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# THE ANTIMICROBIAL ACTIVITIES OF PROBIOTICS *LACTOBACILLUS* SPP. AGAINST MULTIDRUG RESISTANT PATHOGENIC BACTERIA FROM DIABETIC INFECTIONS

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NOOR SUBHI FAYYADH AL-AGELE

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By Noor Subhi Fayyadh AL-AGELE

January 2023

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## ABSTRACT

THE ANTIMICROBIAL ACTIVITIES OF PROBIOTICS *LACTOBACILLUS* SPP. AGAINST MULTIDRUG RESISTANT PATHOGENIC BACTERIA FROM DIABETIC INFECTIONS

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January 2023

Growing scientific evidence supporting the positive role of probiotic microorganisms in human health; nevertheless, more study is required. In the current study, 170 samples were collected among them 100 of them were suffer from diabetes having foot infectious and 70 of them have no diabetic foot infections. The individuals were selected in both genders, aged between 33-78 from hospitals in Diyala province Iraq. After culturing the samples, the results showed that 54 isolates had apositive growth from the 70 samples for diabetic patients without diabetic foot infection as normal flora. while 70 isolates (70%) were positive growth for diabetic foot patients and 30 (30%) were negative growth. Selective media, VITEK 2 compact, biochemical testing, and microscope analysis were employed to identify *K. pneumonia* (%36), *P. aeruginosa* (%16), *E. coli* (%12), *P. mirabilis* (%4) ve *S. epidermidis* (%2). Enterobacteriaceae was most resistant to polymixin B and *P. aeruginosa* was mostly resistant to cefepime, ticarcillin-clavulanic acid and polymixin B. In this study indicated that probiotics have antibacterial activity ranging from 12 mm to 19 mm and anti-biofilm activity ranging from 29% to 50% in Diyala province in Iraq.

**2022, 65 pages**

**Keywords:** *Lactobacillus* spp, Diabetic, Probiotic, Pathogenic bacteria,

# ÖZET

BAZI LOKAL PROBİYOTIKLERİN LACTOBADLLUS'UN DIYABETİK HASTA ENFEKSİYONLARINDAN İZOLE EDİLEN ÇOKLU İLACA DİRENÇLİ PATOJENİK BAKTERİLERIN ANTIMİKROBİYAL AKTİVİTELERİ

Noor Subhi Fayyadh AL-AGELE

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Ocak 2023

Probiyotik mikroorganizmaların insan sağlığı üzerinde pozitif rolünü destekleyen artan sayıda çeşitli bilimsel kanıt olmasına ragmen daha fazla çalışmaya ihtiyaç vardır. Bu çalışma kapsamında, 100'ü diyabetik ayak enfeksiyonunu olan, 70'i ise diyabetik ayak enfeksiyonu olmayan kişilerden olmak üzere, toplam 170 örnek toplanmıştır. Bireyler, Irak'ın Diyala bölgesindeki hastanelerden yaşları 33-78 arasında, kadın ve erkeklerden arasından seçilmiştir. Örnekleri kültürlenmesinden sonra sonuçlar, normal flora olarak diyabetik ayak olmayan diyabetik hastaların 70 örneğinden 54 izolatın üreme açısından pozitif olduğunu, diyabetik ayaklı hastalardan 70 izolatın (%70) ve diyabetik ayaklı hastalardan üreme için pozitif olduğunu gösterilmiştir. 30 izolatın (%30) negatif beslenme kaydedilmiştir *K. pneumonia* (%36), *P. aeruginosa* (%16), *E. coli* (%12), *P. mirabilis* (%4) ve *S. epidermidis* (%2)'i tanımlamak için seçici besiyeri, VITEK 2 kompakt, biyokimyasal test ve mikroskop analiz kullanılmıştır. Enterobacteriaceae en çok polimiksin B'ye, *P. aeruginosa* ise en çok sefepim, tikarsilin-klavulanik asit ve polimiksin B'ye direnç göstermiştir. Bu çalışma probiyotiklerin 12 mm ile 19 mm arasında değişen antibakteriyel aktiviteye sahip olduğunu ve Irak'ın Diyala bölgesinde % 29 ile% 50 arasında değişen anti-biyofilm aktivitesine sahip olduğu belirlenmiştir.

**2022, 65 sayfa**



**Anahtar Kelimeler:** *Lactobacillus spp,* Diyabetik, Probiyotik, Patojenik bakteri

## PREFACE AND ACKNOWLEDGEMENTS

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**Noor Subhi Fayyadh AL-AGELE**
**Çankırı-2023**

## CONTENTS

## LIST OF SYMBOLS

| | |
|---|---|
| - | Minus |
| % | Percent |
| + | Plus |
| °C | Degrees Celsius |
| μL | Microliter |
| L | Liter |
| mg | Milligram |
| min | Minutes |
| mL | Milliliter |
| nm | Nanometre |
| U | Unit |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| AST | Antibacterial susceptibilty test |
| BHI | Brain heart infusion |
| CFS | Cell free supernatant |
| DFI | Diabetic foot infection |
| DFU | Diabetic foot ulcer |
| DM | Diabetes mellitus |
| GRAS | Generally recognized as safe |
| LAB | Lactic acid bacteria |
| MDR | Multidrug resistance |
| MHA | Mueller hinton agar |
| MRSA | Methecillin resistance *Staphylococcus aureus* |
| MTP | Micro titer plate |
| NA | Nutrient agar |
| NB | Nutrient broth |
| PDR | Pan drug reisitance |
| XRD | Extensive drug resistance |

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# 1. INTRODUCTION

Infected diabetic feet consider a public complication and once disruptions occur in the defensive wall of the skin in individuals with diabetes permitting easy access of microbes. Diabetic Foot Ulceration (DFU) which turn out to be infected is consider a main causative pathway to amputation of the lower extremity and identification of contributing microbes and any accomplices is vital in directing anti-microbial treatment (Mutonga 2019).

It is reported that more than 13.9% of adults in Iraq suffer from diabetes, and many do not know that they have the disease The worldwide diabetes occurrence in 2019 is assessed to be 9.3% (463 million persons), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045. (Saeedi *et al.* 2019) . More than a one of three of human population have suffer from ulceration in diabetic feet throughout their lifespan and with 50% of these developing into infested and producing diabetic foot illnesess (DFIs). Fifteen percentages of persons with DFIs need elimination of the lower limb to avoid development of the contamination (Commons *et al.* 2018, International Diabetes Federation 2019). Diabetic foot care is not cheap, with an expected US $8659 once yearly cost per person, thus give emphasis to the significance of premature finding and cure of DFUs/DFIs (Raghav *et al.* 2018).

Probiotics and its metabolites (post-biotics) are considered as adjunct beneficial ways. Probiotics display an abundance of anti micro-organisms' activities (anti-bacterial, anti-biofilm, anti-drug resistance, anti-virulence, co-aggregation, and anti-quorum sensing) via the creation of several incompatible combinations like organic acids, $H_2O_2$, small molecular weight compounds, bio-surfactants, bacteriocins, and bacteriocins like inhibitory elements. Moreover, pprobiotics make stability of the epithelial wall role and definitely modify the immunological system of the host by means of modifiable many Mechanisms of signal transmission (Nataraj and Mallappa 2021).

Pre-clinical and human involvement studies have recommended that probiotics out-compete with numerous pathogenic bacteria by displaying anti-colonization mechanisms by means of defending, competitive, and dis-placement modes. Probiotics involved Lactic Acid Bacteria (LAB) are consider groupings of cocci-orbbacilli in shape, Gram-positive microbes. They are facultative an-aerobes, catalase-negative, motile, do not yield spores, and generate lactic acid as a metabolic end-product throughout the fermentation of the carbohydrates. ProbioticcLAB is categorized as homo-fermentative or hetero-fermentative grounded on the metabolic ways (Nataraj and Mallappa 2021). In homo-fermentative LAB, they depend on sugar fermention to yield chiefly lactic acid in an-aerobic circumstances. In hetero-fermentative LAB, the fermentation of the sugar produces ethanol, $CO_2$, and a smaller amount of lacticbacid (Ayyash *et al.* 2018). LABAcan besides yield anti-microbial combinations in addition lactic and acetic acids, diacetyl and bacteriocins antagonistic to an expansive spectrummof micro-organisms which able to prevent foodtspoilage andppathogenic bacteria (Amelia *et al.* 2020).

The present work aimed to diagnose and characterize the bacteria from foot in patients with diabetic and to investigate the biological activity and anti-biofilm activity of the local probiotics *Lactobacillus* spp. against the pathogenic bacteria. To achieve this aim, the following steps were carried out:

- Comparing several methods, including traditional and commercial methods for the isolation and identification according to the standard methods.
- Detecting the frequency of MDR and XDR occurrence among the isolated clinical bacteria.
- Detection the degrees of resistance toward the antibiotics among all the isolates.
- Detection of some virulence factors by cultural methods.
- Investigate the anti-bacterial activity of local probiotic isolates against the growing and anti-biofilms production of multidrug resistance isolates.

# 2. LITERATURE REVIEW

The skin comprises two major covers: the epidermis, which consists of a specialized layered epithelial tissue of embryonic origin from the ectoderm, and the dermis, which is located under the epidermis and consists of dense connective tissue and vascular of its embryonic origin from the mesoderm. The connection between the epidermis and the dermis is tortuous and irregular, where the dermis forms protrusions that enter indentations within the epidermis. These projections are called dermal papillae. On the other hand, the epidermis forms projections that enter indentations within the dermis, and these projections are called epidermal papillae. Under the dermis there is a layer consisting of loose interstitial connective tissue and adipose tissue, and this layer is called the hypodermic layer or hypodermis layer (Wong *et al.* 2016). The skin is a dividing wall between the internal structures of the body and the external environment, and it also reflects the clinical status of many diseases within the human body. It works to protect the body from ultraviolet rays and mechanical and chemical damage, and it is an immune barrier against the invasion of pathological factors that cause many infections (Fischer and Meyer-Hoffert 2013). A disease that affects diabetics through the appearance of some pathological symptoms in the foot, such as swelling, sores, and wounds because of neuropathy, poor blood circulation, or bacterial infections. This occurs after diabetes or diabetes for a long time, and this occurs because of peripheral neuropathy and insufficient blood circulation to the foot as a result of diabetes (Lehrke and Marx 2017).

## 2.1 Diabetes Mellitus

Diabetes mellitus (DM), or basically diabetes, is a set of disorders described by increase blood glucose level which come from a consequence of defects in the production and/or usage of insulin by patient's body, diabetes and related conditions was consider as a collection of metabolic syndrome (Lehrke and Marx 2017).aDiabetes mellitushhasebeen increased predominant overethe latestedecades, whereethe expectationoof DM occurrence is increased to 592 million globally by 2035 (Quinn *et al.* 2019). The number of deaths in Iraq from diabetes mellitus in 2017 was 6,382, or 3.62% of all deaths, according to the most recent WHO data. The global age-adjusted Death Rate is 40.12 per 100,000 people. Select the full health profile to get a list of additional causes of death, or click the links below. The disorder of DM which is a group of metabolic syndromes and characterized by excessive blood glucose levels caused by insulin production flaws as well as insulin action, or together (Chawla *et al.* 2016). The long term hyperglycemia of diabetes is correlates withulong-term microvascular andgmacrovascular complications, including dysfunctionbin many organs, including the kidney, eyes, nerves, heart, and blooduvessels, which poses a serious challenge to the diabetic patient and his state of health (Hsia *et al.* 2015). According to International Diabetes Federation (IDF), the occurrence of DM (type 1 and type 2) in individuals with age ranging twenty to seventy-nine years has increased to 463 million (9.3% of all population) approximately 7.6% of Iraq population. Impairment or adequate achievement to address the pandemic, diabetes will be predicted 5789million people (10.2% of the population) by 2030. This figure will rise globally to a staggering 700 millionz (10.9%) by 2045 (International DiabetesrFederation 2019).

Bacterial skin infections in patients with diabetes are very common and mostly minor unpleasant infections or be fatal to humans. There infections are mainly causedbby types of Gram-positiveuanduGram-negative bacteria. Cutaneous infections occur through microbial invasion of the layers of the skinaand subcutaneous softotissues, and these tissues are heterogeneous in clinical sites. Infection occurs through clear penetration of the skin barrier, such as wounds. However, infection can occur without penetrating the skinbbarrier, such asmfolliculitis that occurs in hair follicles and boils (Ki and Rotstein 2008, Khadir *et al.* 2018). There is a great diversity in the type and number offmicroorganisms endemic to the diabetic foot, involved in causing the infection either one type of mono-microbial or several types combine to infect one ulcer. Previous studies indicated that most of the bacterial pathogens such as *Pseudomonas aeruginosa, Escherichia coli*, *Proteus* spp. frequently present in diabetic foot ulcers are represented. Superficial wounds of ulcers are accompanied by aerobic microorganisms, while deep areas of wounds include anaerobic microorganisms such as the bacterium *Clostridium perfringens* (Banoo *et al.* 2012).

## 2.2 Causative bacteria

*Staphylococcus epidermis* are a component of the natural flora of the mucous membranes nose, ears and armpits, but some studies showed the presence of this bacteria related to hospital-acquired infections, especially infection of newborn children and immunosuppressed patients and bacteria are the main cause of infection (Pinheiro *et al.* 2015). The bloodstream and infections related to cardiac catheterization and dialysis devices and involved in infections of deep wounds, burns, eye and ear infections and urinary tract infection (Widerström *et al.* 2012).

