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Effect of Probiotic on Motility Factors and Swarming Phenomenon of *Proteus mirabilis*

A thesis

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By

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بسم الله الرحمن الرحيم

فَلَولا فَضلُ اللهِ عَلَيكَ وَرَحمتُهُ لَهَمَّت طَائِفَةٌ مِنهُم ان يُضلوكَ وَمَا يُضِلونَ اِلاَّ انَفُسَهُم وما يَضُرونَكَ مِن شَيء وانزَلَ اللهُ عليكَ الكِتَبَ والحكمَة وَعَلَّمَكَ مَالَم تَكُن تَعَلمُ وَكَانَ فَضَل اللهِ عَلَيَكَ عَظيماً

صدق الله العظيم

سورة النساء {١١٣}

الاهداء

الى

من ارسله الله قدوةً ومعلماً ورحمة للعالمين ... سيدنا محمد (صلى الله عليه وسلم)...

من قضى بهما رب العزة احسانا والديّ الكريمين الذين غرسوا في نفسي الطموح والاصرار...

من اشدد بهم ازري واشركهم في امري ...اخوتي...

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من رافقتني في دربي... غيداء...

الى كل من مدّ لي يد العون والمساعدة الى كل هؤلاء، اهدي ثمرة جهدي المتواضع...

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Reem

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

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Summary

- A total of 140 samples of urine were collected from patients suffering from urinary tract infections (UTI) in Al-Yarmouk and Al-Karama hospitals in Baghdad. Samples were cultured (spreading) on MacConkey agar and blood agar and incubated at 37ºC for 24 hr. Results showed that 89 samples gave positive culture for bacteria, and the primary identification led to detect 20 isolates belong to genum *Proteus*. Occurrence of such isolates was highest in males 70% than in females 30%. Further identification declared that 75% of the isolates were belonged to *Proteus mirabilis* and 25% to *Proteus vulgaris*. Isolates of the first species were chosen for further experiments.
- When sensitivity of the *Proteus mirabilis* 15 isolates were tested against 13 commonly used antibiotic, results showed the ciprofloxacin was the most effective antibiotics against the isolates when resistance percentage of isolates was only 6.6%. While penicillin G, on the other hand, was the least effective one when 93.3% of the isolates resisted it.
- Depending on the antibiotic sensitivity results, one isolate of *Proteus mirabilis* PR15 was selected due to its multi-resistance of antibiotics, to be used for detecting its important virulent factors, namely swarming on the solid medium and motility in the semi-solid medium.
- The yeast *Saccharomyces boulardii* was used after insuring its sensitivity to Cycloheximide to detect its ability in producing inhibitory materials against *Proteus mirabilis* isolate, as well as its effect on the swarming and motility properties of the bacteria. Moreover, ability of the bacteria cells to adhere to the yeast cells was also investigated to avoid bacterial adhesion to the host cells.
- The yeast was propagated in two locally prepared media [dates extract broth (DEB) and dried tea leavies extract broth (DTLEB)] after enriched them with the other necessary nutrients. Growth of the yeast was also

compared in two commercially used artificial media [sabauroud broth (SB) and yeast extract glucose peptone broth (YEGPB)]. To obtain best inhibitory effect, the yeast was propagated into four different liquid media, two ready to use and others locally prepared and two solid ready to use media. Results showed that liquid media were more efficient in exhibiting inhibitory activity of the yeast than the solid media when the first dilution concentrated of liquid media inhibited motility and swarming of the bacteria.

- To improve ability of the yeast in exhibiting inhibitory activity, various media, pH values, temperature and period of incubation were applied. Best activity was obtained when the yeast was propagated, separately, in sabauroud broth (SB) and yeast extract glucose peptone broth (YEGPB) media with pH 4.5 for 48 hr. at 37ºC. But in dates extract broth (DEB) and dried tea leavies extract broth (DTLEB) media with pH 5.5 under same other conditions.
- Minimum inhibitory concentrations (MIC,s) of yeast first diluted concentrated filtrates was determined in each of the four medium used, as well as its values against swarming and motility properties of *Proteus mirabilis* isolate. Results show that the filtrate totally inhibited swarming of the bacteria on the solid medium, and its motility in the semi-solid medium.

List of Contents

List of Tables

List of Plates

List of Abbreviations

Abbreviation	Mean
UTI	Urinary Tract Infection
TSI	Triple Sugar Iron
PAD	Phenylalanine Deaminase
LB	Luria-Bertonia
SB	Sabauroud Broth
YEGPB	Yeast Extract Glucose Peptone Broth
DEB	Dates Extract Broth
DTLEB	Dried Tea Leaf Extract Broth
MIC,s	Minimum Inhibitory Concentration
PR15 Isolate	Proteus mirabilis 15 isolate (used in experiment)
PBS	Phosphate Buffer Saline

CHAPTER ONE

Introduction and Literature Review

Chapter One Introduction and Literature Review

1.1 Introduction

Enterobacteriacea family contains several bacterial genera of health concern. Genus *Proteus* is one of these genera which are known through its two pathogenic species, *Proteus mirabilis* and *Proteus vulgaris*, to cause most of the common hospital-acquired infections of complicated urinary tract (Senior, 1997).

Proteus mirabilis possesses several virulence factors which enable it to be the major cause of urinary tract infection all around the world. One of the important pathogenic infectivity of this bacterium is swarming and motility ability among infected tissues.

During swarming, *Proteus mirabilis* co-ordinately upregulates expression of flagellar gene hierarchy and production of several virulence factors including the haemolysin HmpA, urease and Zap protease (Zunnio *et al*., 1999).

Several investigators and specialists are concerned now on naturally producing means to treat pathogenic causes of various diseases and infections to avoid the side effects and microbial resistance of most commonly used antibiotics and other chemotherapeutic agents. Probiotics are some of such means through the use of some secure microorganisms to retard the pathogenic bacteria.

Saccharomyces boulardii, a non-pathogenic yeast is now used as a probiotic microorganisms which administrated in Western Europe. *In vitro* studies have demonstrated that *Saccharomyces boulardii* exerts antagonistic activity against various bacterial pathogens (Czerucka *et al*., 2000).

Aims of the Study:

- Isolation and characterization of pathogenic *Proteus mirabilis* isolates from urinary tract infection patients.
- Detection of its swarming and diffusion (Motility) activity.
- Detecting the most antibiotics-resistant isolate of *Proteus mirabilis* to be used in the probiotic experiment.
- Detection the inhibitory ability of *Saccharomyces boulardii* against the isolate of *Proteus mirabilis*.
- Determining the minimum inhibitory concentrations of yeast filtrates against swarming and motility of *Proteus mirabilis*.

1.2 Literature Review

1.2.1 Genus *Proteus*:

The variable appearance of *Proteus* bacilli is related to the swarming phenomenon expressed by them. Moreover, this ability seems to play a very important role not only in their colonization on solid surface, but also in the pathogenicity of these bacteria and characterizing it from other members of the family. *Proteus* is rod, motile, gram negative bacilli. They are straight or slightly curved rod cells (0.5-1.0 μm in width) by (1.5-4 μm in length).

According to Bergy's Manual, *Proteus* consists of four species: *Proteus vulgaris*, *Proteus mirabilis*, *Proteus penneri* and *Proteus myxofaciens*, most of them are urease positive and phenylalanine deaminase positive.

The species which encountered in clinical bacteriology is *Proteus mirabilis* (Alder *et al*., 1971; Robert *et al*., 1975).

Larsson in (1978) found that *Proteus* cause urinary tract infection among young boys when as urinary tract infection caused by *Escherichia coli* are more common in girls. Species, such as *Proteus mirabilis*, *Proteus vulgaris* and *Proteus penneri*, under favorable conditions are able to cause pathological problems such as nosocomial infections, wound infections and otitis (Dutton and Ralston, 1957; Chow *et al*., 1979). This could result in auto-infection or transmission of the bacteria from patients in hospital (Chow *et al*., 1979).

First description of the urinary tract infection (UTI) and renal stones was made by Abu-Bakir Al- Razi (known also as Al-Arab Galineous) in the ninth century (A.C.) in Baghdad (Asccher, 1980).

Symptoms of UTI are frequently urination, flank, pain, dysuria, burning while urination and some time fever. In general bacterial infection of the urinary tract is the commonest cause of both community acquired and nosocomial infection in patients admitted to hospital which is occurred secondly after the respiratory tract infections (Shanson, 1982).

First description of *Proteus* as putrefying bacteria was made by Hauser in 1885. *Proteus* have the ability to transform itself into many shapes (Hawkey *et al*., 1986).

Urinary tract infection caused by species belong to *Proteus* are known to be frequently persistent, difficult to treat and even fatal depending on severity of illness in particular patients. The complication of infection in catheterized and non-catheterized patients include the development of urolithasis, urinary tract obstruction, obstruction of urinary catheters, bladder and kidney stones formation and bacteriuria (Scott, 1960; Warren *et al*., 1982; Krajden *et al*., 1987).

Bacteria belonging to the genus *Proteus* represent particular groups of microorganisms belonging to the family Enterobacteriaceae (Brenuer *et al*., 1978; Penner, 1984; Penner, 1992).

Most *Proteus* isolates are free living saprophytes in soil, vegetation, water, sewage and in the intestine in many healthy persons, they occur also in the urinary tract infections, wounds and other sits (Penner, 1992).

Proteus bacilli when present in the kidneys, could cause sever histological damage which is characterized as a cute pylonephritis (Engler *et al*., 1992).

However, *Proteus mirabilis* is the most frequently cause of urinary tract infection in patients with urinary catheters (Warren *et al*., 1987) or with structural abnormalities (Benz *et al*., 1994).

Recommendation on the diagnosis and treatment of this common infection vary according to patient's age and sex, level of risk of infection with resistant pathogens, previous response to therapy, the use of a urinary catheters and pregnancy (Warren, 1996).

Morphologically they are non-fermentative, non-capsuling, non-sporing, faculitative anaerobic, filamentous rods, highly motile and flagellated. Culture characteristics include fishy smell and swarming appearance when grown in non-inhibitory solid media such as nutrient agar and blood agar (Rozalski *et al*., 1997).

Proteus mirabilis is characterized as the most common pathogen which may be explained by its high carriage rate (25%) in human intestine (Chow *et al*., 1979; Rozalski *et al*., 1997).

In general, infections caused by *Proteus* could be subdivided into two categories: hematogenous infections (also Known as systematic infections) and ascending infections in which bacteria colonize step by step the introits, bladder, ureter and at the end, the kidneys (Belas *et al*., 1999).

UTI is characterized by the multiplication rate of microorganisms (mainly bacteria) in any segment of the urinary tract (Connolly and Thorp, 1999).

1.2.2 Virulence Factors of *Proteus mirabilis*:

1.2.2.1 Surface Structures.

Flagella can be detected serologically under electron microscope as well as by Leifson's staining. *Proteus* cell has many flagella which are estimated in

thousands. Such flagella as distributed all over the bacterium surface; a type of flagella arrangement, called peritrichous (Morello *et al*., 1991).

Flagella are the organs of locomotion, their presence can be detected by observing the motility of organism either by a hanging drop preparation or by the spread and movement of bacteria over or through the surface of agar (Morello *et al*., 1991). The flagellar components are known to be sequentially assembled from the cytoplasmic membrane (Shapiro, 1996).

Surface structures, such as flagella and fimbria, of *Proteus*, as a gram negative bacteria, play significant role in causing in the pathogenicity of such bacteria against higher organisms. It's believed that, in particular, the cell surface of *Proteus* is very important to its virulence, particularly their motility, adherence ability, colonization of the urinary tract and formation of stones (Jawetz *et al*., 1998).

A flagellum consists of three parts: the filament, the hook and basal body. The hook basal body structure is embedded in the cell envelope, where as the filament is the only part which is external. The hook and basal body carry different antigens, while filament is composed of a protein called as flagellin which has the power to contract just like myosin but without ATPase activity. The flagellin monomer has a molecular weight of 40,000 daltons and can aggregate to form polymers (Morello *et al*., 1991). Flagellin being the last and major flagellar protein expressed (Senesi *et al*., 2002).

1.2.2.2 Swarming.

The mode of surface translocation, swimming and swarming depend on flagella (Heierichsen, 1972).

The products of large number of genes are believed to take part in the swarming process (Sidorezyk and Zych, 1986).

and Literature Review

Swarmer-cell differentiation and swarming motility are the result of at least four separate phenomena including; the ability to sense cues from the environment; the production of an elongated swarmer cell, the synthesis of vastly increased amount of flagellin, and the coordination multicellular interaction that result in cycle waves of cellular differentiation and dedifferentiation (Belas, 1992).

A defect in any of these events results in either abnormal swarming or a complete lack of motility (Belas, 1992).

Differentiation requires chemical signals as well as the physical signals derived from the inhibition of flagellar rotation. Glutamine is the major extracellular signal which is sensed by a specific transduction mechanism that is independent on the cellular and nutritional amino acid uptake system (Belas, 1992).

Present data showing that ability to invade host urothelial cells is closely coupled to swarming. Differentiated filaments entered urothelial cells within 30 min and were 15 fold more invasive than vegetative cells isolated before differentiation (Allison *et al*., 1992).

