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Antimicrobial activity of Colistin combined with some Probiotics against the clinical isolates of *Acinetobacter baumannii* bacteria

A thesis

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By

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بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

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I certify that this thesis entitled "**Antimicrobial activity of Colistin combined with some Probiotics against the clinical isolates of *Acinetobacter baumannii* bacteria**" was prepared under my supervision at the Department of Biology / College of Science / University of Diyala, as a partial requirement for the degree of Master of Science in Biology.

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Dedication

To the dearest of people and the closest to my heart, to my dear father and my dear mother, who were the helpers and supporters to me, and their blessed supplications had the greatest impact in facilitating the ship of the search until it anchored on permanent success.

To those with whom a gift from fate appeared and they know the meaning of siblinghood, my beloved brothers and sisters.

Munaf 2021

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Summary

Acinetobacter baumannii has emerged as a hazardous nosocomial pathogen due to its resistance to antibiotics and its potential to colonize and cause severe infections among the patients. This study aimed to investigate the antibacterial and anti-biofilm property of probiotics strains the cell-free supernatant (CFS) against *A. baumannii* and demonstrate the synergistic effect of Colistin in combination with the tested probiotic CFS against the clinical isolates of multidrug-resistant *A. baumannii*.

In the current study, 20 (8.69%) clinical isolates of *A. baumannii* were identified from a total of 230 samples collected from different sources, including (burns, wounds and blood). These samples were taken from hospitalized patients and visitors in Baquba Teaching Hospital/ Diyala from September 2020 to the end of December 2020. The bacterial isolates were identified based on the morphological characteristics and initial biochemical tests. Confirmation of diagnosis and antibiotics susceptibility of *A. baumannii* were determined using the VITEK2 system. The tissue culture 96 well microtiter plate was used for the following assays; biofilm formation by *A. baumannii*, coaggregation test, minimum inhibitory concentration (MIC) and minimum biofilm inhibitory concentration (MIC-B) of the tested antimicrobials alone and in combination. Colistin was combined with the tested probiotic CFS to determine the nature of antimicrobial interactions; synergism, antagonism or additive effect using a disk diffusion assay and a checkerboard method.

The results showed that the highest isolation rate was from burns 9 (45%) and wounds infections 6 (30%) while a low percentage was isolated from blood samples 5 (25%). All bacterial isolates (100%) of *A. baumannii* had the ability to form a "strong" biofilm and they were (100%) resistant to the following tested antibiotics; Ampicillin, Piperacillin/Tazobactam,

Cefazolin, Cefoxitin, Ceftazidime, Ceftriaxone, Meropenem, Ciprofloxacin, Levofloxacin and Cefepime. Furthermore, (95%) of clinical isolates were resistance to Imipenem and Amikacin, and (90%) were resistance to Gentamicin and Tobramycin, while (75%) were resistance to Trimethoprim-sulfamethoxazole. We have noticed that all *A. baumannii* isolates (100%) were sensitive to Colistin and (85%) to Tigecycline and Minocycline. The majority 13(65%) of *A. baumannii* isolates were identified as extensively-drug resistant (XDR) and 7(35%) were multi-drug resistant (MDR).

The CFS of *Bacillus subtilis* KATMIR1933 and *Bacillus amyloliquefaciense* B-1895 showed no activity (even at 100% of CFS) against the planktonic cells, but the slight effect on biofilm formation of *A. baumannii*. While a strong anti-microbial activity and anti-biofilm potential were reported against *A. baumannii* when 50% (as MIC and MIC-B) of *Lactobacillus casei* CNCMi 1572 and mixed lactobacilli were applied causing inhibition (100%) of biofilm formation by *A. baumannii*. The study showed that co-aggregation % between the probiotic strains and *A.baumannii* was higher compared to their individual auto-aggregation. The auto-aggregation of *Bacillus amyloliquefaciense* B-1895 was higher than what was noticed in *Bacillus subtilis* KATMIR1933, *L. casei* CNCMi 1572 and mix lactobacilli. Based on the isobologram and the total fractional inhibitory concentration index (∑FIC), the CFS of the tested probiotic strains were synergized with Colistin against both planktonic cells and biofilms of three isolates of *A. baumannii*. The MIC and MIC-B of Colistin in combination with probiotic strains CFS (*Bacillus subtilis* KATMIR1933, *Bacillus amyloliquefaciense* B-1895, *Lactobacillus casei* CNCMi 1572 and mix lactobacilli) were lower compared to using it alone against *A. baumannii* isolates. Through this study, we concluded that The

antimicrobial activity of Colistin is enhanced when it is combined with the CFS of the tested probiotic strains, therefore, a lower concentration of antibiotic will be used, which in turn reduces its negative side effects.

List of contents

List of tables

List of figures

List of Appendix

List of Abbreviations

Abbreviation	Expansion
A.	*Acinetobacter*
ATCC	American Type culture collection
AME	Aminoglycoside-modifying enzymes
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species
LPS	Lipopolysaccharide
OmpA	Outer membrane protein A
NIH	National Institutes of Health
CFS	Cell free supernatants
VAP	Ventilator-associated pneumonia
CLSI	Clinical Laboratories Standards Institute
PLA	Phospholipase A
PLC	Phospholipase C
PLD	Phospholipase D
ICU	Intensive Care Unit
OMV	Outer membrane vesicle

PDR	Pan-drug resistant
PBPs	Penicillin-binding proteins
QS	Quorum sensing
Bap	Biofilm associated protein
EPS	The exopolymeric substances
PNAG	The Extracellular polysaccharide poly-β-(1,6)-N-actylglucosamine
AHLs	Acyl homoserine lactones
MBLs	Metallo-β-lactamases
OXAs	Oxacillinases
MATE	The Multidrug and toxin extrusion family
SMR	The Small multidrug resistance family
RND	The Resistance-nodulation-cell division super family
MFS	The Major facilitator super family
AMP	Antimicrobial Peptides
LAB	Lactic acid bacteria
GRAS	Generally Recognized as Safe
UPEC	Uropathogenic *E. coli*
DNA	Deoxyribose nucleic acid
UTI	Urinary tract infection
CV	Crystal Violet Stain
AST-GN	Antibiotics susceptibility test Gram-negative
ELISA	Enzyme linked immunosorbent assay
LBF	Low biofilm formers
IBF	Intermidiate biofilm formers
HBF	High biofilm formers
PTFE	Polytetrafluoroethylene filter-sterilized
MIC	Minimum inhibitory concentration
MIC-B	Minimum biofilm inhibitory concentration
ΣFIC	The Total fractional inhibitory concentrations
GN ID	Gram-negative identification
MTP	The Micro Titer Plate Method
OD	Optical Density
XDR	Extensive Drug Resistant

1. Introduction

Acinetobacter baumannii is a Gram-negative, obligate aerobe, coccobacillus and one of the most prevalent causative agent of several infections (Martín-Aspas *et al*., 2018). *A. baumannii* causes a range the hospital and community-acquired infections, including skin and soft tissue, urinary tract infections, in addition to meningitis, bacteremia and pneumonia (Morris *et al*., 2019). This pathogen is one of the multidrug-resistant (MDR) ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) (Shin and Eom, 2020). *A. baumannii* is capable to develop resistance mechanisms to a wide range of antimicrobial agents (Ibrahim, 2019). Basically, *A. baumannii* has three main mechanisms of antibiotics resistance, including: (i) disactivating of antibiotics by enzymes, (ii) delaying entrance of antibiotics into the target site of bacteria and (iii) alteration of their target or cellular functions (Hamzeh *et al*., 2019).

Biofilm-forming capability and antibiotic resistance are the most important virulence factors of *A. baumannii* playing an important role in bacterial survival and infection (Raheem *et al*., 2018). Biofilm is defined as a microbial aggregate embedded inside a self-released extracellular polymeric material including proteins, polysaccharides and extracellular DNA (Shin and Eom, 2020).

The antibiotic resistance in Acinetobacter infections had led to the use of the old generation of antibiotics, such as Colistin (Asif *et al*., 2018). Polymyxin E (or Colistin) has recently been used as a “last line” therapeutic substance to control the Gram-negative multi-drug resistant bacteria (Pacheco *et al*., 2019). Colistin is a cationic antimicrobial peptide that affects the lipid A moiety of lipopolysaccharide (LPS) of Gram-

negative bacteria and eventually, disrupting the outer membrane of bacterial pathogens (Farshadzadeh *et al*., 2018).

The monotherapy by antibiotics has been reported as a less effective protocol, in comparison to combination therapy, thus, antimicrobial combinations are recommended by the National Institutes of Health (NIH) as a potential integrative therapeutic option (Mosca *et al*., 2020). Therefore, an effective approach of combinations is urgently required, using the new technologies, to find out and develop alternative and safe strategies in order to encounter bacterial resistance to antibiotics. One of the suggested methods is using probiotics and/or their metabolites as antimicrobial substances in combination with antibiotics to increase the sensitivity of pathogenic strains (Isayenko *et al*., 2020).

Probiotics are known as live microorganisms that provide health benefits when administered in appropriate amounts (Saud *et al*., 2020). Probiotics play a key role in the regulation of the host immune system by stimulating cytokine production and cellular activity and inhibit the colonies of pathogens (Hager *et al*., 2019). In addition, probiotic therapeutic properties are attributed to the production of a variety of antibacterial agents, such as short-chain fatty acids, organic acids (such as lactic, acetic, formic, propionic and butyric acids), ethanol, hydrogen peroxide and bacteriocin (Karacaer *et al*., 2017).

The combination of Colistin, *in vitro*, with the other active antimicrobials that reported have a synergistic effect, is widely used by physicians in critical patients (Asif *et al*., 2018). These effective combinations have several advantages; including lower concentrations of antimicrobials are used with higher activity, in addition to reducing their cost and their toxic threatening side effect on human health (Isayenko *et al*., 2020).

Our study aimed to:

1- evaluate, *in vitro*, the antibacterial and anti-biofilm activity of the probiotic strains and their metabolites cell-free supernatants (CSF) alone and in combination with Colistin against *A. baumannii* using disc diffusion method and broth microdilution assay (Checkerboard assay).

2- In addition, the demonstrat the co-aggregation potential of probiotic strains with *A. baumannii*.

3- Detection of the synergy effect of the Colistin in combination with probiotic CSFs against multidrug-resistant *A. baumannii* .

2 Literatures Review

2.1 *Acinetobacter* spp

Acinetobacter is a Gram-negative, cocco-bacilli, non–lactose fermenter bacteria and growing at a high wide range of temperature 37-44°C (Lin and Lan, 2014). Acinetobacter has the ability to survive in diverse environments and isolated from soil, water and from multiple cases of human infections (Lee *et al*., 2017). It is considered as one of the causative agents of nosocomial infection, long-term survived on the dry non-living surfaces. As a successful pathogen causing hospital-acquired infections, Acinetobacter produces several virulence agents, including biofilm formation, which associate with the bacterial tolerance to the environmental stress factors such as, antibiotics, high temperature and dryness (Lee *et al*., 2017).

2.2 Acinetobacter classification

Bacterial classifications of Acinetobacter have been generally, as shown in table (2-1). The taxonomic classification is given as; Domain: Bacteria, Phylum: Proteobacteria, Class: Gamma Proteobacteria, Order: Pseudomonadales, Family: Moraxellaceae, Genus: Acinetobacter. The species *A. baumannii*, *A. haemolyticus* and *A. calcoaceticus* are of clinical importance (Jung and Park, 2015). The initial landmark classification of Acinetobacter species was based on DNA–DNA hybridization, in which 12 DNA classes or genospecies were distinguished, some of which were formally named as; *Acinetobacter baumannii, Acinetobacter calcoaceticus, Acinetobacter haemolyticus, Acinetobacter johnsonii, Acinetobacter junii and Acinetobacter lwoi*. In 2019, eleven species of Acinetobacter were classified and named while fifteen species were initially described (Vijayakumar *et al*., 2019). The strategies of phenotypic classification are

based on the growth temperatures, hemolysis, acidification of glucose and types of carbon utilizing as a source of energy (Vijayakumar *et al*., 2019).

Table 2-1: Scientific classification of Genus Acinetobacter (Vijayakumar *et al.*, 2019).

Domain:	**Bacteria**
Kingdom:	Eubacteria
Phylum:	Proteobacteria
Class:	Gammaproteobacteria
Order:	Pseudomonadales
Family:	Moraxellaceae
Genus:	Acinetobacter
Species:	*Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, *Acinetobacter haemolyticus*, *Acinetobacter johnsonii*, *Acinetobacter junii* and *Acinetobacter lwoi*

2.3 *Acinetobacter baumannii*

Acinetobacter baumannii is a well-known pathogen associated with hospital-acquired infections due to its extraordinary capability to persist and survive in the hospital environment and posses many of the resistance determinants (Bogdan *et al*., 2017). Thus, controlling methods of infections caused by this pathogen is difficult to maintain (Abdulhasan *et al*., 2016). The development of multi-drug resistant (MDR) *A. baumannii* is one of

the challenging pathways. Biofilms formation by bacterial cells may explain their antimicrobial resistance and survival on the abiotic surfaces with the presence of disinfectants and/or desiccation agents (Ivanković *et al*., 2017). The adhesion capacity of Acinetobacter strains on the surfaces is an initial and important step of bacterial pathogenicity, which is mediated by specific and non-specific factors (M'hamedi *et al*., 2014).

2.4 Morphological and physiological features of *A. baumannii*

During the log (exponential) phase of bacterial growth, they are short, plump, normally 1.0-1.5 μm by 1.5-2.5 μm in size, but evolve into more coccoids in the stationary phase, generally appear in pairs or long chains of various lengths (Jung and Park, 2015). *Acinetobacter baumannii* grow well on the routine laboratory media, such as blood agar, chocolate agar and MacConkey agar. It forms white or cream-coloured, glossy mucoid, non-hemolytic colonies on blood agar and smooth with a diameter of 1–2 mm after incubation for 18–24hrs at 37°C under aerobic conditions. On MacConkey agar, it grows as glossy mucoid and tomb-shaped, the light lavender colour indicating their inability to ferment lactose sugar (Almasaudi, 2018).

2.5 Natural habitats for *A. baumannii*

Acinetobacter spp are free-living bacteria, commonly spread in diverse ecosystems, including; soil, water, wastewater, vegetables and in the animal and human skin (Maravić *et al*., 2016). Most strains of *Acinetobacter* spp, other than *A. baumannii*, were isolated from different body parts of healthy individuals, including; nose, ear, mouth, forehead, trachea, conjunctiva, vagina, perineum, axillae, groin, fingertips and toe webs (Al Atrouni *et al*., 2016). They colocies on beds, curtains, walls, roofs, medical instruments and appliances of the hospital settings. In

addition, it was isolated from the medical staff possessions, tap water sinks, telephones, door handles, hand sanitisers, dispensers, trolleys and cabinets (Kanafani and Kanj, 2014). They have the ability to live on inanimate objects for long periods. Resistance to key antimicrobial drugs and disinfectants and their capacity to live with desiccants are the reasons responsible for their survival in a hospital setting (Evans *et al*., 2013).

2.6 Epidemology

Acinetobacter baumannii is reported primarily as a healthcare-associated pathogen causing several nosocomial infections, including; septicemia, bacteremia, ventilator-associated pneumonia, wound sepsis, endocarditis, meningitis and urinary tract infections (Almasaudi, 2018). Several epidemiological studies have reported the occurrence of MDR *A. baumannii* infections in different regions of the world including Europe, North America, Argentina, Brazil, China, Taiwan, Hong Kong, Japan and Korea (Kanafani and Kanj, 2014). Several cases of MDR *A. baumannii* have been reported at the hospitals of the United Arab Emirates, Bahrain, Saudi Arabia, Palestine and Lebanon (Almasaudi, 2018). The mortality rate of the hospitalized patients, who have MDR *A. baumannii*, reached 26% and increased to 43% in the ICUs (Greene *et al.*, 2016). A recent study in northwestern Ethiopia found that *A. baumannii* was isolated from 9% of hospital bloodstream infections, a 100% were resistant to ampicillin and piperacillin, while 33.3% and 44.5% showed resistance to meropenem and ciprofloxacin, respectively (Motbaino *et al*., 2020). In a study about pediatric meningitis caused by *A. baumannii* in China, the authors found that isolated bacteria from cerebrospinal fluid after neurosurgery were MDR, XDR and PDRs *A. baumannii* causing significantly high mortality (Xiao *et al*., 2019).In other study revealed that a total number of 20 (9.7%) isolates of *Acinetobacter baumannii* were obtained from (207) clinical

specimens in governmental hospitals in Diyala \ Iraq (AL-Dahlaki, 2020). The prevalence of MDR and XDR in another study in China, the isolation percentages of *A. baumannii* from patients with intracranial infection was 33.64% (Pan *et al*., 2018). In the United States and Europe, *A. baumannii* accounted for 8 to 14% of ventilator-associated pneumonia. In Asia, Latin America and some Middle Eastern nations, this pathogen was associated with higher infection percentages (19% to >50%) (Lynch *et al*., 2017). The prevalence of MDR phenotypes among *A. baumannii* that cause hospital-acquired pneumonia and VAP was close to 80% in a recent meta-analysis, involving 29 countries. The highest prevalence was in Central America, Latin America and the Caribbean, while the lowest was in Eastern Asia (Lim *et al*., 2019).

2.7 Pathogenicity of *A. baumannii*

To establish an infection, cell-to-cell adhesion is required to trigger the infection processes. It is reported that the ability of *A. baumannii* to bind to the mucosal cells is not strong compared to the other microorganisms, such as *Pseudomonas aeruginosa*, *Neisseria meningitides*, *Campylobacter*, *Yersinia enterocolitica* and *Helicobacter pylori* (Tibor and Tudofnyegyetem, 2013). The weak adhesion could lead to weak or inability to the invasion of the host cells by *A. baumannii*. However, the bacterial cells possess a hydrophobic potential that enhance them to bind to various surfaces. The hydrophobicity of the surfaces has been shown to be significantly expressed in the bacterial cells isolated from infected compared to the normal skin (Peleg *et al*., 2008). Outer membrane protein A (OmpA) binds to improve adhesion, specifically to respiratory epithelial cells. It is existed in the mitochondria and in the nucleus stimulating the expression of the pro-apoptotic cytochrome c and causing cell death (Schweppe *et al*., 2015). The outer membrane vesicles of bacterial cells contain various virulence-related proteins (protease, phospholipase,

superoxide dismutase and lactase) which secreted at the site of infection and accelerated the local innate immune response, leading to tissue damage. The outer membrane vesicles play a role in increasing biofilm production on abiotic surfaces (Nho *et al*., 2015). The polysaccharide capsule of the pathogenic Gram-negative bacteria can be considered as a virulence agent. It plays an essential role in protecting bacteria from phagocytosis by the innate immune system of the host (Barrie and Gorman, 2016). Lipopolysaccharides (LPSs), another virulence factor, of *A. baumannii* composed of O-antigen, carbohydrate core and lipid A. LPS is an antigenic chemical agent that recruits inflammatory cells and stimulates them to release their cytotoxic substances (Rossi *et al*., 2016).

Quorum sensing is related with the ability of *A. baumannii* to produce biofilms on both biotic and abiotic surfaces and participated in the bacterial resistance mechanisms. To survive under unfavourable conditions, bacterial cells become metabolically inert in the deep layers of biofilms. The lack of antibiotics penetration and their inability to act on the metabolically inactive bacteria greatly increases their virulence (Green *et al*., 2016). *Acinetobacter baumannii* is rarely isolated from urinary tract infection (UTI). It has been found that only 1.6% of UTIs acquired intensive care unit was because of *A. baumannii* which was usually associated with catheter-related colonization (Falagas *et al*., 2015). *Acinetobacter baumannii* has become a major bacterial agent of bloodstream infection in hospitals where intravenous catheters or intra-respiratory devices are frequently used. The mortality rate of bloodstream infections caused by *A. baumannii* approaches 40% (Moubareck and Halat, 2020). Nosocomial meningitis caused by *A. baumannii* remains life-threatening in the intensive care neurosurgery units, with a mortality rate of 70%, especially, in patients on indwelling ventriculostomy tubes or cerebrospinal fistulae (Moubareck and Halat, 2020). *A. baumannii* has been repeatedly isolated from skin and

soft tissue in patients with severe burns, wounds or trauma, for instance, soldiers injured during military operations or victims of natural disasters (Moubareck and Halat, 2020). Generally, ventilator-associated pneumonia (VAP) caused by MDR *A. baumannii* remains a leading cause of high mortality rate (Jaruratanasirikul *et al*., 2019).

2.8 Virulence factors of *Acinetobacter baumannii*

2.8.1 Outer Membrane Proteins (Porins)

Porins are cylindrical proteins that act as special channels/ pores for passing some types of molecules into the cells. These proteins are large enough for passive diffusion (Peleg *et al.*, 2008), and they are located in the outer membranes of bacteria, actinomycetes, mitochondria and chloroplasts. Porins play an important central role in microbial virulence through drug expulsion mechanisms (Rumbo *et al*., 2013). OmpA is an important porin in *A. baumannii* and is involved in antimicrobial resistance, epithelial cell adhesion and biofilm formation (Choi *et al*., 2008). It lowers the cellular permeability to the biocidal agents (Smani *et al*., 2014). Another outer membrane protein of *A. baumannii* is the 33 to 36 kDa Omp protein that acts as a water conduit and its expression is associated with resistance to Carbapenem (Smani *et al*., 2013). Like in Omp 33-36, CarO also plays a key role in Carbapenem resistance in *A. baumannii*; Elevation of CarO expression has been shown to delay pulmonary neutrophil infiltration through attenuating of inflammatory responses in the trachea and lungs, allowing the rapid spread of the bacteria and leading to acute pneumonia (Sato *et al*., 2017). Furthermore, Omp22 was also diagnosed as a novel, conserved and safe antigen to develop and produce effective vaccines for controlling *A. baumannii* infection (Huang *et al.,* 2016).

2.8.2 Capsular Polysaccharides and Lipopolysaccharides (LPS)

The envelope of *A. baumannii* is associated with several factors that are involved in pathogenicity. In this regards, the external capsular polysaccharides and LPS are the pathogenic factors of *A. baumannii*. It was reported that many clinical isolates of *A. baumannii* expressed a surface capsular polysaccharide with a large conserved gene set, called the *K locus*, a site of capsular polysaccharides formation (Geisinger and Isberg, 2015). The packed sugar units of the bacterial capsule produce a barrier against environmental stress, such as dehydration, disinfection, sterilization, antimicrobial agents and the immune defence system including; phagocytosis (Singh *et al*., 2019). Capsular polysaccharides-mutants deficient strains have low self-resistance to peptide antibiotics. In addition, the presence of antibiotics results in increased capsular polysaccharide production (Geisinger and Isberg, 2015).

Lipopolysaccharides are composed of an endotoxin lipid A fragment, an oligosaccharide core and a repetitive O-antigen (Lee *et al*., 2013). In *A. baumannii*, LPS plays an important role in the virulence and survival of bacterial cells (McQueary *et al*., 2012). Some studies indicated that inhibition of LPS synthesis could be used as an effective strategy to discover new antimicrobial to targeting LPS molecules. In addition, several studies have shown that alterations of LPS lead to a reduction in the sensitivity of *A. baumannii* to some important antibiotics, such as Colistin (Chen *et al*., 2015).

2.8.3 Enzymes

Phospholipase is a lipolytic enzyme essential for the metabolism of phospholipids and it is an important virulence factor of many pathogenic bacteria, such as *P. aeruginosa*, *Listeria monocytogenes* and *Clostridium*

perfringens (Flores-Diaz *et al*., 2016). Three classes of phospholipase; phospholipase A (PLA), phospholipase C (PLC) and phospholipase D (PLD) were classified based on their cleavage site. PLA hydrolyzes the fatty acids from the glycerol backbone, PLC cleaves the phospholipid head assembly, while PLD is a transphosphatidylase that only derived from the head group. Degradation of phospholipids affects the stability of host cell membranes and the cleft head assembly can interfere with cellular signalling, causing changes in the host's immune response (Flores-Diaz *et al*., 2016). PLD and PLC were identified as virulence factors in *A. baumannii* (Stahl *et al*., 2015).

2.8.4 Outer Membrane Vesicles (OMVs)

Outer Membrane Vesicles are small globular vesicles, their size ranged from 10 to 300 nm, produced by all Gram-negative bacteria. These vesicles were identified at the different growth conditions, their presence to certain conserved evolutionary mechanisms (Roier *et al*., 2016). It was noticed that vesicles formation is a natural process, although, the exact mechanism of their biogenesis and differentiation are not understood yet (Roer *et al*., 2016). Based on the recent research works, It is unclear if there is a specialized mechine or it is just a natural secretory pathway, as a response to various environmental conditions (Cahill *et al*., 2015). Currently, there are three hypotheses for OMV biogenesis; first, the loss of lipoprotein-peptidoglycan interactions leads to membrane protrusion and vesicle formation. Secondly, the accumulation of denatured proteins and peptidoglycan fragments in the periplasmic space causes membrane swelling. Thirdly, the enrichment of the molecules driving the membrane curvature leads to a vesicle formation (Roier *et al*., 2016). The role of OMVs in the pathogenesis of *A. baumannii* disease was reported, recently. The abundant amounts of OMVs production by some strains of *A.*

baumannii, referred to the more virulence factors which, eventually, promoted a stronger innate immune response and more cytotoxic effect compared to the strain that produces less OMV (Li *et al*., 2015).

2.8.5 Biofilms

Biofilm is a cluster of microorganisms embedded in a self-producing exo-polymeric matrix in a steady-state (Bjarnsholt *et al*., 2018). Historically, microbial biofilms were discovered earlier; however, the clinical importance was not fully recognized until recent time. According to the National Institutes of Health (NIH), biofilms are involved in about 65% and 80% of all microbial infections. Clinically, microbial biofilms, via colonization of implants (artificial heart valves, catheters, joint replacements and medical devices), are associated with hospital-acquired infections. Moreover, biofilm infection was identified in various disorders, for example, diabetes mellitus, dental caries, medical implants and wound infections which broadly affect the quality of life and thus increase the global morbidity rate (Bjarnsholt *et al* ., 2018). In regards to antibiotic resistance, there some biofilm-associated factors involved leading to increase microbial resistance to antimicrobial agents including; biofilm phenotype and genotype. The phenotypic factors are represented by; impairment of drug proliferation due to microbial aggregations, overexpression of the outer material matrix (EPS), alterations in microbial phenotypic. While the genotypic features are the stress responses, and physiological heterogeneity as a result of physical chemistry gradients (Yang *et al*., 2017). The phenotypic and genetic features are activated when bacterial cells produce quorum sensing (QS) signals which is a cell-density-dependent process. QS aids cell-to-cell communication during any undesirable changes in various environmental factors such as temperature, oxygen level, acidity, and the quality of growth medium (Moreno-Gámez

et al., 2017). The cell surface protein directly contributes to the biofilms formation of *A. baumannii* known as Biofilm associated protein (Bap) (Noori *et al*., 2014). Most of the virulence agents of *A. baumannii* are internal (parts of the cell surface) and many are associated with the formation of biofilms (Weber *et al*., 2016). It has shown that capsule polysaccharides influence the formation of microbial biofilms in other organisms. However, little is known about this mechanism (Lee *et al.*, 2013).