*Escherichiaecoli* is the source of hospital-acquired infections, especially in individuals with weak immunity, that forces them to get medicins in abundance, and thus the emergenceeof antibioticwresistant strains (Korzeniewska *et al.* 2013). It is also one of the greatest public bases of disease and death in children suffering from diarrhea. Innall parts of theeworld, especially in developeddnations (Kalantar *et al.* 2011), mostly due to the urinary and genital infections, mainly in pregnant women reaching 10% (Dielubanza and Schaeffer 2011). The cause of the pathogenicity of bacteria is due to the virulence factors that they possess, including the fact that their cell wall contains many lipopolysaccharides, in addition to their secretion of two types of toxins, which are heat-stable endotoxins and non-thermally stable toxins. It also possesses the hemolytic enzyme (hemolysin), which plays a major and important part in its pathogenesis (Huang *et al.* 2012).

*Pseudomona aeruginos*a is among the most imperative and most pathogenic reasons of hospital infections in Iraq. It was isolated from various clinical specimens such as wounds, burns, ear and urinary tract infection (Rasheed *et al.* 2016). Staying in hospitals for long periods can lead to a high rate of infection with these germs, as the percentage can reach 20% within 72 hours, especially for patients with severe burns (Ochoa *et al.* 2013). The germ is one of the opportunistic factors that often accompanies with hospitaltacquiredeinfections that can cause chronic lung disease inflammation in cysticcfibrosismpatients, as well asuurinarywtract infection, inflammation of wounds and burns, and bacteremia. This is due to the germ possesses that many virulence factors secrete enzymes and toxins and the germ has the ability to transmit through water when present in aquatic environments and other sources such as sewage treatment plants (Streeter and Katouli 2016).

*Klebsiella pneumonia* is considered the most significant cause of nosocomial infection relative to community-acquired infections. *K. pneumoniae* nosocomial infections are quiet far more common and possibly more risky as a result of the high evolutionary rate and spread of antibiotics resistance in hospital (Lundbergg *et al.* 2013). *Klebsiellas*speciesaare classified asoopportunisticcpathogens colonization of mucosal surfaces without disease; however, from mucosaew*Klebsiella* perhaps spread to other tissues causing serious infectionsucomprising DFU and chronic pyelonephritis, pneumonia, urinary tract infection, rbloodstream infections1and sepsise (Paczosa and Mecsas 2016).

*Proteus mirabilis* possess various virulence factors that made them one of the most dangerous pathogens for humans. Tese factors include the ability to adhering to the host's surface tissues, and their possession of inertia and movement by flagella. The production of toxins and enzymes such as hemolysin and bacteriocin, and the invasion of the immune system, the bacteria invade patients withhweak immunessystems, such as thosewwith complex wounds and patients with diabetes (Baldo and Rocha 2014).

## 2.3 Virulence factors

Virulence factors (VFs) can be defined as a molecule that formed by bacteria via a diversity of many mechanisms, stimulate infectionmand propagation within aahost. Specific VFs can be created collectively by a speciesoofubacteria, while others possibly will possibly be picked up via mutations or the gaining of mobilergeneticmelements (MGEs) (Bien *et al*. 2011).

An extended hair-like projections which called *pili* could be used by the bacteria in the attaching process toohost cell surface receptorsoor extra-cellular matrix (ECM) proteinss (Webb and Kahler 2008). The microbes can also transfer through trans-cellular spaces to get overeepithelial surface of theehost. When, the bacteria are inside the host cell, it has the ability to escape from up taking by the phagosomes, and the cause damage to the phagosomes via toxins, inhibit phago-lysosome development, or exit phagosomes and stay alive in the intra-cellular environment (Webb and Kahler 2008).

Bacteria typically use quorum sensing to link intracellularly by secreting small chemicals referred to as autoinducers. The auto-inducer is the driving force behind the formation differences of VFs and the establishment of biofilms. Biofilms are communities of microorganisms that attach to biotic and abiotic surfaces with the help of an extracellular substance made up of polysaccharides, proteins, and extracellular DNA. The formation of these biofilms is influenced by quorum sensing and the intra-cellular secondary messenger c-di-GMP system (Hall and Mah, 2017). Lipo-poly-saccharide (LPS), a component of the outer cell membrane of gram-negative bacteria, is considered to be a main contributor to the creation of pro-inflammatory cytokines that trigger endo-toxic shock when recognized by human toll-like receptor 44 (TLR-4) (Ramachandran et al., 2014). This allows the bacteria to exchange nutrients more easily and resist antibiotics when there is a large concentration of bacteria in the environment (Deep *et al*. 2011).

Super-antigens are highly potent toxins produced by Staphylococcus aureus, Streptococcus, Mycoplasma, and Yersinia species. Super-antigens (SAgs) have the ability to bind major histocompatibility complex (MHC) class 2 molecules on antigen-presenting cells (APCs) and T-cells, leading to T-cell activation without the need for antigen processing (Krakauer *et al*. 2019). Consequently, there is activation of exaggeratedccytokine response and in complicated circumstances effects inntoxic shockssyndrome (Carney *et al*. 2014).

## 2.4 Antibiotics Resistance

Antibiotics typically function by binding to a target cell and preventing a crucial metabolic action. The various ways that bacteria can withstand antibiotic action are including prevent antibiotic binding, increasing efflux or decreasing permeability to lessen the antibiotic's intra-cellular accumulation, inactivating the antibiotic through hydrolysis or modification, and metabolically bypassing the cellularrprocess that theeantibiotic was blocking as shown in Figurei2.1 (Bonev and Brown12019).

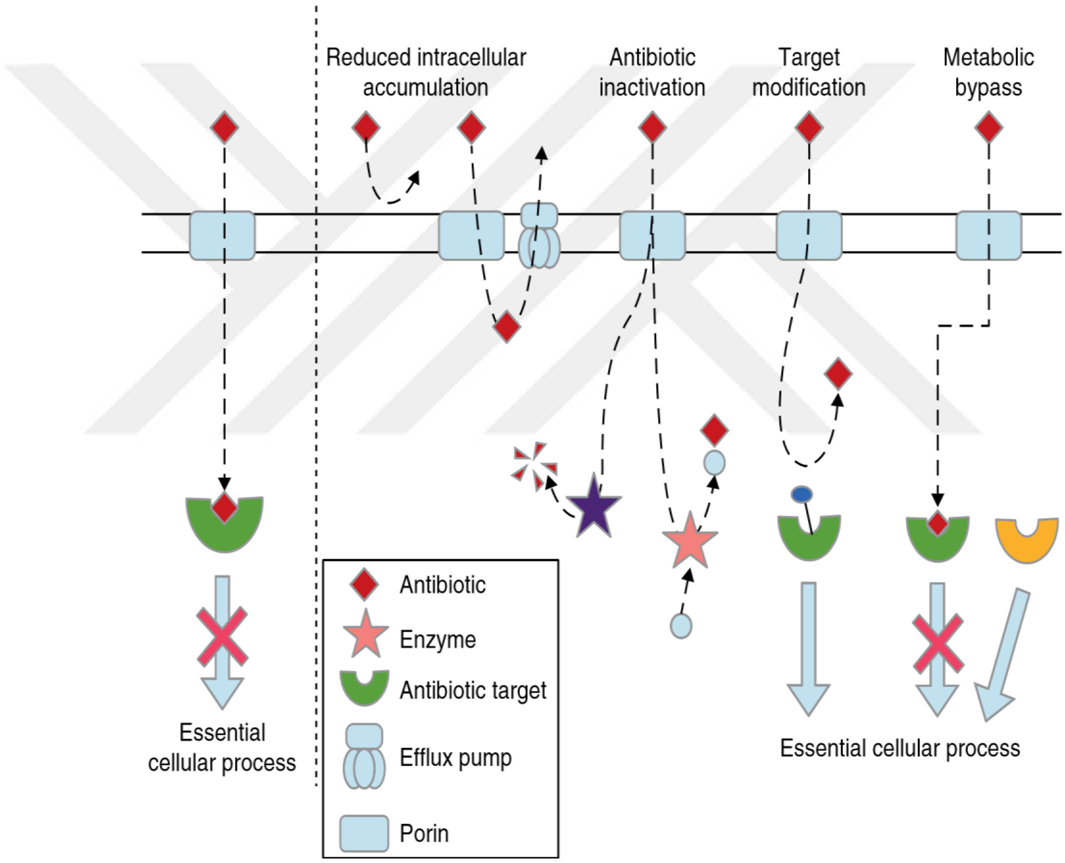


**Figure 2.1** Mechanisms of antibiotics resistance (Bonev and Brown 2019)

## 2.5 Natural Method to ControleBacterialeInfections

Overthe five decades, anti-biotic using as anti-microbial treatment is increasing and anti-biotic resistant bacteria became difficult with population safety (Odonkor and Addo

2011). The cause may be related to the adverse consequences of systemic antibiotic treatment, including hypersensitivity responses, renal, hepatic, and gastrointestinal issues. The goal of Natural Health Remedies is to find non-conventional natural antimicrobial agents that are safe for the body while also eradicating bacterial resistance to antibiotics. Natural treatments for bacterial illnesess like probiotic *Lactobacillus* spp. bacteria developed from the application of herbal compounds and various kinds of honey. Most people consider the rod-shaped bacteria in the genus *Lactobacillus* to be safe (Van *et al.* 2013).

There has been great interest in using nanoparticles as antibiotic substitutes over the last 20 years. Nano-biotechnology is one of the new and exciting fields for LAB applications. Alternatives to chemical and physical methods that are reliable and kind to the environment have been made possible by biological nanoparticle production technologies based on bacteria (Narayanan and Sakthive 2010).

### 2.6 Probiotics

The term probiotic was first suggested by Vergio in 1954 by comparing it with the side effects resulting from the use of antibiotics and other inhibitory substances towards the microorganisms in the gut (Al-Dulaimi 2005). Lacticaacid bacteria is oneiof the greatest imperativesspecies and many beneficialweffects on human health has beenaassociated with the presence of this bacteria in fermented foods. The studies indicated that consumption of lacticmacid bacteria leads touan improvement iniintestinal microbial balance (Khalid 2011). Probiotics were defined in 1992 by Veld and Havennar as a group of the microorganisms that have beneficial effects on human health through the role they play which improves the characteristics of intestinal microorganisms, and it is also known as substances prepared from bacterial cells used have abeneficialweffect on health (Salman *et al.* 2014)

The most crucial characteristics of strains to even be taken into consideration for probiotic use include resistance to bile acidity, mucus adherence, antimicrobial activity against potentially pathogenic bacteria or fungi, the capacity to reduce pathogen adhesion to surfaces, bile salt hydrolase activity, and enhancing probiotic viability. Also, the bacteria used as probiotics must be safe and not cause health risks for the consumer. Lactic acid bacteria were classified as safe from a health point of view, so their use was allowed globally in medical and veterinary applications (Abdel Hassan 2009). It also have to remain alive when passing through the digestive tract through its resistance to acidity stomach and salts and their ability to adhere to the mucous membranes of the intestine, preferably sourced from humans, where they can interact with the host specialized, they use bio-enhancers isolated from humans in dairy products for their ability to imprint in the intestines, yet there is some probiotics whose source of isolation is yoghurt (Salman *et al.* 2014).

There are several mechanisms by which lactic acid bacteria are used as prebiotics to maintain human health through its metabolic activities and the products it produces (Flynn *et al.* 2002). Inhibition of pathogenic microorganisms existed in the alimentary canal through their production for many substances including lactic and aceticcacid, which lowering theppH of the intestines. This leads to decrease in the development of harmful microorganisms that prefer the alkaline environment, or through its production of other inhibitory substances such as $CO_2$ resulting from the fermentation of sugars. Peroxide, the acetaldehyde compound produced by the fermentation of sugars, which is responsible for giving milk a taste and the distinctive flavor (Evivie *et al.,* 2017). Changing the metabolic activity of the microorganisms present in the alimentary canal by increasing the activity of beneficial enzymes that relieve lactose mal-absorption, such as galactosidase enzyme and it break down toxins produced by pathogenic bacteria in the intestines (Salman *et al.* 2014). Studies also indicated that eating beneficial bacteria contributes to stimulating the immune system by promoting the release of antibodies, raising interferon levels, and boosting the number of macrophages and natural killer cells (Agrawal 2005).

