Bacteriological study on *Proteus mirabilis* after subjection to probiotic

A Thesis

Submitted to the Council of the College of Science /Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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B. Sc. Biotechnology/ College of Science/ Al-Nahrain University (2011)

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January 2014 Rbea Al-awale 1435

الإهداء

إلى من علم هذا الكون

كيف يخط بأحلى لون...بلدي العراق...

إلى الطيبة أمي...

إلى العزيز أبي...

إلى سدي الذي يحميني حين تفيض بحار الحياة

احمد أخي...

إلى من اشد بهم أزري

إخوتي وأخواتي ...

إلى أستاذي الدكتور حميد المحترم...

إلى كل من له الفضل في تعليمي

ابتداء من الذي علمني كيف أمسك القلم...

تقى

Acknowledgements

At the beginning, Praise to great ALLAH the Lord of the Universe who gave me the faith and strength to accomplish this work. The Prayers and the peace are upon our Prophet the master of the creatures Mohammed, and his genial modest family and his companions.

Special thanks with the respect and appreciation to my supervisor Professor Dr. Hameed Majeed for his kind follow up throughout the research stages, as well as his continuous support, valuable advices, and providing all required facilities to perform this work.

Great thank for Professor Dr. Abdulwahid B. Al-Shaibani for his support and continuous helpful.

Special thanks and appreciation to Mr. Abd Al-Khaliq Abas, Mrs. Entesar, Mr. Ziyad, Mrs. Beaan, Mrs. Karema, Mrs. Ashnaa, Mr. Ali, Mrs. Amal, Mr. Haeder, Mr. Mohanned, and all the staff in the central public health laboratory for their cooperation during the research period, the knowledge I gained from them and both academic and nonacademic matters have been invaluable and will definitely be beneficial to my future career.

I am very thankful for staff of the Department of Biotechnology at Al-Nahrain University for their appreciable help, especially Mr. Zaid, Mrs. Tamara, Mr. Mohammed Lftaa and all my colleagues. Thanks to my friend sarah in the Department of Biology at Baghdad University for her special helpful.

It is a pleasure to thank my friend Noorhan Sabih (my soul sister), for her support and helpful. And I would like to extend my thankful to my best friends Khtam, Marwaa and Aliaa for their supporting all the time.

Finally, deep thanks and respect should be conveyed to my family for their patience, help and support.

Touka

Supervisors Certification

I, certify that this thesis entitled "**Bacteriological study on *Proteus mirabilis* after subjection to probiotic''** was prepared by **''Touka Hasson Ali''** under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology.

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Committee Certification

We, the examining committee certify that we have read this thesis entitled **"Bacteriological study on *Proteus mirabilis* after subjection to probiotic"** and examined the student **''Touka Hasson Ali''** in its contents and that in our opinion, it is accepted for the Degree of Master of Science in Biotechnology.

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Summary

For the isolation of *Proteus mirabilis,* 85 samples were collected from different locations in Baghdad governorate. These samples include 20 samples of urine, 45 samples of wounds, and 20 samples of chicken and meat. From the overall samples a total of 146 isolates were obtained. These isolates were identified according to their morphological, microscopical characteristic and biochemical tests. Results of identification showed that 15 of the total isolates were belonged to *P. mirabilis.* These results were confirmed by identification using Api20E system.

Pathogenecity of local isolates of *P. mirabilis* were studied by investigating several virulence factors of these isolates of *P. mirabilis* which include invasion and adhesion ability and protease production. Results showed that most of these isolates invaded uroepithelial cells with average range between 1 and 20 bacterial cell/uroepithelial cell, have the adhesion ability with average range between 30 and 55 bacteria/uroepithelial cell, and able to produce protease with specific activity between 14.5 and 0.121 U/mg protein. Among these different isolates, the isolate *P. mirabilis* P15 from urine was selected for further studies because it was the most virulent.

Antibiotic susceptibility of *P. mirabilis*P15 against different antibiotics was examined. Results showed that this isolate was reiststant to Tetracycline, Cefotaxime, Cefepime, Amikacinand, Ciprofloxacin, while it was sensitive to other antibiotics studied.

Plasmid profile of *P. mirabilis* P15 was detected by extraction of plasmid DNA according to alkaline lysis method.

Results of electrophoresis on agarose gel showed that this isolate harboring two plasmids differ in their size conferring to cefotaxime and Amikacin resistance, according to the results of curing experiment by using ethidium bromide.

Another part of this study was focused on the effect of probiotic of lactic acid bacteria on growth and virulence factors of *P. mirabilis* P15. This was achieved first by re-identification of an isolate of *Lactobacillus acidophilus*, then probiotic of fresh culture of this isolate was used to study its effect on *P. mirabilis* P15. Results showed that invasiveness, adhesion, and protease production by *P. mirabilis* P15 were highly influenced and decreased after treatment with probiotic, and the effect was increased with the increase of probiotic concentration.

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List of Abbreviation

Abbreviate	Full name
Api	Analytical Profile Index
Bp	Base pair
BSA	Bovine Serum Albumin
CDSs	Coding Sequences
CNF1	Cytotoxic Necrotizing Factor 1
DNA	Deoxyribonucleic acid
D.W.	Distilled water
EDTA	Ethylene diamine tetra acetic acid
IgA	Immunoglobulin A

IgG	Immunoglobulin G
Kb	Kelo base
KDa	Kelo Dalton
LAB	Lactic Acid Bacteria
Nm	Nanometer
O.D	Optical Density
P. mirabilis	*Proteus mirabilis*
PBS	Phosphate buffered saline
pH	Power of Hydrogen
R-plasmid	Resistant plasmid
Rpm	Revolution per minute
rRNA	ribosomal ribonucleic acid
sIgA	Secretary Immunoglobulin A
Spp.	Species
TCA	Trichloroacetic acid
UEPCs	Uroepithelial cells.
UTI	Urinary Tract Infection
WHO	World Health Organization

Chapter One

Introduction
and
Literature Review

1.1 Introduction

Proteus species, members of the family *Enterobacteriaceae* are motile Gram negative enteric bacteria, they are important pathogens of the urinary tract and are the primary infectious agent in patients with indwelling urinary catheters. Individuals suffering from urinary tract infections caused by *Proteus mirabilis* often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever (Johnson *et al*., 1993 and Burall *et al*., 2004).

Several potential virulence factors of *Proteus* have been studied in relation to its virulence and pathogenicity, including hydrolysis of urea by urease, cell invasiveness, cytotoxicity induced by hemolysins, cleavage of IgA and IgG by proteolytic enzyme and adherence to the uroepithelium mediated by fimbriae (Nucleo *et al*., 2010).

There were many proposed mechanisms and influencing factors for the invasive properties of *P. mirabilis*. Microbial invasion could be facilitated by virulence factors, microbial adherence, and resistance to antimicrobials. Virulence factors assisted pathogens in invasion and resistance of host defences. Bacterial proteins with enzymatic activity (e.g. protease, elastase, collagenase) facilitated localtissue spread. Microbial adherence to surfaces helps microorganisms establish a base to penetrate tissues. The adhesive properties in the *Enterobacteriaceae* were generally mediated by different types of pili (Coker *et al*., 2000).

The ability of *P. mirabilis* to express virulence factors, including urease and haemolysin, and to invade human urothelial cells, is coordinately regulated with swarming differentiation (Liaw *et al*., 2004).

Virulence factors of pathogenic bacteria (adhesins, toxins, invasins, protein secretion systems, and others) may be encoded on chromosomal DNA, bacteriophage DNA, plasmids, or transposons in particular regions of the prokaryotic genome termed pathogenicity islands (Johnson *et al*., 2003).

E.coli, Proteus species and *Pseudomonas* species isolates carrying plasmid of molecular size above 2.1kb. The plasmid were able to move genetic antibiotic resistant materials among various bacterial strains and contribute to overall pathogenic potential of disease causing bacteria (Motta *et al.,* 2003; Yah *et al.,* 2007).

Probiotic is defined as " living organisms which upon ingestion in certain numbers exert health benefits beyond inherent base nutrition" one of the most significant groups of probiotic organisms are the lactic acid bacteria, these bacteria have a long history safe use in food. New strategies devised the use of probiotics as an alternative therapy and prevention of bacterial gastrointestinal and renal infections (Fooks and Gibson, 2002; Bomba *et al.,* 2006). There is a growing interest in probiotics as a safe therapeutic agent through their ability to alleviate food allergies, enhance non-specific and specific immune responses, suppress intestinal infections, and anticarcinogenic activity (Grajek *et al.,* 2005).

Using probiotics has been expanded for controlling Entropathogenic bacterial infection such as that belonging to *Escherichia, Shigella, Salmonella, Proteus* and *Staphylococcus* (Collado *et al.,* 2007).

According to these mentioned above, this study was aimed to:

1. Isolation and identification of *Proteus mirabilis* from different clinical (urine and wound) and meat samples.
2. Detection of virulence factors produced by the bacterial isolates and selection the most virulent isolate.
3. Study the plasmid profile of the most virulent isolate.
4. Curing of plasmid DNA of the selected isolate to detect the relationship between plasmid profile and bacterial virulence factors.
5. Investigation the inhibitory effects of *Lactobacillus acidophilus* filtrate as a probiotic against pathogenecity of selected *P. mirabilis* isolate.

1.2 Literature Review

1.2.1 Genus *Proteus*

Proteus is one of important medical genera belongs to the *Enterobacteriaceae* family. In this family it is placed in the tribe *Proteeae*, together with the genera *Morganella* and *Providencia*. Bacteria in this genus is Gram negative rod, measuring (1.5-3) μm in length and 0.5 μm in diameter, motile by peritrichous flagella, facutatively anaerobic, nonsporforming, uncapsulated, most isolates having fimbriae, it's oder is very strong. The genus *Proteus* currently consists of five species which are *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens* (O'hara *et al.*, 2000).

Proteus applied its name by Hauser in 1885 for their different shapes, from short vegetative swimmer cell to elongated highly flagellated forms referred as swarmer cell. The most important feature which differentiated *Proteus* from other genera in the *Enterobacteriaceae* family is the swarming phenomena (Mobley and Belas, 1995).

Proteus could be isolated from clinical specimen such as urine, wound, and blood and it also found in normal flora in the intestine of healthy human, but it is considered as opportunistic pathogens that causing many infection when moves from their normal site. These kinds of infections are associated with all three opportunistic species, *P. vulgaris*, *P. penneri*, and *P. mirabilis*. However, the last of these is the most common pathogen; this can be explained by its high carriage rate (25%) in human intestines. however, that these bacteria are in general less virulent than the pathogenic *Escherichia coli* strains (Asad and Amna, 2004).

Microorganisms belonging to *Proteus* spp. are widely distributed in the natural environment. They can be found in polluted water and in soil and manure, where they play an important role in decomposing organic matter of animal origin. *Proteus* rods also exhibit proteolytic activity under aerobic and facultatively anaerobic conditions. The oxidative deamination of amino acids

and the ability to hydrolyze urea to ammonia and carbon dioxide are the most representative biochemical properties of these bacteria (Jacobsen *et al.*, 2008).

Proteus have developed several morphological and biochemical features and factors such as fimbriae, flagella, enzymes (urease, proteases, and amino acid deaminases), and toxins (hemolysins and endotoxin), which act individually or in concert during infection (Rozalski *et al.*, 1997).

1.2.2 *Proteus mirabilis*

P. mirabilis is a motile, urea-hydrolyzing, opportunistic pathogen of the human urinary tract that infects patients with indwelling urinary catheters or with postoperative wound infections. The hallmarks of *P. mirabilis* infection include the formation of calcium- and magnesium-rich stones in the bladder and kidney mediated by urease-catalyzed urea hydrolysis and stable and persistent biofilms formed by various fimbriae (Johnson *et al.*, 1993; Rocha *et al.*, 2007).

This organism is not usually a pathogen, but does become a problem when it comes into contact with urea in the urinary tract. From there, infection can spread to other parts of the body. It is one of the species within the *Proteus* genus responsible for causing urinary tract infections in thousands of people each year in hospitals (Nielubowicz *et al.*, 2008).

P. mirabilis infection are difficult to treat and are often fatal as these bacilli are resistant towards a wide spectrum of antibiotics . *P. mirabilis* is increasingly being reported as Extended spectrum ß-lactamas (ESBL) producers (Wu *et al.*, 2006).

P. mirabilis genome codes for at least 10 adhesion factors making this organism extremely sticky and motile. *P. mirabilis* tests indole-negative and it can be easily identifiable in a blood agar plate by the formation of concentric rings of its swarming movement. It also makes a variety of fimbriae. The endotoxins of its lipopolysaccharide membrane elicit an inflammatory response from the host. *P. mirabilis* produces urease, an enzyme that converts urea into

ammonia by the following process: $(NH_2)_2CO \rightarrow 2NH_3 + CO_2$. Infection by *P. mirabilis* can therefore be detected by an alkaline urine sample (pH 8 and up) with large amounts of ammonia (Burall *et al.*, 2004).

Pearson *et al*., (2008) using microarrays analyzed *P. mirabilis* gene expression *in vivo* from experimentally infected mice and showed 471 gene upregulated and 82 downregulated in comparison to the *in vitro* broth culture. Upregulated genes encoded among others mannose-resistant *Proteus*-like fimbriae, urease, and iron uptake systems. Genes encoding flagella were downregulated.

P. mirabilis evades the immune system response during infection thanks to the production of sIgA protease. The best characterized is 54-kDa secreted metalloprotease ZapA (Loomes *et al*., 1990).

The antigens found on the outer membrane of *P. mirabilis* can potentially serve as targets for vaccines. So far, of the 37 identified immuno-reactive antigens, 23 are surface-bound proteins. Studies have shown that 2 iron acquisition proteins increase the virulence of *P. mirabilis* in the urinary tract. Since both of these proteins contribute to pathogenesis, they are good candidates for vaccines. Once an effective vaccine is made for these antigens, further research will determine whether or not these vaccines may be used against other bacteria that cause complicated urinary tract infections, such as *Providencia* and *Morganella* (Li *et al.*, 2004).

Extensive research in the last two decades has identified a significant role for fimbriae in the pathogenesis of *P. mirabilis*. They aid in the process of infection by mediating adhesion, aggregation, hemagglutination, or motility *in Vivo* (Rocha *et al.*, 2007).

1.2.3 Virulence Factors of *P. mirabilis*

Proteus mirabilis have many Virulence Factors shown in figure (1.1) which help in causing the infections

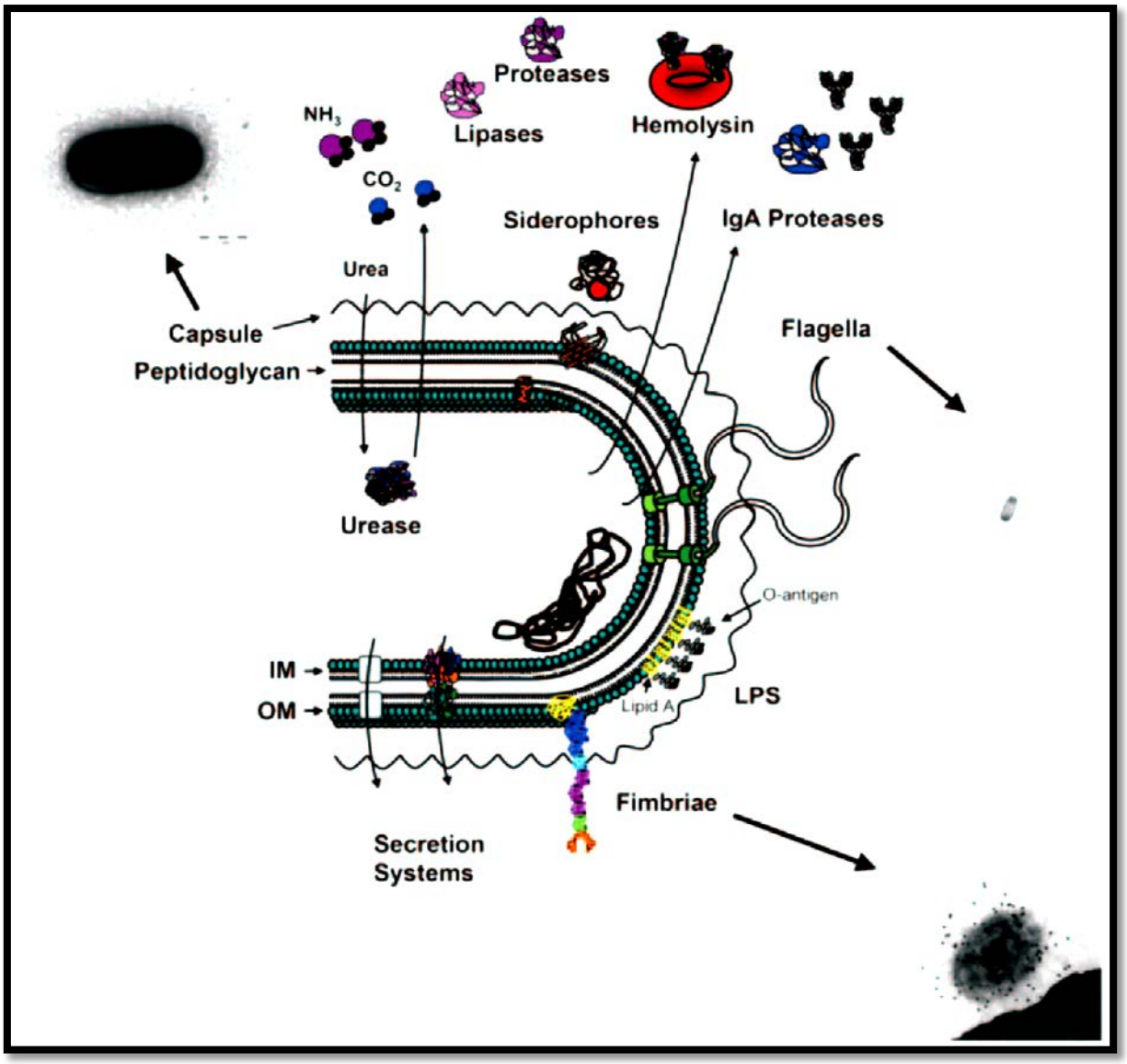

Figure (1.1): Virulence factors of the gram-negative uropathogen *P. mirabilis* (Jones *et al.*, 2004).

1.2.3.1 Fimbriae and Adherence Ability

Fimbriae are hair like appendages attached to bacterial cells, similar to flagella, but fimbriae are considerably shorter and thinner than flagella, fimbriae (or pili) consist of protein called pilin arranged helically around the control core (Funk and Case, 1989).

Pili are of two types each have different function, the first type called common pili, allows a cell to adhere to the surface including the surface of other cells, the second type of pili referred to as sex, functioning to join bacterial cells prior the transfer of DNA from one cell to another (Jawetz *et al.*, 1980).

P. mirabilis form a number of different types of peritrichous fimbriae, some are associated with virulence. Some of the fimbriae can be distinguished morphologically and by their haemagglutinating characteristics, among these mannose-resistant *Proteus*-like fimbriae (MR/P), channeled thick (7-8 nm in diameter) fimbriae and mannose resistant *Klebsiella*-like (MR/K), non channeled thin (4-5 nm in diameter) fimbriae are frequently found. Other fimbriae such as ATF (ambient temperature fimbriae), PMF (*Proteus mirabils* fimbriae) and UCA (uroepithelial cell adhesion) fimbriae are unable to agglutinate erythrocytes (Adegbola *et al.,* 1993; Jansen, 2004).