Proteus is dimorphic bacilli bacteria. When grown in liquid medium, these cells displaying swimming behavior and have a distinct morphology these bacilli referred to as swimmer cells. When transferred to a solid medium, they undergo morphogenesis and swarm over the surface of the solid medium. Such growth is termed as "swarming phenomenon". Swarming as a form of a bacterial movement across the solid surface of artificial or nutural media. They are differentiation as short rods into a septate, multinucleated by 50 to 500 fold increase in the number of nucleoids of the swarmers in proportional to the increase in the length. It was found that a 40 μm swarmer cell has about 20 chromosomes (Gygi *et al*., 1995).

Furthermore, defects in chemotaxis have been shown to result in the abnormal swarming, implicating other metabolites in swarmer-cell differentiation and swarming behavior. Swarm cells are different from normal cells. They produce more virulence factors such as toxins, that lyse red blood cells and capsule that the bacteria must produce in order to swarm (Rahman *et al*., 1999).

A strong correlation was found between the ability to secrete protease. However, some isolates, particularly of *Proteus vulgaris* were non-proteolytic even when they formed swarming growth (Senior, 1999).

Characterization of non swarming mutant has firmly established that all defects in genes encoding subunits of the flagellar export apparatus completely abolish swarming differentiation (Young *et al*., 1999; Haughes, 2002).

The relationship between swarming and virulence has been investigated mostly in gram-negative bacteria (Allison *et al*., 1994; Ederl *et al*., 1996), although several species belonging to the *Bacillus* and *Clostridium* genera have been described to be active swarmers (Henrichsen *et al*., 1972; Macferlane *et al*., 2001; Senesi *et al*., 2002).

Swarming occurred at a wide range of temperatures with optimum being between 25 and 38 °C (Senesi *et al*., 2002).

The swarming process is very critical to the virulence of *Proteus mirabilis* because the expression of virulence determinants such as urease, hemolysin and the IgA metalloprotease which are specific to the swarming bacteria (Sturgill *et al*., 2002).

The relationship between virulence and motility may be demonstrated in the following, in some microorganisms, a substantial increase in the secretion of virulence factors is associated with a specialized form of flagellum-driven motility, the swarming motility that enable bacterial cells to collectively move

across solid surfaces (Allison *et al*., 1992; Eberl *et al*., 1996; Macfarlene *et al*., 2001; Senesi *et al*., 2002).

Recently, transposon-generated mutants lacking flagella have been observed to lose the ability to invade cultured uroepithelial cell and that flagellated non-swarming vegetative cells were also reduced invasive ability, and that flagella are necessary but not sufficient for the ability of *Proteus mirabilis* to invade (Allison *et al*., 1994; Ghelardi *et al*., 2002).

1.2.2.2.1 Regulation of Swarming Motility

Proteus swarmers over solid surfaces and swim through water-filled channels in the presence of iron, and these motilities can be inhibited by heavy metal ions, iron chelators, some chemical materials like sodium azide, boric acid (1:1000), alcohol 6%, phenyl-ethylalcohol, chloral-hydrate or by using Mac-Conkey medium which contain bile salt and Luria agar contain 2% agar-agar and 0.5% of glycerol. Swarming also is inhibited by using some antibiotic like ampicillin, tetracycline and kanamycine and specific osmotic agents.

One of the methods used by *Proteus* to facilitate surface locomotion is production of a polysaccharide slime which acts to reduce surface friction (Wilkerson and Niederhaff, 1995).

Daytor *et al* (1996) observed that swarming motility is dioxygen dependent while swimming motility depends on the respiration. Invasion of urethelial cells, which line the urinary tract, is being modeled in the laboratory with the use of synthetic basement membrane protein matrices.

1.2.2.2.2 Dienes Phenomenon

The genetic basis of the *Proteus mirabilis* swarming phenomenon was studied using transposon mutagenesis. The transponson Tn.5 was used by Belas *et al* (1991) to obtain mutants defective in swarming motility.

When two cultures of *Proteus* cell coming from the same origin are inoculated at different positions on a plate containing enriched medium, the resulting growing swarms coalesce without signs of demarcation. However, when two different strains of coalesce remain separated this is known as the "Dienes Phenomenon" which has been used to determine the identity or non-identity of strains by the epidemiologist (Belas, 1992).

Allison *et al* (1992) have showed that in addition, swarming *Proteus mirabilis* was also exhibited a substantial increase in the activity of intracellular urease, extracellular hemolysin and metalloprotease. Expression of the genes encoding the synthesis of these pathogenic factors is coordinated in swarmer cell.

The ability to invade human uroepithilial cells by *Proteus mirabilis* swarmers was described for the first time by Allison *et al* (1992). Quit recently, they showed that swarming differentiation occurs *in vivo* and that the differentiated cells are the virulent from of *Proteus mirabilis*.

1.2.2.3 Urease.

The role of urease in infection has been studied by several authors (Braude and Siemienski, 1959; Maclaren *et al*., 1968; Maclaren, 1969; Musher *et al*., 1975) and considered as significant virulence factor especially in *Proteus mirabilis* strains.

The enzyme has also been implicated as a factor contributing to the pathogenicity of many bacteria including *Proteus*, *Providencia* and *Morganella* species (Magana-Plaza *et al*, 1967; Penner *et al*, 1967; Senior *et al*., 1980; Rosenstein *et al*., 1981; Jones *et al.*, 1987).

Urease activity of these bacteria is used to distinguish them from other members of the family Enterobacteriaceae (Mobley *et al.*, 1989).

Urease activity has been found in over 200 species of gram-negative and gram-positive bacteria (Griffith *et al*., 1976; Mclean *et al*., 1988; Mobley *et al*., 1990; Mobley *et al*., 1995).

Urea represents main nitrogenous excretory product in human and animals. Urease (urea amido hydrolyase) catalysis the hydrolysis of this compound to yield ammonia and carbon dioxide, which result in an increase in the urine pH (Merlin *et al*., 1988; Mobley *et al*., 1989; Mobley *et al*., 1995).

1.2.2.4 IgA and IgG Proteases.

The most characteristic feature of *Proteus mirabilis* IgA proteases is the cleavage (Immunoglobulin) of Ig heavy chain outside the hing region (Senior *et al*., 1987).

Moreover, Allison *et al* (1992) stated that proteases were also used indirectly, in the demonstration of the differentiation of *Proteus mirabilis* short vegetation rods into filamentous multinucleate and hyperflagellate swarmer cells.

IgA in the form of secretary protein (SIgA) is a dimmer of IgA held by the J-chain and contains the secretary component used to transport the antibody molecule. This SIgA is the predominant Ig in mucus secretion. Its function is to protect mucous membrane and underlying tissue from bacteria and their product, SIgA is resistant to degradation by proteolytic enzymes of many microorg-anisms. However, only a few microbes have been found to synthesize extracellular proteolytic enzymes capable of degrading IgA (Senior *et al*., 1997).

1.2.2.5 Hemolysins.

Emady *et al* (1982) described the relationships between the production of extracellular hemolysin and virulence of *Proteus mirabilis*.

Synthesis of the cytotoxic hemolysis is common among both gram-negative and positive-bacteria. The history investigation of *Proteus* hemolytic activity is very long and starts from beginning the twentieth century. When Wenner and Rettager were unable to find this activity in 1919 whereas Taylor in 1928 observed lysis of erythrocytes in young broth culture of *Proteus* (Mobley *et al*., 1995).

1.2.2.6 Invasiveness.

Invasion of mammalian cells by *Proteus* could be stimulated by urea (Bruade and Siemienki, 1959).

A correlation between invasion and cell associated hemolytic activity with penetration was also observed (Peerbooms *et al*., 1984).

However, others indicated that all *Proteus mirabilis* and *Proteus vulgaris* strains invested, including those isolated from patient with urinary tract infection as well as those from healthy persons, were able to penetrate vero cell (the African green monkey kidneys cells) (Peerbooms *et al*., 1984; Peerbooms *et al*., 1985).

Cell invasiveness, termed cell penetration, is an important step in infection, and has been investigated widely (Peerdooms, 1984; Finaly and Falkow, 1989; Finlay, 1990).

The ability to invade Vero cells as found closely coupled to the swarming phenomenon. It was also shown that invasion by swarmer cells occurred within invasion by vegetative cells, which were internalized more slowly. The cell penetration by swarmers was complemented by over production of protease, urease and cell-surface bound hemolysin (HpmA) (Allison *et al*., 1992).

1.2.2.7 Outer Membrane Proteins.

Several proteins have been characterized in *Proteus mirabilis* outer membrane proteins (OMP) posses immunogenic properties and mitogenic activity for B-cell (Besslor and Henning, 1979).

1.2.2.8 Resistant to Normal Serum.

Proteus mirabilis has the ability to resist to the normal serum (Zunino *et al*., 1999), and it is able to persist in the blood circulation and resistance the lysis by the complement. This resistance is due to the presence of the O-side chains of bacterial LPS. Such virulence factor plays an important role in *Proteus mirabilis* invasiveness and causing bacteremia (Jawetz *et al*., 1998).

1.2.3 Antibiotics Sensitivity of *Proteus mirabilis*:

Many criteria are considered for choice of drug for treatment of urinary tract infection includes; the drug is active against the infecting organisms, non-toxic, the tissue concentration obtained, the effect of pH and possess no or little effect on normal flora of intestine and other regions (Glauser, 1986).

Antibiotic acts on one of the eight essential mechanisms for life of microorganism (Egorov, 1985).

They act as;

A. Inhibiting synthesis of the cell wall such as penicillins.

B. Causing membrane dysfunction such as gramacidins.

C. Inhibiting selectively the synthesis of nucleic acids such as actinomycin.

D. Inhibiting the synthesis of protein such as erythromycin.

E. Inhibiting the synthesis of pyrimidines and purine such as sarcomycin.

F. Inhibiting respiration such as oligmycin.

G. Inhibiting oxidation phosphorylation such as tyrocidins.

H. Having antimetabolic properties.

There are different mechanisms by which microorganisms might exhibit resistance to drug. Some of them are by; production of enzymes that destroy the active drug, changing their permeability to the drug, developing an altered structural target for the drug, and finally, metabolic pathway that microorganisms produce an altered enzyme for continuation to perform its metabolic function but is much less affect by the drug (Jawtz *et al*.,1998).

β-lactam antibiotics effect on the cell wall of sensitive bacteria by inhibiting the enzyme that involve in the formation of peptidoglycan layer which is the main constituent of bacterial cell wall (Duguid *et al*., 1978).

Ampicillin and amoxicillin are active against *Proteus mirabilis* and *Escherichia coli* and this don't mean that it is not sensitive to β-lactamase enzyme which lyses it (Cercenado *et al*., 1990).

Amoxicillin is similar to ampicillin but is better absorbed, less making diarrhea and also give high blood level (Murphy *et al*., 1985).

Pipracillin is a reliable therapy for complicated, non-complicated, and community or hospital acquired urinary tract infection (Sifuentes *et al*., 1996).

The resistance of bacteria to β-lactam is due to the degradation of drug by β-lactamase which is produce by bacterial cell and lack or altered penicillin binding protein (PBP) which is drug receptors on the cell wall and some of these are enzymes involve in transpeptidation (Jawetz *et al*., 1998). β-lactamase inhibits the activity of β-lactam antibiotics by breaking the β-lactam ring of the antibiotic (Arakwa *et al*., 1989).

β-lactamase is encoded by chromosome or plasmid (Wiedemann, 1990).

All aminoglycosides antibiotics inhibit protein synthesis by attaching and inhibiting the function of 30S subunit of the bacterial ribosome (Jawetz *et al*., 1998).

Amikacin is one of the important drugs which belong to aminoglycoside group, this drug constitute the first drug of choice for treatment of infection caused by *Proteus mirabilis* (Al-Talib and Habib, 1986).

Aminoglycoside especially gentamycin used widely in treatment of urinary tract infection so it's considered as drug of choice in this field (Merlin *et al*., 1988). Streptomycin is considered as oldest aminoglycoside drug and also it still drug of choice for treatment of bacteria which resist to other type of drugs, common gram negative bacteria develop resistant to this drug (Mingeot-Leclerco *et al*., 1999). The resistance to aminoglycoside was due to alteration of ribosomal receptor (chromosomal mutant), enzymatic destruction of drug (Plasmid mediated transmissible resistant of clinical important) and lack of permeability to drug molecule and lack active transport in the cell (Jawetz *et al*., 1998).

The mode of action of all quinolone involve inhibition of bacterial DNA synthesis by blocking of the DNA synthesis by acting on DNA gyrase (Jawetz *et al*., 1998).

Quinolone include many antibiotics such as naldixic acid, ciprofloxacin has become one of the most widely prescribed antimicrobial agents and has low incidence of side effects (Mulhall and Bergann, 1995). Chromosomal resistance developed by mutation and involve one of two mechanisms: either an alteration in the A subunit of the target enzyme or change in outer membrane permeability result in decrease drug accumulation (Jawetz *et al*., 1998). Macrolide antibiotics have been widely used to treat various infections, they bind to 23S rRNA on the 50S ribosomal subunit resulting in blockage of transpiptidation and / or translocation (Kawamura *et al*., 2000). The antimicrobial activity of macrolide is broad spectrum antibiotic used against gram-positive and some gram-negative bacteria. Resistance to erythromycin result from an alteration (Methylation) of rRNA receptor, this is under control of transmissible plasmids (Jawetz *et al*.,

1998). Rifampin binds strongly to DNA dependent RNA polymerase and thus inhibits RNA synthesis in bacteria. Rifampin is well absorbed after oral administration, widely distributed in tissue, and excreted mainly through the liver and to lesser extend in the urine. Rifampin resistance is due to alter obtain of RNA polymerase and this caused by mutation (Jawetz *et al*., 1998).