*Lactobacilli* differ from Lactic Acid Bacteria (LAB), a group of microorganisms widely being defined by production of acid lactate as a primary by product of sugar breakdown, it is a diverse collection of organisms with variable scientific categorization. Döderlein's bacillus, which was originally discovered by Döderlein in 1892 in the acidic secretions of pregnant women, has since been identified as a strain of L. *acidophilus* (Satokari et al., 2005). With over 100 speciessnow known, lactobacilli species are taxonomically diverse. Gram+, unable to produce spoes, unable to motile, rod or cocco-bacilli in configuration, and the G-C concentration typically below 50%, are how it is defined. They have strong fermentative capabilities and are facultatively anaerobic, aerotolerant, and acidophilic (Liu *et al.* 2014). Several Lactobacilli culture mediums have been illustrated, but de Man Rogosa Sharpe (MRS) medium is the best and most typical one for lab use (Horn *et al*. 2005). On agar medium, *Lactobacillus* colonies are typically small (2 to 5imm), withwarched, smooth, sheen and opaque colorless entire edges. In a few rare instances, they are yellowish or rose, plainly viscid states. When growing on agar, it contains stray protein or lipid, clearing zones by exo-enzymes are typically not visible. However, most strains only have weak proteolytic activity because of peptidases and proteases that are either adhered to or released from the cell membrane. Ordinarily, chemoorganotrophic *Lactobacillus* ferments carbohydrates to produce lactic acid (Ezema 2013). The optimal temperature for lactobacilli growth is between 30 and 40 °C, and they grow ideal in slightly acidic medium with a minimal pH of 4.5 to 6.4. Depending on the species and strain, growth ends at pH levels between 3.6 and 4.0. Additionally, many strains are comparatively aerotolerant; best growth is achieved in microaerophilicoor anaerobiccconditions; increased $CO_2$ fixation (5%) may stimulate development. Thermophilic lactobacillus may contain a maximal limitation of 55°C and do not develop below 15°C. Most LAB grow best in mesophilic temperatures, with a maximum breaking point of about 40°C. But certain stains can also form at temperatures as low as 5°F and below 15°C. There are no known thermophilic lactobacilli above 55 °C (Tannock 2004).

Bifidobacteria, which are currently present germs, exert a variety of anti-microbial effects on both good and bad bacteria. Probiotics, which colonize the gut flora and change the gut microbiome, are acceptable nonpathogenic bacteria, and significantly impact the host's health through their metabolic processes (Neville and O'Toole 2010). A bacteria must primarily be non-pathogenic (unharmed), tolerant to lower pH, and rich in concentrations of conjugated and deconjugated bile acids to be considered as a probiotic. They were subjected to the immune system's torment and shouldn't have caused the formation of antibodies (Belicová *et al.* 2013). Probiotics offer a variety of health benefits, including as immunomodulatory effects that could reawaken both humoral and cell-mediated immune responses. Increased serum antibody synthesis and circulation, increased cytokine excretion, improved efficacy of ingested vaccinations, and the restoration of immune function in immunocompromised hosts are all confirmed effect (Ezendam and Van Loveren 2006). The improvement of intestinal mobility is one of the physiological benefits attributed to certain bacteria, such as those from the genus Lactobacillus. This improvement can be due to various factors, including the production of short chain fatty acids (SCFAs), bacteriocins, reduction of intestinal pH, competition for nutrients and adhesion sites with pathogens, and other anti-microbial actions (Gill 2003). Members of the genus *Lactobacillus*, including *L. rhamnosus, L. plantarum. L. acidophilus, L. johnsonii, L. reuteri, L. gallinarum*, and *L. crispatus*, are commonly considered safe and are often added to food as probiotics (Jeevaratnam *et al*. 2005).

# 3. MATERIALS AND METHODS

## 3.1 Study design

In this research, 170 clinical cases were collected from October 2021 to January 2022 from two hospitals located in Baqubah City, Diyala Province, Iraq. The hospitals were Baqubah Teaching Hospital and Al-Batool Teaching Hospital. The individuals were selected from both gender and ages ranging from 33-78. There were two group obtaioned in the study: 100 samples from patients with diabetes who had infections in their feet and 70 samples from patients with diabetes who did not, Samples were taken by using cotton disposable swabs from patients and then transferred in disposable containers into the sterile media. In the study, all swabs were collected under sterile conditions, immediately transferred and inoculated onto blood agar and MacConkey agar. The plates were then incubated for 18 to 24 hours at 37°C, and pure growth colonies were cultivated on selective media. The standard procedures were then followed to perform a biochemical examination for identification, diagnosis, and characterization of the bacteria. The following diagram shows the processes of the current investigation (Figure 3.1).

Hospital executives provided an authorized consent. All participants and medical professionals were also made aware of the goal of the work. Patients were made aware that there were no health concerns associated with the study. The trial was fully free to participate in, and all participants had the opportunity to leave at any moment, without having to offer a reason or suffer any detrimental impacts on their treatment or health. The participants were made aware that the data collected would only be used for the purposes of the study, and steps were taken to protect anonymity by coding the data. Oral consent was obtained, and patient information was recorded (Appendix 1).

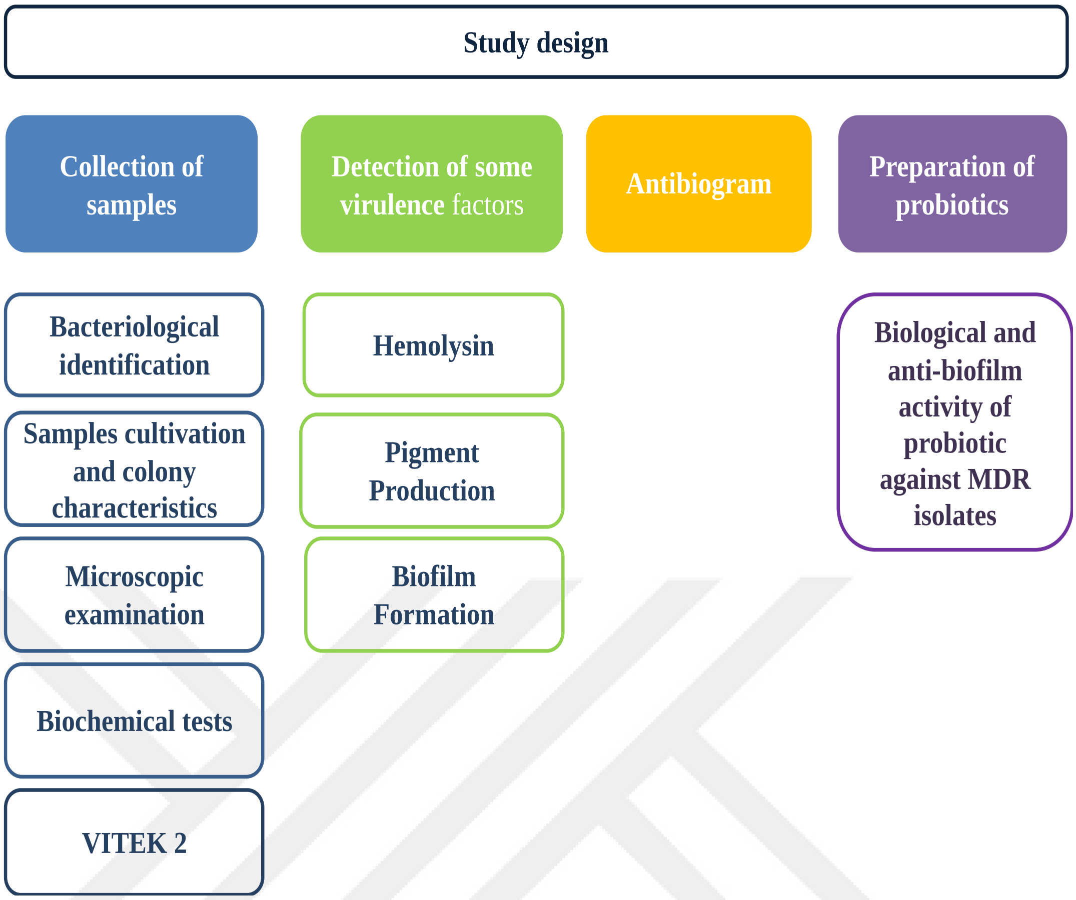


**Figure 3.1** Study design **1**

In the current study, the Antibiotic (Mastgroup / UK) were used in Table 3.2 according to the concentration of Clinical and Laboratory Standards Institute, (2022).

**Table 3.1** Antibiotics

| Antibiotic Class | Antibiotic Type | Symbol | Concentration µg/Disc |
|---|---|---|---|
| ß-lactam combination agent | Piperacillin-Tazobactam | PTZ | 100/10 |
| | Amoxicillin-Clavulanate | AMC | 20/10 |
| | Ticarcillin-Clavulanate | TIM | 75/10 |
| Cephalosporins | Cefepime | CPM | 30 |
| | Ceftriaxone | CRO | 30 |
| Fluoroquinolones | Ciprofloxacin | CIP | 5 |
| | Levofloxacin | LEV | 5 |
| | Ofloxacin | OFX | 5 |
| Aminoglycosides | Gentamicin | GM | 10 |
| | Streptomycin | S | 10 |
| | Netilmicin | NET | 30 |
| | Amikacin | AK | 30 |
| | Tobramycin | TN | 10 |
| Monobactams | Aztreonam | ATM | 30 |
| Carbapenems | Imipenem | IMI | 10 |
| | Meropenem | MEM | 10 |
| Lipopeptides | Polymyxin B | PB | 300 |
| Glycopeptides | Vancomycin | VA | 30 |
| Lipoglycopeptides | Teicoplanin | TEC | 30 |
| Macrolides | Azithromycin | ATH | 15 |
| | Clarithromycin | CLA | 15 |
| Glyopeptides | Vancomycin | VA | 30 |
| Nitrofurans | Nitrofurantoin | NI | 300 |
| Phenicols | Chloramphenicol | C | 30 |
| Folate antagonist | Trimethoprim-Sulfamethoxazole | TS | 1.25/23.75 |
| Lincosamides | Clindamycin | CD | 2 |
| Ansamycins | Rifampin | RF | 5 |
| Tetracyclines | Tetracycline | T | 30 |

In the study, a variety of agar media were employed, including Brain Heart Infusion Broth (BHIB), Blood agar, Muller Hinton agar (MHA), Lactobacillus MRS agar, Nutrient agar, Lactobacillus MRS broth, MacConkey agar, Pseudomonas agar base with CFC supplement, Eosin methylene blue, and mannitol salt agar. The preparation of the culture media followed the instructions provided by the manufacturer for bacteria cultivation and identification. This involved dissolving the powder in distilled water, mixing it thoroughly with hot water while stirring, and sterilizing it in an autoclave for fiften minutes in 121°C and 1 bar of pressure (15 bar/inch 2). The sterilized medium was then added to sterile Petri dishes. To ensure sterility, all petri dishes were incubated at 37°C for 24 hours before being stored at 4°C until use (James *et al.* 2020). Here is a list of how the strains and solution for the current investigation were prepared below:

- **Gram strain**

In the current study, various media were utilized, including Pseudomonas agar, MacConkey agar, and nutrient broth (NB). The Gram staining technique was utilized in the study, using a commercially available kit which comprised of crystal violet stain, Lugol's iodine stain, decolorization solution, and a 0.5% safranine counter-stain. This technique is commonly used in microbiology labs and plays a vital role in categorizing bacteria into two groups, such as gram-positive and gram-negative. (Murray *et al*. 2020).

- **McFarland standart solution**

In this study, the French company Bio Mérieux was utilized to measure the quantity of bacteria, providing an estimate of approximately 1.5108 CFU/mL.

- **Bacterial suspension**

In this study, A bacterial suspension was prepared by taking a single colony grown on MacConkey agar and adding it to (five) mL of distilled water. The turbidity of the suspension was measured using the McFarland standard and was determined to be (1.5108) CFU/mL.

### 3.1.1 Phenotypic identification

In the beginning of the study, initial tests were performed based on the visual characteristics of the bacteria growing on the media. These characteristics included the shape, texture, color, and edge of the colonies, As well as its smell and the color of any dye produced. (Baron *et al*. 2007).

The examination of the growing bacterial cells was carried out by using a multipart light microscope at a magnification of 100x. The sample was obtained from a young colony using a sterile inoculation loop and spreading it on a clean slide. After allowing it to dry and fixing it by heating it on a Bunsen burner, the sample was stained using the Gram stain method. (James *et al.* 2018).

### 3.1.2 Biochemical tests

Bio-chemical tests were used to diagnose bacterial isolates, the used test are listed below:

- **Catalase (slide test)**

Using a clean inoculating needle, we can remove the center of a 24-hour-old, healthy-growing, unpolluted culture colony, mix it with 60 microliters of 3% hydrogen peroxide (H2O2) reagent, and then place it on an uncontaminated slide to see if catalase has developed. In the current study, a 3% concentration of commercial hydrogen peroxide (H2O2) was made from a 15% stock solution and stored in a dark tube. The test was performed to determine the presence of catalase in bacteria. A positive result was indicated by the production of fast bubbles of gas O2, while the lack of a catalase reaction indicated a negative result (Kasprowicz *et al*. 2018).

- **Oxidase**

In the study, a ready-to-use oxidase reagent was employed to identify the presence of cytochrome C in gram-negative bacteria. The reagent consisted of N, N, N, N-tetra-methyl-p-phenylene-diamine di-hydrochloride, which was dissolved in 3 mL of sterile distilled water or saline. To perform the test, a few drops of the oxidase reagent were applied to a piece of filter paper and a sample of the colony under investigation was collected. If the presence of cytochrome C was detected, the color of the filter paper changed to blue or purple within 10 seconds. The oxidase reagent was made by dissolving one gram of N-N-N-tetramethyl-P-phenylene diamine hydrochloride in 100 milliliters of distilled water and stored in a dark container in the refrigerator for use within a week. (Forbes *et al.* 2007).

- **Coagulase**

The test's objective was to distinguish between coagulase-positive and coagulase-negative Staphylococci. To perform the test, a mixture of *Staphylococci* colonies and 0.5 mL of plasma was created. The mixture was then incubated for 4 hours at 35°C, and the tubes were checked to see if any clots had formed. The presence of bound and free coagulase was assessed using the Coagulase (Clumping Factor) assay. (Forbes *et al.* 2007) as described in:

- The suspected bacterial colony was blended with one drop of saltwater, placed on a clean glass slide, and then one drop of human blood plasma was added. This is referred to as the slide coagulase test (Slide method). If a clot is formed, the result is considered positive, while negative results are subjected to a retest using the tube method.