Table 2-2: The most identified virulence factors of *Acinetobacter baumannii*.

Virulence factor	Proposed role in pathogenesis	References
Porin (OmpA, Omp33-36, Omp22, CarO, OprD-like)	• Adherence • Invasion • Induction of apoptosis • Serum resistance • Persistence and biofilm formation	(Huang *et al*., 2016**)**
Capsular polysaccharide	• Growth in serum • Survival in tissue infection • Biofilm formation	(Lees-Miller *et al*., 2013)
Lipopolysaccharide (LPS)	• Serum resistance, • Survival in tissue infection • Evasion of the host immune response	(McConnell *et al*., 2013)
Phospholipase (PLC and PLD)	• Serum resistance, • Invasion, • *In vivo* survival	(Fiester *et al*., 2016**)**
Outer membrane vesicle (OMV)	• Delivery of virulence factors, • Horizontal transfer of antibiotic resistance gene	(Li Z *et al*., 2015**)**
Biofilms	They protect the bacterial cells they harbour against various hazards, such as antimicrobial agents and macrophage attacks, in addition to stress conditions such as desiccation and disinfection.	(Lebeaux *et al*., 2014; Longo *et al*., 2014).

2.9 Step of Biofilm Formation

2.9.1 Microbial Attachment

Acinetobacter baumannii to adhere at the surface initially, they form pili by means of the *CsuA* / BABCDE usher-chaperone assembly system. This system is regulated by a two-component BfmS/BfmR system with a kinase sensor encoded by bfmS and a response regulator encoded by bfmR (Longo *et al*., 2014). It is also believed that the outer membrane protein, OmpA, plays an important role in the binding of bacterial cells to specific surfaces (Longo *et al*., 2014). The Bap plays an important role in cell−cell adhesion, an initial step of establishing newly formed biofilmon the medically relevant materials, such as polystyrene and titanium (Loehfelm *et al*., 2008). The extracellular polysaccharide poly-β-(1,6)-N-actylglucosamine (PNAG), encoded from the pgaABCD operon, is believed to be involved in intercellular binding within the biofilm (Longo *et al*., 2014). Figure (2-1).

2.9.2 Quorum Sensing (QS) Phenomenon

Quorum sensing is a cell-cell communication process in which hormone-like compounds, including acyl-homoserine lactones (AHLs), are produced as a chemical signal responsible for regulating bacterial movement, the formation of biofilms and other physiological activities (Saipriya *et al*., 2019). The QS in *A. baumannii* includes an AbaI promoter and its analogous receptor AbaR. The AbaI, encoded by the *abaI* gene, is a sensor protein that acts as an autoinducer synthase generating AHL molecules, while AbaR acts as a receptor protein, binding to AHL leading into a chain reaction. This reaction causes more AHLs to be produced in the manner of a positive feedback loop, leading to the regulation of biofilm formation (Saipriya *et al*., 2019). Therefore, control of QS phenomenon

should be kept in consideration in order to develop new strategies for preventing bacterial biofilm infection (Wu *et al*., 2015).

2.9.3 The exopolymeric substances production

The exopolymeric substances (EPS) is produced by adherent cells and plays a major role in the formation of microbial biofilm. It is composed mainly of hydrolytic enzymes which stabilize the biofilm structure and prevent the penetration by antibacterial agents (Lewis, 2008). In addition, EPS including; protein, polysaccharides, glycoproteins, glycolipids, extracellular DNA, metal ions, divalent cations and other surface-active components (Yadav *et al*., 2012). The formation of EPS facilitates adherence to biotic or abiotic surfaces, the formation of micro-colonies and the 3-dimensional surface of a mature biofilm (Van Houdt and Michiels, 2010).

2.9.4 Micro-colony formation

After attachment, the bacteria begin to multiply and initiate contact by the formation of quorum-sensing molecules. At a certain stage of quorum-sensing molecules, environmental signals induce the development of EPS and bacteria begin to multiply within the EPS. Biofilm analysis showed that, once the production of EPS has started, the bacteria concentrate on the maturation of the biofilm and generate pili, flagella and fimbriae (Phil, 2014).

2.9.5 Colonization or maturation step

The final stage of biofilm development is maturation, where biofilms grow into a self-organized complex structure comprising a number of micro-environments. The final configuration is a three-dimensional

structure composed of bacteria surrounded by EPS, which has channels for nutrients and water flow (Phil, 2014).

2.9.6 Dispersal

Biofilm dispersal or detachment is due to a number of factors, such as the presence of QS molecules, the availability of nutrients, changes in surface character and physical forces from the surface (Phil, 2014).

Figure 2-1: Summary of biofilm development stages. Biofilm development stages: 1, individual planktonic cells attach to the surface; the attached cells form microcolonies; 2, subpopulations interact with each other during biofilm structure development; macrocolonies are formed in mature biofilms; 3, dead cells accumulate under stressful conditions; , cells are released from the biofilm macro-colonies (adapted from Yang *et al*., 2011).

2.10 Antibiotics

Antibiotics are chemicals or biological substances that kill or inhibit the growth of microbes and eventually, controlling microbial infections. They are classified based on their various features. These antibiotics are either synthetic substances or secondary metabolites produced by different species of microorganisms and have the potential to be bacteriostatic and /or bacteriocidal agents against other microbes (Al-Shuwaikh, 2016). The mechanisms of action (killing or inhibition) of antibiotics in figure (2-2).

2.10.1 Beta-Lactams

The antibiotics of beta-lactams group are characterized by containing a beta-lactam ring which is a part of the basic structure in the various types of antibiotics, including (Penicillins, Cephalosporins, Carbapenems and Monobactam). These antibiotics inhibit the cell wall synthesis and have a fatal effect on bacteria, however, some bacterial species are resistant to this type of antibiotics (Brandt *et al*., 2017). This antibiotic works on inhibiting bacterial cell wall synthesis through binding with the special proteins within the cell membrane, Penicillin Binding Proteins (PBPs), which inhibit transpeptidase, an important enzyme that creates peptide chains leading to a defect in peptidoglycan layer formation (Zervosen *et al*., 2012).

A-Penicillins

Penicillins are divided into:

- Natural penicillins such as penicillin G (Benzylpenicillin) and penicillin V (Phenoxy methyl penicillin).
- Semi-synthetic penicillins which include: extended-spectrum penicillins (Carboxy penicillin such as Ticarcillin and Carbencillin) and Ureidopenicillin (Azlocillin, Piperacillin and Mezlocillin) (Al-Shuwaikh, 2016).

B-Cephalosporins

They are semi-synthetic antibiotics that contain a beta-lactam ring attached to a ring dehydrothiazine, it has broad-spectrum activity against Gram-negative and Gram-positive bacteria. They are used as alternatives therapeutic agent for patients who are Penicillin sensitive (Hancu *et al*., 2013). Cephalosporins are divided into several groups, as explained below,

depending on their chemical composition and antimicrobial effectes against pathogenic microbes:

- **First generation Cephalosporins:** include Cephalothin, Cefazolin, Cephapirin, Cephalexin and Cephradine (Al-Marjani, 2011).
- **Second generation Cephlosporins:** include Cefoxitin and Cefotetan (Metha and Sharma, 2016).
- **Third generation Cephalosporins:** include Ceftibuten, Ceftizoxime, Ceftriaxone, Ceftazidime and Cefotaxime (Metha and Sharma, 2016).
- **Fourth generation Cephalosporins:** including Cefepime (Jawetz and Adelberg, 2016).
- **Fifth generation Cephalosporin:** including Ceftobiprole and Ceftaroline (Bassetti and Matheo, 2013).

C- Carbapenems

Including meropenem and imipenem which have a broad spectrum of action against Gram-positive and -negative bacteria, working on penicillin-binding proteins and inhibiting the synthesis of the cell wall (Metetis, 2016).

D- Monobactam

Aztreonam, the first antibiotic of this group, has a higher antimicrobial activity against the Gram-negative bacteria compared to its weak effect on Gram-positive species (Al-Marjani, 2011).

2.10.2 Aminoglycosides

A group of bacteriocidal antibiotics, such as (Streptomycin, Neomycin, Amikacin, Tobramycin and Gentamicin) work by inhibiting the protein synthesis of the bacterial cells. They bind to the ribosomal unit (30s) ,

causes a change in the amino acid sequences and leading to the production of abnormal proteins that accumulate inside the bacterial cell and thus, stopping the bacterial growth and death (Al-Marjani, 2011).

2.10.3 Polymyxins

Polymyxins are a group of polycationic peptide antibiotics that were discovered more than 60 years ago exhibiting a potent efficacy against most Gram-negative bacteria (Lee *et al*., 2016). Among all five Polymyxins (A– E), only Polymyxin B and E (Colistin), with one amino acid difference, are used clinically. Colistin is a key component and drug of choice used to control MDR *A. baumannii* infections (Cai *et al*., 2012). Colistin or Polymyxin E is a bactericidal drug that disrupts cell membrane like a detergent. Its positively charged cationic region binds to the negatively charged hydrophilic portion of bacterial LPS and lead to loss of cellular membrane integrity and cell death, finally (Nhu *et al*., 2016).

2.10.4 Tetracyclines and Glycylcyclines

These antibiotics are bacteriostatic antibiotic, broad-spectrum against Gram-negative and positive bacteria. Tetracyclines and Glycylcyclines inhibit protein synthesis by preventing the attachment of aminoacyl tRNA to the target ribosome (Maleki *et al*., 2014).

2.10.5 Fluoroquinolones

According to their antimicrobial activity, quinolones are classified into four generations: (i) the 1st generation are acid quinolones are included Nalidixic acid, (ii) the 2nd generation includes fluoroquinolones, Levofloxacin, Norfloxacin and Ciprofloxacin, (iii) the 3rd generation, includes, Grepafloxacin, Gatifloxacin and Sparfloxacin, (iv) the 4th generation involved Moxifloxacin, Trorafloxacin and Gemifloxacin

(Kocsis, 2012). Quinolins preventing cell division through inhibition of the bacterial enzymes (DNA gyrase and topoisomerase IV), those are important for the supercoiling and synthesize of DNA (Correia *et al*., 2017).

2.10.6 Folic Acid Synthesis Inhibitors

Trimethoprim/Sulfamethoxazole, also known as co-trimoxazole, is a folic acid synthesis inhibitor that acts through the prevention of the dihydrofolate pathway, producing a bactericidal effect (Goldberg and Bishara, 2012).

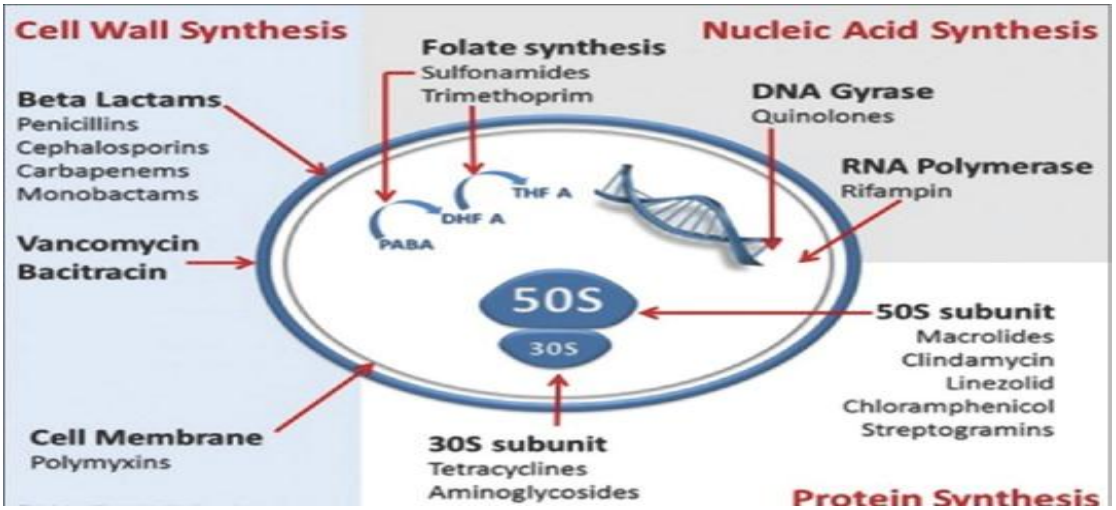


Figure 2-2: Mechanism of antibiotic action (Kapoor *et al*., 2017).

2.11 Mechinsm of antibiotic resistance

Several *A. baumannii* isolates possess different resistance mechanisms to various antimicrobial agents. These species are classified as: (i) multi-drug resistant (MDR) when they are resistant to one antibiotic from three or more antibiotic categories, (ii) extensively-drug resistant (XDR) when they are resistant to one antibiotic from all except two or fewer antibiotic categories, (iii) pan-drug resistant (PDR) when they are resistant to all antibiotic categories (Magiorakos *et al*., 2011). The worldwide distribution

of MDR *A. baumannii* isolates has severely limited the treatment options for controlling their infections nowadays (Zarilli *et al.*, 2013). Treatment of MDR *A. baumannii* infections, as well as other Gram-negative pathogens, is considered as one of the greatest challenges of contemporary medicine (Mehrad *et al.*, 2015). Figuer (2-3).

2.11.1 β-Lactamases

Inactivation of β-lactam antibiotics by β-lactamases is a major mechanism of antibiotic resistance in *A. baumannii*. Based on the sequence homology, β-lactamases are grouped into molecular classes, A, B, C and D (Jeon *et al*., 2015). All four classes of β- lactamases were identified in *A. baumannii*. The studies have shown that *A. baumannii* has a natural competence to incorporate exogenous DNA and its genome which has foreign DNA at high frequencies, involving, a frequent horizontal gene transfer (Touchon *et al*., 2014). Class A β-lactamases are inhibited by clavulanate hydrolyze Penicillins and Cephalosporins more efficiently than Carbapenems (Jeon *et al*., 2015). Unlike the serine-dependent β-lactamases (classes A, C and D), class B β-lactamases are Metallo-β-lactamases (MBLs) that require zinc or another heavy metal for catalysis (Jeon *et al*., 2015). Due to the broad substrate spectrum, MBLs catalyze the hydrolysis of virtually all β-lactam antibiotics including Carbapenems, but not monobactams (Jeon *et al*., 2015). One of the therapeutic crisis associated with class C β-lactamases is the cross (produce) resistance to Cephamycins, Penicillins and Cephalosporins. Also, it was reported that class C β-lactamases are not significantly inhibited β-lactamase inhibitors, such as clavulanic acid was used (Jeon *et al*., 2015). Class D β-lactamases are called OXAs (Oxacillinases) because they commonly hydrolyze isoxazolyl penicillin oxacillin much faster than benzylpenicillin (Jeon *et al*., 2015).

2.11.2 Aminoglycoside-Modifying Enzymes

Aminoglycoside-modifying enzymes are the major mechanism by which *A. baumannii* resistant to aminoglycosides. Aminoglycoside-modifying enzymes can be classified into acetyltransferases, adenyltransferases and phosphotransferases. These enzymes are typically existed on transposable elements and are genetically transferred among pathogenic bacteria (Lin and Lan, 2014). The genes encode for these enzyme include; *aac(6')-Ih*, *aac(3)-Ia*, *aac(3)-IIa*, *aac(6')-Ib*, *aph(3')-Ia* and *aph(3')-VI* which could be incorporated into mobile genetic elements and co-exist with each other (Nowak *et al.*, 2014). Genes encoding AMEs can be transferred as part of gene cassettes in the case of integrons and through conjugation mechanisms (Garneau-Tsodikova and Labby, 2016).

2.11.3 Changing the Target Site

Alteration of target sites, such as penicillin-binding proteins (PBPs) and mutations of DNA gyrase could alter the target sites for antibiotics binding (Lee *et al*., 2017). Overexpression of certain PBPs and mutation in DNA gyrase led to quinolone and tetracycline resistance by *A. baumannii* (Lee *et al* ., 2017). Alteration of the target site of the antibiotic is an essential mechanism of bacterial resistance. In general, this mechanism is based on random point mutations that have a minimal impact on bacterial cell homeostasis, It has been noticed that the 23_S RNA ribosomal mutations in *H. pylori* cause resistance to Clarithromycin (Eghbali *et al.*, 2016). A recent study showed that the mutations of Ser83Leu in the *gyrA* and in the *parC* were the most common types of the topoisomerase genes mutations in *A. baumannii* enhancing their resistance to several antimicrobial agents (Güler and Eraç, 2016).

2.11.4 Decreased Membrane Permeability

Decreasing membrane permeability may increase bacterial antibiotic resistance. The pores of the outer membrane have an essential role in the tolerance the environmental stress and in the virulence potential of *A. baumannii* strains, through mediating the transport of the molecules (Lee *et al*., 2017). Porins found to play a significant role in the mechanism of bacterial resistance. It was reported that reducing the expression of some porins like Caro, Omp22-33 was associated with Carbapenem resistance in *A. baumannii* (Lee *et al*., 2017). In addition to outer membrane proteins, lossing of LPS increases Colistin resistance in *A. baumannii* due to a decrease in membrane integrity (Lee *et al*., 2017). These results provide a brief view and increase our understanding of the mechanisms of antibiotic resistance in the bacterial cell.

2.11.5 Efflux Systems

Efflux pumps usually have 3 components, (a) the pump itself, located at the cytoplasmic membrane; (b) an exit portal (porin channels that pass through the outer membrane) and (c) a linker lipoprotein located between (a) and (b). The efflux pump type resistance-nodulation division (RND) has been illustrated in *A. baumannii* as it is responsible for Aminoglycoside, Quinolones, Tetracyclines, Chloramphenicol, Erythromycin, Trimethoprim and Ethidium bromide resistance (Nowak *et al*., 2015). Bacterial efflux systems are responsible for causing the ejection of potentially toxic compounds outside bacteria to the extracellular environment. Five bacterial efflux pump families have been identified; (1) the multidrug and toxin extrusion family (MATE), (2) the small multidrug resistance family (SMR), (3) the resistance-nodulation-cell division superfamily (RND), (4) the major facilitator superfamily (MFS) and (5) the proteobacterial

antimicrobial compound efflux family (Du *et al*., 2018). In Gram-negative pathogens, the flow pumps play major roles in excreting bile salts, fatty acids, antimicrobial peptides and virulence factors, such as iron acid (Du *et al*., 2018). In Acinetobacter, AceI and AdeABC flow pumps improve resistance to biocides and aminoglycosides, respectively (Liu *et al*., 2018). The AbaF pump was identified in *E. coli* (and recently, in *A. baumannii*), as a novel flow pump associated with fosfomycin resistance (Sharma *et al*., 2016). The EmrAB-TolC flow pump is also present in *A. baumannii* enhancing bacterial resistance to netilmicin, tobramycin and imipenem (Nowak-Zaleska *et al*., 2016). Another report identified three new flow pumps (A1S_1535, A1S_2795 and ABAYE_0913) in *A. baumannii* using the virtual multiplexer assay (Li *et al*., 2016).

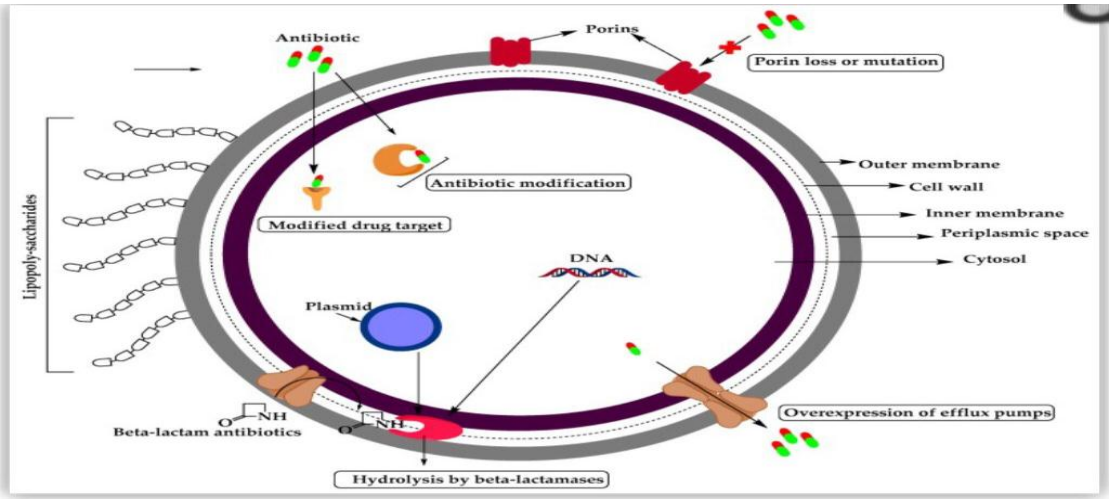


Figure 2-3: Mechanisms of antibiotics resistance in *A. baumannii* (Breijyeh *et al*., 2020).

2.12 Innovative strategies for controlling *A. baumannii* infections

Several studies have been conducted recently, referred to the unusual role of *A. baumannii* strains in rising the incidence of nosocomial infections. In these studies, *A. baumannii* was indirect, associated with morbidity and mortality rates. Analysis of resistance profiles of *A. baumannii* strains showed that the worldwide spread of the MDR

phenomenon to the most popular antibiotics, including the new generations of antibiotics (D'Onofrio *et al*., 2020; Dahdou *et al*., 2017). Recently, the researchers started focusing on combing therapeutic agents. Antibiotics combination could produce a synergistic potential, however, some antibiotics combinations were reported to reflect an antagonistic effect on the bacterial growth, such as a combination of Minocycline/Tigecycline (Castanheira *et al.*, 2014), Colistin/Rifampin (Aydemir *et al.*, 2013). Polymyxin B-combined therapy had minimal effects on controlling nosocomial infection caused by *A. baumannii* (Menegucci *et al*., 2019). Such antibiotics interactions lead to produce a selective pressure that may increase antibiotic resistance of bacterial cells. Therefore, a combination of antibiotic therapy could not be feasible in clinical settings for the long term. Because *A. baumannii* strains showed resistance to almost all tested antibiotics (Adams *et al*., 2009). A new, safe and potential antimicrobial should urgently discover. Therefore, an effective, alternative protocol to control *A. baumannii* infections are required in order to encounter their resistance to antibiotics. The naturally derived antimicrobials alone or in combination with traditional antibiotics, could be the best choice, at this time, to safely prevent pathogenic infections. In this study, we focused on some of the biocontrolling agents that will be presented in this reveiw coulding; the new antimicrobial peptides, probiotic factors, to prevent the spread of MDR of *A. baumannii* strains.

2.12.1 Antimicrobial Peptides (AMP)

Antimicrobial peptides are alternative substances, used instead of antibiotics, to manage the spread of MDR strains. AMP is a class of compounds that a part of innate immunity, serving as a primary barrier against infectious agents, such as viruses, bacteria and fungi (Falanga *et al*., 2016). AMPs are amphipathic molecules with a positive electrical charge

of approximately 11–50 amino acid residues in length (Kumar *et al*., 2018). The key mechanisms of AMPs activity are: (i) destroying the structures of cell wall and membrane, (ii) inhibition of protein and nucleic acids synthesis, and (iii) activation of apoptosis and necrosis (Govender *et al*., 2012). *In vitro* and *in vivo* recent studies found that antimicrobial peptides play an important role in the inhibition of planktonic and biofilm cells growth, eradication of persistent bacterial cells and prevention of the inflammatory processes (Mwangi *et al*., 2019). However, more *in vivo* experiments are required to identify their safety, pharmacokinetic. The antimicrobial function of eight AMPs was evaluated against certain strains of *A. baumannii* (Jakiewicz *et al*., 2019). Natural AMPs may be a starting point for the biosynthesis of AMPs with identical functions and an attractive treatment choice for the prevention of *A. baumannii* infections (Mourtada *et al.*, 2019). Most of the AMPs are susceptibility to enzymatic digestion and some are elevated toxicity, therefore, AMPs are prefered to apply topically but not orally or intravenously (Starr and Wimley, 2017).

2.12.2 Probiotics

Probiotics are defined as live microorganisms that have health benefits on the host when delivered in sufficient quantities (Saud *et al*., 2020). Previous experiments have shown that probiotics participate in controlling the host immune system by promoting the development of cytokines and cellular activation (Hager *et al*., 2019). For example, the capacity of the probiotic *Bifidobacterium breve* to act against MDR infections has been investigated (Asahara *et al*., 2016). This probiotic greatly improves the immune defence system against pathogenic intestinal infections caused by MDR *A baumannii* (Asahara *et al*., 2016). Strains of probiotics may influence the pathogenic microorganisms via a variety of mechanisms, such as enhancing intestinal barrier efficacy, raising the mucin development and

modulating the behaviour of the immune system (Vieco-Saiz *et al*., 2019). In addition to immunity stimulation, probiotics are capable to produce antimicrobial metabolites and compete with pathogens on the nutrients and neutralize toxin excretion or repress the virulence-related genes of pathogenic bacteria (Markowiak and Slizewska, 2017; Rätsep *et al*., 2017). Probiotics should have beneficial characteristics such as non-virulent, tolerant to bile salts and acid, aid in the absorption of nutrients in the gastrointestinal tract, ability to adhere to the cell surface and exhibit antimicrobial activity against human pathogens (Fijan, 2016). Probiotics could be isolated from dairy and non-dairy products. Probiotic-rich milk products include fermented milk, ice cream, cheese, yogurt and butter milk, etc., while the non-dairy products contain probiotics include soy-based products, nutrition bars and cereals, etc (Kechagia *et al*., 2013). Probiotics are now applied into the food and beverage industry. It has been shown that producing of safe antimicrobials and active metabolites by probiotic strains can effectively encounter the growth of certain pathogenic strains (Vieco-Saiz *et al*., 2019). Antimicrobial resistance is currently emerging as a global threat to living organisms. The abuses and overuses of antibiotics to control bacterial infections lead to the development of antibiotic resistance (Saud *et al*., 2019).

