In vitro combinatory antimicrobial effect of crude bacteriocins from *Pediococcus pentosaceus* strains and selected antibiotics against *Pseudomonas aeruginosa* ATCC 10145

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Abstract

Pseudomonas aeruginosa presents a major challenge for treatment in nosocomial infections due to the ability of *P. aeruginosa* to rapidly develop resistance to multiple antipseudomonal drugs during treatment. This high resistance has severely limited the therapeutic options for *P. aeruginosa* infections and caused serious complications such as increase in morbidity and mortality. The increased in multidrug-resistant (MDR) *P. aeruginosa* strains in recent years gave urgency to the discovery of new strategy to combat MDR *P. aeruginosa* infections. Hence, this study explores the efficiency of using crude bacteriocins from *Pediococcus pentosaceus* isolated from local fermented foods and in combination with selected antibiotics to revoke the multi-drug resistance ability of *P. aeruginosa* ATCC 10145. Forty-two lactic acid bacteria (LAB) were isolated from tempeh, tapai ubi and tapai pulut. Only seven isolates successfully inhibited *P. aeruginosa* ATCC 10145 with modified bacteriocin activity that ranging from 31.67 to 126.67 AU.cm/ml. Three LAB, namely TU2, TP1 and TE1 with the highest modified bacteriocin activity were identified as *Pediococcus pentosaceus* via API 50 CHL carbohydrate fermentation test and 16S rDNA sequencing. Crude bacteriocins TU2, TP1 and TE1 demonstrated MIC of 15.63 mg/ml against *P. aeruginosa* ATCC 10145. Meanwhile, *P. aeruginosa* ATCC 10145 displayed high sensitivity towards ciprofloxacin at MIC of 0.25 µg/ml, while remained resistant to tetracycline and chloramphenicol at MIC 32 µg/ml. The fractional inhibitory concentration (FIC) checkerboard assay revealed that the combination respective tetracycline and ciprofloxacin with crude bacteriocins resulted in synergistic interaction in inhibiting *P. aeruginosa* ATCC 10145. While, the combination of crude bacteriocins and chloramphenicol had indifferent effects. Time-kill assay further confirmed the synergistic interaction of combination of crude bacteriocins with tetracycline with bactericidal inhibition detected after 10 h of incubation. However, the combination of crude bacteriocins with chloramphenicol also successfully inhibited *P. aeruginosa* ATCC 10145 totally after 8 h of incubation. Contradict to the result of FIC assay, the time-kill assay revealed that treatment

with ciprofloxacin alone was more effective in killing *P. aeruginosa* ATCC 10145 within 2 h of incubation than in combination with crude bacteriocins. Untreated *P. aeruginosa* ATCC 10145 (control) cells were observed to be rod-shaped, smooth and intact cell walls and membrane without any morphological changes at 4 h and formed clumps of cells after 8 h of incubation when examined under scanning electron microscope (SEM). In contrast, treatment of crude bacteriocin TU2 in combination with all antibiotics (chloramphenicol, ciprofloxacin and tetracycline) resulted in similar cell surface morphological changes of *P. aeruginosa* ATCC 10145 such as formation of pores on the cell membranes at 2 h, cell membranes ruptured and released of the cellular contents at 4 h, and cell lysis occurred, resulting in cell death at 8 h of incubation. However, the combination of crude bacteriocin TU2 with chloramphenicol showed distinct cell surface morphological changes such as cell elongation at 2 h of incubation. Whilst *P. aeruginosa* ATCC 10145 cells revealed to be shorter and formed chains when treated with crude bacteriocin TU2 in combination with ciprofloxacin. When compared the three combinations of bacteriocin TU2 with all antibiotics, only the combination of crude bacteriocin TU2 with chloramphenicol caused 100% killing of *P. aeruginosa* cells in the shortest time at 8 h. Hence, the treatment of crude bacteriocin TU2 with chloramphenicol produced the best combination against *P. aeruginosa* ATCC 10145 in this study. SEM images revealed that the the crude bacteriocins enhanced the effects of antibiotics by forming pores on the target membrane of *P. aeruginosa* ATCC 10145 which resulted in cell death. Further studies need to be carried out to provide insights into the action mechanisms of the combination of crude bacteriocins and antibiotics against *P. aeruginosa* prior to future medical and therapeutic applications.