- The Coagulase assay (tube method) involved mixing a single colony with diluted human plasma (1:6) in a test tube. The presence of free coagulase enzyme was determined by incubating the mixture at 37°C for 1 to 4 hours, and then examining the result. For negative results, the tube was reinsulated for 24 hours before reexamination, with the formation of a clot indicating a positive result.

- **IMVic tests**

All IMiVC biochemical testing were conducted in accordance with (James *et al*. 1996).

- **Indole test**

The capacity of an organism to degrade tryptophan with the aid of tryptophanase and create indole was assessed using the indole test. A bacterial culture was incubated overnight and then grown for 24-48 hours at 37°C, and 0.5 mL of Kovac reagent was added to the culture flask. A positive outcome was evidenced by the immediate appearance of a red band at the surface of the broth.

- **Methyl red test**

Methyl red (MR) testing is used to identify microbes that ferment mixed acids when they come into contact with glucose. Three acids (lactate, acetate, and succinate) are generated in large quantities during mixed acid fermentation. The pH of the medium significantly decreases below 4.4 as a result of the considerable amount of acid. By employing a pH indicator, methyl red can be seen as yellow above pH (5.1) and red at pH. (4.4). The examined bacterial culture was added to MR-VP medium, which was then incubated for 72 hours at 37°C. The broth culture received just a few drops (5–6) of the methyl red solution. An immediate red hue denotes a successful outcome, while a yellow color denotes a failure.

- **Vogas-Proskauer test**

In this test, the capacity of an organism to create acetoin, the major consequence of glucose metabolism, and trace quantities of mixed acids, was examined. The test involved incubating a bacterial culture in the presence of oxygen and a catalytic agent, 40% potassium hydroxide, which converts acetoin into diacetyl and alpha-naphthol to form a red complex. The medium was infused with the cultur, which was then incubated for 24-48 hours at 37°C. Three milliliters of solution 1 and one milliliter of solution 2 were then added. After five minutes, the test was deemed successful if a red hue developed.

- **Citrate Utilization test**

The goal of this experiment was to test whether bacteria could use sodium citrate as their sole carbon source. Inorganic ammonium di-hydrogen phosphate is the sole source of nitrogen that is always available (NH4H2PO4). The studied bacteria were injected into the Simmon's citrate agar slant by streaking with a sterile loop, and they were then cultivated at 37°C for 24-48 h. The color of the medium was changed from green to blue to denote a successful conclusion.

- **Kligler iron agar**

This medium used to check the capability of the bacteria to fermenting lactose and glucose (dextrose). It was prepared according to instructions of (Himedia) company. The isolates were inoculated on Kligler iron agar (KIA) slant by spotting as well as incubation at 37°C for 24-48 h. The color alteration of phenol red from red to yellow indicates positive reaction and elevated the medium indicates to production of gas. Sometimes some microbes yield ($H_2S$) from thiosulphate, and then $H_2S$ reacted with iron-salts to yield a black precipitate.

- **Urease test**

It was made by dissolving 24 grams of the urea agar foundation medium in 950 milliliters of distilled water. 50 mL of urea 40% should be added and thoroughly combined after cooling to around 45°C and autoclaving to sterilize. After that, tilt the test tube after adding 5 mL to it. For 24 hours at 37°C, the culture media was streaked and stabbed at an angle. A favorable outcome is indicated by the hue changing from medium to pink (Forbes *et al.* 2007)

### 3.1.3 Isolation and Identification of the Bacteria

The clinical swabs were cultured by streaking methods on selective and differential media, the bacteria isolated in pure cultuers and- identified initially by culturing on selective and differential media- as follow:

- **Mannitol salt agar**

The identification of *Staphylococci* is achieved by using mannitol salt agar plates that are inoculated with the bacteria. *Staphylococcus* aureus, a pathogenic strain, has the ability to ferment mannitol, which leads to a yellow coloration near the growth on the medium, allowing it to be differentiated from the non-pathogenic *Staphylococcal* species. (James *et al.* 2020).

- **Eosin methylene blue**:

To identify the gut bacteria and differentiate lactose fermenters from non-fermenters, the *E. coli*, lactose, and dyes eosin and methylene blue are employed. The presence of significant amounts of acid produced by *E. coli* causes the dyes to precipitate onto the surface of the colonies, resulting in a metallic green sheen and blue-black color. On the medium, thick, mucoid, and pink colonies are produced by other coliform bacteria such Enterobacter aerogenes. Lactose-intolerant bacteria produce colorless colonies that, due to their transparency, look purple in the media. Additionally, the medium discourages the growth of gram-positive organisms, leading to an abundance of gram-negative growth. (James *et al.* 2020).

- **Pseudomonas agar**

48.4 g of Pseudomonas agar powder, 1000 mL of distilled water, and 10 mL of glycerol were combined to create this medium. The mixture was then sterilized by autoclaving it for 15 minutes at 121°C and a pressure of 15 pound/in2, chilled to 45 °C. A vial of CFC supplement was added to the cooled mixture, which was then poured into sterile Petri dishes. (Baron *et al*. 2007).

### 3.1.4 Identification of the isolates by VITEK2

It was possible to accurately identify isolated bacteria using the VITEK-2 system. With an accuracy of up to 98%, this device's 64 biochemical tests are utilized to diagnose germs, and the investigation's findings can be obtained in as little as 8 hours or less. The stages included were as follows:

- **Preparation of microbial suspension**

A few colonies from a pure bacterial culture were transferred to clear plastic test tubes using a sterile brush, and then suspended in 4 mL of sterile saline solution. Following that, the solution's turbidity was set to 2.0 optical densities.

- **Inoculation of diagnostic card**

The process of identifying the isolate involved using a specialized apparatus that combined a vacuum system with an identification card. The suspension of the isolate was injected into the card using the vacuum apparatus, which was then inserted into a rack known as a cassette. The cassette could accommodate multiple tests and held a test tube containing the isolate's suspension. The cassette was then placed in the vacuum chamber station, where a vacuum was applied to force the suspension into small channels that filled each test well on the card. This process could be done manually or automatically, and the cassette could hold up to 10 or 15 tests.

- **Card sealing and incubation**

A device that seals the card after cutting off the transfer tube and inserting it into the carousel incubator was used to deliver the inoculated card. The incubator carousel has a capacity of (30) or (60) cards. Online incubation is done at (35.5 + 1.0)$^{o}$C for all card types. Every 15 minutes, a single card is taken out of the carousel incubator, returned until the following reading period after being sent to the optical system for reaction readings. Data were collected throughout the entire incubation period at intervals of 15 minutes.

There are two methods that were used to preserve the isolates. The bacterial isolates were first injected, incubated at 37°C for 24 hours, and then kept at 4°C in the refrigerator for one to three months after being found on the slant nutritional agar. Up to three months were spent keeping the bacterial isolates at -20°C on a medium of brain heart infusion broth with 15% glycerol (deep freezing). The medium was created by mixing 8 mL of brain heart infusion broth with 2 mL of glycerol, and it was then autoclaved. A pure bacterial isolated colony was injected into the medium after chilling, and it was then cultured at 37°C for 24 hours. The tubes were kept under deep freezing at -20°C (Forbes *et al.*, 2007)

### 3.1.5 Detection of some virulence factors of *P. aeruginosa* isolates

The following virulence variables were evaluated for in the isolates of the current study:

- **Hemolysin production**

The purpose of the study was to monitor the production of the haemolysin enzyme. The samples were placed on a blood agar substrate, which contained 5% human blood, and allowed to grow for 24 hours at 37°C after being injected. The colonies that grew and exhibited hemolysin positivity, indicated by the complete lysis of red blood cells (as seen in the clear region), were identified as containing the desired enzyme (Nader *et al.* 2017).

- **Pigment Production**

Pseudomonas agar and Nutrient agar medium were used to assess pigment formation. Bacterial isolates were added to the media, and the cultures were kept warm for 224 hours at 37°C (Nader *et al.* 2017).

- **Biofilmiformation**

According to reports, the bio-production was identified using the micro-titer plate method. films for 24 hours, the bacteria were cultured at 37°C after being inoculated onto nutritious broth. Then, using the same medium as the diluent, a McFarland standard No. 0.5 was compared to the broth cultures (Almeida *et al.* 2013). In a 96-well flat-bottomed polystyrene plate, 200 L of the isolate suspension was added to three wells. The plate was then kept in an incubator at 37°C for 24 hours to allow for incubation. After that, the contents of each well were gently shaken and rinsed three times with distilled water, before being thoroughly dried. 200 liters of pure methanol were unable to move the stuck bacterial cells. Following that, 200 liters of 0.5% crystal violet were placed in each well for 15 minutes to mark them. In the experiment, crystal violet was bound to the adhering cells and was not removed even after repeated washings and the addition of 200 L of ethanol per well. A negative control was established by using the absorbance of wells containing only nutritional broth, free of microorganisms. The amount of crystal violet removed by 95% ethanol was measured by determining the OD 630 nm of each well using an ELISA reader, in accordance with the method. The absorbance values indicated the degree of biofilm formed by the studied isolates on the surface of the micro-titer plate. The outcomes were categorized into three groups: non-biofilm producer, moderate biofilm producer, and strong biofilm producer. The absorbance of the grown well was compared with that of the control wells. If the OD of the isolate was equal to or less than the OD of the control, the isolate was considered a non-biofilm producer. The isolate was classified as a moderate biofilm producer if its OD was higher than the control's OD but not twice as high as the control. And the isolate was regarded as a potent biofilm producer if its OD was more than two times that of the control (Tang *et al.* 2011).

### 3.1.6 Anti-biogram

According to the antimicrobial susceptibility testing protocol outlined by the Clinical and Laboratory Standards Institute (2022), the following steps are involved:

- The Kirby-Bauer method was utilized for species that grow quickly on Mueller-Hinton agar plates. The petri dishes were filled with a solvent to a depth of approximately 4 mm.
- The inoculum used was a pure culture, and 2 to 4 related colonies were transferred into 5 mL of sterile saline solution. To obtain an average of 1.5 x 108 cells/mL, the turbidity of the bacterial suspension was compared to that of the McFarland Standard.
- The entire agar surface of the petri dish was inoculated using a sterile cotton swab. The swab was used to streak the surface three times, between each line, turn it at a 50-degree angle. With the cover on, the inoculum was allowed to dry for five to ten minutes.
- Antibacterial disks were applied to MHA with the use of a disk dispenser (MASTTM) (Oxoid™).
- After being cultured for 18 to 24 hours at 37°C, the petri dishes were then examined. To the closest millimeter, the inhibitory zone diameters were measured and reported (Moreno *et al.* 2006).

To identify the presence of *Lactobacillus* spp. in Baqubah city, samples of local fermented and curd food (dairy products) were collected from various local markets. The isolation of *Lactobacillus* spp. was performed using the following steps:

- The serial dilution procedure utilizing MRS Broth and Agar was utilized to acquire the Lactobacillus isolates used in the study as shown in (Figure 3.2). The plates were poured and cultured, and then incubated under microaerophilic conditions in candle jars with 5% Co2 for 48 hours. (Ranganath *et al*. 2012).
- Based on its cultural and biochemical properties, the bacteria were located.

- Gram stain and catalase production were the first tests performed on each isolated strain. Different phenotypic categories were developed based on the isolates' cell shape and colony characteristics on de-Man Rogosa and Sharpe agar (MRS agar).
- For subsequent research, only Gram-positive , catalase-negative , and rod-shaped isolates were chosen.
- The isolates were kept alive on MRS agar slants.

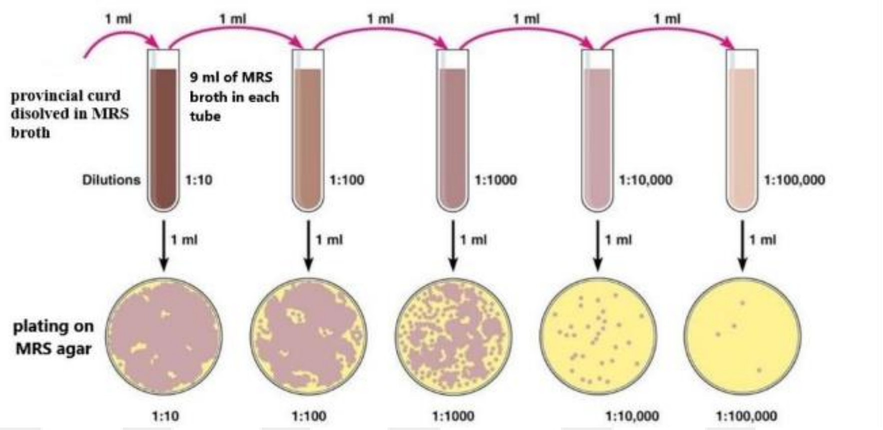


**Figure 3.2** The isolation of Lactobacillus spp was performed using the serial dilution method with MRS broth and agar (Moreno *et al*. 2006)

The supernatant of Lactobacillus spp. was prepared by inoculating a test tube with 10 mL of MRS broth containing 2% of Lactobacillus spp. incubating it for 24 hours at 37°C, and then centrifuging it to extract the cell-free supernatant (CFS). Later, to determine the activity by agar well diffusion methods, the bacterial suspension was prepared as mentioned above, the agar well diffusion method was conducted to determine the activity of *Lactobacillus* spp. as below:

- After performing the streaking technique and comparing with McFarland tube (1.5x108 CFU/mL), 5mm wells were drilled into the Muller Hinton agar plate for each bacterium using a sterilized cork borer.

