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**Ministry of Higher Education and Scientific Research**
**Al-Nahrain University**
**College of Science**
**Department of Biotechnology**

# Isolation and Identification of Klebsiella Accompanying Accident Wounds and Treatment with Probiotics

**A Thesis**

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

**By**

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**Saba**

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# Summary

A total of (100) samples were collected from patients (referred to three hospitals in Baghdad) suffering of accident wounds included traumatic wounds, foot ulcer and burns. The samples were collected from both sexes during the period 10/12/2009 – 14/3/2010. A total of 22 isolates of *Klebsiella* were identified depending on cultural, microscopic and biochemical characterizations. Other bacteria obtained were identified as *Escherichia coli*, *Pseudomonas* spp., *Enterobacter*, *Proteus* and *Citrobacter* in numbers 25, 23, 6, 5 and 2, respectively.

Biochemical and Api-20E system identification revealed that all *Klebsiella* isolates were belonged to the species *K. pneumoniae*.

Antibiotic susceptibility of *K. pneumoniae* isolates against (15) of all commonly used antibiotic was determined through disc-diffusion method. After that, well-diffusion method was used to investigate the resistance of *K. pneumoniae* isolates toward six selected antibiotic solutions. Results declared that, generally, the isolates were resistant to the antibiotics used except their sensitivity to imipenem (in the disc-diffusion method) and meronem (in the well-diffusion method).

Depending on the antibiotic susceptibility findings, only two isolates were selected; one from traumatic wounds and the other from burns, to study the inhibitory effect of probiotics and using combinations of probiotics- antibiotics in different ratios. For this purpose, the inhibitory effect of three filtrates of *Lactobacillus* isolates (*L. acidophilus, L. plantarutm* and *L. gasseri*) and a yeast isolate (*Saccharomyces boulardii*) were determined against the two *K. pneumoniae* isolates. Results showed that *Lactobacillus* filtrates were

more effective in the inhibitory effect on *K. pneumoniae* isolates than the yeast isolate did.

Regarding results of inhibitory effect of probiotics-antibiotics combination, it was found that a ratio of 5:5 each of combinations *L. acidophilus*: amoxicillin, *L. plantarum* : amoxicillin and *S. boulardii* : amoxicillin was the most effective on traumatic wound isolate. Moreover, *L. acidophilus* : amoxicillin in the same ratio gave effective result on the burn isolate.

Concerning combination with ceftriaxone, ratios of 9:1 and 1:9 (*S. boulardii*: ceftriaxone) and ratios 8:2 and 2:8 (*L. gasseri*: ceftriaxone) were the most effective treatments against isolate of traumatic wound. All probiotic filtrates when combined with ceftriaxone in a ratio 1:9 exhibited serous affect against burn isolate. Almost similar effect was given by the combination of the filtrates and ceftriaxone in ratios of 8:2 *L. acidophilus* : ceftriaxone and 2:8 of each of *L. plantarum* : ceftriaxone, *L. gasseri* : ceftriaxone. Effect of ofloxacin on the burn isolate was increased when mixing with all probiotic filtrates in ratios of 1:9 and 2:8 probiotic : antibiotic.

On traumatic wound, results revealed that the combination of probiotic and ofloxacin gave same (or slightly less) inhibitory effect of ofloxacin (alone). Moreover, mixing meronem with probiotic filtrates had no (or negative) effect in all ratios used against both *K. pneumoniae* isolates.

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# List of Abbreviations

| Abbreviation | Term |
|---|---|
| ADH | Arginine dihydrolase |
| BHI | Brain Heart Infusion |
| C3b | Complement protein |
| C5b-C9 | Complement proteins |
| CIT | Citrate |
| CPS | Capsular polysaccharide |
| D.W. | Distilled water |
| DNA | Deoxyribonucliec acid |
| EMB | Eosin Methylene Blue |
| ESBL | Extended Spectrum Beta Lactamase |
| FAO | Food and Agriculture Organization |
| GEL | Gelatin |
| IND | Indole |
| K-antigen | Capsular antigen |
| KIA | Kligler Iron Agar |
| LAB | Lactic acid bacteria |
| LDC | Lysin decarboxylase |
| LPS | Lipopolysaccharide |
| MRHA | Mannose-resistance hemagglutinins |
| MRS | DeManns Rogosa Sharp |
| MSHA | Mnnose-sensetive hemagglutinins |
| NCCL | National Committee for Clinical Laboratory Standard |
| ODC | Ornithin decarboxylase |
| rpm | Revolution per minute |
| SHV | Sulfhydral reagent variable |
| TDA | Tryptophan daaminase |
| TEM | Temoneria |
| URE | Urease |
| VP1-VP2 | Voges-Proskauer |
| WHO | World Health Organization |
| YEPD | Yeast Extract Peptone Dextrose |

| | |
|---|---|
| YNB | Yeast Nitrogen base |

# 1. Introduction and Literature Review:

## 1.1 Introduction

*Klebsiella* is among the five Gram negative pathogens most commonly encountered in hospital-acquired infections, due to it is ubiquitous in nature (Hansen *et al*., 2004). As opportunistic pathogens, *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer from sever underlying diseases such as diabetes mellitus or chronic pulmonary obstruction (Chien-Ko *et al*., 2002).

Nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, which is a common hospital-acquired pathogen causing sever respiratory infections such as pneumonia. Other infections caused by this organism include urinary tract infection, wound infection, abscesses and diarrhea (Feizabadi *et al*., 2007).

Treatment of *Klebsiella* infections is complicated. The over and widespread use of antibiotics has often been held responsible for the occurrence of multiply resistant *Klebsiella* strains in hospitals; leading to produces extended spectrum beta-lactamases (ESBLs) (Kang *et al*., 2004). ESBLs are enzymes encoded by genes on plasmids which can hydrolyze penicillins, cephalosporins and monobactam (Paterson *et al*., 2004). The majority of ESBL isolates are also resistant to other antibiotics as fluoroquinolones and aminoglycosides (Yum *et al*., 2005).

The term probiotic is used to describe microorganisms with antagonistic activity against pathogens (John *et al*., 1997). Probiotics are use to control infection of resistant pathogenic bacteria (Nash *et al*., 2002). So probiotics such as lactic acid bacteria (LAB) can be use as

alternative to the antibiotics due to their production of inhibitory compounds such as organic acid, hydrogen peroxides, diacetyl and bacteriocins and others against pathogenic bacteria (Riaz *et al*., 2010). The yeast, *Saccharomyces boulardii* possesses also a strong direct antagonist effect against number of pathogens (Vandenplas *et al*., 2008).

In addition, probiotic can be used as adjunct with the antibiotics to augment their effect and to reduce the side effects. *Lactobacillus* spp. and *S. boulardii* might be the ideal probiotics to be used for treatment in conjunction with antibiotics (Reid, 2006).

Due to the common occurrence of *Klebsiella* in the accident wound cases, and the safe use of probiotics in the treatment, this study was suggested and aimed to:

- Isolation and identification of *Klebsiella* from accident wounds.
- Identification the most common species in the accident wounds with concentration on *Klebsiella*.
- Identification of probiotic microorganisms.
- Application of the most effective probiotic in treatment of *Klebsiella*.
- Studying the effect of combination of probiotic with antibiotic on *Klebsiella*.
- Selecting the most effective ratio of probiotic-antibiotic combination against *Klebsiella*.

# 1.2 Literature Review

## 1.2.1 *Klebsiella* spp. (Overview):

Cells of *Klebsiella* spp. are Gram negative, rod-shape bacteria, non-motile (except *Klebsiella mobilis*), non-spore forming with a prominent polysaccharide capsule. This capsule encases the entire cell surface, accounts for mucus texture on culture media and it provides resistance against many host defense mechanisms (Cruickshank *et al*., 1975; Greenwood *et al*., 1997). Facultative anaerobic; having both a respiratory and a fermentative type of metabolism. *Klebsiella* is oxidase negative, glucose fermented with acid and gas production (more $CO_2$ is produced than $H_2$), and the Voges-Proskauer test is usually positive (Garrity, 2005).

*Klebsiella* species are ubiquitous in nature; in environment where they are found in surface water, sewage, soil and on plant. In humans *Klebsiella* may be regarded as normal flora in many parts of the colon and intestinal tract and other mucus surface ( Brisse and Duijkeren, 2005). Rosebury (1962) found that because Gram negative bacteria do not find good growth conditions on the human skin, *Klebsiella* spp. are rarely found there and are regarded simply as transient members of the flora (Kloos and Musselwhit, 1975). While Horan *et al*. (1988) declared that *Klebsiella* is among the five Gram negative pathogens most commonly encountered in hospital-acquired infections; common sites include the urinary tract, lower respiratory tract and surgical wound sites. *Klebsiella* spp. primarily attack immunocompromised individuals who are hospitalized and suffer

from sever underlying disease such as diabetes mellitus or chronic pulmonary obstruction (Chien-Ko *et al*., 2002).

Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae* which is the most frequently occurring species (De La *et al*., 1985; Watanakunakorn, 1991; Hansen *et al*., 1998). To much lesser degree *Klebsiella oxytoca* has been isolated from human clinical specimens, much more rarely encountered are *Klebsiella ozaenae* and *Klebsiella rhinoscleromatis* which have been retained as separate species because of their association with specific diseases (Hansen *et al*., 2004).

Due to the extensive spread of antibiotic-resistance strains, especially of extended spectrum β-lactamase (ESBL) producing strains, there has been renewed interest in *Klebsiella* infections (Jacoby and Medeiros, 1991; Bush, 2001).

## 1.2.2 Taxonomy of The Genus *Klebsiella*:

The genus of *Klebsiella* belong to the family *Enterobacteriaceae*. The organisms are named after Edwin Klebs, a nineteenth century German microbiologist (Garrity, 2005). The first *Klebsiella* strain ever described was a capsulated bacillus isolated from patient with rhinosecleroma (Frisch, 1882).

Originally, the medical importance of genus *Klebsiella* led to its being subdivided into three species corresponding to the diseases they caused: *K. pneumoniae, K. ozaenae* and *K. rhinoscleromatis* (Jawetz *et al*., 2010).

As the taxonomy became increasingly refined due to the development of new methods such as numerical taxonomy, the species classification in this genus was continually revised. In time, three main classifications emerged, those of Cowan, Bascomb and Ørskov, as show in table (1-1) (Podschun and Ullmann, 1998).

**Table (1-1):** Species classification of the genus *Klebsiella* by different taxonomic systems (Podschun and Ullmann, 1998).

| **Classification System:** | | |
|---|---|---|
| **Cowan** | **Bascomb** | **Ørskov** |
| *K. aerogenes*<br>*K. edwardsii*<br>sudsp. *edwardsii*<br>subsp. *atlantae*<br>*K. pneumoniae*<br>*K. ozaenae*<br>*K. rhinoscleromatis* | *K. aerogenes* /*oxytoca*/<br>*edwardsii*<br>*K. pneumoniae*<br>*K. ozaenae*<br>*K. rhinoscleromatis*<br>*K.* "unnamed group"<br>*Enterobacter aerogenes* | *K. pneumoniae*<br>subsp. *pneumoniae*<br>subsp. *ozaenae*<br>subsp. *rhinoscleromatis*<br>*K. oxytoca*<br>*K. terrigena*<br>*K. planticola* (syn. *K. trevisanii*)<br>*K. ornithinolytica* |

In the early 1980s, *Klebsiella* isolates from the environment, which had been previously classified as "*Klebsiella*-like organisms" (groups J, K, L and M), were increasingly being classified into provisional taxa (Gavini *et al*., 1977). These groups gave raise to four new species: *K. terrigena* (Lzard *et al*., 1981), *K. ornithinolytica*

(Sakazaki *et al*., 1989), *K. planticola* (Bagley *et al*., 1981) and *K. trevisanii* (Ferragut *et al*., 1983). In 1986, the last two species were combined into one species *K. planticola*, because of their extensive DNA sequence homology (Gavini *et al*., 1986). The pathogenicity of these species for humans has been considered negligible, as they mainly have been isolated from environmental habitats. Recent studies, however, demonstrated that these new species can also be isolated from clinical specimens (Mori *et al*., 1989; Liu *et al*., 1997). They were isolated from the respiratory tract secretions, wound and urine isolates as the next most common (Podschun and Ullmann, 1994).

Finally, species *Enterobacter aerogenes* is often regarded as an eighth member of the genus *Klebsiella* (*Klebsiella mobilis*) (Ørskov, 1984; Hansen *et al*., 2004).

Adoption of a consistent nomenclature has been further complicated by the fact that England and the former commonwealth countries adhere to the classification of Cowan, while the USA prefers Ørskov's classification. Consequently, same bacterium may be called *K. pneumoniae* in one country and *K. aerogenes* in another. Most European countries follow the American example and recognize the worldwide predominant classification of Ørskov (Podschun and Ullmann, 1998).

### 1.2.3 Virulence Factors of *Klebsiella*:

*Klebsiella* virulence factors differ depending on the sites of infection because the host defense mechanisms differ from site to other (Highsmith and Jarvis, 1985).

Several bacterial factors were known to contribute to the pathogenic mechanisms of *Klebsiella* infections (Williams and Tomas, 1990). Podschun and Ullmann (1998) documented that the virulence factors of *Klebsiella* species focuses on five major bacterial factors: capsule, pili, serum resistance, lipopolysaccharids and sidrophores, as shown in figure (1-1).

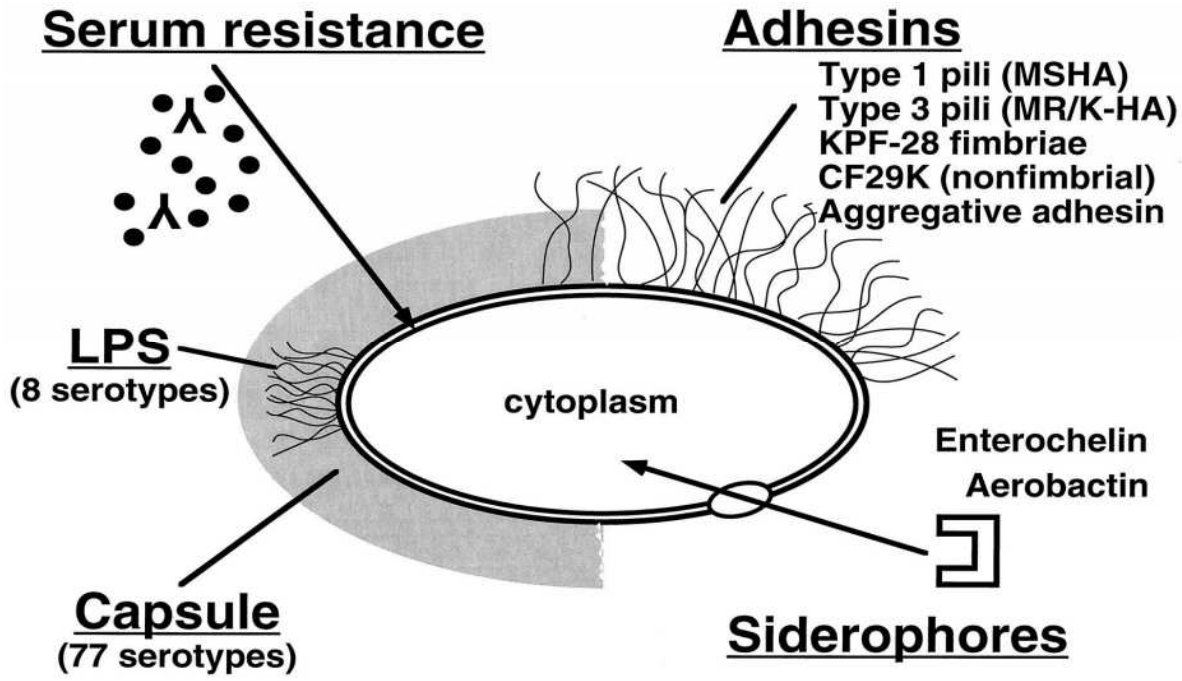



**Figure (1-1): Schematic representation of *Klebsiella* virulence factors** (Podschun and Ullmann, 1998).

### 1.2.3.1 Capsular antigens (K-antigen):

*Klebsiella* cell usually develop prominent capsules composed of complex acidic polysaccharides which are classified into 77 serological types (Ørskov and Ørskov, 1984). Capsules are essential to the virulence of *Klebsiella* (Cryz *et al*., 1984; Highsmith and Jarvis, 1985). The capsular materials form thick bundles of fibrillose structures covering bacterial surface in massive layers (Amako *et al*., 1988).

According to Simoons-Smith *et al*. (1986) and Podschun *et al*. (1992), capsular materials protect the bacterium from phagocytosis by polymorphonuclear granulocytes on the one hand, and prevents killing the bacteria by bactericidal serum factors on the other hand (Williams *et al*., 1983). A part from their antiphagocytic function, *Klebsiella* capsule polysaccharides have been reported to inhibit the differentiation and functional capacity of macrophage *in vitro* (Yokochi *et al*., 1979).

While *Klebsiella* capsular polysaccharide (CPS) were generally considered to mediate virulence properties, Mizuta *et al*. (1983) found that this consideration has recently been abandoned because of the great differences in virulence observed among different capsular types; strains expressing the capsule antigens K1 and K2 were found to be especially virulent, whereas isolates of other serotypes showed little or no virulence. Simoons-Smith *et al*. (1984) also reported that *Klebsiella* strains of serotypes K1, K2, K4 and K5 were more virulent than were those expressing other capsule types.