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Abbreviations

Common abbreviations

AMP	Antimicrobial peptide
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
CF	Cystic fibrosis
CFS	Cell-free supernatant
CLSI	Clinical and Laboratory Standards Institute
CM	Cytoplasmic membrane
CMT	Cytoplasmic membrane transporter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
FIC	Fractional Inhibitory Concentration
FICI	Fractional inhibitory concentration index
GRAS	Generally recognized as safe
Lb.	*Lactobacillus*
LAB	Lactic acid bacteria
MBC	Minimum Bactericidal Concentration
MDR	Multidrug-resistance
MDRO	Multidrug-resistance organisms
MDRP	Multidrug-resistance *Pseudomonas aeruginosa*
MH	Mueller-Hinton
MIC	Minimum Inhibition Concentration
MOH	Ministry of Health
MRS	De Man, Rogosa and Sharpe
NCCLS	National Committee for Clinical Laboratory Standards
NSAR	National Surveillance of Antibiotic Resistance
OM	Outer membrane
OMF	Outer membrane factor

PCR	Polymerase chain reaction
rDNA	Ribosomal DNA
SEM	Scanning electron microscope
TB	Tuberculosis
TBE	Tris/Borate/EDTA buffer solution
TEM	Transmission Electron Microscopy
USFDA	U.S. Food and Drug Administration
XDR	Extensively drug-resistant

Units

℃	Degree Celsius
AU	Arbitrary Unit
AU/ml	Arbitrary Unit per ml
CFU/ml	Colony-forming unit per ml
Cm	Centimeter
g	Gravity
h	Hour
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
µl	Microliter
µm	Micrometer
nm	Nanometer
psi	Pound per square inch
v/v	Volume/volume
w/v	Mass/volume

Chapter 1: Introduction

Antibiotics have been found to cure various kind of infectious diseases that once killed people for decades. However, some serious infections have become more difficult to treat and antibiotic-resistant strains are starting to emerge (Boucher *et al.*, 2009; Savjani *et al.,* 2009). Among the top priority of antibiotic-resistant strains according to the World Health Organization (WHO) are carbapenem-resistant Enterobacteriaceae (CRE), carbapenem-resistant *Acinetobacter baumannii* (CRAB) and carbapenem-resistant *Pseudomonas aeruginosa* (CRPsA) (WHO, 2017). These bacteria are hard to treat due to their high level of resistance and are linked with high mortality (WHO, 2017). The European Centre for Disease Control and Prevention reported mortality ranging from 30-70% in patients with CRE bloodstream infections (ECDC, 2016). In Canada, the incidence of CRE infections increased from 0 before 2007 to 0.33 cases/100,000 population in 2015 (Kohler *et al.,* 2018). Whereas, a study of clinical isolates conducted across the United States showed that the percentage of CRAB isolates increased from 21% in 2003-2005 to 48% in 2009-2012 (Bullens *et al.,* 2018). Meanwhile, a study of clinical isolates from 14 countries in Europe revealed that CRPsA isolates increased from 12.3% in 2010 to 30.6% in 2011 (Walter *et al.,* 2019). These overwhelming increase in antibiotic resistances have become a global health concern and is considered by the WHO to be perhaps the most urgent issue facing medical science.

In 2017, WHO has released the recommendations for the precaution and control of CRE, CRAB and CRPsA in healthcare facilities. The guidelines comprise several strategies that should be implemented in an integrated way. Among the recommendations made by the guideline development group (GDG) panels are good hand hygiene compliance, surveillance of the infections, contact precautions and isolation of the infected patients,

environmental cleaning protocols compliance and surveillance cultures of the environment for CRE, CRAB and CRPsA colonizations (WHO, 2017).

WHO is working closely with the Food and Agriculture Organization of the United Nations and the World Organization for Animal Health in leading global efforts against antimicrobial resistance and support the development of new antimicrobial medicines, vaccines and other tools (WHO, 2017). Furthermore, the global priority of CRE, CRAB and CRPsA for research and development has been emphasized by WHO with drug discovery and development strategies focus urgently on novel antibiotics specifically active against Gram-negative bacteria such as *Pseudomonas aeruginosa* (WHO, 2017).