Tetracyclin is concentrated by susceptible bacteria, and as a result; inhibit protein synthesis by inhibiting binding of aminoacyle tRNA to the 30S subunit of bacterial ribosome (Jawetz *et al*., 1998). Chloromphenicol is a potent inhibitor of protein synthesis in microorganism it blocks the attachment of amino acid to the nascent peptide chain of the 50S unit ribosome by interfering with the action of peptidyl transferase. So the resistance to chloromphenicol is due to destruction of the drug by an enzyme (chloromphenicol acetyl transferase) that is under plasmid control (Jawetz *et al*., 1998).

Co-Trimethoprim-Sulfamethazol (SXT) resistance among urinary tract isolates has recently been reported with an increase frequency in Canada and United State (Zhanel *et al*., 2000).

1.2.4 Probiotic:

The term "Probiotic" was first used to describe a live microbial supplement, which beneficially affects the host by improving its microbial balance (Fuller, 1989).

Since then, researches have looked at possible clinical uses for these agents, Muller *et al* (1995) established a greater understanding of their properties. They described, the term "biotherapeutic agents" as microorganisms with specific therapeutic properties that also inhibit the growth of pathogenic bacteria.

Probiotic are nonpathogenic microorganisms that, when ingested, exert a positive influence on the health or physiology of the host. They can influence

intestinal physiology either directly or indirectly through modulation of the endogenous ecosystem or immune system (McFarland and Elmer, 1995).

A sufficient level of proof to enable probiotic to be used in treatment of gastrointestinal disturbance are the good tolerance of yoghurt compared with milk in subject with primary or secondary lactose maldigestion. Other applications of probiotics were; the use of *Saccharomyces boulardii* and *Enterococcus faecium* to prevent or shorten the duration of antibiotic-associated diarrhea, the use of *Saccharomyces boulardii* to prevent further recurrence of *Clostridium difficile* associated diarrhea and the use of fermented milk containing *Lactobacillus rhamnosus* to shorten the duration of diarrhea in infants with rotaviruse enteritis (and probably also in gastroenteritis of other causes) (Hennequin *et al*., 2001).

Probiotic uses (bacteria and yeast) could be summarized as: a) to improve the microbial balance of the host, b) their special properties that make them useful in fighting infection of mucosal surface such as the gut and vagina, c) different species of *Lactobacilli* have the potential for use in clinical as also the yeast *Saccharomyces boulardii*, d) becoming increasingly a valiable as capsules and dairy based food supplements sold in healthy food stores and some supermarket, e) the relative lack of side effects makes probiotics a possible way of preventing antibiotic associated diarrhea (Marteau *et al*., 2001).

Various mechanisms have been suggested for the action of probiotics some of them are; antagonizing pathogens directly through antimicrobial agents they produce like cytokines and butyric acid, reducing gut pH by stimulating latic acid producing microflora, compete for binding and receptor sites that pathogens occupy, improving immune function and stimulating immunomodulatory cells, producing lactase which aids in lactose digestion, adhering to the site on the gut wall and inhibiting intestinal bacterial enzyme in the synthesis of colonic carcinogens (Otles *et al*., 2003).

1.2.4.1 *Saccharomyces boulardii*.

Saccharomyces boulardii is a saprophytic, thermophylic yeast. It was first isolated from lychee fruit in Indochina and used in France to treat diarrhea, beginning in 1950s. Comparing to most other yeasts, it has an unusually high optimal growth temperature of 37ºC. A lyophilized form was prepared in 1962 by Biocodex Laboratory in France, and mainly used for antibiotic associated diarrhea. It is widely available in Europe, South America and Africa (Blehaut *et al*., 1989; Gedek and Amselgruber, 1990).

Growing applications of *Saccharomyces boulardii* in the prevention and treatment of human septic enteritis was also emphasized (Butes *et al*., 1994).

Saccharomyces boulardii is considered a non-pathogenic, non-colonizing baker's yeast species, which is very closely related to brewer's yeast also known as *Saccharomyces cerevisiae* (Schellenberg *et al*., 1994).

Saccharomyces cerevisiae subtype of *Saccharomyces boulardii* produced ethyalcohol, glycerol, succinic acid, 2-3 butandiol, acetyldehyde, acetic acid, lactic acid (Mcfarland *et al*., 1994).

Yeast need some vitamins for growth like; biotin, riboflavin, folic acid and pantothenic acid which play important role in stimulating the growth of yeast and increasing the production of secondary metabolite under aerobic and anaerobic conditions (Silva, 1996).

Saccharomyces boulardii used to improve the intestinal resistance against bacterial infection. It also have the ability to inhibite the toxin produced by *Vibrio cholerae* by production of proteins that able to binding with specific receptors in the toxin (Brandao *et al*., 1998).

Gastric juice has no effect on the yeast which can grow along the gastrointestinal tract. It is used clinically as an oral lyophilized preparation (McCullough *et al*., 1998).

Saccharomyces boulardii is a unique probiotic in that its known to survive gastric acidity; it is not adversely affected or inhibited by antibiotic and does not alter or adversely affect the normal flora in the bowel. For this reason good friendly probiotic organisms (including *Lactobacillus* and *Biofidobactrerium*) can be taken at the same time as *Saccharomyces boulardii* (Kailasapahthy and Chin, 2000).

This yeast was considered as a good source for pyridoxime, nicotinic acid, pantothenic acid, riboflavin, biotin, thiamin, folic acid and stimulate the innate immunity to secret IgA (Hennequin *et al*., 2001).

There is relationship between diameter of inhibition zone and the concentration of inhibitory substance of *Saccharomyces cerevisiae* subtype of *Saccharomyces boulardii* (Wood and Bevan, 1986; Pfeiffer and Radler, 1982). The inhibitory materials that produced from *Saccharomyces boulardii* are acid proteins with molecular weights ranging between (10,000-120,000) dalton (Kagon, 1983; Brandao *et al*., 1998). Effects of such materials are different according to the type of the sensitive microorganisms that used mechanism of inhibitory material that work against microorganisms and different nature of the receptors that found on the surface of sensitive microorganisms (Schmitt and Radler, 1988).

In vitro production of inhibitory compounds toward known pathogens for the considered species has often been used in the selection of probiotic strains (Hansen *et al*., 1989; Gibson *et al*., 1998; Riquelme *et al*., 1997; Rengpipate *et al*., 1998). A correlation is made between the *in vitro* ability of the probiotic to inhibit bacterial pathogens (*Pseudomonas spp., Vibrio cholerae, Clostridium difficile, Escherichia coli*) (Verschuers *et al*., 2000).

Purification of the inhibitory proteins substance that produced from yeast cells was done by precipitation by ammonium sulphate at ratio 30-70% in saturation and used gel filtration chromatography (Sephadex G100). Then study

the physical and chemical factors such as temperature that affect to the inhibitory substance at different times of periods (Pfeiffer and Radler, 1982).

Chapter Two

Materials and Methods

Chapter Two
Materials and Methods

2.1 Materials

2.1.1 Equipment and Apparatus:

The following equipment and apparatus were used throughout the study:

Equipment	Company (Origin)
Autoclave	Diako (Japan)
Balance	Hanna (Italy)
Bench centrifuge	Beckman (USA)
Refrigerator centrifuge	Harrier (UK)
Compound light microscope	Olympus (Japan)
Distillator	Gallenkamp (UK)
Freeze-Dryer	Vitris (USA)
Hot plate with magnetic stirror	Gallenkamp
Incubator	Ficher-Scientific (USA)
Micropipette	Gelson (France)
Millipore filter (0.22μm)	Millipore and Whattman (England)
Electrical oven	Memmert (Germany)
Pasture pipettes	Witeg (Germany)
pH-meter	Hanna
Power Supplier	LKB (Sweeden)
Sensitive-balance	Mettler
Shaking incubator	Gallenkamp
Shaking Water bath	Techne (UK)
Spectrophotometer	Aurora instrument Ltd. (UK)
Vortex	Seintific industries (USA)

2.1.2 Culture Media:

2.1.2.1 Ready to Use Media.

Medium	Company (Origin)
Blood base agar medium	Oxoid (UK)
Brain –heart infusion agar medium	Oxoid
MacConkey agar medium	Oxoid
Muller-Hinton agar medium	Mast diagnosis (UK)
Nutrient agar medium	Oxoid
Nutrient broth medium	Oxoid
Phenylalanine deaminas (PAD) agar medium	Oxoid
Sabauroud agar medium	Oxoid
Simmon citrate agar medium	Difco (USA)
Triple sugar iron (TSI) agar medium	Difco
Urea agar base medium	Oxoid
Yeast nitrogen base medium	Oxoid

2.1.2.2 Laboratory Prepared Media.

- Blood agar medium
- Dates extract broth (DEB) medium
- Dried tea leaf extract broth (DTLEB) medium
- Indole medium
- Luria-Bertonia (LB) medium
- Motility medium
- Phenylalanine deaminase (PAD) medium
- Sabauroud broth (SB) medium
- Urea agar medium

- Yeast extract glucose peptone (YEGP) medium

2.1.3 Chemicals:

The chemicals used in this study were the following which are classified according to the manufacturing companies.

- **BDH (England)**

  Hydrochloric acid, D-glucose, Agar-Agar, Ethanol, Sucrose, Acetic acid, Methylene blue, Sodium dihydrogen, Isoamylalcohol, Potassium chloride, K_2HPO_4, Na_2HPO_4.

- **Difco (USA)**

  Tryptone, Peptone, Yeast extract, N,N,N,N-tetramethyl-p-phenylene diamine dihydrochloride.

- **Fluka (Switzerland)**

  Methanol, Urea, Sodium hydroxide.

- **Riedel-Dehaeny (Germany)**

  Sodium chloride, p-dimethyl-amino-benzaldehyde.

- **Oxoid (England)**

  Gelatin.

2.1.4 Api 20E Kit (Api Bio-Merieux / France):

The Api kit used for identification of *Proteus mirabilis* was obtained from the Central Health Laboratory in Baghdad, they are composed of:

A) The Galleries:

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

B) Api 20E Reagents:

- Oxidase reagent (1% tetra-methyl-p-phenylene diamine).
- Kovac's reagent (P-dimethyl-amino-banzaldehyde at 4% in HCl isoamyl-alcohol).
- Voge's-proskaur reagent:
  - ✓ VP1 (40 % potassium hydroxide)
  - ✓ VP2 (6 % alphanaphthole)
- Ferric chloride 3-4%.

2.1.5 Antibiotics:

Two forms of antibiotics were used in this study, antibiotic disc and antibiotic powder.

2.1.5.1 Antibiotic Disk.

Antibiotic	Symbol	Concentration (µg / disk)	Company (Origin)
Amikacin	AK	30	AL-Razi Co.- Iraq
Amoxicillin	AMX	10	Al-Razi Co.
Cefatoxime	CTX	30	Oxoid- England
Chloramphenicol	C	30	AL-Razi Co.
Ciprofloxacin	CIP	5	Oxoid
Co-trimethaxole	STX	25	AL-Razi Co.
Gentamycin	GM	10	Oxoid
Naldixic acid	N.A.	30	AL-Razi Co.
Penicillin G	P	10	Al-Razi Co.
Pipracillin	PIP	30	Oxoid
Rifampicin	R.D.	30	AL-Razi Co.
Streptomycin	STE	10	Oxoid
Tetracycline	TE	30	AL-Razi Co.

(NCCLs, 1991).

2.1.5.2 Antibiotic Powder.

Antibiotic	Company (Origin)
Actidion (Cycloheximide)	Fluka (Germany)

2.1.6 Microorganisms Strains:

Microorganisms strains used in this study are tabulated as follows.

Isolate	Source
Escherichia coli ATCC 25922	Department of Biotechnology / College of Science / Al-Nahrain University.
Saccharomyces boulardii	Department of Biology / College of Science / Al-Mustansiriyah University.

2.1.7 Solutions, Buffers and Reagents:

- Antibiotic solution (Actidion)
- Fixative solution
- Kovac's reagent
- Oxidase reagent
- Phosphate buffer saline (PBS)
- Physiological saline solution
- Staining solution

2.2 Methods

2.2.1 Media Preparation:

2.2.1.1 Ready to Use Medium.

The media listed in (2.1.2.1) were prepared according to manufacture information fixed on their containers.

2.2.1.2 Laboratory Prepared Media.

2.2.1.2.1 Blood Agar Medium

It was prepared by adding 5% blood to the previously autoclaved blood agar base. After mixing, the medium was poured into petridishs. This medium was used for identification of *Proteus mirabilis* (Swarming phenomenon).

2.2.1.2.2 Urea Agar Medium

It was prepared by adding proper amounts of urea filtrate to the previously autoclaved urea agar base (Christenses medium). After sterilized, it was dispensed into test tube and let to solidify as slants. This medium was used for urease production.

2.2.1.2.3 Triple Sugar Iron (TSI) Agar and Simon Citrate Agar Media:

They were prepared, adjust to pH=7 and autoclaved. This medium was used for identification of *Proteus mirabilis* isolates.

2.2.1.2.4 Luria-Bertonia (LB) Medium

It was prepared according to Atlas *et al*., (1995) by dissolving tryptone (20 g), yeast extract (5 g) and sodium chloride (5 g) in 1 liter distilled water, after pH was adjusted to 7, it was autoclaved. This medium was used for activation of *Proteus mirabilis* isolates.

