and *L. fermentum* (Lb.Y3), respectively. These results agreed with Al-Yas, (2006) who found that inhibitory effect of LAB against *Helicobacter pylori* increased after 48 hr. of incubation. In another study, *S. aureus* was completely inhibited after treatment with *Lactobacillus* and incubation period of 48 hr. (Guessas *et al.*, 2007).

Ogunbanwo *et al*. (2003) found that maximum bacteriocin production by *Lactobacillus brevis* was achieved after 48 hr. of incubation period, but increasing the period to 72 hr. resulted in decreasing its production.

During their study on evaluating the effect of incubation period on bacteriocin production, Lade *et al.* (2006) observed that the bacteriocin exhibited its maximum activity against the test microorganisms at the end of 48 hrs. of incubation. While, results in table (3-5) showed that *L. casei* (Lb. M2) and *L. plantarum* (Lb.V) isolates exhibited no increasing in diameters of inhibition zones for such period (48 hr.). Similar finding was noticed by Kubba, (2006) who found that 24 hr. of incubation period was enough to show effect against *P. aeruginosa* with an inhibition zone of (15.5) mm, and increasing incubation period to 48 or 72 hr. did not cause any observable effect on the inhibitory activity.

A study by Al-Jeboury, (2005) reported that best inhibitory effect was achieved after 24 hr. of incubation with (18) mm of inhibition zone diameter, and added that increasing incubation period to 48 hr. resulted in less inhibitory effect for LAB isolates.

Elimination of 99% of MRSA by LAB isolates was recorded after 24 hr. of incubation in a study done by Karska-Wysockib *et al*. (2010).

Adversely, results recorded in table (3-5) showed that increasing incubation period to 72 hr. resulted in decreasing in the inhibitory effect of *Lactobacillus* isolates. This result agreed with that obtained by Al-Marsoomy, (2008) and Al-Yas, (2006) who reported that the inhibitory effect of *Lactobacillus* spp. decreased with increasing the incubation period to 72 hr. In another study, *S. aureus* was completely inhibited after 72 hr. of incubation period (Anas *et al*., 2008). Aktypis *et al*. (1998) referred that such variation in the inhibitory effect at different incubation periods might be attributed to the nature of LAB isolates used against the test bacteria itself.

Results mentioned in table (3-5) showed that the 48 hr. incubation period was superior for *Lactobacillus* isolates to exhibit their antagonistic effects against methicillin resistant *Staphylococcus aureus.*

Depending on the results above, two *Lactobacillus* isolates *(L. acidophilus* Lb.G2 and *L. gasseri* Lb.G1) that gave best inhibitory effect against test microorganism were chosen for further studies. Filtrates of the two selected isolates were concentrated to two and three folds in order to improve their antagonistic effect. After each fold, the inhibitory effect against MRSA isolate was evaluated.

In general, it was found that the diameter of inhibition zone was correlated to the filtrate concentration. As is showed in figures (3-2) A and B the two-fold concentrated filtrate cause an increase in the inhibitory effect against the test isolate when the zone diameter raised to (17) mm for *L. acidophilus* and (18) mm for *L. gasseri*.

While, the three-fold concentrated filtrates exhibited the highest inhibitory effect with (21 and 23) mm of zone diameters for both isolates, respectively after 24 hrs. of incubation.

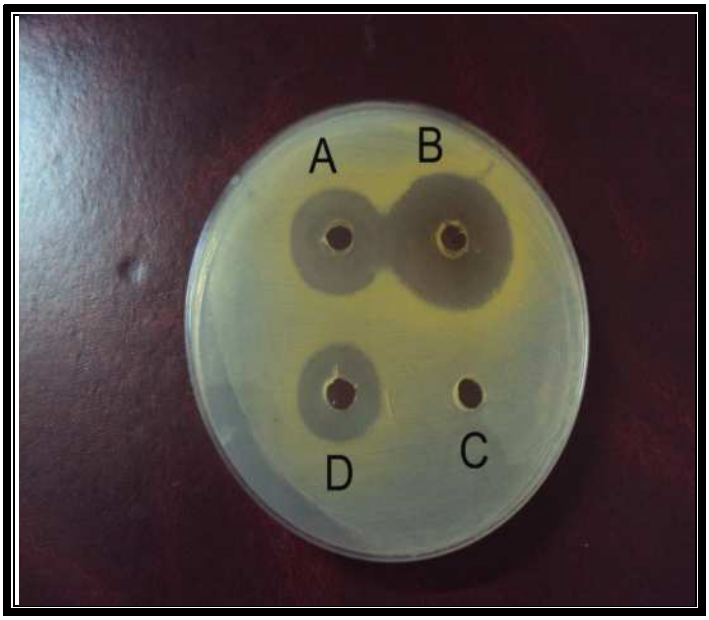


Figure (3-2) A: Inhibition zones of concentrated and unconcentrated filtrates of *Lactobacillus acidophilus* against *Staphylococcus aureus.* Where is: (A) Two-fold (B) Three-fold (C) Unconcentrated filtrate (control) (D) One-fold after 24 hr. of incubation.

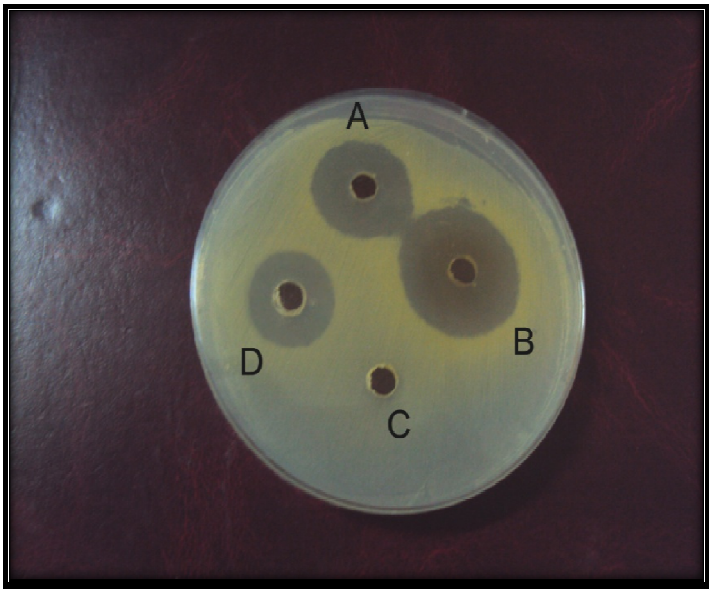


Figure (3-2) B: Inhibition zones of concentrated and unconcentrated filtrates of *Lactobacillus gasseri* against *Staphylococcus aureus.* Where is: (A) Two-fold (B) Three-fold (C) Unconcentrated filtrate (control) (D) One-fold. after 24 hr. of incubation.

Kubba, (2006) found that while one-fold concentrated filtrate of *L. plantarum* gave inhibitory zones between (15-17) mm, the two-fold filtrate gave (18.5) mm. While, the three-fold concentrate caused an increase in the zone diameter to (22) mm. Pfeiffer and Radler, (1982) stated that there was a relationship between the diameter of inhibitory zone and concentration of the inhibitory substances.

3.5 Detection of inhibitory compound of *Lactobacillus* spp.:

In order to characterize the compounds produced by *Lactobacillus* isolates that possess inhibitory activity against *S. aureus* isolate, crude supernatants were assayed for the presence of bacteriocins and organic acids.

3.5.1 Bacteriocin:

Supernatant of *Lactobacillus* isolates were treated with trypsin enzyme in order to determine if bacteriocins are responsible for the inhibitory effect. Results illustrated in figure (3-3) showed that the inhibitory effect of crude supernatant for both isolates of LAB was slightly decreased after treatment with trypsin. Inhibition zones of only (7) mm for *L. acidophilus* and (8) mm for *L. gasseri* were recorded as compared to those resulted by the crude filtrates (12 and 11 mm for the two isolates, respectively).