Bacterial adhesion to epithelial surfaces is thought to be one of the most important virulence factors (Mobley and Chippendle,1990), this capacity is associated with the presence of fimbriae on bacterial cells. It was found that a heavily fimbriated *P. mirabilis* strains cause pyelonephritis with higher efficacy than a lightly fimbriated one did. In contrast to this effect, fimbriae diminish the ability of *P. mirabilis* to infect the renal parenchyma by the hematogenous route, also it renders the pathogen more susceptible to phagocytosis (Rozalski *et al.*,1997).

Specific chemicals located on the tips of fimbriae enable organisms to attach to selected host tissue sites (eg. Urinary tract endothelium). The adherence of bacteria to biological surface is a complex process which often involve lock and key type interaction between bacterial attachment fimbriae and specific complex carbohydrate structure of the host cell membrane (receptors). It was found that bacteria which are high adherence are more virulent than that of less adherent (Rocha *et al.,* 2007).

Strain of *Proteus sp.* attached only to sequamous and not to transitional epithelial cells, where most of *Escherichia coli* tested attached to both cell types (Eden *et al.*,1988).

1.2.3.2 Flagella and swarming motility

Except for non flagellate variants all strains are actively motile by peritrichate flagella, normal and curly forms of flagella sometimes are found together on the same organism, the form of the flagella is influenced by the pH of the media. *P. mirabilis* has several genes coding for flagella but only one type of flagellin , FlaA, a protein of 40kDa is formed, but it may undergo antigenic variation as a result of spontaneous mutation and this may be an important survival mechanism *in vivo* (Belas, 1994). The H antigen determinants are flagellar proteins. Serotyping of *P.mirabilis* distinguished 19 different H antigens. Unlike O antigens, H antigens are destroyed by heating at 100 ºC, ethanol, and dilute HCL. The commonest flagellar antigens are H1, H2, and H3 (Jansen *et al.,* 2003).

Swarming is a characteristic feature of *Proteus* in which a group of cells at the edge of a developing microcolony migrate to uninoculated area of the medium. However it is not unique to *Proteus* and similar behaviour can be found in some other bacteria out of the tribe, such as *Serratia marcescens* and *Vibrio parahaemolyticus* (Jansen *et al.*,2003).

Flagella are also key structures in *Proteus mirabilis* swarming. The flagellar filaments of *P. mirabilis* are thought to act as tactile sensors allowing the cell to sense contact with solid surfaces. It has been suggested that inhibition of flagellar rotation due to increase viscosity of the medium or contact with solid surfaces triggers swarmer cell differentiation in *P. mirabilis* (Jones *et al.*, 2004). In general the presence of flagella on the surface of pathogenic and opportunistic bacteria facilitate the colonization and dissemination from the initial site, the association of motility with the virulence of flagellated *P.mirabilis* bacilli has

been demonstrated. The ability of *P. mirabilis* to express virulence factors, including urease, protease, and hemolysin, and to invade human urothelial cells is coordinately regulated with swarming differentiation (Liaw *et al.,* 2005).

Another aspect associated with the important role of flagellin as a bacterial surface antigen (H antigen). Since flagellin is strongly immunogenic, it can be assumed that at least part of the immunresponse of the host during infection is directed against this antigen, so the possible changes in flagellin antigenicity may enable bacteria to escape the immune response of the infected host, and increase the survival of *P.mirabilis* colonizing the urinary tract, by protecting the bacteria against the action of secreted IgA, because IgA antibodies are directed against bacterial surface antigens, so change in the antigenicity could escape immobilization by antibodies (Belas, 1994).

There are many antiswarming agents which can inhibit the swarming phenomena of *Proteus mirabilis* such as acohol (6%), sodium azide, boric acid and poly nitrophynylglycerol (Liaw *et al.,* 2000).

1.2.3.3 Outer membrane proteins (OMP)

The cell walls of Gram-negative bacteria have an outer membrane situated above a thin peptidoglycan layer. Sandwiched between the outer membrane and the plasma membrane, a concentrated gel-like matrix, the periplasm is found in the periplasmic space. Together the plasma membrane and the cell wall (outer membrane, peptidoglycan layer, and periplasm) constitute the gram-negative envelope (Finlay and Falkow, 1997).

In general, OM proteins (OMP) possess immunogenic properties and mitogenic activity for B cells . Furthermore, OM lipoproteins and their synthetic analogs function as adjuvants and can also activate macrophages to produce tumur necrosis factor, they also are able to mediate the penetration of hydrophilic molecules through the artificial model membranes and protect them from disaggregation by detergents (Piccini *et al.,* 1998; Lin *et al.,* 2002).

The OM of *P. mirabilis* contains three major proteins of 39.0, 36.0, and 17.0 kDa. These proteins correspond the ones previously shown to be OMP of *Proteus* spp. These proteins were highly immunogenic through specific antibodies detection after an experimental UTI in mice. A *P. mirabilis* mutant by transposon mutagenesis that was unable to use heme as the unique iron source, and tracked the transposon interruption to an outer membrane heme receptor gene. The mutant was outcompeted by the wild type in its ability to colonize the urinary tract in the mouse model of UTI, pointing out the importance of the heme receptor in *P. mirabilis* virulence (Lima *et al.,* 2007).

In particular, OmpA is effective as an immunomodulator of the immune response to LPS, greatly enhancing the level of O-specific IgG. This protein is also mitogen for murine B cells *in vitro* and displays strong adjuvant activity. The 39-kDa protein inhibits oxygen radicals, as well as interleukin-1 (IL-1) production, and enhances TNF secretion by LPS-stimulated macrophages (Weber *et al.*, 1993).

1.2.3.4 Lipopolysaccharide (LPS)

The lipopolysaccharide is considered as an important virulence factor of *P. mirabilis.* Lipopolysaccharide is composed of three genetically and structurally distinct regions: O-specific chain (O-antigen, O-specific polysaccharide), the core oligosaccharide and lipid A, which anchors the LPS molecule to the bacterial outer membrane (Raetz *et al.,* 2002). LPS containing all these three regions is produced by smooth forms of bacteria. Rough strains synthesize LPS containing lipid A and the whole core region or only its part (Rozalski, 2008).

The differences in the structure of O-antigens serve as a basis for the serological classification of *Proteus* strains. The serological classification scheme currentlyconsists of 78 serogroups (Drzewiecka and Sidorczyk, 2005; Knirel *et al*., 2011).

Six types of core regions have been identified so far *Proteus* LPS. The characteristic feature which distinguishes the *Proteus* core region from *E. coli* and *Salmonella* cores is the presence of D-galacturonic acid. It was found that galacturonic acid plays an important role in the specificity of *P. mirabilis* LPS. Based on the results of chemical analysis of lipid A, it was found that *P. mirabilis* lipid A has some biological effects as, mitogenic activity, lethal toxicity, and the local Shwartzman reaction (Vinogradov *et al*., 2002).

LPS from *Proteus* showed inhibitory activity toward mouse liver cytochrome P-450. It was also observed that there is significant decrease in respiratory burst intensification and also formation of apoptosis bodies after addition of lipopolysaccharide of *P. mirabilis* (Kaca *et al.,* 1996).

Biologically, LPS are endotoxins, well-known pathogenic factors of Gram-negative bacteria, which cause a broad spectrum of pathophysiological effects such as fever, hypotension, disseminated intravascular coagulation, and lethal shock. Endotoxin can be released from cell surfaces of bacteria during their multiplication, lysis, and death. Such a free LPS is a bioactive molecule and acts through its biological center (lipid A component) on various cell types, of which macrophages and monocytes are the most important. On the other hand, LPS as a bacterial surface antigen is recognized by specific antibodies produced by the host defense system. LPS of pathogenic bacteria contributes to their resistance against bactericidal action of serum and intracellular killing by phagocytes (Kaca *et al*., 2009).

1.2.3.5 Protease of *P. mirabilis*

Strains belong to *P. mirabilis*, associated with human urinary tract infections have previously been shown to secret an extracellular metaloproteinase which cleave both sub classes of immunoglobulin proteins which are IgA and IgG and also non immunoglobulin proteins such as secretory component casein and bovine serum albumin (Belas *et al*., 2004; Jacobsen *et al*., 2008).

Human immunoglobulin A (IgA), which occurs in two isotypic forms, IgA1 and IgA2, is unlike other immunoglobulins in that it exists in a variety of molecular forms, each with a characteristic distribution in various body fluids (Kerr *et al.,* 1995)

Bacterial IgA proteases are distinguished from other proteolytic enzymes by their narrow substrate specificity which is restricted to the IgA1 subclass of Igs produced by humans. IgA proteases cleave the heavy chain of IgA1 isotype at a specific site within a 13-amino-acid praline rich polypeptide segment in the hinge region. Since this sequence is not present in IgA2, this subclass of Ig is resistant to IgA protease action (Senior *et al.*,1999). Most strains of *P. mirabilis* produce an EDTA sensitive metalloproteinase which, unlike the IgA proteinases described above, is able to cleave not only IgA1 but also IgA2, IgG and other non immunoglobulin substrates (Loomes *et al.,* 1999).

It was found that the proteinase of *P.mirabilis* cleave the heavy chain of serum IgA at sits different from those cleaved by the IgA proteinases of pathogenic *Haemophilius* and *Neisseria* species, the latter enzymes cleave the alpha 1 chain exclusively in the hinge region to generate Fab and Fc fragments. In contrast *P.mirabilis* cleave the alpha chain in serum IgA1 in a sequential manner, starting with the removal of the tail piece, the CH3 domain, and finally the CH2 domain, also unlike other proteinases *P. mirabilis* proteinases have the ability to cleave the secretory component. In contrast to other proteinases, the rate of cleavage by the *P. mirabilis* proteinase of the alpha 1 chain in serum IgA1 was higher than that in secretory IgA1 (Almogren *et al.*, 2003).

1.2.3.6 Hemolysin production by *P. mirabilis*

The synthesis of cytotoxic hemolysins is common among both Gram-negative and Gram-positive bacteria. Two distinct hemolysins have been found among *proteus* isolate which are HpmA and HlyA (Pablo *et al*., 2003).

The HpmA which is calcium independent hemolytic activity produced by all strains of *P. mirabilis* and most strain of *P. vulgeris*, while HlyA which is calcium dependent hemolysin activity is not found in *P. mirabilis* but it is found in some strains of *P.* vulagaris. In addition, hemolytic strains of *P. mirabilis* are more virulence than non-hemolytic strains, when injected intravenously into mice. It was found that *P. mirabilis* exhibit hemolysis in detectable amounts in early log phase and that hemolysis reached its maximum in the mid log to late log phase, then the activity rapidly decline, this phenomenon was calcium independent (Swihart and Welch, 1996; Liaw *et al*., 2000).

P. mirabilis HpmA hemolysins was identified as a 166 kDa polypeptide and it was calcium independent hemolysin, this hemolysin appeared to be not stable and it was detectable only if the hemoysin assay was immediately performed on fresh samples (Braun and Focareta, 1991).

P. mirabilis strains synthesizing cell-associated hemolysin had a lower 50 % lethal dose (LD50) than non hemolytic strains when injected intravenously into mice. It was also shown that hemolytic activity is correlated with cell invasiveness of *Proteus* strains. HmpA was also cytotoxic against a variety of target cell lines: human B-cell lymphocyte, human monocyte, Vero and cultured human renal tubular epithelial cells (Mobely *et al.,* 1999).

HlyA hemolysin is produced by *P. vulgaris*, *P. penneri* and *P. hauseri* strains. It is a strongly cytotoxic, calcium dependent 110 kDa extracellular toxin. It is similar to the *E. coli* hemolysin, HlyA, with respect to its predicted amino acid sequence, operon structure, and immunological reactivity (Rozalski and Staczek, 2011).

1.2.3.7 Urease production by *P. mirabilis*

Urease activity has been found in several bacteria, fungi, and plants and has been shown to be an important pathogenic factor for the bacteria *P. mirabilis* (Jones *et al*., 1990; Eaton *et al*., 1991; and Tsuda *et al.*, 1994).

Urease is an important agent responsible for the pathogenesis of *Proteus* in the kidney, it is a cytoplasmic multimeric, nicked metalloenzyme. This enzyme hydrolyzed urea, which is present in urine generating ammonia and carbon dioxide, elevated the pH and result in precipitation of magnesium ammonium phosphate and carbonate_apattite which form stones of kidney and bladder (Dattelbaum *et al.*, 2003; Burall *et al.*, 2004).

It is also found that the increase in pH due to hydrolysis of urea by urease result in decrease biological activity of antibodies and destruction of lyukocytes. The presence of ammonia has been implicated as directly toxic to epithelium of kidney (Jacobsen *et al*., 2008; Nielubowicz and Mobley, 2010).

The urease enzyme is comprised of three structural subunits, Ure A, Ure B, and Ure C, assembled as a homotrimer of individual Ure ABC heterotrimers (means that there is three copies of each subunit). The majority of urease from *P. mirabilis* is present in the soluble cytoplasmic fraction. *P. mirabilis* urease was associated with the periplasm and outer membrane. The active enzyme contains nickel into performed apourease. *P. mirabilis* urease in its native form is a 212- to 280-kDa protein containing three subunits, α, β, and γ (Susan and Mobely, 2001).

1.2.3.8 Siderophores

In the presence of a deficiency of iron, bacteria produce iron chelators, named siderophores, which are excreted to the surroundings; they bind iron and transport it into the bacterial cells by using suitable receptor proteins and appropriate transport mechanisms. The synthesis of siderophores is under the control of chromosomal or plasmid genes. In all kinds of the host-bacterium relationships , the bacteria are in competition with their host for iron. Eukaryotic proteins like transferrin and lactoferrin, with high iron affinity, render prokaryotic cells iron deficient. An efficient production of siderophores may seal

the fate of an invader. From this point of view, they can be considered one of the virulence (invasiveness) factors (Massad *et al*., 1995; Nielubowicz and Mobley, 2010).

Alpha Hydroxyisovaleric acid has been described as a siderophore produced by *P. mirabilis*. Its general significance in amino acid rich media is, however, disputable. The siderophore activity of a-keto acids, products of deamination of amino acids by these bacteria, was studied by Drechsel *et al.* (1994).

1.2.3.9 Invasiveness

Cell invasiveness, also termed cell penetration, is an important step in infection and has been investigated, in relation to *Proteus* rods (Finlay,1990).

The invasion of mammalian cells by *P. mirabilis* rods *in vivo* and *in vitro* was stimulated by urea. It was shown that all *P. mirabilis* and *P. vulgaris* strains investigated, including those from patients with UTI as well as those from healthy persons, were able to penetrate Vero cells (the African green monkey kidney cells). A correlation of cell_associated hemolytic activity with penetration was also observed. A statistically higher Vero cell invasiveness by strongly hemolytic strains of *P. mirabilis*, as compared with *P. vulgaris* strains with low hemolytic activity, was found. all the cell lines used (Vero, HeLa, L-929 mouse fibroblasts, and human blood lymphocytes) were penetrated by *P. mirabilis*, *P. vulgaris*, and *P. penneri*. The maximal invasiveness was observed between 3 and 5 h after incubation of the tested cells with bacteria (Rozalski and Kotełko, 1987).

Chippendale *et al.*, (1994), was study the internalization process of *P. mirabilis* strains by human renal epithelial cells. For this investigation, confluent monolayers of primary cultures of human renal proximal tubular epithelial cells (HRPTEC) were used. It was demonstrated by light and electron microscopy that internalized bacteria were present within membrane-bound vacuoles of kidney cells. Also found that short rod-shaped vegetative forms of *P. mirabilis* internalized within membrane-bound vacuoles were not able to replicate.

Allison *et al.,* (1992) also showed that the ability to invade Vero cells and two human uroepithelial cell lines, was closely coupled to the swarming phenomenon. It was shown that invasion by swarmer cells occurred within 30 min and was about 15 to 20 fold greater after 2 h when compared with invasion by vegetative cells, which were internalized more slowly.

By use of different transposon (Tn*5*) mutants with specific defects in motility and multicellular behavior, it was possible to study the role of the swarming cycle in the invasion process. The nonflagellated mutants, which were nonmotile and nonswarming, were completely noninvasive. Mutants which were motile but defective in swarmer formation (motile, nonswarming mutants) have very limited invasion ability. In this study, it was also shown that non hemolytic mutants were only slightly less able to invade tested cells than was the wild type form. the differentiated swarmer cell filaments of *P. mirabilis* were the most invasive forms of these bacteria and may play a major role in the colonization of human epithelium during UTI. The mechanisms of this process are not clear. High activity in swarmer cells of two important virulence factors, HpmA hemolysin and urease and possibly synthesis of invasins (cell surface proteins) are thought to be crucial to the invasion process and thereby to the pathogenicity of *Proteus* rods (Peerbooms *et al.*,1985).

1.2.4 Genome of *P. mirabilis*

The genome sequence of *P. mirabilis* HI4320 was completed in 2008 by Melanie M. Pearson identifying more than 3,658 coding sequences with 7 rRNA loci. The *P. mirabilis* genome consists of a 4.063-Mb chromosome and a 36,289-nucleotide (nt) plasmid containing 3,685 and 55 CDSs, respectively. The origin of replication was assigned based on the GC deviation of the genome. The *P. mirabilis* chromosome is considerably smaller than the chromosomes of other *Enterobacteriaceae* (average, 4.6 Mb), including uropathogenic strains of *E. coli*

(CFT073, 5.23 Mb; UTI89, 5.21Mb; 536, 4.94 Mb) (Miller *et al.,* 2000). General features of the genome are shown in Table (1.1).

Table (1.1): General features of the *P. mirabilis* genome.

Genetic element	Chromosomal DNA	Plasmid DNA
Size (bp)	4,063,606	36,289
G+C content (%)	38.9	36.21
No. of coding sequences	3,693	53
Avg. gene size (bp)	941	606
No. of insertion elements	15	0
No. of rRNA operons	7	0
No. of tRNA genes	83	0
No. of noncoding RNA genes	18	0
No. of pseudogene	24	0

In addition to the 4-Mb chromosome, *P. mirabilis* HI4320 was also found to carry a plasmid. Not all *P. mirabilis* strains carry this plasmid or other plasmids. The *P. mirabilis* HI4320 plasmid, designated pHI4320, is highly related to R6K from *E. coli* (Kontomichalou *et al.*, 1970) and encodes the π protein and R6K replication proteins.

Interestingly, two independent transposon insertions in one of the plasmid CDSs that encodes a conjugal transfer protein were previously found to alter the virulence of *P. mirabilis.* Since it is clear that this CDS is not involved in virulence, it is possible that the plasmid carries other genes that might contribute to virulence. The analysis of the plasmid sequence did not reveal any obvious or known virulence factors. However, the plasmid carries CDSs encoding a bacteriocin and its immunity system, which may confer a competitive advantage to the bacterium in the urinary tract (Burall *et al.,* 2004).

Within the genome is a genomic island involved in pathogenicity that codes for a type III secretion system comprising 24 genes used to inject bacterial proteins into a host genome. This type III system appears to be incorporated through horizontal gene transfer and is noted for its relatively smaller G+C content compared with the rest of the genome.

Genes encoding several previously identified regulators of *P. mirabilis* flagellar motility were also located in the chromosome, including *umoA*, *umoB*, *umoC*, *umoD* , *rssBA*, *wosA*, and the *rcsBCD* regulatory system composed of genes encoding the sensor kinase RcsC, the phosphotransfer intermediate protein RcsD, and the response regulator RcsB (Takeda *et al.*, 2001; Lai *et al.*, 2005; Clemmer and Rather, 2007; Hatt and Rather, 2008).

The genome sequence encodes 17 different types of fimbriae Included in this fimbriae genes is an apparent duplication of the *mrp* operon immediately adjacent to *mrpI*. The duplicated *mrp* operon does not appear to have an invertible element in its promoter (Li *et al.*, 2002).