Lactobacillus (lactic acid bacteria (LAB)) and bifidobacterium are the most important microbial genera widely used as probiotic preparations (Sanchez *et al*., 2017). The most widely used are those of the genus Lactobacillus and Bifidobacterium, which are commensal bacteria living in or on human bodies (Novik and Savich, 2020). These strains play a key role in balancing the immune system, stabilizing gut microbiota and increasing absorption of nutrients (Sanchez *et al*., 2017). Furthermore, probiotic bacteria are enhancing lactose fermentation in patients that Suffer

from poor lactose fermentation, relieving constipation and increasing vitamin and mineral absorption (Novik and Savich, 2020). Recently, more light was focused on the probiotics due to their capability of producing antimicrobial effects against several pathogens (Abdelhamid *et al*., 2018). The *in vitro* studies have concluded that positive outcomes against enteropathogens are correlated with the use of LABs, specifically Lactobacillus species (Kaur *et al*., 2018). Lactobacilli are known as desirable intestinal microflora (Shokryazdan *et al*., 2014). Several species of probiotics have been approved as (Generally Recognized as Safe) GRAS status, recognized microorganisms or microbial derivatives as safe for use in the food industry (Cui *et al*., 2017; Gabliardi *et al*., 2018). Lactobacillus is typically found in low molecular oxygen tension conditions, such as humans' intestinal and urinary tracts and sharing their habitat with many pathogenic microorganisms, including pathogenic enterobacteria (Ruiz *et al*., 2017). This beneficial microorganism producing organic acids, hydrogen peroxide, biosurfactants and bacteriocins (Fernandes *et al*., 2019). Lactic and/or acetic acids produce low acidity environment (Fijan, 2014). Acids produced by LAB enter the cytoplasm of pathogenic bacteria minimizing intracellular pH and interfering with cellular metabolic processes (Hughes and Webber, 2017). In addition, these acids increase the permeability of the outer membrane of Gram-negative bacteria, compromising their integrity and could improve the action of other antimicrobial substances such as, bacteriocins (Gálvez *et al*., 2010). The hydrogen peroxide formed by many strains of Lactobacillus is also capable of inducing stress in the outer membrane of some bacteria, such as uropathogenic *E. coli* (UPEC) through modifying the structures of fimbriae and prevents its ability to adhere to bacterial cells (Costa *et al*., 2012). Lactobacillus species develop various exo-metabolites, such as EPS, bacteriocins (Sharma *et al*., 2018). Polysaccharides developed by LAB

have reported reflecting an anti-biofilm potential (Kim and Kim, 2009), stimulate the immune defence system and produce antioxidant effects (Pan and Mei, 2010). The EPS extracted from Lactobacillus spp showed antimicrobial activity against both Gram-positive (e.g., *Listeria monocytogenes* and *S. aureus*) and Gram-negative bacteria (e.g., *P. aeruginosa* and *Salmonella typhymurium*). The findings showed that the capacity to remove biofilms is linked to the EPS concentration (Mahdhi *et al*., 2017). In several studies, the anti-biofilm activity of bacteriocins-produced by probiotics was demonstrated. *L. brevis* DF01 bacteriocin prevents the development of biofilms but does not eliminate existing *E. coli* and *S. typhimurium* biofilms (Kim *et al*., 2019). Lactobacillus species also inhibit biofilm of some yeast such as *Candida albicans* by producing exometabolites and lead to inhibition of the initial stage of colonization (Matsubara *et al*., 2016).

Probiotic microorganisms involved Bacilli strains. *Bacillus subtilis* is widely distributed in nature and enters the human body with drinking water and food, some researchers indicated that these bacterial species are a natural inhibition of intestinal microbiota (Savustianenko, 2016). Subsequent studies shed the light on the isolation of several structured antimicrobials agents from bacilli actively working against Gram-positive and Gram-negative microorganisms and fungi (Sumi *et al*., 2015). Subtilosin A a cyclic bacteriocin (antibiotic protein) synthesized by *B. subtilis*, is an important probiotic derivative. It has a net cationic charge that is usually located at surface receptors rather than electrostatic binding to bacterial cells. Subtilosin showed an antimicrobial activity against *Gardnerella vaginalis* and *L. monocytogens*. Moreover, the antibiotic effect of subtilosin aginst *G. vaginals* alone and in combination with some natural antimicrobial agents was noticed (Algburi *et al*., 2015). Algburi *et al*., (2017), reported that subtilosin has anti-QS effect against *E. coli*

O157:H7, *L. monocytogenes* and *G. vaginalis* ATCC 14018. Subtilosin contributed to the inhibition of 60% of *E. coli*, 80% of the *L. monocytogens* and 90% of *G. vaginal* biofilms (Algburi *et al*., 2017). Similarly, sonorensin, a bacteriocin extracted from *Bacillus sonorensis* MT93, was able to reduce *S. aureus* biofilms cell viability, prevents the binding and development of biofilm cells and induces the thinning of mature biofilms (Chopra *et al*., 2015). Sporobacterin is a spore suspension of the probiotic *B. Subtilis* strain 534 with a 7% aqueous solution of sodium chloride. This suspension has been used in Russia for the past thirty years, especially for the prevention and treatment of postoperative bacterial and fungal infections in high-tech surgery, including organs transplantation (Efremenkoova *et al*., 2019).

2.13 Combanition strategies of Probiotic and antibiotic

The emerging of antibiotic-resistance of various species of microorganisms raises concerns in many countries and attracted scientists to develop alternative medications (Pizzolato-Cezar *et al*., 2019). The low efficacy of the existing methods to correct this micro-ecological issue makes it necessary to continue searching for the most effective ways which are not risky on the host microbiome, physiology, metabolism and signalling pathway. Recently, several studies have been done on the derivatives of probiotics; their structural components and metabolites. These studies have been developed as “metabiotics”, “biological drugs”, “biogenecs”, “cell-free supernatants” (CFS), “postbiotics”, “heat-killed probiotics” or “pharmacobiotics” (Singh *et al*., 2018). The combined approach is distinguished by the use of two or more antimicrobials for controlling infections. The advantages of this combination are: (i) decreasing the toxic side effects, (ii) reducing the cost of treatment and (iii) using low concentrations of each antimicrobial compared to using them

separately (Mulani *et al*., 2019). The efficacy of antimicrobial combinations has been confirmed in a model of pneumonia caused by the poly-resistant strain of *A. baumannii*. The synergized antimicrobials cause a decrease in the bacterial load in the lungs and an increase in the host survival (Dillon *et al*., 2019). Some research studies revealed that using reuterin and lactoferrin, as naturally derived antimicrobials, exhibited higher antimicrobial activity against microorganisms compared to their individual effects (Montiel *et al*., 2015). Other publications reported synergic activity when probiotics were combined with antibiotics against several pathogens (Lainson, 2017). The antimicrobial potential of certain antibiotics was augmented when they were combined with nisin, a bacteriocin produced by *Streptococcus lactis*. These antibiotics include: Penicillin, Ampicillin, Gentamicin, Kanamycin, Roxithromycin, Streptomycin, Vancomycin, Chloramphenicol, Cefuroxime, Cefazolin, Ceftriaxone, Cefepime, Ciprofloxacin, Imipenem and Linezolid (Tong, 2014). Biologically active substances *L. rhamnosus* GG and *S. boulardii*, can reduce the required concentration of antibiotics, prolonging their use, and suspend the likelihood of the pathogens developing resistance to microorganisms (Isayenko, 2019).

3. Materials and Methods

3.1 Materials

3.1.1 Devices and Instruments

The devices and instruments which used are listed in the table (3-1).

Table 3-1: Devices and Instrument.

ID	Apparatus and equipment	Company	Origin
1	Autoclave	Labtach	Korean
2	Blue tips, 1000 µl	Afco	China
3	Cold speed Centrifuge	Nuva	Turkey
4	Densichek TM	Biomerix	France
5	Digital camera	Samsung	Vietnam
6	Electrical balance	Precisa	Switzland
7	ELISA reader and washer	Human	Germany
8	Hot plate with magnetic stirrer	IKA	Germany
9	Incubator	Nuva	Turkey
10	Laminar air flow hood	Nuva	Turkey
11	Light microscope	Olympus	Japan
12	Micropipette	Brand	Germany
13	Microscope slides	Alaseraco	China
14	Millipore filter (0.45µm)	Chm Lab	Germany
15	Multi -channel pipette	Human	Germany
16	Oven	Nuva	Turkey
17	Petri dishes	AFCO	Jordan
18	pH meter	Jenwey	UK
19	Screw capped	AFCO	Jordan
20	Tissue culture plate 96 wells flat bottom	AFCO	Jordan
21	Transport swab without media	AFCO	Jordan
22	Vitek 2 System compact®	Biomerix	France
23	Volumetric flasks	LAB	Germany
24	Vortex	Labcoo	Germany
25	Water bath	Memmert	Germany
26	Water distillatory	Nuva	Turkey
27	Yellow tips, 200 µl	AFCO	China

3.1.2 Chemicals and Reagents

The important materials and substances in the current study were listed in table (3-2).

Table 3-2: Chemicals and biological materials used in this study.

ID	Chemical and biological material	Company	Origin
1	Ethyl Alcohol (96%)	Taiba	Iraq
2	Crystal violet	Syrbio	Syria
3	Glycerol	BDH	England
4	Gram stain kit	Syrbio	Syria
5	Glucose anhydrous	Himdia	India
6	Hydrogen peroxide (H_2O_2) 3%	AL-mothalath AL –massy	Iraq
7	Immersions oil	BDH	England
8	Kovac's reagent	Connectivity and vaccines	Bagdad
9	Macfarland	Biomerix	France
10	Methyl red staining	BDH	England
11	Normal Saline (Sterile)	PSI	Saudi Arabia
12	Oxidase reagent	Himdia	India
13	Phosphate Buffer Saline (PBS)	BDH	England
14	Urea	Fluka	Switzerland
15	Vogaes-prskour Reagent	BDH	England

3.1.3 Probiotic agents

The following Antimicrobial agents were used in this study are listed in the table (3-3), appendixes (1).

Table 3-3: Probiotics agent.

Id	Agent	Origin
1	*Bacillus subtilis* KATMIR 1933	Isolated form Yougrt Prechased from YogoFarm Market, U.S.A
2	*Bacillus amyloliquefaciense* B-1895	Isolated from New Jersy soil, Johnson Park, U.S.A
3	*Lactobacillus casei* DG CNCM I-1572	Prepared powder, ENTEROLACTIS®, Duo, Spain
4	Mix of lactobacillus(*Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus bulgaricus, Lactobacillus brevis, Lactobacillus reuteri, Lactobacillus salivarius, Lactobacillus fermentum, Lactobacillus gasseri, Lactobacillus rhamnosus, Bifidobacterium lactis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis* and *Bifidobacterium longum*)	Graden of life LLC, U.S.A

3.1.4 Culture media

Table (3-4), presents the culture media that were used in this study.

Table 3-4: The culture media used in this study.

Id	Media	Company	Origin
1	Blood agar base	Hi media	India
2	Brain heart infusion agar	Oxoid	England
3	Brain heart infusion broth	Oxoid	England
4	MacConkey agar	Hi media	India
5	Man Rogosa Sharpe (MRS) agar	Liofilchem	Italy
6	Man Rogosa Sharpe (MRS) broth	Liofilchem	Italy
7	Methy red-Voges-Proskauer (MR-VP) broth	Hi media	India
8	Mueller-Hinton agar	Oxoid	England
9	Peptone water	Tmmedia	India
10	Simmon's citrate agar	Hi media	India
11	Triple Sugar Iron Agar	Hi media	India
12	Urea agar base	Hi media	India

3.1.5 Antibiotics

Antibiotic powders that were used in the current study are listed in table (3-5), appendix (2), while antibiotic discs are listed in table (3-6).

Table 3-5: Antibiotic powders used in the present work.

ID	Antibiotic powders	Weight (mg)	Company	Origin
1	Colistin methanesulfonate	384 mg	Kocak Farma	Turkey

Table 3-6: Antibiotic discs used in the present work.

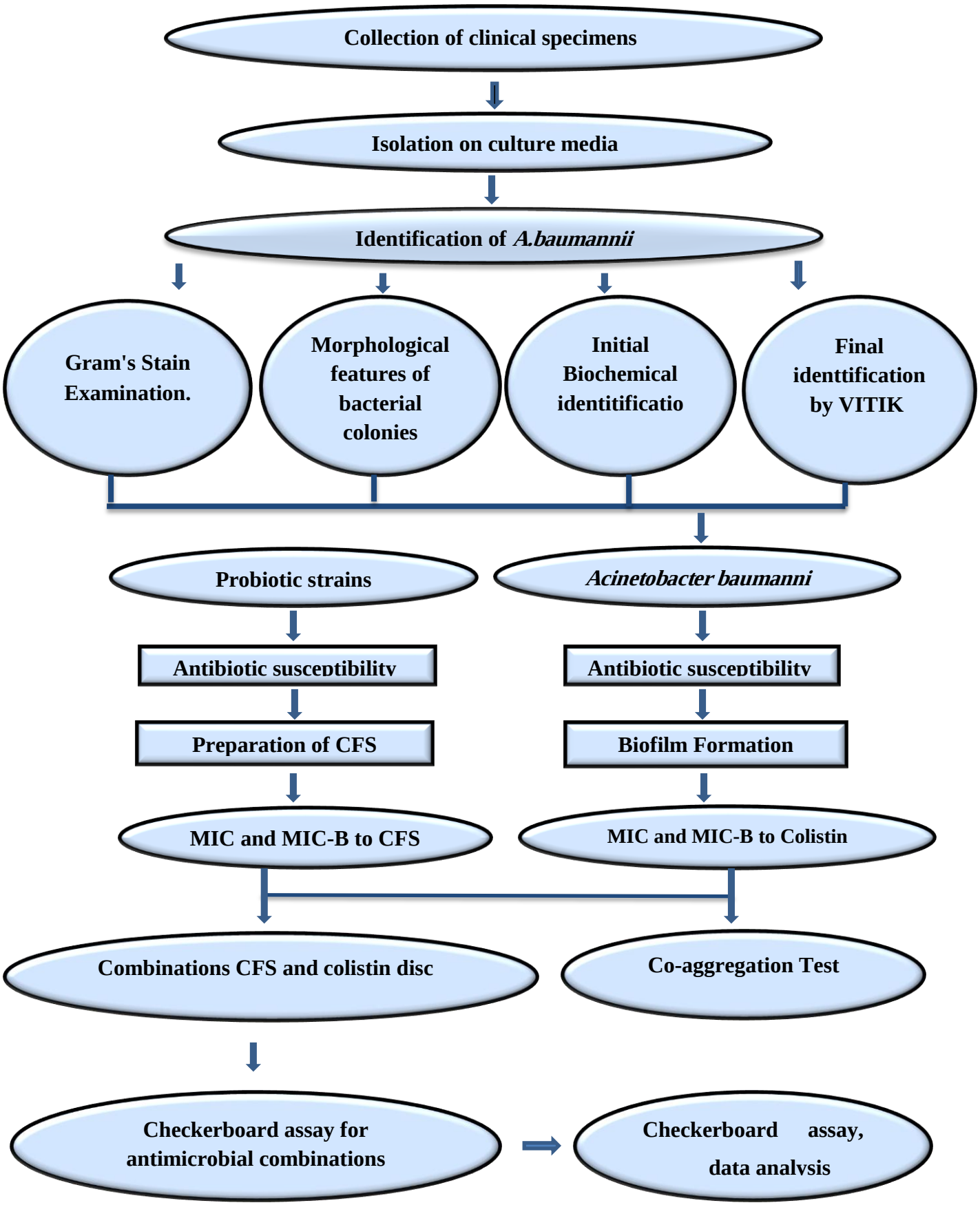
ID	Antibiotic discs	Code	Disc potency (µg/disc)	Company	Origin
1	Amikacin	AK	30	Mast Group	U.K
2	Colistin	COL	25	Mast Group	U.K
3	Cefoxitin	FOX	30	Mast Group	U.K
4	Cefotaxime	CTX	30	Mast Group	U.K
5	Meropenem	MEM	10	Mast Group	U.K
6	Trimethoprim-sulfamethoxazole	TS	1.25/23.75	Mast Group	U.K

3.1.6 Diagnostic and susceptibility kits

Table 3-7: Vitek 2 system used in the present work.

ID	TOOL	Company	Origin
1	Vitek 2 system (GN) Card	Biomerix	France
2	Vitek 2 system (AST-GN) Card	Biomerix	France

3.2 Methods: (*In vitro* Design)



3.2.1 Preparation of Culture Media

All culture media offered in the table (3-4) except (Blood agar, Peptone water, Urea Agar and Man Rogosa Sharpe MRS broth) were prepared according to the manufacturing company's instructions. The ingredients were dissolved in distilled water (DW), the pH was adjusted to 7.2 ± 0.2, then heated in the water bath to completely dissolve all the ingredients. The media was sterilized at 121˚C for 15min at 15pounds/inch^2 using autoclave, subsequently dispensed into sterile Petri dishes; otherwise, the media were incubated for 24hrs at 37˚C to confirm sterility (Harley and Prescott, 2002).

A- Blood Agar Medium

This type of medium was prepared according to manufacturing company instructions (40 gm/L) sterilized by autoclaving and cooled to 45-50°C. Then, 5-10% of sterilized fresh human blood was added. Blood agar was used for isolation most pathogenic bacteria to identify their capability for blood hemolysis (Atlas and Snyder, 2006).

B- Man Rogosa Sharpe (MRS) Medium

Man Rogosa Sharpe media was prepared according to the manufacturing company instructions by dissolving 10 gm peptone, 10 gm beef extract, 5 gm yeast extract, 20 gm glucose, 1 ml tween 80, 2 gm K2HPO$_4$, 2 gm triammonium citrate, 200 mg MgSO$_4$.7H$_2$O, 50 mg MnSO$_4$.4H$_2$O and 5 gm of sodium acetate hydrate in 900 ml of DW, with adjusted pH to 6.2, volume was completed to 1 litre with DW. The media was brought to boil on magnetic hot plate stirrer by a magnetic bar at 100°C until constituents are being dissolved completely and sterilized by autoclaving. Then, the media dispensed into sterile Petri dishes and incubated for 24hrs at 37˚C to ensure sterility last stored at 4°C until use (Mahmood and Hameed, 2018).

C- Peptone Water Medium

This medium was prepared by dissolving 5 g of NaCl and 10 g of peptone in 1 litre of DW. The pH was adjusted to 7.4 and distributed into sterile tubes and then sterilized by autoclave for 15 min at 121°C. It was used for detecting the bacterial capacity to produce indole from tryptophan (Atlas and Snyder, 2006).

D-Urea Agar Medium

Urea agar was prepared in 95 ml of DW by dissolving 2.4 g of urea base agar, then sterilizing the medium and left to cool at 45°C. After that, 5 ml of urea was added at (40 %), then pouring it into sterile tubes, left to solidified, This culture medium was used to detect bacteria's ability to produce urease (Forbes *et al*., 2007).

3.2.2 Preparation of Reagents and Solutions

3.2.2.1 Reagents

3.2.2.1.1 Catalase reagent

Hydrogen peroxide H_2O_2 (3%) was prepared from the stock solution (15%) and kept at a dark place as reported by (Forbes *et al*., 2007).

3.2.2.1.2 Oxidase reagent

In this assay, the *N,N,N,N*- tetramethyl-p-phenylene diamine dihydrochloride reagent powder was dissolved in DW or phosphate buffer saline (PBS), then, immediately stored at a dark container. This test was used to detect cytochrome c existing in specific Gram-negative bacteria (Shields and Cathcart, 2010).

3.2.2.1.3 Kovac's Reagent

The reagent was prepared to detect indole production. Kovac's reagent was prepared by dissolving 5 gm of P-dimethyl amino benz aldehyde

(DMAB) in 75 ml (amyl-alcohol). In addition, 25 ml of concentrated HCL added to this mixture (McFaddin, 2000).

3.2.2.2 Solutions

3.2.2.2.1 Antibiotic Stock Solutions

The antibiotic stock solutions have been prepared with a final concentration of 20000 µg/ml as primarily stock. A 100 mg of Colistin antibiotic was dissolved in 5 ml sterilized DW. The solution was filtered by a Millipore filter (0.45 µm) and stored in the refrigerator at 4°C. The concentrations (50 µg/ml), which were selected and applied in our experiments, were calculated based on the following equations: $C_1*V_1= C_2*V_2$ (CLSI, 2019).

3.2.2.2.2 Phosphate Buffer Saline (PBS)

Solution A: prepared by adding 1.39 g of sodium phosphate dihydrogen (NaH_2PO4) to 100 ml of DW.

Solution B: prepared by dissolving 3.58 gm of sodium phosphate hydrogen ($Na_2HPO_4.12H_2O$) in 100 ml DW. The PBS was obtained by mixing 61 ml of solution B with 39 ml of solution A. Then, the volume was completed to 100 ml by adding DW (pH = 7.2). Subsequently, it was autoclaved at 121ºC under 15 pounds/inch for 15 min and placed at 4ºC.

3.2.2.2.3 Solutions for Biofilm Phenotypic Detection

The following solutions were prepared according to (Badmasti *et al.*, 2015):

- **Crystal Violet Stain (1%)**

It was prepared by adding 20gm of crystal violet stain powder into 100 ml Alcohol (Ethanol 95%) as Stock Solutions and dilute with ammonium oxalate (0.8 g) in (80 ml) from DW.

- **Ethanol/Acetone (95:5, v/v)**

To prepare this solution, 5 ml of acetone was mixed with 95 ml of absolute ethanol.

3.2.3 Sterilization methods

Three methods of sterilization were used:

A) Moist-heat sterilization (Autoclaving)

Microbial culture media, reagents and solutions were sterilized by autoclaving for 15 min (15 pounds/inch2) at 121°C, unless otherwise stated.

B) Dry-heat sterilization (oven)

An electric oven was used to sterilize glassware for 3hrs at 180°C and for fixation of biofilm of *A. baumannii*.

C) Millipore Filters (0.45µm) sterilization

Used to sterilize antibiotic solution and the cell-free supernatants (CFS) of probiotics.

3.2.4 Samples Collection

A total of 230 clinical samples (wounds, burns and blood) were collected from Baqubah Teaching Hospital and Its Consultative Clinic. The samples were collected starting from the beginning of September till the end of December 2020, from both genders at age ranged from 10 to 70 years old. Cotton sterile swabs were used to take the sample (wounds and burns). As for blood samples, they were collected and cultured by the following steps:

- A tourniquet was applied and location of vein was determined by touch, then the tourniquet was released while the skin was being prepared for venipuncture, and a bactericidal disinfectant (2%tincture of iodine, and 70% alcohol) was used at the venipuncture site and the skin was cleaned in concentric circles and increasing diameter. The disinfectant was allowed to

evaporate on the skin surface before blood was withdrawn for at least 30 seconds. The skin was not touched after preparation.

- The tourniquet was reapplied, venipuncture was performed and 10 ml of blood was withdrawn, then 5ml of the blood was transferred into blood culture bottles.
- Blood culture had been by used BACT\Alert 3D system was used. This system is closed for continuous monitoring of the blood culture and employed to detect bacteremia and fungemea. This is possible because the blood stream is usually a sterile environment. Inoculated bottles were placed in the instrument where they were incubated and continuously monitored for the presence of microorganisms' growth. When there was a bacterial growth, the device emitted a signal, the device was pointing when there was no microorganism growth, and the result was regarded negative and the bottle came out of the device (depending on the manufactures instructions).

Before sample collection, a questionary form about the patient information was used included: name, age, gender, source of samples and other related information, appendix (3). The samples were cultured for diagnostic purposes immediately after sampling.

3.2.5 Isolation of *Acinetobacter baumannii* isolates

Blood agar and MacConkey agar were used for samples inoculation using a direct streaking method. The agar plates were incubated for 24hrs at 37ºC. Non-hemolytic, vague creamy colonies were sub-cultured on Blood agar plates and incubated for 24hrs at 37ºC. Non-lactose fermenting colonies were re-cultured on additional MacConkey agar plates to obtain a pure isolated colonies. An initial biochemical tests were performed in order to primary identify the bacterial Species (Cappuccino and Welsh, 2018).

3.2.6 Activation of probiotic isolates

Frozen stocks of *B.* subtilis KATMIRA1933, *B. amyloliquefaciens* B-1895 and poder (50 mg) from *Lactobacillus casei* DG CNCM I-1572 and Mix Lactobicillus agent were inoculated by sterial loop in Man, Rogosa and Sharpe (MRS) medium and incubated aerobically for 24 h at 37 °C.

3.2.7 Identification of *Acinetobacter baumannii*

3.2.7.1 Gram's Stain Technique

According to (Finegold and Marten, 1982), all bacterial isolates were stained using Gram's staining method. The bacterial cells were observed under the light microscope by taking one bacterial colony and transported to the microscopic slide. The shape and cell arrangement were observed with oil immersion lens.

3.2.7.2 Morphological Examination

Morphological examination based on certain characteristics, including colony colour, shape, edges and texture (as primary diagnostic tests) of isolates growth was observed on Brain heart infusion agar (BHI), Blood agar and MacConkey agar (Biswas and Rather, 2019).

3.2.7.3 Growth at 44ºC

The isolates were streaked on BHI agar and incubated for 24hrs at 44ºC. The results showed that the ability of the tested bacteria to grow at 44ºC. This test was used to distinguish *A. baumannii* (which able to grow at 44ºC) from other *Acinetobacter* species which unable to grow at this temperature (Dijkshoorn *et al.*, 2011).

3.2.7.4 Biochemical Tests

3.2.7.4.1 Oxidase Test

It is carried out to detect the bacterial ability to release the oxidase enzyme. It was done by saturating a filter paper with an oxidase reagent (1

% of -N-N-N-N-tetramethyl para-phenylenediamine dihydrochloride). A single isolated colony was transferred to a piece of filter paper by a wooden stick. Then, 2-3 drops of oxidase reagent were added to the filter paper. The change in colour to dark purple within 20-30 seconds indicates a positive test. Oxidase-negative bacteria will not produce a change in colour (Biswas and Rather, 2019).