Pseudomonas aeruginosa is a well-known opportunistic pathogen and frequently related to nosocomial infections and ventilator-associated pneumonia (Barbier *et al*., 2013). *P. aeruginosa* presents a great challenge in the clinical environment because of its multidrug-resistance (MDR) (El Zowalaty *et al*., 2015) and hence the focus of current study. As a matter of fact, numerous studies highlighted the link between multidrug-resistance with increased morbidity and mortality, increased length of hospital stay and higher hospital costs (Slama, 2008; Tumbarello *et al*., 2011). Hence, novel substances and innovative therapies are continuously being discovered to overcome the challenges and rapid development of the drug-resistant infections caused by *P. aeruginosa*.

Bacteriocin has been proposed as a promising natural antimicrobial peptide (AMP) as an alternative to replace and to enhance the efficiency of antibiotics with the purpose of treating multiple drugs resistance pathogens (Yang *et al*., 2014). Bacteriocin is generally defined as peptide or protein ribosomally synthesized by bacteria that inhibit or kill other related or unrelated microorganisms (Cotter *et al.,* 2005). Since the majority of bacteriocin produced by lactic acid bacteria (LAB) have GRAS (Generally Recognized as Safe) status and they are easily digested by human gastrointestinal tract,

bacteriocins are attracting considerable interest as safe alternatives to conventional antibiotics which have many adverse effects to human (Silva *et al.,* 2018). Minahk *et al*. (2004) reported that the bacteriostatic activity of some clinical antibiotics were strongly increased in the presence of sublethal concentrations of bacteriocin. Hence, this study hypothesizes that bacteriocins produced by *Pediococcus pentosaceus* strains isolated from local fermented food could be used as putative anti-pseudomonal agents and might induce synergistic antagonism with selected antibiotics to reverse the antibiotic resistance ability of *P. aeruginosa*.

1.1 Objectives

This study is in line with the Malaysian Action Plan on Antimicrobial Resistance or known as MyAP-AMR (2017-2021) which has been established by the Malaysian government due to the awareness of the impact of antimicrobial resistance (AMR) across the population in Malaysia. Solutions are urgently required for the growing number of infections caused by multidrug-resistant *P. aeruginosa*. So far, to the author's knowledge, very few studies have been conducted involving combinations of bacteriocin from *Pediococcus pentosaceus* with antibiotics against other pathogens. Hence, this study explores the efficiency of using crude bacteriocins from *Pd. pentosaceus* isolated from local fermented foods and in combination with selected antibiotics to revoke the multi-drug resistance ability of *P. aeruginosa* ATCC 10145.

The specific objectives of this study were:

1. To isolate and screen for putative bacteriocin-producing lactic acid bacteria (LAB) from traditional fermented foods
2. To determine the synergistic interactions between crude bacteriocins and selected antibiotics against multidrug-resistant *P. aeruginosa* ATCC 10145.

3. Effect of crude bacteriocins and selected antibiotics on the cell surface morphological changes on *P. aeruginosa* ATCC 10145

Chapter 2: Literature Review

2.1 Background of multidrug-resistance organisms

Multidrug-resistance organisms (MDRO) refer to pathogens that have become *in vitro* resistant to multiple antimicrobial drugs (Magiorakos *et al.*, 2012) and was first detected in *Escherichia coli*, *Salmonella* and *Shigella* in the late 1950s to early 1960s (Levy, 2001). The condition of patients infected with MDRO tend to be worse than the patients infected with more drug-susceptible organisms (Vardakas *et al*., 2013). The MDRO have a huge impact on modern medicine and might affect medical treatment such as cancer care, transplantation and surgical procedures (Perez & Van Duin, 2013).

Indeed, some strains have become resistant to almost all the commonly prescribed antibiotics. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) was found to be resistant not only to methicillin (which was developed to fight against penicillinase-producing *S. aureus*) but also to aminoglycosides, macrolides, tetracycline, chloramphenicol and lincosamides. Thus, MRSA was said to be responsible for major sources of hospital-acquired infections (Lencastre *et al*., 2007).