2.2.1.2.5 Phenylalanine Deaminase (PAD) Medium

It was prepared according to Holt *et al*., (1994) by dissolving tryptone (15 g) and L-phenylalanine (10 g) in 1 liter distilled water. After pH was adjusted to 7, it was autoclaved. The medium was dispensed in (10) ml amounts into sterile tubes which were made as slant. This medium was used for identification of *Proteus mirabilis* isolates.

2.2.1.2.6 Indole Medium

It was prepared by autoclaving the tryptone (15 g) in 1 liter after adjusting the pH to 7. Medium was autoclaving dispensed, aseptically in (10) ml amounts into sterile test tubes. After incubation, 10 drops of Kovac's reagent were added directly to the culture tube. This medium was used to identification of *Proteus mirabilis* isolates (negative result).

2.2.1.2.7 Motility Medium

It was prepared according to the Cruikshank *et al*., (1975) by dissolving nutrient broth (8 g) and agar-agar (4 g) in 1 liter of distilled water, pH was adjusted to 7 then the medium was autoclaved.

2.2.1.2.8 Yeast Extract Glucose Peptone (YEGP) Medium

It was prepared according to Barnett *et al*., (1990) by dissolving glucose (20 g), peptone (10 g), yeast extract (10 g), and agar-agar (18 g) in 1 liter of distilled water. Before autoclaving pH of the medium was adjusted to 5.5. This medium was used as a broth to grow the yeast isolates and obtaining their filtrates.

2.2.1.2.9 Sabauroud Broth (SB) Medium

It was prepared by dissolving glucose (20 g) and peptone (10 g) in 1 liter of distilled water. After autoclaving pH was adjusted to 5.5. This medium was used for activation and preparing the filtrates of yeast isolate.

2.2.1.2.10 Dates Extract Broth (DEB) Medium

It was prepared by boiling (100 g) of dates with (500 ml) of tap water for 1 hr. After that, the filtrate was obtaining throughout gauze pieces which was filtrated again by filter paper (Whattman No. 1). Then, autoclave and pH was adjusted to (4, 4.5, 5 and 5.5).

2.2.1.2.11 Dried Tea Leavies Extract Broth (DTLEB) Medium

It was prepared by soaking (10 g) of dried leaf tea into (500 ml) of tap water, then boiling for 1 hr. After that, it was filtrated throughout gauze pieces to get the filtrate which was refiltrated again by filter paper (Whattman No. 1). After that, (5 g) of glucose was added and the volume was completed to (500 ml) by distilled water, pH of the medium was adjusted to (4, 4.5, 5 and 5.5) after autoclave.

2.2.2 Solutions, Buffers and Reagents Preparation:

2.2.2.1 Solutions and Buffers.

2.2.2.1.1 Physiological Saline Solution

It was prepared according to Atlas *et al*., (1995) by dissolving (0.85 g) of NaCl in 1 liter of distilled water. After that, pH was adjusted to 7 and the medium was sterilized by the autoclave. It was used for dilution.

2.2.2.1.2 Staining Solution of Yeast

It was prepared according to Atlas *et al*., (1995) by dissolving (0.3 g) of methylene blue powder in (30 ml) of ethanol (95 % (v/v)).

2.2.2.1.3 Fixative Solution

It was prepared according to Atlas *et al*., (1995) by mixing (30 ml) of methanol with (10 ml) of acetic acid. It was used for fixation the yeast when staining by methylene blue.

2.2.2.1.4 Phosphate Buffer Saline (PBS)

It was prepared according to Gruikshank *et al*., (1975) as follow:

After dissolving (8 g) NaCl, (0.2 g) KCl, (0.2 g) KH_2PO_4 and (1.15 g) Na_2HPO_4 in distilled water was sterilized by the autoclave, it was used for preserve the cells (yeast and bacterial cells).

2.2.2.2 Reagents Preparation.

2.2.2.2.1 Oxidase Test Reagent (Baron *et al*., 1994)

A solution of 1% N,N,N,N-tetramethyl-p-phenylene diamine dihydro-chloride was prepared in sterile distilled water were needed.

2.2.2.2.2 Kovac's Reagent (Colle *et al*., 1996)

It was prepared by dissolving (1 g) of P-dimethyl-amino-banzaldehyde into (15 ml) of isoamylalcohol, then (5 ml) of concentrated HCl was carefully and gradually added. It was used for indole test.

2.2.3 Sterilization:

2.2.3.1 Moist-Heat Sterilization.

Media and solutions were sterilized by the autoclave at 121°C (15 lb / in^2) for 15 min.

2.2.3.2 Dry-Heat Sterilization.

Electrical oven was used to sterilized glasswere at 180°C for 2 hr.

2.2.3.3 Membrane Sterilization (Filtration).

Millipore filters (0.22 μm in diameter) were used to sterilize the antibiotic solutions and the filtrates of yeast growth (probiotic) *Saccharomyces boulardii*.

2.2.4 Urine Samples Collection:

Midstream urine samples were collected in 5-ml sterile tubes from patients refereed to the Al-Yarmok and Al-Karama Hospitals, during the period from

1 / 10 / 2003 – 1 / 2 / 2004. A total of (140) samples were collected and transported to the laboratory within 1 hr of collection.

2.2.5 Culturing Urine Samples:

A loopfull of undiluted urine sample was spreaded on MacConkey agar and blood agar plates. After that plates were incubated for overnight at 37°C, single colonies characterized by non-lactose fermented, giving negative reaction on oxidase test and making swarming on blood agar. The procedure was repeated several times to get pure cultures, for further diagnosis.

2.2.6 Maintenance of Bacterial Isolates:

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

2.2.6.1 Short-Term Storage.

Bacterial isolates were maintained for periods of few weeks on MacConkey agar plates. The plates were tightly wrapped in parafilm papers and stored at 4ºC until use.

2.2.6.2 Medium-Term Storage.

Bacterial isolates were maintained as stap cultures for periods of few months. Such culture were prepared in small screw-capped bottles containing (10) ml of nutrient agar each, then stored at 4ºC until use.

2.2.7 Maintenance of Yeast Isolate:

Maintenance of yeast culture was performed according to Branett *et al*., (1990) as follow:

The isolated colony was activated on yeast extract glucose peptone broth (YEGPB) and incubated at 30 °C for 24 hr. A loopfull of the yeast culture was transferred to (10 ml) of yeast extract glucose peptone agar (YEGPA) slant, then

incubated at 30°C for 24 hr. After that, it was stored at 4°C. The yeast cultures were reactivated and persevered after each two weeks period.

2.2.8 Identification of *Proteus mirabilis:*

2.2.8.1 Samples Culturing.

A loopfull of each undiluted urine sample was spreaded on MacConkey agar and incubated at 37°C for 24 hr. After incubation, growth was observed as well as ability to ferment sugars contained in the medium. Non-fermentable colonies were reinoculated on blood agar, then incubated under same condition. Waved growth (swarming), color, and odor of the grown colonies were observed (Atlas *et al*., 1995).

2.2.8.2 Microscopic Examination.

A loopfull of *Proteus* isolates was fixed on a glass microscopic slide, then stained by Gram stain to examine cell shape, grouping, motility (by Hunging drop method) and spore forming (Atlas *et al*., 1995).

2.2.8.3 Biochemical Tests.

2.2.8.3.1 Urease Test (Atlas *et al*., 1995)

Urease activity was detected by inoculating the surface of the Christensen urea agar slants with the bacterial growth, and incubating at 37°C for 24 hr. After incubation, red-violet color was checked as the indicator of positive test, while negative test was by the yellow-orange color.

2.2.8.3.2 Triple Sugar Iron (TSI) Test (Atlas *et al*., 1995)

Bacterial isolates were cultured on TSI agar slants by stapping and streaking on the slant surface, then incubated for 24-48 hr at 37°C.

Changing medium color from red to yellow indicated acid formation, while appearance of black precipitation indicate ferric sulfate formation. Pushing the agar to the top indicates CO_2 formation.

2.2.8.3.3 Citrate Utilization Test (Atlas *et al*., 1995)

Bacterial isolates were cultured on simmon citrate agar slants by stapping and streaking on slant surface, then incubated for 24-48 hr at 37°C. Changing the medium color from green to blue this indicate utilize citrate as its sole carbon-source (positive reaction).

2.2.8.3.4 Indole Formation Test (Colle *et al*., 1996)

Indole broth medium was inoculated with a drop of overnight isolate culture grown on nutrient broth, then incubated at 37°C for 24 hr. The subsequent development of pink color at the surface of this medium after addition 10 drops of Kovac's reagent (2.2.2.2.2) of each tube indicated the indole ring formation (positive reaction).

2.2.8.3.5 Phenylalanine Deaminase (PAD) Test (Senior, 1997)

Ten ml of PAD medium was inoculated in a test tube by the bacterial suspension, incubated at 37°C for 24 hr. After incubation, positive result was detected by changing medium color from green to blue.

2.2.8.3.6 Oxidase Test (Atlas *et al*., 1995)

This test was done by a filter paper (Whattman No.1) moistened by a few drops of a freshly prepared oxidase reagent (2.2.2.2.1), then aseptically, a clump of cells from slant growth was picked up with a sterile wooden stick and smeared on the moistened paper. The development of a violet or purple color within (10) second indicates a positive test.

2.2.9 Identification of *Proteus mirabilis* by Api 20E:

Identification was carried out by subculturing of selected colonies grown on Luria-Bertonia agar into Api 20E microtubes galleries for Enterobacteriaceae.

This system is designed for the performance of more than 20 standard biochemical tests from a single colony grown on the medium. Each test in this minimized system is performed within a sterile plastic microtube which containing appropriate substrates and was fixed to an impermeable plastic strip (gallery), each gallery contains 20 microtubes including the biochemical test and sugar fermentaion. Inoculation of the galleries was done with sterile pasture pipette and (5) ml of tap water dispensed into tray to provide humidity atmosphere, then incubated at 37°C for 24 hr. After that, the reagents were added for reading the galleries. Each positive reaction was given a value 1, 2 and 4 according to the position of the test in its group, so, a value from 0 to 7 digit was observed, then looked up in the index for the identification (appendix 1).

2.2.10 Antibiotics Sensitivity Test of *Proteus mirabilis* (Baron *et al.*, 1994):

2.2.10.1 Disk Diffusion Test.

Ten ml of nutrient broth medium was inoculated with 0.1 ml bacterial isolates suspension. The culture was incubated at 37°C to mid log phase (O. $D._{600}$ about 0.35) giving ($1*10^8$) cell / ml. After that, 0.1 ml of the inoculated broth was transferred to Muller-Hinton agar plates. A sterile cotton swab was used to streak the inoculum on the plate surface in 3 different plane (by rotation the plate approximately 60° each time to obtain an even distribution of the inoculum). The inoculated plates were then placed at room temperature for 10 minutes to allow absorption of excess moister. With sterile forceps, the antibiotic disks were placed on the inoculated plates, then incubated the plates at 37°C for 18 hr in an inverted position. After incubation, the diameters of inhibition zones were measured by a ruler in millimeter. Results were compared according to the National Committee for Clinical Laboratory Standard (NCCLs, 1991).

2.2.11 Identification Tests of *Saccharomyces boulardii*:

2.2.11.1 Actidion (Cycloheximide) Sensitivity and Adhesion Tests.

The solution of actidion was prepared according to Frazier, (1985) by mixing two subsolutions, First; it was prepared by dissolving 10 mg of actidion powder in 90 ml of distilled water and filtered. The second solution; it was prepared by dissolving 6.7 g of yeast nitrogen base and 1 g of glucose in 100 ml of distilled water and filtrated. Then, 4.5 ml from the first solution was mixed with 0.5 ml of the second solution in a sterilized test tube. After inoculation with the yeast culture, it was incubated at 30°C for 3 weeks, the growth of the yeast was observed weekly, and tube which gave no growth after three weeks was considered to be very sensitive to the antibiotic (Actidion). Also study include investigation of yeast as trapped to *Proteus mirabilis* by making a suspension of yeast and *Proteus mirabilis* in BPS and incubate for one hr and viewing under oil immersion objective (1000x).

2.2.12 Determining Inhibitory Effect of *Saccharomyces boulardii*:

2.2.12.1 On Solid Medium.

A culture of *Saccharomyces boulardii* was grown on yeast extract glucose peptone agar (YEGPA) and sabauroud agar (SA), it was incubated under aerobic condition at 30°C for 24 hr (Wood and Bevan, 1986). After incubation, disks from the yeast growth were obtained by cock-porer (5 mm in diameter), and put on the surface of nutrient agar plate that was previously inoculated with (0.1) ml of overnight bacterial culture by a spreader, then incubated at 37°C for 24 hr. After that, the inhibition zone around the disk of yeast culture was measured and estimated in (mm). Same procedure was completed for to other incubation periods (48 and 72 hr) to determine the optimum incubation period that gives greater inhibition zone.

2.2.12.2 In Liquid Medium.

Sterilized sabauroud broth was inoculated by 2% of *Saccharomyces boulardii* culture, after adjusted the pH to 4, 4.5, 5 and 5.5, then incubated, aerobically, at 30°C for 48 hr. After incubation, then culture was centrifuged at 3000 rpm for 15 min to get supernatant which contained the filtrate of grown yeast cells, then it was filtered by millipor filter unit (0.22μm). Well test method mentioned by Wilkins, (1949) was followed through using nutrient agar medium plate which was previously inoculated with 0.1 ml of the bacterial culture by spreading method. Well (5 mm in diameter) was made in the center of inoculated plates and filled with 0.5 ml of yeast filtrate, then incubated at 37°C for 24, 48 and 72 hr respectively. The inhibition zone around the well was measured by diameter (mm) and compared with the control (without yeast filtrate). The filtrate was concentrated by Freeze-dryer and the well test method was repeated for (first dilution, second dilution and third dilution) to investigate the effect of concentrated filtrate against the test bacteria. Same procedure was repeated by growing the yeast in other media (yeast extract glucose peptone broth, dates extract broth, and dried tea leavies extract broth) after adjusting the pH to 4, 4.5, 5 and 5.5 for the determination of optimum pH giving greater inhibition zone results.