3.2.7.4.2 Catalase Test

The catalase production test was done by picking up the centre of the 24hrs growth pure culture colony and mixed with a drop of 3% hydrogen peroxide (H_2O_2) reagent on a clean glass slide. The appearance of immediate bubbles (gas liberation O_2) pointing to as a positive result of this test, while bacteria gave no gas bubbles refer to a negative result (Biswas and Rather, 2019).

3.2.7.4.3 Lactose Fermentation Test

This test was used to distinguish the Gram-negative bacteria which have the ability to lactose fermentation on MacConkey agar containing lactose sugar. The bacterial isolates were streaked on the MacConkey and incubated for 24hrs at 37^{o}C under aerobic condition. To identify lactose fermentation, the medium turns into yellow at alkaline pH (no lactose fermentation) but pink colour at acidic pH when lactose is fermented (Biswas and Rather, 2019).

3.2.7.4.4 Urease production Test

This test was used to detect the bacterial potential for urea analysis and the production of ammonia and carbon dioxide. Urea agar slant was inoculated by a sterile loop with the bacteria tested and incubating for 24hrs at 37°C. The yellow colour indicates a negative result, while the pink colour indicates a positive result (Cappuccino and Welsh, 2018).

3.2.7.4.5 Triple Sugar–Iron (TSI) Test

This is a test used for the differentiation of whether a bacteria ferment glucose, lactose and sucrose with or without gas releasing and hydrogen sulfide (H_2S) production. A single colony of *A. baumannii* isolate outgrowth on BHI agar subculture picked up by the disinfected sterile loop. Thereafter inoculated by stabbing the surface centre of the Triple Sugar–Iron medium to the bottom of the tube. Then incubated for 24hrs at 37˚C. After that, the results were reported (Cappuccino and Welsh, 2018).

3.2.7.4.6 IMVIC Tests (Cappuccino and Welsh, 2018)

- **Indole Test**

Peptone water was inoculated with bacterial cells. After incubation, 5 drops of Kovac's reagent was added. A positive result was indicated by the formation of a red surface ring.

- **Methyl Red Test**

Methy red-Voges-Proskauer (MR-VP) medium was inoculated with bacterial cells and incubated for 24hrs at 37^{o}C aerobically; a few drops of methyl red solution were applied to the culture of the broth. A favourable outcome is shown by the changing in the medium colour from yellow to red.

- **Voges-Proskauer Test**

Methy red-Voges-Proskauer (MR-VP) medium was inoculated and incubated with bacterial culture, then 0.6 ml of VP1 (alpha-naphthol) and 0.2 ml of VP2 (KOH) were added; a positive result was indicated by the formation of red colour after 15 min.

- **Citrate Utilization Test**

Simmon citrate slants have been inoculated with the bacterial cells, then incubated for 24hrs at 37°C. A positive result is the conversion of the

medium colour from green to blue due to the use of sodium citrate as a carbon source leading to a change in the pH of the inoculated culture medium.

3.2.7.5 Confirmation of Bacterial Identification using VITEK 2 Compact System

The VITEK 2 Compact system is an automated microbiological system utilizing growth-based technology. It is characterized by accommodating the colourimetric reagent cards that are incubated and interpreted automatically, as shown in the appendix (4). It was used to confirm the identification of *A. baumannii*. The VITEK 2 System is an identification system, which depends on the biochemical reactions between the bacterial isolates suspended in their solutions and the culture media included in the VITEK 2 identification cards, to identify the bacterial isolates. The selected bacterial isolates were inoculated on MacConky agar plates and then incubated overnight at 37°C under aerobic conditions. Based on the manufactures instructions, single colonies (3-5) were selected and suspended in PBS solution. The turbidity of the bacterial suspension was adjusted with VITEK Densichek to match the McFarland 0.5 standard in 0.45% sodium chloride. Then VITEK 2 ID-GN (Gram Negative) card and the bacterial suspension tubes were manually loaded into the VITEK-2 system. Following steps in the software were done according to the manufacturer's instructions (BioMerieux, France).

3.2.8 Preservation of Bacterial isolates

3.2.8.1 Short term preservation

To preserve the bacterial isolates for a short time (1 to 3) months in the case of slants, respectively, A single colony of *A. baumannii* was streaked on BHI agar slant (screw-capped tube) and incubated for 24hrs at 35ºC$\pm$2

aerobically, then, stored in the refrigerator at 4°C for a short time (Vandepitte *et al*., 2003).

3.2.8.2 Long term preservation

To maintain bacterial isolates for a long time (up to three months), the isolated bacteria were inoculated on culture media containing 20% glycerol. The medium was prepared by adding 2 ml of glycerol 99.9% to 8 ml of BHI broth, dispensed into the small screw-capped bottle and sterilized by autoclaving. After the cooling of the mixture, the tubes were inoculated by one pure isolated colony and incubated for 24hrs at 37^{o}C. The tubes were stored in a deep-freezing (Vandepitte *et al*., 2003).

3.2.9 Antibiotic susceptibility Test

The antibiotics susceptibility of *A. baumannii* isolates was performed using antibiotics susceptibility test Gram-negative (AST-GN) card inserted into the VITEC 2 system. The antibiotic-resistance profile against the selected isolates was evaluated by the Kirby-Bauer method, which was performed according to Clinical and Laboratory Standards Institute (CLSI, 2020) and following **(**Cappuccino and Welsh, 2018) with minor modifications. Briefly, 3-5 colonies grown of *A. baumanni* on BHI plates were transferred by a sterile inoculating loop to a tube containing 5 ml of BHI broth, and 3-5 colonies grown of probiotic strains on the MRS plates were transferred by a sterile inoculating loop to a tube containing 5 ml of MRS broth. Using a spectrophotometer (Densichek TM), the bacterial growth was diluted and adjusted to an optical density (OD_{630}) of 0.1 which correlated with 1.5×10^{8} CFU/ml. A 100 µl aliquots of the last bacterial dilution (1.5×10^{8} CFU/ml) was inoculated by streaking on to Müeller Hinton agar to *A. baumanni* and MRS agar to probiotics strains at three directions. The tested antibiotics were chosen based on the recommendation of a local physician, as commonly prescribed

antimicrobials for *A. baumanni* infections. The tested antibiotic discs including: Amikacin (30 µg), Colistin (25 µg), Cefoxitin (30 µg), Cefotaxime (30 µg), Meropenem (10 µg) and Trimethoprim-sulfamethoxazole (1.25/23.75 µg). These antibiotics were placed on the previously inoculated Müeller Hinton agar with *A. baumanni* and MRS agar with probiotics strains. The agar plates incubated aerobically for 24hrs at 37°C. The diameter of each zone of inhibition was measured in millimetres (mm). Bacterial resistance and/or sensitivity to the tested antibiotics was determined based on the standard chart approved by (CLSI, 2020). Please see table (2-8).

Table 3-8: The Diameters of inhibition zone; Interpretive Standards for *Acinetobacter* spp. according to (CLSI, 2020).

ID	Antibiotic discs	Code	Diameter of inhibition zone (mm)		
			Resistant	Intermediate	Susceptible
1	Amikacin	AK	14≤	15-16	≥17
2	Cefotaxime	CTX	14≤	15-22	≥23
3	Meropenem	MEM	14≤	15-17	≥18
4	Trimethoprim-Sulfamethoxazole	SXT	10≤	11-15	16≥
5	Colistin	COL	-	-	-
6	Cefoxitin	FOX	≤13	14-20	≥21

3.2.10 Biofilm Formation Assay Susceptible

According to Ghellai *et al*., (2014), the biofilm formation test was carried out as follows:

- A twenty µl of overnight bacterial culture growth was used (1.5×10^8 CFU/ml), to inoculate the flat-bottom of tissue culture 96 wells microplate containing 180 µl of BHI broth that supplemented by 1% glucose (BHIG).
- The negative control was 200 µl of BHIG broth only.
- All the plates were sealed with parafilm and incubated for 24hrs at 37°C under aerobic conditions.
- After incubation, all unattached bacterial cells were removed by pipetting and the wells were washed three times with PBS pH=7.1.
- The microplate was dried for 60 min at 60°C by the oven, then 100 µl of crystal violet solution (0.1%) was added into the treated wells and left for 20 min. After that, the residue of crystal violet was removed.
- Each well was washed by PBS three times to remove the unbounded crystal violet dye. The plate was left to dry at room temperature for 15 min.
- Then, 200 µl of ethanol 95% was added to each well and incubated for 30 min at 4°C.
- The absorbance was measured for each well, including the negative control well, at 630 nm using an ELISA reader.

The results were classified based on the absorbance, into 3 categories: low biofilm formers (LBF), Intermediate biofilm formers (IBF), and high biofilm formers (HBF), this classification is demonstrated according to the below table (Tang *et al*., 2011).

Table 3-9: Classification of *A. baumannii* as according to the strength of biofilm formation.

"OD" ≤ "ODc"	Non-biofilm
"ODc < OD ≤ 2 x ODc"	Moderately biofilm producer
"2 x ODc < OD"	Strong biofilm producer

* "OD" Mean optical density reader average of biofilm mass of bacterial isolates.

* "ODc" Mean optical density reader average of negative control.

3.2.11 Preparation of CFS of the tested Probiotics

The cell-free supernatant (CFS) of the studied probiotic strains were prepared as previously described by Sutyak *et al*., (2008). These selected strains include: *Bacillus subtilis* KATMIRA1933, *Bacillus amyloliquefaciens* B-1895, *Lactobacillus casei* DG CNCM I-1572 and the mix lactobacilli. These strains were inoculated into MRS broth and incubated aerobically for 24hrs at 37°C. The cells were removed by centrifugation (4480 *rpm* for 30 min at 4°C). The collected supernatants were filter-sterilized using a millipore 0.45 µm polytetrafluoroethylene (PTFE) syringe filter and kept in sterile tubes at 4°C until it was used. This is considered a stock solution (100 %) of CSF probiotic strains.

3.2.12 Antibiotics discs in combination with CFS of Probiotics

The antimicrobial combination in the disc was performed according to CLSI, (2016). Briefly, the Kirby-Bauer method, was modified to identify *A. baumanni* sensitivity to the antibiotics in combination with CFS of the tested probiotics in the table (2-3). The bacterial suspension of *A. baumanni* in BHI broth was diluted and adjusted to 1.5×10^8 CFU/ml using a spectrophotometer (Densichek TM), to an OD_{630} of 0.1. Then, a swab saturated with the bacterial suspension (1.5×10^8 CFU/ml) was streaked over the BHI agar plate at 3 directions. The entire surface of the dish was covered with bacterial cells. Each antibiotic disc was separately saturated with 20 µl of CFS of the tested probiotics. Sterile forceps were used to pick up the antibiotic discs. Three types of discs were prepared: An antibiotic disc only, an antibiotic disc saturated with probiotic's CFS, and a blank disc saturated with probiotic CFS only. A blank disc was used as a control. All discs were placed on the surface of BHI agar which was previously inoculated with the isolated pathogen. The antibiotic discs were placed 15 mm from the edge of the petri dish. The agar plates were left for 30 min, until the antibiotic was diffused from the discs into the surrounding agar

surface and then incubated aerobically for 24hrs at 37°C. After incubation, the bacterial growth prevention was evaluated by measurement of inhibition zones.

3.2.13 Coaggregation Test

The evaluation of the tested probiotics' auto-aggregation and coaggregation with *A. baumannii* was performed according to Cisar *et al*., (1979) with some modifications. Briefly, the bacterial cultures were harvested from the planktonically grown cells incubated at 37°C by centrifugation (4480 *rpm*, 15 min, 23 °C); the participated cells were washed with sterile PBS twice. After the second wash, the harvested cells were re-suspended in PBS and their optical density (OD_{630}) was adjusted to 0.25. In a 96-well microtiter plate, 100 µl of each washed probiotic cells were mixed with 100 µl of washed *A. baumanni*. As controls, 200 µl of each single species of bacterial cells (monoculture) was added in separate wells. The plate was incubated at 37°C and the measurements of OD_{630} were taken once at 0, 4 and 24hrs. The coaggregation percentages were calculated based on the below equation. Each experiment was performed in triplicates. Samples of 100 µl were taken at 0, 4 and 24 hrs and stained with Gram staining and observed microscopically for coaggregation.

- **Mathematical Analyses**

In each mixture (Probiotics and *A. baumannii*), the percent of bacterial coaggregation were evaluated as described by (Ledder *et al*., 2008) using the following equation:

$$coaggregation\,\% = (\frac{x - y}{x}) \times 100$$

where *x* is the before incubation value and *y* is the after incubation value per time point.

- **Microscopy**

The bacterial coaggregation was identified on the slides at the 0, 4 and 24 hrs. Bacterial interactions were stained with Gram stain and examined using the 100x/1.25 oil objective. Photographs were taken and processed using the Kopacam, NIS-Elements D3.0 software. The amount of coaggregation was visually analyzed and scored with a scoring system following Algburi *et al.*, (2020), with 0 being the absence of coaggregation.

3.2.14 Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) determination was performed according to Sutyak Noll *et al*., (2012), with minor modifications. Briefly, the 100 mg of Colistin was dissolved in 5 ml of sterile DW to obtain 20000 µg/ml as a stock solution, A 500 µl of stock solution was taken and transferred into 9.5 ml of BHI broth to obtain on 1000 µg/ml as primarily stock solution. A series of two-folds dilution of final stock solution (1000 µg/ml) of Colistin and the CFSs of the tested probiotic strains were performed with an appropriate volume of fresh BHI broth into the 96 well microplate with a final volume of 100 µl. The concentrations (25, 12.5, 6.25, 3.13, 1.6 and 0.8 µg/ml) to Colistin and (50, 25, 12.5 and 6.25 %) to the CFSs of the tested probiotic strains which were selected and applied in our experiments. The overnight culture of *A. baumanni* at $3\pm2x10^8$ CFU /ml was diluted in BHI broth to a final concentration of $1.5x10^8$ CFU /ml. From the diluted bacterial cells, 100 µl were transferred to each well containing 100 µl of pre-determined concentrations of both; Colisin and probiotic CFSs. The Plates were incubated under aerobic conditions for 24-36hrs at 37°C. The non-adherent cells (180 µl) were pipetted and transferred to a new 96 wells microplate and their turbidity was measured. The growth kinetics in the treated wells were evaluated using a microplate

spectrophotometer (ELISA reader) Diagnostic Automation at OD_{630}. The MIC was defined according to (CLSI, 2020).

3.2.15 Determination of Minimum Biofilm Inhibitory Concentration (MIC-B)

Minimum biofilm inhibitory concentration (MIC-B) was performed as was described in the study of Sutyak Noll *et al.,* (2012), with minor modifications. Briefly, the antibiotic Colistin 100 mg was dissolved in 5 ml DW to obtain 20000 µg/ml, as a stock solution. Then, a 500 µl of stock solution (20000 µg/ml) was taken and transfer to 9.5 ml of BHIG broth to obtain 1000 µg/ml as primarily stock. A series of two-folds dilution of Colistin was performed with an appropriate volume of fresh BHIG broth into the 96 well tissue culture microplate. The CFS of the selected probiotics (*B. subtilis* KATMIRA1933, *B. amyloliquefaciens* B-1895, *L. casie* DG CNCM I-1572 and mix lactobacilli) were also two-folds diluted with an appropriate volume of BHIG broth into a 96-well tissue culture plate. The concentrations (25, 12.5, 6.25, 3.13, 1.6 and 0.8 µg/ml) to Colistin and (50, 25, 12.5 and 6.25 %) to the CFSs of the tested probiotic strains which were selected and applied in our experiments. The final volume of antimicrobial agents diluted into the broth was 100 µl in each well. The overnight cell culture of *A. baumanni* at $3\pm2x10^8$ CFU/ml was diluted in BHIG broth to a final concentration of $1.5x10^8$ CFU/ml. From the final dilution of bacterial cells, 100 µl were separately transferred into the wells containing pre-determined concentrations of Colistin and probiotic's CFS. The Plates were incubated under aerobic conditions for 24-36hrs at 37°C. The non-adherent cells (180 µl) were removed. The wells were then gently washed three times with 200 µl of PBS. The biofilm was fixed by heating for 60 min at 60°C and stained with crystal violet (CV). Into each treated well, 100 µl of 0.1% CV was added over the biofilm and

left for 20 min at room temperature. To remove the residue (unbounded) of CV, each well was then rinsed 3-4 times with 200 µl of PBS and left at room temperature for 15 min to dry. To solubilize the CV-stained biofilm, 200 µl of 95% (v/v) ethanol/water was added into each well, and the microplate was incubated for 30 min at 4°C. After incubation, 100 µl were transferred from each well into a new sterile microtiter plate. The absorbance measurement was made using an automated absorbance (ELISA reader) at 630 nm to determine the MIC-B.

3.2.16 Checkerboard assay for antimicrobial combinations

To evaluate the antimicrobial potential of the selected probiotic CFSs in combination with Colistin against planktonic and biofilm cells of *A. baumannii*, a checkerboard assay was performed following Draper *et al*., (2013), with minor modifications. Briefly, the planktonic cells 24hrs growth of *A. baumannii*, was diluted to achieve $1.5x10^8$ CFU/ml. Each antimicrobial agent was diluted two-folds with BHI broth (to determine MIC) or BHIG broth (to determine MIC-B) into two separate 96-well microplates as following; From each dilution of Antimicrobial A (CFS of one probiotic strain), 50 µl was taken and added horizontally over 50 µl of antimicrobial B (Colistin). The concentrations (25, 12.5, 6.25, 3.13, 1.6 and 0.8 µg/ml) to Colistin and (50, 25, 12.5 and 6.25 %) to the CFSs of the tested probiotic strains which were selected and applied in our experiments. Then, 100 µl of *A. baumannii* suspension ($1.5x10^8$ CFU/ml) was separately added to the pre-determined concentration of antimicrobial combinations. The MIC and MIC-B of each antimicrobial combinations were determined after 24hrs of incubation. A 200 µl of the final bacterial suspension ($1.5x10^8$ CFU/ml) was added in duplicate, as a positive control. After incubation, the non-adherent cells (180 µl) were pipetted and transferred to a new 96 wells microplate and their turbidity was measured. The growth kinetics in the

treated wells were evaluated using a microplate spectrophotometer (ELISA reader) at OD_{630} to determine the MIC of antimicrobials combination. In order to determine MIC-B, the wells were gently washed three times with 200 µl of PBS. As previously explained, the biofilm was fixed, stained with crystal violet (CV) and the absorbance measured at 630 nm using (ELISA reader) to determine the MIC-B. Isobolograms were used to analyze the nature of antimicrobial combinations; synergistic, antagonistic or additive activity against the planktonic cells following the procedure of Hall *et al*., (1983). The total fractional inhibitory concentrations index (ΣFIC) were determined to evaluate the anti-biofilm potential of antimicrobial combinations against *A. baumannii*. using the following equations:

ΣFIC = FICA + FICB

FICA = (CA/MIC-B A) , FICB= (CB/MIC-B B),

where MIC-B A and MIC-B B are the MIC-B of drugs A and B alone, respectively, CA and CB are the concentrations of the drugs in combination, respectively. A FIC index of < 0.5 indicates synergism, > 0.5–1 indicates additive effects, > 1 to < 2 indifference, and ≥ 2 is considered to be antagonism (EUCAST, 2000).

3.2.17 Checkerboard assay, data analysis

Isobolograms were used to compare the MIC values of each antimicrobial agent alone with its MIC values in combinations with other antimicrobials agent. The point on the axis (X) refers to MIC values of the first antimicrobial agent with the coordinates (0, x) and the point on the axis (Y) represents the MIC values of the second antimicrobial agent with the coordinates (y, 0) when they are used alone. The two MIC values are connected by a dashed line (228). The MICs of each antimicrobial combination are plotted as dots on the graph. Results are expressed according to the locations of these dots from the line that connects MICs of the first and second antimicrobials. When the MIC values are located above

the line, the combination of the two antimicrobials are antagonized, but when these dots of interaction are under the line, the combination of the two antimicrobials synergized against the tested microorganism. An additive effect is observed when these dots are located on the line (Weeks *et al*., 2019).

3.2.18 Statistical analysis

All the data collected in this study were sorted according to graph pad prism V5 software. The laboratory request form was checked for the desired test. In this study, Two-way Anova test were done to establish the effect of probiotic bacteria and colistin on bacterial growth between variables and test the inhibition. P values of < 0.05 were considered statistically significant. Whereas, Sigma plot V11 software was used to draw the graphs of isobolograms of the antimicrobials combinations against the planktonic cells of the isolated *A. baumannii*.

4. Results and Discussion

4.1 Isolation of *Acinetobacter baumannii*

the current study, 20 (8.69%) isolates of *A. baumannii* were identified from 230 samples of different clinical sources; burns, wounds and blood. Swabs and samples were taken from both out-patients and hospitalized patients of Baqubah Teaching Hospital/ Diyala. The research was carried out from September till the end of December, 2020. The sample collection included both genders of different ages, appendix (3).

The positive growth samples were 205 from a total of 230 samples from different sources, only 25 samples showed no growth as appeared in figure (4-1).

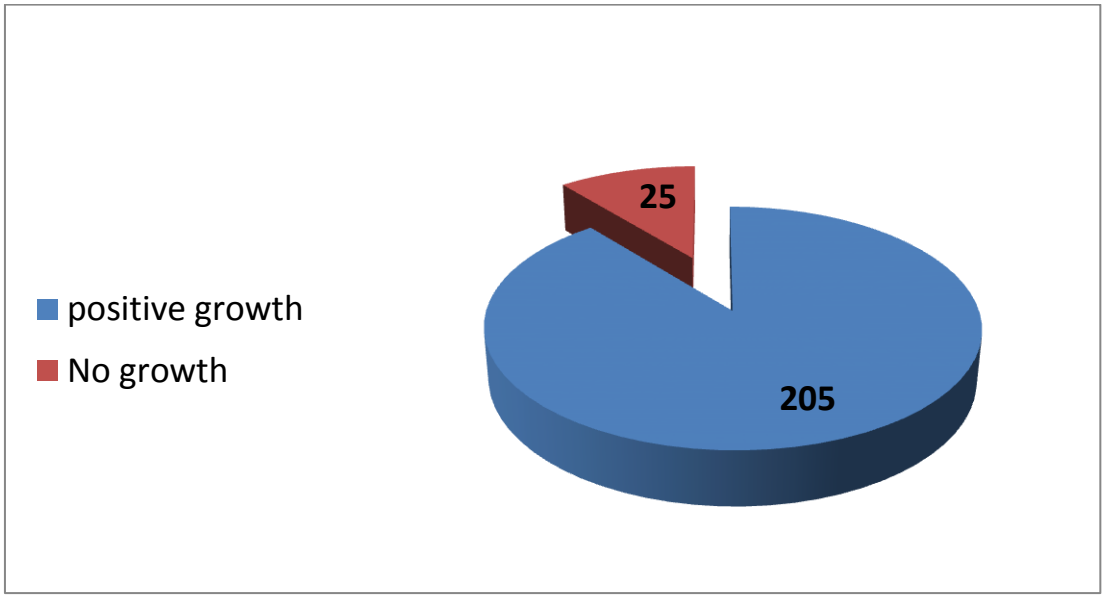


Figure 4-1: The number of clinical samples positive and no growth.

4.2 Identification of *Acinetobacter baumannii*

4.2.1 Microscopic Examination

Microscopic morphology showed that 20 isolates of *A. baumannii* appeared gram-negative as shown in figure (4-2). In general, their shape

varied from bacilli to cocco-bacilli. According to their growth phase, diplococcus and some had short chains (Asif *et al*., 2018).

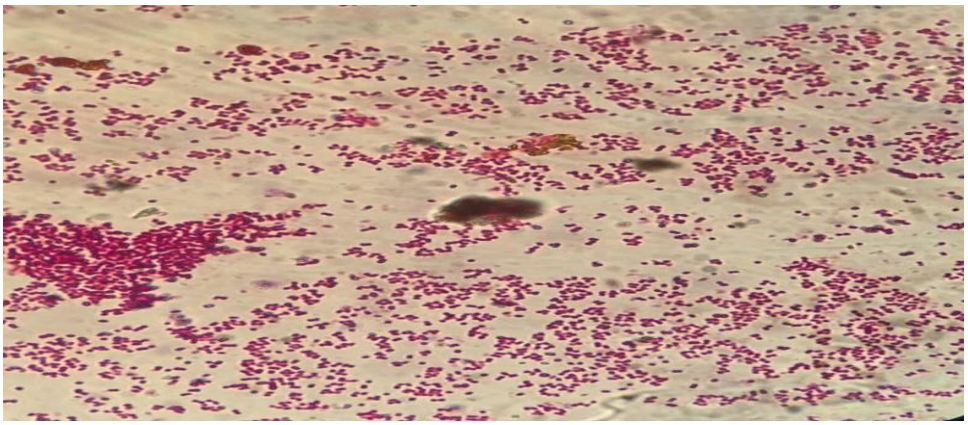


Figure 4-2: Gram stain of *A. baumannii* cells.

4.2.2 Morphological Characterization

The collected samples were preliminarily cultured on blood agar and MacConkey agar plates. The clinical isolates that grown on blood agar were small colonies, smooth, opaque, raised, creamy and non-hemolytic colonies. On MacConkey, the bacterial colonies were pale pinkish colonies with small size and regular edges (AL-Dahlaki, 2020). All isolates were grown at temperatures between 37°C and 44°C. The ability to grow at 44°C is an important characteristic that distinguishes *A. baumannii* from the rest of its species (Asif *et al*., 2018).

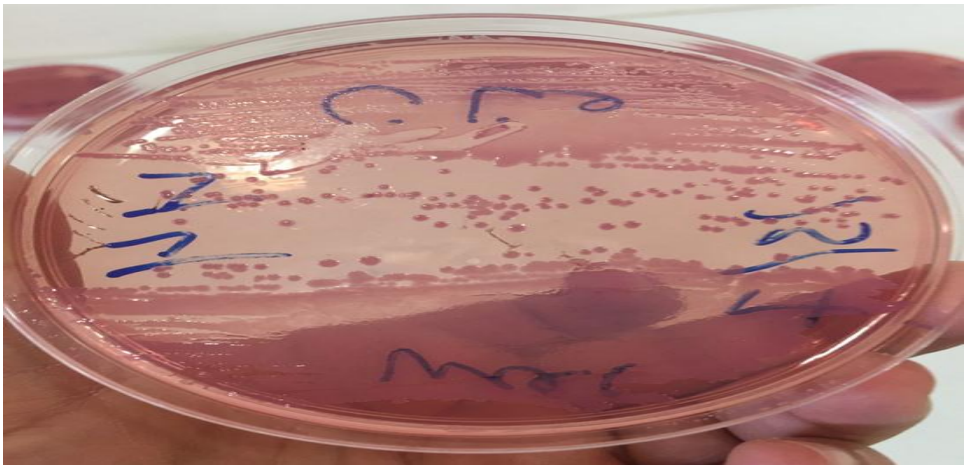


Figure 4-3: *Acinetobacter baumannii* on MacConky Agar.