Podschun and Ullmann (1992) reported that the degree of virulence conferred by a particular K-antigen might be connected to the mannose content of the CPS, *Klebsiella* serotypes containing mannose repeating sequences kill by macrophages with mannose receptor, such as K7 and K21 which is low virulence because both of them contain these repeating sequences (Ofek *et al*., 1993). In contrast, Ofek *et al*. (1993) reported that strains that lack these repeating sequences are not recognized by macrophages and hence phagocytosis dose not take place. This model is consistent with the

marked virulence of K2 which completely lacks mannose repeating sequences.

Thus, *Klebsiella* strains bearing capsule types devoid of these sequences should be closely associated with infectious disease (Kabha *et al*., 1995).

### 1.2.3.2 Pili:

Pili are nonflagellar, filamentous projections on the bacterial surface, they consist of polymeric globular protein subunits (pillin) (Jones and Isaacson, 1983).

The first step in the infectious process is the adherence in which microorganisms must come as close as possible to host mucosal surfaces and maintain this proximity by attaching to the host cell (Lai *et al*., 2000).

Ottow (1975) explained that pili are demonstrated mainly on the basis to their ability to agglutinate erythrocytes of different animal species. Depending on weather the reaction is inhibited by D-mannose, these adhesions are designated as mannose-sensitive or mannose-resistance hemagglutinins (MSHA and MRHA) respectively (Old *et al*., 1985). The different types of pili described in enterobacteria, there are two predominant types in *Klebsiella* spp. (Podschun *et al*., 1987; Dworkin, 2006).

**Type1 (common) pili:** which are the best investigated of the bacterial adhesions. They are mannose-sensitive hemgglutinins (MSHA) which agglutinate guinea pig erythrocytes. Clegg and Gerlach (1987) found that type1 pili mediate bacterial colonization of the urogenital and respiratory tracts.

**Type3 pili:** this type of pili agglutinate only erythrocytes that have been treated with tannin so it is called mannose-resistance hemagglutination (MRHA) (Clegg and Gerlach, 1987). Hornick *et al*. (1992) and Tarkkanen *et al*. (1997) declared that strains of *Klebsiella pneumoniae* expressing type 3 pili adhere to endothelial cells and epithelia of the respiratory tract.

### 1.2.3.3 Serum resistance:

In addition to the phagocytosis by polymorphonuclear granulocytes, the bactericidal effect of serum is the first line of defense by the host against invading microorganisms (Podschun and Ullmann, 1998). In response to this host defense, pathogenic microorganisms developed strategies to counter the serum bactericidal effect (Olling, 1977). Taylor (1983) considered the exact mechanism underlying bacterial serum resistance as unknown.

### 1.2.3.4 Lipopolysaccharide (LPS):

Capsule polysaccharides may cover and mask the underlying LPS and exhibited a surface structure that dose not activate complement cascade (Jawetz *et al*., 1998). The O side chains of the LPS may reach through the capsule layer and be exposed to the exterior milieu in certain *Klebsiella* capsule types (Tomas *et al*., 1988). Since LPS is generally able to activate complement cascade, C3b is subsequently deposited onto the LPS molecules. However, since it is fixed preferentially to the longest O-polysaccharide side chains, C3b is far from the bacterial cell membrane. Thus, the formation of the lytic membrane attack complex (C5b – C9) is prevented, and subsequent membrane damage and cell death do not take place (Alberti *et al*., 1996).

### 1.2.3.5 Siderophores:

Many bacteria attempt to secure their supply of iron in the host by secreting high-affinity, low-molecular-weight iron chelators, called sidrophores, that are capable of competitively taking up iron bound to host proteins (Griffiths *et al*., 1988). Griffiths (1987) documented that iron is an essential factor in bacterial growth, functioning mainly as a redox catalyst in proteins participating in oxygen and electron transport processes. Bullen *et al*. (1978) reported that the supply of free iron available to bacteria in the host milieu is extremely low, since this element is bound intracellularly to proteins (such as hemoglobin, ferritin, hemosiderin and myoglobin) and extracellularly to high-affinity iron-binding proteins (such as lactoferrin and transferrin). Under iron-deficient conditions, for instance in the host milieu, enterobacteria synthesize a variety of siderophores, which belong to two different chemical groups enterobactin and aerobactin. In the genus *Klebsiella*, the production including both enterobactin and aerobactin (Khimji and Miles, 1978) .

## 1.2.4 Resistance to Antimicrobial Medication:

Treatment of *Klebsiella* infections is complicated in cases in which causative organisms produce extended spectrum beta-lactamases (ESBLs) (Paterson *et al*., 2004). During the past two decades, Kang *et al*. (2004) found that broad-spectrum cephalosporins antibiotics have been used world wide, and antibiotic-resistant strains that produce extended spectrum β-lactamase (ESBL) have emerged among the *Enterobacteriaceae* family. In addition, Selden *et al*. (1971) and Tullus *et al*. (1988) declared that the widespread use or overuse of antibiotics had often been held responsible for the occurrence of multiply resistant

*Klebsiella* strains in hospitals. In the 1970s, Christensen *et al*. (1972) and Curie *et al*. (1978) reported that strains producing ESBLs were chiefly aminoglycoside-resistant. Also Bauernfeind *et al*. (1993) and French *et al*. (1996) documented that strains producing ESBLs were resistant to extended- spectrum cephalosporins.

Bradford (2001) explained that ESBLs are enzymes encoded by genes on plasmids which results in easy transfer of ESBL enzymes to other bacterial species. Jacoby and Sutton (1991) declared that in many cases, these plasmids also harbor other antimicrobial resistance genes. Therefore, it is common for organisms with ESBL phenotype to express multiple resistance to aminoglycosides, trimetheprim-sulfamethoxazole and tetracyclines (Feizabadi *et al*., 2008). Also Yum *et al*. (2005) found that ESBLs are enzymes which can hydrolyze penicillins as well as cephalosporins.

Originally, ESBL enzymes were derived from the widespread TEM and SHV β-lactamase family, over 110 derivatives of TEM β-lactamases and more than 63 derivatives of SHV β-lactamases are known (Hawkey and Munday, 2004).

Both Bradford (2001) and Feizabadi *et al*. (2007) documented that the ESBL producing isolates of *K. pneumoniae* has created major problem in antibiotic therapy due to lack of effective drugs against some multi resistance isolates. It is generally thought that patients having infections caused by an ESBL-producing organisms are at increased risk of treatment failure with an extended spectrum β-lactam antibiotics (Feizabadi *et al*., 2008).

## 1.2.5 Wound Infection:

Wound infection has been regarded as the most common nosocomial infection (Dionigi *et al*., 2001). A wound is a breach in the skin, and the exposure of subcutaneous tissue following loss of skin integrity which provides a moist, warm and nutritive environment that is conducive to microbial colonization and proliferation (Shittu *et al*., 2002). Characteristic local responses are a purulent discharge or painful spreading erythema indicative of cellulitis around a wound (CDC. 1992; Peel, 1992).

Davis *et al*. (1992) found that wounds could be broadly categorized as having either an acute or a chronic etiology. Acute wounds are caused by external damage to intact skin and include surgical wounds, bites, burns, minor cuts and abrasions, and more sever traumatic wounds such as lacerations and those caused by crush or gunshot injuries (Davis *et al*., 1992). Chronic wounds such as leg ulcers, foot ulcers are most frequently caused by endogenous mechanisms associated with a predisposing condition that ultimately compromises the integrity of dermal and epidermal tissue (Davis *et al*., 1992).

Banjara *et al*. (2003) reported that their were many factors thought to affect the susceptibility of any wound to infection, some of which strongly predispose to wound infection. These factors include pre-existing illness, length of operation, wound class and wound contamination (Bowler *et al*., 2001). Other factors such as extremes of age, malignancy, metabolic diseases, malnutrition, immune-suppression, cigarette smoking and long duration of preoperative hospitalization are not considered as independent risk factors for wound infection (Sawyer and Pruett, 1994).

Duerden (1994) explained that wounds contaminants are likely to originate from three main sources the environment, the surrounding skin and endogenous sources.

Banjara *et al*. (2003) found that surgical wound and skin infections account for one third of the nosocomial infections among surgical patients. Brachman (1981) declared that Gram negative organisms (like *E. coli*, *K. pneumoniae* and *Pseudomonas* spp.) were more prevalent than Gram positive organisms in surgical wound infection. While Bhatt and Lakhey (2008) and Akinjogunla *et al*. (2009) on the other side reported that *Staphylococcus aureus* was the predominant microorganisms followed by *Pseudomonas* spp., *E. coli* and *K. pneumoniae* in automobile accident wound and other infected wounds. But Kehinde *et al*. (2004) found that *Klebsiella spp*. was the most frequent pathogens followed by *Pseudomonas* spp., *S. aureus* in infected burn wounds. Controlling of wound infections has become more challenging due to widespread bacterial resistant to antibiotic (Akinjogunla *et al*., 2009).

## 1.2.6 Probiotics:

### 1.2.6.1 History:

The actual of probiotic concept belongs to Lily and Stillwell in 1965, after which probiotics are characterized as "microorganisms that promote growth of other microorganisms" (Lily and Stillwell, 1965). In 1974, Parker talks about a food supplement for livestock and improve name of probiotics as "organisms and substances that helps the microbial ecosystem" (Parker, 1974). Their importance was highlighted by Fuller in 1989 who described probiotics as live

microorganisms with beneficial effects on host body, improving intestinal microbial balance (Fuller, 1989). The universal meaning of the term "probiotic" was established by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United States. These two organizations defined probiotics as "live microorganisms which when administrated in adequate amounts, have a beneficial effect on health of the host organisms" (Corcionivosch *et al*., 2010).

### 1.2.6.2 Features of probiotics:

A good probiotic agent needs to be non-pathogenic, nontoxic, resistant to gastric acid, adhere to gut epithelial tissue and produces antibacterial substances (Wadher *et al*., 2010). It should persist, albeit for short periods in the gastrointestinal tract influencing metabolic activities like cholesterol assimilation, lactose activities and vitamin production (Suvarana and Boby, 2005).

### 1.2.6.3 Mode of probiotics action:

Probiotic microorganisms are considered to support the host health, so that there are many studies trying to explain the mechanisms of action of probiotics. These mechanisms are listed below briefly (Castagliuoluolo *et al*., 1999; Salminen *et al*., 1999; Rolfe, 2000; Çak1r, 2003; Wadher, 2010).

- Production of inhibitory substances: Production of some organic acids, hydrogen peroxide and bacteriocins which are inhibitory to both Gram positive and Gram negative bacteria.

- Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition, probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites.
- Competition for nutrients: Probiotics inhibit the pathogens by consuming the nutrients which pathogen need.
- Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease.
- Degradation of toxin receptor: Because of the degradation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii* a probiotic protects the host against *Clostridium difficile* intestinal disease.

Some other studies on the mechanisms suggested suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks *et al*., 1999; Corcionivoschi *et al*., 2010).

### 1.2.6.4 Probiotic microorganisms:

Probiotics are used for long times in food ingredients for human and also to feed the animals without any side effects (Holzapfel *et al*., 2001). Also probiotics are acceptable because of being naturally in the intestinal tract of healthy human and in foods (Çakır, 2003). The most commonly microorganisms used as probiotic preparations are shown in table (1-2).

The most commonly utilized probiotic preparations include specific strains (of either alone or in combination) *Lactobacilli*, *Streptococci* and *Bifidobacteria* (Fuller, 1991). John *et al*. (1997) reported that these three genera are important components of the gastrointestinal flora, are considered to be harmless, and might be capable of preventing of pathogenic bacteria. *Lactobacilli* are perhaps the most well known of these favorable microorganisms. Fuller and Gibson (1997) documented that number of *Lactobacilli* species reside in the human intestine in a symbiotic relationship with each other and with other microorganisms. They are generally considered essential for maintaining gut micro floral health; however, it is the overall balance of the various microorganisms which is ultimately of most importance (Dunne *et al*., 2001).

In addition, Ducluzeau and Bensaada (1982) reported that *S. boulardii*, a yeast species similar to brewer's yeast, has demonstrated a direct antagonistic effect *in vivo* in mice against *Candida albicans*, *C. krusei* and *C. pseudotropicals* strains. Results from experimental animals also showed *S. boulardii* inhibits the action of cholera toxin on enterocytes (Dias *et al*., 1995).

**Table (1-2):** Microorganisms considered as probiotics (Holzapfel *et al*., 2001).

| ***Lactobacillus* spp.** | ***Bifidobacterium* spp.** | **Other lactic acid bacteria** | **Nonlactic acid bacteria** |
|---|---|---|---|
| *L.acidophilus*<br>*L.amylovos* | *B. adolescentis*<br>*B. animalis* | *Enterococcus faecalis* | *Bacillus cereus var. toyoi* |

| | | | |
|---|---|---|---|
| *L. casei*<br>*L. crispatus*<br>*L.delbrueci*<br>subsp.<br>*bulgaricus*<br>*L.gallinaru*<br>*L. gasseri*<br>*L. johnsonii*<br>*L. paracasei*<br>*L. plantarum*<br>*L. reuteri*<br>*L.rhamnoss* | *B. bifidum*<br>*B. breve*<br>*B. infantis*<br>*B. lactis*<br>*B. longum* | *Enterococcus*<br>*faecium*<br>*Lactococcu lactis*<br>*Leuconstoc*<br>*mesenteroides*<br>*Pediococcus*<br>*acidilactici*<br>*Sporolactobacills*<br>*inulinus*<br>*Streptococcus*<br>*thermophilus* | *Escherichia coli*<br>strain nissle<br>*Propionibacterim*<br>*freudenreichii*<br>*Saccharomyces*<br>*cerevisiae*<br>*Saccharomyces*<br>*boulardii* |

## 1.2.7 Effect of Probiotics on Pathogenic Bacteria:

Probiotics in theory can be composed of any live microbe. A large number of probiotics belongs to the *Lactobacillus* or *Bifidobacterium* genera. Also popular is *Saccharomyces boulardii* (yeast). (Sanders, 2009).

*Lactobacillus* group of bacteria is famous for its uses as probiotic and in food preservation. It has own reputation due to its production of inhibitory compounds such as organic acid , hydrogen peroxide, diacetyl and bacteriocins (Brooks *et al*., 1998).

Bacteriocins are proteins inhibiting other bacteria living in the same ecological place. So, *Lactobacilli* use it as a weapon for its survival (Todorov, 2009). Moghaddam *et al*. (2006) reported that bacteriocins had been withdrawn special interest of microbiologist for

the control of pathogenic bacteria. Some investigations had been declared the ability of bacteriocins to inhibit pathogenic bacteria like *E. coli*, *Pseudomonas* and *Klebsiella* (Ogunshe *et al*., 2007; Karthikeyan *et al*., 2009; Raja *et al*., 2010). De Souza *et al*. (2005) reported that among the *Lactobacillus* species, *L. acidophilus* and *L. plantarum* had been extensively utilized as probiotics cultures in dairy and pharmaceutical products.

In addition the antimicrobial effect of *Lactobacillus* spp., Vandenplas *et al*. (2008) demonstrated that *S. boulardii* had been a strong direct antagonist effect against a number of pathogens. *In vitro* studies had shown that *S. boulardii* reduced growth of *C. albicans*, *E. coli*, *Shigella, Salmonella typhimurium*, *P. aeroginosa, S. aureus* (Czerucka *et al*., 2002). Czerucka *et al*. (2007) found that several studies indicated two main mechanisms of action of *S. boulardii* against enteric pathogens: production of factors that compete with bacterial toxins and modulation of the host cell signaling pathways implicated in proinflammatory response during bacterial infection.

### 1.2.8 Probiotics as Alternative and Adjuncts to Antibiotics:

Development of the antibiotic-resistant strains of bacteria especially *Klebsiella* spp. producing extended spectrum beta-lactamase (Pitout *et al*., 2005; Kluytmans-Vandenbergh *et al*., 2005), which cause

the majority of infections, responsible for the biggest problems because of mounting drug resistance. Finegold (1986) and Reid *et al.* (1990) explained that the major problem associated with antibiotics, in addition to the side effects, is causing destruction to the normal microbiota. Additionally, use of antibiotics predisposing an individual to the development of intestinal, urinary tract or vaginal infections (John *et al*., 1997).

The term of probiotic (opposite of antibiotics) is relatively new and refer to organisms which have a significant influence on the treatment and prevention of disease (McFarland and Elmer, 1995; Corcionivoschi *et al*., 2010). John *et al*., (1997) declared that *Lactobacillus* spp. and *S. boulardii* might be the ideal probiotics to give in conjunction with antibiotics.

Reid (2006) reported that there are perhaps three areas in which probiotics may acts as adjuncts to the antibiotics. Probiotic may:

- Reducing the risk of antibiotic-induced super infections in the gut and vagina;
- Secreting antibacterial substances that lower the pathogenic bacterial populations locally and at distant mucosal sites, and disrupt biofilms, making it easier for antibiotics to function; and
- Enhancing generalized mucosal immunity, which in turn aids in the eradication of the organisms at the mucosal site.