2.2.12.2.1 Preparation of Stock Solution of Yeast Filtrate

After propagation of yeast *Saccharomyces boulardii* in each culture media used (SB, YEGPB, DEB, and DTLEB) at 30ºC for 48 hr, the filtrate obtained after centrifuge were concentrated to be converted until to powder in the Lyophilizer. Quantitively, (118.1, 260, 170.4 and 131.2) mg powder, respectively were obtained for each of 200 ml of the above mentioned media, respectively. Then each of these powders was redissolved in 10 ml of distilled water, these are considered to be stock solution of the yeast filtrate. Each of

these stock filtrates (SB, YEGPB, DEB and DTLEB) was diluted three times as follows:

First dilution: 0.5 ml of stock solution + 1 ml of non inoculate same medium.

Second dilution: 0.5 ml of first dilution + 1 ml of non inoculate same medium.

Third dilution: 0.5 ml of second dilution + 1 ml of non inoculate same medium.

2.2.13 Determination of Minimum Inhibitory Concentrations (MIC,s) for Concentrated Filtrates against *Proteus mirabilis*:

Serial dilutions (10 ml each) of three-fold concentrated filtrate of yeast *Saccharomyces boulardii* previously propagated in four media [yeast extract glucose peptone broth (YEGPB), Sabauroud broth (SB), dates extract broth (DEB), and dried tea leavies extract broth (DTLEB)] were prepared by using nutrient broth for dilution. After the following dilutions 0:10; 1:9; 2:8; 3:7; 4:6; 5:5; 6:4; 7:3; 8:2; 9:1 were prepared in test tubes as filtrate : nutrient broth, they were inoculated with 0.1 ml of *Proteus mirabilis* PR15 isolate culture, then incubated for overnight at 37ºC. Growth intensity of each tube was observed and recorded as; light (+), medium (++), heavy (+++), and no growth (–). Growth was estimated by using spectrophotometer 20, and optical density (OD_{600}) was read for each dilution, results were matched with the growth intensities.

2.2.14 Determination of Yeast Filtrate Effect on *Proteus mirabilis* Swarming:

After MIC of first dilution concentrated yeast filtrate was estimated in YEGP broth, the volume of filtrate obtained (in ml) for this purpose was mixed (in a sterile Petridish) with a volume from brain-heart infusion agar which gave a total volume of 10 ml. After the resulting medium was solidified, a drop (by a loop) of overnight culture of *Proteus mirabilis* PR15 isolate was put on its surface and incubated at 37ºC for 24 hr. After incubation, swarming of the isolate was estimated (in mm) and compared with that of the control (without yeast filtrate).

2.2.15 Determination of Yeast Filtrate Effect on *Proteus mirabilis* Motility:

After MIC of the first dilution concentrated yeast filtrate was estimated in YEGP broth, the volume of filtrate obtained (in ml) for this purpose was mixed (in a test tube) with a volume from the motility medium (semi-solid). After the resulting medium was left to semi-solidified, a needle (after immersion in the overnight culture of *Proteus mirabilis* PR15 isolate was stabbed into it, then the test tube was incubated at 37ºC for 24 hr. After incubation, motility of the bacterial was observed and compared with that of the control.

Chapter Three

Results and Discussion

3.1 Isolation and Identification of *Proteus* Isolates

3.1.1 Isolation of *Proteus* Isolates:

Results of 140 midstream urine samples collected from patients suffering from symptoms referred as urinary tract infection showed that (89) samples (63.6%) of them gave positive growth on MacConkey agar and blood agar. Such finding was nearly comes in agreement with the results of AL-Bayati (1999) and Younis (1986) who found that the percentages of positive culture of urine samples were (84%) and (83%), respectively. But they were higher than that found by Obi *et al* (1996) which was only (27%). The reason for such variable in the percentages may be owed to the differences in sample size and number, hospital site, season and medications taken before sampling.

Results also showed that 20 isolates of the positive culture were belonged to the genus *Proteus*, with an isolation percentage of 22.5% from the total urinary tract infection. A nearly result was recorded by Kareem (2001) when he found that the percentages of *Proteus spp.* isolates occurrence in inpatients and outpatients cases were 26.8% and 27.7%, respectively. Saeed (1993), on the other hand, detected lower percentage when only 10.5% of the urinary tract infection cases was belonged to *Proteus*. Adversely, higher finding was obtained by Warren *et al* (1982) who found that the isolation percentage of *Proteus* was 38% from the total urinary tract infection cases they tested.

Proteus isolates were found in both sexes of patients, but they were quietly high in male when 14 out of the total 20 isolates were obtained from male samples (70%), while only 6 isolates (30%) were belonged to the female. In this regard, results of other investigators were different, for instance, AL-Murjany (2000) was found that percentage of *Proteus* isolates in male was 63.5%, while AL-Taiee (2000) found it by only 90% compared to 36.5% and 10% for female,

respectively for both investigators. The reason for such differences in *Proteus* isolation percentage between the two sexes may be related to the presence of preputical sac in males which is considered as important container for *Proteus* that allowing the bacteria to raise up through its motility then causing urinary tract infection by ascending route. Stamy (1975) insisted that killer effect of the vaginal fluid, which has a low pH, is considered to be as a selection pressure against *Proteus.*

3.1.2 Identification of *Proteus* Isolates:

3.1.2.1 Cultural Examination.

Cultural examination of the suspected isolates showed that their colonies were pale in appearance on MacConkey agar (as non-lactose fermenters), while on blood agar the colonies were circular with entire edges, had fishy smell, and showed swarming motility. Such characteristics are came in accordance with those of genus *Proteus.*

3.1.2.2 Microscopical Examination.

When part of a suspected colony grown on MacConkey agar was smeared on a microscopical slide and stained by gram staining technique, cells were found as small rods, grouped mainly singly, gram-negative and non-sporformers. When hanging drop preparation was made, cells were motile which mean that they posses factors of movement, the flagella.

3.1.2.3 Biochemical Tests.

Table (3.1) shows that the suspected isolates of *Proteus* were belonged to only two species; *Proteus mirabilis* and *Proteus vulgaris*. Fifteen of the (20) *Proteus* isolates gave clearly positive results for urease and PAD tests, positive or negative results for citrate utilization and H_2S production, but they were negative to the oxidase and indole tests. They are concieded with those given by

to *Proteus mirabilis*. However, *Proteus vulgaris* was represented by only (5) isolates when such isolate gave positive result to H_2S production, indole, urease and PAD tests, positive or negative results to citrate utilization, but negative to the oxidase test.

Table (3.1): Biochemical tests differentiating between *Proteus mirabilis* and *Proteus vulgaris* spp.

Isolate	Biochemical test							No. of isolates
	Oxidase	Citrate Utilize	H_2S production	Indole	Urease	TSI	PAD	
Proteus mirabilis	–	+/–	+/–	–	+	ALK/A	+	15
Proteus vulgaris	–	+/–	+	+	+	ALK/A	+	5

+/– = Positive or negative result
ALK/A = Alkaline / acid
PAD = Phenylalanine deaminase
TSI = Triple sugar iron
H_2S = Hydrogen sulfide

3.1.3 Identification of *Proteus mirabilis* by Api 20E:

Identification of *Proteus mirabilis* was also confirmed by using Api 20E system. Results of such test, which is illustrated in plate (3.1), insured that (15) of the isolates were belonged to this species (same isolates which were previously identified by the biochemical tests as *Proteus mirabilis*). While the remaining isolates (5) were belonged to *Proteus vulgaris*.

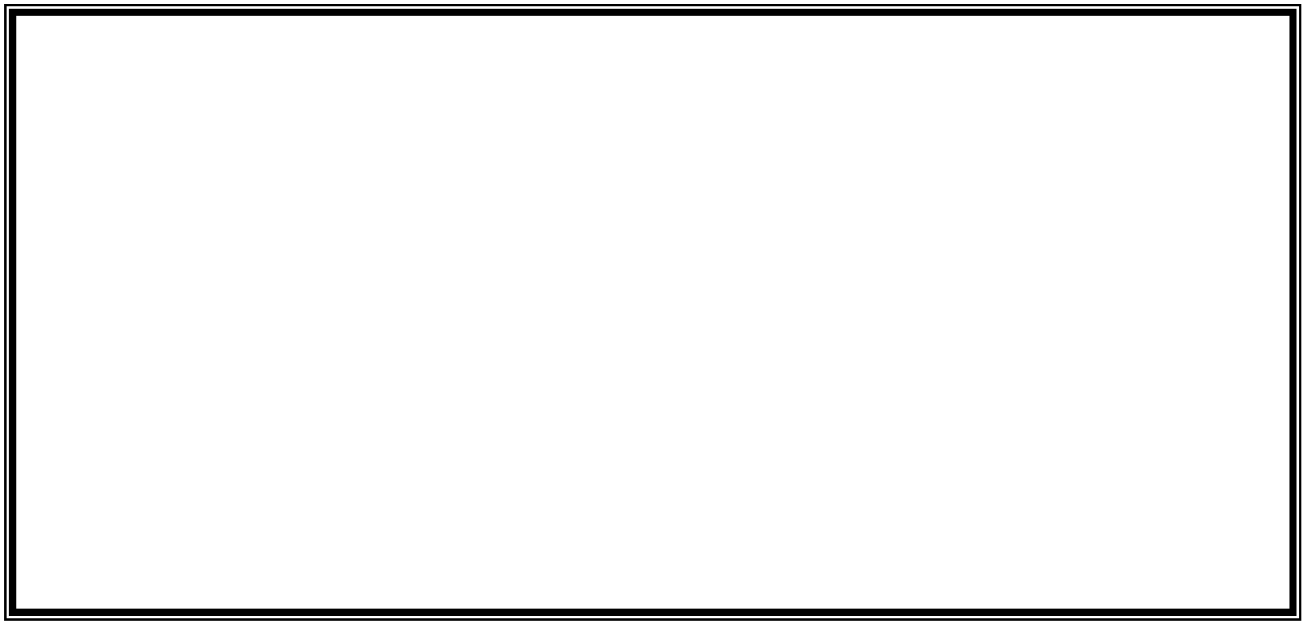

Plate (3.1). Api 20E system results for characterization of *Proteus mirabilis.*
Upper list: Control; **Down list:** for *Proteus mirabilis* which shows that (ODC, CIT, URE, TDA, GEL, GLU) are positive results, while (ONPG, ADH, LDC, H_2S, IND, VP, MAN, INO, SOR, RHA, SAC, MEL, AMY, ARA) are negative.

3.2 Antibiotic Sensitivity of *Proteus mirabilis*

The emergence of prevalence of antibiotic resistance strains is considered as a major therapeutic problem, and that could be explained by several hypothesis such as, the influence of excessive and / or inappropriate antibiotic use (Sotto *et al*., 2001), transmission of resistant isolates between people, consumption of food from animal that had received antibiotic and greater mobility of individuals world wide have also contributed to the extension of antibiotic resistance (Blanco *et al*., 1997). In this study, effect of 13 different antibiotics were applied against 15 isolates of *Proteus mirabilis* by using

standard disk diffusion method. Results, which resembled the inhibition zones, were compared with those fixed by NCCLs (1991).

Table (3.2) shows that, generally a vast of resistance was detected among the *Proteus mirabilis* isolates against the antibiotics used. It was found that the more effective antibiotics against isolate was the ciprofloxacin when 14 isolates were sensitive to it while only (one) isolate was resistant. Followed by amikacin which was resisted by 3 isolates compared to 12 isolates sensitive. Adversely, the less effective of the antibiotics was penicillin G when it was resisted by almost all (except one) isolates. Followed by tetracycline when only (two) isolates were sensitive to it, while the remaining 13 isolates were resistant. However, effect of other antibiotics were falling between the above mentioned antibiotics. Percentages of resistant isolates are included in table (3.3) which shows that all β-lactam antibiotics group used (Penicillin G, amoxicillin and pipracillin) had been resisted by very high percentages of the isolates 93.3, 80 and 66.6%, respectively. Followed by the tetracycline group when (86.6%) of the isolates were resistant to tetracycline. Next to previous two group, comes streptomycin (from aminoglycoside group) with 80% percentage of resistance, then chloramphenicol in a percentage of 73.3%. Closed results were recorded by other investigators, Kareem (2001) found that the resistant percentage to penicillin G was 90%, while AL-Taiee (2000) and AL-Tamimi (2003) found non of the *Proteus mirabilis* isolates they tested was sensitive the penicillin G, which means a (100%) resistant to such antibiotic.

It well known that resistant of wide type of bacteria, and even within strains of the same type, to the β-lactam group of antibiotics still (and continuous) to be one of the serious problems facing physicians and other related specialists. It was found that always large amounts and numbers of the resistant gene are transferred to bacterial isolates that produce β-lactamase (Oliver *et al*., 1999).