4.2.3 Biochemical Identification

The results showed that all tested isolates were positive to catalase production and simmons citrate test and variable in regards to urease production, table (4-1). While, they were negative oxidase test, voges Proskauer, methyl red and indole tests. Due to its inability to consume sugars, their growth in Triple Sugar–Iron (TSI) was alkaline with no gases production. The isolates showed no lactose fermentation on MacConkey.

Table 4-1: Initial identification of *A. baumannii* using some manual biochemical tests.

NO	Biochemical tests	Results
1	Oxidase test	-
2	Catalase production test	+
3	Methyl red	-
4	Simmons citrate	+
5	Voges- Proskauer	-
6	Indole production	-
7	Urease production	Variable
8	Triple Sugar–Iron	Alkaline slant /No change bottom, No gas, No H_2S
9	Lactose fermentation	Non-lactose fermentation

4.2.4 Confirmation of bacterial identification using the VITEK 2 system

The final diagnosis of the clinical isolates was achieved by the VITEK 2 system using Gram-negative Identification (GN ID) card. In general, the sensitivity of VITEK 2 system outcomes is up to 99%, in which 47 biochemical tests were applied belonging to Gram-negative bacteria within 5-8 hrs. All the results obtained were *A. baumannii*, compatible with the

phenotypic and initial biochemical characteristics described earlier, appendix (5).

4.3. Distribution of the specimens

4.3.1 Distribution of the specimens according to hospitalized and out-patients and genders

The results as in table (4-2) displayed the number of samples collected from hospitalized patients, which were higher than those collected from out-patients. Our results were in agreement with a study in Iraq by Kareem, (2019), that showed 72.6% were hospitalized patients and only 27.4% were out-patients; male and female. The study of Raut *et al*., (2020), presented that female gave nearly half the number of samples (51.4%) versus (48.6%) of the samples that obtained from the male.

Table 4-2: The percentages of clinical samples taken from out-patients and hospitalized patients and from both genders.

The Patients	No. and (%) of samples	No. and (%) of *A. baumannii* isolates	Male	Female
Out Patients	63 (27.4%)	4 (1.73%)	27 (11.74%)	36 (15.65%)
Hospitalized Patients	167 (72.6%)	16 (6.96%)	96 (41.74%)	71 (30.87%)
Total	230 (100%)	20 (8.69%)	123 (53.48%)	107 (46.52%)

4.3.2 Distribution of the specimens according to sources

The results of the table (4-3), presented that the clinical isolates of *A. baumannii* obtained from burns, blood and wounds were 9 (45%), 6 (30%) and 5 (25%), respectively. This results were closed to Ghaima *et al*., (2016), findings who showed that 96 isolates of *A. baumannii* were obtained; 65 were isolated from burns and 31 clinical isolates were from wounds. AL-Dahlaki, (2020), found that *A. baumannii* was isolated in a

high percentage from burn 8 (40%), blood 7 (35%) and wound 4 (20%) .Recently, it has been noticed an increase in the number of multidrug-resistant clinical isolates of *A. baumannii* was (90%) that obtained from burns and wounds (Wang and Wang, 2016). Several factors associate with the high prevalence of *A. baumannii* isolation, such as the acquisition of the nosocomial pathogens by patients, long term hospitalization with the complicating infections, delay administration of antimicrobial agents and/or patients have immunosuppressive factors (Hadid, 2015).

Table 4-3: Distribution of the samples according to their sources.

Sample Sources	Total No. of Samples	No. and % of the positive *A. baumannii* Isolates
Burns	53 (23%)	9 (45%)
Blood	67 (29%)	6 (30%)
Wound	110 (48%)	5 (25%)
Total	230 (100%)	20 (100%)

4.4 Antibiotics Susceptibility Test

4.4.1 Antibiotics Susceptibility test of *A. baumannii*

The antibiotics susceptibility of *A. baumannii* isolates was performed using an antibiotics susceptibility Gram-negative (AST-GN) card which was inserted into the VITEC 2 system. This technique is recommended by the clinical and laboratory standards institute guidelines (CLSI, 2020).

In this test, 18 antimicrobial agents were evaluated against the 20 clinical isolates of *A. baumannii*. The obtained data showed that all clinical isolates were highly resistant to the most tested antibiotics, table (4-4). The result found that all isolated *A. baumannii* (100%) were completely resistant to 10 tested antibiotics, table (4-4). Whereas, 95% of isolates were resistant to Imipenem and Amikacin, 90% of them were resistant to

Gentamicin and Tobramycin and 75% were resistant to Trimethoprim-sulfamethoxazole. In regards to sensitivity, all isolates were sensitive to Colistin and 85% were sensitive to Tigecycline and Minocycline, appendix (7).

Table 4-4: The numbers and percentage of antibiotics resistant and sensitive isolatesto of *A. baumannii*.

Antibiotics Family	Antibiotics Type	Sensitive Isolates	Resistant Isolates	Intermediate Isolates
		No. and %	No. and %	No. and %
Penicillins	Ampicillin	0 (0%)	20 (100%)	0 (0%)
B-lactam inhibitors	Piperacillin /Tazobactam	0 (0%)	20 (100%)	0 (0%)
Cephalosporins	Cefazolin	0 (0%)	20 (100%)	0 (0%)
	Cefoxitin	0 (0%)	20 (100%)	0 (0%)
	Ceftazidime	0 (0%)	20 (100%)	0 (0%)
	Ceftriaxone	0 (0%)	20 (100%)	0 (0%)
	Cefepime	0 (0%)	20 (100%)	0 (0%)
Carbapenems	Imipenem	1 (5%)	19 (95%)	0 (0%)
	Meropenem	0 (0%)	20 (100%)	0 (0%)
Aminoglycosides	Amikacin	0 (0%)	19 (95%)	1 (5%)
	Gentamicin	2 (10%)	18 (90%)	0 (0%)
	Tobramycin	2 (10%)	18 (90%)	0 (0%)
Tetracyclines	Tigecycline	17 (85%)	1 (5%)	2 (10%)
	Minocycline	17 (85%)	1 (5%)	2 (10%)
Fluoroquinolones	Ciprofloxacin	0 (0.0)	20 (100%)	0 (0%)
	Levofloxacin	0 (0.0)	20 (100%)	0 (0%)
Folate pathway antagonists	Trimethoprim /sulfamethoxazole	5 (25%)	15 (75%)	0 (0%)
Lipopeptides	Colistin	20 (100%)	0 (0%)	0 (0%)

The results of this study demonstrated that most of the selected isolates were resistant to β-lactam antibiotic classes, the clinical isolates were resistant to Imipenem and Meropenem by 95% and 100%, respectively, table (4-4). In a similar study, AL-Dahlaki, (2020), found that *A. baumannii* were resistance to Carbapenems, Imipenem and Meropenem (95%).

The clinical isolates under a current study showed high resistance to Ampicillin and Piperacillin/Tazobactam (100%), these results were in agreement with a local study of that found that *A. baumannii* resistance to (Piperacillin/Tazobactam) was 100% (AL-Dahlaki, 2020). Furthermore, (Pal *at el*., 2017) found that (100%) of *A. baumannii* were resistant to Ampicillin while 82.4% were resistant to (Piperacillin/Tazobactam). Raut *et al*., (2020), found that 93.8% of *A. baumannii* were surviving in the presence of Ampicillin.

All clinical isolates (100%) could grow with the presence of Cephalosporins. These data were in agreement with Kareem, (2019), in his local study, and Pal *at el*., (2017), who both found that all bacterial isolates (100%) were resistance to Cephalosporins.

Resistance to β-lactam antibiotics is often related to β-lactamases production, which catalyzes the hydrolysis of the amide bond in the β-lactam ring and modifying the antibiotic to an inactive form. Moreover, bacterial resistance to β-lactams could possibly refer to the alteration in penicillin-binding proteins, changes in outer membrane porins (decreased permeability), and expulsion of antibiotics out of the cell through efflux pump (Asif *at el*., 2018).

All isolates were susceptible to Colistin (100%); whereas only 85% were susceptible to Tigecycline and Minocycline. These findings were closed to Rahimi *et al*., (2018), who reported that all the isolates of *A. baumannii* were sensitive to Colistin (100%), followed by Tigecycline

(94%) and Minocycline (73%). Another study found that *A. baumanii* isolates were completely susceptible to Colistin and Tigecycline (100%) (Raut *et al*., 2020).

The antibiotic Colistin was widely used to control the MDR isolates (Raut *et al*., 2020). Polymyxin E is a bactericidal substance that disrupts bacterial cell membrane. It is a positively charged and cationic region binds to the negatively charged hydrophilic portion of bacterial LPS and leading to loss of cellular membrane integrity and kill the pathogenic bacteria (Nhu *et al*., 2016). Tetracyclines and Glycylcyclines inhibit protein synthesis by preventing the attachment of aminoacyl tRNA to the target ribosome (Maleki *et al*., 2014). The presence of both genes *TetA* and *TetB* in *A. baumannii* sp. *TetB* is responsible to control the efflux of both antibiotics inside the bacterial cell (Almasaudi, 2016).

In regards to Trimethoprim /Sulfamethoxazole, we identified that these antibiotics inhibited the growth of 25% of the total tested isolates, table (4-4). These results were closed to a local study of (Al-shammary *et al*., 2017) who found that the resistance percentage of bacterial isolates to Trimethoprim/Sulfamethoxazole were around 73.33%. Due to the presence of dihydrofolate reductases (DHFR and FolA), MDR *A. baumannii* isolates were resistant to trimethoprim (Taitt *et al*., 2014).

It is obvious from the table (4-4), that all *A. baumannii* isolates (100%) were highly resistant to Fluoroquinolones. These results were in agreement with the study of (AL-Kadmy *et al.*, 2018) who found that *A. baumannii* resistance % to Fluoroquinolones were (100%). Fluoroquinolones are broad-spectrum antibiotics that use to prevent wide varieties of bacterial infections. In addition, they are significantly contributing to the rapid increase of bacterial resistance over the past years (Raut *et al*., 2020). A major mechanism for resistance to quinolones was found in both genes (*gyrA* and *parC*) mutation, which cause the phenotypic changes in DNA

gyrase and topoisomerase IV and lead to reduce the antibiotic affinity (Ugolotti *et al*., 2016). Chromosomal DNA encodes drug influx and efflux systems, it mediates the expression reduction of OMPs and increasing the bacterial resistance to quinolones (Charrier *et al*., 2016). Plasmid-encoded quinolone resistance such as *qnrA, qnrB* and *qnrS* have also been identified in *A. baumannii* which protect its DNA by inhibiting the binding of quinolones to DNA gyrase and topoisomerase (Ling *et al*., 2016).

In these study, the bacterial isolates showed a high resistance (95%, 90% and 90%) to some Aminoglycosides; Amikacin, Gentamicin and Tobramycin, respectively, table (4-4). These findings were closely related to AL-Dahlaki, (2020), who found that all *A. baumannii* isolates were resistant to Gentamicin and Amikacin. Additionally, AL-Kadmy *et al.*, (2018) found that bacterial resistance to Tobramycin with more than 90%.

Aminoglycoside resistance by *A. baumannii* species is related to the production of aminoglycoside-modifying enzymes (AMEs), including acetyltransferases, adenyltransferases and phosphotransferases. AMEs are altering the corresponding functional groups of aminoglycosides and disrupting the binding capacity of these antibiotics at their ribosomal target sites (Moubareck and Halat, 2020). Antibiotic resistance to aminoglycosides is also related to 16S rRNA methylase genes such as *armA, rmtA, rmtB, rmtC* and *rmtD,* which altering the target-binding site for aminoglycosides within the 30S ribosomal subunit. Unlike AMEs, methylases induce high-level resistance across all clinically useful Aminoglycosides, including Gentamicin, Tobramycin and Amikacin (Moubareck and Halat, 2020).

4.4.2 Antibiotics Susceptibility of Probiotic Strains

The antibiotic susceptibility of the tested probiotic strains was performed using the disk diffusion method (Kirby-Bauer). Six antibiotics were used (materials and method chapter, table (3-6)).

Data in the table (4-5), showed a high susceptibility level by the tested probiotic strains to the majority of the selected antibiotics, appendix (6). Medically, Amikacin, Cefoxitin, Cefotaxime, Meropenem and Trimethoprim-sulfamethoxazole are broad-spectrum antibiotics (Taher *et al*., 2019). The probiotic isolates showed high resistance to Colistin. Polymyxin E is non-ribosomal peptides produced by *Bacillus polymyxa*, a soil bacterium, as a secondary metabolite. It is a highly bactericidal molecule for Gram-negative bacteria (Pacheco *et al*., 2019). Based on the above-mentioned data, Colistin was chosen, in this study, and combined with the probiotics CFS to identify the nature of antimicrobial interactions against *A. baumannii*.

Table 4-5: Diameter of inhibition Zone of probiotic strains by antibiotics.

Probiotic	**Diameter of inhibition Zone around the antibiotic disks**					
	AK	**COL**	**FOX**	**CTX**	**MEM**	**TS**
Bacillus subtilis KATMIR1933(BSK)	22 mm	zero	35 mm	40 mm	37 mm	37mm
Bacillus amyloliquefaciense B-1895 (BAB)	14 mm	zero	28 mm	28 mm	35 mm	30mm
Lactobacillus caseia CNCMi 1572 (L.C)	20 mm	zero	15 mm	30 mm	35 mm	33mm
Mix lactobacilli (M.L)	18 mm	zero	13 mm	40 mm	35 mm	20mm

(AK) Amikacin 30 mg, (COL) Colistin 25mg, (FOX) Cefoxitin 30mg, (CTX) Cefotaxime 30 mg, (MEM) Meropenem 10 mg, (TS) Trimethoprim-sulfamethoxazole 1.25/23.75 mg.

4.5 Multidrug Resistance (MDR) *A. baumannii* Isolates

According to CLSI guidelines, *A. baumannii* isolates were classified into three major categories: MDR, XDR and PDR. This classification was applied in eighteenth different antibiotype patterns designated arbitrarily from (Ab1 to Ab20) anti-biotypes (CLSI, 2020). A complete picture of antibiotic-resistant level for each tested isolates was done according to the criteria revealed by (Magiorakos *et al*., 2011).

Table (4-6), showed that only seven isolates (35%) of *A. baumannii* were classified as MDR since they were resistant to ≥3 of all antimicrobial categories. Thirteen isolates (65%) of *A. baumannii* were classified as XDR since they were resistant to all antimicrobial categories except two or fewer antibiotics belong to the same category. These results agree with the study of Rahimi *et al*., (2018), who found that 76% of the *A. baumannii* isolates exhibited the XDR phenotype.

The high emergence rates of MDR and XDR strains of *A. baumannii* make it one of the top seven pathogens which are threatening to the medical and health systems (Pfalzgraff, 2018). Previous studies have demonstrated an increasing trend in the emergence of XDR strains over the last decade in Iran (Bahador *et al*., 2015).

One of the important mechanisms in the development of the MDR strains of *A. baumannii* is biofilms formation, which could explain their outstanding resistance to antibiotics, survival properties, owing to protection from disinfectants and/or desiccation on the abiotic surfaces (Ivanković *et al*., 2017; Ryu *et al*., 2017).

Table 4-6: Distribution of *A. baumannii* isolates according to multidrug-resistant categories.

Categories	NO. of isolates	No.and %
MDR	Ab3, Ab4, Ab5, Ab6, Ab9, Ab11, Ab16	N=7 (35%)
XDR	Ab1, Ab2, Ab7, Ab8, Ab10, Ab12, Ab13, Ab14, Ab15, Ab17, Ab18, Ab19, Ab20	N=13 (65%)
PDR	**********	N=0 (0%)

4.6 Phenotypic Detection of Biofilm Formation

The Micro Titer Plate method (MTP) was used to detect biofilm production by *A. baumannii*, figure (4-4) and table (4-7).

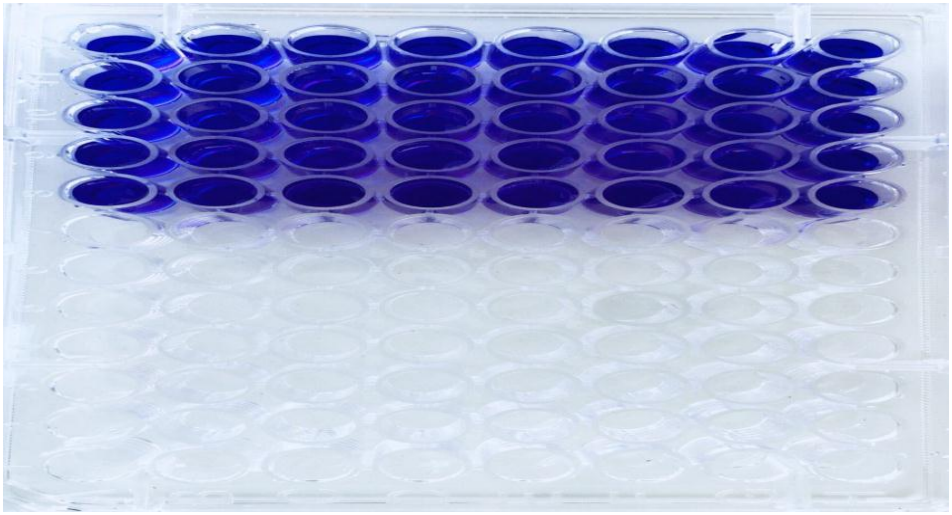


Figure 4-4: Micro-titer plate for quantification of biofilm mass.

In the current study, all the isolated *A. baumannii* isolates were capable of forming biofilms. These were in agreement with the study of Ahmad and Mohammad, (2019), who found that all (100%) of *A. baumannii* isolates had an ability to biofilm formation.

Table 4-7: The strength of biofilm formed by the isolated *A. baumannii*.

Sample No.	Absorbency at 630 nm	Biofilm formation compared to (ODc=0.058)(2*ODc=0.116)	Sample No.	Absorbency at 630 nm	Biofilm formation compared to (ODc=0.058)(2*ODc=0.116)
Ab1	0.128	Strong	Ab11	0.123	Strong
Ab2	0.152	Strong	Ab12	0.158	Strong
Ab3	0.132	Strong	Ab13	0.149	Strong
Ab4	0.143	Strong	Ab14	0.136	Strong
Ab5	0.131	Strong	Ab15	0.133	Strong
Ab6	0.129	Strong	Ab16	0.154	Strong
Ab7	0.155	Strong	Ab17	0.129	Strong
Ab8	0.145	Strong	Ab18	0.127	Strong
Ab9	0.138	Strong	Ab19	0.137	Strong
Ab10	0.144	Strong	Ab20	0.141	Strong

AC=0.035, Ab: *A. baumannnii*

The current study showed that all *A. baumannii* isolates formed a strong biofilm, table (4-7), as the biofilm stained-crystal violet absorbance values ranged from 0.123-0.158 nm. A similar study that found that all of the clinical isolates of bacteria formed strong biofilms on abiotic surfaces (AL-Kadmy *et al*., 2018).

Many bacterial pathogens, including *A. baumannii*, can form matrix-enclosed communities, referring to biofilm formation. The potential for biofilm formation by *A. baumannii* isolates is possibly played an important role in the survival and persistence of bacterial infection in the presence of the environmental stress factors (Runci *et al*., 2017).

The virulence factors of *A. baumannii*, including: biofilm-associated protein (Bap), efflux system (AdeABC, AdeFGH and AdeIJK), quorum

sensing system and pili are involved in biofilm formation and associated with the pathogenicity of *A. baumannii* (Eze *et al*., 2018). Extracellular polymeric substances (EPS) in biofilms are responsible for cells-cells and cells-surface binding and participate in the development of biofilm structure and maturation (Mohamed *et al.*, 2018).

4.7 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentrations of Colistin and probiotic CSF were determined against three isolates of *A. baumannii* coded as; Ab7, Ab8 and Ab9 which were MDR and strong biofilm former isolates of wounds, burns and blood infections, respectively. The MIC was determined via using the broth micro-dilution method using BHIB as a culture medium. After the incubation period (24 hrs), the absorbency was recorded by using an ELISA reader (630 nm),

It is clear from figure (4-5) that MIC value was determined based on the last dilution in the well of MTP gave no growth using naked eyes.

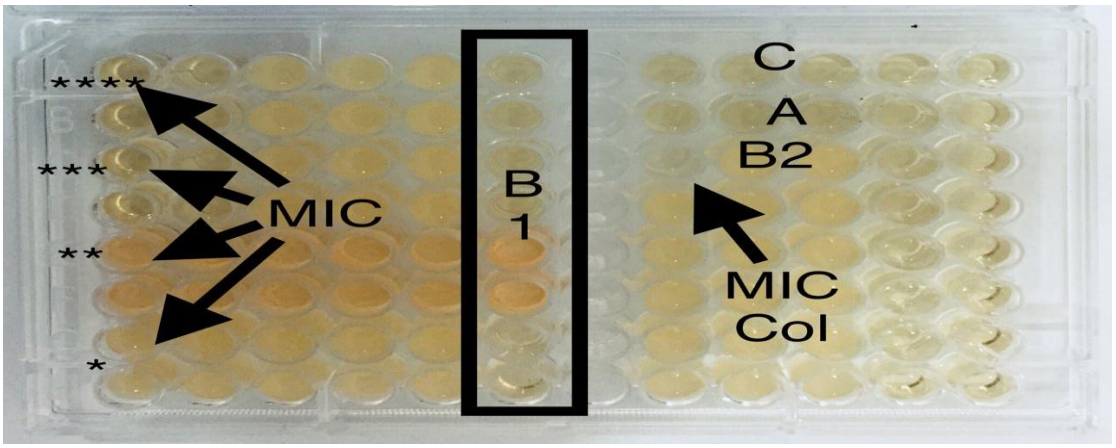


Figure 4-5: Determination of MIC using microtiter plate, MIC probiotic L.M (****), L.C (***), BAB (**) and BSK (*), MIC Col (Colistin), A: control-ve (broth +Antibiotic), B1=control +ve (probiotics growth), B2=control +ve (*A. baumannii* growth) and C=control –ve (only broth).

In general, all bacterial isolates showed a significant inhibition when 3.13 µg/ml of Colistin was used, figure (4-6). Regarding the MIC of Colistin, it was 3.13 µg/ml for Ab9. Whereas, 6.25 µg/ml for both Ab7 and Ab8 isolates. These concentrations inhibited the bacterial growth

significantly ($p \leq 0.001$). These results were very closed with a study had been done by Sato *et al*., (2018), who reported that MIC values of Colistin against *A. baumannii* were 4 µg/ml. However, another study found that MIC values of Colistin against *A. baumannii* ATCC 17978 were 1 µg/ml (Lin *et al*., 2020). The variation of MIC values reported in the different studies might be related to the source and manufacture of Colistin; in this study, we used Colistin methanesulfonate while the above-mentioned studies used pure Colistin. In addition to the difference in the source of the isolates, where (Ab 7, Ab 8) are XDR and (Ab 9) is MDR.

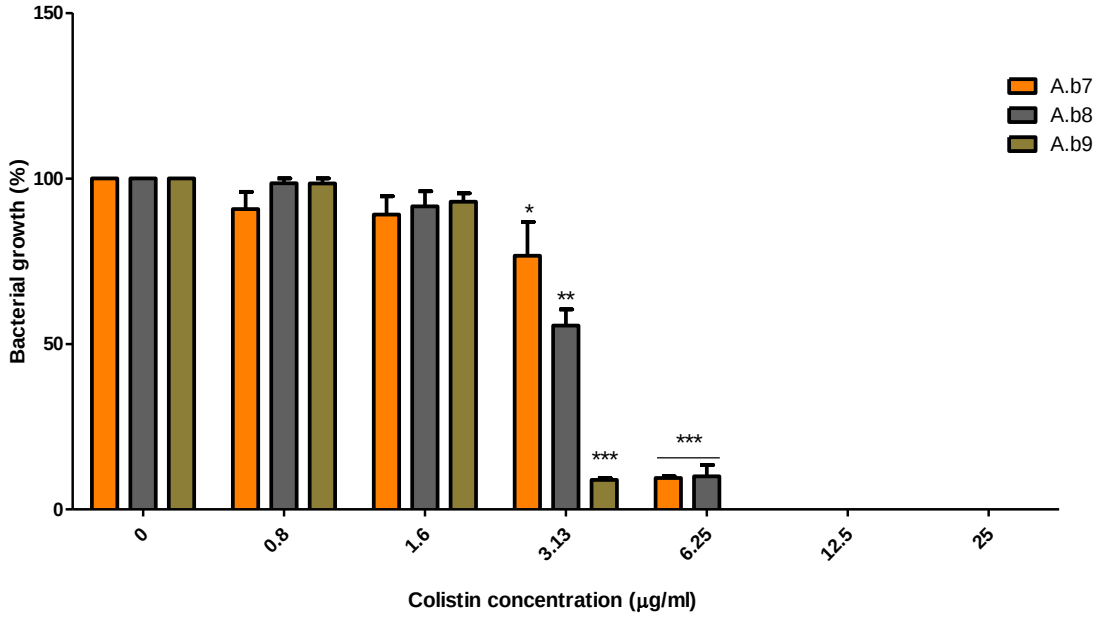


Figure 4-6: Antibacterial activity of Colistin against *A. baumannii* isolates (Ab7, Ab8 and Ab9). Data represented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

The MIC values could not be determined for the CFS of *Bacillus subtilis* KATMIR 1933 against three clinical isolates, even when the highest concentration (50%) was used. Compared to the control (bacterial growth without treatment), only the isolate Ab8 showed a significant ($P \leq 0.05$) growth inhibition at the concentration of 25 µg/ml. Whereas, the

isolates Ab8 and Ab9 were inhibited significantly ($p \leq 0.01$) at the concentration of 50% both. While isolate Ab7 did not affect any concentration of this experiment. Figure (4-7).