- 100 microliters of the CFS that containing the probiotic of *Lactobacillus* spp. was adds to each well and at 37°C, the plates were incubated for 24 hours.

The effectiveness of the probiotic was assessed by measuring the millimeter-scale inhibitory diameter of each concentration (Devi *et al*. 2013). The crystal violet stain was used to evaluate the anti-biofilm activity of the cell-free supernatant (Merino *et al.* 2019). With a few adjustments, 106 CFU/well of microbes were added to 96-well plates with CFSs (100/100 microliter). The medium was taken out after 24 hours of incubation at 37°C, and the wells were carefully cleaned with distilled water to get rid of any remaining planktonic cells. Then, 1% crystal violet solution was added to each well. The wells were dyed for 30 minutes with crystal violet from the biofilm, and any leftover color was removed by dissolving the crystal violet in a solution of 30% methanol and 10% acetic acid. In a micro-plate reader, the absorbance was determined at 570 nanometers. Calculating the variations in biofilm formation before and after CFS treatment allowed for the determination of the biofilm formation degree.

## 3.2 Statistical Analysis

To compare the effect on the percent value chi square test was done by using SPSS version 26.0. To obtain *p* value and all statistical tests were performed at 0.05 significance level. Post-test *p* values are as follows: $p < 0.05$: $p < 0.01$: $p < 0.001$.

# 4. RESULTS AND DISCUSSION

## 4.1 Bacterial Isolation

The isolation of bacteria was divided into two groups, control group with 70 samples without foot infections and 100 samples from patients with diabetes. 30 was negative growth

### 4.1.1 Bacterial isolation from non-infected foot diabetic patients

A total of 70 samples were taken persons without foot infections and have diabetes (Table 4.1). The results showed that 54 (77.1%) samples were positive growth and 16 (22.9%) were negative; the isolates were *S. epidermidis*, *S. aureus* and *E. coli* which were 31 (44.2%), 18 (25.7%) and 5 (7.1%) respectively, this result was in agreement with (Salman, 2018) who also found that the percentage of bacteria as a normal flora that isolated from non-infected foot in diabetes patients was 76.6%.

**Table 4.1** Normal flora isolated from non-infected patients

| Bacteria | Isolated Bacteria | Percentage (%) |
|---|---|---|
| *Staphylococcus epidermidis* | 31 | 44.2 |
| *Staphylococcus aureus* | 18 | 25.7 |
| *Escherichia coli* | 5 | 7.1 |
| Total | 54 | 77.1 |

The isolation of *S. aureus* bacteria within the current study can be expected due to the presence of the normal flora of the skin (25-30%) (Fitz-Gibbon *et al.* 2013), but it is an opportunistic bacteria that varies in its infection from simple skin lesions to dangerous diseases that threaten the life of the host (Koboyshi *et al* 2015). Our knowledge of the areas of settlement of normal flora in the host's body will increase our understanding of infection with pathogenic bacterial species. The presence of these organisms in normal conditions on human skin is beneficial by hindering invading pathogenic microbes from settling the surfaces of the skin and tissues and competing for nutrients, but they sometimes behave as opportunistic behavior under certain conditions and became pathogenic if it changes its natural location in the body or increases its numbers above the normal level of its presence (Carroll *et al.* 2015). There are types of opportunistic bacteria that are often a component of the natural flora that attack the body of immunocompromised patients and differ in disease events, some of them invade the body and spread within the living body, as well as their ability to disrupts the defense mechanisms through the production of enzymes and toxins that destroy tissues and facilitates the spread of the bacteria inside the body (Mastromarino *et al.* 2013).

### 4.1.2 Bacterial isolation from diabetic foot infections

A overall of 100 samples were obtained from patients with foot diabetic infections which were identified by biochemical tests (Table 4.2, Table 4.3), culture on selective and differential media and confirmed by VITEK 2 system. The results showed that the positive growth for bacteria was 70 isolates (70%), difference between gram negative and gram positive isolates was significant (<0.001) and it was in agreement with (Macdonald *et al.* 2021), it was also in agreement with (Shahi and Kumar 2016) who reported that the proportion of gram negative was the highest which was 97% while the gram positive was 3%.

**Table 4.2** Biochemical tests for Gram negative bacteria

| **Biochemical Tests** | ***P. aeruginosa*** | ***E. coli*** | ***K. pneumoniae*** | ***P. mirabilis*** |
|---|---|---|---|---|
| Gram stain | - | - | - | - |
| Test for oxidase | + | - | - | - |
| Test for Catalase | + | + | + | + |
| Methyl red | - | + | - | + |
| Citrate utilization | + | - | + | + |
| Voges-Proskauner | - | - | + | - |
| Indol production | - | + | - | - |
| Urease production | -/+ | - | + | - |
| Kliglar iron agar | K/K | A/A | A/A Gas | K/A |

**Table 4.3** Biochemical tests for Gram positive bacteria

| Biochemical Test | | | | |
|---|---|---|---|---|
| **The Isolates** | **Catalase** | **Coagulase** | **Oxidase** | **Gram stain** |
| *Staphylococcus epidermidis* | + | - | - | + |

Two isolates were belonging to the gram positive and 68 belonging to gram negative. Among the microorganisms, *K. pneumonia* had been isolated the most (36%) followed by *P. aeruginosa*, *E. coli*, *Proteus spp.* and *S. epidermidis* (16%, 12%, 4% and 2% respectively). The positive growth was 89%,found that *Pseudomonas* spp. was the microorganism that had been isolated the most which was 23% followed by *E. coli* and *Klebsiella* which were 20% and 17% respectivel (Table 4.4). The last result was in line with the previous study that percentage of *Proteus* spp. was 3%, (Abd El Mohsen 2020).

**Table 4.4** Frequency of bacteria

| Types of the bacteria | Isolate | Number | Percentage (%) | Total No. and % No. (%) |
|---|---|---|---|---|
| | *S. epidermidis* | 2 | 2 % | 2 % |
| Gram Negative | *E. coli* | 12 | 12 % | 68 (68 %) |
| | *K. pneumonia* | 36 | 36 % | |
| | *Proteus* spp. | 4 | 4 % | |
| | *P. aeruginosa* | 16 | 16 % | |
| | *P*- value < 0.000 | | | 70 (70 %) |

According to a meta-analysis (1980 to 2019) for aerobic bacteria done by Macdonald *et al.* (2021), 6736 clinical samples from 57 investigations that were established for just aerobic growth were described, of which 5945 (88.3%) were positive growth.

*S. epidermidis* was also conducted in one study as the predominant isolated bacteria in gram positive, although *S. epidermidis* is a natural element of the skin, it can cause serious infections when there are foreign things present, such as prosthetic devices and wound infections (Sadeghpour Heravi *et al.* 2019). The most prevalent bacteria found in DFI isolates were *Proteus*, *E. coli*, *Enterobacter*, *Pseudomonas* and *Klebsiella* (Smith *et al.* 2016).

Bacterial isolates identification had been confirmed for each *S. epidermidis*, *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *P. mirabilis* by using Vitek2 system (Biomeriux) with a high-accuracy diagnosis of 98%. The findings of the present investigation revealed that the highest proportion was in patients >50 year which was 89.2% (Figure 4.1) . The uppermost frequency of DFI was reported in the age set above 60 years (Ahmadishooli *et al.* 2020), while another study reported that 15.3%, 34.6%, 30.7% and 19.2% of patients having DFI in the age group 35-44, 45-54, 55-65, and 65-74 respectively (Khadir *et al.* 2018). A study indicated that 6.5% of patients with DFI were in the age group 18-30 while 13%, 35% and 45% of patients with DFI were in the age group 30-40, 40-50 and 50-65 respectively (Abd-El Mohsen 2020). Accordingly, most patients with DFI were in their fifth and sixth years old (50.6% and 24.1%) (Mutonga 2019).

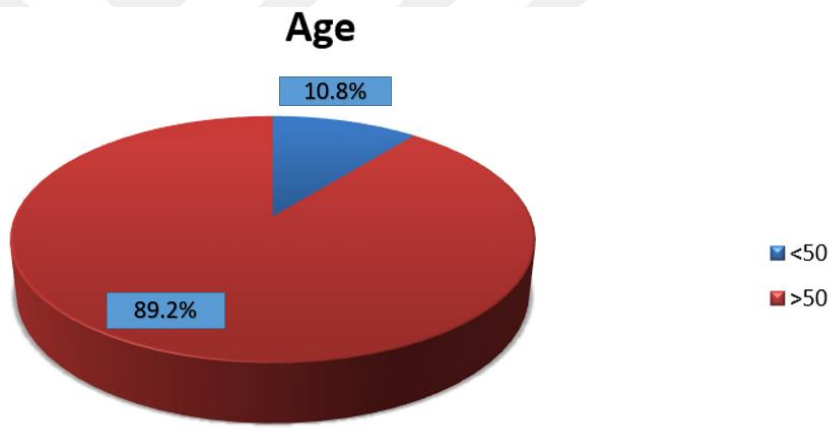


**Figure 4.1** Distribution of DFI according to age

The findings of the present investigation revealed that 89 (68.5%) of patients were males while 41 (31.5%) were females (Figure 4.2). The current study was agreed with previous study that males consist the highest proportion of patients with DFI which was 75.96% while the female's proportion was 24.04% (Khadir *et al.* 2018). The reason might be because of the different physical strengths between men and women during the daily life in region and the movement of mans foot led to increase the pressure on it and afect on the foot area. Another study indicated that males have a significant frequency of diabetic foot infection 88.8% than females 11.2% (Mukhtar *et al.* 2020). The current study, on the other hand, was disagree with (Abd-El Mohsen 2020) that 46.5% of patients were males and 53.5% were females, accordingly 45.9% of patients were males and 54.1% were females (Mutonga 2019).

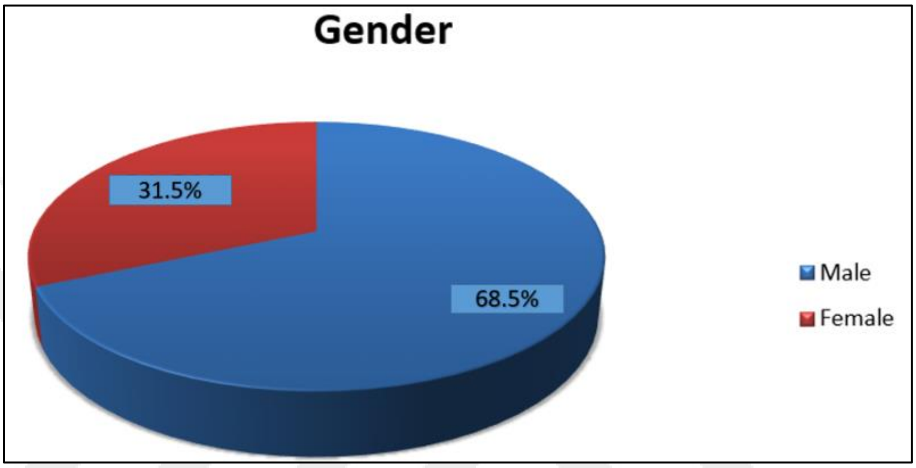


**Figure 4.2** Distribution of DFI in line with gender

Findings from the current study revealed that many patients that having DFI were living in urban area (71.5%) comparing with those living in rural area (28.5%) with significant difference (< 0.001). This result agreed with (Mutonga 2019) who reported that 68.3% of patients with DFI were living in urban and 31.7% were living in rural area, while it was disagree (Abd-El Mohsen 2020) who reported that 42% of patients with DFI were living in urban and 58% were living in rural area (Figure 4.3).

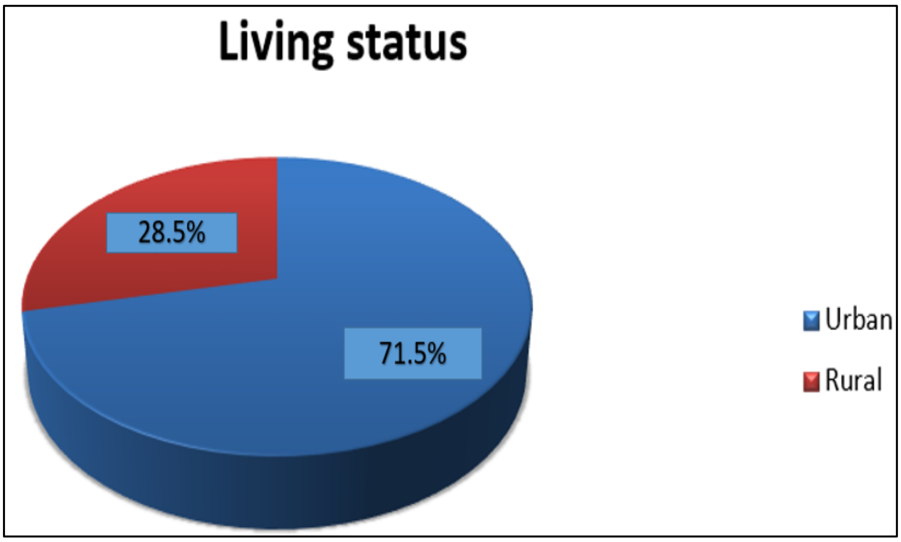


**Figure 4.3** Distribution of DFI according to living status

Findings from the current study revealed that the majority of patients with DFI were without or have minimal education 74.5%, the highest proportion was in patients without or having informal education which was 39.4% followed by primary, secondary and collage which was 36.1%, 19.2% and 6.15% respectively, these results show that utilizing a substantial difference, the infection rate decreases with increased education levels. (< 0.001) and was agree with (Abd-El Mohsen 2020) who reported that 39.5% of patients with DFI have no education and 32.5% were just reading and writing, while it was 16.5%, 12.5, and 8% for primary, secondary and collage respectively. (Muntago 2019) studies show that 60% of diabetic foot patients have little or no formal education (Figure 4.4). A significant problem with diabetic foot infections is illiteracy, a lack of health education, and the predominance of false beliefs. Interestingly, 40% to 50% of diabetic foot infections can be avoided with education and good foot hygiene (Shigidi and Abdelgafar 2013).