According to Papp-Wallace *et al*. (2011), almost all members of Family Enterobacteriaceae were used to be susceptible to antibiotic carbapenems. However, several studies clearly showed that resistance to carbapenems is escalating worldwide (Chouchani *et al*., 2011; Gopalakrishnan & Sureshkumar, 2010; Nicasio *et al*., 2008). In the early 2000s, carbapenem resistance in *Klebsiella pneumoniae* and other Enterobacteriaceae strains were uncommon in North America. However, carbapenem-resistance Enterobacteriaceae (CRE) began to spread throughout USA after initial outbreaks occurred in hospitals in the Northeast in New York City. By 2009–2010, the National Health-care Safety Network from the Centers for Disease Control and Prevention (CDC) revealed that 12.8% of *Klebsiella*

pneumoniae isolates associated with bloodstream infections were resistant to carbapenems (Sievert *et al*., 2013).

Recently, the WHO has referred *Pseudomonas aeruginosa* as one of the bacterial species in which there is a critical need for the development of the new antibiotics to treat *P. aeruginosa* infections (Tacconelli *et al*., 2018). *P. aeruginosa* displayed resistances against a variety of antibiotics such as aminoglycosides, quinolones and β-lactams (Hancock & Speert, 2000), hence this study focuses on the multidrug-resistant *P. aeruginosa*.

2.2 Multidrug-resistant (MDR) *P. aeruginosa*

Pseudomonas infections are diseases originated from bacteria of the genus *Pseudomonas* and are found widely in the nature such as in soil, water and plant (Noura *et al.,* 2009). The most common type of bacteria strains causing infections in human is called *Pseudomonas aeruginosa* (CDC, 2013). *P. aeruginosa* is a Gram-negative, rod-shaped and monoflagellated bacterium that grows well at 25°C to 37°C (Wu *et al.*, 2015). It is a ubiquitous microorganism which can tolerate a variety of environmental conditions and survive on minimal nutritional requirements allowing this organism to remain in both community and hospital settings (Lister *et al*., 2009). In the hospital, *P. aeruginosa* can be isolated from a variety of sources including respiratory therapy equipment, soap, sinks, mops and medicines (Pollack, 1995).

From 1992 to 1997, data from National Nosocomial Infections Surveillance System showed that *P. aeruginosa* was responsible for 21% of pneumonias, 10% of urinary tract infections, 3% of bloodstream infections and 13% of eye, nose and throat infections within ICUs in the United States (Richards *et al.,* 1999). *P. aeruginosa* was found to be resistant to several antipseudomonal drugs such as β-Lactams, fluoroquinolones and aminoglycosides (Lister *et al.,* 2009).

Previously, *P. aeruginosa* is the main bacteria that is responsible for nosocomial infections and accounted for 10% of all hospital-acquired

infections (Morrison *et al*., 1984). Recently, a latest study by Raman *et al*. (2018) showed that MDR *P. aeruginosa* was responsible for 13–19% of healthcare-associated infections in the US in the present year. *P. aeruginosa* is resistant to almost all antibiotics, which seriously challenges the treatment of immunocompromised individuals and can result in death (Levy, 1998).

In a study conducted by Raman *et al*. (2018), it was demonstrated that patient-to-patient transmission of resistant strains as well as newly acquired resistance due to previous antibiotic exposure were among the factors that contributed to the increasing level of resistance in MDR *P. aeruginosa*. Bodro and her co-workers (2015) revealed that 49 out of 318 bacteremia infection cases in solid organ transplant recipients were caused by *P. aeruginosa*. Moreover, thirty-one strains (63%) were found to be extensively drug-resistant (XDR) *P. aeruginosa.*

In a retrospective cohort study performed by Cao *et al.* (2004), it was demonstrated that of 44 cases of multidrug-resistant *Pseudomonas aeruginosa* (MDRP) infections, 20 patients died directly from *P. aeruginosa* infections. This study also concluded that mechanical ventilation and antibiotic resistance switch were predictive factors of the outcomes of MDRP infections.