This result indicating that bacteriocin was not the major factor responsible for the inhibitory effect of the two isolates against *S. aureus*. In this regard, Savadogo *et al*. (2004) found that the inhibitory effect of LAB supernatant was lost after treatment with trypsin enzyme.

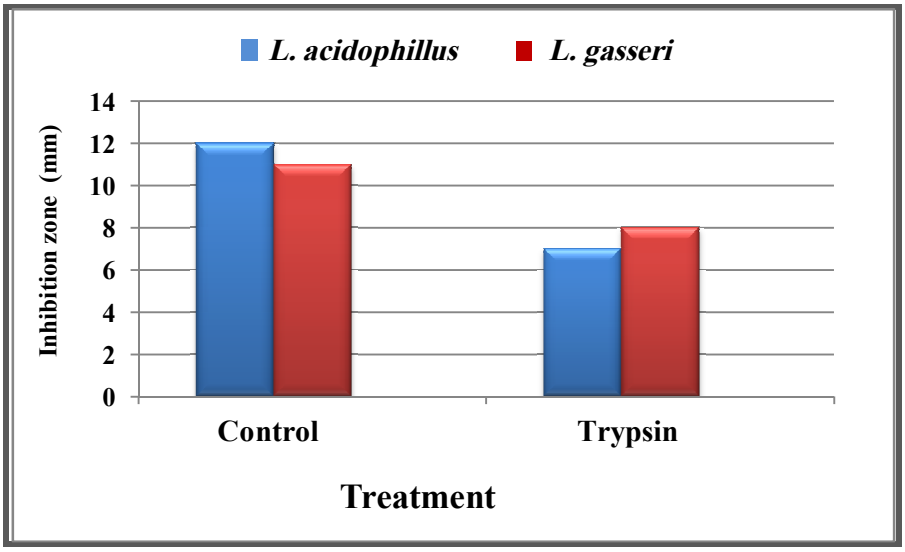


Figure 3-3: Inhibitory effect of *L. acidophilus* and *L. gasseri* crude filtrates against *Staphylococcus aureus* after treatment with trypsin for 24 hr.

Also, Radovanovic and Katic, (2009) reported that bacteriocin was not the major factor responsible for the inhibition of *S. aureus*. On the other hand, Olasupo *et al.* (1997) found that bacteriocin-producing *Lactobacillus* strains obtained from fermented foods were more active against *S. aureus* than those of the non-fermented food origin.

3.5.2 Organic acids:

To remove the effect of organic acids, pH of the crude supernatants of *L. acidophilus* and *L. gasseri* was adjusted to 7 by the addition of NaOH (1 M). Results demonstrated in figure (3-4) showed that no inhibition zones were observed after treatment with NaOH for both *L. acidophilus* and *L. gasseri*, indicating that the isolates inhibited growth of *S. aureus* through the production of organic acids or the synergism effect of both bacteriocin and organic acids. Similar conclusion was achieved by Charlier *et al.* (2009) who found that organic acids produced by *Lactobacillus* spp. were the main factor affecting *S. aureus* growth. Also, Elzbieta and Zdzislawa, (2004) mentioned that acids were the most effective inhibitory factor against *S. aureus*.

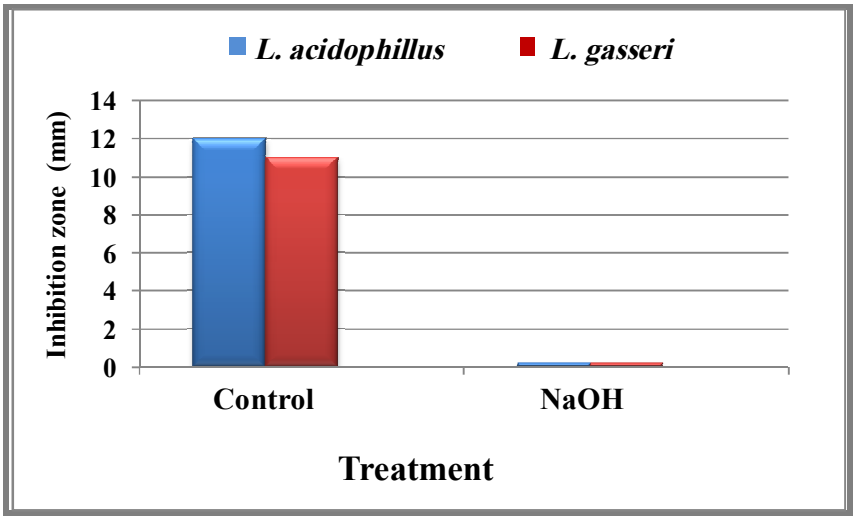


Figure 3-4: Inhibitory effect of *L. acidophilus* and *L. gasseri* crude filtrates against *Staphylococcus aureus* after treatment with NaOH.

In contrast, Guessas *et al*. (2007) reported that *Lactobacillus* isolated from raw goat's milk inhibited growth of *S. aureus* through production of bacteriocins. Ogawa *et al*. (2001) declared that organic acids mainly, lactic acid possesses potent bactericidal activity.

3.6 Antibiotics susceptibility of *Lactobacillus* isolates:

Antibiotics resistancey exist among some lactic acid bacteria including those used as probiotic microorganisms (Salminen *et al.,* 1998). Such resistancey is considered to be an important public health problem due to the possibility of transferring to other opportunistic or pathogenic bacteria (Ammor *et al*., 2008).

The two isolates (*L. acidophilus* and *L. gasseri)* which were previously chosen due to their superior antagonistic effect were subjected to antibiotics susceptibility test using the standard disk diffusion method. Results in table (3-6) showed in general, that a vast of resistancey was reported among *Lactobacillus* isolates against the antibiotics used in this study. It was found that the isolates were most resistance to the two antibiotics inhibiting bacterial

protein synthesis (streptomycin and gentamycin) when zero and (1-2) mm in inhibition zones were recorded respectively, for both isolates.

The two isolates were also resistant to the DNA replication- inhibitor antibiotics, nalidixic acid and ciprofloxacin. While, these two isolates varied in there sensitivity towards tetracycline, trimethoprim and cephalexin antibiotics, When the inhibition zones of *L. gasseri* were (7, 8, 11) mm, and of *L. acidophilus* (10, 11, 7) mm for the three antibiotics, respectively.

Table 3-6: Antibiotics susceptibility of *Lactobacillus gasseri* and *Lactobacillus acidophilus* recorded as diameter of inhibition zones (mm).

Antibiotic	Sym.	Conc. (µg/ml)	*L. gasseri* (Lb.G1)	*L. acidophilus* (Lb.G2)
Rifampicin	RA	5	Int	Int
Streptomycin	S	10	R	R
Ciprofloxacin	CIP	5	R	R
Chloramphenicol	C	30	S	S
Cephalexin	CL	30	Int	R
Cephotaxime	CTX	30	S	S
Tetracycline	TE	30	R	R
Amoxicillin	AX	25	S	S
Gentamycin	CN	10	R	R
Ampicillin	AM	10	R	R
Penicillin	P	10	S	Int
Trimethoprim	TMP	5	R	Int
Nalidixic acid	NA	30	R	R

(R): resist, (S): sensitive and (Int**): intermediate.**

On the other hand, both isolates were highly sensitive to amoxicillin when the inhibiting zones were (22, 17) mm for *L. acidophilus* and *L. gasseri,* respectively. Also, these isolates showed major sensitivity toward chloramphenicol which interferes with the protein synthesis, it gave inhibitory zones of (17) and (20) mm in diameter against *L. gasseri* and *L. acidophilus,* respectively.

Liasi *et al*. (2009) reported that all *Lactobacillus* isolates obtained from fermented foods in their study were resistant to gentamycin, streptomycin, nalidixic acid and trimethoprim but sensitive to penicillin and chloramphenicol.

Rifampicin had almost same effect on both isolates with (11) mm of inhibition diameter. Regarding cephotaxime, (a cell wall synthesis inhibitor) both isolates were less sensitive to it than chloramphenicol when the inhibition zones reached (15) mm for *L. gasseri* and (18) mm for *L. acidophilus*.