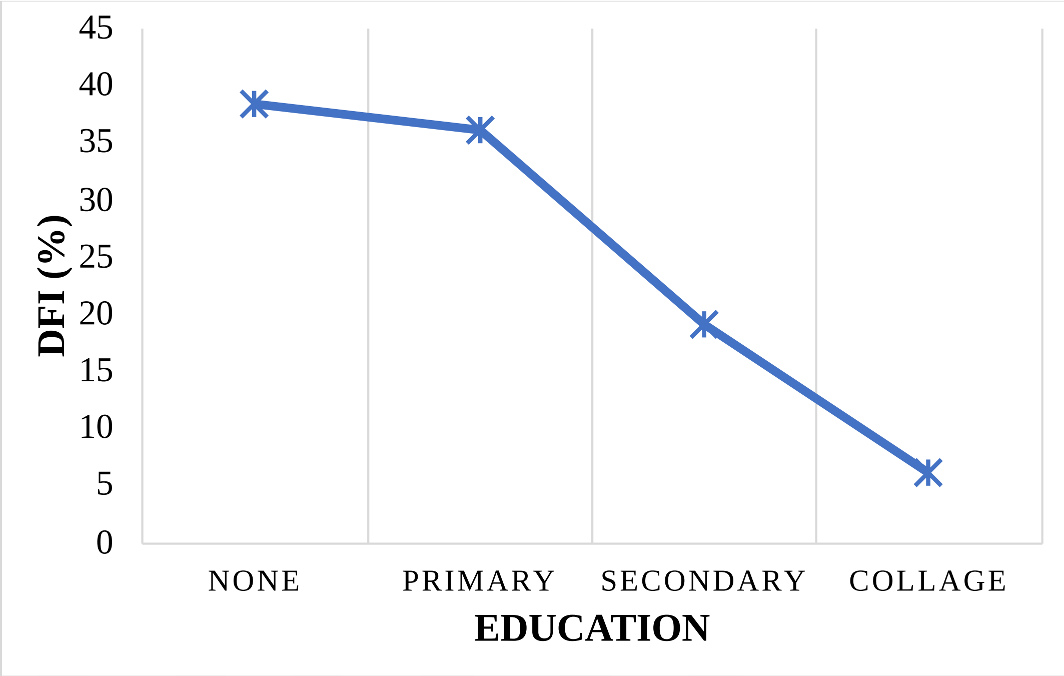


**Figure 4.4** Distribution of DFI according to Education level

## 4.2 Investigation of Virulence Factor

Hemolysis on blood agar was conducted for *P. aeruginosa* isolates to detect the beta hemolysis by observing the clear zone around the colony. The outcomes of the present study displayed that all these isolates were positive production to this virulence factors, it is a possible virulence agent that bacteria create that might be harmful to people's health. Hemolysin damages the cell membrane of red blood cells, which is thought to be responsible for a number of activities in the host cell. Heme, which contains iron and is appreciated for its visual appeal in red blood cells (RBCs), is released into the environment when RBCs deteriorate, allowing bacteria to gather free ironHemolysin has a number of impacts on tissue, including causing damage, promoting bacterial growth, extracting nutrients from the host, and potentially modifying host signaling pathways which can influence various processes like host cell survival, inflammatory responses, and changes in cytoskeletal dynamics (Reda *et al.* 2017).

*The P. aeruginosa* isolates were observed to produce pigment on nutrient agar, with all of the isolates generating a bluish-green color. *P. aeruginosa* is also capable of producing other pigments, including the blue-green pigment pyocyanin, commonly referred to as "blue pus," which can be seen on the surface of the petri dish during growth. Additionally, the organism can produce pyorubin, a red pigment, and pyromelanin, a black pigment (Abdulrahman *et al.* 2020).

Pyocyanin, which is part of the phenazine family due to its phenazine nucleus and role as a virulence factor, is a product of secondary metabolism. It acts as a biosignal molecule, impacting gene expression and promoting the health of growing bacteria, and is integral to the formation of biofilm. With antibacterial and antifungal properties, pyocyanin also causes oxidative stress that alters the host's mitochondrial electron transport system and can damage tissues, particularly oxygenated tissues like the lung, through oxidation (Aykac *et al.* 2017).

#### 4.2.1 Biofilm formation

Due to the biofilm's inherent resilience to anti-microbial treatments and the ability to select different phenotypic variants, biofilm development is another virulence factor. Using the microtiter plate method, the ability to form biofilms was used to evaluate all isolates (70). The sticky biofilms are stained using the micro-titer Plate technique (MTP), a colorimetric method, and then counted using an absorbance micro-titer plate reader. On abiotic surfaces, it is utilized to study the first phases of biofilm formation. The present study's findings include provided in (Table 4.5), and they reveal that only 13% of the isolates were non-biofilm producers, with yields ranging from substantially to moderately greater than those of the negative control. Of the isolates that generated biofilm, 34% were highly biofilm producers and 53% were moderate producers.

**Table 4.5** Biofilm formation. The abreviations refer to: SE= *S. epidermidis* / PA= *P. aeruginosa* / KP= *K. pneumonia* / EC= *E. coli* / PM= *P. mirabilis* / N= negative / M= moderate / S= strong.

| No. of isolate | Absorbance at 360 nm | Biofilm level compared to (ODc=0.040) | No. of isolate | Absorbance at 360 nm | Biofilm level compared to (ODc=0.040) | No. of isolate | Absorbance at 360 nm | Biofilm level compared to (ODc=0.040) |
|---|---|---|---|---|---|---|---|---|
| SE31 | 0.059 | M | EC8 | 0.054 | M | EC33 | 0.063 | M |
| SE32 | 0.037 | N | KP9 | 0.048 | M | PM34 | 0.074 | M |
| PA1 | 0.074 | M | EC10 | 0.052 | M | EC35 | 0.056 | M |
| PA2 | 0.230 | S | KP11 | 0.037 | N | EC36 | 0.148 | S |
| PA3 | 0.159 | S | KP12 | 0.041 | M | PM37 | 0.067 | M |
| PA4 | 0.257 | S | KP13 | 0.038 | N | KP38 | 0.038 | N |
| PA5 | 0.212 | S | KP14 | 0.070 | M | EC39 | 0.123 | S |
| PA6 | 0.053 | M | KP15 | 0.053 | M | KP40 | 0.122 | S |
| PA7 | 0.169 | S | KP16 | 0.064 | M | EC41 | 0.065 | M |
| PA8 | 0.177 | S | KP17 | 0.044 | M | PM42 | 0.065 | M |
| PA9 | 0.073 | M | KP18 | 0.051 | M | KP43 | 0.069 | M |
| PA10 | 0.058 | M | KP19 | 0.047 | M | KP44 | 0.088 | S |
| PA11 | 0.134 | S | KP20 | 0.048 | M | KP45 | 0.159 | S |
| PA12 | 0.059 | M | KP21 | 0.066 | M | KP46 | 0.152 | S |
| PA13 | 0.241 | S | KP22 | 0.038 | N | EC47 | 0.119 | S |
| PA14 | 0.053 | M | KP23 | 0.038 | N | PM48 | 0.053 | M |
| PA15 | 0.141 | S | KP24 | 0.153 | S | KP49 | 0.127 | S |
| PA16 | 0.270 | S | KP25 | 0.152 | S | KP50 | 0.037 | N |
| KP1 | 0.075 | M | KP26 | 0.064 | M | KP51 | 0.038 | N |
| KP2 | 0.059 | M | KP27 | 0.092 | S | EC52 | 0.044 | M |
| EC3 | 0.095 | S | EC28 | 0.045 | M | | | |
| KP4 | 0.103 | S | KP29 | 0.041 | M | | | |
| KP5 | 0.126 | S | KP30 | 0.073 | M | | | |
| KP6 | 0.063 | M | KP31 | 0.034 | N | | | |
| EC7 | 0.058 | M | KP32 | 0.043 | M | | | |

According to MTPs, the composition of a biofilm varies depending on how much of a substance's surface it has. For instance, the membrane developed on a microtiter plate's polystyrene surface is more efficient than one made of silica. The medium, the detection method, the incubation circumstances, and the type of surface employed for that procedure are just a few of the variables that affect how biofilms form (Wojnicz *et al.* 2015). Numerous factors could be to blame for the variations in biofilm density between the isolates in the current investigation. The capability of each strain to form biofilms is influenced by several factors, such as differences in the number of cells that can effectively adhere and the type and amount of quorum-sensing signaling molecules produced by each strain. Variations in the production of ß-Lactamase between strains have also been linked to increased biofilm formation, with strains producing multiple forms of the enzyme showing higher biofilm formation compared to those producing only one type. The microtiter plate method is considered a reliable, simple, and sensitive approach for detecting biofilm formation, as it can accurately detect the formation of biofilms in their early stages. This technique offers steady conditions for investigating the numerous elements required for biofilm development, including pili, flagella, and genes responsible for exopolysaccharide synthesis. (Heydari and Eftekhar 2014; Obaid, 2019)

Antibiotics are unable to completely eliminate mixtures that are wrapped in biofilm, a self-produced extracellular matrix. Thanks to the barrier of the matrix, it can adhere to a surface in the environment. This coating affects how resistant bacteria are to medications. The presence of biofilms offers ten to a thousand times greater resistance to antibiotics, making chronic infections persistent (Ciofu and Tolker-Nielsen 2019). The results of the current study indicate high rates of resistance among isolates with a high capacity for biofilm formation, which is shown to offer 1,000-fold better resistance to antibiotics than planktonic bacteria (Cepas and Soto 2020).

## 4.3 Anti-Biogram

Antibacterial susceptibilty tests were performed for all isolated bacteria by Kirby Bauer disk diffusion method.

### 4.3.1 Anti-biogram for *Staphylococcus* epidermidis

Two *Staphylococcus epidermidis* isolates were tested for antibiotic susceptibility. against, 12 kinds of antibiotics Ofloxacin (5 µg), Ciprofloxacin (5 µg), Clarithromycin

(15 µg), Teicoplanin (30 µg), Gentamicin (10 µg), Vancomycin (5 µg), Nitrofurantoin (300 µg), Chloramphenicol (30 µg), Trimethoprim-sulfamethoxazole (1.25/23.75 µg), Clindamycin (2 µg), Rifampin (5 µg) and Tetracycline (30 µg) pertaining to ten distinct classes (Table 4.6). The Kirby Bauer technique, a common agar disk diffusion method, was employed in the (Clinical and Laboratory Standards Institute 2022). For *S. epidermidis*, Ofloxacin, Ciprofloxacin, Clarithromycin, Gentamicin and Tetracycline were the most effective antibiotics (0% resistance) while Teicoplanin, Vancomycin, Nitrofurantoin, Chloramphenicol, Clindamycin, Trimethoprim-Sulfamethoxazole and Rifampin were resisting in 100% of S. epidermidis isolates. These findings were in agreement with (Mutonga 2019) who reported that 100% of the isolates were sensitive to GM, while it was disagree with him in the hand of CD, NI, RF, TEC and VA which were sensitive by 100%. (Khadir *et al.* 2018) reported that CIP, OFX, RF and VA were resisted in 28%, 42%, 0%, and 42% of *S. epidermidis* isolates respectively. There may be differences in results because of differences in the number of *S. epidermidis* isolates (Table 4.6). Antibiotic resistance has emerged as a result of geographical variances, irregular use, and needless medication use in several places, making effective treatment challenging. (Rastogi *et al.* 2017). The presence of these organisms in normal conditions on human skin is beneficial by hindering invading pathogenic microbes from settling the surfaces of the skin and tissues and competing for nutrients, but they sometimes behave an opportunistic behavior under certain conditions, and it is satisfactory if it changes its natural location in the body or increases its numbers above the normal level of its presence (Jawetiz *et al.* 2016). There are types of opportunistic bacteria that are often part of the natural flora that attack the body of patients who are immunocompromised and differ in disease events (Mastromarino *et al.* 2013).