# 2. Materials and Methods

## 2.1 Materials:

### 2.1.1 Apparatus and Equipments:

The following apparatus and equipments were used in this study:

| Apparatus or equipment | Company (Origin) |
|---|---|
| Anaerobic jar | Rod Well (England) |
| Autoclave | Express (Germany) |
| Compound light microscopic | Olympus (Japan) |
| Cooling centrifuge | Sigma (USA) |
| Digital balance | Ohans (France) |
| Electric oven | GallenKamp (England) |
| Filter papers | Whatman No.1 (England) |
| Forceps | Olympus |
| Incubator | GallenKamp |
| Laminar flow hood | Memmert (Germany) |
| Micropipette | Gilson (France) |
| Millipore filters | Sartorius (Germany) |
| pH-meter | Radiometer (Denmark) |
| Shaker incubator | GLF (Germany) |
| Water distiller | GLF |

## 2.1.2 Chemical and Biological Materials:

| Materials | Company (Origin) |
|---|---|
| Api 20E | Biomerieux (France) |
| Ammonium sulfate | BDH (England) |
| Aniline blue | |
| Calcium carbonate | |
| Dextrose | |
| Barium chloride | |
| Diammonium citrate | |
| $KH_2PO_4$ | |
| Glucose | |
| Glycerol | |
| L-histidine | |
| Lactic acid | |
| Lactose | |
| $MgSO_4.7H_2O$ | |
| $MnSO_4.4H_2O$ | |
| Peptone | |
| Phenol crystals | |
| Sodium acetate hydrate | |
| Succenic acid | |
| Sulfuric acid | |
| Sucrose | |

| | |
|---|---|
| Mannose | |
| Manitol | |
| Xylose | |
| Raffinose | |
| Arabinose | |
| Galactose | |
| Bromocresol purple | Biolife (Italy) |
| Litmus | |
| Skim milk | |
| Agar-agar | Himedia (India) |
| Yeast extract | |
| Ethanol | Local market (Iraq) |
| Sodium hydroxide | Merck (Germany) |
| Isoamyl alcohol | Oxoid (England) |
| p-dimethylamine-benzaldehyde | |
| Gelatin | |
| Sodium chloride | |
| Meat extract | |
| Tween 80 | |
| Hydrochloric acid | Sigma (USA) |
| Ribose | |
| Tetramethyl-p-phenylene diamine | |

## 2.1.3 Culture Media:

### 2.1.3.1 Ready-to-use media:

The following media were prepared and autoclaved after adjusting the pH as mentioned on their containers by the manufacturing companies:

| Medium | Company (Origin) |
|---|---|
| Nutrient agar | Difco (USA) |
| Nutrient broth | |
| Kligler Iron Agar (KIA) | |
| Simmon citrate agar | |
| Eosin Methylene Blue (EMB) | Himedia (India) |
| Brain Heart Infusion broth (BHI) | |
| MacConkey agar | Oxoid (England) |
| Sabouraud broth | |
| Yeast Nitrogen Base (YNB) | |

### 2.1.3.2 Laboratory-prepared media:

- Peptone broth medium
- Urea agar base medium
- Motility semisolid medium
- DeMan, Rogonsa and Sharpe (MRS) agar
- DeMan, Rogonsa and Sharpe (MRS) broth
- MRS-$CaCO_3$ agar
- Litmus milk medium
- Gelatin medium

- Sugar fermentation medium
- Yeast Extract Peptone Dextrose medium (YEPD)
- Minimal medium

## 2.1.4 Probiotic Isolates:

| Isolate | Source |
|---|---|
| *Lactobacillus acidophilus* | Biotechnology Department/Al-Nahrain University (from vagina) |
| *Lactobacillus gasseri* | |
| *Lactobacillus plantarum* | |
| *Saccharomyces boulardii* | Biocodex (France) |

## 2.1.5 Antibiotics:

### 2.1.5.1 Antibiotic discs (Bioanalyse/Turkey):

| Antibiotic | Disc symbol | Concentration (µg) |
|---|---|---|
| Amikacin | AK | 30 |
| Amoxicillin | Ax | 25 |
| Amoxicillin-clavulanic acid | AMC | 30 |
| Ampicillin | Am | 10 |
| Aztreonam | ATM | 30 |
| Carbincillin | Py | 10 |
| Ceftazidime | CAZ | 30 |
| Ceftriaxone | CRO | 30 |

| | | |
|---|---|---|
| Cephalothin | CF | 30 |
| Ciprofloxacin | Cip | 5 |
| Doxycycline | Do | 30 |
| Gentamicin | CN | 10 |
| Imipenem | IPM | 10 |
| Ticarcillin-cavulanic acid | TIM | 85 |
| Tobramycin | Tob | 10 |

### 2.1.5.2 Antibiotic powders:

The following ready-to-use antibiotic powders were dissolved in distilled water and prepared to obtain solutions with concentrations 100 mg/ml, each:

| **Antibiotic** | **Company (Origin)** |
|---|---|
| Amikacin | Zhejiang Ruixin Pharmaceutical (Germany) |
| Amoxicillin (Moxyzyl) | Mission Vivacare Limited (India) |
| Ceftriaxone | Zhongshan (China) |
| Ciprofloxacin (Ciproxene) | Midical Bahri (Syria) |
| Imipenem (Meronem) | Dainippon Sumitomo (UK) |
| Ofloxacin | Indofarma (Indonesia) |

# 2.2 Methods:

## 2.2.1 Sterilizing methods (Baily *et al.*, 1990):

Three methods of sterilization were used:

### 2.2.1.1 Wet-heat sterilization (autoclaving):

Microbial culture media, solutions, buffers, and reagents were sterilized by the autoclave at 121˚C (15 Ib/inch$^2$) for 15 min unless otherwise stated.

### 2.2.1.2 Dry-heat sterilization (oven):

Electric oven was used to sterilize glassware at 180˚C for 3 hrs.

### 2.2.1.3 Membrane filtration:

Bacterial filtrates, sugar solutions, and antibiotic solutions were sterilized throughout (0.45) and (0.22) µm in diameter millipore filters, and Whatman No.1 filter papers.

## 2.2.2 Preparation of Reagents, Stains and Solutions:

### 2.2.2.1 Reagents:

- **Kovac's Reagent:** (Collee *et al*.,1996)

It was prepared by dissolving 5 g of p-dimethlamine-benzaldehyde in 75 ml of isoamyl alcohol, then 25 ml of HCI (6 %) was added, and stored in a glass - Stoppard bottle in refrigerator.

- **Oxidase reagent:** (Collee *et al*., 1996)

This reagent was prepared by dissolving 1 g of tetramethyl-p-phenylene diamine dihydrochloride powder in 100 ml D.W.

- **Catalase reagent:** (Collee *et al*., 1996)

This reagent was prepared by mixing 3 ml of hydrogen peroxide solution (22%) with 97 ml D.W.

### 2.2.2.2 Stains:

- **Gram stain:**

Ready to use stains and reagents (crystal violet, gram iodine, ethanol 95%, safranin stain) were obtained prepared from Fluka / Switzerland, which were used for staining bacterial isolates for microscopic examination.

- **Lactophenol- cotton blue stain:** (Atlas *et al*.,1995)

It was used for the staining of fungi, for microscopic examination, and prepared from the following ingredients:

| | |
|---|---|
| Lactic acid | 20 ml |
| Phenol crystals | 20 g |
| Glycerol | 40 ml |
| D.W | 20 ml |
| Cotton blue or Aniline blue (Poirier's blue) | 0.05 g |

Phenol was dissolved in lactic acid, glycerol and distilled water by gentle heating, then aniline blue was added. The mixture was left for 24 hrs at room temperature, then filtered through filter paper (Whatman filter paper No.1).

### 2.2.2.3 Solutions:

- **Sugar solutions:** (Harrigan and MacCance, 1976)

Sugars used in *Lactobacillus* fermentation tests were prepared by dissolving suitable carbon source (glucose, ribose, sucrose, lactose, mannose, mannitol, xylose, raffinose, arabinose and galactose), separately, in D.W. All sugar solutions were sterilized by membrane filtration throughout 0.22 µm millipore filter.

- **Normal saline:** (Brown and Poxton, 1996)

It was prepared by dissolving of 0.85 g NaCl in l00 ml of distilled water and sterilized by autoclaving.

- **Turbidity standard solution:** (Vandepitte *et al*., 1991)

Mcfarland No. 0.5 ($1\times10^8$ CFU/ml) solution was prepared by mixing 0.6 ml of barium chloride with 99.4 ml of sulfuric acid, then the solution was preserved in dark place.

## 2.2.3 Laboratory-Prepared Media :

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

This medium was used for identification of *Klebsiella* spp., and prepared by dissolving 5 g of peptone in l00 ml of distilled water. It was distributed in test tubes (5 ml each) before sterilized by autoclaving, then stored at 4 ºC until use.

- **Urea agar base medium:** (Atlas *et al*., 1995)

It was prepared by dissolving 24 g of urea base in 50 ml of distilled water, pH was adjusted to (6.5-7.0) and sterilized by autoclaving. Then 50 ml of 20% urea solution previously sterilized by using millipore filter unit 0.22 µm were added aseptically, after that it was placed in sterile tubes to solidify as slant. This medium was used for identification of *Klebsiella* spp.

- **Motility semi solid medium:** (Cruickshank *et al*., 1975)

It was used for identification of *Klebsiella* spp., which is prepared by dissolving 0.8 g of nutrient broth powder and 0.4 g of Agar-agar in 100 ml of distilled water. After preparation the medium was dispensed in test tubes (5 ml to each one) and sterilized by autoclaving.

- **DeMan, Rogonsa and Sharpe (MRS) agar:** (DeMan *et al*., 1960)

The medium was used for growth and identification of *Lactobacillus* spp. and prepared from the following ingredients:

| | |
|---|---|
| Peptone | 10 g |
| Meat extract | 10 g |
| Yeast extract | 10 g |
| $KH_2PO_4$ | 5 g |
| Glucose | 20 g |
| Diammonium citrate | 2 g |
| Sodium acetate | 5 g |
| $MgSO_4$ | 0.5 g |
| $MnSO_4$ | 0.2 g |
| Tween 80 | 0.1 ml |
| Agar- Agar | 15 g |
| D.W. | 1 L |

All ingredients above were mixed and dissolved in 950 ml D.W., then pH was adjusted to 6.2 using glacial acetic acid. Volume was completed with D.W. to 1000 ml before sterilizing medium by the autoclave.

- **DeMan, Rogonsa, and Sharpe (MRS) broth:** (DeMan *et al*., 1960)

It was used for growth and identification of *Lactobacillus* spp. and containing all the ingredients of the MRS without agar.

- **MRS - $CaCO_3$ agar:** (Harrigan and MacCance, 1976)

This medium was used for identification of *Lactobacillus* spp. and prepared by adding 1 g of $CaCO_3$ to each 100 ml of MRS agar, then medium was sterilized by the autoclave

- **Litmus milk:** (Baron and Finegold, 1994)

It was used for identification of *Lactobacillus* spp. and prepared as follows:

| | |
|---|---|
| Skim milk | 100 g |
| Litmus | 5 g |
| D. W. | 1000 ml |

All ingredients were mixed and dissolved in 1000 ml D.W., then sterilized by the autoclave at 121˚C for 5 min.

- **Gelatin medium:** (Collee *et al*., 1996)

It was used for identification of *Lactobacillus* spp. and prepared by adding 12 g of gelatin to each 100 ml of brain heart infusion broth, then medium was sterilized by the autoclave.

- **Sugar fermentation medium:** (Harrigan and MacCance, 1976)

This medium was used for identification of *Lactobacillus* spp. prepared by using MRS broth free from glucose and meat extract but with the addition of 0.005% (V/V) bromocresol purple. After pH was adjusted to 6.2, the medium was distributed in test tubes, then sterilized by the autoclave. Later on, each sugar solution was aseptically added separately, to the medium to obtain a final concentration of 2%.

- **Yeast Extract PeptoneDextrose medium(YEPD):**(Barnett *et al*., 1985)

This medium was used for growth of *Saccharomyces boulardii*. It is composed from the following ingredients:

| | |
|---|---|
| Yeast extract | 15 g |
| Peptone | 10 g |
| Dextrose | 20 g |
| Agar | 18 g |
| D. W. | 1000 ml |

All ingredients were mixed and dissolved in 1000 ml of distilled water, before sterilized by autoclaving.

- **Minimal medium (Mm):** (Halvorson, 1958)

It was used for growth of *Saccharomyces boulardii.* and composed from the following ingredients :

| | |
|---|---|
| Yeast nitrogen base | 1.6 g |
| Succinic acid | 5 g |
| NaOH | 6 g |
| Ammonium sulfate | 1 g |
| D.W | 1000 ml |

All ingredients were mixed and dissolved in 1000 ml of distilled water, then the medium was sterilized by autoclaving.

## 2.2.4 Samples Collection :

Samples were collected from patients suffering from acute and chronic wounds with purulent discharge or painful spreading erythema

around wounds including continuous abscesses, traumatic wounds, foot ulcer and burns. A total of 100 patients from all age groups attending Al-Yarmook, Al-Kadhmiaa and Al-Kindy Teaching Hospital in the period between 10/12/2009 to 14/3/2010 were included in the study. Pus was collected from wounds by sterile disposable cotton swabs and immediately inoculated onto MacConkey agar plates before incubated at 37 ºC for 24 hrs. Questioner forma was prepared and filled for each patient including the name, date, address, sex, age, occupation, complaint, short notes about his illness, duration and the cause of illness condition of patients.

## 2.2.5 *Klebsiella* Isolation (Holt *et al*., 1994):

The collected samples were immediately inoculated onto MacConkey agar plates and incubated at 37 ºC for 24 hrs, then the colonies with pink color and mucous texture were subcultured onto eosin methylene blue (EMB) plates for isolation of *Klebsiella* spp.

## 2.2.6 Identification of *Klebsiella* spp.:

Identification of suspected isolates was done according to the colony morphology, Staining reaction and biochemical tests (Atlas *et al*.,1995; Macfaddin, 2000; Garrity, 2005).

### 2.2.6.1 Staining reaction:

Smears were prepared from the suspected colonies on clean glass slides and fixed by heat, then stained by Gram stain to examine cells shape, grouping, gram reaction microscopically (Atlas *et al*., 1995).

### 2.2.6.2 Biochemical tests :

Following tests were performed according to (Macfaddin, 2000; Garrity, 2005).

**2.2.6.2.1 Indole test:**

Peptone broth into the test tubes were prepared as in item (2.2.3) inoculated with fresh culture of bacteria before incubated at 37 ºC for 24 hrs. A portion of 0.5 ml Kovac's reagent was added for each test tubes. Appearance of red ring at the top of the broth indicates a positive result.

**2.2.6.2.2 Simmon citrate test:**

Simmon citrate slants inoculated (with a sterilized needle) by streaking the slant agar with organisms, then tubes were incubated at 37 ºC for 24 hrs. Growth and changing medium color from green to blue indicates a positive result.

**2.2.6.2.3 Sugars fermentation and gas production test:**

Kligler iron agar (KIA) slant tubes were inoculated with an inoculating needle by streaking the slant, then tubes were incubated at 37 ºC for 24 hrs. Results were recorded as follows:

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

**2.2.6.2.4 Urease test:**

Urea agar slants were prepared as in item (2.2.3) and inoculated with fresh culture of bacteria, then incubated at 37 ºC for 24 hrs. Changing medium color to purple - pink indicates a positive result.

#### 2.2.6.2.5 Motility test

Semi solid motility medium which was prepared as in item (2.2.3) was inoculated by single stabbing with a needle inoculated with the suspected colony, then incubated at 37 ºC for 24 hrs. Movement away from the stab line or a hazy appearance through the medium indicated a motile organism.

## 2.2.7 Api Identification of *Klebsiella* Isolates:

The reagents and indicators (IND,TDA,VP1-VP2), which were used in Api-20E system had been prepared according to the Manufacturer's instruction kit of (BioMrrieux), all isolates were examined by this kit as following:

### 2.2.7.1 Preparation of the strips:

Five milliliters of tap water were placed into the Api incubation tray, then Api test strip was withdrawn from the sealed package and placed into the incubation tray.

### 2.2.7.2 Preparation of the inoculums:

Aseptically, one suspected colony was picked from EMB agar plate with a sterilized loop and transferred to a test tube containing 5 ml of sterile normal saline solution which was prepared as in (2.2.2.3). After shaking well, tube was recapped before comparing its turbidity with the McFarland No.0.5 solution which was prepared as in (2.2.2.3).

### 2.2.7.3 Inoculation of strips prepare:

Bacterial suspension was transferred to the Api test strip by a sterile pasture pipette, the Api strip was tilted and the microtube was

filled by placing the pipette tip against the side of the cupule, both the tube and cupule sections of the **CIT**,**VP** and **GEL** microtubes were filled.

But the cupule sections of **ADH**, **LDH**, **ODC**, **$H_2S$** and **URE** microtubes were filled completely with sterilize mineral oil to prepare anaerobic conditions. Then the test strip was incubated at 37ºC for 24 hrs. After incubation the reagents were added to **TDA**, **IND** and **VP** microtubes.

## 2.2.8 Maintenance of *Klebsiella* Isolates:(Maniatis *et al*., 1982)

### 2.2.8.1 Short term storage:

Bacterial isolates were maintained for a period of few weeks by subcultured on MacConkey agar plates, wrapped tightly with parafilm and stored at 4 ºC.

### 2.2.8.2 Medium term storage:

Tubes that contained (5 – 8) ml of sterile nutrient agar in slants position were cultured with the bacterial isolate, then incubated at 37°C overnight, before stored at 4°C.

### 2.2.8.3 Long term storage:

A single colony of *Klebsiella* was used to inoculate a test tube contained 10 ml of sterile brain heart infusion broth and incubated at 37°C for 24 hrs, then 8.5 ml of the cell suspension was mixed with 1.5 ml of glycerol (20%), and stored at (-20) °C.