P. mirabilis HI4320 is known to be tetracycline resistant and displays intermediate resistance to chloramphenicol. Genes conferring these resistance traits were located in the genome; *tetAJ* encodes a tetracycline resistance protein, and *cat* encodes chloramphenicol acetyltransferase (Pearson *et al.*, 2008).

1.2.5 Plasmids of *P. mirabilis*

Proteus species harbors a wide range of varied molecular weights antibiotic resistant plasmids (23.1kb). *Proteus* species have varied multi-drug resistant markers, of which some are encoded on transferable plasmids. The frequency of transfer of the resistant plasmids ranged from 2x10-4 to4x10-2 per donor cells. It was therefore evident that antibiotics resistant plasmids markers among *Proteus* species could easily be transferred by conjugation. This is because the resistant patterns of the transconjugants were similar to the resistant pattern of the donor cells (Yukata *et al.*, 2004).

Not all strains of *P. mirabilis* contain a plasmid, this strain was attenuated *in vivo*. A possible genetic basis for its attenuation may be the instability of its large plasmid, which has substantial changes in its restriction profile, independent of the insertion event. Attenuation due to transposon insertion within the plasmid suggests that the plasmid is necessary for full virulence (Norma *et al.*, 2004).

Wide spread of plasmids resistance genes among *Proteus* species that 44% of antibiotic resistance were plasmid mediated, 32% by chromosome, while 24% of the resistance pattern to antibiotics could not be ascertained. Adeniyi *et al*., (2006) reported that *E.coli*, *Proteus* species and *Pseudomonas* isolates caring plasmid of molecular size above 2.1kb. Other investigators also detected two plasmids harbored by some of analyzed strains of *Proteus mirabilis* of about 6 and 93kb (Stankowska *et al.*, 2008).

Dharmadhikari and Peshwe, (2009) confirmed the location of antibiotic markers on R-plasmid by treating the cells with curing agents. A novel plasmid-mediated 16S rRNA methylase, RmtC, in a clinical *P. mirabilis* isolate that demonstrated an extraordinarily high level of aminoglycoside resistance like actinomycetes (Yan *et al.*, 2004). A new plasmid-mediated quinolone resistance gene, *qnrC*, was found on and cloned from a transferable plasmid, pHS10, in a clinical isolate of *P. mirabilis* (Jacoby *et al.*, 2008).

The *in vitro* analysis challenging transfer experiments was the inability of plasmids reception by the standard strain and the rate at which the plasmids are lost during samples analysis. It was observed that successive sub-cultures of the *Proteus* species resulted in rapid lost of the plasmids than from freshly analyzed samples (Miranda *et al.*, 2004).

The inability of some of the *Proteus* species to exhibit particular resistant pattern after curing indicated that resistance to some antibiotics were plasmids or chromosome mediated or both plasmids and chromosome mediated (Enabulele *et al.*, 1993).

According to Miranda *et al.*, (2004), the clinically derived *Proteus* species plasmids do not belong to distinct plasmid lineages but exhibit evidence of broad scale inter-plasmid gene transfer probably involving a range of mechanisms such as recombination, transposition and integration.

1.2.6 Probiotic

The term "Probiotic" derives from the Greek meaning "for life" opposed to 'antibiotics' which means 'against life'. The concept of probiotics was first reported by Elie Metcgnikoff in 1907, while its name was introduced in 1965 by Lilley and Stillwell as an antonym to antibiotic. Consuming of fermented milk products was responsible for life prolongation, through controlling intestinal microflora to sustain normal balance between pathogenic and non pathogenic bacteria (Weese, 2001).

Probiotics are defined as live microbial food supplements or compounds of microorganisms which having beneficial effects on human health. Probiotic microorganisms include strains of lactic acid bacteria (e.g. *Lactobacillus* and *Bifidobacterium*), *Clostridium butyricum, streptococcus salivarius*, non pathogenic strain of *E. coli* (e.g. *E. coli* Nissle 1917), and *saccharomyces boulardii* yeast (Haish and Varghese, 2006).

The term 'probiotic' firstly used in 1965 by Lilly and Stillwell to describe substances which stimulate the growth of other microorganisms. After this year the word 'probiotic' was used in different meaning according to its mechanism and the effects on human health. The meaning was improved to the closest one we use today by Parker in 1974. Parker defined 'probiotic' as 'substances and organisms which contribute to intestinal microbial balance'. In 1989, the meaning use today was improved by Fuller. Thus, probiotic is a live microbial supplement which affects host's health positively by improving its intestinal microbial balance. Then this definition was broadened by Havenaar and Huis

in't Veld in 1992 including mono or mixed culture of live microorganisms which applied for animal and man (Sanders, 2003; Guarner *et al.*, 2005). In the following years lots of researchers studied on probiotics and made so much definition. They are:

1. 'Living microorganisms, which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition' by Shaafasma, 1996.
2. 'A microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity, as well as improving nutritional and microbial balance in the intestinal tract' by Naidu *et al.*, 1999.
3. 'A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host' by Schrezenmeir and Devrese, 2001.
4. The most proper definition published by FAO / WHO in October, (2001) was "probiotics are live microorganisms which when administrated in adequate amounts confer a health benefits on the host" (Khan and Ansari, 2007).

   Lee and salminen, (2009) suggested that the health effects of probiotics are:

   - Prevention of rotavirus-induced or antibiotic-associated diarrhea as well as alleviation of lactose intolerance symptoms.
   - Reduction of the concentration of cancer-promoting enzymes.
   - Prevention and alleviation of gastrointestinal tract problems in healthy people.
   - Prevention of respiratory tract infections.
   - Beneficial effects on inflammatory diseases of the gastrointestinal tract (*Helicobacter pylori* infection).
   - Normalization of stool passage in subjects with an irritable colon.
   - Prevention of allergies and atopic diseases in infants.

- Prevention as well as treatment of urogenital infections.
- Treat colitis.
- Enhance specific and nonspecific immune response.
- Reduce chance of infection from common pathogens (*Sallmonella*, *Shigella*, *Proteus*, etc.).
- Synthesize nutrients (folic acid, niacin, riboflavin, vitamins B1and B2).

1.2.6.1 The Mechanism of Action of Probiotics

1.2.6.1.1 Adherence and colonization of the gut

Probiotic microorganisms are able to adhere to epithelial cells because they have anti-adhesive effects thereby blocking adherence of pathogens (Lin *et al.*, 2008). Anti adhesive effects might be due to competitive exclusion for the same receptor by the probiotics and the pathogens, secretion of proteins that destroy the receptor, induction of biosurfactants, establishing a biofilm, and production of receptor analogues (Mack, 2003; Oelschlaeger, 2010).

1.2.6.1.2 Competition for limiting resources

Almost all bacteria need iron as an essential element with the exception of *Lactobacillus* which didn't need iron in their natural habitat (Weinberg, 1997). *Lb. acidophilus* and *Lb. delbrueckii* were able to bind ferric hydroxide at their surface making it unavailable to pathogenic microorganisms. This mechanism is of crucial advantage in competition with other microorganisms which depend on iron (Elli *et al.,* 2000).

1.2.6.1.3 Anti –invasive effects

Not only adhesion but also invasion of epithelial cells is an important property for full pathogenicity of many gut pathogens; the ability to inhibit bacterial invasion of gut epithelial cells by pathogens is rather wide spread among probiotics (Hess *et al.*, 2004). Some researchs confirmed that some

probiotics (like *Lactobacillus* and *Bifidobacterium* strain Bb12) had the ability to secrete factors which interfere with the invasion of host epithelial cells by *p. mirabilis* (Ingrassia *et al.*, 2005; Botes *et al.*, 2008).

1.2.6.1.4 Immunomodulatory effects

Many probiotic strains were able to stimulate production of immunoglobulin A (IgA) that help in maintaining homoral immunity of the intestine by binding to the antigen and limiting their access to epithelium. Children at age of (2-5) years who were vaccinated with rotavirus and received *Lb. rhammnosus* GG showed an increase in production of IgA producing cells (Isolauri *et al.*, 1995). Probiotics are usually absorbed orally. They stimulate the gastro intestinal immunity by enhancing the specific and non specific immune response, inhibiting the pathogen growth, and translocation which will reduce the chance of infection from common pathogens, for example *Proteus*, *Salmonalla*, *shigella*, etc. (Delcenserie *et al.*, 2008).

1.2.6.1.5 Production of antimicrobial substance

Lactic acid bacteria (LAB) including *Lb. acidophilus* had the ability to inhibit growth of Gram negative and Gram positive bacteria (Wohlgemuth *et al.*, 2009). This is due to their ability to produce organic acids (lactic acid and acetic acid), hydrogen peroxide, bacteriocens, bacteriocines like substance, and possibly biosurfactant (Bierbaum and Sahl, 2009; Oelschlaeger, 2010).

1.2.6.2 Lactic Acid Bacteria as Probiotics

Lactic acid bacteria constitute a heterogeneous group of industrially important bacteria that are used to produce fermented foods and beverages, using various substrates, such as milk, vegetables, cereals, meat, cocoa beans etc.. During the last few decades, development of the functional food concept and, more specifically, the application of certain LAB strains as life vaccines,

pro- and prebiotics, nutraceuticals, have created new perspectives for LAB research and human consumption, attracting the attention of both food scientists and health professionals (Ouwehand, 1998; Ur-Rasid *et al* ., 2007).

Scientific evidences supported the role of probiotics LAB in many positive health effects because of the long history in safe use of diary strains of LAB.These strains were considered as commercial microorganism with no pathogenic potential (Soomro *et al.,* 2002). Many researches like Isolauria *et al.,* 1991; Salminen and Deighton, 1992 stated that the LAB resembling large proportion from normal flora in the intestinal and gut. LAB strains that demonstrate a wide spectrum of antimicrobial characteristic including; acid and bile resistance, to low pH, antimicrobial systems (ex: bacteriocin, lactic acid and peroxide), adhesion to various types of pathogens and immunomodulatory effects (Cases and Dobrogosz, 2000; Tambekar *et al.,* 2009).

There are many mechanisms in which LAB protects the intestinal tract including; decreasing pH value, adherence to the intestinal cell wall, production of inhibitory material (bacteriocin), production of antitoxin and ability to still life (Deraz *et al.,* 2005).

Lactic acid bacteria produce β-galactosidase and lactic dehydrogenase (LDH) to convert lactose to lactic acid. The product is reported to have some physiological benefits on human: it enhances the digestibility of milk proteins; it improves absorption of calcium, phosphorous and iron; it can stimulate secretion of gastric juices; it accelerates the peristalsis and it serves as a source of energy in the process of respiration (Maldonado *et al.*, 2005; Wee *et al.*, 2006).

1.2.6.2.1 Inhibitory compound produced by LAB

1.2.6.2.1.1 Acetaldehyde

Acetaldehyde is produced by hetrofermentaive LAB that possess the enzyme therioninaldolase which converts the amino acid threonine to acetaldehyde and

glycine. Because of LAB inability to consume it, the acetaldehyde will accumulate and exerts its inhibitory effect against some of the pathogenic bacteria, e.g. *E. coli*, *proteus mirabilis S. typhimurium, and staphylococcus aureus* (Piard and Desmazeuud, 1991).

1.2.6.2.1.2 Carbon dioxide (CO_2)

Carbon dioxide is produce by the hetrofermentative LAB by fermenting sugars that provide anaerobic conditions which will in turn inhibit growth of the obligate aerobic microorganisms, lowering the pH, interfering with the cellular enzymes reactions, and destroying the cellular membrane (Adams and Nicolaides, 1997).

1.2.6.2.1.3 Organic acid

Organic acids such as pyruvate, helps in lowering the pH and inhibits growth of many pathogenic Gram negative and positive bacteria. However lactic acid and acetic acid have a synergistic inhibitory effect against *E. coli* and *Proteus* (Naidu *et al.*, 1999; Servin, 2004).

1.2.6.2.1.4 Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is produced in presence of oxygen and accumulates due to the inability of LAB to produce the catalase enzyme. Hydrogen peroxide has an inhibitory effect against many Gram negative and Gram positive bacteria because of its oxidative effects; it also breaks cellular nucleic acids and proteins (Naidu *et al.*, 1999; Kullisaar *et al.*, 2002).

1.2.6.2.1.5 Bacteriocins

Bacteriocins produced by LAB, *Lactobacillus spp.* are extracellular ribosomally synthesized peptides, usually ranging in size from 20 to 60 amino acids with bactericidal activity, and are immune to their own action including

acidocin and lacticin produce from *Lb. acidophilus*, plantaricin C which is abroad-spectrum bacteriocins from *Lb. plantarum* and Gassericin from *Lb. gasseri* (Tamime, 2005). Bacteriocins produced by *Lb. acidophilus* inhibited several enteropathogens *in vivo* and *in vitro* (Coconnier *et al.*, 2000).

1.2.6.2.2 Adherence properties of LAB

Gut colonization with *Lactobacilli* starts within the first week of life (Saminen *et al.*, 1995). To exert their probiotic effects, maintenance of LAB in the gastrointestinal tract is necessary to prevent their rapid removal by contraction of the gut. The ability of LAB to adhere to mucosal surface could confer a competitive advantage, important for bacterial maintenance in human gastrointestinal tract. The ability to adhere to mucosa is required for long term colonization and persistence in the gut (Gill *et al.*, 2001).

The ability to adhere to epithelial surfaces is considered an indispensable pre-requisite of probiotic strains in order to colonise and then to exert health promoting effects. Bacterial adhesion is initially based on non-specific physical interactions between two surfaces (like hydrophobic interaction), which then enable specific interactions between adhesins (usually proteins) and complementary receptors (Kos *et al.*, 2003; Canzi *et al.*, 2005).

Lactobacillus spp. Adhere to human intestinal cells via mechanisms that involve different combinations of carbohydrate and protein factors on the bacteria and eukaryotic cell surface (Lee *et al.*, 2000). Some microorganisms are able to bind to epithelial cells of the gastrointestinal tract through lectins present in their surface structures. Lectins are carbohydrate-binding proteins or glycoproteins from non-immune origin which agglutinate cells with receptors (Gusilis *et al.*, 2002).

Several authors observed a good correlation between adhesion ability and cell surface hydrophobicity (Canzi *et al.*, 2005). Adherence was assessed by counting the number of bacteria adhered to the intact epithelial cells. Such

adherence may promote colonization of the vaginal epithelium through formation of a bacterial "film" that tends to exclude pathogens from the mucosa (Reid and Burton, 2002; Pascual *et al.,* 2008). The adhesion of *Lactobacillus* strains to intestinal epithelial cells *in vitro* upregulated the *MUC3* gene, stimulating the production of mucus. The upregulation of mucin encoding genes, particularly during enteric infections is another Important mechanism of action by probiotics (Hooper *et al.,* 2001; Mack *et al.,* 2003).

Lb, acidophilus and *Bifidobacterium* were able to adhere to cells analoge to human intestinal cells and prevented the adhesion of *E. coli, Yersinia pesudotuberculosis, P. mirabilis* and *S. typhimurium* (Macfarlane and Cummings, 1999). Another study showed that *Lb. crispatus* expressed an S-layer protein was able to inhibit adhesion of *E. coli* to the cell matrix basement membrane (Horie *et al.,* 2002).

1.2.6.3 Genetically Modified Probiotics

The use of new techniques provided by the molecular biology offers the possibility that genetic screening may lead to identification of new probiotic strains to demonstrate multiple beneficial effects in difficult environmental conditions (Nicolae *et al.,* 2010).

In the first human trial with genetically engineered therapeutic bacteria, Crohn's disease patients were treated with modified *L. lactis* in which the thymidylate synthase gene was replaced with a synthetic sequence encoding human IL-10. When the modified bacteria are deprived of thymine or thymidine, they are not viable. Neither thymine nor thymidine is readily available in the external environment, thereby limiting the viability of the excreted organism. The treatment was safe, disease activity was reduced, and the modified bacteria were biologically contained. Therefore, bacterial-based topical delivery of biologically active proteins represents a highly promising and safe therapeutic strategy for combating mucosal diseases (Braat *et al.,* 2006).

There are clear research directions leading to a better understanding of *Lactobacillus and Bifidobacterium* role: correlation of phenotype and genotype characteristics influencing their functionality, molecular detection methods and systems for genetic transfer. Other lines of research focus on genetic modifications to improve existing characteristics of probiotic bacteria. For example expression of a gene that encodes an amylase in *L. amylovorus* in a strain used in the manufacture of silage, as *L. plantarum*, resulted in enhanced ability to degrade starch (Fitzsimons *et al.*, 1994).

Another example is to introduce a gene that encodes a glutamate dehydrogenase involved in catabolism of *Peptostrptococcus asaccharolyticus* in *L. lactis* in order to allow these microorganisms to produce α-Ketoglutarate from glutamate, an amino acid present in high quantities in cheese (Rijnen *et al.*, 1999).

Recombinant "designer" probiotics that express molecular mimics of host toxin receptors on their surface are being investigated for their ability to bind bacterial toxins, thereby preventing enteric infections (Paton *et al.*, 2006). In a recent study, a chimeric lipopolysaccharide containing a glycosylated lipid that mimics the cholera toxin receptor was expressed into nonpathogenic *E. coli* CWG308 (Focareta *et al.*, 2006). The recombinant probiotic could bind cholera toxin, inhibit its cytotoxicity, and also protect infant mice from challenge with *Vibro cholerae.*

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipments

The following apparatus and equipments were used in this study:

Equipment	Company/ Origin
Anaerobic jar	Rod Well / England
Autoclave	Express/Germany
Balance	Satorius/ Germany
Centrifuge, Cooling centrifuge	Hermile Z200A /Germany
Compound light microscope	Olympus/ Japan
Distillator, Shaker incubator	GFL/ Germany
Electrophoresis Unit	Bio Rad/ Italy
Incubator	Sanyo / Germany
Laminar air flow hood	Sanyo / Germany
Micropipettes	Ependrof / Germany
Microwave	Kenwood / China
Millipore filter unit	Millipore corp / USA
Oven, Hot plate with magnetic stirrer	Gallenkamp / England
pH-meter	Martini/ Germany
Refrigerator	Ariston / Japan
Sensitive balance	Denver/ Germany
Spectrophotometer	Aurora instruments Ltd / England
U V Transilaminator	Vilber Lourmat/ France
Vortex	Stuart/ England
Water bath	Grant/ England

2.1.2 Chemicals and Biological materials

The following chemicals and biological materials were used in this study:

Material	Company/ Origin
Brillent blue	Analar / U.K.
Agar-Agar, Litmus, Skim milk, TCA	Biolife / Italy
Acetic acid, Ammonium sulfate, Glucose Glycerol, HCl, Isoamyle alcohol, K2HPO4, KCl, KH2PO4, Mannitol, MgSO4.7H2O, Na2HPO4, Sodium acetate hydrate	BDH / England
Diamine dihyrochloride, N,N,N,N-Tetramethyl-p-phenylene	Difico / England
Diammonium citrate, Ethylene diamine tetra-acetic acid, Hydrogen peroxide, Mannose, NaOH, Tris-hydrochloride, Tryptone	Fluka / Switzerland
Crystal Violet, Peptone, Urea,Yeast extract	Himedia / India
Gelatin, Meat extract, Tween 80	Oxoid / England
Bromo phenol blue, Boric acid, Methanol, MnSO4.4H2O, p-dimethyl-amino-benzaldehyde, Sodum Cholride	Riedel-DeHaeny / Germany
Agarose, Casein, Ethanol, Ethidium bromide, Phosphate Buffer Saline	Sigma / USA

2.1.3 Bacterial Isolate

An isolate identified as *L. acidophilus* was obtained from the Center of Market research and consumer protection / Baghdad university.