This study also shows that cefatoxime (a third generation antibiotic belonged to the cephalosporins) possess the lowest resistant percentage (33.3%) of the isolates. Such result falls between those found by AL-Taiee (2000) who found it as 52% and AL-Tamimi (2003) with a percentage of 24.4%.

Table (3.3): Percentages of antibiotic resistant among *Proteus mirabilis* isolates.

Antibiotic Group and Member	Resistant isolates	
	Number	Percentage (%)
β-lactam penicillin		
Penicillin G (P)	14	93.3
Amoxicillin (AMX)	12	80
Pipracillin (PIP)	10	66.6
Cephalosporin		
Cefatoxime (CTX)	5	33.3
Aminoglycosides		
Gentamycin (GM)	5	33.3
Streptomycin (STE)	12	80
Amikacin (AK)	3	20
Quinolones		
Naldixic acid (N.A.)	4	26.6
Ciprofloxacin (CIP)	1	6.6

Tetracycline		
Tetracycline (TE)	13	86.6
Others		
Rifampicin (R.D.)	6	40
Chroamphenicol (C)	11	73.3
Co-Trimethaxol (SXT)	6	40

Resistant to aminoglycosides in this study were found to be variable. Amikacin, for example, was the strongest one when it shows a resistant of 20%, while AL-Tamimi (2003) found that resistant among *Proteus mirabilis* as (15.5%).

In general, bacterial resistance to the aminoglycosides may be related to the production of modifying enzyme which alter the aminoglycoside and prevent it from binding to the ribosomes of the cells (Jawetz *et al*., 1998).

Regarding high resistant to tetracycline among the isolates of present study which reached 86.6%, the investigators, such as AL-Talib and Habib (1986), AL-Murjany (2000) and AL-Tamimi (2003) found that *Proteus mirabilis* isolates were very resistant to the tetracycline when the percentage achieved (96%) and (68.9%), respectively.

Merlin *et al*., (1988) found that Enterobacteriaceae were very resistant to the tetracycline and it is related to the resistant genes which are carried by plasmid, and these genes could be transferred among Enterobacteriaceae members from one to each other.

Quinolines, on the other hand shows good effect on *Proteus mirabilis* isolates when most isolates were found sensitive to these antibiotics. For example, ciprofloxacin show a resistant of only (6.6%). This result is closed to

that found by AL-Murjany (2000) when found that resistant among *Proteus mirabilis* to the ciprofloxacin was (4.7%), and AL-Tamimi (2003) who found it as 8%. Although, this antibiotic was newly used in treatment but resistant to it was, developed as a result of common and wide less use of the antibiotic (Rice *et al*., 1992).

3.3 *Saccharomyces boulardii* Identification and Adhesion

Isolate of *Saccharomyces boulardii* was obtained from AL-Mustansiriyah University / College of Science, it was rediagnosted against sensitivity to actidion (Cycloheximide) antibiotic, when non growth of *Saccharomyces boulardii* was observed after 3 weeks period due to its sensitivity to this antibiotic.

Green and Gray (1950) detects that *Saccharomyces cerevisiae* subtype of *Saccharomyces boulardii* yeast is sensitive to the actidion antibiotic at concentration 0.01 mg / ml, so that it's used in culturing media to separate bacteria from the wine and beer.

Also the ability of *Saccharomyces boulardii*, as trap for *Proteus mirabilis*, was tested. Plate (3.2) shows that cells of bacterial isolate (PR15) of *Proteus mirabilis* (rod shape) were adhered to the surface of *Saccharomyces boulardii* cells (oval shape) after staining by methylene blue stain.

In vitro studies have demonstrated that *Saccharomyces boulardii* exerts antagonistic activity against various bacterial pathogens (Brugier and Patte, 1975; Czerucka *et al*., 2000). Other studies have reported the adhesion of the *Salmonella typhimurium,* enteropathogenic *Escherichia coli* and enterorrhagic

Escherichia coli cells to the cells of *Saccharomyces boulardii* (Gedek, 1999; Czerucka *et al*., 2000).

Plate (3.2). Microscopical examination of adhesion of *Proteus mirabilis* cells (rods) to the surface of *Saccharomyces boulardii* cells (large ovals) under the oil immersion objective (1000x).

3.4 Inhibitory Effect of *Saccharomyces boulardii*

Depending on the nature of medium used to evaluate the inhibitory effect of *Saccharomyces boulardii* on illustrated as follow:

3.4.1 On Solid Medium:

Results of table (3.4) indicate that the inhibitory effect of *Saccharomyces boulardii* when grown on sabauroud agar (SA) supplemented with yeast extract (YEGPA) gave larger inhibition zones than when grown only on the SA after the three periods of incubation (24, 48 and 72 hr) at 37ºC. Inhibition zone was measured after 24 hr of incubation, and gave 9 mm when grown on SA and 10.9 mm on yeast extract glucose peptone agar (YEGPA). After 48 hr of incubation best results were obtained when the inhibition zone increased to 10 mm on SA and 12 mm on YEGPA, plate (3.3).

Incubation period of 72 hr had adversed effect when the inhibition zone decreased to 7 mm on SA and 8.5 mm when grown on YEGPA.

Silva (1996) found that, yeast cells are able to produce inhibitory factors that kill sensitive cells at 28ºC but fail to do so in 18ºC, but this also dependent on the incubation period and medium uses. He found that supplying the medium with yeast extract increased production of the inhibitory factors, while incubation in the shaking incubator reduces such factors.

Table (3.4): Effect of incubation time (hr) on the inhibitory effect of *Saccharomyces boulardii* grown at two different media against *Proteus mirabilis* isolate PR15.

Incubation time (hr)	Diameter of Inhibition zone (mm)	
	SA	YEGPA
24	9	10.9
48	10	12
72	7	8.5

SA = Sabauroud agar; YEGPA = Yeast extract glucose peptone agar

Plate (3.3). Inhibitory effects of Saccharomyces boulardii against Proteus mirabilis isolate PR15 when grown on nutrient agar medium at 37ºC for 24 hr.

SA= Sabauroud agar; **YEGPA=** Yeast extract glucose peptone agar.

3.4.2 In Liquid Medium:

3.4.2.1 In Sabauroud Broth (SB) Medium.

Results of the table (3.5) show the inhibitory effect of the *Saccharomyces boulardii* yeast filtrates after grown in SB medium at different pH values. In the third dilution concentrated filtrate (14.76 mg / ml), highest inhibition zone was recorded at pH 4.5 when it reached 14 mm compared to these of 12, 11 and 9 mm for pH values of 5, 4 and 5.5, respectively. When the filtrate of the yeast was concentrated twice (second dilution), which contained 29.5 mg / ml, highest inhibition zone was also recorded for the same pH value 4.5 when the inhibition zones increased to 19 mm compared to 15 mm for both 4 and 5 pH values and (14) mm for the pH value of 5.5. However, first dilution concentration of the

filtrate (59 mg / ml) gave almost similar pattern of results when the highest inhibition zone (21 mm) was achieved at pH 4.5 also and the lowest (17 mm) at pH 5.5.

Depending on the above findings, it is clearly concluded that best inhibitory effect by the yeast *Saccharomyces boulardii* was exhibited against *Proteus mirabilis* PR15 isolate in the sabauroud medium with a pH value of 4.5. Adversely, pH value of 5.5 resulted in the lowest inhibitory activity of the yeast among all times of dilution of the filtrates used.

Several investigators insisted that pH values in the rang of (4 to 5) is considered to be favorable by the yeast to produce killing factors and to preserve its stability (Heard and Fleet, 1987; Mickalcakova and Repova, 1992).

Table (3.5): Effect of *Saccharomyces boulardii* concentrated filtrates (first dilution, second dilution and third dilution) against *Proteus mirabilis* isolate PR15 grown in sabauroud broth medium at different pH values after incubation for 48 hr at 37ºC.

Concentrated filtrate	pH	Diameter of inhibition zone (mm)
First dilution	4	19
	4.5	21
	5	18
	5.5	17

Second dilution	4	15
	4.5	19
	5	15
	5.5	14
Third dilution	4	11
	4.5	14
	5	12
	5.5	9

3.4.2.2 In Yeast Extract Glucose Peptone Broth (YEGPB) Medium.

Table (3.6) shows the inhibitory effect of the *Saccharomyces boulardii* filtrate after grown in YEGPB medium at different pH values. In the third dilution concentrated filtrate (32.5 mg / ml), highest inhibition zone was recorded at pH 4.5 when it reached (18) mm, compared to those of (15, 11 and 10) mm for pH values of (4, 5 and 5.5), respectively. When filtrate of the yeast was concentrated twice (second dilution), which contained 65 mg / ml, highest inhibition zone (24 mm) was also recorded for the same pH value 4.5, compared to those of (21, 17 and 16) mm for pH values of (4, 5 and 5.5), respectively.

However, first dilution concentration of the filtrate (130 mg / ml) gave almost similar pattern of results when the highest inhibition zone (25 mm) was achieved at pH 4.5, compare to (21) mm at pH 5.5.

Depending on the above findings, it is clearly concluded that best inhibitory effect given by the yeast *Saccharomyces boulardii* was exhibited against *Proteus mirabilis* isolate PR15 in the yeast extract glucose peptone broth medium with a pH of 4.5. Adversely, pH value of 5.5 resulted in the lowest inhibitory activity of the yeast among all times of dilutions of the filtrates used.

Plate (3.4) shows that best inhibitory effect of (one-fold, two-fold and three-fold) concentrated filtrates of *Saccharomyces boulardii* against *Proteus mirabilis* isolate PR15 was recorded after at 37ºC for 48 hr. Wood and Bevan (1968) stated that yeast extract stimulates the producer cell to produce large amount of inhibitory materials, while addition of gelatin increase stability of the inhibitory materials.

Table (3.6): Effect of *Saccharomyces boulardii* concentrated filtrates (first, second and third dilution) against *Proteus mirabilis* isolate PR15 grown in yeast extract glucose peptone broth medium at different pH values after incubation for 48 hr at 37ºC.

Concentrated filtrate	pH	Diameter of inhibition zone (mm)

First dilution	4	24.3
	4.5	25
	5	24
	5.5	21
Second dilution	4	21
	4.5	24
	5	17
	5.5	16
Third dilution	4	15
	4.5	18
	5	11
	5.5	10

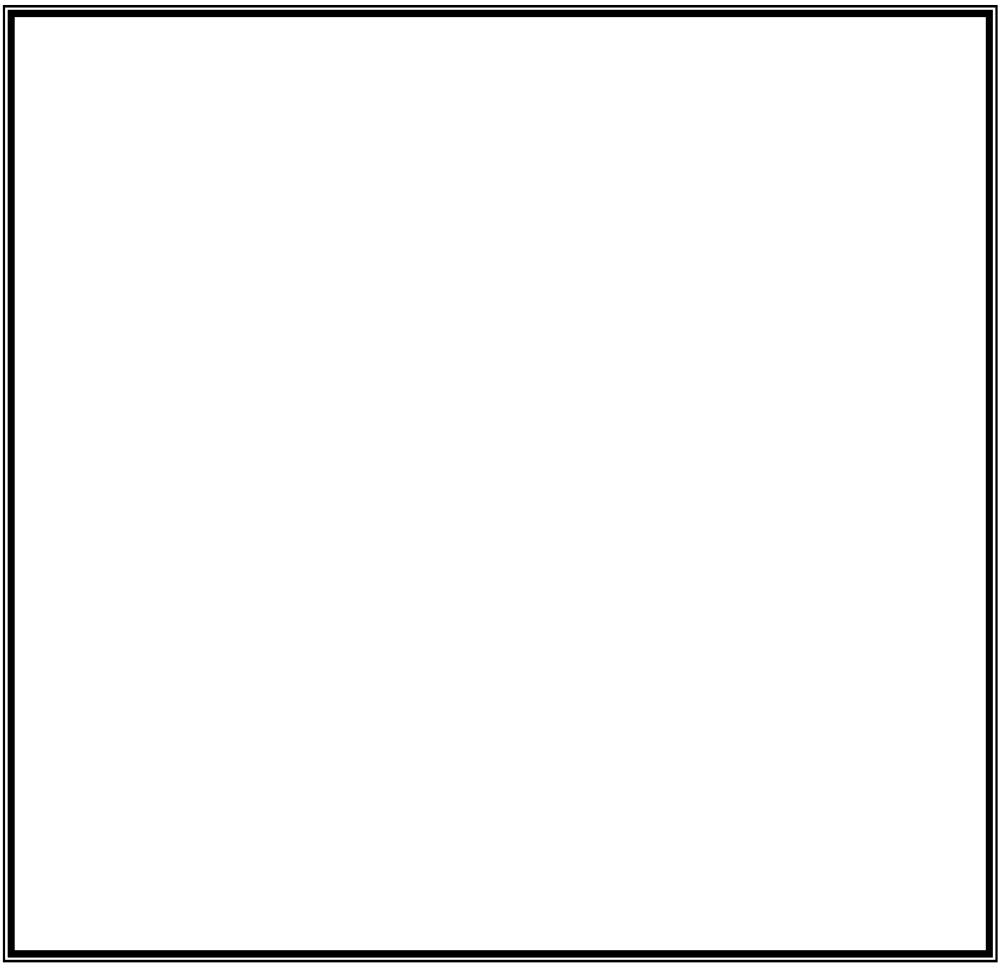

Plate (3.4). Inhibitory effect of *Saccharomyces boulardii* grown in yeast extract glucose peptone broth medium at pH 4.5 against *Proteus mirabilis* isolate PR15 after incubation for 48 hr at 37ºC.