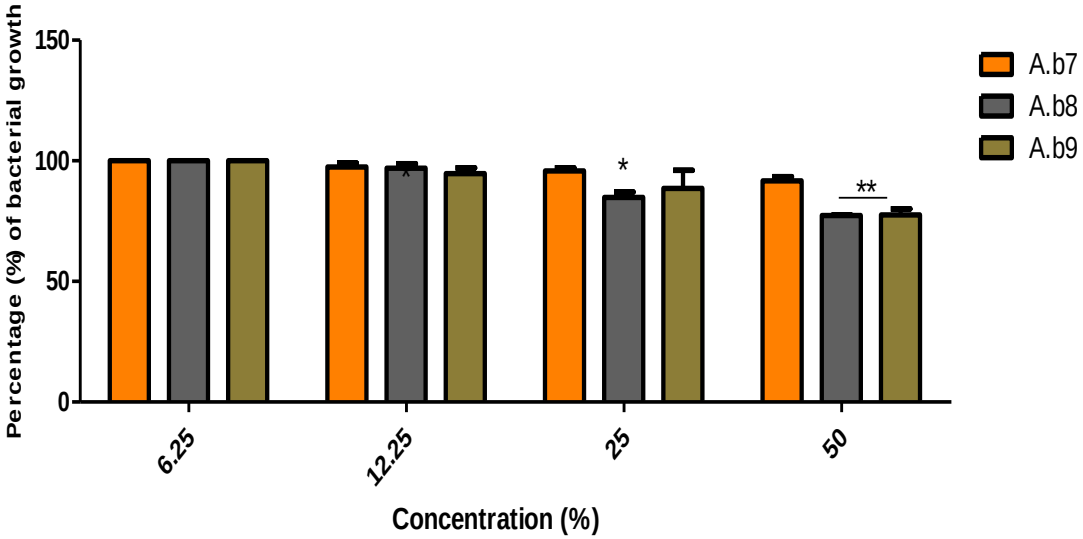


Figure 4-7: Antibacterial activity of *Bacillus subtilis* KATMIR 1933 CFS against *A. baumannii* isolates growth (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

The MIC value of the CFS *Bacillus amyloliquefaciense* B-1895 was not identified against the three isolates. The growth inhibition of the selected isolates displayed a significant decrease (with exception of isolate Ab7) at the concentration of 12.5%. Whereas, all bacterial isolates were inhibited dramatically ($p \leq 0.001$) with a higher concentration of *B. amyloliquefaciense* B-1895 CFS. Figure (4-8).

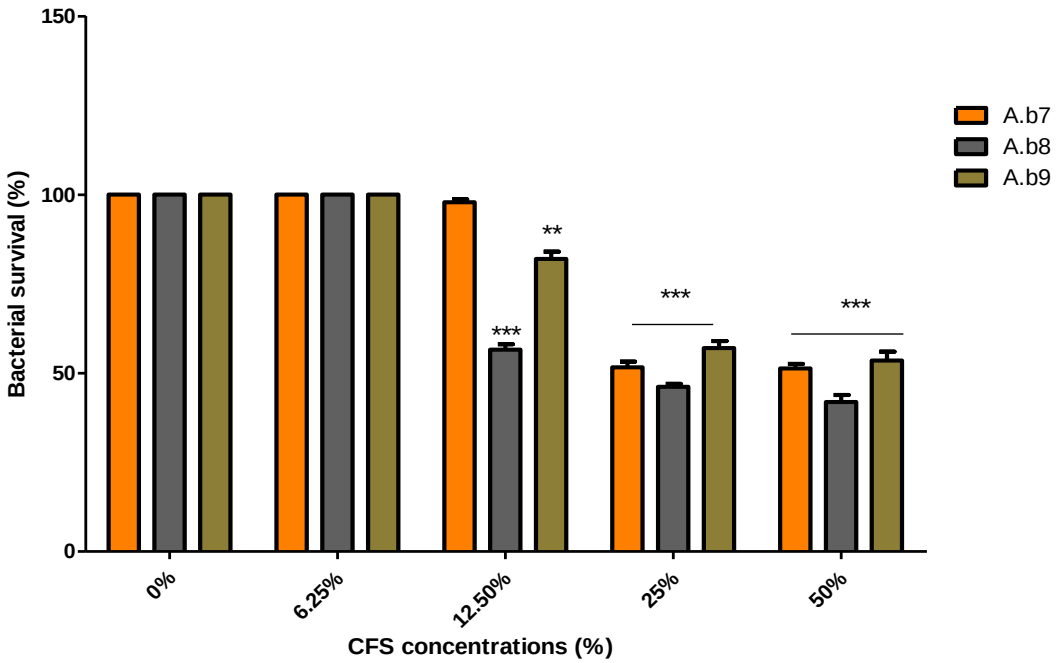


Figure 4-8: Antibacterial activity of *Bacillus amyloliquefaciens* B-1895 CFS against *A. baumannii* isolates growth (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

Figure (4-9), illustrated that all the isolates showed a growth inhibition when 12.5% and 25% *Lactobacillus casei* DG CNCM I-1572 CFS was used. However, only isolate Ab8 showed significant inhibition at both concentrations 12.5% and 25% (p≤0.01) as a comparison with control, while at 50% (which was determined as MIC) all the isolates growth was completely inhibited.

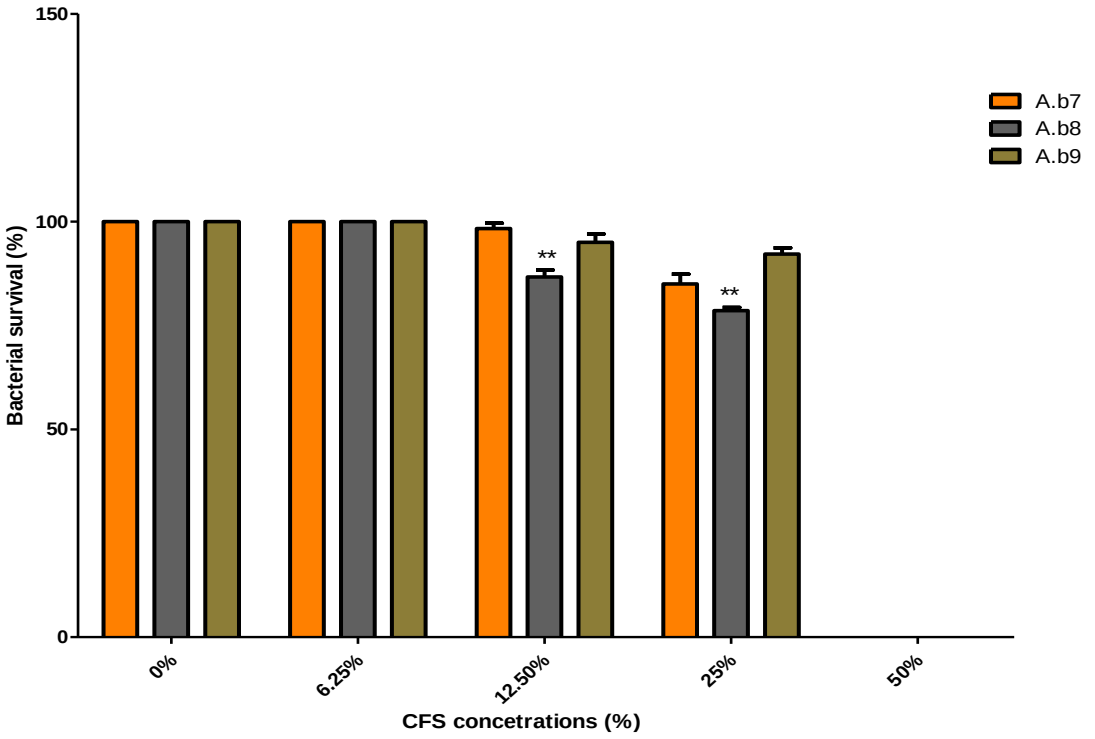


Figure 4-9: Antibacterial activity of *L. casie* CFS against *A. baumannii* isolates growth (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

A mix of lactobacilli CFS that used in the current study showed a significant decrease in a bacterial growth at the concentrations of 12.5 %, however, the isolate Ab8 was not influenced by the probiotic CFS at 12.5%. Whereas, 25% of probiotic CFS could significantly inhibit the growth. At 50%, as MIC value, of the mix of lactobacilli CFS, the bacterial growth was completely inhibited, figure (4-10).

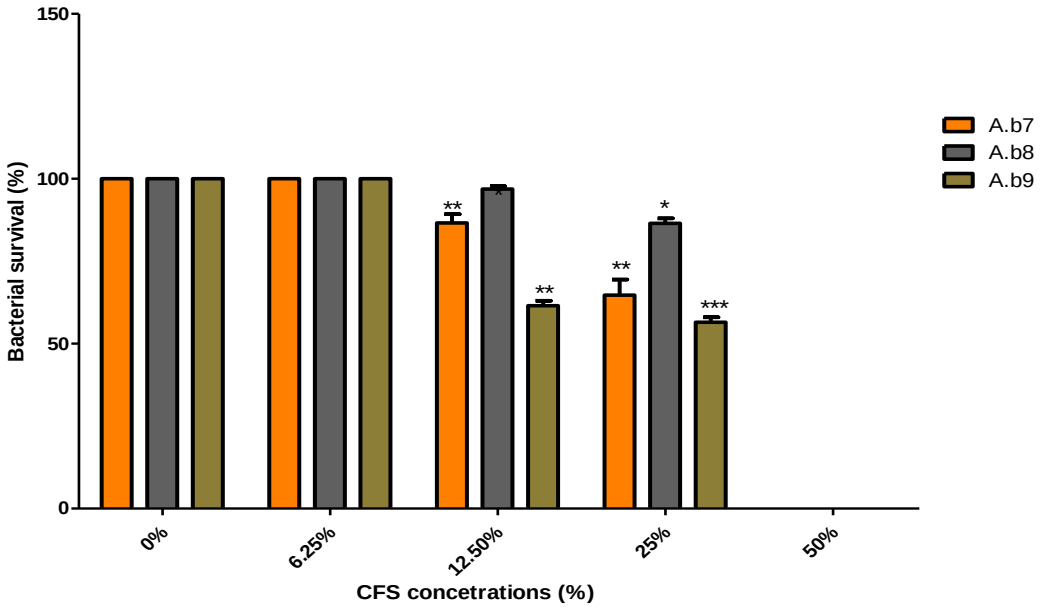


Figure 4-10: Antibacterial activity of mixed lactobacilli CFS against *A. baumannii* isolates growth (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

The antimicrobial activity of CSF of some probiotics against *A. baumannii* was reported in several publications Shin and Eom, (2020), identified an antimicrobial activity of *C. butyricum* CFS against *A. baumannii* strains. They found that 50% of *C. butyricum* CFS was drastically inhibited 98.51% of the planktonic cell growth of *A. baumannii* ATCC 19606.

Another study showed that lactobacilli had an inhibition growth effect on both *A. baumannii* and *P. aeruginosa* (Soltan *et al*., 2017). *Bacillus subtilis* 534 is capable to produce active substances against clinical isolates. These substances have different molecular targets cell of pathogenic microorganisms (Efremenkova *et al*., 2019). During their stationary phase of growth, lactobacilli and bacilli strain simultaneously secrete organic acids such as bacteriocins and biosurfactants. Bacteriocins are small antimicrobial peptides produced by numerous lactic acid bacteria (Lashin *et al*., 2017). These peptides create holes (pores) in the phospholipid bilayer

of bacteria and disrupt the cytoplasmic membrane generating the proton motive force. Also, they prevent the probable growth and metastasis of bacteria through immunologic and non-immunologic mechanisms (Fozouni *et al*., 2019). Acids generated by Probiotic strains penetrate the cytoplasm of bacteria' pathogen , lowering intracellular pH and interfering with metabolic processes (Hughes and Webber, 2017).

4.8 Minimal Biofilm Inhibitory Concentration (MIC-B)

The minimal biofilm inhibitory concentration (MIC-B) is defined as the concentration of an antimicrobial that inhibits either 50% (MIC-B50) or 90% (MIC-B90) of biofilm growth compared to untreated control (Chapot-Chartier and Kulakauskas, 2014). MIC-B concentrations were determined as described by (Algburi *et al*., 2017), using the broth microtitre dilution standard method and the biofilm mass was stained with crystal violet solution (0.1%), figure (4-11).

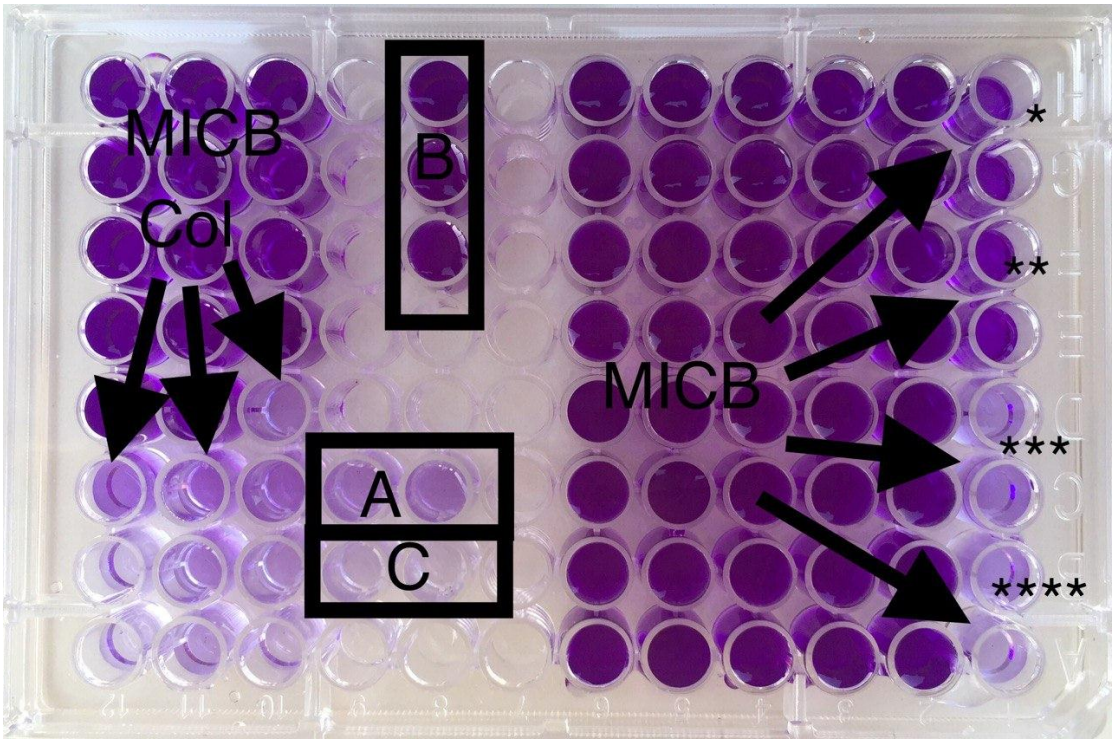


Figure 4-11: Determination of MIC-B using microtiter plate, MIC-B probiotic L.M (****), L.C (***), BAB (**) and BSK (*), MIC Col (Colistin), A: control-ve (broth +Antibiotic), B=control +ve (*A. baumannii* growth) and C=control –ve (only broth).

As appeared in figure (4-12), all isolates displayed a biofilm formation inhibition at 1.65 and 3.13 µg/ml which were significant compared to the positive control. The MIC-B of Colistin was 3.13 µg/ml, against Ab9 isolate that removed (100%) of biofilm formation but 6.25 µg/ml against both Ab7 and Ab8 with a significant reduction ($p \leq 0.001$) in biofilm formation by 85% and 83.4% respectively, figure (4-12).

The results of the current study agree with the study of (Lin *et al*. 2020) who found that MIC-B of Colistin against *A. baumannii* was 8.192 µg/ml. In the study of Eze *et al*., (2018), huge differences were noticed in the MBIC of Colistin against *A. baumannii* isolates that displayed increased their biofilm formation.

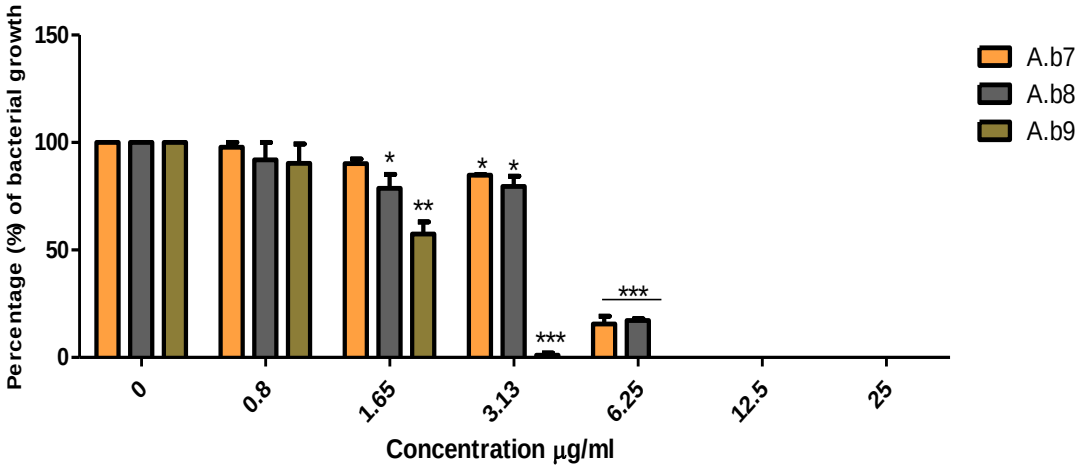


Figure 4-12: Anti-biofilm activity of Colistin against *A. baumannii* isolates (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

The MIC-B of CFS of *Bacillus subtilis* KATMIR 1933 was not determined against the selected isolates, even when the highest concentration 50% was used. From figure (4-13), 50% CFS of *Bacillus subtilis* KATMIR 1933 prevented 7.3%, 23.3% and 22.5% of the biofilm formation by Ab7, Ab8 and Ab9, respectively with a significant difference ($p \leq 0.01$).

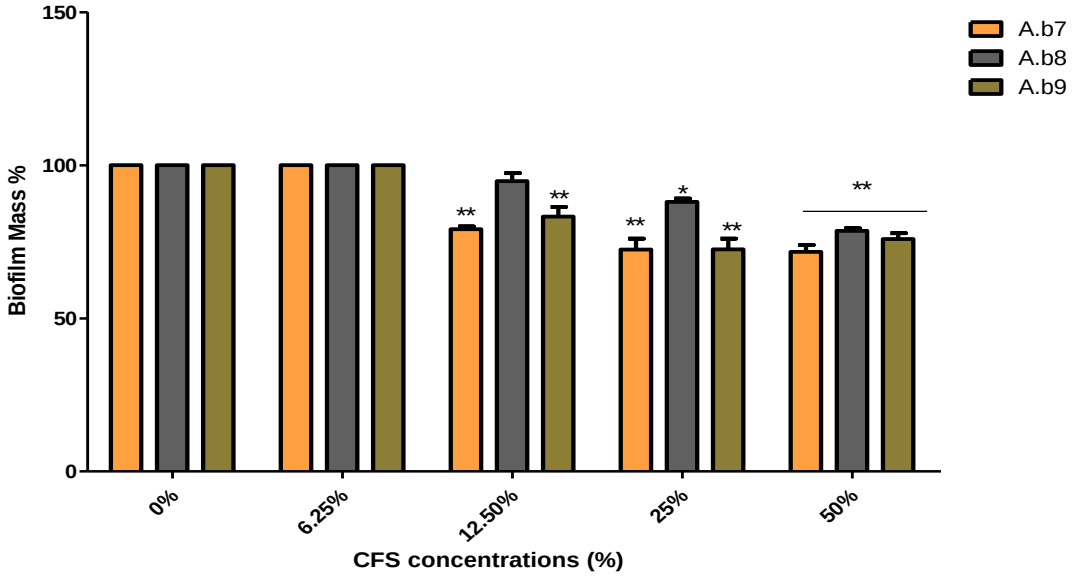


Figure 4-13: Anti-biofilm activity of *Bacillus subtilis* KATMIR 1933 CFS against *A. baumannii* isolates (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

Similarly, no MIC-B was determined, however, we noticed that *Bacillus amyloliquefaciense* B-1895 CFS produced a slight reduction in biofilm formation at 12.5% and 25%, figure (4-14). At 50%, A significant ($p \leq 0.01$) reduction (48.7 %) was reported in biofilm formed by the isolate Ab7. In the same regards, 50% CFS of *Bacillus amyloliquefaciense* B-1895 was significantly ($p \leq 0.05$) removed 58.4% and 53.6% of biofilm formation by Ab8 and Ab9, respectively.

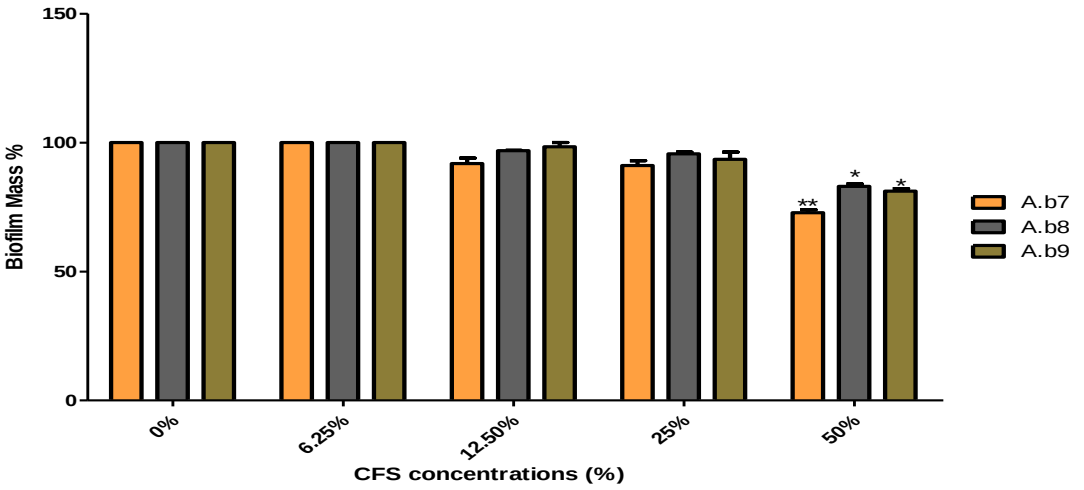


Figure 4-14: Anti-biofilm activity of *Bacillus amyloliquefaciens* B-1895 CFS against *A. baumannii* isolates (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

A slight inhibition was only noticed in Ab7 when *Lactobacillus casei* DG CNCM I-1572 CFS was applied at 12.5% and 25%. At 50%, as a MIC-B, a significant removal (100%) was reported in biofilm formed by the tested bacterial isolates, figure (4-15) (p≤0.001).

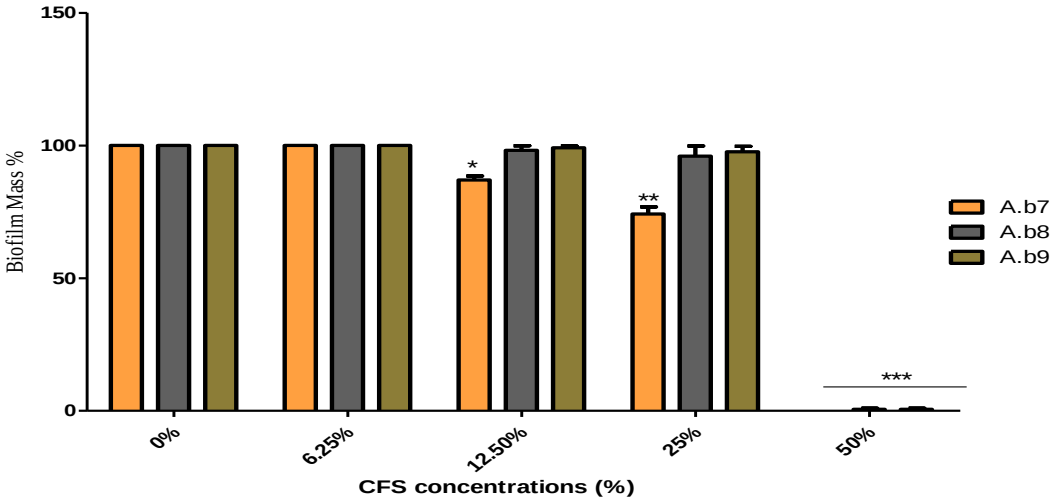


Figure 4-15: Anti-biofilm activity of *L. casie* CFS against *A. baumannii* isolates (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

Similarly, the MIC-B of the mixed lactobacilli CFS against the selected isolates was 50% at which, all the biofilms layers have been removed significantly ($p \leq 0.001$). Figure (4-16).