A similar finding was obtained by Patel and Goyal, (2010) who found that LAB isolates were sensitive to chloramphenicol, cefotaxime and tetracycline. Results obtained by Modzelewska-Kapitulal *et al.* (2008) showed similar range of susceptibility when all *Lactobacillus* species that have probiotic properties were resistant to nalidixic acid but sensitive towards rifampicin and chloramphenicol.

In contrast, the *Lactobacillus* isolates were variable in their sensitivity towards the penicillin when *L. gasseri* was inhibited by (15) mm and *L. acidophilus* by (12) mm zone diameters. When compared with other studies, Vescovo *et al*. (1982) found that all LAB isolates were sensitive to penicillin except one (*L. acidophilus*) was resistant. While, Korhonen *et al.* (2010) reported that *Lactobacillus* isolates were susceptible to many cell wall synthesis inhibitors like penicillin and ampicillin.

A study by Gupta *et al.* (1995) suggested that LAB cultures should be tested for their sensitivity towards the commonly used chemotherapeutic agents to eliminate starter failure during manufacture of cultured milk products.

In order to recolonization and rebalancing the intestinal microflora, LAB should resist several antibiotics if it wants to be used as probiotic dietary supplements (Sullivan and Nord, 2002).

3.7 Mutagenesis of *Lactobacillus*:

The two *Lactobacillus* isolates (*L. gasseri* and *L. acidophilus*) were subjected to chemical and physical mutagenesis in order to improve their inhibitory effect against *S. aureus*.

3.7.1 Chemical mutagenesis:

Two types of chemical mutagens (ethidium bromide and acridin orange) were used:

3.7.1.1 Mutagenesis by ethidium bromide:

Cell suspensions of each of (*L. acidophilus* and *L. gasseri*) isolates were incubated with different concentrations of ethidium bromide for 30 min at 37˚C. Results illustrated in figure (3-5) showed that the killing effect of ethidium bromide was increased with the increase in ethidium bromide concentration. The total viable count of *L. acidophilus* was decreased from $259x10^6$ CFU/ml (100%) before treatment to 94.5%, 64%, 30%, 5%, 3.4% after incubation with ethidium bromide in concentrations of 50, 100, 150, 200, 250 µg/ml, respectively.

Depending on the survival curve of ethidium bromide mutagenesis for both isolates, survivals of the 10% or less were screened to obtain the probiotic over producing mutants. By selecting 91 mutants randomly of *L. acidophilus* results mentioned in table (3-7) and appendix (1) showed that 9 of these mutants were the most efficient in probiotic production because their inhibitory effect against *S. aureus* was raised to (15.5) mm in comparison with that of the wild type (12 mm).

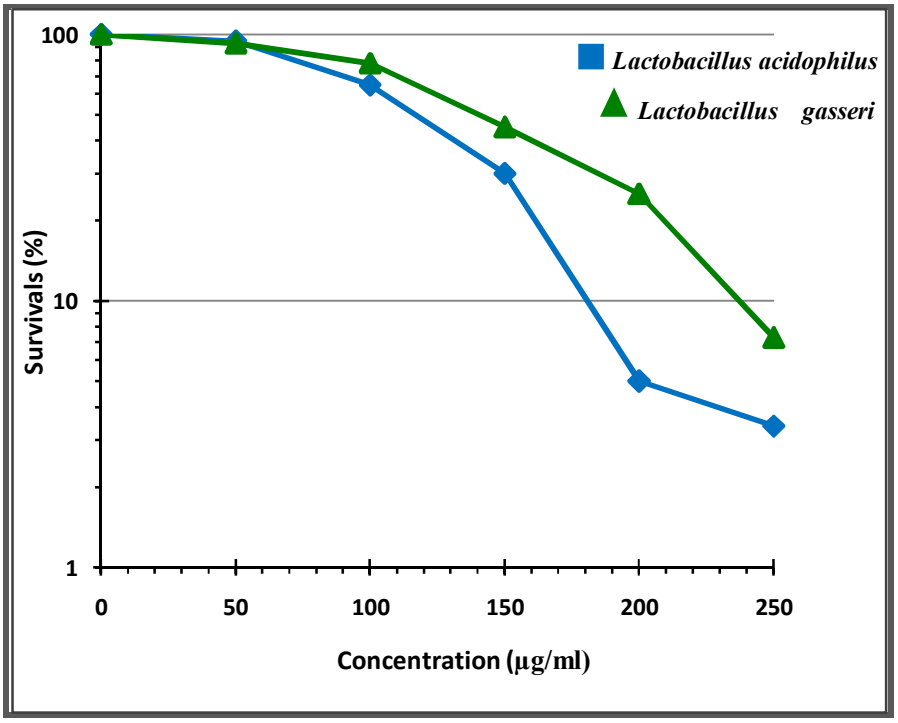


Figure 3-5: Survival curve of Lactobacillus isolates after treatment with five concentations of ethidium bromide.

On the other hand, 65 mutants of *L. acidophilus* were found to have inhibitory effect against *S. aureus* expressed as zones of diameter ranged between (5-12) mm which is less than that of the wild type (12 mm). Ten mutants out of the total (91) of *L. acidophilus* recorded inhibition zones with less than (5) mm in diameter. While, 7 mutants were similar to the control in their inhibitiory effect.

As it was showed in figure (3-6) it was found that the *L. acidophilus* mutant La-m 24 was the most efficient in its inhibitory effect against *S. aureus* because the inhibition zone was increased to (15.5) mm compared with the wild-type (12 mm).

Table 3-7: Distribution of *Lactobacillus acidophilus* mutants according to their inhibitory effect against *S. aureus* after ethidium bromide mutagenesis.

Group of mutants	Percentage of whole	Range of inhibition * zone (mm)
65	71%	5-12
10	11%	<5
7	7.6%	= 12
9	10%	>12

***inhibition zone of the wild type *L. acidophilus* against *S. aureus* was 12mm.**

Lactobacillus gasseri isolate was also incubated with Et-Br at different concentrations for 30 min. to induce genetic mutations which may leads to increase the ability of the isolate in probiotics production. Results mentioned in figure (3-5) showed that the viable count of the bacterial isolate was decreased from $353x10^6$ (100%) before treatment to 92.6%, 77.9%, 45%, 25%, 7.3% after incubation with ethidium bromide in concentrations of 50, 100, 150, 200, 250 µg/ml, respectively.

In the case of *L. gasseri*, 85 mutants were selected randomly and screened according to their inhibitory effect against *S. aueus*. Results mentioned in table (3-8) and appendix (1) revealed that 6 mutants were the most efficient because of the increase of inhibitory effect to (14) mm in diameter than that of the wild type (11mm). In contrast, the inhibitory zones diameter of 52 *L. gasseri* mutants ranged between (5-11) mm which is less than the wild-type (11mm).

While, there are other 16 mutants were decreased in there inhibitory effect because of the decrease in the diameter zones to less than (5) mm, and 11 mutants were similar to the wild-type (11 mm) in their inhibitory effect.

Table 3-8: Distribution of *L. gasseri* mutants according to their inhibitory effect against *S. aureus* after ethidium bromide mutagenesis.

Group of mutants	Percentage of whole	Range of inhibition* zone (mm)
52	61%	5-11
16	19%	<5
11	13%	= 11
6	7%	>11

***inhibition zone of the wild type *L. gasseri* against *S. aureus* was 11mm.**

As it was showed in figure (3-6) it was found that the *L. gasseri* mutant Lg-m 13 was the most efficient in its inhibitory effect against *S. aureus* because the inhibition zone was increased to (14) mm compared with the wild-type (11 mm). Almost similar results were obtained by Sudi *et al.* (2008) who found that mutagenesis of *Lactobacillus bulgaricus* and *Streptococcus thermophillas* (isolated from local yogurt) by ethidium bromide resulted in selecting mutants characterized by high acids production.