In another retrospective cohort study conducted by Palavutitotai *et al*. (2018), it was revealed that the prevalence of XDR-*Pseudomonas aeruginosa* infections represented almost a quarter of *P. aeruginosa* hospital-acquired infections and manifested a higher mortality. In this study, it was shown that out of 255 patients with *P. aeruginosa* infections, 56 patients were due to XDR-*P. aeruginosa* and 32 patients to MDR-*P. aeruginosa.*

From these published reports, it can be concluded that *P. aeruginosa* presents a major challenge for treatment in nosocomial infections. The ability of *P. aeruginosa* to rapidly develop resistance to multiple antipseudomonal drugs during the course of treatment have severely limit the therapeutic

options for *P. aeruginosa* infections and caused serious complications such as increase in morbidity and mortality. The increased in MDR *P. aeruginosa* strains in recent years gave urgency to the discovery of new antibiotic to combat *P. aeruginosa* infections. Hence, this study focuses on the production of novel bacteriocin from traditional fermented foods which could potentially be used as antipseudomonal agent to overcome the *P. aeruginosa* infections in immunocompromised population.

2.2.1 MDR *Pseudomonas aeruginosa* cases in Malaysia

In year 2000, the Malaysian government has established the National Surveillance of Antibiotic Resistance (NSAR) programmes in Malaysian hospitals (MOH, 2017). According to the NSAR, *Acinetobacter baumanii* resistant to meropenem has risen from 49% in 2008 to 61% in 2016 (MOH, 2017). Meanwhile, the CRE Surveillance in 2016 reported an alarming increase in the number of cases from 28 cases in 2011 to more than 800 cases in 2016. Analysis of these cases showed that 95% of the patients have history of antibiotic exposure and out of these, 50.6% has history of antibiotic exposure of more than 7 days (MOH, 2017).

Due to the rising problems of antimicrobial resistance (AMR) in Malaysian hospital and their implications to the community, the Ministry of Health Malaysia (MOH) has developed the Malaysian Action Plan on AMR (MyAP-AMR) to address the AMR situation in Malaysia. The MyAP-AMR outlines the views of stakeholders from across the sectors of human and animal health for priority areas such as; public awareness and education, surveillance and research, infection prevention and control, and appropriate use of antimicrobials (MOH, 2017).

In a study conducted by Al-Kabsi *et al.* (2011), a total of 88 clinical isolates of *P. aeruginosa* were collected randomly between April 2009 and March 2010 from the University of Malaya Medical Center. In this study, it was revealed that *P. aeruginosa* isolated from various clinical samples have lost

susceptibility and showed increasing resistance to gentamicin with 94.3%, followed by ciprofloxacin with 92%, ceftazidime with 89.8%, imipenem with 73.9% and amikacin with 50%. Most of the clinical isolates had a high level of resistance to examined antibiotics due to the inappropriate and incorrect administration of antibiotics to the patients.

In a study conducted by Pathmanathan *et al*. (2009), a total of 97 consecutive clinical isolates of *P. aeruginosa* were collected between October 2007 and December 2007 at the Kuala Lumpur Hospital. It was demonstrated that piperacillin-tazobactam was the most active antimicrobial agent with 91.8% susceptibility, followed by the aminoglycosides (amikacin, 86.6% and gentamicin, 84.5%), the quinolone (ciprofloxacin, 83.5%) and the beta-lactams (cefepime, 80.4%, ceftazidime, 80.4%, imipenem, 79.4% and meropenem, 77.3%). Furthermore, the incidence of multidrug-resistance was found to be 19.6% (19 out of 97 isolates).

A retrospective study on 48 clinical isolates from 6 public hospitals in Malaysia was carried out by Lim and his co-workers (2009). It was demonstrated that 69% of the *P. aeruginosa* isolates analyzed were multidrug-resistant. Most of the *P. aeruginosa* isolates were resistant to tetracycline (73%) and chloramphenicol (60%). Apart from that, it was also found that less than 20% of the isolates were resistant to ciprofloxacin. Clearly based on these reports, the threat of AMR of *P. aeruginosa* in Malaysia is growing at an alarming rate with the misuse of antibiotics as the main cause of resistance. The implementation of MyAP-AMR nationwide may prevent and curb the spread of AMR in Malaysian community. One of the expected outcomes of the action plan includes reduction of the volume of antibiotic used for human consumption (MOH, 2017).

2.2.2 Antibiotic resistance mechanisms of *P. aeruginosa*

Generally, antibiotic resistance mechanisms of *P. aeruginosa* can be divided into two types: intrinsic resistance and acquired resistance. Intrinsic

resistance refers to resistance that is a consequence of a large selection of genetically-encoded mechanisms which may decrease the effectiveness of a specific antibiotic (Blair *et al*., 2015). Meanwhile, acquired mechanism refers to resistance that is achieved via the acquisition of additional mechanisms or is a consequence of mutational events under selective pressure (Poole, 2011).