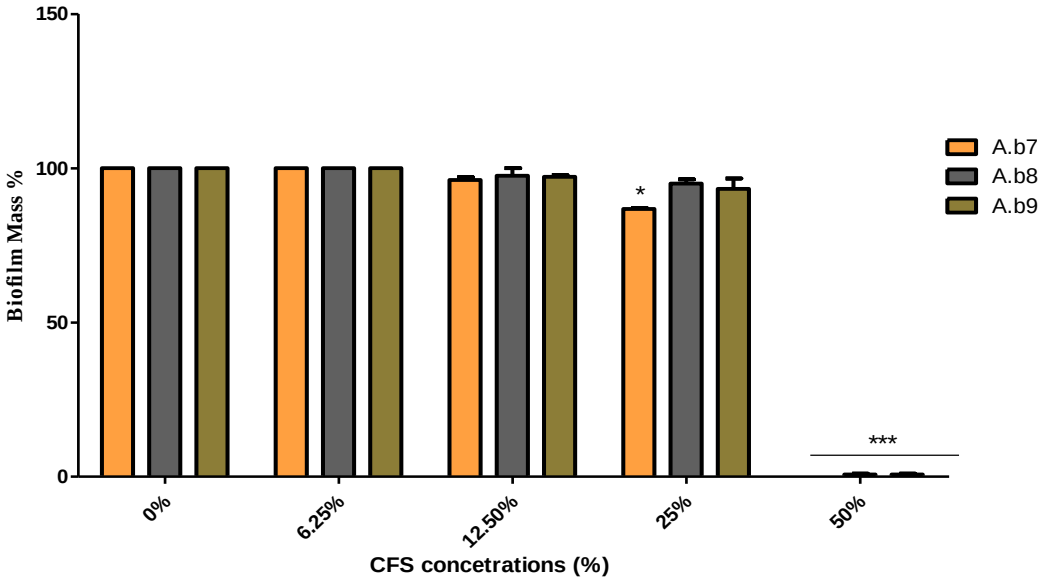


Figure 4-16: Anti-biofilm activity of mixed lactobacilli CFS against *A. baumannii* isolates (Ab7, Ab8 and Ab9). Data presented as mean MIC±SEM (µg/ml) to three independent experiments. Asterisks refer to significance levels: * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Several studies were performed shadding the light on the anti-biofilm potential of probiotic species against pathogenic bacteria. Aminnezhad and Kasra-Kermanshahi, (2014), reported that CFS of *L. casei* produced a biofilm inhibitory effect on *P. aeruginosa*, reducing more than 87% of the intact biofilm. Moreover, Sadri *et al*., (2016), stated that CFS of *L. casei* and *L. acidophilus* showed a moderate anti-biofilm potential against two pathogenic strains of *E. coli* preventing 46.7% and 25.3% of their biofilm, respectively. It was mentioned that *L. plantarum* CFS inhibited the biofilm formation of *P. aeroginusa* strains by 71% to 89% (Zamani *et al.*, 2017). In addition, the authors observed that 52.2% of biofilm was inhibited when *E. coli* strains were exposed to CFS of the tested lactobacilli. In regards to *A. baumannii*, Shin and Eom, (2020), found that when CFS of *C. butyricum*

(12.5%, 25% and 50%) were applied, 33.97%, 43.17% and 99.65%, of biofilm mass was reduced, respectively. The CFS obtained from lactobacilli species contained various biologically active compounds including exopolysaccharides and proteins. The CFS of *L. plantarum* Strongly inhibited biofilm formation due to the effect of several digestive enzymes released within CFS. These enzymes associated with a disruption of the preformed biofilm (Zamani *et al.*, 2017). The amphipathic interaction between the biofilm and butyric acid, which was released from the probiotic, could facilitate by the significant water content (97%) of the biofilms (Cordeiro *et al.*, 2019). Probiotics CFS can also include bacteriocins, which are involved in antimicrobial action. For example, subtilosin from *Bacillus subtilis* inhibits biofilm formation by inhibiting bacterial quorum sensing (Algburi *et al.*, 2017). Exopolysaccharide generated by *Lactobacillus acidophilus* demonstrated anti-biofilm activity against a wide variety of Gram-positive and Gram-negative bacteria by influencing the expression of genes involved in chemotaxis, auto-aggregation and co-aggregation (Stefania *et al.*, 2017), or by modification of bacterial cell surfaces and thus prevention of initial attachment (Zamani et al., 2017).

4.9 Auto-aggregation and Co-aggregation

Kinetic measurements of auto-aggregation and co-aggregation of the probiotic strains with three isolates of *A. baumannii* were determined at 0, 4hrs and 24hrs time period using an automated microtiter plate reader to quantitatively evaluate the potential of bacterial aggregation using ELIZA reader at a wavelength of 630nm.

After 4hrs of incubation, the percentage of auto-aggregation of *B. amyloliquefaciens* B-1895, *Bacillus subtilis* KATMIR 1933, *Lactobacillus casei* DG CNCM I-1572 and the mixed of lactobacilli were 25.5%, 22.76%, 0 % and 0%, respectively. We noticed that after 24hrs, the

percentages of auto-aggregation were increased as follows: *B. amyloliquefaciens* B-1895, *Bacillus subtilis* KATMIR 1933, *Lactobacillus casei* DG CNCM I-1572 and mixed lactobacilli were 95.7%, 82.4%, 8 % and 43.9 %, respectively, table (4-8).

Table 4-8: Auto-aggregation of the tested Probiotic strains at 4hrs and 24hrs of incubation.

Bacterial Strains	**Auto-aggregation%**	
	Time	
	4hrs	**24hrs**
Bacillus amyloliquefaciens B-1895	25.5%	95.7%
Bacillus subtilis KATMIR 1933	22.76%	82.4%
Lactobacillus casei DG CNCM I-1572	0 %	8 %
Mixed lactobacilli	0 %	43.9 %
A. baumannii isolates; (Ab7, Ab8 and Ab9)	(24.5%, 20.58%, 28.97%)	(0.48%, 8.4%, 30.5%)

The percentages of auto-aggregation values of *A. baumannii* isolates; Ab7, Ab8 and Ab9 after 4hrs were 24.5%, 20.58% and 28.97%, respectively. Whereas, after 24hrs, they were 0.48%, 8.4% and 30.5%, respectively, table (4-8).

The co-aggregation was reported after 4hrs and 24hrs of bacterial incubation. After 4hrs, the high co-aggregation percentage was observed when *Bacillus subtilis* KATMIR 1933 was mixed with Ab7, Ab8 and Ab9, as following: 33.43%, 31.89% and 34%, respectively. When *Bacillus amyloliquefaciens* B-1895 was added to Ab7, Ab8 and Ab9, the co-aggregation percentages were 23.98%, 29.39% and 17.15%, respectively. In addition, mix of lactobacilli were co-aggregated with *A. baumannii*

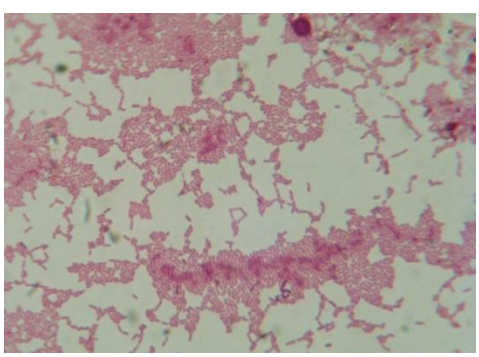
isolates with 5.78%, 17.7% and 17.59%, respectively, table (4-9). Low co-aggregation percentages weas reported when *Lactobacillus casei* DG CNCM I-1572 was mixed with *A. baumannii* isolates as follows: 8.77%, 5.75% and 0%, respectively.

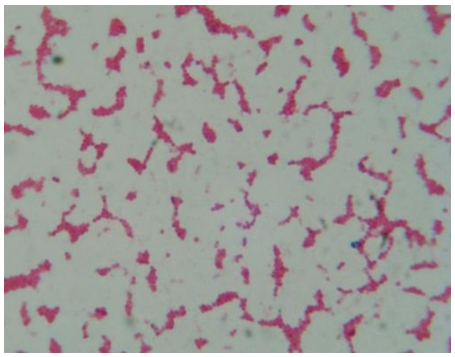
After 24hrs incubation, the percentages of co-aggregation were higher compared to 4hrs. *Bacillus subtilis* KATMIR 1933 was co-aggregated with *A. baumannii* Ab7, Ab8 and Ab9 at 60.1%, 53.16% and 62.8%, respectively. Moreover, the co-aggregation of *Bacillus amyloliquefaciens* B-1895 with *A. baumannii* (Ab7, Ab8 and Ab9) were 50.57%, 55.64% and 50.67%, respectively. Whereas the co-aggregation of mix of lactobacilli with Ab7, Ab8 and Ab9 were 26.53%, 30.81% and 39.38%, respectively. The lowest co-aggregation percentages were observed in the combination of *Lactobacillus casei* DG CNCM I-1572 with *A. baumannii* isolates which were 24%, 27.1% and 21%, respectively, table (4-9).

Table 4-9: Co-aggregation % of probiotic strains with *A. baumannii* isolates; Ab7, Ab8 and Ab9 at 4hrs and 24hrs of incubation.

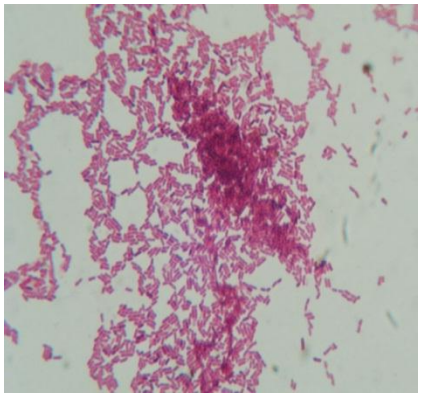
Bacterial Strains	**Co-aggregation%**	
	Time	
	4hrs	**24hrs**
Bacillus amyloliquefaciens B-1895 with (Ab7, Ab8 and Ab9	(23.98%, 29.39% and 17.15%)	(50.57%, 55.64% and 50.67%)
Bacillus subtilis KATMIR 1933 with (Ab7, Ab8 and Ab9)	(33.43%, 31.89% and 34%)	(60.1%, 53.16% and 62.8%)
Lactobacillus casei DG CNCM I-1572 with (Ab7, Ab8 and Ab9)	(8.77%, 5.75% and 0%)	(24%, 27.1% and 21%)
Mixed lactobacilli with (Ab7, Ab8 and Ab9)	(5.78%, 17.7% and 17.59%)	(26.53%, 30.81% and 39.38%)
A. baumannii isolates; (Ab7, Ab8 and Ab9)	(24.5%, 20.58%, 28.97%)	(0.48%, 8.4%, 30.5%)

The heigh co-aggregation is appeared in (24 hrs) by using Microscopic after gram stain used as shown in figure (4-17).





A-*Bacillus amyloliquefaciens B-1895* B- *A. baumannii* some auto-aggregation(24 hrs) some auto-aggregation (4 hrs).



C-*Bacillus amyloliquefaciens B*-1895 with *A. baumannii* Co-aggregation (24 hrs)

Figure 4-17: Auto and co-aggregation of tested Probiotic strains with *A. baumannii.*

The results of the current study showed the highest co-aggregation ratios of the tested probiotic strains with *A. baumannii* (Ab7, Ab8 and Ab9) was after 4hrs and 24hrs of incubation. Similarly, AL-Azawi, (2019), reported a high co-aggregation of *P. mirabilis* causing UTI with *B. amyloliquefaciens* B-1895 and *B. subtilis* KATMIRA1933 after 24hrs incubation, was 67.7% .

Co-aggregation of probiotic strains with the pathogenic bacterial lead to disruption of the preformed biofilms through competition of interacted bacterial cells to the attached surfaces (Zamani *et al*., 2017).

4.10 The synergism between Colistin and Probiotic strains (Kirby-Bauer methods)

The susceptibility of the *A. baumannii* isolates to Colistin, alone and in combination with the CFS of tested probiotic strains was determined, first, using the disk diffusion method (Kirby-Bauer). Antimicrobial activity was determined based on the diameter of the inhibition zone (mm).

In general, all the results showed a significant increase in the diameters of the bacterial growth inhibition zones of *A. baumannii* when CFS of probiotic strains was combined with Colistin as a comparison with using Colistin alone, figure (4-18) and appendix (8). Colistin, alone produced an inhibition zone around the isolates Ab7 and Ab8 (11 mm for each), while the isolate Ab9 displayed 10 mm of growth inhibition zone around the same antibiotic disk, figure (4-18).

The diameters of *A. baumannii* growth inhibition zones were 12, 14 and 16 mm for Ab7, Ab8 and Ab9, respectively, were treated with CFS of *Bacillus subtilis* KATMIR 1933 and Colistin. In the same regards, the zone of bacterial growth inhibition was significantly increased ($p \leq 0.01$) (13mm) when Colistin disk was combined with CFS of *Bacillus amyloliquefaciens* B-1895 and CFS of mixed lactobacilli. Furthermore, the zones growth inhibition of *A. baumannii* Ab7, Ab8 and Ab9, were 11, 12 and 13 mm when CFS of *Lactobacillus casei* DG CNCM I-1572 was added to Colistin disk.

The results of the current study are in agreement with the study of Isayenko *et al*., (2020) they found an increase in the diameter of growth inhibition zones of *A. baumannii* when antibiotics were combined with the metabolite complexes of *Lactobacillus rhamnosus* and *Saccharomyces boulardii* using the modified disk-diffusion method.

Furthermore, an earlier study by Algburi *et al*., (2020), who identified a complementary activity against the tested methicillin-resistant

staphylococcus aureus via a combination of cefotaxime with the CFS of *Bacillus subtilis* KATMIRA1933 and *Bacillus amyloliquefaciens* B-1895.

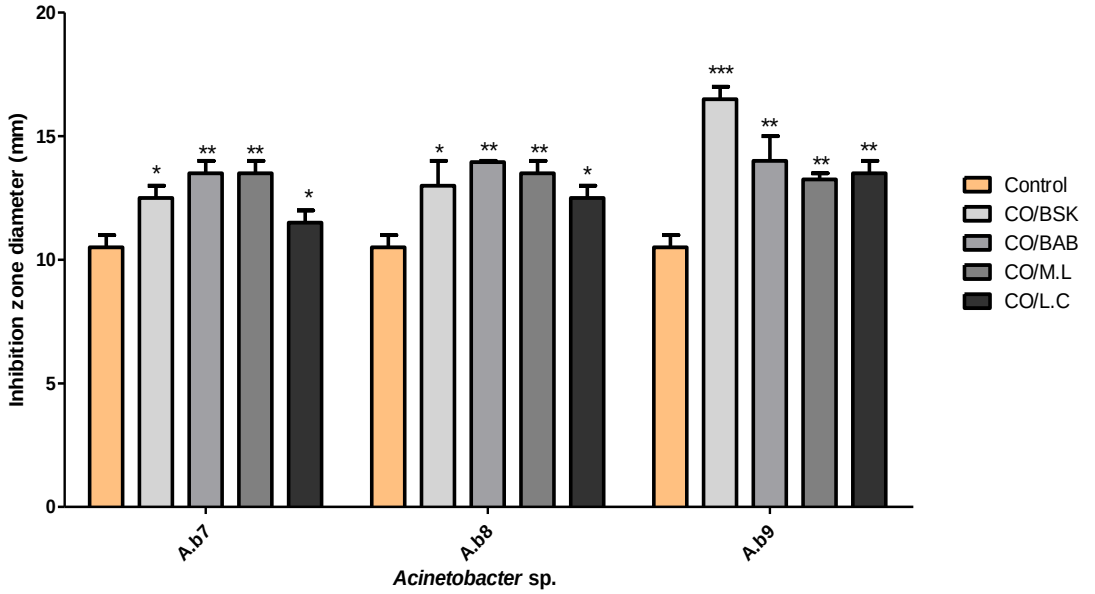


Figure 4-18: Combination Colistin with the tested probiotcs strains CFS against *A. baumannii* by disc method. Data presented as mean ±SEM to three independent experiments. Asterisks refer to significance levels: * p≤ 0.05, ** p≤ 0.01 and *** p ≤ 0.001.

4.11 The synergistic effects of Colistin with Probiotic strains against the planktonic and biofilm cells of *A. baumannii*

These data regarding the synergistic effects between Colistin and CSF of the tested probiotic strains against the planktonic and biofilm cells of the three isolates of *A. baumannii* were determined using checkerboard. This method was used to evaluate the antimicrobial combinations and according to Algburi *et al*., (2017). After 24-36hrs incubation for the microtiter plate, the values in the figure (4-19) were obtained using ELISA reader at wavelength 630 nm.

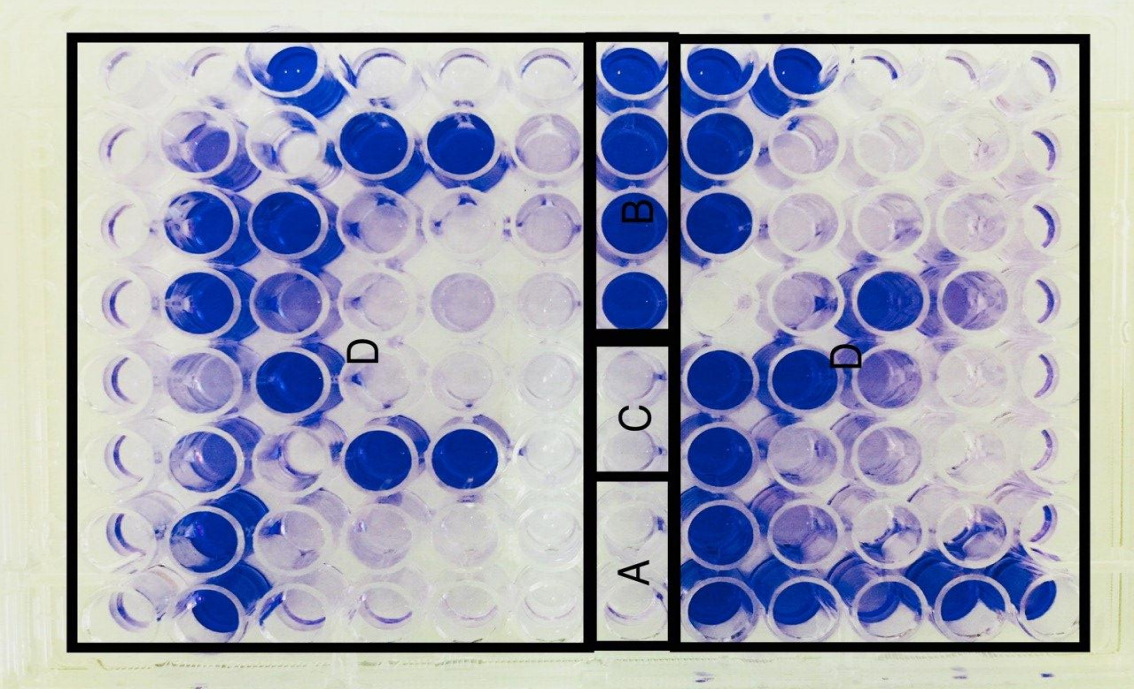


Figure 4-19: Antimicrobial combination on microtiter plate, A: control-ve (broth +Antibiotic), B=control +ve (bacterial growth), C=control –ve (only broth) and D= combination probiotic CSF+ Antibiotic).

According to the current study, the antimicrobial activity of Colistin was complimented with the CFS of probiotic strains against both planktonic cells and biofilm of three isolates of *A. baumannii*. The results noticed that the MIC and the MIC-B values of Colistin in combination with probiotic strains were lower, compared to with Colistin when used alone.

Isobologram was used to evaluate the combination of CFS of tested probiotics with Colistin against planktonic cells while the total fractional inhibitory concentration index (∑FIC) was used to assess the nature of antimicrobial combinations against biofilm cells.

When Colistin was combined with the CFS of probiotic strains against *A. baumannii* isolate Ab7, A synergistic activity was reported; the MICs of combinations were 3.1 µg/ml for Colistin when 1.6% and 3.1% of CFS of *Bacillus subtilis* KATMIR 1933 was used, figure (4-20). The ∑FIC was 0.527 when the same combinations were used against biofilm cells,

appendix (9). Also, a synergism was identified when Colistin mixed with CFS of *Bacillus amyloliquefaciens*. The MICs of combinations were 3.1 µg/ml for Colistin when 1.6% and 3.1% of CFS of *Bacillus amyloliquefaciens* B-1895 was used, figure (4-20). The ∑FIC was 0.511 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 3.1 and 1.6 µg/ml for Colistin when 6.25% and 3.1% of CFS of *Lactobacillus casei* DG CNCM I-1572 was, respectively used, figure (4-20). The ∑FIC was 0.318 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 3.1 and 0.8 µg/ml for Colistin when 1.6% and 12.5% of CFS of mix lactobacilli was used, figure (4-20). The ∑FIC was 0.378 when the same combinations were used against biofilm cells, appendix (9).

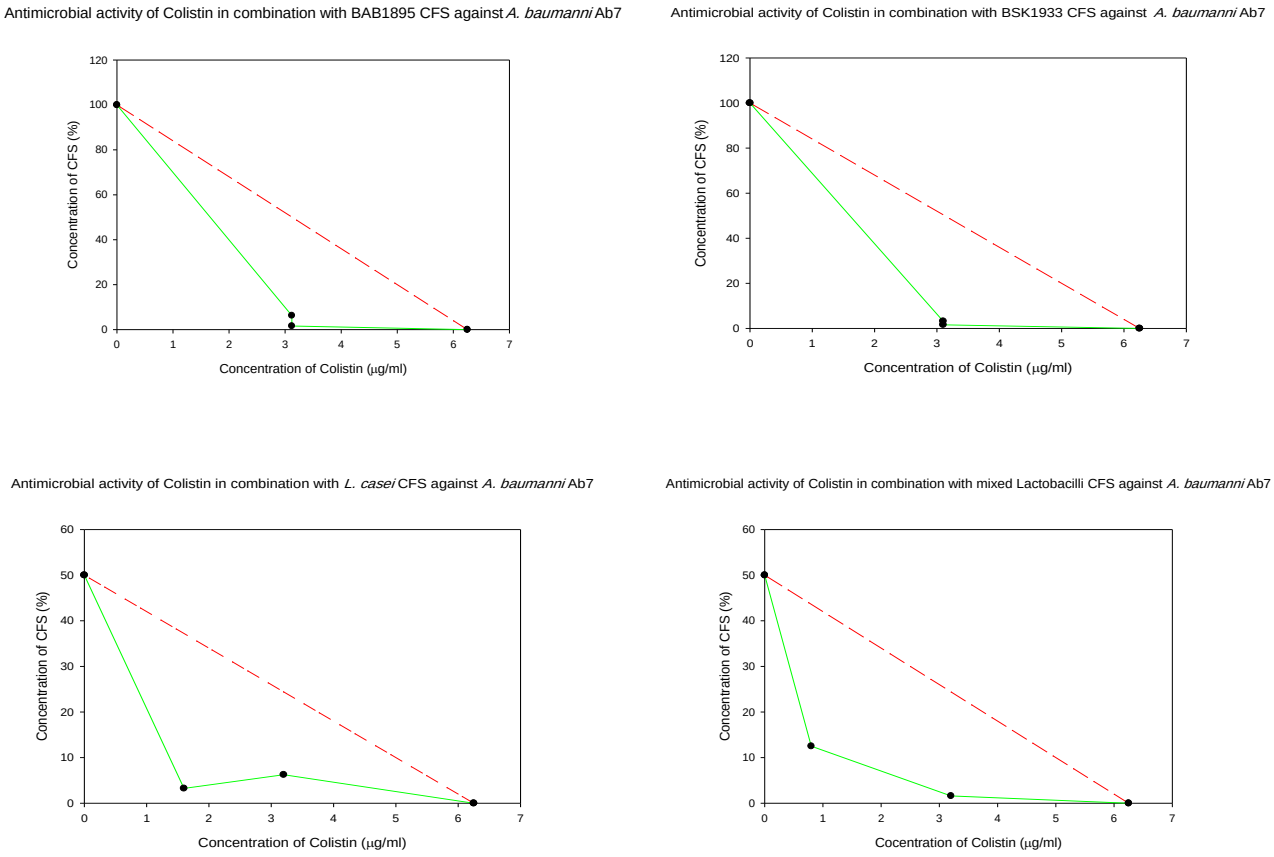
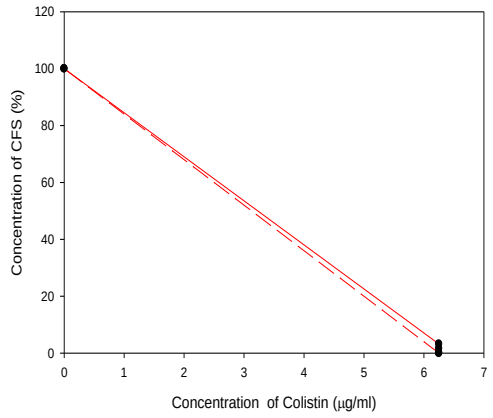


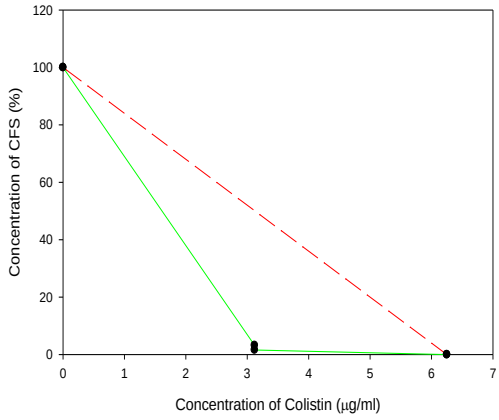
Figure 4-20: Isobolograms of Colistin with probiotics strains CFS against planktonic cells and biofilm form of (Ab7) *A. baumannii*.

When Colistin was combined with the CFS of probiotic strains against *A. baumannii* isolate Ab8, A synergistic activity was reported except combination with the CFS of *Bacillus amyloliquefaciens* B-1895; the MICs of combinations were 3.1 µg/ml for Colistin when 1.6% and 3.1% of CFS of *Bacillus subtilis* KATMIR 1933 was used, figure (4-21). The ∑FIC was 0.516 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 6.25 µg/ml for Colistin when 1.6% and 3.1% of CFS of *Bacillus amyloliquefaciens* B-1895 was used, figure (4-21). The ∑FIC was 1.016 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 3.1 and 1.6 µg/ml for Colistin when 1.6% of CFS of *Lactobacillus casei* DG CNCM I-1572 was used, Figure (4-21). The ∑FIC was 0.288 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 3.1 and 1.6 µg/ml for Colistin when 1.6% and 12.5% of CFS of mix lactobacilli was used, figure (4-21). The ∑FIC was 0.266 when the same combinations were used against biofilm cells, appendix (9).