Ethidium bromide dye is a well-known reagent that mainly used for nucleic acids detection that binds specifically by the intercalation between base pairs of the double stranded DNAs leading to the enhancement of the striking fluorescence of Et.Br-DNA complexes (Dragan *et al*., 2009). Inhibition of DNA replication and RNA transcription enzymes is found to be another consequence of this intercalation (Luedtke *et al*., 2003). Ethidium bromide is also known by its several biological effects e.g. anti-trypanosomal and antibacterial such as inhibiting growth of *E. coli* and *Bacillus cereus* (Tomchick and Mandel, 1964).

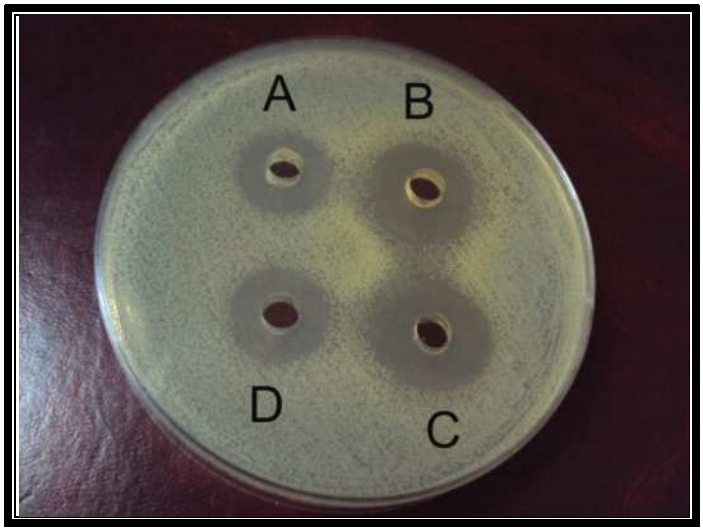


Figure 3-6: Inhibition zones of *L. acidophilus* and *L. gasseri* against *S. aureus* after mutagenesis by ethidium bromide. Where is: (A) Wild isolate of *L. gasseri* (B) Lg-m 13 (D) Wild isolate of *L. acidophilus* (C) La-m 24.

Exposing LAB to random mutagenic conditions such as UV light or chemicals like N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), or ethyl methyl sulphate followed by specific screening for desired traits may result in the identification of new strains with improved utility to pharmaceutical and food industries (Sybesma *et al*., 2006).

3.7.1.2 Mutagenesis by acridine orange:

Another chemical mutagen (acridine orange) was used for mutagenesis of *Lactobacillus* isolates. According to figure (3-7), treatment with acridin orange decrease the viable count of *L. gasseri* from $281x10^6$ CFU/ml (100%) for the control treatment to 86.4%, 63.7%, 30.6%, 22.4% and 7.4%

after subjection to 25, 50, 75, 100 and 125 µg/ml of acridine orange respectively. While, the total viable count of *L. acidophilus* was decreased from $451X10^6$ CFU/ml in the control (100%) to 89.3%, 51.4%, 35.6%, 20.3% and 8.6% after treatment with 25, 50, 75, 100 and 125 µg/ml of this mutagen respectively.

According to the survival curve of acridine orange for both isolates, survivals of 10% or less were screened to obtain the over probiotic produced mutants. A total of 97 *L. acidophilus* mutants and 94 mutants from *L. gasseri* were selected randomly and screened according to their inhibitory effect against *S. aureus*. Results of mutagenesis by acridine orange showed that there is no any effect on increasing their ability in probiotic production and increasing its inhibitory effect against *S. aureus*, hence the most selected mutants kept their inhibitory effect or in some of them was decreased.

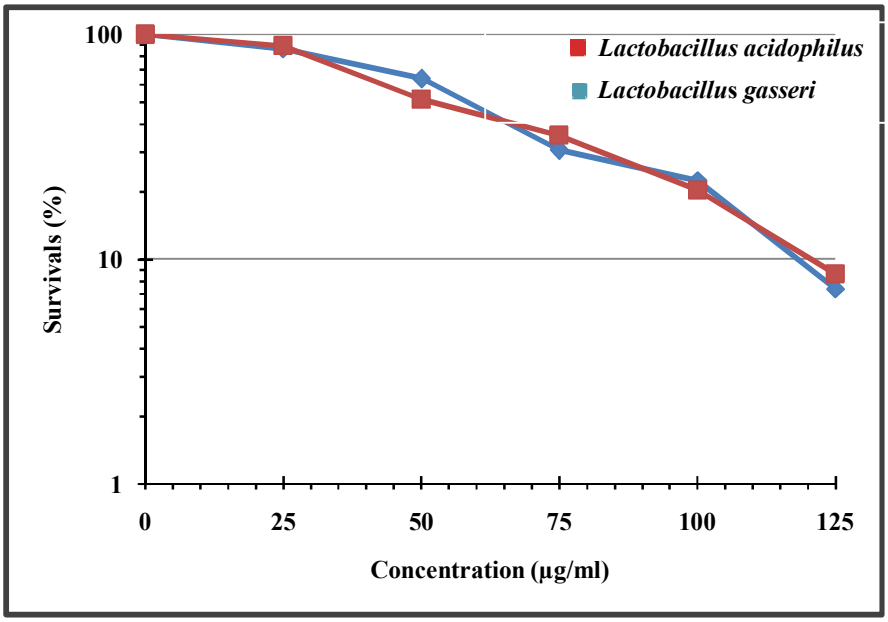


Figure 3-7: Survival curve of *Lactobacillus* isolates after treatment with five concentrations of acridin orange.

Results of mutagenesis by acridine orange showed that there is no any effect on increasing their ability in probiotic production and increasing its inhibitory effect against *S. aureus*, hence the most selected mutants kept their inhibitory effect or in some of them was decreased. Such finding came in accordance with that obtained by Al-Aubaidy, (2006) who found that mutagenesis of LAB isolates by acridine orange had no effect on their inhibitory activity against the

test microorganisms. In contrast, Margino *et al*. (1998) reported that isolates of LAB mutated by acridine orange had increased their antibacterial activity compared with the not mutated isolates.

3.7.2 Physical mutagenesis:

Cultures of *L. acidophilus* and *L. gasseri* were subjected to irradiation by uv ray at a dose of 10 J/m^2 for different periods of time (5, 10, 15, 20 and 25 sec.). Results illustrated in figure (3-8) showed that irradiation of the *Lactobacillus acidophilus* by uv ray decreased its viable count from 283x10^6 (100%) at zero time to 57%, 45%, 15%, 4.2% and 1.4% after 5, 10, 15, 20 and 25 sec. of uv exposure.