**Table 4.6** Antibacterial susceptibility test of *Staphylococcus epidermidis* (R= Resist / S= Sensitive / I= Intermediate)

| Antimicrobial Agent | Resistance Pattern | | | | | |
|---|---|---|---|---|---|---|
| | **R** | **%** | **S** | **%** | **I** | **%** |
| **OFX** | 0 | 0% | 2 | 100 | 0 | 0 |
| **CIP** | 0 | 0% | 2 | 100 | 0 | 0 |
| **CLA** | 0 | 0% | 1 | 50 | 1 | 50 |
| **TEC** | 2 | 100% | 0 | 0 | 0 | 0 |
| **GM** | 0 | 0% | 2 | 100 | 0 | 0 |
| **VA** | 2 | 100% | 0 | 0 | 0 | 0 |
| **NI** | 2 | 100% | 0 | 0 | 0 | 0 |
| **C** | 2 | 100% | 0 | 0 | 0 | 0 |
| **TS** | 2 | 100% | 0 | 0 | 0 | 0 |
| **CD** | 2 | 100% | 0 | 0 | 0 | 0 |
| **RF** | 2 | 100% | 0 | 0 | 0 | 0 |
| **T** | 0 | 0% | 2 | 100 | 0 | 0 |

### 4.3.2 Anti-biogram for *Enterobacteriaceae*

Enterobacteriaceae 52 isolates were tested for antibiotic resistance to 12 different types of antibiotics Ceftriaxone (30µg), Aztreonam (30µg), piperacillin-Tazobactam (100/10 µg), Amoxicillin-Clavulanic acid (20/10 µg), Imipenem (10 µg), Polymixin B (300 µg), Ofloxacin (5µg), Gentamicin (10µg), Tobramycin (10µg), Streptomycin (10 µg), Azithromycin (15 µg) and Chloramphenicol (30 µg) related to 9 different classes. The Kirby Bauer technique, a common agar disk diffusion method, was employed in the (Clinical and Laboratory Standards Institute 2022). Ceftriaxone (CRO) and Polymixin B (PB) were the most resisted antibiotics by 100%, these results were agreed with (Khadir *et al.* 2018) who reported a high percentage of resistance 83%, while it was disagree with (Mutonga 2019) who indicate that only 33.3% of *K. pneumonia* isolates were resist. The most effective antibiotic against isolates of K. pneumonia was ofloxacin (OFX), yet 16.7% of the isolates were resistant. Aztreonam (ATM) was resist in 50% *K. pneumonia* isolates, (Mutonga 2019) indicate that 33.3% of *K. pneumonia* isolates were resist to ATM. Piperacillin-Tazobactam (PTZ) was resist in 33.3% of *K. pneumonia* isolates, this result was agreed with (Khadir *et al.* 2018, Mutonga 2019) who reported that 25% and 33.3% of *K. pneumonia* isolates were resist. Amoxicillin-Clavulanic acid (AMC) was resisted in 72.2%, this result was agreed with (Mutonga 2019, Albadri 2021) who found that 66.7% and 77.7% of *K. pneumonia* isolates were resist, it was disagree with (Hefni *et al.* 2013) who found that 100% of *K. pneumonia* isolates were sensitive. Imipenem (IMI) was resisted in 38.9% and this result was disagree with (Hefni *et al*. 2013, Ahmadishooli *et al.* 2020) who found that IMI was sensitive in 100%. Gentamicin (GM) and Tobramycin were resisted in 66.7% and 88.9% of *K. pneumonia* isolates and these findings were agree with (Khadir *et al.* 2018) who found that 66% of *K. pneumonia* isolates were resist to GM and disagree with (Ahmadishooli *et al.* 2020) who found that 100% of *K. pneumonia* isolates were sensitive to GM. Streptomycin (S) and Azithromycin (ATH) were resist in 61.1% and 72.2% of *K. pneumonia* isolates. Chloramphenicol (C) was resisted in 27.8% of *K. pneumonia* isolates and this result was agreed with (Hefni *et al.* 2013) who reported that 20% of *K. pneumonia* isolates were resist and 80% were sensitive (Table 4.7).

**Table 4.7** Antibacterial susceptibility test of *Klebsiella pneumonia* (R= Resist / S= Sensitive / I= Intermediate)

| **Antimicrobial Agent** | **Resistance Pattern** | | | | | | ***P*-Value** |
|---|---|---|---|---|---|---|---|
| | **R** | **%** | **S** | **%** | **I** | **%** | |
| **CRO** | 36 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **ATM** | 18 | 50 | 18 | 50 | 0 | 0 | > 0.05 |
| **PTZ** | 12 | 33.3 | 20 | 55.6 | 4 | 11.1 | < 0.05 |
| **AMC** | 26 | 72.2 | 10 | 27.8 | 0 | 0 | < 0.05 |
| **IMI** | 14 | 38.9 | 18 | 50 | 4 | 11.1 | < 0.05 |
| **PB** | 36 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **OFX** | 6 | 16.7 | 30 | 83.3 | 0 | 0 | < 0.001 |
| **GM** | 24 | 66.7 | 12 | 33.3 | 0 | 0 | < 0.05 |
| **TN** | 32 | 88.9 | 4 | 11.1 | 0 | 0 | < 0.001 |
| **S** | 22 | 61.1 | 14 | 38.9 | 0 | 0 | > 0.05 |
| **ATH** | 26 | 72.2 | 10 | 27.8 | 0 | 0 | < 0.05 |
| **C** | 10 | 27.8 | 24 | 66.7 | 2 | 5.5 | < 0.001 |

For *E. coli*, Imipenem was the most effective antibiotic which 100% of *E. coli* isolates were sensitive, this result agreed with (Abd Al-Hamead *et al.* 2013) who found the same result and disagree with (Albadri 2021) who found that the sensitivity was 70%. Ceftriaxone, Amoxicillin-Clavulanic acid, Polymixin B and Tobramycin were resist in 100% of *E. coli* isolates, these results were disagree with (Khadir *et al.* 2018, Mutonga 2019) who reported that CRO, AMC were resist in 35%, 75% and 69.2%, 53,8% respectively. Aztreonam and Ofloxacin were resist in 16.7% of *E. coli* isolates, (Ahmadishooli *et al.* 2020) found that 100% of *E. coli* isolates were sensitive to ATM while (Mutonga 2019) reported that 84.6% of *E. coli* isolates were resist and these results consider non-compliant with the result of the current study. Piperacillin-Tazobactam, Gentamicin, Streptomycin and Azithromycin were resisted in 33.3% of *E. coli* isolates, (Khadir *et al.* 2018) reported that PTZ and GM were resist in 17% and 64%, (Ahmadishooli *et al.* 2020) indicated that PTZ and GM were resist in 28% and 30%. Chloramphenicol was resisted in 50% of *E. coli* isolates (Table 4.8).

**Table 4.8** Antibacterial susceptibility test of *Escherichia coli* (R= Resist / S= Sensitive /I= Intermediate).

| **Antimicrobial Agent** | **Resistance Pattern** | | | | | | ***P- Value*** |
|---|---|---|---|---|---|---|---|
| | **R** | **%** | **S** | **%** | **I** | **%** | |
| **CRO** | 12 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **ATM** | 2 | 16.7 | 10 | 83.3 | 0 | 0 | < 0.05 |
| **PTZ** | 4 | 33.3 | 8 | 66.7 | 0 | 0 | > 0.05 |
| **AMC** | 12 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **IMI** | 0 | 0 | 12 | 100 | 0 | 0 | < 0.001 |
| **PB** | 12 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **OFX** | 2 | 16.7 | 10 | 83.3 | 0 | 0 | < 0.05 |
| **GM** | 8 | 66.7 | 4 | 33.3 | 0 | 0 | > 0.05 |
| **TN** | 12 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **S** | 4 | 33.3 | 6 | 50 | 2 | 16.7 | > 0.05 |
| **ATH** | 4 | 33.3 | 8 | 66.7 | 0 | 0 | > 0.05 |
| **C** | 6 | 50 | 6 | 50 | 0 | 0 | > 0.05 |

For *P. mirabilis*, Amoxicillin-Clavulanic acid, Polymixin B, Tobramycin, Streptomycin and Azithromycin were resist in 100% of *P. mirabilis* isolates, Ceftriaxone, Gentamicin and Chloramphenicol were resist in 50% of *P. mirabilis* while Aztreonam, Piperacillin-Tazobactam, Imipenem and Ofloxacin which antibiotics had the best results against *P. mirabilis* isolates which were sensitive in 100%. A study showed that *P. mirabilis* isolates were resist to AMC, CRO and GM in 23%, 70% and 47% respectively and it indicated that IMI and PTZ were sensitive in 100% of *P. mirabilis* (Khadir *et al.* 2018) which is in line with the present study. Another study reported that 86% of *P. mirabilis* isolates were resist to AUG while 43% were resist to IMI (Shaaban *et al.* 2020).

However, *P. mirabilis* isolates were sensitive to CRO, IMI and PTZ (that 100%, 100% and 33.3%, respectively) (Ahmadishooli *et al.* 2020) which is diffirent than the current study. Another study showed that. the *P. mirabilis* isolates were sensitive in 100% for AUG, 90% for C, 90% for GM and 95% for IMI (Hefni *et al.* 2013) as observed in the current findings (Table 4.9).

**Table 4.9** Antibacterial susceptibility test of *Proteus mirabilis* (R= Resist / S= Sensitive/ I= Intermediate)

| **Antimicrobial Agent** | **Resistance Pattern** | | | | | | ***P*-Value** |
|---|---|---|---|---|---|---|---|
| | **R** | **%** | **S** | **%** | **I** | **%** | |
| **CRO** | 2 | 50 | 2 | 50 | 0 | 0 | > 0.05 |
| **ATM** | 0 | 0 | 4 | 100 | 0 | 0 | < 0.001 |
| **PTZ** | 0 | 0 | 4 | 100 | 0 | 0 | < 0.001 |
| **AMC** | 4 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **IMI** | 0 | 0 | 2 | 50 | 2 | 50 | > 0.05 |
| **PB** | 4 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **OFX** | 0 | 0 | 4 | 100 | 0 | 0 | < 0.001 |
| **GM** | 2 | 50 | 0 | 0 | 2 | 50 | > 0.05 |
| **TN** | 4 | 50 | 0 | 0 | 0 | 0 | < 0.001 |
| **S** | 4 | 50 | 0 | 0 | 0 | 0 | < 0.001 |
| **ATH** | 4 | 50 | 0 | 0 | 0 | 0 | < 0.001 |
| **C** | 2 | 50 | 2 | 50 | 0 | 0 | > 0.05 |

### 4.3.3 Anti-biogram for *P. aeruginosa*

From the 16 isolates of *P. aeruginosa* were tested for antibiotic resistance to 12 different types of antibiotics: Amikacin (30 µg), Levofloxacin (5 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Polymixin B (300 µg), Netilmicin (30 µg), Aztreonam (30 µg), Cefepime (30 µg), Imipenem (10 µg), Meropenem (10 µg), piperacillin-Tazobactam (100/10 µg) and Ticarcillin-Clavulanic acid (75/10 µg) related to 7 different classes. Cefepime (CPM), Ticarcillin-Clavulanic acid (TIM) and Polymixin B (PB) were the most resisted antibiotics by 100%, and these results were disagreed with (Khadir *et al.* 2018, Ahmadishooli *et al.* 2020) who discovered that 35% and 0% of *P.*

*aeruginosa* isolates were resist to CPM. Furthermore, 98% of *P. aeruginosa* isolates were sensitive to PB unlike the findings of (Hefni *et al.* 2013). Imipenem (IMI) and Meropenem (MEM) were the most effective antibiotics (100%). *P. aeruginosa* isolates were sensitive that the results were agree with (Hefni *et al.* 2013) who also reported the same findings while it was incompatible with (Khadir *et al.* 2018) who found that 16% and 12% of *P. aeruginosa* isolates were resist to IMI and MEM respectively. Amikacin (AK) and Piperacillin-Tazobactam (PTZ) were resist in 25% of *P. aeruginosa* isolates, furthermore conflict with (Ahmadishooli *et al.* 2020) who found that the resistance of AK 0% and PTZ 100%. Levofloxacin (LEV) and Gentamicin (GM) were resisted in 12.5% of *P. aeruginosa* isolates, (Hefni *et al.* 2013) found that 90% and 100% of were sensitive to LEV and GM respectively. Ciprofloxacin (CIP) was resisted in 43.8% of *P. aeruginosa* isolates, furthermore conflict with (Khadir *et al.* 2018) who found that 61% of *P. aeruginosa* isolates were resist. Netilmicin (NET) and Aztreonam (ATM) were resist in 37.5% of *P. aeruginosa* isolates, furthermore conflict with (Hefni *et al.* 2013) who found that 99% of *P. aeruginosa* isolates were sensitive to NET (Table 4.10).