Antimicrobial activity of Colistin in conbination with BAB-1895 CFS against *A. baumanni* Ab8



Antimicrobial activity of Colistin in conbination with BSK1933 CFS against *A. baumanni* Ab8



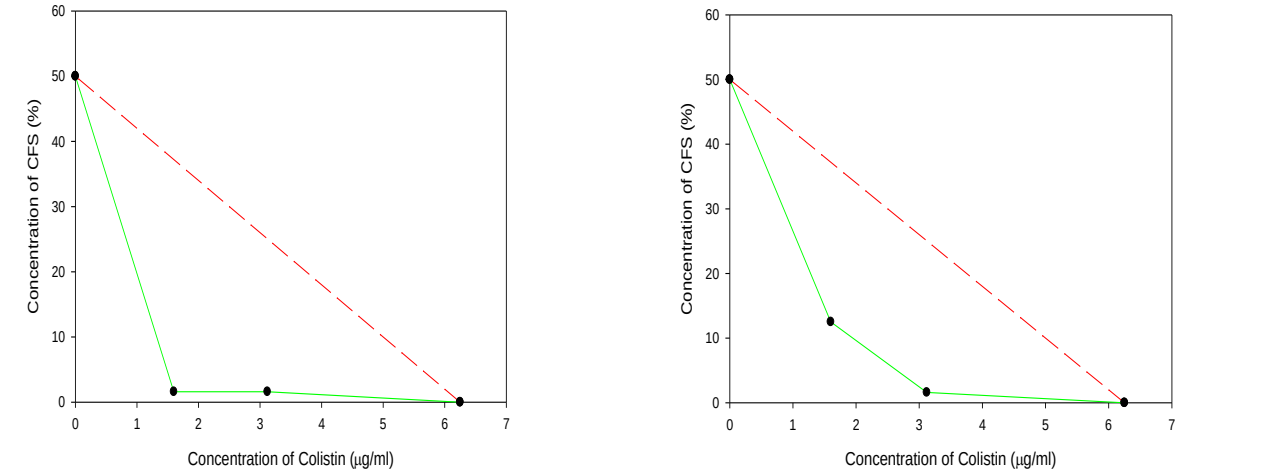


Figure 4-21: Isobolograms of Colistin with probiotics strains CFS against planktonic cells and biofilm form of (Ab8) *A. baumannii*.

When Colistin was combined with the CFS of probiotic strains against *A. baumannii* isolate Ab9, A synergistic activity was reported except CFS of *Bacillus subtilis* KATMIR 1933 was not a synergistic activity with Colistin against biofilm formation of *A. baumannii* isolate Ab9; the MICs of combinations were 1.6 µg/ml for Colistin when 1.6% and 3.1% of CFS of *Bacillus subtilis* KATMIR 1933 was used, figure (4-22). The ∑FIC was 1.016 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 1.6 µg/ml for Colistin when 3.1% and 12.5% of CFS of *Bacillus amyloliquefaciens* B-1895 was used, figure (4-22). The ∑FIC was 0.272 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 1.6 and 0.4 µg/ml for Colistin when 6.25% and 12.5 of CFS of *Lactobacillus casei* DG CNCM I-1572 was used, figure (4-22). The ∑FIC was 0.381 when the same combinations were used against biofilm cells, appendix (9). The MICs of combinations were 0.4 and 0.2 µg/ml for Colistin when 12.5% of CFS of mix lactobacilli was used, figure (4-22). The ∑FIC was 0.266 when the same combinations were used against biofilm cells, appendix (9).

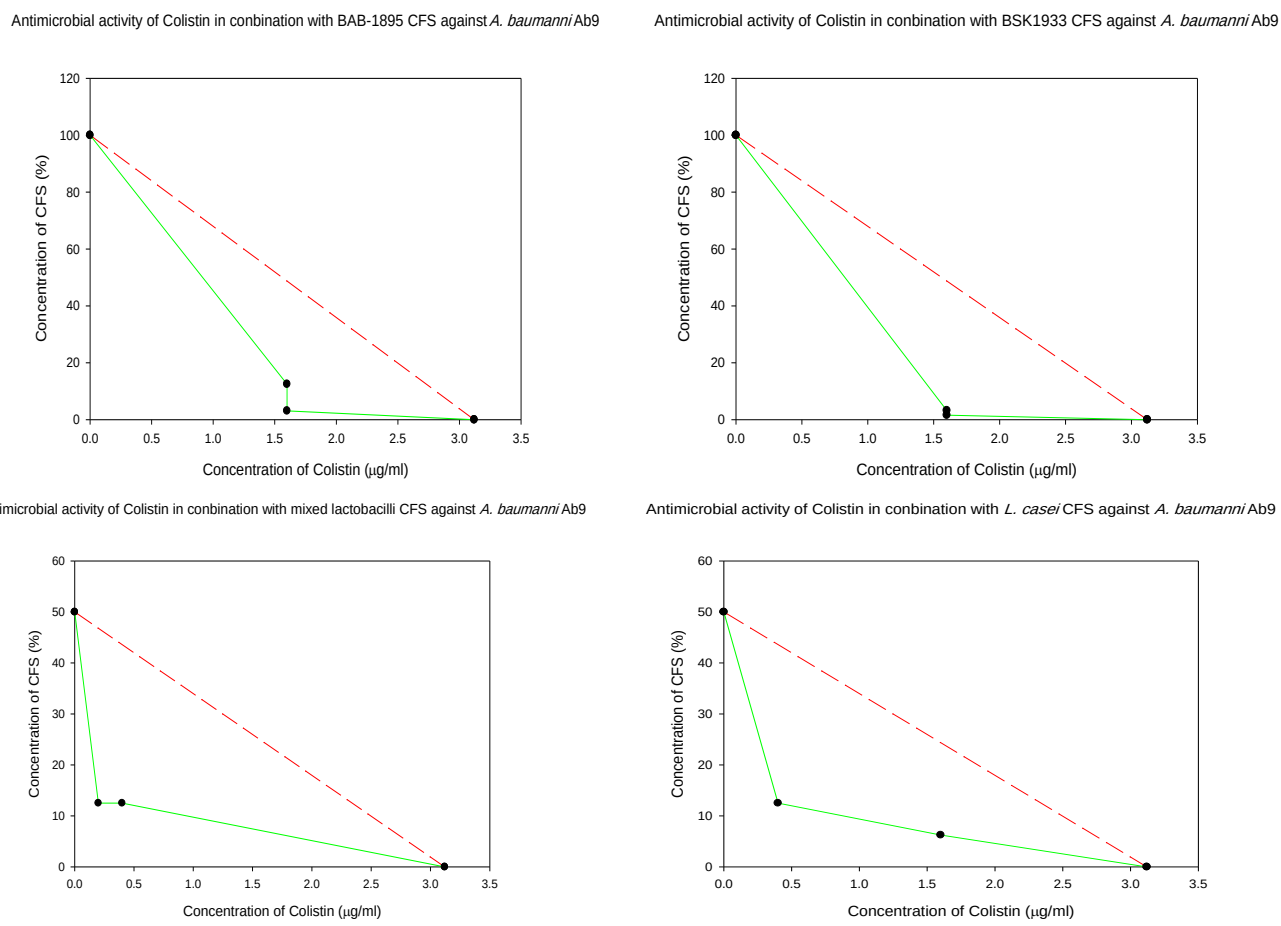


Figure 4-22: Isobolograms of Colistin with probiotics strains CFS against planktonic cells and biofilm form of (Ab9) *A. baumannii*.

These data agree with the study of Mathur *et al*., (2018), who determined an anti-biofilm activity of nisin and polymyxins against *P. aeruginosa*: Inhibition of *P. aeruginosa* biofilm formation in the presence of nisin alone was (1/3× MIC), Colistin alone (1/2×MIC) and (1/5×MIC) when nisin was combined with Colistin.

The acquired resistance of pathogenic bacteria makes it difficult to select an appropriate antimicrobial agent. The clinical use of Colistin is increased, but, as the last line of therapeutic antibiotics because of its nephrotoxicity and neurotoxicity (Ballini *et al.*, 2019). Colistin synergism with other antimicrobials is an attractive way for the choice of combination therapy, especially in care units where patients are critically ill and the resistant strains of pathogens are commonly isolated and difficult to

control. Currently, in patients with MDR gram-negative, Colistin in combination therapy turned out to be more successful than Colistin monotherapy (Mosca *et al.*, 2020).

Products of Probiotic strains in combination therapy are attractive to scientists due to their production of antibacterial agents. The synergistic interactions of biologically active substances of probiotic microorganisms with antibiotics can increase their antimicrobial activity to be applied in the industrial preparations, reduce the required concentrations of both combined antibiotics, and avoid the development of bacterial resistance. The above-mentioned advantages of combined therapy are very important for the future extending the usage of the existing antimicrobials (Isayenko, 2019). Taking probiotics concurrently with antibiotics may reduce the threatening effect of using antibiotics alone in high concentrations, such as avoiding the risk of developing antibiotic-related dysbiosis (Rowles, 2017) Lactic acid bacteria, including species of genus Lactobacillus and Bacillus, have been associated with the prevention or dispersion of biofilms formed by pathogenic microorganisms. This effect is often associated with the production of antimicrobial substances such as organic acids, bacteriocins, hydrogen peroxide and biosurfactants (Fernandes *et al.*, 2019). The synergistic effect occurs when the organic products of the probiotic strains destroy the cell wall and create pores in it (Fozouni *et al.*, 2019), thus facilitating the arrival and action of colistin on the bacterial cell membrane (Nhu *et al.*, 2016).

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Appendix (1): Antimicrobial agents of (A) *Lactobacillus casei* DG CNCM I-1572 and (B) Mix of lactobacilli.

Appendix (2): Colistin methanesulfonate (CMS) vial.

Appendix (3): Patients information form.

Sample NO.:

Patient name:

Patient age:

Gender:

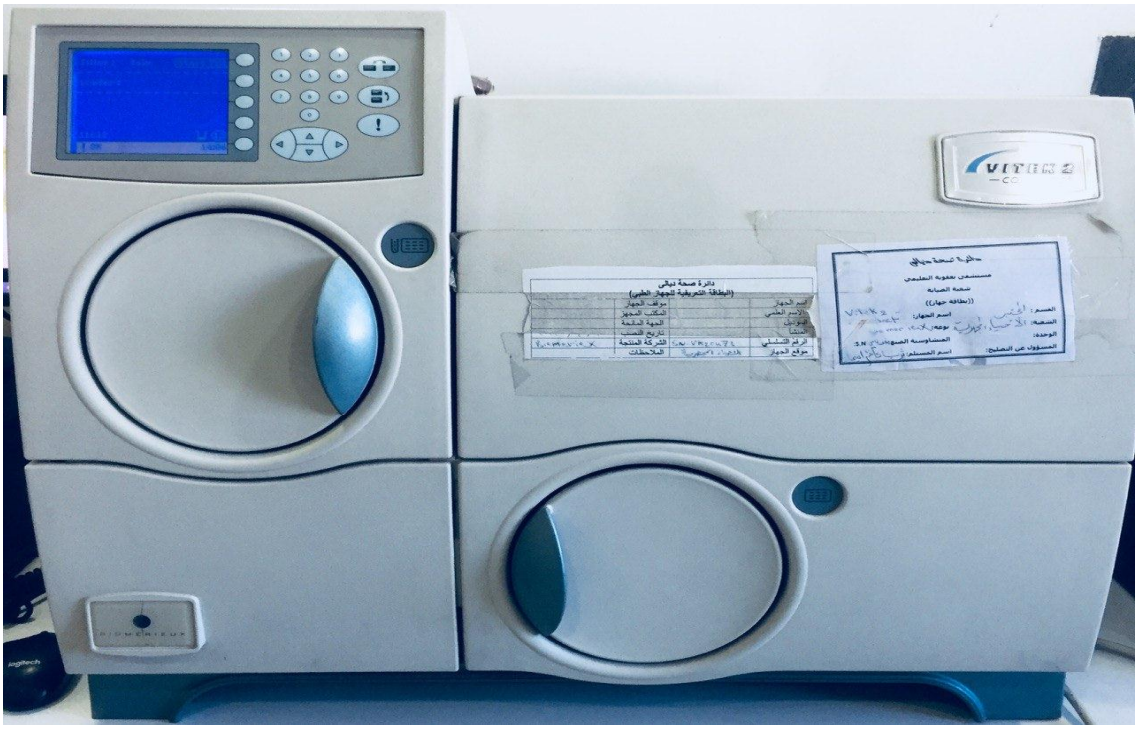
Address:

Sample type:

Isolation place:

Collection date:

Appendix (4): The VITEK 2 system.



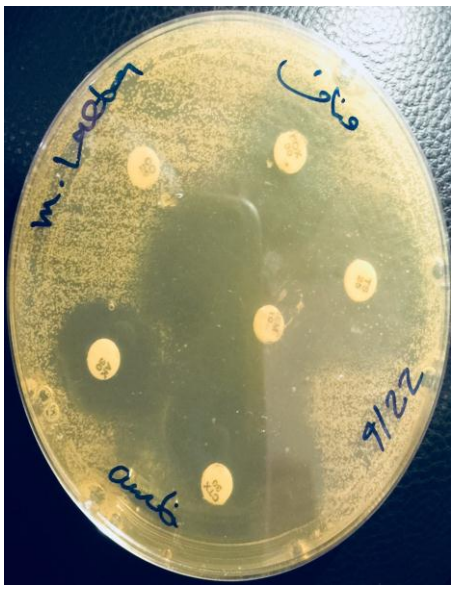
Appendix (5): Biochemical identification of *A. baumannii* using the VITEK-2 system.

Test type	Result	Test type	Result	Test type	Result	Test type	Result	Test type	Result	Test type	Result
APPA	-	ADO	-	PyrA	-	IARL	-	Dcel	+	BGAL	-
H2S	-	BNAG	-	AGLTp	-	dGLU	+	GGT	-	OFF	-
BGLU	-	dMAL	-	dMAN	-	dMNE	+	BXYL	-	BAlap	-
ProA	-	LIP	-	PLE	-	TyrA	+	URE	-	dSOR	-
SAC	-	dTAG	-	dTRE	-	CIT	+	MNT	-	5KG	-
ILATk	+	AGLU	-	SUCT	+	NAGA	-	AGAL	-	PHOS	-
GlyA	-	ODC	-	LDC	-	IHISa	+	CMT	+	BGUR	-
O129R	+	GGAA	-	IMLTA	-	ELLM	-	ILATa	-		

+ Positive result, - Negative result

Appendix (6): Antibiotics Susceptibility to probiotic strains by the disk diffusion method.

(A) *Bacillus subtilis* KATMIR 1933



(B) *Lactobacillus casei* DG CNCM I-1572

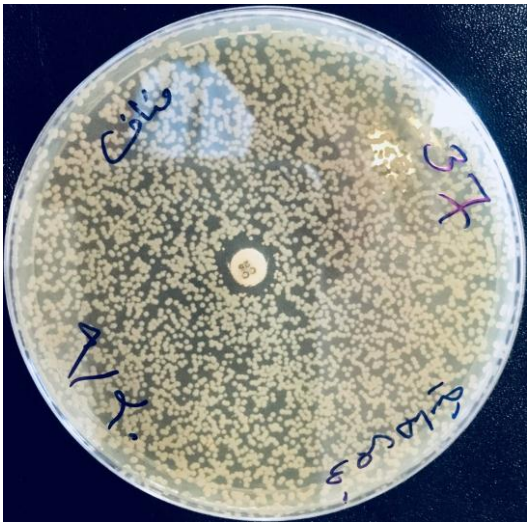
(AK) Amikacin 30 mg, (COL) Colistin 25mg, (FOX) Cefoxitin 30mg, (CTX) Cefotaxime 30 mg, (MEM) Meropenem 10 mg, (TS) Trimethoprim-sulfamethoxazole 1.25/23.75 mg.

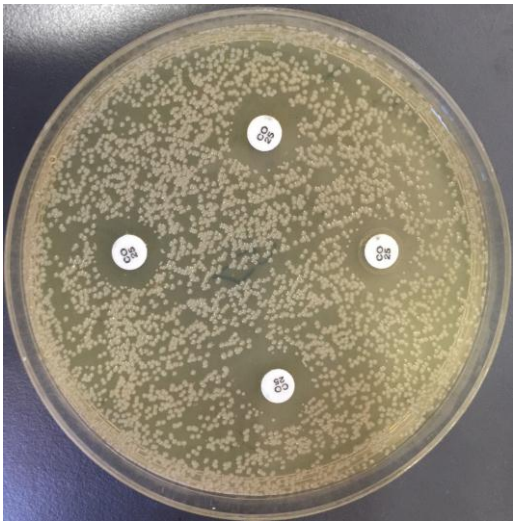
Appendix (7): The result of antibiotics susceptibility test.

Antibiotic																		
ID	AMP	PTZ	CZ	FOX	CAZ	CRO	CPM	IMP	AK	GM	CIP	LEV	INN	TS	MIN	COL	MEM	TOB
1	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	320 R	1 S	0.5 S	16 R	16 R
2	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	320 R	1 S	0.5 S	16 R	16 R
3	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	160 R	1 S	0.5 S	16 R	1 S
4	32 R	128 R	64 R	64 R	64 R	64 R	8 R	16 R	64 R	16 R	4 R	8 R	0.5 S	160 S	1 S	0.5 S	16 R	16 R
5	32 R	128 R	64 R	64 R	16 R	64 R	8 R	16 R	32 I	16 R	4 R	8 R	2 S	20 S	1 S	0.5 S	16 R	16 R
6	32 R	128 R	64 R	64 R	64 R	64 R	64 R	1 S	64 R	16 R	4 R	8 R	0.5 S	320 S	1 S	0.5 S	16 R	16 R
7	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	2 R	4 R	8 R	8 I	20 R	8 I	0.5 S	16 R	16 R
8	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	2 R	4 R	8 R	8 I	20 R	8 I	0.5 S	16 R	16 R
9	32 R	128 R	64 R	64 R	16 R	64 R	64 R	16 R	64 R	16 S	4 R	8 R	1 S	20 S	1 S	0.5 S	16 R	16 R
10	32 R	128 R	64 R	64 R	64 R	64 R	32 R	16 R	64 R	16 R	4 R	8 R	0.5 S	160 R	1 S	0.5 S	16 R	16 R
11	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 S	4 R	8 R	1 S	320 R	1 S	0.5 S	16 R	16 R
12	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	160 R	1 S	0.5 S	16 R	16 R
13	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	1 S	320 R	1 S	0.5 S	16 R	16 R
14	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	320 R	1 S	0.5 S	16 R	16 R
15	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	16 R	320 R	16 R	0.5 S	16 R	16 R
16	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	4 S	4 R	8 R	0.5 S	20 S	1 S	0.5 S	16 R	1 S
17	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	2 S	160 R	1 S	0.5 S	16 R	16 R
18	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	160 R	1 S	0.5 S	16 R	16 R
19	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	0.5 S	160 R	1 S	0.5 S	16 R	16 R
20	32 R	128 R	64 R	64 R	64 R	64 R	64 R	16 R	64 R	16 R	4 R	8 R	2 S	160 R	1 S	0.5 S	16 R	16 R

Ampicillin(AMP),Piperacillin/Tazobactam(PTZ),Cefazolin(CZ),Cefoxitin(FOX),Ceftazidime(CAZ),Ceftriaxone(CRO),Cefepime(CPM),Impenem(IMP),Aikacin(AK),Gentamicin(GM),Ciprofloxacin(CIP),Levofloxacin(LEV),Tigeccline(INN),Trimethoprim/sulfamethoxazole(TS),Minocycline(MIN),Clistin(COL),Meropenem(MEM),Tobramycin(TOB).

Appendix (8): Colistin Combination with CFS of the Tested probiotic Strains by disc zone inhibition.





(A)Alone Colistin (B) Combination Colistin with probiotic

Appendix (9): The nature of antimicrobial combinations against biofilm of *A. baumanni* isolates.

Bacterial Isolates	Antimicrobial combinations	∑ FIC values	Results
A. baumannii Ab7	CO + CFS of BSK	0.527	Synergistic
	CO + CFS of BAB	0.511	Synergistic
	CO + CFS of LC	0.318	Synergistic
	CO + CFS of ML	0.378	Synergistic
A. baumannii Ab8	CO + CFS of BSK	0.516	Synergistic
	CO + CFS of BAB	1.016	Non Synergistic
	CO + CFS of LC	0.288	Synergistic
	CO + CFS of ML	0.266	Synergistic
A. baumannii Ab9	CO + CFS of BSK	1.016	Non Synergistic
	CO + CFS of BAB	0.272	Synergistic
	CO + CFS of LC	0.381	Synergistic
	CO + CFS of ML	0.266	Synergistic

الخلاصة

ظهرت بكتيريا الراكدة البومانية كمسبب مرضي خطير في المستشفيات بسبب مقاومتها للمضادات الحيوية وقدرتها على الاستعمار والتسبب في التهابات شديدة للمرضى. هدفت هذه الدراسة إلى التحقيق في الخاصية المضادة للبكتيريا والمضادة للغشاء الحيوي لراشح بكتيريا المعززات الحيوية ضد الراكدة البومانية وإثبات التأثير التآزري للكولستن مع راشح بكتريا المعززات الحيوية المنتخبة في هذه الدراسة ضد العزلات السريرية للراكدة البومانية المتعددة المقاومة للأدوية.

تم تشخيص ٢٠ (٨,٦٩٪) عزلة سريرية للراكدة البومانية من إجمالي ٢٣٠ عينة تم جمعها من مصادر مختلفة شملت الحروق والجروح والدم. أخذت هذه العينات من المرضى المراجعين والمقيمين في مستشفى بعقوبة التعليمي/ديالى خلال شهر أيلول٢٠٢٠ حتى نهاية كانون الاول٢٠٢٠. تم تشخيص العزلات البكتيرية بناءً على الخصائص المظهرية والاختبارات الكيميوحيوية الأولية. تم تأكيد التشخيص وفحص الحساسية للمضادات الحيوية للراكدة البومانية باستخدام جهاز VITEK 2.

تم استخدام الاطباق الدقيقه ذات٩٦حفره للتجارب الاتية : تكوين الغشاء الحيوي بواسطة الراكدة البومانية واختبار التجمع المشترك فضلا عن تحديد التركيز المثبط الأدنى (MIC) والتركيز الأدنى المثبط للغشاء الحيوي البكتيري (MIC-B) للمضادات الميكروبية المستخدمة في هذه الدراسة وحدها أو مجتمعة. تم خلط الكولستن مع راشح بكتيريا المعززات الحيوية المختبرة لتحديد طبيعة التفاعلات للمضادات المستخدمة (التآزر أو التضاد أو التأثير الإضافي) باستخدام اختبار انتشار القرص وطريقة رقعة الشطرنج.

أظهرت النتائج أن أعلى معدل عزل للبكتيريا المرضية كان من الحروق ٩ (٤٥٪) وعدوى الجروح ٦ (٣٠٪) بينما تم عزل نسبة منخفضة من عينات الدم ٥ (٢٥٪). جميع العزلات البكتيرية (١٠٠٪) من الراكدة البومانية كانت لديها القدرة على تكوين غشاء الحيوي "قوي" اضافة الى انها كانت (١٠٠٪) مقاومة للمضادات الحيوية الاتية : أمبيسلين وبيبراسيلين/تازوباكتام وسيفازولين وسيفوكسيتين وسيفتازيديم وسيفترياكسون وميروبينيم وسيبروفلوكساسين وليفوفلوكساسين وسيفيبيم. فضلا عن انه كانت (٩٥٪) من العزلات السريرية مقاومة للإيميبينيم والأميكاسين و (٩٠٪) منها كانت مقاومة لـ الجنتامايسين وتوبرامايسين. بينما (٧٥٪) منها كانت مقاومة لـترايميثوبريم - سلفاميثاكزازول. لقد لاحظنا أن جميع عزلات الراكدة

البومانية (١٠٠%) كانت حساسة للكولستن و(٨٥٪) منها كانت حساسة تايجيسايكلين ومونوسايكلين. تم تحديد غالبية ١٣ (٦٥٪) من عزلات الراكدة البومانية على أنها مقاومة للأدوية على نطاق واسع (XDR) و٧ (٣٥%) منها كانت مقاومة للأدوية المتعددة (MDR).

لم يظهر الراشح البكتيري للمعززات الحيوية الاتية : *Bacillus subtilis* KATMIR1933 و*Bacillus amyloliquefaciense* B-1895 أي نشاط (حتى عند استخدام ١٠٠٪) ضد عوالق بكتيريا الراكدة البومانية ولكن كان تأثيره طفيفًا على تكوين الأغشية الحيوية للراكدة البومانية. في حين تم تسجيل نشاط مضاد للميكروبات قوي وكذلك تاثير مضاد للغشاء الحيوي المكون من قِبل الراكدة البومانية عندما تم استخدام ٥٠ ٪ (MIC وMIC-B) من الراشح البكتيري من خلايا عصيات اللبنية *Lactobacillus casei* CNCMi 1572 والعصيات اللبنية المخلوطة mixed lactobacilli مما تسبب في تثبيط (١٠٠٪) من الأغشية الحيوية المكونة بواسطة الراكدة البومانية.

أظهرت الدراسة أن النسبة المئوية للتجمع المشترك (Co-aggregation) بين سلالات المعززات الحيوية والراكدة البومانية كانت أعلى مقارنة بالتجميع الذاتي (Auto-aggregation). كان التجميع الذاتي لعصيات *Bacillus amyloliquefaciense* B-1895 أعلى مما لوحظ في *Bacillus subtilis* KATMIR1933 و*L. casei* CNCMi 1572 وكذلك الـ mix lactobacilli.

بناءًا على تطبيق الـ isobologram وإجمالي التركيز المثبط الجزئي (∑FIC) فان الراشح البكتيري من خلايا سلالات المعززات الحيوية كان متآزرا مع الكولستن ضد كلا من عوالق الخلايا والاغشيه الحيوية لثلاث عزلات من الراكدة البومانية المختارة في هذه الدراسة والمعزولة من الجروح والحروق والدم. كان MIC وMIC-B للكولستن بالاشتراك مع الراشح البكتيري من سلالات المعززات الحيوية (*Bacillus subtilis* KATMIR1933 و *Bacillus amyloliquefaciense* B-1895 و*Lactobacillus casei* CNCMi 1572 و mix lactobacilli) أقل مقارنة باستخدامه بمفرده ضد عزلات الراكدة البومانية.

من خلال هذه الدراسة توصلنا إلى أن كولستن تزداد فعاليته المضاده للمايكروبات عندما يمزج بالراشح البكتيري لسلالات المعززات الحيوية وبالتالي سيتم استخدام تركيز أقل من هذا المضاد الحيوي مما يقلل بدوره من الآثار الجانبية التي يسببها الكولستن.

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة ديالى

كلية العلوم

قسم علوم الحياة

النشاط المضاد للميكروبات للكولستن ممزوجاً مع بعض المعززات الحيوية ضد العزلات السريرية لبكتريا الراكدة البومانية

رسالة مقدمة الى

مجلس كلية العلوم – جامعة ديالى

وهي جزء من متطلبات نيل درجة الماجستير في علوم الحياة

من قبل الطالب

منـاف ثـامـر صالـح الـدلـيـمي

بكالوريوس علوم حياة / جامعة ديالى (٢٠١٠)

بإشراف

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