2.1.4 Antibiotics

The following antibiotic discs were used in this study:

Antibiotic	Symbol	Con. (µg/disc)	Diameter of Inhibition zone (mm)			Company (origin)
			R	I	S	
Ampicillin	AMP	25	≤13	14-16	≥17	AL - Razzi (Iraq)
Aztreonam	AT	30	≤17	18-20	≥21	AL – Razzi
Piparacillin	PI	100	≤17	18-20	≥21	AL – Razzi
Ticarcillin	TI	75	≤14	15-19	≥20	AL – Razzi
Ticracillin / Clavulanic acid	TCC	75/10	≤14	15-19	≥20	AL – Razzi
Amikacin	AK	30	≤14	15-16	≥17	Bioanalyse (Turkey)
Cefepime	FEP	30	≤14	15-17	≥18	Bioanalyse
Cefotaxime	CTX	5	≤22	23-25	≥26	Bioanalyse
Chloramphenicol	C	10	≤12	13-17	≥18	Bioanalyse
Ciprofloxacin	CIP	5	≤15	16-20	≥21	Bioanalyse
Gentamicin	CN	30	≤12	13-14	≥15	Bioanalyse
Imipenem	IPM	10	≤19	20-22	≥23	Bioanalyse
Nalidixic acid	NA	30	≤13	14-18	≥19	Bioanalyse
Tetracycline	TE	30	≤11	12-14	≥15	Bioanalyse
Trimethoprim	TMP	5	≤10	11-15	≥16	Bioanalyse

R: Resist, S: Sensitive, I: Intermediate

2.1.5 Media

2.1.5.1 Ready to use media

The following media were prepared according to the instructions of manufacturer companies and sterilized by autoclaving:

Medium	Company/ Origin
Brain heart infusion broth Nutrient broth Phenylalanine deaminase Muller Hinton Agar XLD agar Tetrathionate Broth Rappaport-Vassiliadis broth Skim milk agar medium	Oxoid / England
Blood agar base Kligler Iron Agar Nutrient agar Simmon citrate agar Urea agar base	Difco / U.S.A
MacConky agar	BDH / England
Litmus milk	Biolife / Italy

2.1.5.2 Laboratory Prepared Media

The Following medium were prepared (as will follow later) in the laboratory: Blood agar, Urea agar, Phenylalanine deaminase, Luria-Bertonia, peptone water, Semi- solid agar medium, DeMan and Regosa sharpe broth medium (MRS), DeMan and Regosa sharpe agar medium, litmus milk, and Gelatin medium.

2.1.6 Kits

2.1.6.1 Api 20 E kit (Api Bio merieux, lyon, France)

Api 20 E kit consists of:-

A. Galleries: The gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients.

B. Api 20 E Reagent:

- Kovac's reagent (p-dimethyl amino benzaldehyde at 4% in HCl isoamyl alcohol).
- Oxidase reagent (1% tetra-metheyl-p-phenyle-diamine)
- Voges – proskauer reagent:
  - VP1 (40% potassium hydroxide).
  - VP2 (5% alpha - naphthol).
- Ferric chloride 3.4 %.

2.1.6.2 DNA-Spin™ plasmid DNA Purification Kit (Intron, Korea):

DNA-Spin™ plasmid DNA Purification Kit consists of:

- Resuspension buffer.
- Lysis buffer.
- Washing buffer A.
- Washing buffer B.
- Elution buffer.
- RNase A.
- LysisViewer.
- Columns.
- Collection tube.

2.1.6.3 DNA Ladder (1 kb) (Promega, USA)

2.2 Methods

2.2.1 Preparation of Laboratory Prepared Medium

2.2.1.1 Blood Agar medium

This medium was prepared according to Atlas *et al.*, (1995) by dissolving 40 g of blood base agar in 1000 ml D.W. and sterilized by autoclaving and cooled to 45°C, then 5% blood sterilized by filtration was added, mixed gently and plated in Petri dishes.

2.2.1.2 Urea Agar medium

Urea agar medium was prepared by dissolving 24 g of urea agar base to 950 ml of distilled water, pH was adjusted to 6.8-7.0 and sterilized by autoclaving. After cooling to 50°C, 50 ml of 20% urea solution (sterilized by filtration) was added, mixed gently then medium was distributed into sterile test tubes and left to solidify in slant position (Collee *et al*., 1996)**.**

2.2.1.3 Phenylalanine Deaminase (PAD) medium

PAD medium was prepared according to Holt *et al.*, (1994) by dissolving 15 g of trypton and 10 g of L- phenylalanine in 1 liter of distilled water, then pH was adjusted to 7.0 and sterilized by autoclaving.

2.2.1.4 Gelatin medium

It was prepared according to Collee *et al.*, (1996) by adding 12g of gelatin to 100 ml of brain heart infusion broth, then medium was sterilized by autoclaving and stored until use.

2.2.1.5 Luria-Bertani Broth (LB) medium

It was prepared according to Atlas *et al.,* (1995) by dissolving trypton (10 g), yeast extract (5 g) and sodium chloride (5 g) in 1 liter of D.W., pH was adjusted to 7.0 and sterilized by autoclaving.

2.2.1.6 DeMan and Regosa sharpe agar medium (MRS) (DeMan *et al.*, 1960)

This medium was prepared to be consisted of the followings:

Component	Weight
Peptone	10 (g)
Meat extract	10 (g)
Yeast extract	10 (g)
KH_2PO_4	5 (g)
Glucose	20 (g)
Diammonium citrate	2 (g)
Sodium acetate	5 (g)
$MgSO_4$	0.5 (g)
$MnSO_4$	0.2 (g)
Agar-Agar	15 (g)
Tween 80	0.1 (ml)

All components were dissolved in 950 ml of D.W., pH was adjusted to 6, then volume was complete to 1000 ml and sterilized by autoclaving.

2.2.1.7 DeMan and Regosa sharpe broth medium (MRS)

This medium was prepared according to DeMan *et al.*, (1960); by containing all the components of the MRS agar medium without agar.

2.2.1.8 Pepton water

This medium was prepared according to Collee *et al*., (1996) by dissolving 20 g of peptone and 5 g of Sodium chloride in 900 ml of distilled water, pH was adjusted to 7, mixed thoroughly, then volume was completed to 1000 ml and distributed into test tubes and sterilized by autoclaving.

2.2.1.9 Semi- solid agar medium

This medium was prepared to be consisting of the following components: glucose (20 g), yeast extract (2 g), peptone (3 g) and agar (5 g). All components were dissolved in 900 ml of distilled water, pH was adjusted to 7, then volume was completed to 1000 ml, sterilized by autoclaving, and left to solidify in vertical position (KoBy and Ronald, 1974).

2.2.1.10 Litmus milk medium

This medium was prepared according to Baron and Finegold, (1994) by dissolving 100 g skim milk and 5 g Litmus in 1000 ml D.W., then sterilized by autoclaving for 5 minutes.

2.2.2 Solutions and Buffers

2.2.2.1 Antibiotic solutions

Antibiotic solutions were prepared according to Sambrook and Russell, (2001), sterilized by filtration and stored at -20 ºC.

2.2.2.1.1 Ampicilin solution

This solution was prepared as stock solution of 25 mg/ml of sodium salt of ampicillin in D.W.

2.2.2.1.2 Tetracycline solution

It was prepared as stock solution of 12.5 mg/ml of tetracycline hydrochloride in ethanol/ water (50% V/V).

2.2.2.1.3 Gentamicin solution (100 µg/ml)

This solution was prepared by mixing 250 µl of gentamicin stock solution (40 mg/ml) with 100 ml of D.W.

2.2.2.2 Fixative solution

Fixative solution prepared by mixing 30 ml of methanol with 10 ml of acetic acid (Iawhi *et al.*, 1982).

2.2.2.3 Casein solution (1%)

It was prepared by dissolving 1g of casein in 100 ml of 0.1 M Tris-HCl buffer solution (Atlas *et al*., 1995).

2.2.2.4 Phosphate Buffer Saline

It was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, and 1.15 g of Na_2HPO_4 in 950 ml of D.W. pH was adjusted to 7 and sterilized by autoclaving (Cruckshank *et al*.,1975).

2.2.2.5 Tris-HCl buffer (0.1M), pH 8 (Atlas *et al*., 1995).

2.2.2.6 Trichloroacetic acid 5%

It was prepared by dissolving 5 g of TCA in 90 ml of D.W., and then the volume was completed to 100 ml with D.W., and stored at 4 ºC (Atlas *et al*., 1995).

2.2.2.7 Bovine Serum Albumin

It was prepred by dissolving 10 mg of BSA in 10 ml Tris-HCl buffer pH 8 (Bradford, 1979).

2.2.2.8 Ethidium bromide stock solution (10mg/ml)

This solution was prepared according to Sambrook and Russell (2001) by dissolving 1 g of ethidium bromide in 100 ml of distilled water by stirring on magnetic stirrer until complete dissolving, then it was filtered through wattman filter paper No.1 and stored in dark bottle at 4 ºC until use.

2.2.2.10 Loading buffer

This solution was prepared according to Sambrook and Russell (2001) by dissolving 25 g of bromophenol blue and 40 g of sucrose in 80 ml of distilled water, then volume was completed to 100 ml with D.W., and stored at 4 ºC until use.

2.2.2.11 Tris-Borate-EDTA buffer solution

Tris-Borate-EDTA (TBE) Buffer solution was prepared according to Sambrook and Russell (2001) by dissolving 54 g of Tris-HCl and 27.5 g boric acid in 900 ml of D.W., then 20 ml of 0.5 M EDTA was added, and the volume was completed to 1000 ml with distilled water, pH was adjusted to 8 and sterilized by autoclaving.

2.2.3 Reagents

2.2.3.1 Oxidase reagent

Oxidase reagent was freshly prepared by dissolving 1 g of tetramethyl-p-phenylenediamine dihydrochloride in 100 ml D.W. in dark bottle and kept at 4 ºC until use (Atlas *et. al*., 1995).

2.2.3.2 Catalase reagent

Catalase reagent was prepared by mixing 3 ml of hydrogen peroxide solution (22%) with 97 ml D.W. (collee *et al.,* 1996).

2.2.3.3 Kovac's reagent

This reagent was prepared according to Atlas *et. al*. (1995) by dissolving 10 g of ρ –Dimethyl-aminobenzaldehyde in 150 ml of isoamyl alcohol with heating in water bath at 50◦C, and then 50 ml of hydrochloric acid was added slowly, mixed gently and stored at 4◦C until use.

2.2.4 Stains

2.2.4.1 Gram stain

Gram stain solution were prepared according to Duguid, (1996).

2.2.4.2 Coomassie Brilliant Blue G-250

It was prepared by dissolving 0.1 g of brilliant blue in 50 ml of 95% ethanol, then 100 ml of 85% phosphoric acid was added, and the volume was completed to 1litter with D.W. (Atlas *et al.*, 1995).

2.2.5 Sterilization methods (Colline and Lyne, 1987)

2.2.5.1 Moist heat sterilization (Autoclaving)

Media, buffers and solutions were sterilized by autoclaving at 121°C and (15Ib/in2) for 15 minutes, except some heat sensitive solutions.

2.2.5.2 Dry heat sterilization

Glassware and some other tools were sterilized in oven at 180 °C for 2 hours.

2.2.5.3 Membrane sterilization (Filtration)

heat sensitive solutions were sterilized by using 0.22 μm millipore filters.

2.2.6 Collection of Samples

2.2.6.1 Urine Sample

Urine samples specimens were collected in sterile tubes from patient of AL-Yarmok hospital in Baghdad during period from 11/2012 to 2/2013.

2.2.6.2 Wound Samples

Swabs of wound samples were taken in sterile transport swab from patient of Al- Yarmok hospital and from the Specialist Center for Endocrinology and Diabetes in Baghdad during period from 11/2012 to 2/2013.

2.2.6.3 Meat samples

Samples of chicken and meat were collected from local markets in Baghdad governorate during the period from 11 ⁄2012 to 2⁄2013. These samples were transferred quickly in sterilized containers and nylon bags to the lab for further analysis.

2.2.7 Isolation of *P. mirabilis*

2.2.7.1 From urine samples

For the isolation of *P. mirabilis* from urine samples, loopfull of undiluted mild stream urine was taken and spread on blood agar and MaConkey agar plates. Plates were then incubated for 16 hrs at 35°C, then suspected colonies were selected and subjected to identification according to their morphological and culture characteristics and biochemical tests.

2.2.7.2 From wound samples

Swabs taken from wound samples were used to inoculate blood agar medium and McConkey agar medium by streaking, and incubated at 35°C for 18 hrs, then the suspected colonies were selected and subjected to identification according to their morphological and culture characteristics and biochemical tests

2.2.7.3 From meat samples

One gram of each sample was homogenized in 99 ml of nutrient broth. After incubation at 35 °C for 24 hrs., one ml was inoculated into a tube containing 10 ml of Rappaport-Vassiliadis (RV) broth and was incubated at 35°C for overnight. Another one ml from same pre-enrichment culture was inoculated into 10 ml of Tetrathionate broth and incubated at 35 °C for overnight, then loopfull of sample was taken and spread on XLD agar, blood agar and MaConkey agar plates. Plates were then incubated for 24 hrs. at 35 °C,

then the suspected colonies were selected and subjected to identification according to their morphological and culture characteristics and biochemical tests. This technique recommended by the International Organization for Standardization (ISO), (1998).

2.2.8 Identification of *P. mirabilis*

2.2.8.1 Microscopical and morphological characteristics (Atlas *et al.*, 1995)

Morphological characteristics (size, shape, edge, color, and margin) of the bacterial isolates were examind. Microscopicl characteristics were also examimed by transferring a loop full of bacterial suspension and fixed on a clean slide to study the Gram stain to examine Gram reaction and spore forming under light microscope.

2.2.8.2 Biochemical Tests

2.2.8.2.1 Oxidase Test

This test was achieved by using moistened filter paper with few drops of freshly prepared solution of tetramethyl-ρ-phenylene diamine dihydrochloride. Aseptically, clump of cells was picked up from slant of each bacterial isolate with a sterile wooden stick applicator and smeared on the moistened paper. Development of a violet or purple color within 10 seconds indicates a positive result (Atlas *et al.*, 1995).

2.2.8.2.2 Motility Test

A semi-solid agar medium was inoculated with each bacterial isolate using a straight wire to make single stab down the center of the tube to about half the depth of the medium. Motile bacteria typically give diffuse, hazy growth that spreads throughout the medium rendering it slightly opaque (Collee *et al.*, 1996).

2.2.8.2.3 Urease Test

Urease test was achieved by streaking Christensen urea agar slants with each bacterial isolate, and incubated at 35 °C for 24 hrs. Appearance of a red-violet color indicates a positive result (Atlas *et al*., 1995).

2.2.8.2.4 Indole Test

Indole test was achieved by inoculating test tubes containing Peptone water with fresh culture of each bacterial isolate and incubated at 35°C for 24hrs., and then 50 µl of Kovacs reagent was added and mixed gently. Appearance of a red ring on the surface of the liquid medium indicates a positive result (Collee *et al*., 1996).

2.2.8.2.5 Catalase Test

A single colony of each bacterial isolate was smeared onto a clean glass microscopical slide with a sterile wooden stick applicator, then drop of hydrogen peroxide was placed onto the colony. Production of gaseous bubbles indicates a positive result (Atlas *et al.*, 1995).

2.2.8.2.6 Citrate Utilization Test

Citrate Utilization test was achieved by inoculating fresh culture of each bacterial isolate, and incubated for 24 hrs at 35 °C. Changing the color of media from green to blue indicates a positive result (Atlas *et al*., 1995).

2.2.8.2.7 Phenylalanine Deaminase Test

Phenylalanine Deaminase test was achieved by inoculating of 2 ml of phenylalanine broth in test tube with 50 µl of fresh culture of each bacterial isolate and incubated at 35 °C for 24 hr. Color change to green indicate a positive result (Senior, 1997).

2.2.8.8 Kligler Iron Agar (KIA) Test

Kligler iron agar test was achieved by inoculating KIA agar slants by stapping, and incubated for 24 hrs at 35 °C. The results was read as follows: (Atlas *et al.,* 1995)

- Alkaline/Alkaline → Red/Red
- Alkaline/Acid → Red/Yellow
- Acid/Acid → Yellow/Yellow
- H_2S production → Black precipitation
- Gas production → Bubbles formation

2.2.8.3 Identification by using Api 20E (Overman *et al.,* 1985)

1. Preparation of galleries

Five ml of D.W. dispensed into incubation tray to provide a humid atmosphere during incubation.

2. Preparation of bacterial suspension

Loopful of fresh culture of each bacterial isolate grown for 24 hrs at 35 °C on MacConke agar medium was suspended in 5 ml of D.W. in test tubes, mixed thoroughly and used to inoculate the galaries.

3. Reading of the galleries

All the reactions not requiring reagent were recorded first, then the following reagents were added to the corresponding microtubes:-

a- One drop of 3.4% ferric chloride to the TDA microtube.

b- One drop of Kovac,s reagent to the IND microtube.

c- One drop of Vogas – Proskauer reagent to VP microtube.

The biochemical reaction performed by the API 20E and their interpretation are listed in appendix I.

2.2.9 Maintenance of *P. mirabilis*

Maintenance of bacterial isolates was preformed according to Maintiatis *et al.*, (1982) as follow:

2.2.9.1 Short term storage

Isolates of bacteria were maintained for few weeks on MaConkey agar plates, then plates were tightly warped with parafilm, and stored at 4 °C.

2.2.9.2 Medium term storage

Isolates of bacteria were maintained by stabbing on nutrient agar medium small screw- capped bottles and stored at 4 ºC.

2.2.9.3 Long Term storage

Bacterial can be maintained for many years in medium containing 15 % glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of glycerol to 8.5 ml of exponential growth of bacterial isolates in small screw-capped bottles and stored at – 20 °C.

2.2.10 Identification of *L. acidophilus*

Local isolate of *L. acidophilus* was re-identified according to its microscopical and morphological characteristics and biochemical tests.

2.2.10.1 Microscopical and Morphological Characteristics

Fresh culture of *L. acidophilus* was examined under light microscope to study its Gram stain, cell shape, grouping and spore forming (Harely and Prescot, 1996).

2.2.10.2 Biochemical Tests

2.2.10.2.1 Catalase test

This test was performed as in item (2.2.8.2.5)

2.2.10.2.2 Oxidase test

Oxidase test was achieved as in item (2.2.8.2.1)

2.2.10.2.3 Gelatinase test

gelatinase test was achieved by stabbing gelatin medium prepared in item (2.2.1.9) with fresh culture of the bacterial isolate and incubated anaerobically at 35 ºC for 3 days, then tubes was placed in refrigerator at 4 ºC for one hour. Liquefying of the medium indicates a positive result (Baron and Finegold, 1994).

2.2.10.2.4 Acid and curd production

Fresh culture of *L. acidophilus* was used to inoculate litmus milk medium and incubated anaerobically at 35 ºC for 48 hrs. Changing the color to pink or purple and decreasing pH value indicate a positive result (Forbes *et al.,* 2002).

2.2.10.2.5 Growth at 45 ºC

Fresh culture of *L. acidophilus* isolate was used to inculcate tubes containing MRS-broth medium and anaerobically incubated at 45 ºC for 42 hrs. Turbid growth indicate a positive result (Buck and Gilliland, 1995).

2.2.11 Maintenance of *L. acidophilus* (Baron and Fingold, 1994)

2.2.11.1 Short time storage

Lactobacillus isolate were cultured on MRS slant by stabbing or streaking and incubated at 35 ºC for 24 hrs, then maintained at 4 ºC.

2.2.11.2 Long time storage

Test tubes with 10 ml sterile MRS broth were used for growing the bacteria at 35 ºC for 24 hrs, then sterile glycerol (20%) was added and mixed by vortex and stored at -20 ºC.