## 2.2.9 Antibiotic Sensitivity Test:

### 2.2.9.1 Disc-diffusion method :

The antibiotic susceptibility pattern of *Klebsiella* isolates were studied by standard disk diffusion method according to National Committee for Clinical Laboratory Standards (2002) as follows:

- Five ml of sterile brain heart infusion broth were inoculated with 0.1 ml of the fresh culture of each tested *Klebsiella* isolate and incubated at 37 ºC 24 hrs.
- A sterile cotton swab is dipped into the inoculom and swabbed evenly across the surface of nutrient agar plate.
- The inoculated plates were placed at room temperature for 30 min to allow absorption of excess moisture, then the antibacterial – containing disks were placed firmly on the inoculated plates with a forceps to ensure contact with the agar before incubated at 37ºC for 24 hrs.
- After incubation, diameters (mm) of the inhibition zones were measured and compared with that of standards of the NCCLs.

### 2.2.9.2 Well-diffusion method :

Five ml of sterile brain heart infusion broth were inoculated with 0.l ml of the fresh culture of each tested *Klebsiella* isolate and incubated at 37ºC 24 hrs. A sterile cotton swab was dipped into the inoculom and swabbed evenly across the surface of nutrient agar plate.

The well diffusion method that described by (Gupta *et al*.,1998) was used. By a cork borer, 5 mm diameter (5) wells were made on nutrient agar surface (previously inoculated with isolate), then each well was filled with 50 µl of antibiotic solution and incubation at 37ºC for 24 hrs. After incubation, diameters (mm) of each inhibition zone was

measured and compared with the control well that contain distilled water only.

## 2.2.10 *Lactobacillus* Isolates.

### 2.2.10.1 Identification of *Lactobacillus* spp.:

#### 2.2.10.1.1 Morphological characteristics:

Isolates of *Lactobacillus* spp. mentioned in item (2.1.4) were at first identified according to morphological and cultural characteristics (shape, color, size, edges and height) of colony on MRS agar plates.

#### 2.2.10.1.2 Microscopic examination:

A loopfull of fresh culture of each *Lactobacillus* isolate was fixed on a glass slide, then stained by Gram's stain and examined under the oil – immersion lens of a compound light microscope for cells shape, grouping, gram reaction (Harely and Prescott, 1996).

#### 2.2.10.1.3 Biochemical tests:

- **Growth on MRS Agar containing $CaCO_3$:**

MRS agar medium containing 1% $CaCO_3$ was streaked by 0.1 ml of *Lactobacillus spp.* isolates culture, and incubated anaerobically at 37 $^oC$ for 24 hrs. Appearance of clear zone around the bacterial colonies due to utilizing of $CaCO_3$ represents a positive result (Harrigan and MacCance, 1976).

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

This test was performed by adding drops of hydrogen peroxide (3%) over a mass of bacterial cells placed on a microscopic slide. Production of gaseous bubbles indicates a positive result.

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

A single colony was transferred using a sterilized wooden stick on to a filter paper, then a drop of the oxidase reagent was added. Changing colony color to dark purple indicates a positive result.

- **Gelatinase test:** (Baron and Finegold, 1994)

The test detects ability of the bacterial isolates to liquefy gelatin by stabbing bacteria into tubes contained gelatin medium. After incubation anaerobically at 37°C for 3 days, tubes were placed in the refrigerator at 4°C for an hour. The positive result was observed when gelatin is liquefied.

- **Acid and curd production in litmus milk:** (Forbes *et al.*, 2002)

Litmus milk was used for determining the action of bacteria on milk. *Lactobacillus* isolates were inoculated in the litmus milk medium and incubated at 37°C for 48 hrs. Positive result was recorded by presence of pink color, curd production and decreasing in pH value.

- **Sugar fermentation test:** (Collins and Lyne, 1985)

Tubes containing sugar fermentation medium were inoculated with 1% of *Lactobacillus* isolates, and incubated anaerobically at 37°C for 7 days. Changing in the bromocresol purple indicator color from purple to yellow indicates a positive result.

- **Growth at 15°C and 45 °C:** (Buck and Gilliland, 1995)

*Lactobacillus* isolates were inoculated in tubes contained MRS – broth and incubated at 15°C and 45°C anaerobically for 24 hrs. After that, growth was observed through turbidity as a positive result and compared with the control culture which has been incubated at 37°C.

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

- **Daily working culture:**

MRS broth was inoculated by the *Lactobacillus* isolates, and incubated at 37 ºC for 24 hrs in anaerobic jar. After incubation, l % of $CaCo_3$ was added to the tubes and stored at 4 ºC.

- **Stock culture:**

*Lactobacillus* isolate was cultured in MRS broth medium for 24 hrs at 37 ºC under anaerobic conditions, then 1 ml of a freshly prepared of bacterial culture was added to Bejo bottles containing 20% glycerol, and stored at -20 ºC.

## 2.2.11 *Saccharomyces boulardii*:

### 2.2.11.1 Activation of *S. boulardii*:

*Saccharomyces boulardii* was activated by cultured in sabouraud broth then subcultured onto sabouraud agar plate and incubated at 28 ºC for 24 hrs. Then loop full of the growing colonies was transferred on a glass slide, then stained with lactophenol-cotton blue, and examined under 40x magnification power of the compound light microscope (Barnett *et al*.,1990).

The identified yeast was grown in sabouraud slant, then a loop full of the yeast isolates grown on the surface transferred to inoculate 50 ml of YEPD broth in 250 ml conical flasks as in (2.2.3) before culture was incubated in shaking incubator at 28ºC for 48 hrs.

### 2.2.11.2 Activation of yeast to produce antimicrobial substance:

The yeast isolate was grown in the minimal medium (prepared as in item 2.2.3) (Halvorson, 1958), with the addition of 20 g glucose, 10 g yeast extract, 10 g peptone, 0.05 g gelatin and 30 mg histidine, then the medium was distributed in 100 ml conical flask (each contained 50 ml), before sterilized by the autoclave. After that the flask was inoculated with 1 ml of yeast isolate suspension that already grown for 48 hrs, and incubated at 28 ºC for 24 hrs.

### 2.2.11.3 Maintenance of *S. boulardii*: (Barnett *et al*.,1990)

- Yeast isolate was grown in YEPD agar medium for 48 hrs at 30ºC, then preserved in 4ºC with the consideration of renewing the cultures each two months.
- Yeast isolate was preserved also by suspending yeast cells that were already grown on YEPD slant in 5 ml of YEPD broth, then 4 ml of the suspension were transferred into a test tube containing 4 ml of glycerol and mixed thoroughly. Then mixture was preserved by freezing at -20 ºC.

## 2.2.12 Inhibition Effect of Probiotic Filtrates Against *Klebsiella* Isolates:

### 2.2.12.1 Unconcentrated filtrates :

The filtrates of *Lactobacillus* spp. and *S. boulardii* were obtained by preparing batch culture from each probiotic isolates which mentioned in item (2.1.4) each of LAB isolates was growing in MRS broth with pH 6 then incubated under anaerobic condition at 37 ºC for 24 hrs (Lewus *et al*.,1991). While *S. boulardii* was growing in minimal medium in item (2.2.11.2) (Wilkins, l949). After that, the isolates were centrifuged at (6000 rpm) for 10 min to obtain cell - culture -free liquid, which was

sterilized by filtration using (0.45) and (0.22) µm millipore filter units (Piard *et al*.,1990 and Martinez- Gonzalez *et al*., 2004).

The well diffusion method described by Gupta *et al*., (1998) was used to determine the inhibitory effect of *Lactobacillus* spp. and *S. boulardii* filtrates. A portion of 0.1 ml of *Klebsiella* culture was spread on nutrient agar plate by a sterile glass spreader, 5 mm diameter (5) wells were made in the medium by a cork borer, then each well was filled with 50 µl of the filtrate and incubated at 37ºC for 24 hrs. Inhibition zone diameter was measured and compared with the control wells that contained MRS broth only and the other contain sabouraud broth (Martinez - Gonzalez *et al*., 2004).

### 2.2.12.2 Concentrated filtrates :

Concentrated LAB and *S. boulardii* filtrates were used by putting 100 ml of unconcentrated filtrate in the oven at 40-45ºC until volume decreased to 50 ml (one-fold filtrate). The evaporation of filtrate continued in the oven until reducing the volume to 25 ml (two-ford filtrate). A three - fold filtrate was prepared by reducing the volume to 12.5 ml. Inhibition zones were compared with that of the unconcentrated filtrate.

## 2.2.13 Testing of Probiotic-Antibiotic Mixture Effect Against *Klebsiella* Isolates:

Serial ratios of probiotic - antibiotic mixtures were prepared by mixing different concentrations of three fold concentrated probiotic filtrates (*L.* spp. and *S. boulardii* filtrates) with antibiotic solutions. A single type of each probiotic filtrates was mixed in a sterile test tube with a single type of each antibiotic solution listed in (2.1.5.2) to

give a total volume a one ml of mixture as fallows: (1:9), (2:8), (3:7), (4:6), (5:5), (6:4), (7:3), (8:2), (9:1).

The well diffusion method described by (Gupta *et al*., 1998) was used to determine the inhibitory effect of probiotic-antibiotic mixture.

*Klebsiella* cultures were prepared by spreading 0.1 ml of *Klebsiella* isolates broth on the surface of nutrient agar plates, then the 5 wells were made by using cork borer with a diameter 5 mm and a depth 5 mm, each well was filled with 50 µl of probiotic - antibiotic mixture, then the plates were incubated at 37 ºC for 24 hrs and the inhibition zones diameters (mm) were measured and compared with the control wells that one of them contain MRS broth only while the other contain sabouraud broth only.

# 3. Results and Discussion

## 3.1 Isolation of Bacteria from Wounds:

A total of 100 samples were collected from patients suffering of various accident wounds refer to three hospitals in Baghdad. As shows in figure (3-1), 60% of the samples were from traumatic wounds, while the rest of samples from burns (22%) and foot ulcers (18%).

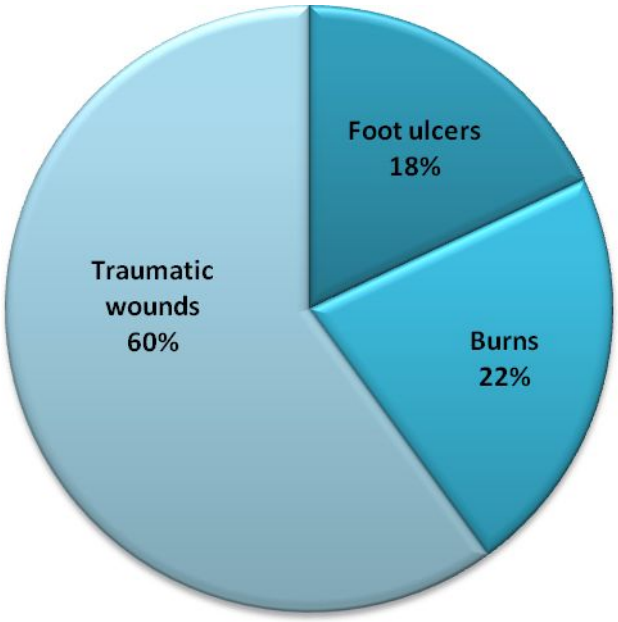



**Figure (3-1): Percentages of samples distributed according to their source of isolation.**

Regarding gender of patients, source of 67% of the samples were from males patients and 33% from females (table 3-1). Very high percentage (76.66%) of traumatic wounds were recorded by males patients compared to only 23.33% for females. Foot ulcer cases were also higher in males (61.11%) than in females (38.88%). Akinjogunla *et al.*

(2009) agreed with the findings of this study when they found that male represent high percentage in accident wounds cases than female.

Adversely, burn wounds were more frequent in females than in males patients with percentages of 54.54% and 45.45% respectively. Such results came close to the results of Kehinde *et al*. (2004).

**Table (3-1):** Numbers and percentages of samples according to wound types
and gender of patients.

| Isolation source | Male | | Female | | Percentage of total (%) |
|---|---|---|---|---|---|
| | No. | % | No. | % | |
| Traumatic wound | 46 | 76.66 | 14 | 23.33 | 60 |
| Burn | 10 | 45.45 | 12 | 54.54 | 22 |
| Foot ulcer | 11 | 61.11 | 7 | 38.88 | 18 |
| Total | 67 | 67 | 33 | 33 | 100 |

Table (3-2) shows the distribution of accident wound cases according to the age group of patients. Results shows that highest cases of traumatic wound (17 cases) were recorded by age group of (21-30) year and the lowest (6 cases) by the over 50 year of age group. These results agreement with Akinjogunla *et al*. (2009) findings.

For burn wounds, age group (31-40) year was the highest with (7) cases out of a total of 22 cases. In a similar study documented by Kehinde *et al*. (2004) fond that burn wound were significantly more frequent in age group (21-40) year.

On the other hand, foot ulcer was detected in only last two elder age groups (41-50) and >50 year with number of (5) and (13) cases respectively, other age groups were free of such accidents. Such results came close to those of Murugan *et al* (2010) findings. The same table also declared that all age groups were closed in their total numbers of accident wounds.

**Table (3-2):** Numbers of accident wound cases distributed according to the patients age group.

| Age group (year) | Traumatic wound | Burn | Foot ulcer | Total |
|---|---|---|---|---|
| ≤ 20 | 11 | 4 | - | 15 |
| 21-30 | 17 | 5 | - | 22 |
| 31-40 | 16 | 7 | - | 23 |
| 41-50 | 10 | 4 | 5 | 19 |
| > 50 | 6 | 2 | 13 | 21 |
| Total | 60 | 22 | 18 | 100 |

# 3.2 Identification of Bacterial Isolates:

## 3.2.1 Identification of *Klebsiella*:

Primary identification of suspected *Klebsiella* isolates were done by culturing on MacConkey agar plates. After incubation, 77 samples gave positive results for bacterial growth. Despite the majority of bacterial growth cases came from traumatic wound, but percentages of

occurrence in foot ulcer and burn cases were highest (88.88% and 86.36% respectively), than that of traumatic wound (70%).

A total of 83 bacterial isolates were obtained from the 77 samples tested. Moreover, 77.27% of the isolates from traumatic wound gave pink growth on the medium which are suspected to be *Klebsiella*, compared to 22.72% colorless isolates. In burn 68.18% of the isolates suspected to be *Klebsiella* which gave pink growth and the other (31.81%) were colorless isolates, while in foot ulcer only 29.41% of the isolates gave pink growth on MacConkey agar, and the rests 70.58% were colorless(table 3-3).

**Table (3-3):** Numbers and percentages of bacterial isolates obtained from accident wound cases.

| **Isolation source** | **No. of sample** | **Sample positive for bacterial Growth** | | **No. of bacterial isolate** | **Pink growth isolate** | | **Colorless growth isolate** | |
|---|---|---|---|---|---|---|---|---|
| | | **No.** | **%** | | **No.** | **%** | **No.** | **%** |
| Traumatic wound | 60 | 42 | 70 | 44 | 34 | 77.27 | 10 | 22.72 |
| Burn | 22 | 19 | 86.36 | 22 | 15 | 68.18 | 7 | 31.81 |
| Foot ulcer | 18 | 16 | 88.88 | 17 | 5 | 29.41 | 12 | 70.58 |
| Total | 100 | 77 | 77 | 83 | 54 | 65.06 | 29 | 34.93 |

The (54) bacterial isolates which produced pink colonies (growth) on MacConkey agar were subjected to the identification by cultural,

microscopic and biochemical characteristics. Results obtained are illustrated as follows:

### 3.2.1.1 Cultural characterization:

Identification of the (54) isolates were performed at first depending on the properties of colonies grown on the surface of both MacConkey and eosin methylene blue (EMB) agar. On MacConkey agar , *Klebsiella* colonies were pink in color, mucoid texture with large size (1.5-2.5 mm). Accordingly, out of the (54) isolates (53) isolates had properties similar to that of *Klebsiella* were suspected to belong to *Klebsiella*, such characteristics came in agreement with Collee *et al.* (1996).

In the identification of *Klebsiella*, a selective enrichment technique including culturing of samples on MacConkey agar was used. This medium contains bile salts and crystal violet which promotes growth of *Enterobacteriaceae* and related enteric Gram negative rods, in addition to suppresses growth of Gram positive bacteria and some fastidious Gram negative bacteria. Lactose in this medium is the sole carbon source that differentiates between lactose-fermenting bacteria and non lactose-fermenting bacteria. The first is characterized by producing pink colonies due to the conversion of neutral red indicator dye when it is below pH 6.8. Adversely, the non lactose bacterial growth appears colorless or transparent (Holt *et al*., 1994).

On the EMB agar only (30) isolates of the (54) were suspected to be belonged to *Klebsiella*. This medium is usually used to differentiate between *Klebsiella* and *E. coli*; because the aniline dyes (eosin and methylene blue) in this medium combines to form a precipitate at acidic pH and appearing as a metallic green sheen, thus serving as indicators for

acid production. Therefore, *Klebsiella* colonies appear in pink color but *E. coli* colonies be dark surrounded by a green metallic sheen due to the highly amount of acid produced by fermentation (Atlas *et al*.,1995).

### 3.2.1.2 Microscopic characterization:

The (30) isolates were identified depending on their Gram reaction and microscopic characteristics. From the suspected *Klebsiella* isolates, only (29) isolates were found to be Gram negative, small straight rods and arranged singly but messily in pairs under the compound light microscope. Such characteristics are usually considered on *Klebsiella*, as described by Garrity (2005).