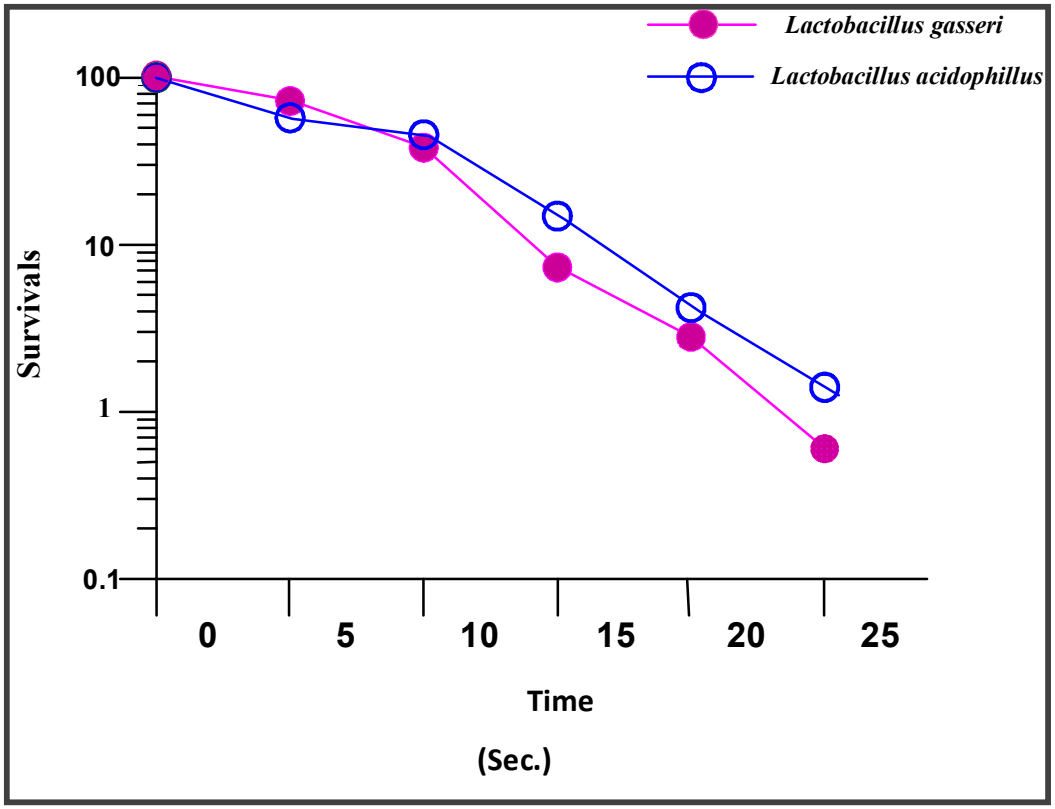


Figure 3-8: Survival curve of *Lactobacillus* isolates after subjection to UV radiation for different periods.

Eighty four of *L. acidophilus* mutants were selected and screened depending on their inhibitory effect against *S. aureus*. Results mentioned in the table (3-9) and appendix (2) showed that 15 of *L. acidophilus* mutants were considered as efficient mutants in probiotic production because their inhibitory effect against *S. aureus* was increased due to the increase of inhibition zones to 16.5 mm compared with that of wild-type (12 mm).

Fifty mutants out of the total (84) ranged between 5-12 mm in their inhibitory effect by comparison with the wild-type (12 mm). On the other hand, 11 *L. acidophilus* mutants recorded less than 5 mm inhibition diameter. The rest 8 mutants were similar to the control (12 mm) in their inhibitory effect.

Table 3-9: Distribution of *Lactobacillus acidophilus* mutants according to their inhibitory effect against *S. aureus* after ultra violate mutagenesis.

Group of mutants	Percentage of whole	Range of inhibition* zone (mm)
50	59.5%	5-12
11	13%	<5
8	9.5%	= 12
15	18%	>12

***inhibition zone of the wild type *L. acidophilus* against *S. aureus* was 12mm.**

On the other hand, the viable count of *L. gasseri* isolate decreased from $312x10^6$ at zero time to 72.4%, 38%, 7.3%, 2.8% and 0.6% after uv exposure for 5, 10, 15, 20 and 25 sec. Regarding *L. gasseri* a total of 88 mutants were selected randomly and screened according to their antagonistic effect on *S. aureus*. Results indicated in table (3-10) and appendix (2) showed that 11 mutants were considered the most efficient mutants when their inhibition zones

raised to 15 mm than that of the wild type (11mm). Out of the total selected mutants 55 were ranged between 5-11 mm in their inhibitory effect. On the other hand, the inhibitory effect of 14 *L. gasseri* mutants was less than 5 mm in diameter. The inhibitory effect of the rest 8 mutants was similar to that in the wild type (11 mm).

Table 3-10: Distribution of *Lactobacillus gasseri* mutants according to their inhibitory effect against *S. aureus* after ultra violate mutagenesis.

Group of mutants	Percentage of whole	Range of inhibition* zone (mm)
55	62.5%	5-11
14	16%	<5
8	9%	= 11
11	12.5%	>11

***inhibition zone of the wild type *L. gasseri* against *S. aureus* was 11mm.**

Results indicated in figure (3-9) showed that the inhibitory effect of *Lactobacillus* isolates against test microorganism was highly affected by UV mutagenesis, when the inhibition zone of the *L. gasseri* mutant (Lg-m 20) was raised to 15 mm after it was 11 mm for the control. While, results mentioned in figure (3-10) revealed that the inhibition zone of *L. acidophilus* mutant (La-m 51) that formed against *S. aureus* raised to 16.5 mm compared with 12 mm for the control (not treated).

These results above confirm that mutagenesis of LAB isolates led to the improvement of inhibitory activity against the test bacteria in comparison to the wild-type (not mutated).

Moat *et al.* (2002) mentioned that all types of mutations may be induced directly or indirectly according to the type of mutagens. Directly, by modifying the purine or pyrimidine bases, causing errors in base pairing that results in replication errors such as in the chemical mutagen (nitrosoguanidin). While, the indirect effect is caused by the faulty repair mechanisms which is called, misrepair, such as in physical mutagen ultraviolet irradiation.

Feng *et al.* (2005) found that treating *Lactobacillus buchneri* CF 10 (a starter strain) with UV radiation resulted in increasing production of its inhibitory compounds. This was also confirmed by Li-Yuan *et al.* (2007) when they noticed that UV mutagenesis of *Lactococcus lactis* V528 led to high productivity of bacteriocin-like substances.

Also, a mutated strain from *Lactobacillus casei* subsp. *rhaminosus* with high-yield L-lactic acid was obtained by Wei *et al*. (2009) after mutagenesis by microwave irradiation.

In contrast, Upreti and Handsdill, (1975) found that the productivity of Lactocin 27 from *L. helveticus* LP 27 was not induced by using UV radiation. As well as Ogunbanwo *et al*. (2003) who reported that using UV radiation and Mitomycin C had no effect on the activity of bacteriocins produced by *L. plantarum* F1 and *L. brevis* OG1. While, Margino *et al*. (1998) reported increases in the antibacterial substances produced by *Lactobacillus plantarum* TGR-2 against *S. aureus* after mutagenesis by UV radiation.

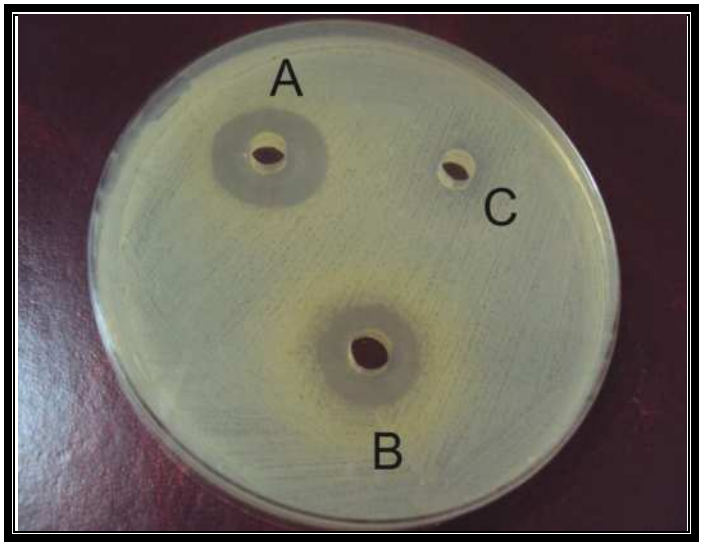


Figure 3-9: Inhibitory activity of *Lactobacillus gasseri* against *S. aureus* after sujection to UV radiation for different periods. Where is: (A) Lg-m 20 (B) wild isolate (C) Control (MRS broth).