**Table 4.10** Antibacterial susceptibility test of *Pseudomonas aeruginosa* (R= Resist / S= Sensitive / I= Intermediate)

| **Antimicrobial Agent** | **Resistance Pattern** | | | | | | ***P-Value*** |
|---|---|---|---|---|---|---|---|
| | **R** | **%** | **S** | **%** | **I** | **%** | |
| **AK** | 4 | 25 | 12 | 75 | 0 | 0 | < 0.05 |
| **LEV** | 2 | 12.5 | 14 | 87.5 | 0 | 0 | < 0.01 |
| **GM** | 2 | 12.5 | 14 | 87.5 | 0 | 0 | < 0.01 |
| **CIP** | 7 | 43.8 | 9 | 56.2 | 0 | 0 | > 0.05 |
| **PB** | 16 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **NET** | 6 | 37.5 | 10 | 62.5 | 0 | 0 | > 0.05 |
| **ATM** | 6 | 37.5 | 8 | 50 | 2 | 12.5 | > 0.05 |
| **CPM** | 16 | 100 | 0 | 0 | 0 | 0 | < 0.001 |
| **IMI** | 0 | 0 | 16 | 100 | 0 | 0 | < 0.001 |
| **MEM** | 0 | 0 | 16 | 100 | 0 | 0 | < 0.001 |
| **PTZ** | 4 | 25 | 10 | 62.5 | 2 | 12.5 | < 0.05 |
| **TIM** | 16 | 100 | 0 | 0 | 0 | 0 | < 0.001 |

### 4.3.4 Multi-Drug Resistance (MRD) isolates

A bacterial isolate that displays resistance to three or more distinct kinds of antibiotics as well as at least one additional medicament is referred to be "multi-drug resistant" (MDR). Bacterial isolates that are resistant to at least one agent from each antimicrobial category, but not more than two, are considered to have "extensive drug resistance" (XDR), meaning they are only susceptible to one or two groups of antibiotics. "Pan-drug resistance" (PDR) is a well-known term that refers to a bacterium's insensitivity to all antibiotics across all antimicrobial categories (Hawkey *et al.* 2018). To validate the proper categorization of bacteria, it is necessary to test bacterial isolates against a comprehensive or near-comprehensive range of antimicrobial agents within their respective categories. The results of the current study reveal a high prevalence of multi-drug resistant (MDR) isolates at 86%, with 8.5% of isolates having extensive drug resistance (XDR) as defined by resistance to one or two groups of antibiotics (Table 4.11). These findings point to a growing trend of bacterial resistance to antibiotics, as previously noted in studies such as (Shahi and Kumar 2016) which reported 26.7% of bacteria isolated from DFI as MDR (Magiorakos *et al.* 2012).

**Table 4.11** Resistance pattern of isolated bacteria

| **Bacteria** | **Resistance Pattern** | ***P- Value*** |
|---|---|---|
| **Gram positive** (*S. epidermidis.*) | MDR: 2 (100%) | 0.001 |
| | XDR: 0 (0%) | |
| | PDR: 0 (0%) | |
| | SEN: 0 (0%) | |
| **Gram negative** (*K. pneumonia, E. coli, P. aeruginosa* and *P. mirabilis*) | MDR: 58 (85.2%) | 0.001 |
| | XDR: 6 (8.8%) | |
| | PDR: 0 (0%) | |
| | SEN: 4 (5.8%) | |
| Total | MDR: 60 (86%) | 0.001 |
| | XDR: 6 (8.5%) | |
| | PDR: 0 (0%) | |
| | SEN: 4 (5.7%) | |

### 4.4 The Antibacterial and Anti-Biofilm of Probiotics Against Isolated Bacteria

The lactobacillus spp. was isolated from local dairy products in MRS agar media at 37°C under an-aerobic conditions for 48 hours.

#### 4.4.1 Isolation and identification of probiotic lactic acid bacteria

In sterile containers, fifty samples of local yogurt were gathered from the markets in Baquba city. Each yogurt sample was diluted to the appropriate quantities with normal saline in sections of ten milliliters. The dilutions were incubated anaerobically at 37°C for 48 hours after being plated on MRS agar in a volume of 0.1 ml. Using colony morphology and a variety of biochemical tests, including Gram staining, catalase, oxidase, growth at various temperatures, sugar fermentation and motility test, endospore formation,milk coagulation activities, 0.4% bacteriostatic phenol tolerance test, and 1-10% NaCl tolerance test, the purified colonies were checked for *Lactobacilli* species.

A fresh overnight culture of Lactobacillus at 1% (v/v) was injected into MRS broth with a range of pH from 2.5 to 8.5 in order to determine the best conditions for growth. Concentrated acetic acid (99%) and 5 N NaOH were used to change the pH. The infected broths were incubated for 24 hours under anaerobic conditions at 37°C with 10% CO2. A spectrophometer was used to compare the optical density at 560 nm (OD) of the bacteria's growth after 24 hours of incubation to the un-inoculated broth.

The *lactobacillus* spp. was isolated from local dairy products in MRS agar media at 37°C under an-aerobic conditions for 48 hours. Named Lactobacillus spp., the isolated bacterium came from a local yogurt. The colonies of LAB on MRS agar were pale, round in shape, soft, mucoid, convex and surrounded by a clear zone as a result of dissolving $CaCO_3$ which was a outcome of generating lactic acid that analyzed calcium carbonate.

According to biochemical results of *Lactobacillus*, two species were identify which were *L. plantarum* and *L. reuteri* were shown in (Table 4.13). The whole outcomes of microscopic morphology and bio-chemical features of the strains were shown in (Table 4.12). The isolates looked as catalase and oxidase-negative. Wholly of the isolates were glucose fermenter.

**Table 4.12** Biochemical test for *Lactobacillus* spp.

| **Tests** | ***L. reuteri*** | ***L. plantarum*** |
|---|---|---|
| Catalase | (-) | (-) |
| Motility | (+) | (+) |
| Oxidase | (-) | (-) |
| **Sugar Fermentation** | | |
| Lactose | (+) | (+) |
| Mannose | (-) | (+) |
| Raffinose | (-) | (+) |
| galactose | (+) | (+) |
| Sucrose) | (+) | (+) |
| Sorbitol | (-) | (+) |
| Glucose | (+) | (+) |

The result showed that both *Lactobacillus Plantarum* and *L. reuteri* were more tolerant and resistant to 0.1% bile salt with a gradual decrease of viable cells in 1.0% bile salt (Table 4.13). Additionally, these isolates were able to tolerate the increased pH and could grow well at pH ranging between 2-5.

**Table 4.13** Some characters of lactic acid bacteria as probiotics

| **Characters** | ***L. reuteri*** | ***L. plantarum*** |
|---|---|---|
| Bile salt tolerance | | |
| (0.1%) | (+++) | (+++) |
| (0.5%) | (++) | (++) |
| (1%) | (+) | (-) |
| Acid tolerance | | |
| (pH 2) | (++) | (++) |
| (pH 3) | (+++) | (+++) |
| (pH 5) | (+++) | (+++) |

### 4.4.2 Antibacterial and anti-biofilm activity of probiotics against isolated bacteria

The antibacterial activity of the cell free supernatant probiotic (CFS) was determined against MDR or XDR isolates that produced biofilm by agar well diffusion method. The result of the current study showed good efficacy as shown in Table 4.14 and Figure 4.5.

**Table 4.14** Antibacterial and anti-biofilm activity of probiotics

| Isolate | Antibacterial Activity (mm) | Anti-biofilm (%) | Isolate | Antibacterial Activity (mm) | Anti-biofilm (%) |
|---|---|---|---|---|---|
| EC3 | 16 | 50 | PA11 | 14 | 39 |
| EC36 | 19 | 34 | KP17 | 17 | 44 |
| EC39 | 14 | 39 | KP20 | 18 | 45 |
| EC47 | 18 | 41 | KP45 | 16 | 31 |
| PA2 | 14 | 33 | KP46 | 15 | 33 |
| PA4 | 15 | 41 | PM42 | Resist | 29 |
| PA5 | 13 | 30 | PM34 | 12 | 41 |

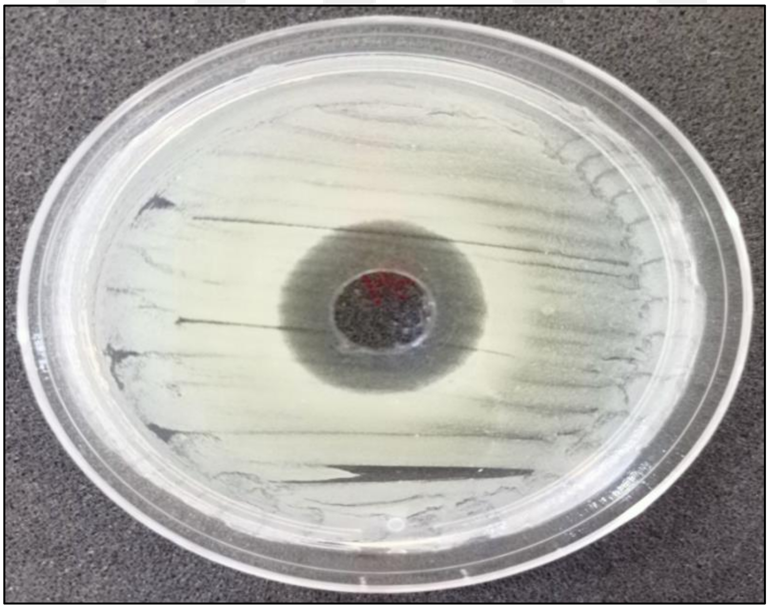

**Figure 4.5** Antimicrobial activity of probiotics

The findings were disagreed with (Karunaratne and Sartaj 2021) who found that *P. aeruginosa* and *E. coli* isolates were resist to the probiotic. The probiotics as Lactobacilli, have been used for the treatment of burn infections and have been shown to interfere with activity of *Psoudomonas aeruginosa* (Shokri *et al.* 2018). Different species of *Lactobacillus* have antibacterial activity against *Proteus* spp, *P. aeruginosa* and *E. coli* isolates (Amelia *et al.* 2020) and this indicated that a widest inhibition zone was observed with *E. coli* (23.28 mm) (Shaaban *et al.* 2020). A study also indicated that the probiotic has an inhibitory effect 12 mm to 20 mm against *P. Mirabilis* (Morales *et al*. 2022).

However, (Ghane *et al.* 2020) reported that the CFS of all LAB strains resulted in more than 30% inhibition of biofilm formation by *E. coli* isolates and reached to 50%. At the present time, inhibition of bio-film development by pathogenic bacteria has turn out to be a gorgeous therapeutic aim (Bjarnsholt *et al.* 2013). Biofilms make the resistance to the act of anti-microbial agents as most of the available anti-microbial agents can be kill or inhibit only the planktonic bacteria. Consequently, probiotic strains which have together the anti-biofilm properties and anti-microbial actions against pathogenic bacteria can be of clinically important. Diverse species of lactobacilli are known to yield substances with anti-microbial features, comprising small molecular weight substances, anti-microbial peptides (Bacterioc-ins) and organic acids (Somashekaraiah *et al.* 2019).

The anti-biofilm activity of *lactobacilli* species in contrast to harmful bacteria has been explored previously. It has been indicated that cell free supernatant of fecal lactobacilli isolates was able to prevent the *Vibrio cholerae* biofilm development by more than 90% (Kaur *et al.* 2018). Likewise, the anti-biofilm activity of *Lactobacillus pentosus (L. pentosus) and Lactobacillus plantarum (L. plantarum)* CFS against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* has been recognized (Ghane *et al.* 2020).

A study showed that the CFSs displayed an anti-biofilm outcome against pathogenic bacteria ranging from 44%-52% (Lee *et al.* 2021). Since the development of this structure makes pathogens more resistant to environmental pressure and anti-microbial agents, biofilms are linked to infection (Monteagudo-Mera *et al.* 2019). As previously mentioned, the CFSs of probiotics include a variety of combinations with anti-microbial characteristics that agitate the pathogens or components of the bi-film matrix. The CFS of Lactobacillus salivarius, Lactobacillus acidophilus LA5, and Lactobacillus casei 431 were shown to have anti-biofilm action against L. monocytogenes, whereas the CFS of Lactobacillus paracasei DTA81 and L. paracasei DTA93 prevented E. coli from forming biofilms (Tarrah *et al*. 2019). The CFS of LAB is made up of oxygen metabolites, saturated fatty acids functioning as bio-surfactants, hydrogen peroxide, exo-polysaccharide, and other substances, as well as its metabolites that can act as anti-biofilm agents (Moradi *et al.* 2019). The findings of the current study indicate that the impact of CFS on the formation of biofilm and growth inhibition varied. This variability may be due to the structure of the biofilm, the concentration of CFS, and the way it acts on bacterial cells or penetrates the matrix barrier.

# 5. CONCLUSIONS AND RECOMMENDATIONS

The follow conclusions may be drawn from the results of the current research. The following conclusions may be drawn from the findings of the current research:

- *K. pneumonia* isolates were the highest isolated bacteria.
- *Enterobacteriaceae* and *P. aeruginosa* isolates have high resistance to Polymixin B.
- The two main concerns in preventing the spread of drug-resistant bacteria in hospitals are the increase in multi-drug resistant isolates and the implementation of effective infection control measures.
- Probiotics have antibacterial and anti-biofilm activity against MDR and XDR isolates.

The following suggestions can be formulated accodrding to the outcomes of the present investigation, after analyzing the literature and several prior trials:

- Regular research to track the frequency and degree of resistance of pathogenic bacteria.
- Genetic investigations of the bacteria to recognize the strategy of resistant in order to combat the widespread and multi-resistant of these isolates.
- Raising health awareness by urging people not to take antibiotics excessively, carelessly, or without a prescription
- Determination of the anti-microbial activity of probiotic on different harmful bacterial species.
- The requirement to conduct a culture and susceptibility tests prior to writing a patient an antibiotic prescription.

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## APPENDICES

**APPENDIX 1. Ethical Statement**

**APPENDIX 2. Questionnaire**

## APPENDIX 1. Ethical Statement

## APPENDIX 2. Questionnaire

| NO. OF SAMPLE | GENDER | AGE | LIVING | EDUCATION |
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