### 3.2.1.3 Biochemical characterization:

Five biochemical tests were used for further identification of *Klebsiella* isolates from other lactose fermenting isolates. *Klebsiella* showed positive reactions for citrate and urease, but was negative for indole and motility tests. In Kligler Iron Agar (KIA) test, *Klebsiella* isolates turned the color of both the slant and butt to yellow with bubbles formation but without $H_2S$ production. These results were agreed with these declared by Garrity (2005).

In the indole test, ability to hydrolyze tryptophan to indole is a characteristic of certain enteric bacteria possessing the enzyme tryptophanase; an enzyme that decomposes amino acid tryptophan to indole, pyruvic acid and water. Indole negative bacteria such as *Klebsiella* produced no tryptophanase, so that when Kovac's reagent was added to a broth free of indole, a red ring will not be formed at the top of the broth (Collee *et al*.,1996).

Utilization of citrate is one of several important physiological test used to diagnose members of *Enterobacteriaceae*, so that the citrate in Simmon citrate medium is important to detect weather is able to grow on it as a unique carbon and energy source. In addition, Simmon's medium also contains bromothymol blue as a pH indicator. Growth of bacteria in this medium leads to produce acid and changing medium color from green to blue, reflecting it as positive citrate test, like *Klebsiella* (Macfaddin, 2000).

In the urease test, urease enzyme catalyzes the breakdown of urea, and the bacteria that can produce this enzyme is able to detoxify the waste products and to drive metabolic energy from its utilization which change the medium color from yellow to purple-pink, indicating urease positive test. *Klebsiella* can produce urease enzyme and gives urease positive test (Atlas *et al*.,1995).

Regarding KIA test, it differentiates the genera of *Enterobacteriaceae* from each other based on their carbohydrate fermentation patterns and $H_2S$ production. KIA slants contain 1% lactose and 1% glucose. The pH indicator (phenol red) changed the medium color from orange-red to yellow in the presence of acids. KIA also contains sodium thiosulfate, a substrate for $H_2S$ production, and ferrous sulfate which produces black precipitate to differentiate $H_2S$ producing bacteria from others.

In this study, *Klebsiella* isolates produced acidic slant (yellow) and acid butt (yellow) accompanied by gas production, but without black precipitate formation, which indicates that lactose and glucose fermentation had occurred and no $H_2S$ was produced (Garrity, 2005).

In the motility test, the movement of the growth away from the stab line or a hazy appearance through the semisolid medium indicates that the bacteria are motile. But the linear growth means negative result a property which *Klebsiella* is characterized by (Gwendolyn, 1988).

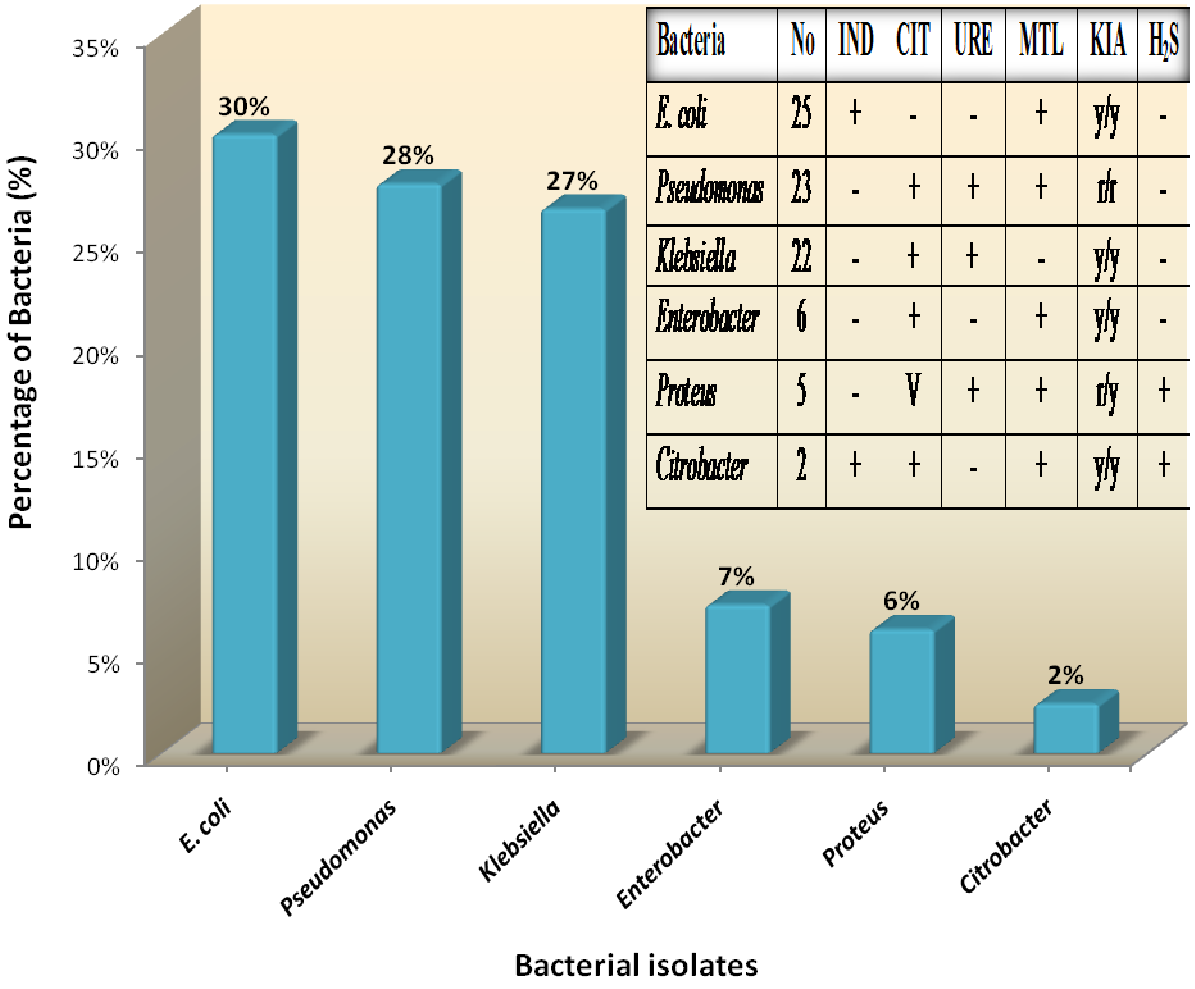
Results of biochemical characterization revealed that *Klebsiella* was represented (26.5%) from isolates. The percentage of *Klebsiella* occurrence in the patients of accident wound is closed to that reported by Cryz *et al*. (1984) who found that the risk of infection with *Klebsiella* spp. in several hospitalized patients including neonates, patients receiving respiratory therapy, urological and burn wound patients was increased. Podschun and Ullmann (1998) documented the principal pathogenic reservoirs for transmission of *Klebsiella* as the gastrointestinal tract and the hands of hospital personnel which increase the likelihood of person-to person transmission, in addition to the contaminated equipment which promoting the spread of *Klebsiella* spp.

*Klebsiella* isolates which were previously identified by the conventional biochemical tests were reidentified by using Api-20E system. Results revealed that all *Klebsiella* isolates were belonged to *K. pneumoniae* species. Dominance of *K. pneumoniae* among all other species was supported by the report documented by Hansen (1997) who found that this species was the most frequently occurring among other species, when its account for 75% of *Klebsiella* species in the hospitals. Horan *et al*. (1988) reported that nosocomial *Klebsiella* infections are caused mainly by *K. pneumoniae*, the medically most important species of the genus.

## 3.2.2 Identification of Other Bacteria:

Numbers and percentages of bacterial genera obtained from the (100) samples of accident wounds are illustrated in figure (3-2). The highest percentage (30.12%) of isolates recorded by *E. coli*, followed by *Pseudomonas* with 27.71%. *Klebsiella* came in the third place when its percentage accounted for 26.5%. Other bacterial isolates (*Enterobacter*, *Proteus* and *Citrobacter*) were occurred in percentages 7.22%, 6.02% and 2.4%, respectively. In a similar study documented by Bhatt and Lakhey (2008) found that *E. coli* was the most predominant Gram negative bacteria in wound infection in percentage (23.9%), followed by *Pseudomonas* (19.9%), *Klebsiella* (13%), *Citrobacter* (5.6%) and *Proteus* (3%).

Other genera were also detected and identified depending on the conventional biochemical tests. *E. coli* isolates were indole and motility positive but urease and Simmon citrate were negative, and had able to ferment both lactose and glucose, and produced gas and acids which change the color of both slant and butt in KIA to yellow but without $H_2S$ production (Macfaddin, 2000).




| Bacteria | No | IND | CIT | URE | MTL | KIA | $H_2S$ |
|---|---|---|---|---|---|---|---|
| *E. coli* | 25 | + | - | - | + | y/y | - |
| *Pseudomonas* | 23 | - | + | + | + | r/r | - |
| *Klebsiella* | 22 | - | + | + | - | y/y | - |
| *Enterobacter* | 6 | - | + | - | + | y/y | - |
| *Proteus* | 5 | - | V | + | + | r/y | + |
| *Citrobacter* | 2 | + | + | - | + | y/y | + |

**Figure (3-2): Percentages of genera of bacteria obtained from patients suffering of accident wounds. No; Number, IND; Indole, CIT; Simmon citrate, URE; Urease, MTL; Motility, KIA; Kligler iron agar, (-); negative, (+); positive, y; yellow, r; red.**

*Pseudomonas* showed positivity to Simmon citrate, urease and motility tests, but indole negative and could not ferment both lactose and glucose so the color of KIA remain red. *Enterobacter* similar to the *Pseudomonas* reactions in indole, Simmon citrate and motility, but unlike it in urease which is negative and in KIA; *Enterobacter* can ferment both lactose and glucose and change the color of slant and butt to yellow. *Proteus* exhibited positive reaction in urease and motility, but negative

reaction in indole and variable reaction in Simmon citrate. In addition, it can ferment glucose but not lactose so it is turned the color of butt to yellow but remain slant red. *Citrobacter* had positive reaction to indole, Simmon citrate and motility tests, but showed negative reaction to urease test. In KIA *Citrobacter* can turned of the color of slant and butt to yellow with black precipitation due to $H_2S$ production (Garrity, 2005).

## 3.3 Occurrence of Bacterial Isolates in Wound Cases:

As shown in figure (3-3), *Klebsiella* was the predominant bacteria in burn cases (54.54%) followed by *Enterobacter* (9%), *E. coli* (4.54%) and *Proteus* (4.54%). In traumatic wounds *E. coli* was more prevalent bacteria with 33.33% followed by *Pseudomonas* (18.33%) , *Klebsiella* (16.66%), *Enterobacter* (5%), *Proteus* (5%) and *Citrobacter* (3.33%).

Kehinde *et al*. (2004) agreed with the findings of this study when they found that *Klebsiella* was the dominant bacteria in burn cases, but with a lower percentage (34.4%) of occurrence. Another study on wound isolates by Haque and Salam (2010) found that *Klebsiella* was also predominant (33.33%) in burn cases, while *E. coli* came first in traumatic wound cases (33.85%).

In the foot ulcer cases, *Klebsiella* was not detected while *Pseudomonas* recorded the highest percentage (66.66%) among others *E. coli*, *Enterobacter* and *Proteus* with percentages 22.22%, 5.5% and 5.5%, respectively. Such results came lower than those of Murugan *et al*. (2010) who also documented that *Pseudomonas* was the most commonly bacteria isolated from foot ulcer cases when it constituted 81.08%.

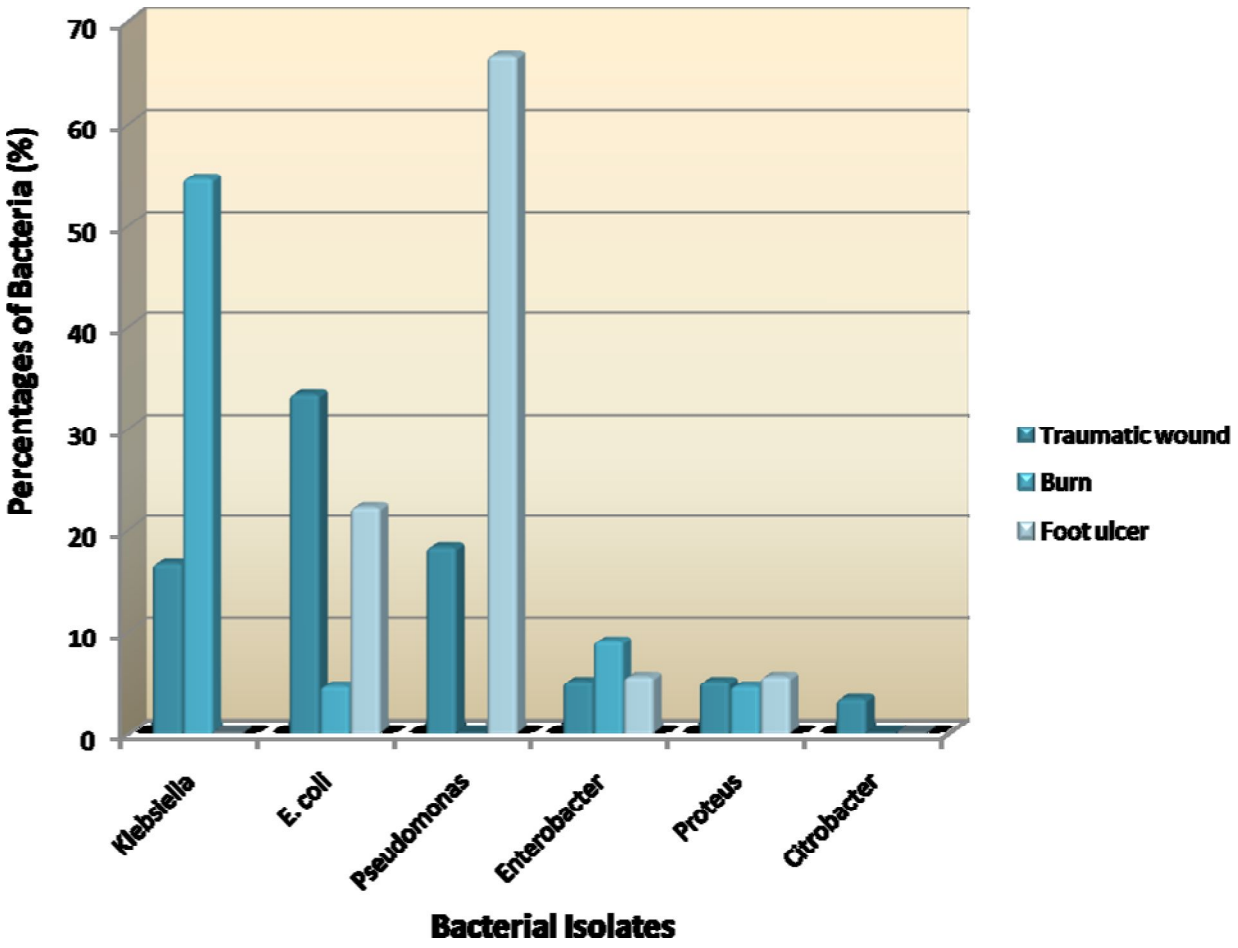



**Figure (3-3): Distribution of bacterial isolates according to the type of accident wounds.**

## 3.4 Antibiotic Susceptibility of *Klebsiella*:

### 3.4.1 Disc-Diffusion Method :

Susceptibility of the 22 isolates of *K. pneumoniae* was examined towards 15 different antibiotics using disc diffusion method

recommended by the National Committee for Clinical Laboratory Standards (NCCLs) guideline.

Results declared that with exception of imipenem, all isolates were completely resistant to other 14 antibiotics used.

Resistance of all *Klebsiella* isolates to the penicillin group (ampicillin, amoxicillin, amoxicillin-clvulanic acid, carbicillin) and to the cephalosporin group (ceftazidime, ceftriaxone, cephalothin) may be related to possessing of β- lactamase enzymes (penicillinase and cephalosporinase) which are able to the inactivate pencillins and cephalosprins through cleavage β-lactam ring of the drug (Levinson and Jawetz, 2000; Stock and Wiedmenn, 2001). Furthermore, Meyer *et al*. (1993) documented that TEM-1 and SHV-1 β-lactamase enzymes in *Klebsiella* also cause resistance to ampicillin. In this regard, Rice *et al*. (1996) and Shiappa *et al*. (1996) documented that their *K. pneumoniae* isolates were totally resistant to the carbincillin.

Resistance of *K. pneumoniae* isolates to the aminoglycosids (amikacin, gentamycin, tobramycin), monobactam (aztreonam) and tetracycline (doxycyclin) was reported in this study. In this regard, Feizabadi *et al*. (2007) found that *Klebsiella* isolates producing extended spectrum β-lactamase enzymes were resistant to aminoglycosids and monobactam. Adversely, Marranzano (1996) found that 90% of the *K. pneumoniae* isolates were sensitive to the tetracycline. Such variation in the susceptibility may due to the abuse treatment lead to elevate resistances to antibiotics (Rice *et al*., 1990).

The over use of antibiotic and widespread use of antimicrobial therapy has often been held responsible for the occurrence of multiply

resistant *Klebsiella* strains in hospitals, especially in persons receiving broad spectrum or multiple antibiotics (Selden *et al*., 1971; Tullus *et al*., 1988). So that the antibiotic use must be regulated in the hospitals to prevent misuse and over use of antibiotics.