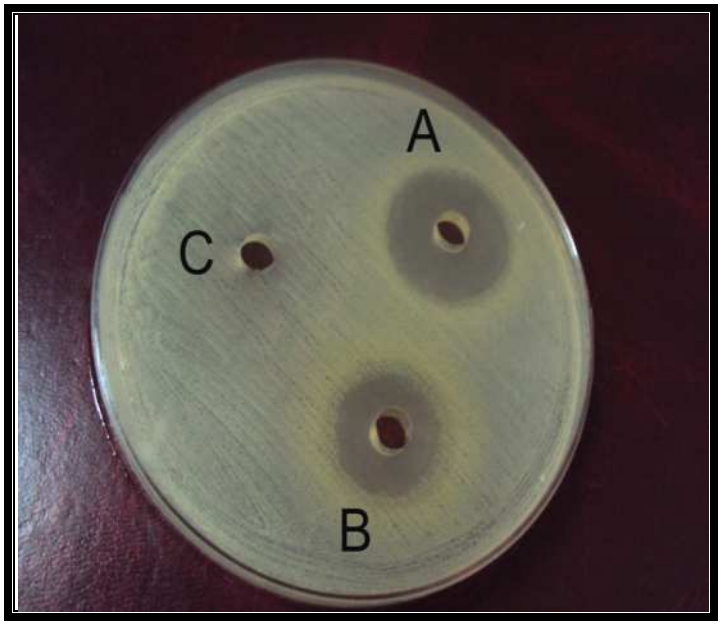


Figure 3-10: Inhibitory activity of *Lactobacillus acidophilus* against *S. aureus* after sujection to UV radiation for different periods. Where is: (A) La-m 51 (B) wild isolate (C) Control (MRS broth).

3.8 Plasmid profile of *Lactobacillus*:

Plasmid DNA in lactic acid bacteria is not always easily detected. This may be due to the growth temperature, nature of the strain, and the isolation procedures used (Kumar *et al*., 2011).

Sewaki *et al*. (2001) stated that plasmid profiling of lactic acid bacteria is considered to be as a useful technique for both food and pharmaceutical industries. As shown in figure (3-11), each of the two isolates and their mutants had the same plasmid profile because each of *L. acidophilus* and *L. gasseri* isolates and their mutants carry one plasmid DNA band.

The plasmid miniprep extraction kit (Promega, USA) was exploited for the isolation of plasmid DNA from *Lactobacillus* isolates. Same isolating protocol was used by Kumar *et al*. (2010) for isolating single plasmid of 19.91 kb in size from two *L. acidophilus* isolates that showed antifungal properties.

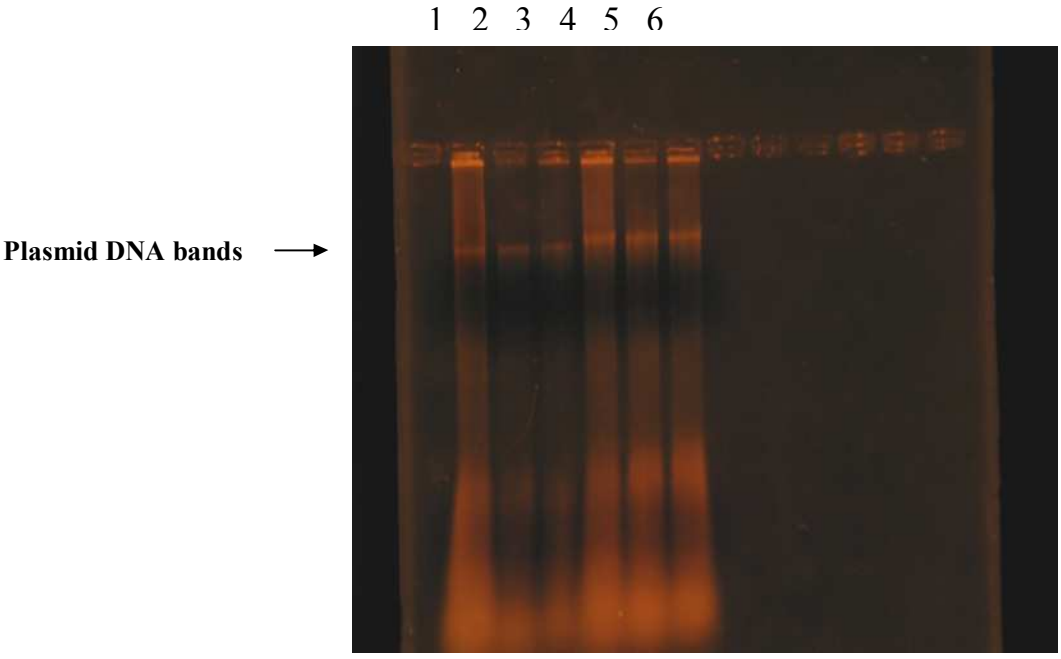


Figure 3-11: Plasmid profile of *L. acidophilus* and *L. gasseri* isolates and their mutants after electrophoresis on 7% agarose gel for 2 hrs.

Lane (1): Wild type of *L. acidophilus*. Lane (2) and Lane (3) its mutants by E.B and UV respectively. Lane (4): Wild type of *L. gasseri* Lane (5) and Lane (6) its mutants by E.B and UV respectively.

Also, the plasmid extraction kit was used by Auputinan *et al*. (2010) for isolating plasmids from twelve *Lactobacillus* spp. from fermented food origin. In contrast, plasmid-free strains of *Lactobacillus* spp. were reported by Todorov and Dicks, (2005) by using plasmid extraction kit.

Presence of plasmids in *L*. *acidophilus* was reported by Klaenhammer and Sutherland, (1980) and also by Van derVossen *et al*. (1994) who concluded that acidocin B production is encoded by the 14 kb plasmid pCV461 in *Lactobacillus acidophilus* M46 strain.

On the other hand, plasmid-free strains of *L*. *acidophilus* were reported by Sewaki *et al*. (2001). While, Soomro and Masud, (2007) stated that most of *L*. *acidophilus* strains which had been isolated from fermented milk were carried single and large plasmids of 20 to 23 kb in size. While, results reported by Roussel *et al*. (1993) showed that *L*. *gasseri* CNRZ222 had a plasmid of 150 kb in size. Almost similar result was obtained by Martín *et al*. (2008) who isolated *L*. *gasseri* from vagina with one plasmid.


Chapter four
Conclusions
and
Recommendations

4. Conclusions and Recommendations

4.1. Conclusions

1- *Lactobacillus* isolates which obtained from local sources possessed inhibitory effect against methicillin resistant *Staphyloccus aureus*.

2- Inhibitory effect of *Lactobacillus* isolates was better when they grew in broth medium than on the solid one.

3- *L. acidophilus* and *L. gasseri* isolates were the most efficient in their inhibitory effect among other *Lactobacillus* isolates.

4- Two and three-fold concentrated filtrates of *L. acidophilus* and *L. gasseri* isolates led to higher antagonistic effect against *S. aureus* by compression with the non-concentrated filtrates.