2.2.12 Detection of *P. mirabilis* Virulence Factors

2.2.12.1 Adherence and Invasion assay

2.2.12.1.1 Preparation of *P. mirabilis* Suspension

P. mirabilis was grown by inoculating 10 ml of nutrient broth with 0.1 ml of overnight bacterial culture and incubated at 35 ºC for 18 hrs. Cells of bacteria were harvested from 10 ml of mid-exponential phase ($O.D_{600}$ about o.6 giving 1×10^9 cells / ml) by centrifugation (1000 rpm, 20 min), washed twice with PBS and resuspended in the same buffer (Iwahi *et al.,* 1982).

2.2.12.1.2 Preparation of Epithelial Cells

Uroepithelial cells were collected from urine of healthy females by centrifugation at 1000 rpm for 5 minutes, then cells were washed three times with PBS before resusapension in PBS (Chan *et al.,* 1984)

2.2.12.1.3 Adherence Test (Iwahi *et al.,* 1982)

- Aliquot of 0.2 ml of bacterial suspension was added to 0.2 ml of epithelial cell suspension and 0.1 ml of PBS and incubated at 35 ºC for one hour.
- Unattached bacteria to uroepithelial cells were removed by centrifugation in PBS at 1000 rpm for 10 min.
- Pellet was reuspended in 0.5 ml of PBS, then A drop of suspension was placed onto a microscopal slide, air – dried , fixed with methanol : acetic acid (3:1) and stained with crystal violate. The adherent bacteria to epithelial cells were observed by the compound light microscope.
- A Control of only the epithelial cells was included.

2.2.12.1.4 Invasion Test

A gentamicin resistances assay was used as a measure to determine the cells invasion mediated by *P. mirabilis* local isolates. 0.5 ml of bacterial suspension was added to 0.5 ml of epithelial cell suspension and incubated at 35 ºC for 3 hrs. Cells were washed twice with PBS to remove free bacteria and 100 µl of gentamicin solution was added, mixed thoroughly and incubated at 35 ºC for 2 hrs. to kill accessible bacteria. A series dilution was made, 100 µl of the final suspention was plated on blood agar medium and incubated for 24 hrs at 35 ºC and then numerate bacterial cells to reveal bacterial survival in epithelial cells (Rejiv *et al.*, 2002).

2.2.12.2 Production of Protease

2.2.12.2.1 Semi Quantitative Screening (Sneath *et al*., 1986)

Local isolates were streaked on nutrient agar medium and incubated at 35 °C for 24 hrs. A single colony was then taken and placed on the center of skim milk agar plate, then Plates were incubated at 35 °C for 48 hrs. Ability of bacterial isolate of *P. mirabilis* to produce protease was estimated by measuring the diameter of hydrolysis zones around colony.

2.2.12.2 Quantitative Screening (Manachini *et al*., 1989)

- After the appropriate incubation period, bacterial culture was centrifuged at 15000 rpm for 20 min, at 4°C. Supernatant was assayed for proteolytic activity by casein digestion method.
- Activity of protease was assayed in triplicate by measuring the release of TCA soluble peptides from 1% (w/v) casein solution. The assay mixture consisted of 0.8 ml casein solution and 0.2 ml of enzyme solution (crude filtrate), and incubated at 35°C for 30 min.
- The reaction was terminated by addition 1 ml of TCA reagent, incubated in ice bath for 10-15 min, and centrifuged at maximum speed for 20 min.

- Control treatment was achieved by using the same steps except the addition of 1ml of TCA reagent into casein solution before the addition of 0.2 ml of enzyme solution.
- The absorbance was measured at 280 nm since one unit (U) of enzyme activity was defined as the amount of enzyme required to increase in absorbance at 280 nm equal to 0.01 in one minute under experimental conditions according to the following equations (Whitaker and Bernard, 1972):

$$\text{Enzyme activity (U/ml)} = \frac{\text{absorbance at 280 nm}}{0.01 \times 30 \times 0.2}$$

0.01: Constant

30: Reaction time (min)

0.2: enzyme volume (ml)

$$\text{Specific activity(U/mg)} = \frac{\text{activity (U / ml)}}{\text{protein concentration (mg / ml)}}$$

2.2.12.3 Determination of Protein Concentration

Protein concentration was determined according to the method described by Bradford, (1979) and as the follow:

- Stander curve of Bovine serum albumin (BSA) was plotted by using different concentration from BSA stock solution (as prepared in item 2.2.2.1.12).
- Then 2.5 ml of Coomassi brilliant blue G-250 dye was added, mixed and left to stand for 2 min at room temperature.
- The absorbance at 595 nm was measured; the blank was prepared from 0.5 ml of Tris-HCl buffer and 2.5 ml of the dye reagent.
- A standard curve was plotted between the BSA concentrations against the corresponding absorbance of BSA.

- Protein concentration was estimated by mixing 0.1 ml of the test sample 0.4 ml of Tris-HCl and 2.5 ml of Coomassi brilliant blue G-250 dye, and left to stand for 2 min at room temperature before measuring the absorbance at 595 nm.

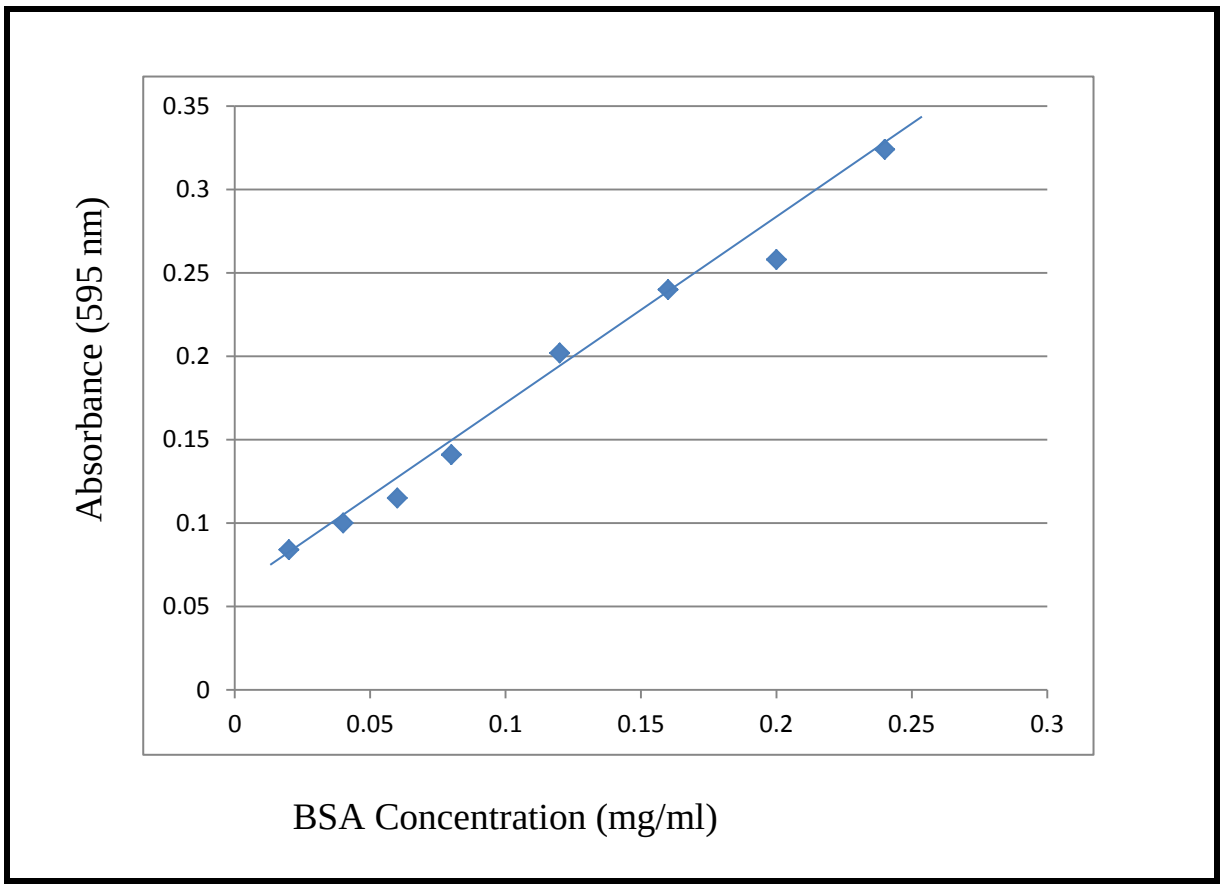

Figure (2-1): Bovine Serum Albumin Standard Curve for determination of protein concentration

2.2.13 Antibiotic Susceptibility Test

Disc diffusion method was achieved according to Atlas *et al.*, (1995) to investigate antibiotic susceptibility of the selected isolate against different antibiotics. A sterile cotton swab was dipped in fresh bacterial growth and streaked on Mullar Hinton agar plates three times by rotating the plate approximately 60° to ensure even distribution, then discs of different antibiotics

were applied on medium surface and incubated at 35 ºC for 24 hr. The zone of inhibition around antibiotic disks were measured and compared with Clinical and Laboratory Standards Institute (CLSI), (2005).

2.2.14 Extraction of Plasmid DNA

Extraction of plasmid DNA was achieved according to alkaline lysis method described by Sambrook and Maniatis, (1989) by using the protocol of DNA-Spin™ plasmid DNA Purification Kit, as follow:

1. Aliquot of 5 ml of fresh culture of the selected isolate of *P. mirabilis* (with 100 µl of Tetracycline solution and 100 µl of ampicilin solution) was centrifuged at 13000 rpm for 30 sec. at 25 ºC, then supernatant was discarded.
2. Bacterial pellet was resuspended in 250µl of resuspension buffer by vortexing.
3. A portion of 250 µl of lysis buffer solution was added to the resuspended bacterial cells and mixed 10 times by inverting and incubated for 3min. at 25 ºC.
4. A portion of 350 µl of neutralization buffer solution was added and gently mixed by inverting 10 times and incubated in ice bath for 5 min. Centrifuged at 13000 rpm for 10 min. at 4ºC. while waiting, the centrifugation, column was inserted into the collection tube.
5. After centrifugation, supernatant was transferred promptly into the spin column, and recentrifuged at 13000 rpm for 1 min., the column was removed from the collection tube, and the filtrate in collection tube was discard, then spin column was placed back in the same collection tube.
6. A portion of 500 µl of washing buffer A was added and centrifuged at 13000 rpm for 1 min. the column was removed from collection tube, filtrate was discarded, then the spin column was placed back in the same collection tube.

7. A portion of 700 µl of washing buffer B was added, centrifuged at 13000 rpm for 1 min. and treated as in previous step, then centrifuged at 13000 rpm for 1 min. to dry the filter membrane.
8. The column was placed into a clean and sterile centrifuge tube, then 50 µl of elution buffer was added to the upper reservoir of the column, and let to stand for 1min., then tube was centrifuged at 13000 rpm for 1min.

2.2.15 Agarose Gel Electrophoresis

Plasmid profile for the selected isolate of *P. mirabilis* was detected on agarose gel (0.8 %) according to Maniatis *et al.*, (1982) . Gel was run horizontally in 1 X TBE buffer. Electrophoretic buffer was added to cover the gel. Samples of DNA extracted from the selected isolate were mixed with loading buffer (1:10 v/v) and loaded into the wells and run for 1 hours at 5 V/cm, then agarose gel was stained with ethidium bromide by immersing in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 minutes. DNA bands were visualized by UV transilluminator. Gel was de-stained using distilled water for 30- 60 min. to get rid of background before photographing of DNA bands.

2.2.16 Curing of Plasmid DNA

Curing experiment was performed by using Ethidium bromide according to Trevors, (1986) and Salzono *et al*., (1992) and as follow:

- Single colony of the selected isolate of *P. mirabilis* was used to inoculate 5 ml of nutrient broth and incubated at 35 ºC for 18 hours. Then 0.1 ml a liquates was taken from the growth culture and transferred to inoculate universal tubes containing 5 ml of nutrient broth with different concentrations of ethidium bromide in each tube (50, 100, 200, 300, 400, 500, 600, 800, 900, 950, 1000 and 1600 µg/ml). All the tubes were incubated with shaking (150 rpm) at 35 ºC for 24 hours.

- Growth density of different tubes was measured and compared with the control to determine the effect of curing agent at different concentrations on bacterial growth.
- Samples were taken from the sublethal concentration of ethidium bromide, then Serial dilutions were achieved, and spread on MacConky agar plates, and incubated at 35 ºC for 24 hours.

2.2.17 Detection of Cured Colonies

Cured colonies were detected by selecting randomly 100 colony from the MacConky agar plates and were examined for antibiotic resistance as in item (2.2.13) in which the discs of antibiotics that wild isolate was resist were applied on medium surface and incubated at 35 ºC for 24 hrs., then colonies that lost their resistance to one or more antibiotics were selected to examine their plasmid profile after treatment with curing agent (Trevors, 1986; Salzono *et al*., 1992).

2.2.18 Detrmining of inhibitory effect of *L. acidophilus* against *P. mirabilis*

2.2.18.1 On sold medium

L. acidophilus isolates that was already grown in MRS broth for 24 hrs. were cultured by streaking on MRS agar, and incubated anaerobically at 35 ºC for 48 hrs. Five mm discs of *Lactobacillus* culture were made by a cork borer and placed on the surface of nutrient agar plates that already spread with 0.1 ml of *P. mirabilis* culture, then incubated at 35 ºC for 24 hrs. Inhibition zone diameter (mm) was measured (Al-Kassab and Al-Khafaji, 1992).

2.2.18.2 In liquid medium

MRS broth was inculcated by *L. acidophilus* then incubated anarobically at 35 ºC for 48 hrs. After incubation the culture was centrifuged at 6000 rpm for 15 min. it was filtrated through Millipore filter unit (0.22 µm) (Lewus *et al.*, 1991).

The well diffusion method described by Gupta *et al.,* (1998) was used to determine the inhibitory effect of *L. acidophilus* filtrate. 0.1 ml of *P. mirabilis* broth was spread on nutrient agar using a sterilized glass spreader, a 5 mm diameter well was made on surface of medium by a cork borer, then this well was filed with 100 µl of the filtrate and incubated at 35 ºC for 24 hrs. After incubation, inhibition zone diameter (mm) was measured and compared with control well that contained MRS broth only (Vignolo *et al.,* 1993).

2.2.19 Effect of *L. acidophilus* Filtrate on virulence Factors of *P. mirabilis*

Fresh culture of *L. acidophilus* grown anaerobically in MRS broth medium (pH 6) for 48 hrs. at 35 ºC was centrifuged at 6000 rpm for 15 min. to get cell free supernatant (which was considered probiotic) and sterilized by filtration through Millipore filter unit (0.22µm) according to Martinez-Gonzalez *et al.*, (2004). The final volume of the filtrate was distributed to the following concentrations:

_ One-fold concentration (15 ml)

_ Two-fold concentration (10 ml)

_ Three-fold concentration (5 ml)

Each concentration was added to 20 ml of fresh culture of the selected isolate of *P. mirabilis* grown in nutrient broth ($O.D_{600}$ about o.2) and incubated at 37 ºC for 24 hrs. After incubation virulence factors was assayed as in items (2.2.12.1) and (2.2.12.2) to study the effect of probiotic on virulence factors of locally isolated *P. mirabilis.*

Chapter Three

Results
and
Discussion

3. Results and Discussion

3.1 Isolation of *P. mirabilis*

In order to isolate*P. mirabilis*, different samples (clinical and meat samples) were collected from different locations in Baghdad goverment during the period from 11-2012 to 2-2013. Results indicated in table (3-1) showed that 65 of clinical samples (urine samples from patients with urinary tract infection and wound samples) and 20 of food samples (chicken and meet) were collected to isolate *P. mirabilis* .

Table (3.1): Bacterial isolates obtained from different clinical and meat samples

Source of sample	No. of sample	No. of isolates	No. of *Proteus spp*. Isolates
Urine	20	5	1
Wounds	45	95	12
Chicken and meat	20	46	13
Total	85	146	26

From all of these samples, a total of 146 different isolates were obtained, these isolates were cultured on blood and MacConky agar medium as a differential medium to differentiate *Proteusspp*. from clinical samples, and on blood, MacConky agar and XLD agar medium for chicken and meat samples.

Among the obtained isolates, 26 isolates were suspected to be belonging to *Proteus* spp. according to their morphological and cultural characteristics.

3.2 Identification of *P. mirabilis*

The selected isolates were first identified according to their cultural, morphological characteristics.

3.2.1 Cultural Characteristics

The suspected isolates were cultured on MacConky agar medium for 24 hrs. at 35 ºC. these isolates appear as pale colonies and have swarming motility on blood agar medium.

3.2.2 Morphological Characteristics

The suspected isolates were examined under oil immersion objective lens of the light compound microscope after staining with gram stain. Results showed that these isolates appeared as Gram negative rods (purple), and non spore formers.

3.2.3 Biochemical Tests

Several biochemical tests were achieved to identify the isolates of *Proteus* spp. Results mentioned in table (3-2) showed that 15 of these isolates were positive for catalase, urease, phenylalanine-deaminase, motility and triple sugar iron, while they gave negative results for oxidase and indole tests. Further more they were varying in their ability to utilize simon citrate.

According to these results of biochemical tests, which were agreed with Al-jeboury, (2005) and Stankawska *et al*., (2008), it could be concluded that these 15 isolates were belonging to *P. mirabilis.*

Table (3.2):Biochemical tests for identification of *Proteus mirabilis* local isolates

Isolate / Symbol / Test		P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
Oxidase		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine-deaminase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Simon citrate		-	+	+	-	-	-	-	-	+	+	+	+	+	-	+
Kligler iron agar	**Slant/ Butt**	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A	K/ A
	H2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	CO2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(-): Negative results, (+): positive results,K/A: alkaline/acid

3.2.4 Identification of *P. mirabilis* by using Api 20E system

To confirm the identification of the 15 isolates of *P. mirabilis*, these isolates were further identified according to the results of biochemical tests by using Api 20E system. Results shown in appendix (2) and figure (3.1) mentioned that these isolates were negative for ONPG, ADH, LDC,MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA while positive for ODC, CIT, H2S, URE, TDA, IND, VIP, GEL, and GLU.

Figure (3.1): Identification of local isolates of *P. mirabilis* using API 20E system

3.3 Detection of the virulence Factors Produced by *P. mirabilis* Isolates

Virulence factors produced by local isolates of *P. mirabilis* were investigated. These virulence factors include adhesion and invasion and protease production in different media

3.3.1Adherence and Invasion

Ability of *Proteus mirabilis* local isolates to adhere to uroepithelial cells is considered as an important virulence factor in pathogenesis of urinary tract infections (Mobley and Chippendle, 1990).

Adherence ability of *P. mirabilis* to uroepithelial cells was detected under oil-immersion lens of the light compound microscope as shown in figure (3.2). This figure shows the uroepithelium obtained from healthy female after infection with *P. mirabilis* isolates. It appeares as rods and adhere to the uroepithelium by pili. Results indicated in table (3.3) showed that the highest number of adhering bacteria to UEP was ranged from 30-55 bacterial cell / uroepithelial cell.

These results almost in agreement with Perez-Serrano *et al* ., (1997) who found that the adherence range of *P.mirabilis* to UEPCs ranged between 45-55 bacteria/cell.

In other study on the adhesion of *P. mirabilis* to uroepithelial cell Al-kabby (2007) found that 31.1 of *P. mirabilis* was adhered to uroepithelial cells.