Sensitivity of all *K. pneumoniae* isolates to the imipenem came in agreement with results obtained by other investigators. Livemore *et al*. (2001) reported that carbapenem (imipenem) antibiotics have strong activity against ESBLs from *Klebsiella* spp. Feizabadi *et al*. (2007) observed that *K. pneumoniae* isolated from respiratory was sensitive against imipenem. In another study *K. pneumoniae* isolated from different clinical specimens was susceptible to imipenem (Feizabadi *et al*., 2008).

## 3.4.2 Well-Diffusion Method:

Depending on the results obtained by disc diffusion method six antibiotic solutions (amoxicillin, ceftriaxone, amikacin, ciprofloxacin, ofloxacin, meronem) were selected to be used in the further experiments, after evaluation by well diffusion method.

Results in table (3-4) shows that meronem solution had a strongest effect on traumatic wound isolates when the diameter of inhibition zone reached to 35 mm. On burn isolates, meronem gave a maximum diameter of inhibition zone (28 mm).

As a carbapenem, meronem belongs to the β-lactam antibiotics which inhibit synthesis of bacterial cell wall. Such antibiotics considered to be resistance to the β-lactamase enzymes produced by multiple-resistant strains like *Klebsiella* (Brook *et al*.,2001).

While both ciprofloxacin and ofloxacin antibiotics belonging to the second generation of quinolins which inhibit bacterial DNA synthesis, table (3-4) declared that ofloxacin had a strong effect on *K. pneumoniae* isolates when compared with the ciprofloxacin. Such difference may be due to the widely use of ciprofloxacin in the hospitals which lead to develop new bacterial strains resistant to this antibiotic. Akinjogunla *et al*. (2009) found that ofloxacin was most effective on *K. pneumoniae* isolated from automobile accident wounds than ciprofloxacin. In this study, *K. pneumoniae* isolates obtained from traumatic wounds ($WK_1$, $WK_2$, $WK_3$, $WK_4$, $WK_5$, $WK_6$, $WK_7$, $WK_8$, $WK_9$, $WK_{10}$) were highly affected by ofloxacin when maximum inhibition zone reached to (30) mm. Less effect of ofloxacin was reported by bum isolates ($BK1_{11}$, $BK_{12}$, $BK_{13}$, $BK_{14}$, $BK_{15}$, $BK_{16}$, $BK_{17}$, $BK_{18}$, $BK_{19}$, $BK_{20}$, $BK_{21}$, $BK_{22}$) with highest inhibition zone reached to (23) mm.

Sensitivity of the isolates to amikacin also differed depend on the type of accident wounds. Its effect on the traumatic wound isolates reached to (23) mm diameter of inhibition zone, while it had no effect on the burn isolates. This may be due to the antibiotic medication previously used which led to develop more resistant strains (Kehinde *et al*., 2004). Ceftriaxone had no considerable effect on both traumatic wounds and burns isolates, while amoxicillin had no any effect on all isolates.

Two *K. pneumoniae* isolates were selected, they were $WK_2$ (from traumatic wound isolates) and $BK_{12}$ (from burn isolates). Then 4 of 6 antibiotic solutions were selected for their effect on the isolates (amoxicillin, ceftriaxone, ofloxacin, meronem). The isolates and antibiotic solutions were used in further experiment to determine the

effect of probiotics and probiotic-antibiotic combinations on *K. pneumoniae* isolates.

**Table (3-4):** Antibiotic susceptibility of *Klebsiella* isolates by using well-diffusion method, estimated as diameters of the inhibition zones (mm)*.

| Antibiotic / Isolate | ME | OF | Cip | CRO | AK | AX |
|---|---|---|---|---|---|---|
| $WK_1$ | 30 | 30 | 15 | 15 | 15 | 0 |
| $WK_2$ | 30 | 35 | 15 | 15 | 15 | 0 |
| $WK_3$ | 33 | 30 | 10 | 13 | 13 | 0 |
| $WK_4$ | 30 | 30 | 15 | 13 | 13 | 0 |
| $WK_5$ | 30 | 30 | 12 | 12 | 12 | 0 |
| $WK_6$ | 32 | 32 | 10 | 15 | 15 | 0 |
| $WK_7$ | 32 | 30 | 10 | 15 | 21 | 0 |
| $WK_8$ | 30 | 30 | 10 | 13 | 23 | 0 |
| $WK_9$ | 30 | 32 | 15 | 13 | 22 | 0 |
| $WK_{10}$ | 30 | 32 | 15 | 13 | 22 | 0 |
| $BK_{11}$ | 25 | 23 | 15 | 13 | 0 | 0 |
| $BK_{12}$ | 28 | 20 | 14 | 12 | 0 | 0 |
| $BK_{13}$ | 28 | 20 | 12 | 10 | 0 | 0 |
| $BK_{14}$ | 26 | 21 | 14 | 9 | 0 | 0 |
| $BK_{15}$ | 25 | 20 | 13 | 10 | 0 | 0 |
| $BK_{16}$ | 28 | 22 | 13 | 10 | 0 | 0 |
| $BK_{17}$ | 28 | 22 | 12 | 10 | 0 | 0 |

| | | | | | | |
|---|---|---|---|---|---|---|
| $BK_{18}$ | 25 | 23 | 14 | 10 | 0 | 0 |
| $BK_{19}$ | 28 | 20 | 14 | 10 | 0 | 0 |
| $BK_{20}$ | 26 | 20 | 13 | 10 | 0 | 0 |
| $BK_{21}$ | 26 | 22 | 13 | 10 | 0 | 0 |
| $BK_{22}$ | 24 | 22 | 12 | 9 | 0 | 0 |

**(*):** Diameter was calculated after subtracting the diameter of the well (5 mm). **ME;** Meronem, **OF;** Ofloxacin, **Cip;** Ciprofloxacin, **CRO;** Ceftriaxone, **AK;** Amikacin, **AX;** Amoxicillin.

# 3.5 Identification of Probiotic Isolates:

## 3.5.1 *Lactobacillus* spp.:

Lactic acid bacterial isolates in this study were obtained from Biotechnology Department at the College of Science/Al-Nahrain University. They were reidentified according to their cultural, microscopic and biochemical characteristics. Results are expressed as follows:

### 3.5.1.1 Cultural characterization:

Colonies of *Lactobacillus* isolates grown on MRS agar medium appears white to pale in color, 2-5 mm in diameter, round shape, soft, mucoid, convex and having smooth edges. On MRS agar containing $CaCO_3$, a clear zone around *Lactobacillus* colonies was observed, this due to the acid produced by these *Lactobacillus* isolates which dissolved the $CaCO_3$. Such cultural characteristics are concerned with those of *Lactobacillus* species (Jawetz *et al*., 1998).

### 3.5.1.2 Microscopic characterization:

After *Lactobacillus* colonies were Gram stained and examined. Results revealed that they were Gram positive, short and sometimes long bacilli, clustered in long and short chain. These results come in accordance with the corresponding microscopic characteristics that mentioned by Hammes and Vogal (1995).

### 3.5.1.3 Biochemical characterization:

Results indicated in table (3-5) shows that all *Lactobacillus* isolates (*L. acidophilus*, *L. plantarum* and *L. gasseri*) gave negative results for catalase, oxidase and gelatinase tests, because of their inability to produce catalase (that reduce hydrogen peroxide to water and oxygen gas bubbles), cytochrome oxidase (that oxidase tetramethyl-p-phenylenediamine dihydrochloride), and gelatinase (that hydrolyze gelatine), respectively, while these three isolates gave a positive result for litmus milk test because of there ability to produce rennin that ferments lactose to lactic acid, and lowering pH of litmus milk medium causing clot formation. These results were agreed to those observed by Holt and Krieg (1984).

The same table (3-5) shows that *L. plantarum* isolate was able to grow at 15˚C but not at 45˚C because it grouped under mesophillic group of *Lactobacillus*. While *L. acidophilus* and *L. gasseri* isolates were able to grow at 45˚C but not at 15˚C because they are grouped under thermophilic group of *Lactobacillus*. Such biochemical findings were similar to those mentioned by Kandler and Weiss (1986).

**Table (3-5):** Results of biochemical tests of *Lactobacillus* isolates.

| Test | *L. acidophilus* | *L. plantarum* | *L. gasseri* |
|---|---|---|---|
| Catalase | - | - | - |
| Oxidase | - | - | - |
| Gelatinase | - | - | - |
| Acid and curd | + | + | + |
| Growth at 15ºC | - | + | - |
| Growth at 45ºC | + | - | + |

On the other hand, table (3-6) reveal that the first isolate appeared to be *L. acidophilus* through their ability to ferment glucose, sucrose, lactose, mannose and galactose, but unable to ferment arabinose, mannitol, ribose, raffinose and xylose as reported by Carr *et al*. (2002).

Regarding to second isolate, their carbon fermentation patterns similar to that of *L. plantarum* by its ability to ferment all carbon sources except xylose as mentioned by Hammes and Vogal (1995).

While the third isolate appeared to be *L. gasseri* through their ability to ferment glucose, sucrose, mannose, rafinnose and galactose, but unable to do so with arabinose, xylose, mannitol, ribose and lactose sugars (Carr *et al*., 2002).

**Table (3-6):** Ability of *Lactobacillus* isolates to ferment carbon sources.

| **Sugar** | ***L. acidophilus*** | ***L. plantarum*** | ***L. gasseri*** |
|---|---|---|---|
| Arabinose | - | + | - |
| Ribose | - | + | - |
| Galactose | + | + | + |
| Glucose | + | + | + |
| Mannose | + | + | + |
| Xylose | - | - | - |
| Lactose | + | + | - |
| Sucrose | + | + | + |
| Raffinose | - | + | + |
| Mannitol | - | + | - |

**(-)**: negative
**(+)**: positive

## 3.5.2 *Saccharomyces boulardii*:

*Saccharomyces boulardii* which obtained in this study as a compacted capsule (Biocodex / France) was reidentified according to its cultural and microscopic characterizations.

Appearance of colonies on sabouraud agar was round, convex, soft, white to cream with regular edges as described by Barnett *et al*. (1985).

Under compound light microscope *S. boulardii* cells were ovoid and some of the cells were with budding. The cells were single form and others clustered like a beehive.

## 3.6 Inhibition Effect of Probiotics Against *Klebsiella*:

Well diffusion method was used to evaluate the inhibitory effect of probiotics filtrates *Lactobacillus* isolates (*L. acidophilus*, L. *plantarum*, *L. gasseri*) and yeast (*S. boulardii*). Probiotic isolates were activated by growth for 24 hrs, *Lactobacillus* isolates at 37ºC under anaerobic condition in MRS broth and *S. boulardii* at 28ºC in the minimal medium.

The unconcentrated filtrate of the probiotics isolates showed no inhibitory effect on *K. pneumoniae* isolates, and this may be due to the low concentration of the active compounds (Barefoot and Kaenhammer, 1983).

Results of inhibitory effect for concentrated filtrates of *L. acidophilus*, *L. plantarum*, *L. gasseri* and *S. boulardii* against *K. pneumoniae* were demonstrated in table (3-7). One-fold, Two-fold and three-fold prepared by concentrating the unconcentrated filtrates. Results revealed that the one-fold concentrated filtrates of *Lactobacillus* and *S. boulardii* had no inhibitory effect against *K. pneumoniae* isolates. Adversely, the two-fold filtrates of probiotics gave inhibition zone diameters of (5), (8), (6) and (1) mm (after subtraction 5 mm) for $WK_2$ isolate by *L. acidophilus*, *L. plantarum*, *L. gasseri* and *S. boulardii*, respectively. Regarding the $BK_{12}$ two-fold filtrates showed less effect with a maximum inhibition zone of 6 mm by the *L. acidophilus* filtrate. Three-fold concentrated filtrates resulted in the highest inhibitory effect after 24 hrs of incubation when the maximum inhibition zone reached to (17) mm by *L. plantarum* on $WK_2$ and 10 mm on $BK_{12}$ isolate by each by *L. acidophilus* and *L. plantarum*.

Same table (3-7) also illustrated that the three-fold concentrated filtrates of *Lactobacillus* isolates exerted more inhibitory effect on *K. pneumoniae* isolates than *S. boulardii* isolate, this was agreed with Corcionivoschi *et al*. (2010) who found in their study that strains of lactic acid bacteria were effective in removing or stopping the activity of pathogenic bacteria. Such result may be related with type of the active compounds produced, in which *Lactobacillus* isolates secrete number of inhibitory compounds such as organic acids, hydrogen peroxides, diacetyl and bactriocins which considered to be effective (Riaz *et al*., 2010).

**Table (3-7):** Inhibitory effect of concentrated filtrates of probiotic microorganisms against *K. pneumoniae* isolates estimated by diameter of inhibition zone (mm).

| **Isolate symbol** | **Concentrated filtrate** | **Diameter of inhibition zone (mm)*** | | | |
|---|---|---|---|---|---|
| | | **L. a.** | **L. p.** | **L. g.** | **S. b.** |
| $WK_2$ | One-fold | - | - | - | - |
| | Two-fold | 5 | 8 | 6 | 1 |
| | Three-fold | 10 | 17 | 15 | 3 |
| $BK_{12}$ | One-fold | - | - | - | - |
| | Two-fold | 6 | 5 | 4 | 1 |
| | Three-fold | 10 | 10 | 8 | 5 |

**(*):** Diameter was calculated after subtracting the diameter of the well (5mm). **L. a.;** *L. acidophilus*, **L. p.;** *L. plantarum*, **L. g.;** *L. gasseri*, **S. b.;** *S. boulardii.*

The results also revealed that *L. plantarum* was more effective on the traumatic wound isolate than the others two *Lactobacillus* isolates.

These results agreed with that results reported by Karthikeyan and Santosh (2009) that *L. plantarum* bacteriocin (plantaricin) showed broad range of antibacterial activity against some major food born pathogens. In addition, *K. pneumoniae* which isolated from burn exerted more resistance, such resistant may be due to the misuse of broad spectrum antibiotics (Zhang and Zhao, 2003). In burn cases, treatment was started with ampicillin as the first course after that changed to ampiclox (ampicillin and cloxacillin) and finally in case of septicemia cefuroxime were widely used, In addition to that tetracycline, gentamycin and chloramphenicol antibiotics ointment were used for face bum treatment.

The overuse of antibiotics for controlling infection as several investigators have demonstrated a close association between previous use of antibiotics and the emergence of subsequent antibiotic resistance in both Gram negative and Gram positive bacteria (Kollef and Fraser, 2001; Rasool *et al*., 2003), such resistance may be lead to the resistant of burn isolate to probiotic filtrates.

## 3.7 Effect of Probiotic-Antibiotic Combination on *Klebsiella*:

To determine the combination effect of probiotic and antibiotic on the two *K. pneumoniae* isolates ($WK_2$ and $BK_{12}$), serial ratios were prepared from the probiotic three-fold filtrates (*L. acidophilus*, *L. plantarum*, *L. gasseri* and *S boulardii*), and the four selected antibiotic solutions (amoxicillin, ceftriaxone, meronem and ofloxacin).

In the first experiment, each of the antibiotics and probiotics was applied individually against the two *K. pneumoniae* isolates. Results shows in table (3-8) revealed that regarding to *K. pneumoniae* ($WK_2$)

isolate, highest inhibition zone (17 mm) of probiotic filtrate was given by *L. plantarum* isolate. While that of antibiotic (35 mm) was produced by ofloxacin. Concerning isolate $BK_{12}$ of *K. pneumoniae*, *L. plantarum* also was the most effective when it resulted in (10 mm) of inhibition zone. Meronem was found to be the most effective antibiotic when the diameter of inhibition zone reached to 28 mm.

**Table (3-8):** Inhibitory effect of probiotic filtrates and antibiotic solutions against *K. pneumoniae* isolates estimated by diameter of inhibition zones (mm)*.

| Isolate symbol | Three-fold concentrated filtrate | | | | Antibiotic | | | |
|---|---|---|---|---|---|---|---|---|
| | L. a. | L. p. | L. g. | S. b. | ME | OF | CRO | AX |
| $WK_2$ | 10 | 17 | 15 | 3 | 30 | 35 | 15 | 0 |
| $BK_{12}$ | 10 | 10 | 8 | 5 | 28 | 20 | 12 | 0 |

(*): Diameter was calculated after subtracting the diameter of the well (5 mm). **ME;** Meronem, **OF;** Ofloxacin, **CRO;** Ceftriaxone, **AX;** Amoxicillin, **L.a.;** *L. acidophilus*, **L.p.;** *L. plantarum*, **L.g.;** *L. gasseri*, **S.b.;** *S. boulardii.*

Results of probiotic-antibiotic combination are illustrated as follows: The effect of ceftriaxone on $WK_2$ isolate increased when it was mixed with *S. boulardii* filtrate in ratio 1:9 and 9:1, when the inhibition zone reached to 23 mm, also its effect increased when it was mixed with *L. gasseri* in ratio 8:2 and 2:8 in which the diameter of inhibition zone reached to 20 mm, as shown in figure (3-4). Concerning to $BK_{12}$ isolate, ceftriaxone effect was increased when mixed with all probiotic filtrates in ratio 1:9 with inhibition zone 15 mm. Also *L. plantarum* and *L. gasseri* increased the effect of ceftriaxone in ratio 2:8 when the inhibition zone

reached to 15 mm. The same diameter of inhibition zone recorded when it was mixed with *L. acidophilus* in ratio 8:2, as shows in figure (3-5).