2.2.2.1 Intrinsic resistance mechanisms of *P. aeruginosa*

P. aeruginosa is intrinsically resistance to many classes of antimicrobial agents. The high intrinsic antibiotic resistance of *P. aeruginosa* is due to several mechanisms: a low outer membrane permeability, expression of efflux pumps that expel antibiotics out of the cell and the production of antibiotic-inactivating enzymes (Breidenstein *et al*., 2011).

a. The outer membrane as a barrier

According to Lambert (2002), the outer membrane of *P. aeruginosa* serves as a semipermeable barrier to the penetration of antibiotics by limiting the rate of penetration of small hydrophilic molecules and eliminating larger molecules. Porins play an important physiological role in the transport of sugars, amino acids, phosphates, divalent cations and siderophores (Hancock and Brinkman, 2002) into the cell.

Certain hydrophilic antibiotics such as β-lactams, aminoglycosides, tetracyclines and some fluoroquinolones have been demonstrated to diffuse into the outer membrane via porin channels (Nikaido, 1989). The pathways for penetration of β-lactams and quinolones through porin channels in the outer membrane (OM), whilst aminoglycosides and colistin promote their own uptake by interacting with the lipopolysaccharide (LPS) on the outer face of the OM. Not surprisingly, the loss of specific porin channels can decrease the susceptibility of *P. aeruginosa* to certain antibacterial agents (Nikaido, 1989). According to Trias & Nikaido (1990), porin OprD is a substrate-specific porin that has been shown to facilitate the diffusion of basic amino acids, small

peptides and carbapenems into the cell membrane of *P. aeruginosa.* Several studies revealed that OprD appears to serve as the preferred portal of entry for the carbapenems and loss of OprD from the outer membrane significantly decreases the susceptibility of *P. aeruginosa* to available carbapenems (Kohler *et al.,* 1999; Mushtaq *et al.,* 2004).

b. The role of efflux systems in resistance

While the loss of porins such as OprD appeared to be an effective barrier for antibiotic entry into the cell, a depletion in drug accumulation can also be achieved via membrane-associated pumps or also known as efflux system. Many of the compounds such as antibiotic that can pass through the OM are actively transported out of the cell again by efflux pumps activated by the proton motive force (Kumar & Schweizer, 2005). The most clinically relevant multidrug efflux systems in Gram-negative bacteria are those of the resistance-nodulation-cell division (RND) family (Lister *et al.,* 2009). *P. aeruginosa* expresses several RND-type multidrug efflux systems such as: MexAB-OprM, MexXY-OprM, MexCD-OprJ and MexEF-OprN (Poole, 2011). These pumps are described in *P. aeruginosa* strains and contribute strongly to a reduced susceptibility towards antibiotics and act synergistically with the low permeability of outer membrane (Lister *et al.,* 2009).

In 1993, the first RND-type multidrug efflux system of *P. aeruginosa* MexAB-OprM was discovered. It was the first genetic evidence that an efflux operon was involved in multiple antibiotic resistance in *P. aeruginosa* (Poole *et al.*, 1993). This study also demonstrated that the resistance-nodulation-division (RND) pumps is a tripartite complex consisting of a periplasmic membrane fusion protein (MFP) (such as mexA, mexC, mexF and mexY), an outer membrane factor (OMF) (such as oprM, oprJ and oprN) and a cytoplasmic membrane transporter (CMT). This complex forms a channel acrossing the entire membrane, allowing for the transportation of antibiotics from the periplasmic space and cytoplasm to the extracellular environment (Lister *et*

al., 2009). Antibiotics which have entered the cell are collected from the cytoplasmic membrane and expelled from the cells through the porin. The MexAB-OprM system has been demonstrated to contribute to the high intrinsic antibiotic resistance of *P. aeruginosa* and hyperexpression of these efflux genes was found to be responsible for the elevated multidrug resistance in *P. aeruginosa* strains *nalB* mutants (Li *et al.,* 1995). Kohler *et al.* (1996) demonstrated that the MexAB-OprM multidrug efflux system is mainly responsible for the intrinsic resistance of *P. aeruginosa* to fluoroquinolones, tetracyclines, chloramphenicol, β-lactams and β-lactamase inhibitors, macrolides, novobiocin, trimethoprim and sulfonamides.