Gram-negative uropathogens produce an assortment of adhesins including those attached to the tip of hair-like projections, known as fimbriae or pili, as well as adhesins anchored directly within bacterial cell membranes, known as non fimbrial adhesins. There was tight relation between invasion and adherence, once initial attachment and permanent adherence commenced on the surface of uroepithelial cells, the establishment uropathogenic *E. coli* infection occurs through the colonization of the bladder by the invasion of host cells and the subsequent formation of biofilms (Mulvey, 2002).

Adhesion mediated by fimbriae is a crucial step for successful bacterial colonization of the urinary tract mucosa and has an important role in the pathogenesis of UTI. *Proteus* spp. can express several types of fimbriae, Mannose-resistant *Proteus*-like Fimbriae (MR/P) contribute significantly to colonization of UTI and increase the risk of development of acute pyelonephritis (Zunino *et al.,* 2007), while the *P. mirabilis* fimbriae (PMF) are involvement in both bladder and kidney colonization by *P. mirabilis* and this fimbriae was widely distributed among *P. mirabilis* isolated from different origins (Zunino *et al.,* 2003).

On the other hand, results indicated in table (3.4) showed the ability of bacterial cells of *P. mirabilis* local isolates to invade uroepithelial cell. These bacterial cells that gain entry to the uroepithelial cell are not susceptible to the gentamicin when it is added to the suspension and ranged between (1-20 cells).

Depending on our results it could be concluded that different isolates *of P. mirabilis* exhibited high invasion capability to epithelial cell and survive intracellularly (Wells *et al.,* 1999).

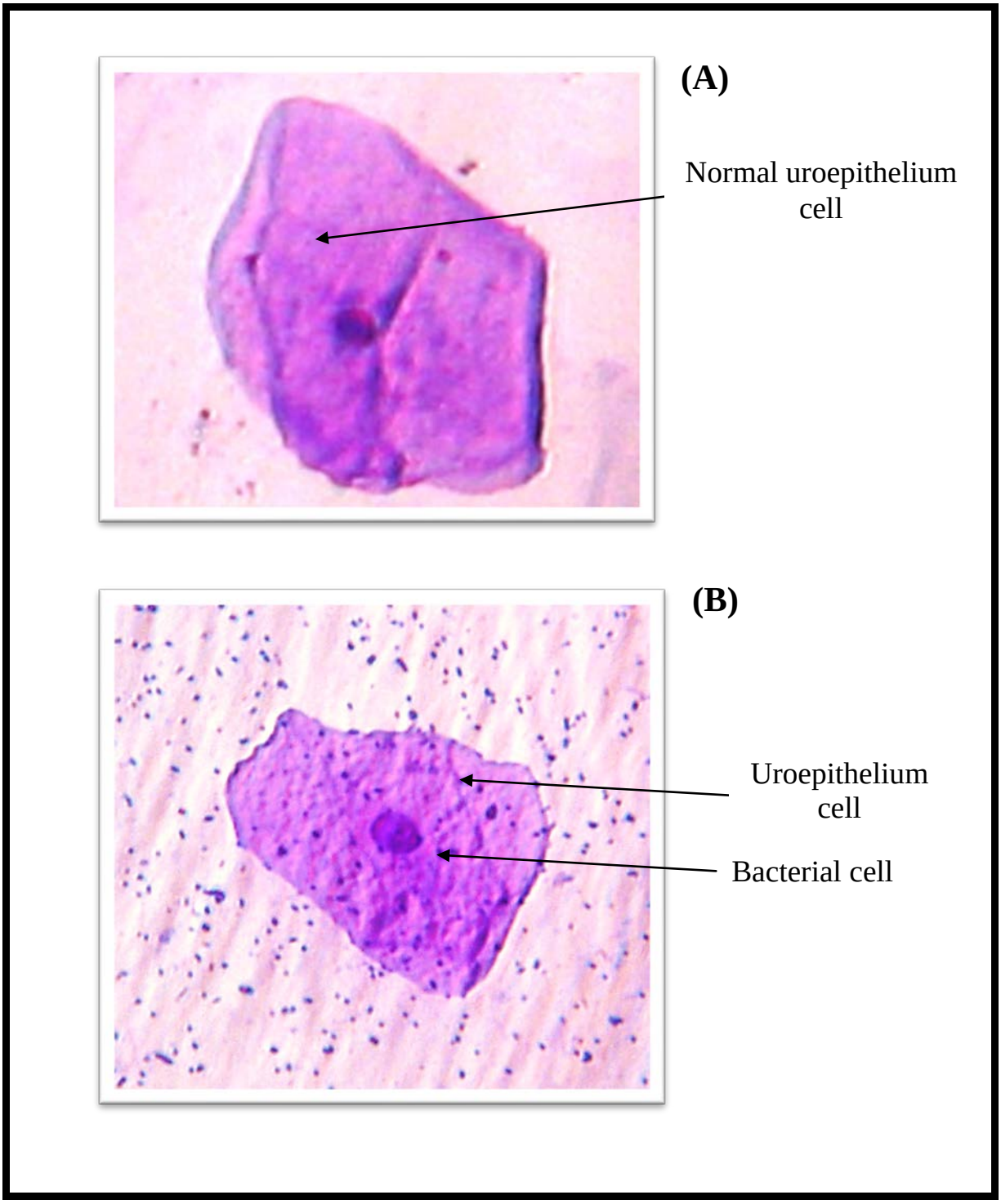

Figure (3.2): Adherence of *Proteus mirabilis* to uroepithelium cells

(A): Normal uroepithelium cell

(B): Uroepithelium cells after adherence of bacterial cells of *P. mirabilis*

Table (3.3): Adhesion average of *P. mirabilis* to uroepithelial cells

Isolate	Adhesion average (Bacterial cell / uroepithelial cell)
P. mirabilis P1	30
P. mirabilis P2	33
P. mirabilis P3	33
P. mirabilis P4	35
P. mirabilis P5	30
P. mirabilis P6	33
P. mirabilis P7	32
P. mirabilis P8	38
P. mirabilis P9	42
P. mirabilis P10	32
P. mirabilis P11	40
P. mirabilis P12	45
P. mirabilis P13	35
P. mirabilis P14	38
P. mirabilis P15	55

The invasion into urothelial and intestinal cells shows different aspects, resulting in single-membrane and double-membrane protection, respectively. This protection induces a relative resistance of *P. mirabilis* to the antibiotics cotrimoxazole and amoxicillin-clavulanic acid. Gentamicin and ciprofloxacin are the the most effective antibiotics in the presence of epithelial cells, and nitrofurantoin also seems to be more effective in the presence of these cells. *P.mirabilis* thrives better inside the cell when there are antibiotics in the culture medium and better outside the cell when there is no selection pressure from antibiotics. Resistance to cotrimoxazole and nitrofurantoin is also often found clinically(Daza *et al.*, 2001).

Table (3.4): Number of bacterial cells of *P. mirabilis* local isolates invade uroepithelial cells

Isolate	No. of Invade cells / uroepithelial cell
P. mirabilis P1	–
P. mirabilis P2	1
P. mirabilis P3	14
P. mirabilis P4	3
P. mirabilis P5	–
P. mirabilis P7	–
P. mirabilis P8	4
P. mirabilis P9	16
P. mirabilis P10	11
P. mirabilis P11	12
P. mirabilis P12	16
P. mirabilis P13	8
P. mirabilis P14	–
P. mirabilis P15	20

This result came in agreement with those mentioned by Jacobsen *et al.*, (2008) who found that the invasion, cytotoxicity and biofilm formation of *P. mirabilis* and *Escherichia coli* (Uropathogenic *E. coli* UPEC strains) have been observed *in vitro* and *in vivo* to be internalized by bladder and renal epithelial cells. Several adhesins and toxins have been implicated to be involved in the process of invasion, including type 1 fimbriae, S pili, P pili, and CNF1. Type 1 fimbria-mediated invasion is dependent upon FimH expression (Martinez *et al.*, 2000). *E. coli* strains that express adhesins have been observed to invade epithelial cells including Caco-2 intestinal cells (Nowicki *et al.*, 2001).

P. mirabilis strains, as a group, were internalized, but less so than *Salmonella typhimurium* (a well-established intracellular pathogen) that HpmA hemolysin is responsible for this invasion is evident from specific observations. The hemolytic titer of *P. mirabilis* strains correlates with cell destruction (Alamuri *et al.*, 2009).

3.3.2 Screening the ability of *P. mirabilis* isolates in protease production

Two methods for screening the ability of the local isolates of *P. mirabilis* for protease production as indicator for the virulence and pathogenesity of these isolates. The first was semi-quantitative screening which depends on the formation of zone of hydrolysis around colonies on skim milk agar medium, and the second was the quantitative screening which depends on the determination of specific activity of protease produced by these local isolates of *P. mirabilis*.

3.3.2.1 Semi- quantitative screening

Semi-quantitative screenings for protease production by the local isolates of *P. mirabilis* was achieved by detecting the ability of these isolates to produce protease enzyme and the formation of zone of hydrolysis around each colony when grown on skim milk agar medium. Results mentioned in table (3.5) and figure (3.3) showed that these isolates were able to hydrolyze skim milk agar medium around each colony and forming halo of hydrolysis with variable degrees.

Results mentioned in table (3.5) also showed that the diameter of zone of hydrolysis ranged between 6 and 12mm for different isolates, among them the isolates *P. mirabilis* P15 (isolated from urine) was the most efficient in protease production because it gives the highest diameter of hydrolysis (12 mm) on skim milk agar. On the other hand results mentioned in table (3.5) showed that the other isolates of *P. mirabilis* were less efficient in protease production due to the lower formation of zones of hydrolysis around their colonies.

Table (3.5): Diameter of clear zones around colonies of *P. mirabilis* isolates on skim milk agar medium after incubation for 24 hrs at 35°C.

Isolate	Diameter (mm)
P. mirabilis P1	5
P. mirabilis P2	6
P. mirabilis P3	5
P. mirabilis P4	6
P. mirabilis P5	7
P. mirabilis P6	6
P. mirabilis P7	5
P. mirabilis P8	5
P. mirabilis P9	8
P. mirabilis P10	7
P. mirabilis P11	7
P. mirabilis P12	8
P. mirabilis P13	5
P. mirabilis P14	7
P. mirabilis P15	14

It is well known that protease is a proteolytic enzyme that responsible for hydrolyzing proteins by attacking peptide bounds in the basic structure ofproteins, so protease produced by bacterial isolates attacked casein (the constituent of skim milk protein) in the culture medium and forming a halo of hydrolysis around each colony (Viji *et al.*, 2011).

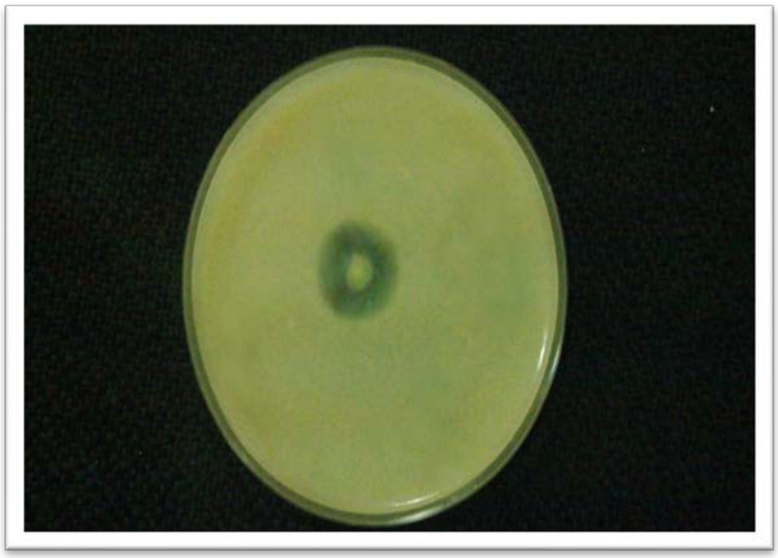

Figure (3.3): Protolytic activity of protease produced by *P. mirabilis* on skim milk agar medium after incubation at 35°C for 24h.

The experimental studies reported by Folasade and Ajele, (2005) indicated that proteases produced by nine *Bacillus* strains on 10% Skim milk agar plates. Similarly, *Pseudomonas* strain was used to produce protease in M.S. medium (Gaillard *et al.*, 2005), Reilly and Day, (1983) also confirmed the production of protease by using the bacterial strain *Aeromonas hydrophilla*. David and Mortimer, (1977) stated that yeast *Saccharomyces cerevisiae* was used to produce protease enzyme.

3.3.2.2 Quantitative screening for protease production by local isolates of *P. mirabilis*

Local isolates of *P. mirabilis* were screened quantitatively to examine their ability in protease production. This was achieved by growing each of the 15 isolates in protease production broth medium for 24 hours at 35°C, then they were centrifuged and the specific activity of protease in crude filtrates was determined. Results indicated in table (3.6) showed that all the isolates were

protease producers with variable degrees. Specific activity of protease in culture filtrates was ranged between 14.5-0.121 U/mg protein.

Among them, *P. mirabilis* P15 was the most efficient in protease production because the specific activity of protease in crude filtrate of this isolate was 14.5 U/mg protein, while the specific activity of protease in culture filtrates of the other isolates were ranged between 0.12 and 14.5 U/mg protein. According to these results, the isolate P15 was regarded as the most virulent because of its high ability in protease production.

The differences in the ability of the isolates to produce protease are due to genetic variations of the genes responsible for the production of protease (Whealer *et al*., 1991). The environmental factors such as pH and temperature are mostly influencing the enzyme production (Esakkiraj *et al.,* 2007).

According to these results of the detection of virulence factors for the bacterial isolates of *P. mirabilis*, the isolate P15 was regarded as the most virulent isolate among the other local isolates of *P. mirabilis* and selected for further studies.

3.4 Antibiotic susceptibility of *P. mirabilis*

The standard disk diffusion method was used to determine the susceptibility of *P. mirabilis* P15 to several antibiotics. The susceptibility of *P. mirabilis* P15 against 15 antibiotics were studied. Results indicated in table (3.7) showed that this isolate was resistant to five antibiotics which they were tetracycline, cefotaxime, cefepime, amikacin and ciprofloxacin while it was intermediate to both of chloramphenicol and piparacillin and it was sensitive to other eight antibiotics which they were gentamicin, trimethoprim, imipenem, nalidixic acid, ampicillin, ticarcillin, aztreonam, ticracillin / clavulanic acid.

Table (3.6): Quantitative screening for protease production by *P. mirabilis* local isolates

Isolate	Specific activity (U/mg)
P. mirabilis P1	0.121
P. mirabilis P2	2.11
P. mirabilis P3	5.03
P. mirabilis P4	1.32
P. mirabilis P5	3.07
P. mirabilis P6	3.86
P. mirabilis P7	2.04
P. mirabilis P8	1.96
P. mirabilis P9	6.04
P. mirabilis P10	12.03
P. mirabilis P11	7.89
P. mirabilis P12	4.55
P. mirabilis P13	5.03
P. mirabilis P14	3.20
P. mirabilis P15	14.50

Resistance trait to these five antibiotics may be encoded by chromosomal and/ or plasmid genes. This resistance could be attributed to degradation of these antibiotics by β-lactamase enzyme produced by the isolates or may be to lack of penicillin binding protein on bacterial cell wall or due to the change in outer membrane permeability of bacterial cell to the drug (Avesion *et al*., 2000).

Table (3.7): Antibiotic susceptibly of locally isolated *P. mirabilis* P15

Antibiotic	Symbol	Susceptibility
Amikacin	AK	R
Chloramphenicol	C	I
Gentamicin	CN	S
Cefepime	FEP	R
Trimethoprim	TMP	S
Imipenem	IPM	S
Nalidixic acid	NA	S
Ciprofloxacin	CIP	R
Tetracycline	TE	R
Cefotaxime	CTX	R
Ampicillin	AMP	S
Ticarcillin	TI	S
Aztreonam	AT	S
Piparacillin	PI	I
Ticracillin / Clavulanic acid	TCC	S

R: Resist, S: Sensitive, I: Intermediate

Resistance of *P. mirabilis* to β-lactam antibiotics was evident in this study, it may be due to the possessing of β-lactamase by the isolate that may be encoded by transferable plasmid found in various Enterobacterceae members such as *E. coli, P. mirabilis, Klebsiella pneumonia* and *Salmonella typhimurium* (Bret *et al.,* 1998). In general bacteria resist aminoglycoside such as amikacin by producing modifying enzymes which alter the aminoglycoside and prevent it from binding to ribosome (Jawetz *et al.,* 1998).

In the current study, *P. mirabilis* was sensitive to Imipenem, and this result was in agreement with Decre *et al.,* (2002), Luzzaro *et al.,* (2006), who found that *P. mirabilis* strains were all sensitive to Imipenem. This may be attributed to the inability of *P. mirabilis* and other organisms to produce enzymes that degrade or inactivate this antibiotic. In other study Johansen *et al.,* (2006), found that 40% of *P.mirabilis* isolates were sensitive to Nalidixic acid, and this may be due to different antibiotic prescribed and subsequently different percentages of sensitivities. Further more it was found that 60% of *P. mirabilis* isolates were sensitive to Trimethoprim, and 56 % of were sensitive to Piperacillin (Jones *et al.,* 2004; Hung *et al.,* 2006).

High gentamicin resistance rate was mentioned by Stratchounski *et al*. (1998) who found that only 56% of *Proteus* isolates were susceptible to gentamicin. High resistance to tetracycline was found among the isolates as 80 % was recorded, and this was due to resistance genes which carried by plasmid (Merlin *et al.,* 1988). Al-Murjany (2000) was found that the *P. mirabilis* isolates were vary in their resistance to tetracycline with a resistance percentage of 96 %.

3.5 Plasmid profile of *P.mirabilis*

To assess the relationship between plasmids and virulence factors of *P. mirabilis* P15, plasmid profile of locally isolated *P. mirabilis* P15 was studied by extraction of plasmid DNA according to the alkaline lysis method described by Sambrook and Maniatis, (1989) by using the protocol of DNA-Spin™ plasmid DNA Purification Kit.

According to previous studies, it is well known that most Gram negative bacteria are harboring plasmids that are responsible for antibiotic resistance and some virulence factors (Virve *et al*., 2004).

Results mentioned in figure (3.4) showed that this isolate have two plasmid DNA bands after extraction of plasmid DNA and electrophoresis on agarose gel. This result was in agreement with the finding of Adeniyi *et al*., (2006) who reported that *E.coli, Proteus* spp. and *Pseudomonas* species isolates carrying plasmid of molecular size above 2.1 kb. Other investigators also detected two plasmids harbored by many strains of *Proteus mirabilis* of about 6 and 93 kb (Stankowska *et al.,* 2008).

These two plasmids of *P. mirabilis* 15 may be responsible for the resistance to one or more antibiotics that it resist (tetracycline, cefotaxime, cefepime, amikacin and ciprofloxacin) or may responsible to one or more of the verulance factors (adherence and invasion and protease production).

On other hand results mentioned in figure (3.4) showed that these two plasmids are small in size, one of them was about 8000 bp and other about 6000 bp in compared with the size of DNA ladder marker fragments run on the same agarose gel.

Khan and Musharaf, (2004) demonstrated that *P. mirabilis* which was able to transfer resistance to ampicillin, kanamycin, nalidixic Acid and chloramphenicol, contained conjugative plasmid with molecular size close to (12.2) kb. According to Yah *et al.,* (2007) majority of multidrug resistance of gram negative bacteria were resistant to 10 antibiotics and the plasmids molecular weight were estimated to be above 2.1 kb.