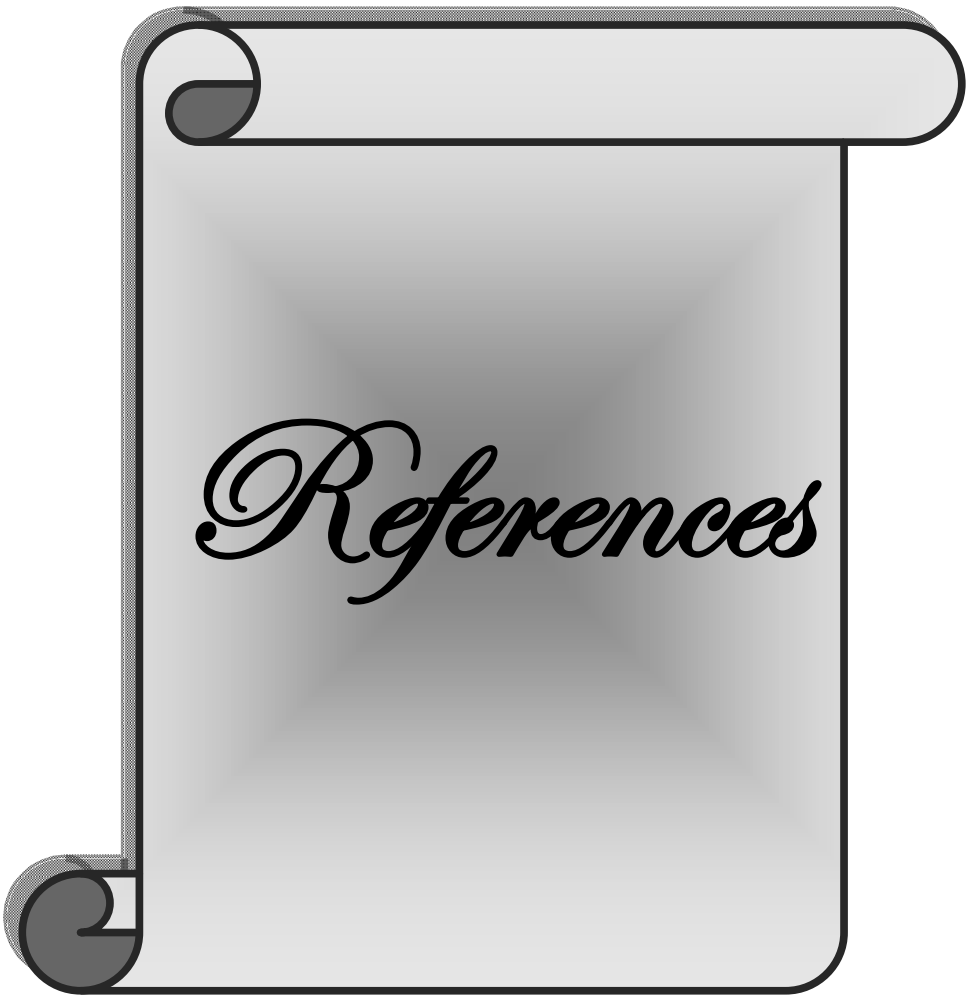
5- Organic acids were the major factor responsible for the inhibitory effect of both *L. acidophilus* and *L. gasseri* isolates against methicillin resistant *S. aureus* (MRSA).

6- Mutagenesis of the two isolates by ethidium bromide and ultra violate led to increase their antagonistic effect against MRSA, while mutagenesis with acridin orange did not do so.

8- Plasmid profile of both *Lactobacillus* isolates (*L. acidophilus* and *L. gasseri*) and their mutants revealed that each isolate carried only one plasmid band.

4.2 **Recommendations:**

1. Investigating the probiotic effects of mutant *Lactobacillus* spp. against other pathogenic bacteria *in vivo*.
2. Subjecting *Lactobacillus* spp. to more genetic studies, especially those bacteriocins producing.
3. Attempting to develop concentrated therapeutic supplements from *Lactobacillus* species.
4. Using other mutagenesis techniques to improve the inhibitory effect of *Lactobacillus* spp. against pathogenic bacteria.
5. Design cloning vector from *Lactobacillus* plasmids for production of therapeutic compounds.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Appendix

Appendix 1: Inhibition zones of the crude filtrate of mutants and wild type of *L. acidophilus* and *L. gasseri* against *S. aureus* after ethidium bromide mutagenesis.

L. acidophilus mutants	Inhibition zones (mm)	*L. gasseri* mutants	Inhibition zones (mm)
La-m1	8	Lg-m1	9
La-m2	5	Lg-m2	10.5
La-m3	14	Lg-m3	11
La-m4	11	Lg-m4	12
La-m5	6	Lg-m5	8
La-m6	4.5	Lg-m6	-
La-m7	-	Lg-m7	5
La-m8	7.5	Lg-m8	7.5
La-m9	6	Lg-m9	9
La-m10	5.5	Lg-m10	10.5
La-m11	5	Lg-m11	-
La-m12	6.5	Lg-m12	11
La-m13	9	Lg-m13	14
La-m14	10.5	Lg-m14	6.5
La-m15	8	Lg-m15	9
La-m16	12	Lg-m16	-
La-m17	11	Lg-m17	7
La-m18	9	Lg-m18	11
La-m19	7	Lg-m19	5
La-m20	-	Lg-m20	-
La-m21	9.5	Lg-m21	13.5
La-m22	10	Lg-m22	11

La-m23	13	Lg-m23	6
La-m24	15.5	Lg-m24	9
La-m25	7.3	Lg-m25	10
La-m26	5	Lg-m26	-
La-m27	-	Lg-m27	9.5
La-m28	11.5	Lg-m28	7
La-m29	11	Lg-m29	5.5
La-m30	8.5	Lg-m30	5
La-m31	7	Lg-m31	-
La-m32	-	Lg-m32	4.5
La-m33	14.5	Lg-m33	6
La-m34	10	Lg-m34	11
La-m35	5	Lg-m35	14
La-m36	7	Lg-m36	10.5
La-m37	13.5	Lg-m37	6
La-m38	10	Lg-m38	9.5
La-m39	9	Lg-m39	5
La-m40	12	Lg-m40	7
La-m41	-	Lg-m41	-
La-m42	5	Lg-m42	7.5
La-m43	7	Lg-m43	-
La-m44	12	Lg-m44	-
La-m45	8	Lg-m45	10
La-m46	5	Lg-m46	13
La-m47	-	Lg-m47	10.5

La-m48	14	Lg-m48	5
La-m49	15.5	Lg-m49	7
La-m50	4.5	Lg-m50	10
La-m51	7	Lg-m51	11
La-m52	12	Lg-m52	9.5
La-m53	6	Lg-m53	-
La-m54	10	Lg-m54	14
La-m55	-	Lg-m55	11
La-m56	9.5	Lg-m56	7.5
La-m57	6	Lg-m57	-
La-m58	-	Lg-m58	8
La-m59	10.5	Lg-m59	5.5
La-m60	8	Lg-m60	6
La-m61	12	Lg-m61	7
La-m62	11	Lg-m62	9
La-m63	10.5	Lg-m63	7
La-m64	11	Lg-m64	11
La-m65	5	Lg-m65	8.5
La-m66	10.5	Lg-m66	11
La-m67	13	Lg-m67	-
La-m68	6	Lg-m68	5
La-m69	6.5	Lg-m69	7
La-m70	5	Lg-m70	-
La-m71	9	Lg-m71	-
La-m 72	7	Lg-m72	10

La-m73	7.5	Lg-m73	11
La-m74	6	Lg-m74	5
La-m75	9	Lg-m75	11.5
La-m76	15	Lg-m76	-*
La-m77	9	Lg-m77	-
La-m78	12	Lg-m78	6
La-m79	11	Lg-m79	9
La-m80	7.5	Lg-m80	8.5
La-m81	9	Lg-m81	10.5
La-m82	-	Lg-m82	9
La-m83	-	Lg-m83	5.5
La-m84	12	Lg-m84	7
La-m85	10	Lg-m85	8.5
La-m86	6	**(Wild type)**	**11mm**
La-m87	8		
La-m88	7		
La-m89	10.5		
La-m90	11		
La-m91	8.5		
(Wild type)	**12 mm**		

*** (-) referred to inhibition zones less than (5 mm).**

Appendix 2: Inhibition zones of the crude filtrate of mutants and wild type of *L. acidophilus* and *L. gasseri* against *S. aureus* after ultra violate mutagenesis.