The efflux pumps have different substrates specificities and their productions and activities can be increased by many factors commonly present in infections such as high inocula of bacteria, low pH and stationary-phase growth (Aeschlimann, 2003). MexAB-OprM is the only pump in *P. aeruginosa* that is constitutively expressed and can transport most of the antibiotic families whereas the others are more selective and are induced under specific conditions (Housseini *et al.,* 2018). Meanwhile, MexCD-OprJ was demonstrated to extrude a variety of antimicrobial agents including fluoroquinolones, β-lactams, chloramphenicol, tetracycline, novobiocin, trimethoprim and macrolides (Gotoh *et al.,* 1998). According to Ziha-Zarifi *et al.* (1999), the investigation of resistance mechanisms of *P. aeruginosa* revealed that MexXY-OprM multidrug efflux system extruded aminoglycosides whilst MexEF-OprN efflux system expelled carbapenems and quinolones.

c. Antibiotic-inactivating enzymes

The production of antibiotic-inactivating enzymes that break down or modify antibiotics is one of the major mechanisms of intrinsic resistance in bacteria. *P. aeruginosa* could produce enzymes such as β-lactamases and aminoglycoside-modifying enzymes which may hydrolyzed the chemical bonds of antibiotics (Poole, 2005; Wolter & Lister, 2013).

Like other Gram-negative bacteria, *P. aeruginosa* possesses an inducible *ampC*gene encoding the hydrolytic enzyme β-lactamase. This enzyme can break the amide bond of β-lactam ring, leading to inactivation of β-lactam antibiotics (Wright, 2005).

Berrazeg *et al.* (2015) reported that the class C β-lactamase produced by *P. aeruginosa* has been shown to inhibit antipseudomonal cephalosporins, a class of β-lactams. In another study, some *P. aeruginosa* isolates have been found to produce extended-spectrum-β-lactamases (ESBLs) which confer a high degree of resistance to the majority of β-lactam antibiotics, including penicillins, cephalosporins and aztreonam (Paterson & Bonomo, 2005; Rawat & Nair, 2010).

2.2.2.2 Acquired resistance mechanisms of *P. aeruginosa*

Bacteria can acquire resistance in two ways: either through mutational changes or acquisition of resistance genes via horizontal gene transfer (Munita & Arias, 2016). This happens when bacteria changes in a way that protects it from the antibiotic.

a. Resistance by mutations

A study conducted by Mandsberg *et al.* (2009) demonstrated that inactivation of the DNA oxidative repair system increase mutation frequencies in *P. aeruginosa* leading to enhanced enzyme β-lactamase production and overexpression of the MexCD-OprJ efflux pump. In another study, it was demonstrated that spontaneous mutations could affect the expression and function of specific porin, thereby reducing bacterial membrane permeability and increasing antibiotic resistance (Fernandez & Hancock, 2012).

b. Mechanism of horizontal gene transfer

Antibiotic resistance genes can be carried out on plasmids, transposons, integrons or prophages and bacteria can acquire these antibiotic resistance genes via horizontal gene transfer from the same or different bacterial

species (Breidenstein *et al.,* 2011). The main mechanisms of horizontal gene transfer involve transformation, transduction and conjugation (Arber, 2014). Acquisition of aminoglycoside and β-lactam resistance genes has been reported in *P. aeruginosa*. According to Hong *et al.* (2015), six types of *P. aeruginosa* metallo-beta-lactamases (MBLs) which belong to class B β-lactamases that hydrolyze most β-lactam-based antibiotics have been described in their study. The genes for these *P. aeruginosa* MBLs have been detected being carried by genetic elements including integrons and plasmids. Furthermore, multiple antibiotic resistance genes can be carried in a single integron. Poirel and his co-workers (2001) have identified two novel aminoglycoside resistance genes, *aacA29a* and *aacA29b* which are located at the 5′ and 3′ end of the carbapenem-hydrolyzing β-lactamase VIM-2 gene cassette respectively, in class I integrons of *P. aeruginosa* clinical isolates.