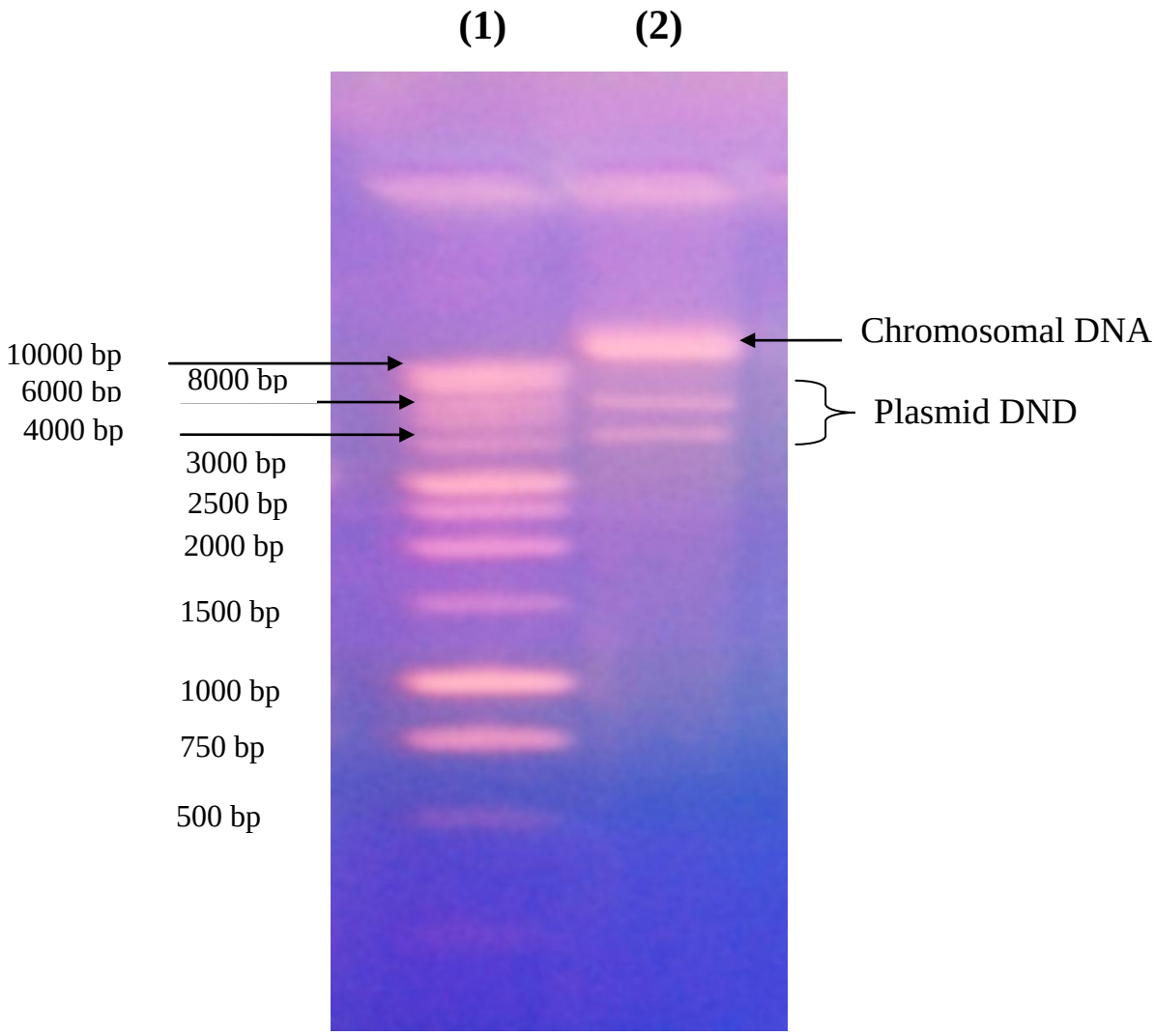

Figure (3.4): Gel electrophoresis of plasmid DNA extracted from locally isolated *P. mirabilis* P15 on agarose gel (0.8%) in TBE buffer at 5 V/cm.
Lane (1): 1 kb DNA ladder marker
Lane (2): *P. mirabilis* P15

3.6 Curing of plasmid DNA

To determine whether the genes responsible for virulence factors and antibiotic resistance are chromosomally located or encoded by plasmid. curing experiment for *P. mirabilis* P15 was achieved using ethidium bromide as a curing agent with different concentrations in culture media ranged between 50 to 1600µg/ml.

Results indicated in table (3.8) showed that the highest concentration of ethidium bromide that allows the growth of *P. mirabilis* P15 was 1000 µg/ml, then appropriate dilutions were done and 100 µl of the approperate dilution was taken and spread on MacConkey agar medium in order to investigate cured colonies, then a total of 100 colonies were selected and antibiotic sensitivity of these colonies were examined using the antibiotics that wild type was resist (Tetracycline, Cefotaxime, Cefepime, Amikacin and Ciprofloxacin) in order to detect the cured colonies who lost their ability to conferring the resistance phenotypes to these antibiotics.

Results showed that 20 colonies out of 100 colonies, became sensitive to three antibiotics (cefotaxime, tetracycline, and amikacin) as shown in table (3.9), one of these colonies was selected randomly and examined for the presence of its own plasmids by extraction of plasmid DNA and electrophoresis on agarose gel.

Result mentioned in figure (3.5) showed that cured cells of *P. mirabilis* P15 was lost their own plasmids. This results confirmed that plasmids of *P. mirabilis* P15 are responsible for resistance to cefotaxime, tetracycline, and amikacin, while the resistance to cefepime and ciprofloxacin was chromosomally encoded.

The result revealed that the β-lactamase gens located on plasmids for the analyzed strains. This result was in agreement with the finding of other investigators (Dharmadhikari and Peshwe, 2009) were they confirmed the location of antibiotic markers on R-plasmid by treating the cells with curing agents. The characterization of various plasmid mediated TEM-type β-lactamase in Proteus mirabilis are evidence of the wide diversity of β-lactamases produced by this species and of its possible role as β-lactamase-encoding plasmid reservoir (Bonnet *et al*., 1999).

Table (3.8): Effect of different concentrations of Ethidium Bromide on growth of *P. mirabilis* P15 after incubation in nutrient broth medium at 35 for 24 hrs.

concentrations (µg/ml)	Growth
0	+++
50	+++
100	++
200	++
300	++
400	+
500	+
600	+
800	+
950	±
1000	±
1600	–

(–): no growth , (±):slight growth, (+):moderate growth, (++):good growth, (+++):heavy growth.

Table (3.9): Antibiotic susceptibly of cured cell *P. mirabilis* P15 in compared with wild-type

Antibiotic	Wild-type	Cured
Cefotaxime	R	S
Tetracycline	R	S
Amikacin	R	S
Cefepime	R	R
Ciprofloxacin	R	R

R: Resist, S: Sensitive

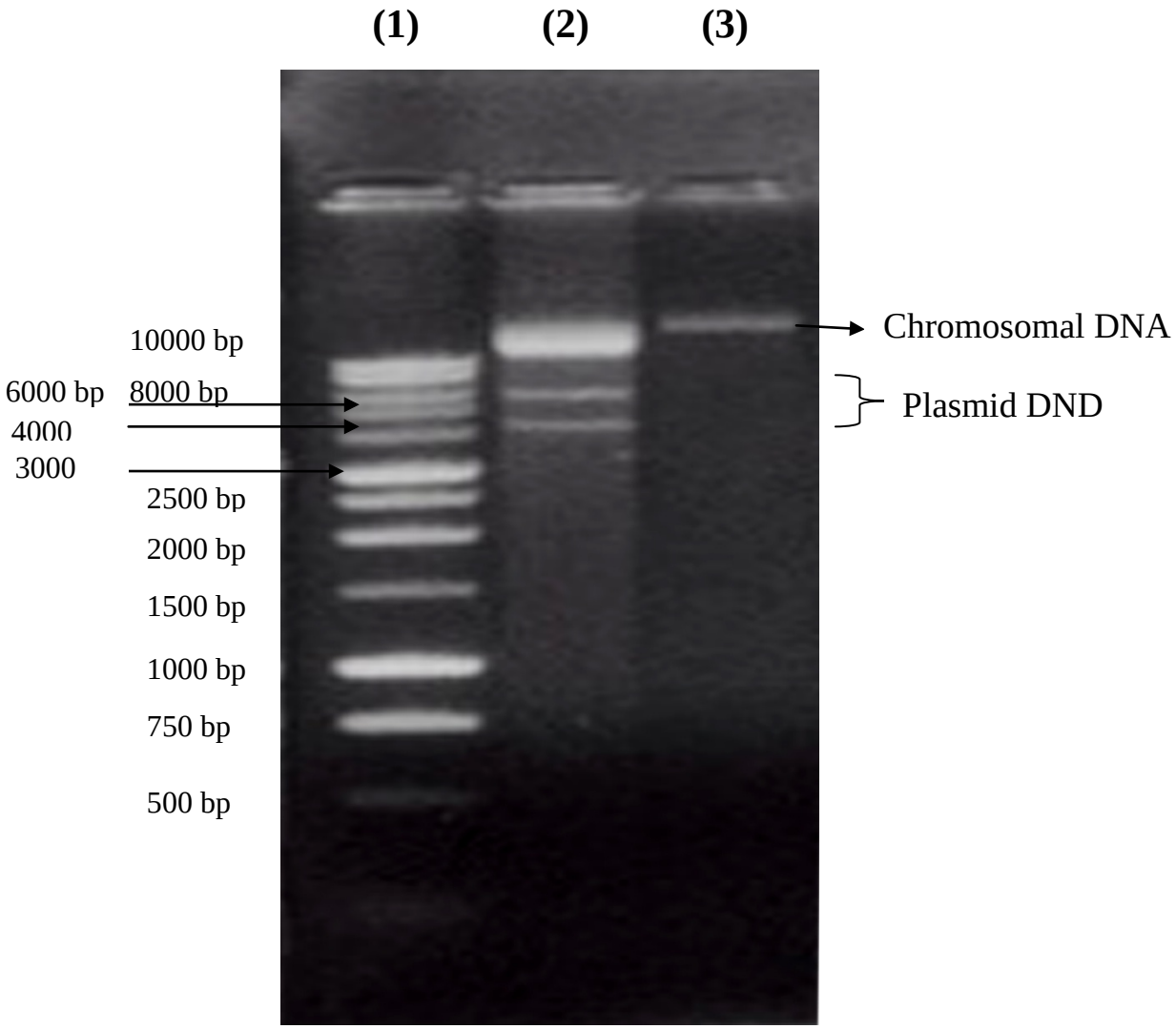

Figure (3.5): Plasmid profile of wild type and cured cells of *P. mirabilis* P15 after extraction and electrophoreses on agarose gel (0.8%) at (5 v/cm) for 1.5 hrs

Lane (1): 1 kb DNA ladder marker

Lane (2): *P. mirabilis* P15 (wild-type)

Lane (3): *P. mirabilis* P15 (cured)

3.7 Detection of Virulence Factors of Cured *P. mirabilis*

Virulence factors produced by cured *P. mirabilis* P15 were detected by studying the ability of cured cells in invasion and adherence, and protease production. Woodward *et al.,* (1989) have described serovars containing plasmids of various molecular size which did not contain the genes associated with virulence. The search for the plasmid content of strains was often used in the study of epidemiological markers. While remaining of some interest, this approach does not provide any information as to the potential virulence of strains, since on the one hand the presence of plasmids does not necessarily imply the presence of the plasmid gene associated with mouse virulence, and on the other hand the plasmid/virulence combination is not inevitably linked to the molecular weights of the plasmids observed (Shah *et al.,* 1991).

Results showed that cured cells of *P. mirabilis* P15 were still able to adhere to uroepithilial cells with average of 35-45 bacterial cell / uroepithelial cell. Results also showed that these cured cells of *P. mirabilis* P15 were also still able to invade uroepithelial cells with the same ability of wild-type.

Hull *et al.*, (1981) demonstrated that the genes encoding different classes of uropathogenic mannose resistance fimbriae are chromosomal determinants, while El-Baghdady *et al.,* (2009) observed that the elimination of plasmids of *Proteus* spp. by acridine orange resulted in loss of adhesion capability of isolates and the pathogens lost their resistance to different antibiotics such as cifteriaxone and amikacin.

These results agreement with Sahly *et al.,* (2004) who found that the elimination of the plasmid that encoded extended spectrum β-lactamaseESBL production did not influence the ability of strains to adhere to or invade the epithelial cells, indicating that the invasion potential of *Klebsiella* is chromosomal rather than plasmid mediated, contradicting previous reports indicating an R plasmid-restricted increase in adhesive properties. Alternatively, the adhesion/invasion of the strains in the studies cited above might be mediated

by fambrial adhesions. on other hand, The virulence-associated plasmids of *Salmonella typhimurium*were not essential to vero-cell invasion by these strains (Gulig and Curtiss, 1987)

Results of curing of plasmids of *P. mirabilis* P15 showed that gene responsible for protease production was chromosomally located due to that ethidium bromide at different concentrations was unable to affect the protease production gene. The gene encoding this enzyme, zapA, has been cloned and its sequence determined in *P. mirabilis* (Wassif *et al.*, 1995), on the other hand, the gene responsible for protease production may be located on the chromosomal DNA of *Pseudomonas aeruginosa* isolates (Guzzo *et al*.,1990).

3.8 Effect of Probiotic of *L. acidophilus* on Growth and Virulence Factor of *P. mirabilis* P15

To study the effect of probiotic of lactic acid bacteria (LAB) on virulence factors of *P. mirabilis* P15, an isolate of *L. acidophilus* was re-identified according to its cultural, morphological and biochemical characteristics as follows:

3.8.1 Cultural characteristics

Results of cultural identification of *L. acidophilus* show that colonies of this bacteria LAB on MRS agar medium were appeared as pale, round shape, soft, mucoid, convex and surrounded by zone of hydrolysis as a result of dissolving medium component of calcium carbonate.

3.8.2 Morphological characteristics

Morphological characteristics of *L. acidophilus* were examined. Results revealed that it was gram positive, short or long bacilli, clustered in long and short chain, and non-spore forming.

3.8.3 Biochemical tests

Biochemical characteristics of *L. acidophilus* isolate was studied. Results of identification indicated in table (3.10) that this isolate was negative to catalase, because it was unable to reduce hydrogen peroxide to water and oxygen, negative for oxidase because it lacks to cytochrome oxidase that oxidize tetramethyl-p-phenylenediamine, and negative for gelatenase that hydrolyze gelatin, while it gave a positive result for litmus milk test because of its ability to ferment lactose to lactic acid, and lowering pH of litmus milk medium causing clot formation. Moreover this isolate was unable to grow at 45 ºC.

These results confirmed that this isolate was *L. acidophilus* as mentioned by Kandler and Weiss, (1986) and Nigatu and Cashe, (1994).

3.8.4 Inhibitory effect of *L. acidophilus* against *P. mirabilis* (*in vitro*)

To estimate the effect of *L. acidophilus* against *P. mirabilis* bacteria different methods which depending on nature of media were used (on sold medium (MRS agar) and in liquid medium (MRS broth) as following:

3.8.4.1 Inhibitory effect of *L. acidophilus* cells

Figure (3.6) shows the antimicrobial activity of *L. acidophilus* against *P. mirabilis* on sold medium according to their inhibition zones diameter. Result revealed that the inhibitory zone diameter was 14 mm given by isolate *L. acidophilus* after 24 hrs. of incubation.

This may due to production of inhibitory compounds by *Lactobacillus* spp. especially organic acids, and bacteriocins. These results agreed with Jacobsen *et al.,* (1999) who found that the antagonist activity of lactic acid bacteria against diarrheal causing bacteria might be referred to its ability in producing organic acids which lower pH, bactriocins, and competition on the nutrients with the pathogenic bacteria.

Table (3.10): Results of biochemical testes for identification of *L. acidophilus*

Biochemical test	The results
Oxidase	-
Catalase	-
Gelatenase	-
Acid and curd production	+
Growth at 45 ºC	-

(-): Negative results, (+): positive results

Figure (3.6): Inhibitory effect of *L. acidophilus* isolate against *P. mirabilis* on sold medium after 24 hrs. incubation

The variability in inhibition of LAB against pathogenic bacteria were related with the type of pathogen bacteria, type of inhibitory substance and its quantity and ability for distribution in media (Jacobsen *et al.,* 1999). Garver and Murians (1994) recoded that production of inhabited materials by LAB is dependent on the medium used for growth, and they found that tween 80 induced the production of protein (bacteriocin) by increasing the activity of bacteria.

Jin *et al.,* (1996) found that a strain of *Lactobacillus* isolated from chickens was able to inhabited the growth of *E. coli* and *Salmonella* spp. Rolfe (2000) pointed out the ability of LAB to inhabit *in vitro* growth of many enteric pathogens causing broad range of gastrointestinal disorders in both human and animal.

L. acidophilus showed the highest inhibitory effect against *P. mirabilis* as mentioned by Fernandez *et al.,* (2002) who provide that *L. acidophilus* and *L. gasseri* (of human origin) were able to inhabit growth of many enteric pathogens including *Salmonella, Proteus, Listeria and Campylobacter*. Also Hutt *et al.,* (2006) noticed that the antagonist activity of five probiotic bacteria and two bifidobacteria strains against *E. coli, Salmonella enteric* and *Shigella sonneiin vitro* in solid and liquid medium.

3.8.4.2 Inhibitory effect of *L. acidophilus* filtrate

The filtrate of *L. acidophilus* isolate was used to investigate its inhibitory effect against *P. mirabilis* as shown in figure (3.7). Inhibitory effect of filtrate obtained from *L. acidophilus* grown in MRS broth measured by the well diffusion method to determine the inhibition activity of *L. acidophilus* filtrate, grown at 48 hrs. by filling the well which made in nutrient agar plates that have been cultured by tested isolate with the filtrate of LAB isolate.

Figure (3.7) showed that the inhibition zone diameter 20 mm was recorded by using the *L. acidophilus* filtrate as compared to a zone of 14 mm obtained by *L. acidophilus* cells on solid medium after 24 hrs. incubation time.

Figure (3.7): Inhibitory effect of *L. acidophilus* against *P. mirabilis* when grown on MRS broth medium after 24 hrs. incubation

It's clear that MRS broth is better stimulator for inhibitory product than on MRS agar and that explained by (Gupta *et al.,* 1998) who recorded that the MRS broth which was stimulated inhibitory effect against Gram-posative bacteria (*Staphylococcus aureus, Bacillus subtilis*) and gram-negative bacteria (*E. coli,Klebsiella spp*.) when inhibition zone diameter ranged between (13-19 mm).

Probiotic strains have inhabited pathogenic bacteria both *in vitro* and *in vivo* through several different mechanisms; throughout the production of inhibitory compounds (e.g. bacteriocin), reduction of pH through short chain fatty acid production, which could themselves be directly inhibitory to certain pathogens, competition for nutrients and adhesion sits on the gut wall, modulation of the immune response and regulating coloncyte gene expression (Fooks and Gibson, 2002).

A similar finding was obtained by Kingamkono *et al.,* (1994) and Olukoya *et al.,* (1994) who noticed the disappearance of *S. typhimurium* and *Shigella flexneri* from the medium when subjected to *Lactobacillus*. Mishra and Lambert, (1996) also noticed the killing action of the bacteriocins as they bind with the cytoplasmic membrane, effects its permeability, and cause death of the sensitive cell.

Acetic acid and lactic acid are manly produced by LAB; they effect the cytoplasmic membrane and diffuse in the cytoplasm quickly and cause bacterial death (Adams and Nicolaides, 1997 and Ogawa *et al.,* 2001).

3.8.5 Effect of *L. acidophilus* filtarate on invasion of *P. mirabilis*

It was found that probiotic acts as suppressor to pathogenic bacteria, affects as barrier function, antagonism throughout the production of antimicrobial substances (acids, hydrogen peroxide and bacteriocins) and modulation of the immune system. These mechanisms vary according to the specific strain or combination of strains used, the presence of prebiotics (a non-digestible food ingredient which beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon having the potential to improve host health) and the condition that is being treated the patient (Pascual *et al.,* 2009).