3.4.2.3 In Dates Extract Broth (DEB) Medium.

Results of the table (3.7) show the inhibitory effect of the *Saccharomyces boulardii* yeast filtrates after growing in DEB medium at different pH values. In

the third dilution concentrated filtrate (21.3 mg / ml), highest inhibition zone was recorded at pH 5.5 when it reached (14.9) mm, compared to those of (14, 13 and 12) mm for pH values of (5, 4.5 and 4), respectively. Second dilution concentration filtrate (42.6 mg / ml) resulted also in highest inhibition (19.9 mm) zone at pH value 5.5, compared to (16, 15 and 14) mm for pH values of (5, 4.5 and 4), respectively. However, highest inhibition zone (21 mm) was given by first dilution (85.2 mg / ml) concentrated filtrate gave at pH 5.5 in comparison to (7.5) mm as the lowest which was given at pH 4.

From the just mentioned results, it is obvious that best inhibitory effect by the yeast *Saccharomyces boulardii* was exhibited against *Proteus mirabilis* isolate PR15 in the dates extract broth medium when pH of this medium was adjusted to 5.5. Adversely, pH lower value of 4, resulted in the least inhibitory activity of the yeast among all times of dilutions of the filtrates tested.

Table (3.7): Effect of *Saccharomyces boulardii* concentrated filtrates (first, second and third dilution) against *Proteus mirabilis* isolate

PR15 grown in dates extract broth medium at different pH values after incubation for 48 hr at 37ºC.

Concentrated filtrate	pH	Diameter of inhibition zone (mm)
First dilution	4	7.5
	4.5	18
	5	19
	5.5	21
Second dilution	4	14
	4.5	15
	5	16
	5.5	19.9
Third dilution	4	12
	4.5	13
	5	14
	5.5	14.9

Nearly results were recorded by AL-Zubaiedy (2001) when found that pH 6 was the optimal in giving best inhibition zone in dates extract broth (DEB) medium by *Saccharomyces cerevisiae* subtype of *Saccharomyces boulardii* against each of *Escherichia coli*, *Bacillus subtilis, Staphylococcus aureus, Salmonella typhimurium*, *Pseudomonas aeroginosa* and *Klebsiella spp.* after 48 hr incubation at 37ºC with diameters (22, 16, 30, 25, 29 and 25) mm, respectively.

Results of third, second and first concentrated filtrates of yeast *Saccharomyces boulardii* are best illustrated (in plate 3.5) against isolate PR15 of *Proteus mirabilis* after grown in dates extract broth medium and incubation at 37ºC for 48 hr. Inhibition zone diameter are increasing as the fold of concentration increases.

3.4.2.4 In Dried Tea Leavies Extract Broth (DTLEB) Medium.

Results of the table (3.8) show the inhibitory effect of the *Saccharomyces boulardii* yeast filtrates after grown in DTLEB medium at different pH values. Highest inhibition zones were recorded at pH 5.5 for all the three *Saccharomyces boulardii* concentrated filtrates third dilution (16.4 mg / ml), second dilution (32.8 mg / ml) and first dilution (65.6 mg / ml) when the diameters reached (10.5, 13.5 and 17) mm, respectively. Same medium with a pH value of 4 was responsible for giving the lowest inhibition zone diameter when they decreased to only (7, 10 and 14.7) mm, respectively, to the above concentrated filtrates. However, all high, low and other inhibition zone diameters were recorded after incubation for 48 hr at 37ºC, plate (3.6).

Results of the affect of *Saccharomyces boulardii* concentrated filtrates after grown in the DTLEB medium at various pH values lead to the conclusion that best inhibitory effect was exhibited by all yeast concentrates against *Proteus mirabilis* isolate PR15 was achieved when pH of the medium was adjusted to 5.5. However, result was nearly with AL-Zubaidy (2001) when found that pH 6 was optimal in giving best inhibition zone in dried tea leavies extract broth (DTLEB) medium by *Saccharomyces cervisiae* subtype of *Saccharomyces boulardii* against each of *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Salmonella typhimurium,* and *Klebsiella spp.* after 48 hr incubation at 37ºC with diameter (21, 21, 20, 22 and 20) mm, respectively.

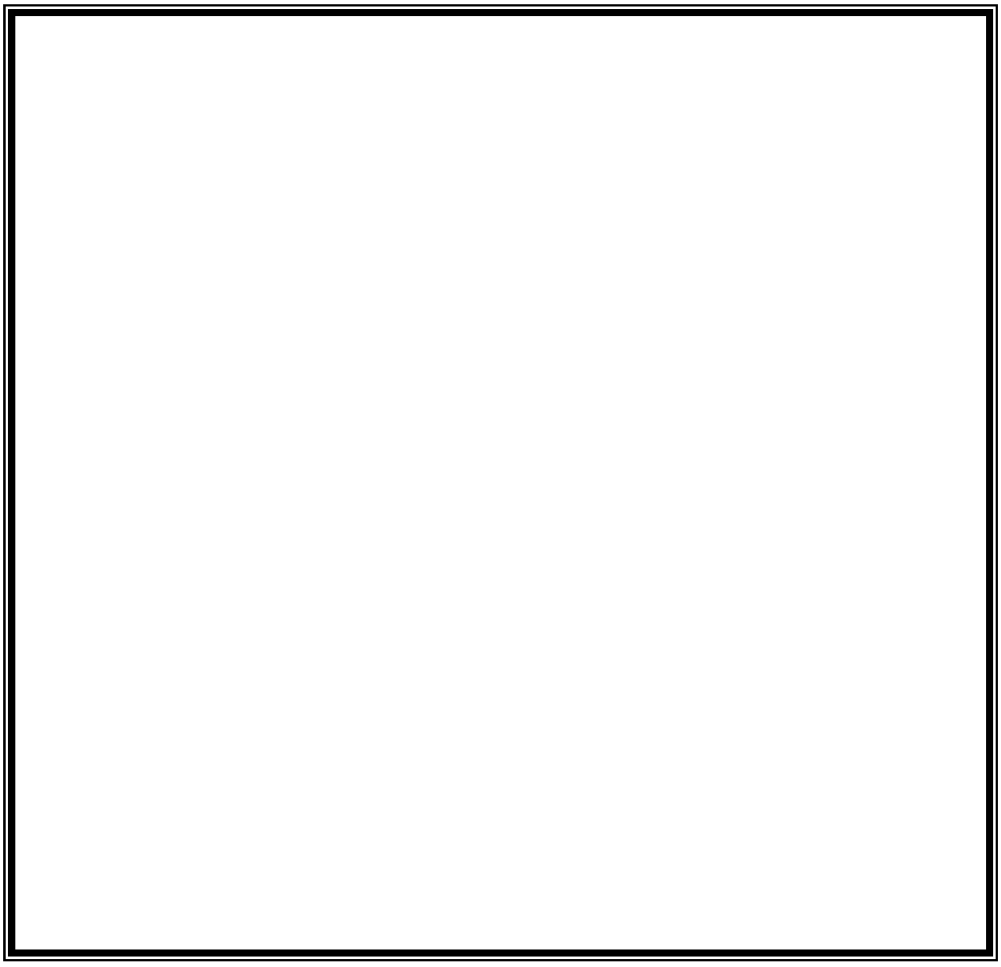

Plate (3.5). Inhibition effect of *Saccharomyces boulardii* grown in dates extract broth medium at pH 5.5 against *Proteus mirabilis* isolate of PR15 after incubation for 48 hr at 37ºC.

Table (3.8): Effect of *Saccharomyces boulardii* concentrated filtrates (first, second and third dilutions) against *Proteus mirabilis* isolate PR15 grown in dried tea leavies extract broth medium at different pH values after incubation for 48 hr at 37ºC.

Concentrated filtrate	pH	Diameter of inhibition zone (mm)
First dilution	4	14.7
	4.5	15
	5	16
	5.5	17
Second dilution	4	10
	4.5	11
	5	13
	5.5	13.5
Third dilution	4	7
	4.5	9.1
	5	8
	5.5	10.5

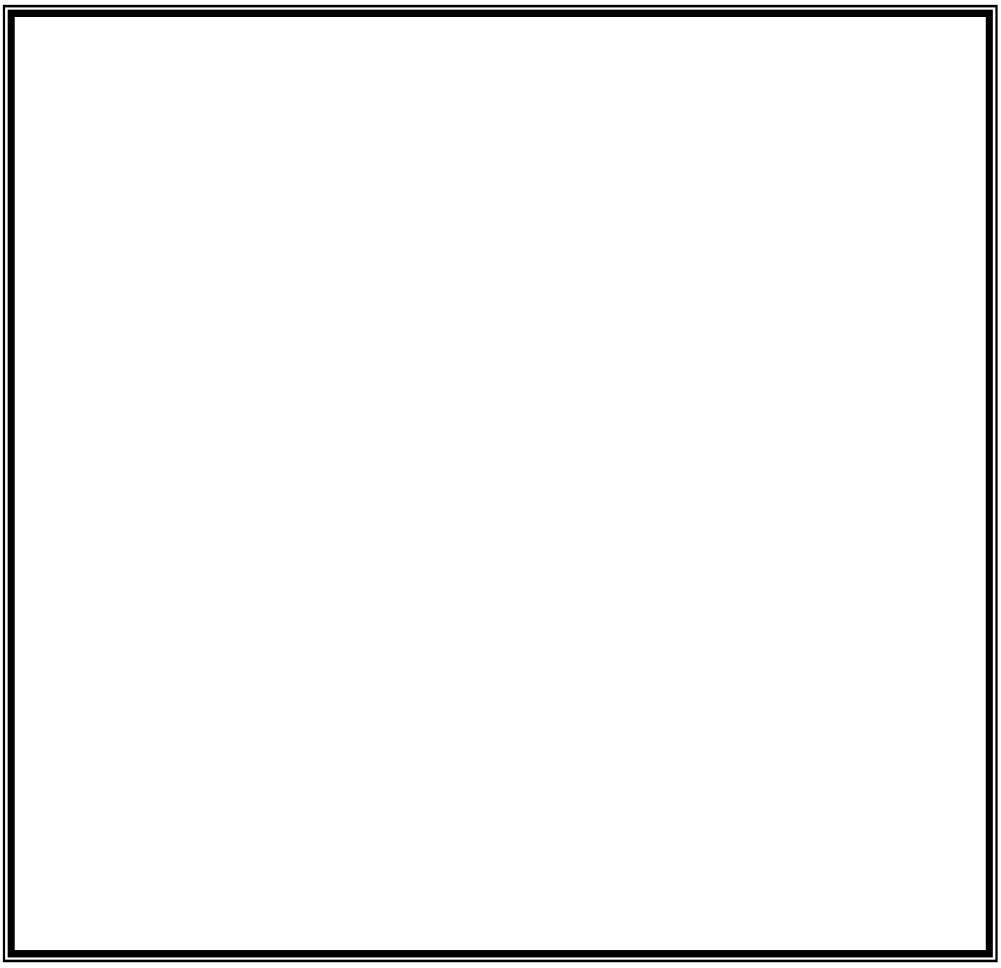

Plate (3.6). Inhibitory effect of *Saccharomyces boulardii* grown on dried tea leavies extract broth medium at pH 5.5 against *Proteus mirabilis* isolate of PR15 after incubation for 48 hr at 37ºC.

Among the four media used, yeast extract glucose peptone broth (YEGPB) medium was the most efficient medium in producing inhibitory effect of yeast filtrate at pH 4.5 after incubation for 48 hr at 37ºC, followed by the medium sabauroud broth (SB) under same conditions, then by dates extract broth (DEB) and dried tea leavies extract broth media, respectively, but at pH 5.5 and same other conditions.

3.5 Determining Minimum Inhibitory Concentrations (MIC,s) of Concentrated Filtrates against *Proteus mirabilis* Isolate

Minimum inhibitory concentration (MIC), as it is defined by Atlas *et al*., (1995), is the least concentration that prevent the clear growth of bacteria after incubation at 37ºC for overnight.

There are many factors that influence in MIC estimation, volume of bacterial inoculum (the value of MIC increased upon increasing this volume), pH, temperature and nature of cell wall (Nikaido, 1989). First dilution of the concentrated filtrates of the four media [yeast extract glucose peptone broth (YEGPB), Sabauroud broth (SB), dates extract broth (DEB) and dried tea leavies extract broth (DTLEB)], respectively, then serial dilutions were made as previously mentioned (2.2.13) to determine the MIC for each of the four medium.

Table (3.9) contains, MIC,s of the concentrated filtrates of yeast *Saccharomyces boulardii* in the four different media. Result of the table declared that the first two concentrations (1:9 and 2:8) had no observed effect against *Proteus mirabilis* PR15 isolate when heavy growth of this bacteria was noticed after incubation in all four media. But the growth was decreased at the following two concentrations (3:7 and 4:6) to the medium level for all media used. However, growth of *Proteus mirabilis* isolate was sharply decreased when concentrations of 5:5 was used in all four media.

With the concentration of 6:4 the situation was different when no growth was observed for *Proteus mirabilis* isolate in (YEGPB and SB) but light growth in the remaining to media (DEB and DTLEB) used. The three last concentrations of the yeast concentrated filtrates (7:3, 8:2 and 9:1) were quite enough to retard any growth of *Proteus mirabilis* isolate.

Relating to the just mentioned finding it may be concluded that filtrate concentration of 5:5 is the MIC for PR15 isolate of *Proteus mirabilis* in yeast extract glucose peptone broth and sabauroud broth media, and 6:4 concentration for same isolate but in the date extract broth and dried tea leavies extract broth media.