***L. acidophilus* mutants**	**Inhibition zones (mm)**	***L. gasseri* mutants**	**Inhibition zones (mm)**
La-m1	13.5	Lg-m1	15
La-m2	14	Lg-m2	7
La-m3	10	Lg-m3	-
La-m4	5	Lg-m4	-
La-m5	7.5	Lg-m5	5.5
La-m6	9	Lg-m6	9.5
La-m7	8	Lg-m7	7
La-m8	10.5	Lg-m8	9
La-m9	9	Lg-m9	10
La-m10	7	Lg-m10	15
La-m11	7	Lg-m11	11
La-m12	6	Lg-m12	9
La-m13	5.5	Lg-m13	7
La-m14	11	Lg-m14	14.5
La-m15	10	Lg-m15	11
La-m16	-	Lg-m16	11
La-m17	8	Lg-m17	8
La-m18	11.5	Lg-m18	9
La-m19	6	Lg-m19	-
La-m20	12	Lg-m20	15
La-m21	16	Lg-m21	5
La-m22	9	Lg-m22	10

La-m23	10	Lg-m23	15.5
La-m24	-	Lg-m24	7.5
La-m25	6	Lg-m25	10
La-m26	8	Lg-m26	13
La-m27	9.5	Lg-m27	11
La-m28	14	Lg-m28	8.5
La-m29	14.5	Lg-m29	5
La-m30	7	Lg-m30	-
La-m31	5.5	Lg-m31	8
La-m32	15	Lg-m32	10.5
La-m33	12	Lg-m33	-
La-m34	16	Lg-m34	6
La-m35	13.5	Lg-m35	10
La-m36	-	Lg-m36	9
La-m37	9	Lg-m37	9
La-m38	5	Lg-m38	5
La-m39	-	Lg-m39	15
La-m40	12	Lg-m40	9.5
La-m41	10	Lg-m41	6
La-m42	14	Lg-m42	14
La-m43	11	Lg-m43	9
La-m44	9.5	Lg-m44	11
La-m45	7	Lg-m45	6
La-m46	-	Lg-m46	13.5
La-m47	7	Lg-m47	10.5

La-m48	8	Lg-m48	11
La-m49	14	Lg-m49	5.5
La-m50	12	Lg-m50	-
La-m51	16.5	Lg-m51	6.5
La-m52	10	Lg-m52	11
La-m53	12	Lg-m53	7
La-m54	6	Lg-m54	9
La-m55	5	Lg-m55	6
La-m56	-	Lg-m56	14
La-m57	11	Lg-m57	7
La-m58	6	Lg-m58	8
La-m59	10	Lg-m59	5
La-m60	9	Lg-m60	-
La-m61	15	Lg-m61	8.5
La-m62	-	Lg-m62	10
La-m63	6	Lg-m63	9.5
La-m64	-	Lg-m64	-
La-m65	5	Lg-m65	-
La-m66	14.5	Lg-m66	8
La-m67	7	Lg-m67	6
La-m68	12	Lg-m68	6
La-m69	10.5	Lg-m69	7.5
La-m70	8.5	Lg-m70	-
La-m71	-	Lg-m71	5
La-m72	16.5	Lg-m72	8

La-m73	5.5	Lg-m73	10
La-m74	12	Lg-m74	9
La-m75	10	Lg-m75	5
La-m76	-	Lg-m76	6
La-m77	7.5	Lg-m77	-
La-m78	9	Lg-m78	14
La-m79	-	Lg-m79	11
La-m80	8	Lg-m80	-
La-m81	12	Lg-m81	-
La-m82	10.5	Lg-m82	7.5
La-m83	10	Lg-m83	6
La-m84	16.5	Lg-m84	-
(Wild type)	**12 mm**	Lg-m85	5.5
		Lg-m86	7
		Lg-m87	9.5
		Lg-m88	10.5
		(Wild type)	**11mm**

*** (-) referred to inhibition zones less than (5 mm).**

الخلاصة

امكن الحصول من الحليب الخام والزبادي والخل والمهبل على ستة عشر عزلة من بكتريا *Lactobacillus* spp. بواسطة زرعها اولا على وسط MRS الصلب قبل اخضاعها للفحوصات المجهرية والزرعية والكيموحيوية. وبغية أختبار فعاليتها التثبيطية ضد بكتريا المكورات العنقودية المقاومة للمثيليسين (بكتريا ألأختبار), فقد تمت تنمية جميع العزلات مرة على وسط اكار MRS وأخرى في مرق نفس الوسط. اظهرت النتائج ان التأثير التثبيطي للعزلات المنماة في الوسط السائل كان اعلى من تلك على الوسط الصلب, واعتمادا على ذلك, فقد اختير الوسط السائل لتنمية العزلات في التجارب اللاحقة.

تم الحصول على رواشح العزلات بعد تنميتها قي الوسط السائل ومن ثم تركيزها لمرة واحدة. لم تشير النتائج الى حصول تأثير تثبيطي للرواشح غير المركزة ضد بكتريا ألأختبار, فيما حصل ذلك لتلك المركزة لمرة واحدة لاسيما بعد تنميتها لمدة 24 ساعة. ومن بين العزلات الستة عشر, اعطت العزلتان *Lactobacillus gasseri* (Lb.G1) *Lactobacillus acidophilus* (Lb.G2) اللتان مصدرهما المهبل افضل فعالية تثبيطية مما ادى الى استخدامهما في التجارب اللاحقة.

ازداد التأثير التثبيطي لراشحي العزلتين المركزين لمرتين وذلك عندما بلغ قطرمنطقة التثبيط (17) ملم للعزلة *Lactobacillus acidophilus* و(18) ملم *Lactobacillus gasseri* بعد ان كان على التوالي (12) و (11) ملم لراشحي نفس العزلتين المركزين لمرة واحدة وذلك بعد التنمية على درجة حرارة 37° م لمدة 24 ساعة. ومن جهة اخرى, فقد اعطى راشحي العزلتين المركزين لثلاث مرات افضل فعالية بقطري منطقتي تثبيط بلغتا (21) و (23) ملم على التوالي.

لدى اختبار تأثير المركبات التي تنتجها بكتريا اللاكتيك, اظهرت النتائج ان الحوامض العضوية هي المسؤول الرئيس عن الفعالية التثبيطية ضد بكتريا ألأختبار.

اشارت النتائج التي تم الحصول عليها من اختبار حساسية عزلات اللاكتيك تجاه (13) مضادا حيويا انها كانت مقاومة لل streptomycin, ciprofloxacin, gentamycin, ampicillin وnalidixic acid لكنها حساسة لchloramphinicol, cephotaxime و amoxicillin , فيما تغايرت حساسيتها تجاه بقية المضادات المستخدمة.

بعد تعريض العزلتين (في اعلاه) للتطفير بغية الحصول على طفرات ذات فعالية تثبيطية افضل ضد بكتريا ألأختبار, ادى التطفير فيزياويا باستخدام ألأشعة فوق البنفسجية الى زيادة التأثير التثبيطي ضد بكتريا المكورات العنقودية الذهبية وذلك بزيادة قطر منطقتي التثبيط الى (16,5) ملم بالنسبة لعزلة *L. acidophilus* والى (15) ملم ل*L. gasseri*. ومن جهة اخرى, ادى التطفير الكيمياوي ببروميد ألأثيديوم الى زيادة التأثير ضد بكتريا ألأختبار وبقطري منطقتي تثبيط بلغا (15.5) و (14) ملم للعزلتين, على التوالي, مقارنة بمعاملة سيطرتهما (12و 11) ملم. فيما لم يؤدي التطفير الكيمياوي بالأكريدين البرتقالي الى اي تحسن في الفعالية التثبيطية لكلتا العزلتين.

دلت النتائج التي امكن الحصول عليها من عزل وترحيل المحتوى البلازميدي لعزلات بكتريا اللاكتيك وطافراتها, ان النسق البلازميدي كان متماثلا وذلك من خلال حمل كل عزلة لحزمة بلازميدية واحدة.

بسم الله الرحمن الرحيم

﴿كَمَا أَرْسَلْنَا فِيكُمْ رَسُولاً مِّنكُمْ يَتْلُو

عَلَيْكُمْ آيَاتِنَا وَيُزَكِّيكُمْ وَيُعَلِّمُكُمُ الْكِتَابَ

وَالْحِكْمَةَ وَيُعَلِّمُكُم مَّا لَمْ تَكُونُواْ

تَعْلَمُونَ﴾

صدق الله العظيم

سورة البقرة (١٥١)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

تعريض بكتريا *Lactobacillus* المعزولة محلياً للتطفير لتحسين فعاليتها التثبيطية ضد بكتريا *Staphylococcus aureus*

رسالة

مقدمة الى كلية العلوم –جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الإحيائية

من قبل

ابتسام سالم مهدي

بكلوريوس بايولوجي– كلية العلوم– الجامعة المستنصرية–2003

بإشراف

أ.د. عبد الواحد باقر الشيباني **م.د. شيماء حسين**

ذي الحجة- 1432 تشرين الأول-2011