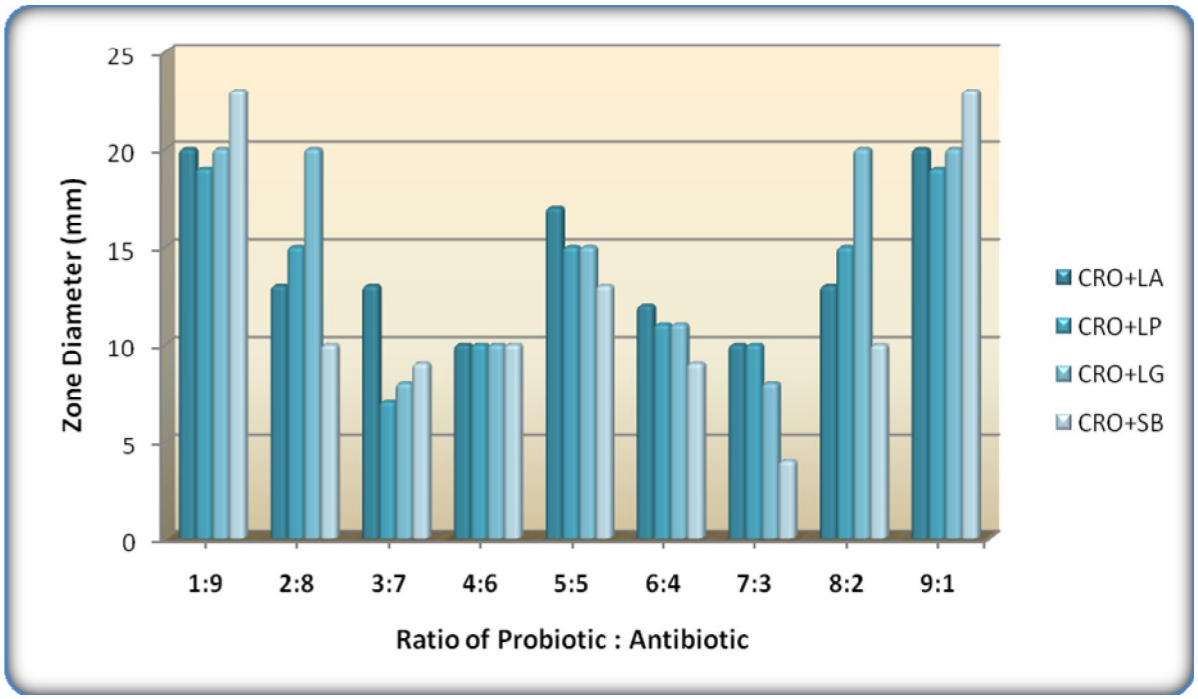



**Figure (3-4): Effect of ceftriaxone-probiotic filtrates mixtures on $WK_2$ isolate estimated by diameter of inhibition zone (mm). CRO; Ceftriaxone, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii***

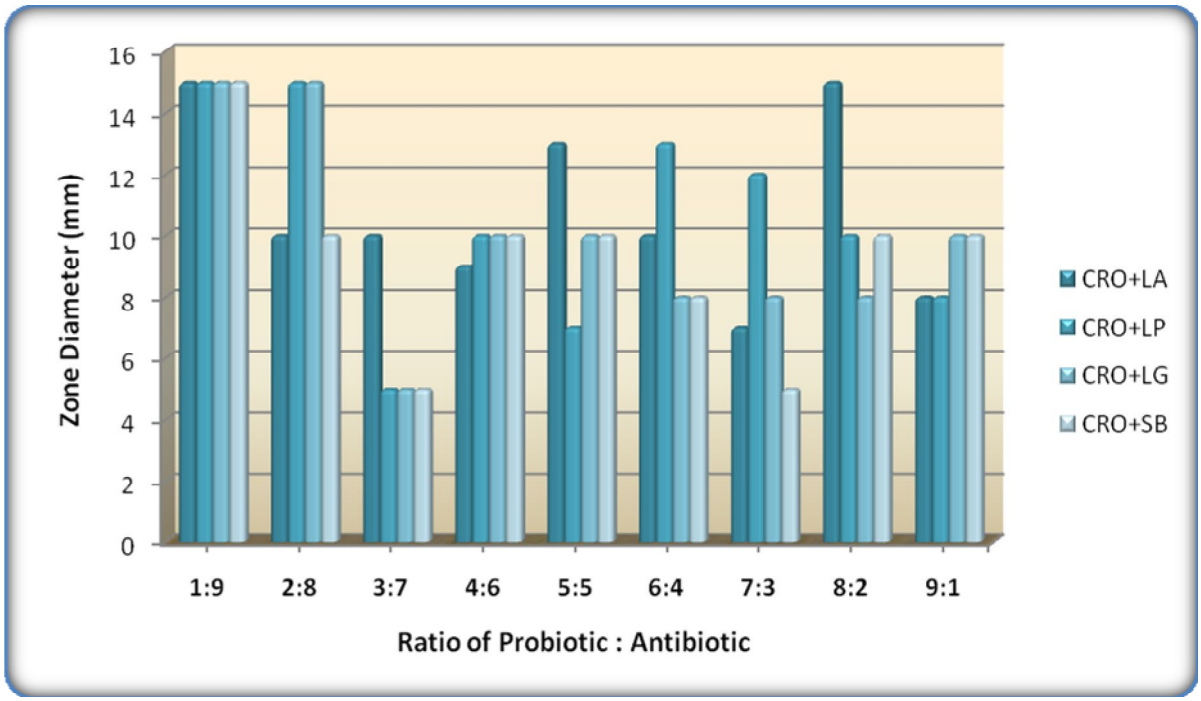



**Figure (3-5): Effect of ceftriaxone-probiotic filtrates mixture on $BK_{12}$ isolate estimated by diameter of inhibition zone (mm). P; Probiotic, CRO;Ceftriaxone, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii***

Figure (3-6) declared that the mixture between probiotic filtrates and amoxicillin showed observable effect in the increased of the antibiotic effect on $WK_2$ isolate in ratio 5:5, when the inhibition zone reached to 15 mm when it was mixed with *L. acidophilus*, *L. plantarum* and *S. boulordii*. The mixture containing *L. acidophilus* and amoxicillin in the same ratio, also gave 15 mm of inhibition zone on $BK_{12}$ isolate. But the other solutions had no considerable effect on both isolates , as shown in figure (3-7).

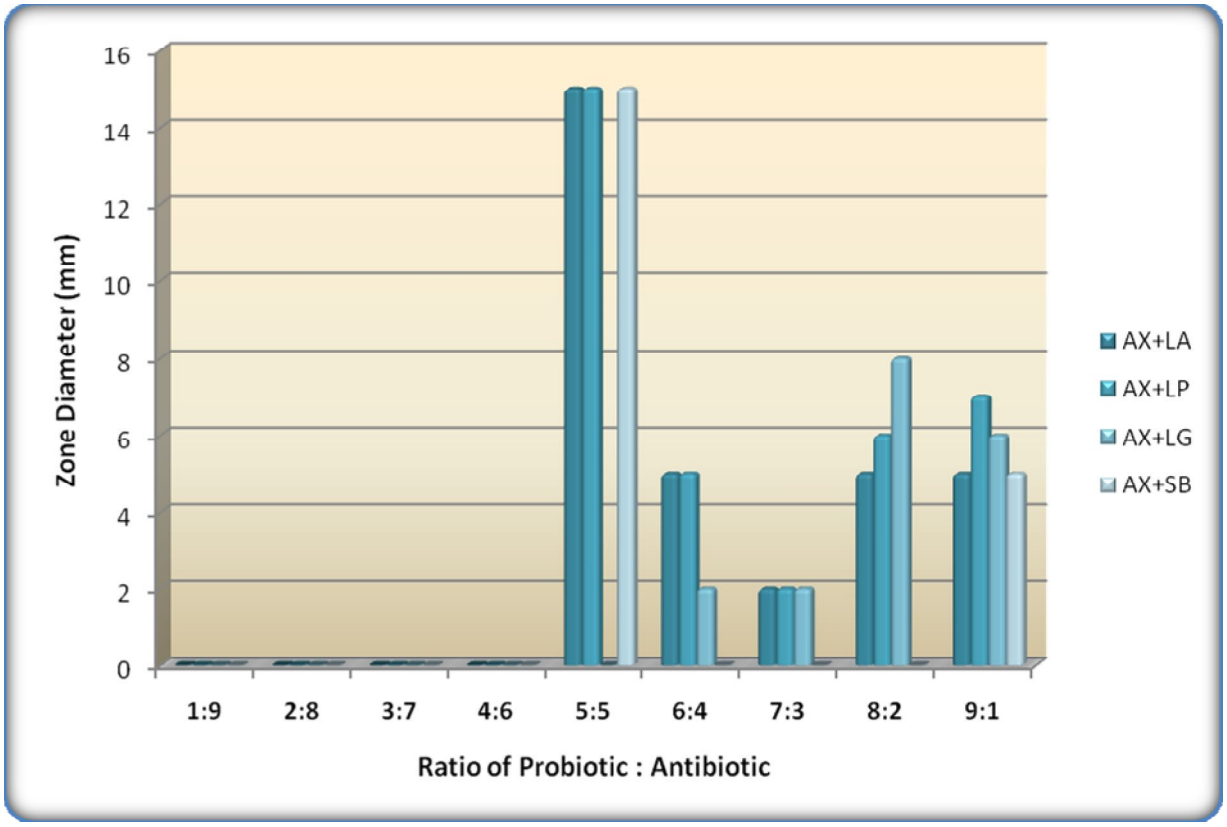



**Figure (3-6): Effect of amoxicillin-probiotic filtrates mixture on $WK_2$ isolate estimated by diameter of inhibition zones (mm). AX; Amoxicillin, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii*.**

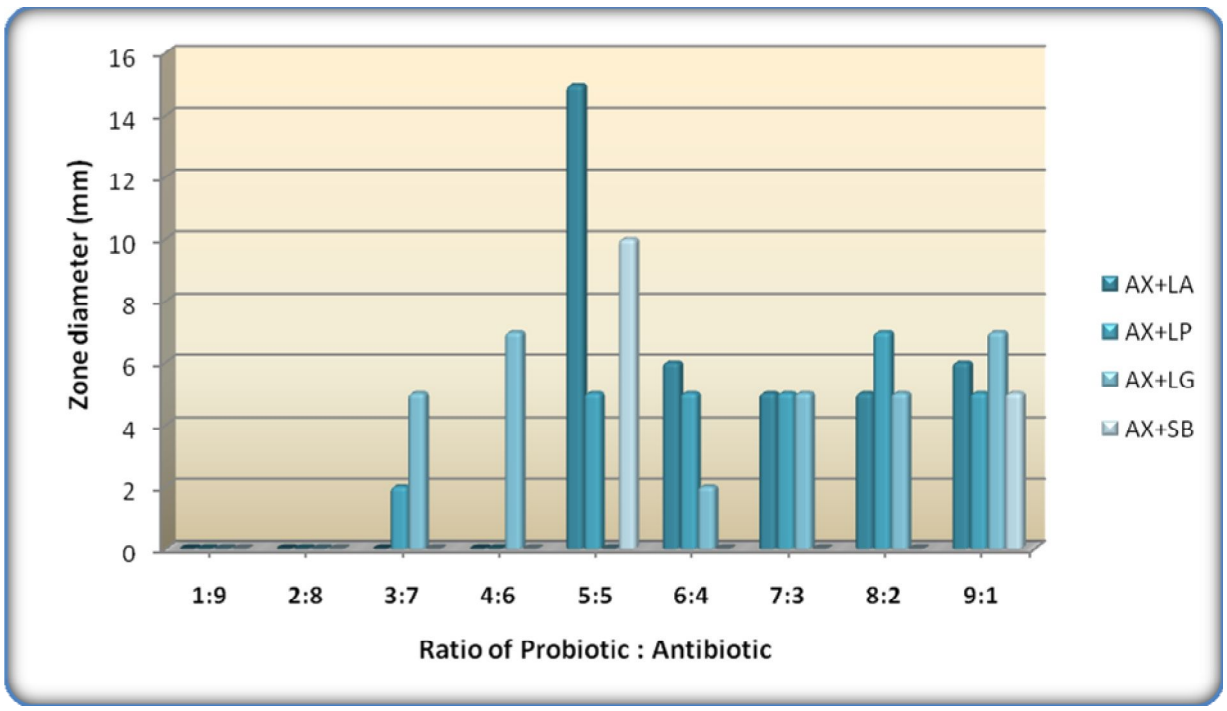


**Figure (3-7): Effect of amoxicillin-probiotic mixtures on $BK_{12}$ isolate estimated by diameter of inhibition zone (mm). AX; Amoxicillin, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii*.**

While the mixture between probiotic filtrates and ofloxacin increased the effect of antibiotic on the burn isolate ($BK_{12}$) in ratio 1:9 when the diameter of inhibition zone reached to 25, 25, 22, 23 mm when the antibiotic was mixed with *L. acidophilus*, *L. plantarum*, *L. gasseri* and *S. boulardii*, respectively, and in ratio 2:8 when the diameter of inhibition zone reached to 22, 22, 23 mm when it was mixed with *L. acidophilus*, *L. plantarum* and *L. gasseri*. But ofloxacin with *S. boulardii* in this ratio gave the same effect of antibiotic alone. Also mixture of all probiotics with ofloxacin in ratio 8:2 had the same effect of antibiotic alone. The mixture of probiotics with ofloxacin in the other ratio decreased the antibiotic effect as shown in figure (3-8).

But this mixture between probiotic filtrates and ofloxacin decreased the effect of antibiotic when applied on traumatic wound isolate in all solutions as shows in figure (3-9).

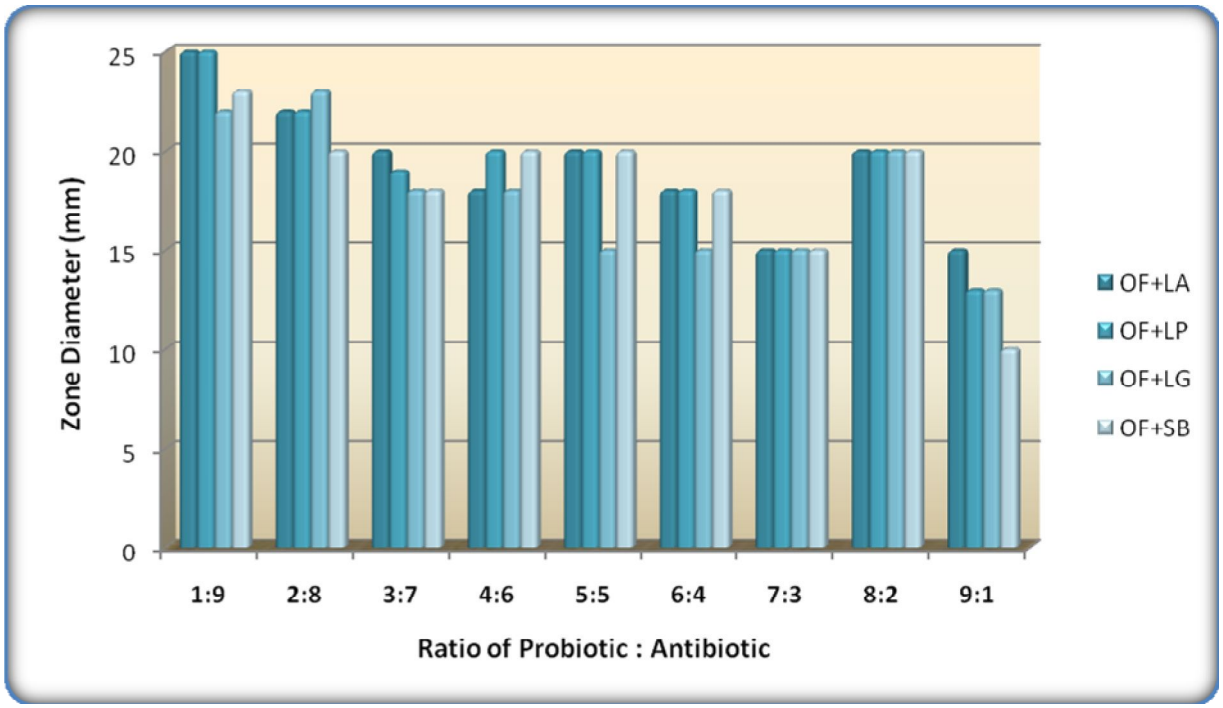



**Figure (3-8): Effect of ofloxacin-probiotic filterates mixtures on $BK_{12}$ isolate estimated by diameter of inhibition zone (mm). OF; Ofloxacin, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii*.**