Table (3.9): Minimum inhibitory concentrations of first dilution concentration filtrate of *Saccharomyces boulardii* against *Proteus mirabilis* isolate PR15.

Media	Concentration of filtrate: (Concentrate: Medium)									
	Control 0:10	1:9	2:8	3:7	4:6	5:5	6:4	7:3	8:2	9:1
YEGPB	+++	+++	+++	++	++	+	–	–	–	–
SB	+++	+++	+++	++	++	+	–	–	–	–
DEB	+++	+++	+++	++	++	+	+	–	–	–

DTLEB	+++	+++	+++	++	++	+	+	–	–	–

YEGPB= yeast extract glucose peptone broth**; SB=** sabauroud broth**; DEB=** dates extract broth**; DTLEB=** dried tea leavies extract broth**; –:** no growth (0.105-0.131); **+:** light growth (0.198-0.210); **++:** medium growth (0.259-0.271)**; +++:** heavy growth (0.278-0.292)**.**

These results insured the probiotics role of the yeast in prevention of entric infections, Perdiagon *et al* (1991) found that low dose of probiotic being enough for protection against intestinal infections by increasing IgA secretion into the intestinal lumen, thus providing adequate defenses for the mucosal surface. Probiotic are used to treat disturbed intestinal microflora and increased gut permeabililty, which are characteristics of many intestinal disoreders, such as acute rotaviral diarrhea, food allergy, colon disorders, metabolic changes during pelvic radiotherapy, and changes associated with colon cancer development, in all such conditions, altered intestinal microflora, impaired gut barrier, and different types of intestinal inflammation are present. Effective probiotic bacteria are able to survive gastric conditions and colonize the intestine, at least temporarily (Salminen *et al*., 1996).

3.6 Effect of *Saccharomyces boulardii* Concentrated Filtrate on Swarming and Motility of *Proteus mirabilis*.

Effect of concentrated filtrate of *Saccharomyces boulardii* was investigated against (PR15 isolate) of *Proteus mirabilis* by using the concentrations given the MIC,s in the previous item (3.5) for the four media used.

For this purpose a concentration 6:4 was used for media (DEB and DTLEB) and 5:5 for (YEGPB and SB) media. Results illustrated on plates (3.7 and 3.8) show that there was an obvious effect for the concentrate filtrates of the yeast on swarming and motility of PR15 isolate of the bacteria when the

diameter of swarming was reduced to only (29 mm) after it was occupied almost all the diameter of the petridish (88 mm).

Rabaan *et al* (2001) pointed out chemical signals (Chemotaxis) which are responsible for motion of the bacteria will be decreased due to the effect of probiotic which has an antagonistic activity to the biological process of the bacterial isolate.

While, Kawamura *et al* (2000) related this to the inhibition motion by macrolids, which have a relationship with the losses of gene expression of flagellin, in addition to inhibiting the synthesis of signals responsible for motion of the bacteria and then to paralyze motion.

Plate (3.7). Probiotic effect of *Saccharomyces boulardii* concentrate filtrate (5:5) of yeast extract glucose peptone broth on the swarming phenomenon of *Proteus mirabilis* PR15 isolate on Brain-Heart infusion agar as a solid medium after incubation for 24 hr at 37ºC.

Above: control (medium without probiotic); **Below:** (medium + probiotic).

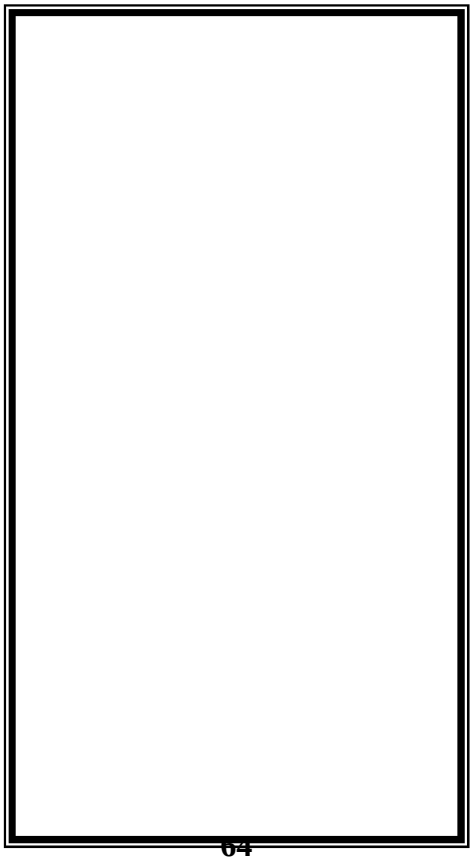

Plate (3.8). Probiotic effect of *Saccharomyces boulardii* concentrate filtrate (5:5) of yeast extract glucose peptone broth on the motilily phenomenon of *Proteus mirabilis* PR15 isolate on motility medium as a semi-solid medium after incubation for 24 hr at 37ºC.

Left: control (medium without probiotic); **Right**: (medium + probiotic).

Chapter Four

Conclusions and Recommendations

Chapter Four Conclusions and Recommendations

Conclusions:

1. Isolates of *Proteus mirabilis* varied in their resistant to the (13) antibiotics used in the study.
2. The yeast *Saccharomyces boulardii* was able to produce better inhibitory substances in the liqiud media rather than in the solid media.
3. First dilution concentrated filtrate of the yeast possessed very noticable inhibitory effect against swarming of *Proteus mirabilis* in the solid medium, in addition to limit motility of the bacteria in the semi-solid medium.

Recommendations:

1. Further studies are needed to investigate the effect of *Saccharomyces boulardii* on other virulence factors of *Proteus mirabilis*.
2. Studies are needed to extract and purify the most effective metabolites produced by the yeast *Saccharomyces boulardii* against the swarming and motility properties of *Proteus mirabilis*.
3. *In vivo* (experimental animals) study of the biological effect of the inhibitory substances produced by the yeast after infecting such animals by the pathogenic bacteria.

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Appendix -1-

Interpretation of Api 20 E Reactions

(Biomerienx, Api 20 System)

Microtube	Positive	Negative
ONPG	Yellow	Colorless
ADH	Red-Orange	Yellow
LDC	Orange	Yellow
ODC	Red-Orange	Yellow
CIT	Blue-Green / Green	Pale green / Yellow
H_2S	Black deposit	Colorless / Grayish
URE	Red-Orange	Yellow
TDA	Dark-Brown	Yellow
IND	Red Ring	Colorless
VP	Pink-Red	Colorless
GEL	Diffusion of Black Pigment	No diffusion
GLU	Yellow	Blue / Blue-green
MAN	Yellow	Blue / Blue-green
INO	Yellow	Blue / Blue-green
SOR	Yellow	Blue / Blue-green
RHA	Yellow	Blue / Blue-green
SAC	Yellow	Blue / Blue-green
MEL	Yellow	Blue / Blue-green
AMY	Yellow	Blue / Blue-green
ARA	Yellow	Blue / Blue-green
OX	Violet / Dark purple	Colorless / Light purple

الخلاصة

- جمعت ١٤٠ عينة ادرار من مرضى يعانون من اصابات التهاب المجاري البولية في مستشفيي اليرموك والكرامة ببغداد، ثم زرعت بطريقة النشر على وسطي اكار مكونكي واكار الدم وحضنت بحرارة ٣٧م° لمدة ٢٤ ساعة. اظهرت النتائج ان ٨٩ عينة منها اعطت نتيجة موجبة لتواجد البكتريا، فيما ادى التشخيص الاولي الى الكشف عن ٢٠عزلة تابعة الى جنس *Proteus*. وكانت نسبة تواجد هذه العزلات في الذكور ٧٠% اعلى منها في الاناث ٣٠%. وبعد التشخيص النهائي ساد النوع *Proteus mirabilis* بنسبة بلغت ٧٥% مقارنة بالنوع *Proteus vulgaris* الذي بلغت نسبة ٢٥%. وتم اختيار عزلات من النوع الاول للتجارب اللاحقة.
- لدى اختبار حساسية ١٥ عزلة تعود للنوع *Proteus mirabilis* لثلاثة عشر مضاداً حيوياً شائعة الاستخدام، اظهرت النتائج ان السبروفلوكساسلين كان افضل المضادات تاثيراً عندما لم تتجاوز نسبة المقاومة له ٦،٦ %. بينما كان البنسلين اقلها تاثيراً اذ بلغت نسبة مقاومته ٩٣،٣% من العزلات.
- اعتمادا على نتائج فحص الحساسية للمضادات الحيوية فقد اختبرت عزلة واحدة وهي (PR15) لاظهارها صفة المقاومة المتعددة للمضادات الحيوية، ليتم اعتمادها للتحري عن عوامل الضراوة المهمة لهذه البكتريا ولا سيما ظاهرة االانثيال (Swarming) على الوسط الصلب والحركة (Motility) في الوسط شبه الصلب.
- استخدمت عزلة من خميرة *Saccharomyces boulardii*، بعد ان تم التاكد من حساسيتها ضد المضاد الحيوي سايكلوهيكساميد واختبرت قدرتها على انتاج مواد مضادة لنمو البكتريا *Proteus mirabilis* (PR15) ولا سيما تاثيرها على ظاهرتي الانثيال والحركة. فضلا عن اختبار قدرة الخلايا البكتيرية المرضية على الالتصاق بخلايا الخميرة للتخلص من التصاق البكتريا بخلايا العائل.
- بغية الحصول على افضل فعالية تثبيطية فقد نميت الخميرة في اربعة اوساط زرعية سائلة (اثنان جاهزان والاخران محضران محليا) وعلى وسطين صلبين جاهزين، اظهرت النتائج افضلية الاوساط السائلة عن الصلبة في اعطاء الفعالية التثبيطية وذلك عندما ادى راشح الخميرة (الذي تم الحصول عليه من التنمية في الوسط السائل) والمركز لثلاث مرات الى تثبيط حركة وانثيال البكتريا.
- حددت الظروف المثلى الممكنة لزيادة فعالية المواد المثبطة وشملت هذه الظروف الاس الهيدروجيني، درجة الحرارة، فترة الحضن ونوع الوسط الزرعي، اذ امكن الحصول على افضل فعالية للمواد المثبطة عند تنميتها في وسطي سابرود وخلاصة الخميرة-ببتون-كلوكوز السائلين باس هيدروجيني ٤،٥ وفي وسطي خلاصة التمر وخلاصة اوراق الشاي السائلين باس هيدروجيني ٥،٥ لمدة ٤٨ ساعة بدرجة ٣٧م°.

- حدد التركيز المثبط الادنى (MIC,s) لراشح الخميرة المنماة في كل وسط والمركز لثلاث مرات، كما واختبرت فعالية هذا الراشح المركز في كل وسط على ظاهرة الانثيال (Swarming) وعلى الحركة (Motility) واظهرت النتائج ان هنالك تاثير واضح في شل حركة الانثيال البكتيري (Swarming) على الطبق وتحديد انتشار الحركة (Motility) في انبوب الاختبار.

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قسم التقانة الاحيائية

تاثير Probiotic على عوامل الحركة وظاهرة الانثيال لبكتريا *Proteus mirabilis*

رسالة

مقدمة الى كلية العلوم جامعة النهرين

وهي جزء من متطلبات نيل درجة ماجستير علوم في التقانة الاحيائية

من قبل

ريم وليد يونس زين العابدين

بكلوريوس تقانة احيائية جامعة النهرين ٢٠٠٢

ربيع الثاني ١٤٢٦

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Table (3-2): Sensitivity of 15 isolates of *Proteus mirabilis* to 13 antibiotics.

Symbol of Isolate	Antibiotic												
	P	**AMX**	**PIP**	**CTX**	**GM**	**STE**	**AK**	**TE**	**N.A.**	**CIP**	**R.D.**	**C**	**SXT**
PR1	R	R	R	S	R	R	S	R	S	S	R	R	S
PR2	R	R	R	R	R	R	S	R	R	S	S	S	S
PR3	R	R	R	S	S	R	S	R	S	S	R	R	R
PR4	R	R	S	R	S	S	S	R	S	S	S	R	S
PR5	R	R	R	S	S	R	S	R	S	S	R	R	R
PR6	R	R	R	S	R	R	R	R	S	S	R	R	S
PR7	R	S	S	R	S	S	S	S	S	S	S	S	S
PR8	R	R	R	S	S	R	S	R	S	S	S	R	R
PR9	R	S	S	R	S	R	S	R	R	S	R	S	S
PR10	R	R	R	S	S	R	S	R	S	S	S	R	R
PR11	S	S	S	R	S	S	S	S	S	S	S	S	S
PR12	R	R	R	S	R	R	R	R	R	S	S	R	R
PR13	R	R	R	S	S	R	S	R	S	S	S	R	S
PR14	R	R	S	S	S	R	S	R	S	S	R	R	S
PR15	R	R	R	S	R	R	R	R	R	R	S	R	R

S= Sensitive; **R=** Resistance; **P=** Penicillin G; **AMX=** Amoxicillin; **PIP=** Pipracillin; **CTX=** Cefatoxime; **GM=** Gentamycin; **STE=** Streptomycin; **AK=** Amikacin; **TE=** Tetracycline; **N.A.=** Naldixic acid; **CIP=** Ciprofoxacin; **R.D.=** Rifampicin; **C=** Chloramphenicol; **SXT=** Co-trimethaxole.