L. acidophilus isolate was used to study the effect of probiotic produced by this isolate on virulence factors of *P. mirabilis* P15. By prepared three concentration from this filtrate (5 ml, 10 ml and 15 ml) each volume was added to 20 ml of fresh culture of the of *P. mirabilis* P15 grown in nutrient broth (O.D $_{600}$ about 0.2) and incubated at 35 ºC for 24 hrs.

Three concentration of *L. acidophilus* probiotic were used to determine their effect on the invasion and adhesion of *P. mirabilis* P15. Results indicated in table (3.11) showed that increasing of probiotic concentration decrease the invasion ability of *P. mirabilis* P15 to uroepithelial cells.

The invasion was completely lost when bacterial cells of *P. mirabilis* was treated with two fold and three fold concentrations of the probiotic, while there is only 6 bacterial cells / uroepithelial cell were occurred after the treatment with one fold concentration of probiotic in comparison with 20 cells / uroepithelial cell for untreated *P. mirabilis* P15 (control positive).

Table (3.11): Effect of concentrated filtrates of *L. acidophilus* against virulence factors of *P. mirabilis*

Virulence Factors	Untreated *P. mirabilis*	One-fold con. of *L.acidophilus* filtrate	Two-fold con. of *L.acidophilus* filtrate	Three-fold con. of *L. acidophilus* Filtrate
Invasion (Bacterial cells/ uroepithelial cell)	20	6	—	—
Adhesion (Bacterial cells/ uroepithelial cell)	50	35	15	5
Protease (diameter of clear zone (mm) on skim milk)	14	6	—	—
Specific activity (U/mg)	14.50	9.50	7.04	0.266

Because of prevention of counteract the epithelial cells dysfunction induced by interoinvasive *P. mirabilis*, and the effect of *L. acidophilus* probiotic to induce interruption of the early interactions of *P. mirabilis* to the uroepithelial cells. These results were also described by Mack *et al.,* (1999) who found that part of the beneficial effect of *L. plantarum* and *L. ramnosus* was mediated by induction of mucin genes in intestinal epithelial cells, thus preventing adherence and invasion of enteropathogenic *E coli*.

These findings explain the importance of investigating diversity of host-microorganism interactions when profiling probiotics. Probiotics which display different characteristic phenotypes may be valuable as therapeutic tools under different pathophysiological conditions by preventing invasion and extensive colonization, probiotics considerably reduce the infective load and thus the oxidative stress that is in part responsible for the mucosal inflammation induced by enteric pathogens. Only live probiotics were shown to have beneficial effects on epithelial barrier function when added by themselves, and to prevent a number of deleterious consequences otherwise bring out by infection of the epithelium with an invasive pathogen (Resta-Lenert and Barrett, 2002)

LAB strains that maintain adhesive properties and the ability to colonize the human gastrointestinal tract may hinder the association or invasion between the epithelial cells and the pathogenic bacteria (Hudault *et al.,* 1997; Coconnier *et al.,* 2000; Jankowska *et al.,* 2008).

3.8.6 Effect of *L. acidophilus* filtarate on adhesion of *P. mirabilis*

The initial step in the infection of host cells by *P. mirabilis* P15 is the adhesion step, so interference with adhesion process cause a prevention of infection (Mobley and Chippendle, 1990).

Results indicated in table (3.11) also showed that the adherence ability of *P. mirabilis* P15 to uroepithelial cells was also decreased after treatment of bacterial cells with *L. acidophilus* probiotic in different concentrations. The adhesion ability of *P. mirabilis* P15 was decreased to 35, 15 and 5 bacterial cell \ uroepithelial cell after treatment with one, two and three concentrations of probiotic respectively.

Results mentioned in figure (3.8) confirmed these findings. It was show that adhesion of bacterial cells of *P. mirabilis* P15 to uroepithelial cells were decreased in comparison with the adhesion ability of untreated cells.

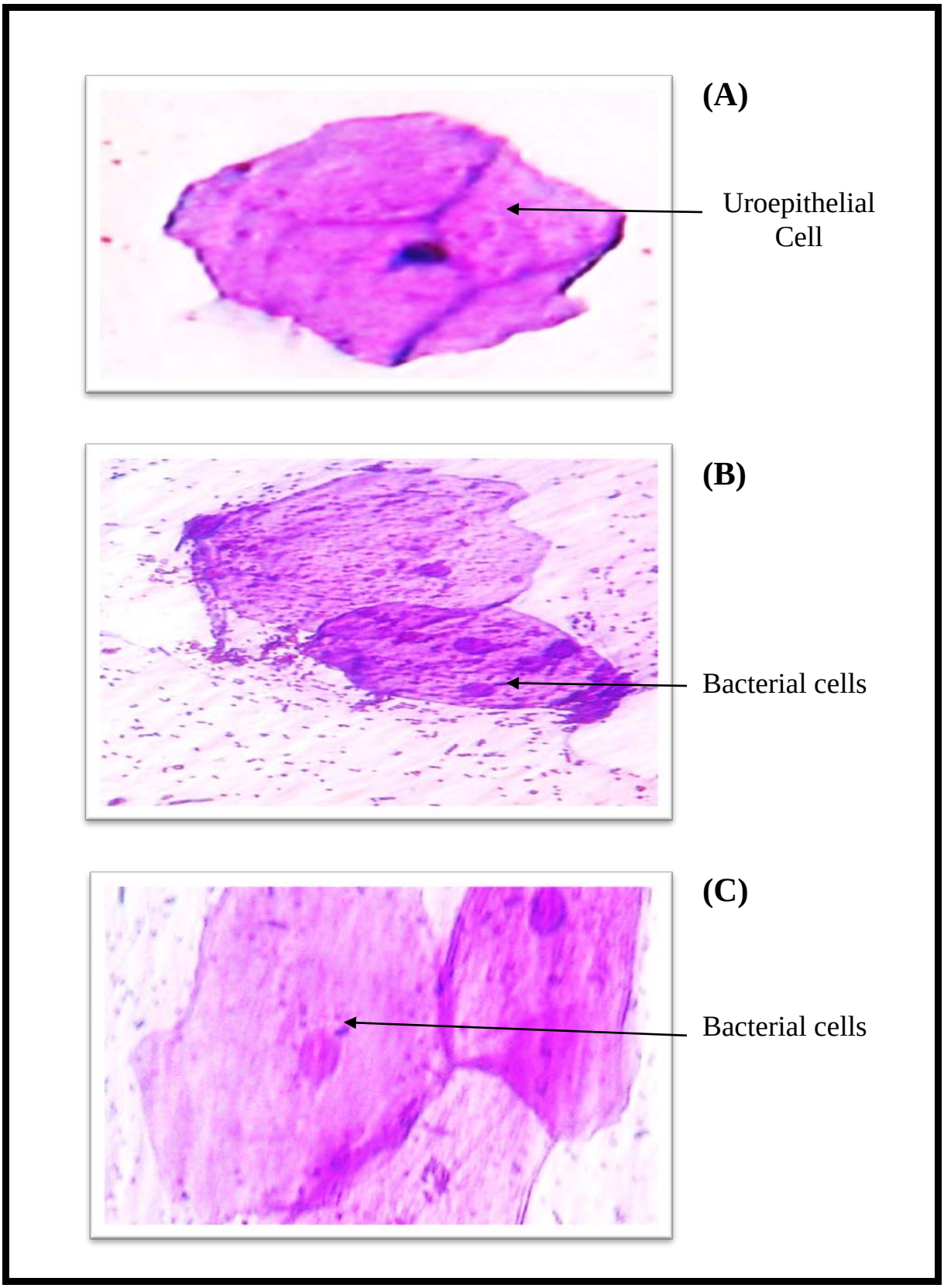

Figure (3.8): Effect of *L. acidophilus* filtrate on adhesion ability of *P. mirabilis* P15 to uroepithilial cells. (A): uroepithileal cell (control negative); (B): Adhesion of bacterial cells without treatment with probiotic (control positive); (C): Adhesion of bacterial cells after treatment with probiotic

This was may be due to the effects of inhibitory substances found in the filtrates of the *L. acidophilus* such as H_2O_2, bacteriocins and organic acids in addition to the acidic pH of probiotic which affects growth of gram negative bacteria by altering some surface structures (like pili), leading to prevent bacterial cells from adhesion to UEPCs without killing the bacteria (Michail *et al.,* 2003).Other studies reported that partial and complete inactivation of adherence of several gram negative uropathogenes.

On other hand, the inhibitory effect of probiotic of *Lactobacillus casei* on *E. coli* was examined and it was found that the inhibitory effect was not caused by the bacteriophage or hydrogen peroxide but due to the coaggregation of *E. coli* and *L. casei* in urine which was occurred after 20 hr at 37ºC (Reid *et al.,* 1990).

The prevalence of inhibitory-producing LAB on the uropathogenes and the ability of LAB to interact closely with the uropathogenes seem to constitute an important host defense mechanism against infection. Probiotics microorganisms compete with pathogens for nutrients and physical space (Johannsen, 2003).

Velraeds *et al.,* (1996) reported that precoating of LAB strains reduced the binding of uropathogenic coagulase-negative *Staphylococci* and *E. coli* to 8 bacteria/cell, So biosurfactant surlactin as released by *Lactobacillus* isolates may open the way to the development of antiadhesive biologic coating against *Enterococcus faecalis*, they reported a decrease in the percent of adhering *Enterococcus* which was reach to approximately 70%.

Some bacteria can inhibit and prevent the colonization by pathogenic microorganisms by means of a mechanism of strict obstruction or blocking of specific receptors . *L. plantarum* have been shown to competitively inhibit the attachment of enteropathogenic *E. coli* 0157H7 to HT-29 human colonic cancer cells (Michail *et al.*, 1997; Alvarez-Olmos and Oberhelman, 2001).

3.8.7 Effect of *L. acidophilus* filtarate on Protease Production of *P. mirabilis*

Protease is another virulence factor of *P. mirabilis*. effect of three concentrations of *L. acidophilus* probiotic on protease production by *P. mirabilis* was studied. Results indicated in table (3.11) showed that ability of protease production was decreased with the increase of probiotic concentration according to the decrease of zone of hydrolysis on skim milk agar plates to 6 mm after treatment of bacterial cells with one fold concentration of probiotic in comparison with 14 mm for the untreated cells, while there was completely inhibition of protease (no hydrolysis zone) on skim milk agar plates after treatment of bacterial cells of *P. mirabilis* with two and three fold concentration of probiotic.

Results mentioned in table (3.11) also showed that the specific activity of protease produced by *P. mirabilis* was decreased to 9.5, 7.04 and 0.26 U / mg Protein after the treatment with *L. acidophilus* probiotic with the concentration of one fold, two fold and three fold respectively.

Lactic acid bacteria have an important role in the inhibition of food-borne pathogenic and spoilage microorganisms with antimicrobial metabolites, including lactic acid, acetic acid, and other organic acids, hydrogen peroxide, bacteriocins and bacteriocin-like substances. Lactic acid and acetic acids cause a reduction in pH , hydrogen peroxide is a non-stable thermodynamic compound and destroys bacterial enzymatic activity (Juven *etal*., 1992).

Hydrogen peroxide (H_2O_2) is produced by most LAB in the presence of oxygen. LAB are unable to produce catalase; therefore, they cannot degrade hydrogen peroxide that, after accumulation, develops its oxidative properties with the production of powerful oxidants such as singlet oxygen, superoxide radicals, and the hydroxyl radical. Reactive oxygen species can cause irreversible damage to a number of cell components such as enzymes, membrane constituents and DNA (Schurman, 2001; Strus *et al.*, 2005).

Chapter Four

Conclusions and Recommendations

4. Conclusions and Recommendations

4.1 Conclusions

1. Local isolates of *P. mirabilis* are able to produce different virulence factors such as invasion and adhesion production and protease production with variable degrees.
2. Local isolate of *P. mirabilis* harboring two plasmids responsible for antibiotic resistance.
3. Virulence factors of *P. mirabilis* are chromosomally encoded, while many antibiotic resistance genes were located on plasmid DNA and the other were chromosomally encoded.
4. Probiotic of *Lactobacillus acidophilus* has effective role in decreasing the ability of production virulence factors of *P. mirabilis*.

4.2 Recommendation

1. Study the genetic polymorphisim of local isolates of *P. mirabilis* by using polymerase chain reaction.
2. Cloning of *P. mirabilis* P15 protease gene into *E.coli* or other protease producing bacteria for large scale production of this enzyme.
3. Extraction, purification and characterization of inhibitory substances produced by the probiotic LAB isolates.
4. Investigating the effect of LAB inhibitory substances against other virulence factors of *P. mirabilis* such as urease or swarming.
5. *In vivo* studies about the effect of LAB isolates against *P. mirabilis*.

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Appendix

Appendix I: The biochemical reaction performed by the API 20E and their interpretation

Test	Substrate	Enzyme reaction	Result	
			Positive	Negative
ONPG	Ortho – nitro Phenyl-galactosidase	Beta-galactosidase	Yellow	Colorless
ADH	Arginine	Arginine dehydrolase	Red-Orange	Yellow
LDC	Lysine	Lysine decarboxylase	Orange	Yellow
ODC	Ornithine	Ornithine decarboxylase	Red- Orange	Yellow
CIT	Sodium citrate	Citrate utilization	Blue-Green / Green	Pale Green / Yellow
H2S	Sod-Thio-ulphate	H_2S production	Black Deposite	Colorless / Grayish
URE	Urea	Ureas	Red-Orange	Yellow
TDA	Tryptophane	Tryptophane deaminase	Dark Brown	Yellow
IND	Tryptophane	Indol production	Red Ring	Yellow Ring
VP	Sodium pyruvate	Aceton production	Pink / Red	Colorless
GEL	Gelatin	Gelatinase	Diffusion of Black Pigment	No diffusion
GLU	Glucose	Fermentation oxidation	Yellow	Blue/Blue-Green
MAN	Mannitol	Fermentation oxidation	Yellow	Blue/Blue-Green
INO	Inositol	Fermentation oxidation	Yellow	Blue/Blue-Green
SOR	Sorbitol	Fermentation oxidation	Yellow	Blue/Blue-Green
RHA	Rhamnose	Fermentation oxidation	Yellow	Blue/Blue-Green
SAC	Sucrose	Fermentation oxidation	Yellow	Blue/Blue-Green
MEL	Melibiose	Fermentation oxidation	Yellow	Blue/Blue-Green
AMY	Amygdalin	Fermentation oxidation	Yellow	Blue/Blue-Green
ARA	Arabinose	Fermentation oxidation	Yellow	Blue/Blue-Green

Appendix (2): Biochemical tests for identifying local isolates of *P. mirabilis* using Api 20E system.

Isolate / Symbol / Test	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
ONPG	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ADH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LDC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ODC	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CIT	-	+	+	-	-	-	-	-	+	+	+	+	+	-	+
H_2S	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
URE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TDA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
IND	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	-	+	+	-	+	+	+	-	+	+	+	+	-	+	+
GEL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GLU	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MAN	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
INO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RHA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MEL	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ARA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

(-): Negative results, (+): positive results

حيث تم اولا إعادة تشخيص عزلة محلية من بكتريا *Lactobacillus acidophilus*، ثم الحصول على المعزز الحيوي من المزرعة البكتيرية النشطة لدراسة تأثيره التثبيطي على العزلة البكتيرية *P. mirabilis* P15. وقد أظهرت النتائج وجود تأثير كبير للمعزز الحيوي لبكتريا *L. acidophilus* على عوامل الضراوة حيث اختزلت قابلية اختراق الخلايا الطلائية والالتصاق بها وإنتاج إنزيم البروتييز للعزلة البكتيرية *P. mirabilis* P15، وتزداد كفاءة التأثير بزيادة تركيز المعزز الحيوي.

الخلاصة

لعزل بكتريا *Proteus mirabilis* فقد جمعت ٨٥ عينة من مصادر مختلفة في محافظة بغداد شملت، ٢٠ عينة إدرار و ٤٥ عينة جروح و ٢٠ عينة لحوم حمراء وبيضاء (دجاج).ومن المجموع الكلي لتلك العينات فقد تم الحصول على١٤٦عزلة بكتيرية تم تشخيصها وفقا لخصائصها المظهرية والمجهرية والكيموحيوية، وقد اشارت نتائج هذه الاختبارات الى ان ١٥ عزلة من بين العدد الكلي لتلك العزلات كانت *P. mirabilis* وقد تم تأكيد تشخيصها باستخدام العدة التشخيصية Api-20E.

درست امراضية عزلات بكتريا *P. mirabilis* وذلك بالتحري عن عوامل الضراوة لتلك العزلات والتي شملت عوامل الالتصاق والاختراق وقابليتها على إنتاج إنزيم البروتييز. وقد أظهرت النتائج قدرة تلك العزلات على الالتصاق بالخلايا الطلائية بمدى يتراوح بين ٣٠ و ٥٥ خلية بكتيرية/خلية طلائية، وقابليتها على الاختراق بمدى يتراوح بين ١ و ٢٠ خلية بكتيرية/خلية طلائية، فضلا عن قابليتها على إنتاج إنزيم البروتييز بفعالية نوعية تراوحت بين ٠,١٢ و ١٤,٥وحدة/ملغم بروتين.وقد تميزت من بين تلك العزلات البكتيرية العزلة *P. mirabilis* P15 المعزولة من عينة الادرار بضراوتها العالية لقدرتها العالية على إنتاج عوامل الضراوة.

اختبرت حساسية العزلة *P. mirabilis* P15 ضد ١٥ نوع من مضادات الحياة، وقد أظهرت النتائج أن هذه العزلة كانت مقاومة للتتراسايكلين والسيوفوتاكسيم والسيفبيم والاميكاسين والسيبروفلاكسين، بينما كانت حساسة لبقية المضادات المدروسة.

درس المحتوى البلازميدي للعزلة *P. mirabilis* P15 باستخلاص الدنا البلازميدي بطريقة التحلل القاعدي. وقد أظهرت نتائج الترحيل الكهربائي على هلام الاكاروز امتلاك هذه العزلة بلازميديين مختلفين في الحجم يشفران لصفة المقاومة للسيفوتاكسيم والاميكاسين وفقا لنتائج تحييد الدنا البلازميدي باستخدام مادة بروميد الاثيديوم.

تم التركيز في الجزء الاخر من هذه الدراسة على تأثير المعزز الحيوي (probiotic) للبكتريا حامض اللاكتيك على نمو وعوامل ضراوة العزلة *P. mirabilis* P15 .

بسم الله الرحمن الرحيم

اقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ (١) خَلَقَ الإِنسَانَ مِنْ عَلَقٍ (٢)
اقْرَأْ وَرَبُّكَ الأَكْرَمُ (٣) الَّذِي عَلَّمَ بِالْقَلَمِ (٤) عَلَّمَ الإِنسَانَ
مَا لَمْ يَعْلَمْ (٥)

صدق الله العظيم

سورة العلق

الآية (١-٥)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الإحيائية

دراسة بكتريولوجية على *Proteus mirabilis* بعد تعريضها للمعززات الحيوية

رسالة

مقدمة إلى مجلس كلية العلوم/جامعة النهرين كجزء من متطلبات نيل درجة الماجستير في العلوم/ التقانة الإحيائية

من قبل

تقى حسون علي

بكالوريوس تقانة إحيائية / جامعة النهرين (٢٠١١)

بأشراف

أ.د. حميد مجيد جاسم

ربيع الأول ١٤٣٥ كانون الثاني ٢٠١٤