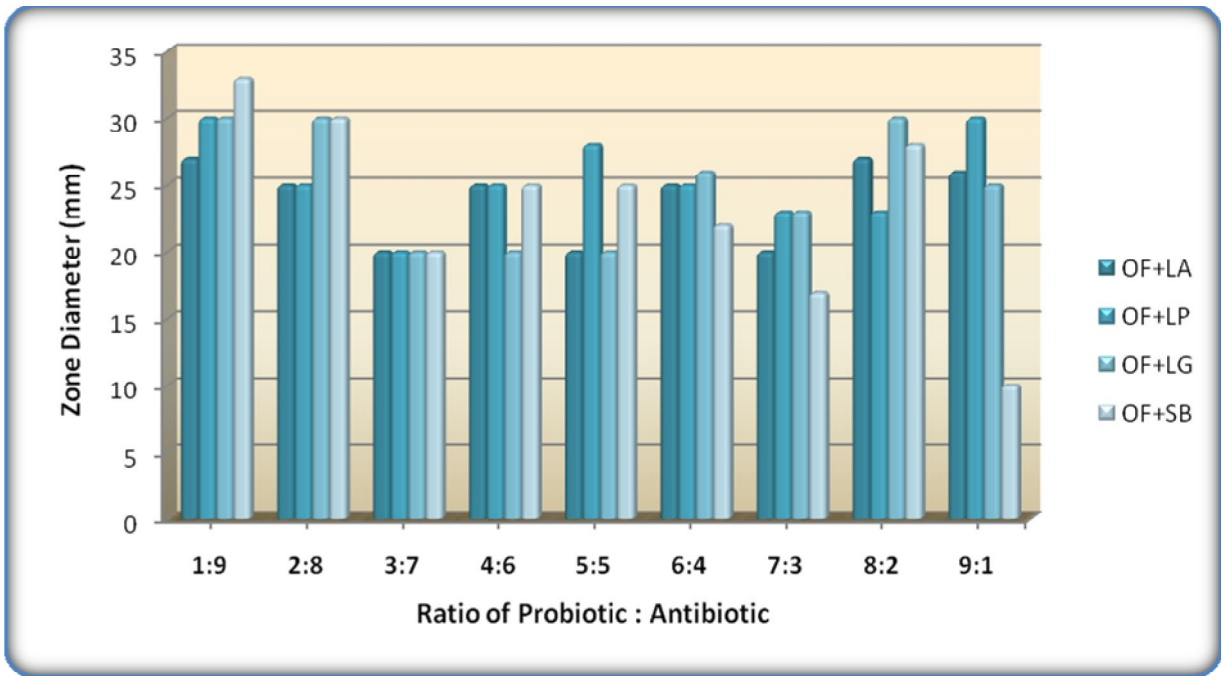



**Figure (3-9): Effect of ofloxacin-probiotic filterates mixtures on $WK_2$ isolate estimated by diameter of inhibition zone (mm). OF; Ofloxacin, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii*.**

Figure (3-10) shows that effect of the mixture between probiotics and meronem on wound isolate gave the same effect when the antibiotic was alone, this could be observed in the solution contain *L. gasseri* and meronem in ratio 1:9 and in the solutions contains *L. acidophilus* and meronem, *S. boulardii* and meronem in ratio 5:5 were the inhibition zone reached to 30 mm. While the other mixture decreased the effect of antibiotic. But the mixture of meronem and probiotic filtrates decreased the effect of antibiotic on burn isolate in all solutions as shown in figure (3-11).

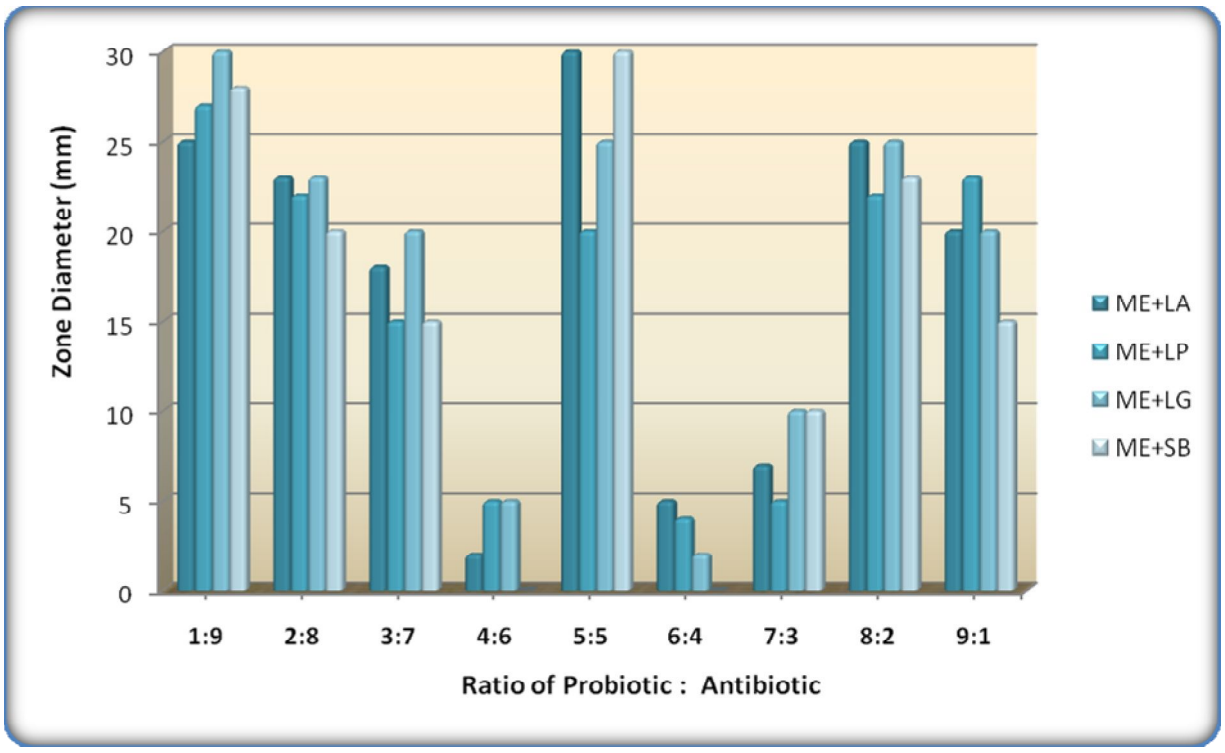



**Figure (3-10): The inhibition effect of meronem-probiotic filtrates mixtures on $WK_2$ isolate by diameter of inhibition zones (mm). ME; Meronem, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii*.**

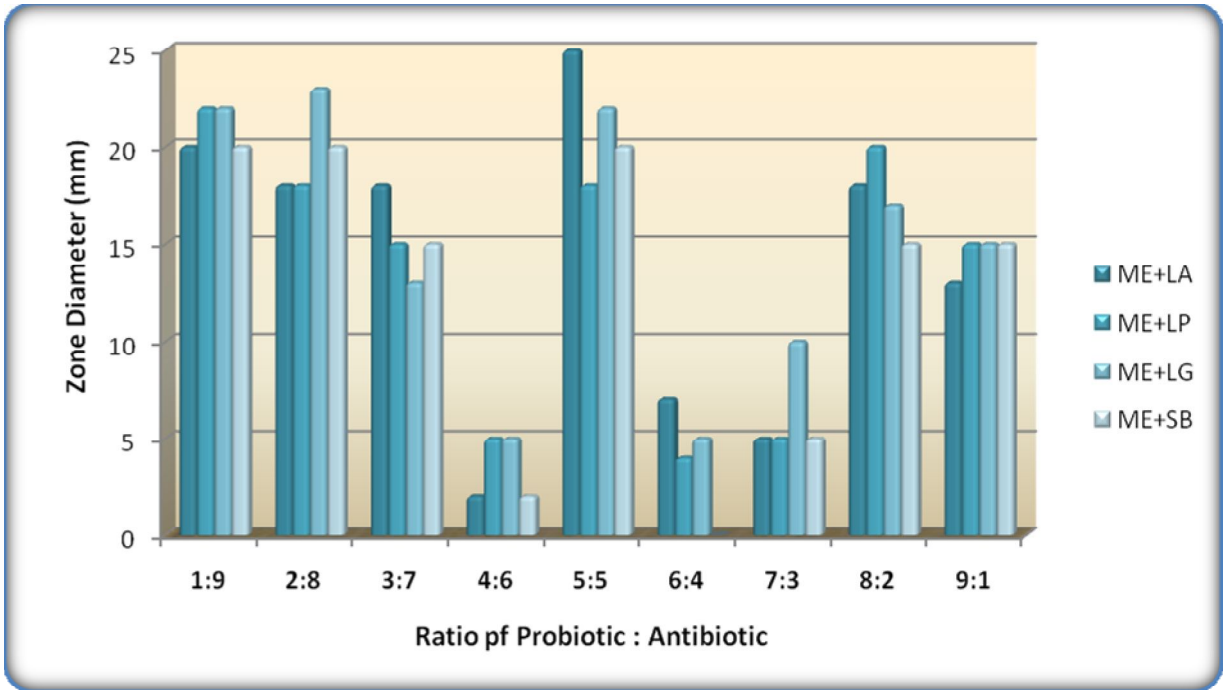



**Figure (3-11): The inhibition effect of meronem-probiotic filtrates mixtures on $BK_{12}$ isolate by diameter of inhibition zones (mm). ME; Meronem, LA; *L. acidophilus*, LP; *L. plantarum*, LG; *L. gasseri*, SB; *S. boulardii*.**

Previous results declared that probiotic filtrates enhanced the effect of antibiotics (amoxicillin, cefiriaxone and ofloxacin) on both $WK_2$ and $BK_{12}$ isolates mainly in ratio like 1:9, 2:8, 5:5, 8:2 and 9:1 (probiotic:antibiotic), but the mixtures in the other ratios decreased the antibiotics effect. On the other hand, probiotic filtrates showed either no effect or negative effect on the meronem activity. In this regard, there is no publications were available.

In general Reid (2006) documented that probiotic strains can act as adjuncts to the antibiotic therapy by reducing adverse effects and at the same time improving antibiotic function. Moreover, John *et al*. (1997) found that *Lactobacillus* spp. and *S. boulardii* might be the ideal probiotics in conjugation with antibiotics.

The synergistic effect of probiotic filtrates with each of ceftriaxone and amoxicillin, individually. may be due to their similar mechanisms of action by interacting with phospholipids of the bacteria cell membrane, therefore, cell permeability and disrupting osmotic integrity will be increased (Giacometti *et al*., 1999). On the other hand, probiotic filtrates increase the permeability of bacterial cell membrane and allow ofloxacin to enter the bacterial cell and disrupt bacterial DNA synthesis (Giacomettt *et al*., 1999).

# 4.Conclusions and Recommendations

## 4.1 Conclusions:

- *Klebsiella* was the most common bacteria found in burns than in traumatic wounds while it was not found in the foot ulcers.
- All *Klebsiella* isolates found to be belonged to the species *K. pneumoniae*
- The isolates were resistant to all antibiotic in the disc diffusion method except imipenem.
- *K. pneumoniae* of wounds were sensitive to both meronem and ofloxacin antibiotic solutions, while those of burns were sensitive to only meronem solution.
- *K. pneumoniae* of burns exhibited higher resistant than those of traumatic wounds.
- While probiotics (*Lactobacillus* spp. and *Saccharomyces boulardii*) filtrates had inhibitory effect on *K. pneumoniae* isolates, *Lactobacillus* spp.(*L. acidophilus, L. plantarum, L. gasseri*) were found to give more inhibitory effect on *Klebsiella* isolates than *S. boulardii*.
- Combination between some antibiotic solutions with probiotic filtrates gave better effect on *Klebsiella* isolates.

## 4.2 Recommendations:

- Studying the effect of probiotics on the *Klebsiella in vivo*.
- Further studying on effect of probiotics-antibiotics combinations *in vivo*.
- Extraction, purification and identification of the inhibitory substances produced by *L. acidophilus*, *L. plantarum*, *L. gasseri* and *S. boulardii* able to inhibit *Klebsiella* growth as well as in the synergistic uses with antibiotics.

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# الملخص

تم جمع مائة عينة من مرضى (ممن راجعوا ثلاثة مستشفيات في بغداد) يعانون من اصابات الحوادث تضم جروح رضية، تقرحات القدم والحروق. جمعت العينات من كلا الجنسين خلال الفترة ٢٠٠٩/١٢/١٠ الى ٢٠١٠/٣/١٤. شخصت ٢٢ عزلة منها بكونها تعود الى الجنس *Klebsiella* اعتمادا على الصفات الزرعية، المجهرية والكيموحيوية. فيما شخصت العزلات المتبقية على انها تعود الى *E. coli*، *Pseudomonas*، *Enterobacter*، *Proteus* و *Citrobacter* بأعداد بلغت ٢٥، ٢٣، ٦، ٥ و ٢ على التوالي.

اضهرت نتائج التشخيص الكيموحيوي ونضام التشخيص بعدة ال Api-20E بأن جميع عزلات *Klebsiella* كانت تعود الى النوع *K. pneumoniae*.

تم تحديد حساسية عزلات *K. pneumoniae* تجاه (١٥) مضادا حيويا شائعا بأستخدام طريقة الأقراص. تلا ذلك استخدام طريقة الأنتشار في الحفر للتحري عن حساسية عزلات *K. pneumoniae* تجاه محاليل (٦) مضادات حيوية والتي تم اختيارها اعتمادا على نتائج طريقة الأقراص. اضهرت النتائج مقاومة العزلات المرضية للمضادات الحيوية المستخدمة باستثناء حساسيتها تجاه imipenem (بطريقة الأقراص) و meronem (بطريقة الأنتشار في الحفر).

اعتمادا على نتائج حساسية العزلات للمضادات الحيوية، فقد تم اختيار عزلتين من *Klebsiella* احدهما من الجروح الرضية والاخرى من الحروق وذلك لدراسة التأثير التثبيطي لرواشح ال probiotics لوحدها فضلا عن مزجها بنسب مختلفة مع المضادات الستة المستخدمة. ولهذا الغرض فقد تم تحديد التأثير التثبيطي لرواشح ثلاثة عزلات تعود لبكتريا *Lactobacillus* (*L. acidophilus*، *L. plantarum*، *L. gasseri*) و عزلة خميرة *S. boulardii* ضد عزلتي *K. pneumoniae* المرضية. اضهرت النتائج بأن رواشح عزلات *Lactobacillus* كانت الأكفأ في تأثيرها من عزلة الخميرة.

فيما يخص التأثير التثبيطي للمزج بين رواشح ال probiotics و المضادات الحيوية، اشارت النتائج الى ان مزج (٥) اجزاء من كل من الرواشح الاربعة مع (٥) اجزاء من مضاد amoxicillin (على انفراد) اي بنسبة (٥:٥) كانت الأكثر تأثيرا على عزلة الجروح الرضية، بينما اعطى المزج بين *L. acidophilus* : amoxicillin بنفس النسبة افضل تأثير تثبيطي على عزلة الحروق.

فيما يخص تأثير ceftriaxone فقد ازداد تأثير هذا المضاد على عزلة الجروح الرضية عند مزجه مع *S. boulardii* وبالنسبتين ١:٩ و ٩:١ من amoxicillin : *S. boulardii* فضلا عن النسبتين ٨:٢ و ٢:٨ من ceftriaxone : *L. gasseri*. ادت جميع رواشح ال probiotics الى زيادة التأثير التثبيطي لمضاد ceftriaxone على عزلة الحروق لاسيما عند النسبة ٩:١ antibiotic:probiotic، بالأضافة الى المزج بنسب ٢:٨ من *L. acidophilus* : ceftriaxone ٨:٢ من كلا من ceftriaxone : *L. plantarum*، ceftriaxone : *L. gasseri* سببت زيادة التأثير التثبيطي للمضاد على عزلة الحروق. فيما ازداد التأثير التثبيطي لمضاد ofloxacin على عزلة الحروق عند مزجه مع جميع رواشح ال probiotic وبنسبتي ١:٩ و ٢:٨ راشح : مضاد.

اضهرت النتائج عدم حصول اية زيادة في التأثير التثبيطي لمضاد ofloxacin ضد عزلة الجروح الرضية عند مزجه مع جميع الرواشح (كلا على انفراد) وبجميع النسب المستخدمة. فيما ادى مزج المضاد هذا مع الرواشح احيانا الى خفض الفعالية التثبيطية له على عزلة الجروح الرضية. كذلك الحال بالنسبة للمزج بين رواشح ال probiotics مع مضاد meronem لم يسبب اية زيادة في التأثير التثبيطي للمضاد واحيانا ادى مزج المضاد مع الرواشح الى خفض تأثيره التثبيطي على كلتا العزلتين وبجميع النسب.

# الإهداء

**إلى من حملتني وهنا على وهن ...**

أمي العزيزة

**إلى الصدر الذي ألجأ إليه من هموم حياتي ...**

أبي العزيز

**إلى من اشد بهم أزري وأشركهم في أمري ...**

أخوتي و أخواتي

**إلى من أرى من خلال عينيه مستقبلي وطموحي ...**

زوجي حيدر

**إليكم اهدي ثمرة جهدي ...**

**صبا**

بسم الله الرحمن الرحيم

﴿ يُؤْتِي الْحِكْمَةَ مَن يَشَاءُ وَمَن يُؤْتَ

الْحِكْمَةَ فَقَدْ أُوتِيَ خَيْرًا كَثِيرًا وَمَا يَذَّكَّرُ

إِلَّا أُولُو الْأَلْبَابِ ﴾

صدق الله العظيم

سورة البقرة، آلأية (٢٦٩)

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

# عزل وتشخيص بكتريا الكلبسيلا من جروح الحوادث ومعالجتها بواسطة الأحياء المجهرية العلاجية

**رسالة**

**مقدمة الى كلية العلوم/جامعة النهرين كجزء من متطلبات نيل درجة الماجستير في العلوم/التقانة الأحيائية**

**من قبل**

**صبا رعد جعفر**

**بكلوريوس تقانة أحيائية/جامعة النهرين (٢٠٠٨)**

**بأشراف**

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تشرين الثاني : ٢٠١١