2.3 Background of traditional fermented foods

Fermented foods are obtained through the action of microorganisms, namely bacteria, yeasts and fungi and their enzymes, in a process referred to as fermentation (Frias *et al*., 2016). Fermentation is a slow decomposition process of organic substances induced by microorganisms or enzymes that essentially convert carbohydrates to alcohols or organic acids (FAO, 1998). Fermentation is the oldest known form of food biotechnology, which has been practiced for thousands of years by the ancient people and has long been used to preserve and enhance the shell-life, flavour and texture of foods (Hutkins, 2018).

The importance of fermented foods has gained considerable attention since 1907, when Nobel Prize laureate Elie Metchnikoff, assessed the beneficial effects of fermented milks on the longevity of Bulgarian populations and introduced the concept of probiotics (Hemarajata & Versalovic, 2013). According to WHO, probiotics are termed as live microorganisms which when administered in adequate amounts confer a health benefit to the host (WHO,

2006). The consumption of fermented foods that rich in probiotics can bring many advantages to human and has emerged as important dietary strategy for improving human health (Marco *et al*., 2017).

2.3.1 Fermented cassava, fermented glutinous rice and fermented soybean cake

In this study, three different types of fermented foods that are commonly found in Malaysia were used; fermented cassava "tapai ubi", fermented glutinous rice "tapai pulut" and fermented soybean cake "tempeh". There are various names for fermented cassava, such as "tape ketella", as it is commonly called by Indonesians, "tape telo" in Java and "peujeum" in Sudan whereas, fermented glutinous rice is commonly called "tape ketan" in Indonesia and "lao chao" by the Chinese (Halim *et al.,* 2014). "Tapai" is a popular dessert or snack that is produced in the region on a cottage industry scale by traditional manufacturers or at home for family consumption. It is a perishable product and has to be consumed immediately within 3 to 4 days after the optimum stage of fermentation (Law *et al.*, 2011).

"Tempeh" is a fermented soybean product originally made by Javanese people through fermentation with *Rhizopus* species and its popularity has increased due to its superior nutritive qualities (Matsumo & Imai, 1990). Fermented food such as "tempeh" form an important source of protein, calories and vitamins besides being freely available, cheap and requiring no special storage conditions (Law *et al*., 2011). Among the health benefits of the long consumption of "tempeh" are bowel health, menopausal health and protection against cardiovascular disease (Astuti *et al*., 2000).

The traditional fermented foods such as fermented cassava, fermented glutinous rice and fermented soybean cake are generally fermented by LAB such as *Lactobacillus plantarum*, *L. pentosus*, *L. brevis*, *L. fermentum*, *L. casei*, *L. kimchi*, *L. fallax*, *Weisella confuse*, *W. cibaria* and *Pediococcus*

pentosaceus, which are considered as the probiotic source of the food practice (Anandharaj & Sivasankari, 2013).

2.4 *Pediococcus pentosaceus*

In current study, several strains of *Pediococcus pentosaceus* were isolated from local fermented foods. *Pd. pentosaceus* is Gram-positive, facultatively anaerobic and non-motile member of the industrially important lactic acid bacteria (Schlegel, 1993). The formation of tetrads via cell division in two perpendicular directions in a single plane is a distinctive characteristic of *Pediococci* (Simpson and Taguchi, 1995).

Like other lactic acid bacteria, *Pd. pentosaceus* are acid tolerant, cannot synthesize porphyrins and purely homofermentative which possess a strictly fermentative metabolism with lactic acid as the major metabolic end product (Axelsson, 1998).

2.4.1 Importance of *Pediococcus pentosaceus*

Pd. pentosaceus is a member of lactic acid bacteria group that is widely present in foods and commonly used as bio-preservatives, in addition to scent, texture and flavor enhancers. In the last decade, various studies on *Pd. pentosaceus* have been published and *Pd. pentosaceus* was found to exhibit probiotic properties (Dubey *et al.,* 2015) and cholesterol-lowering activity (Guardamagna *et al.,* 2014). Published study also revealed that *Pd. pentosaceus* could produce bacteriocin which may exert antimicrobial effect against pathogenic bacteria (Cotter *et al.,* 2012).

In a study conducted by Huang and his co-workers (2014), it was suggested that *Pd. pentosaceus* strain 4012 (LAB4012) isolated from cobia intestine can be a dietary probiotic for groupers in modulating the immunity and protecting the groupers from *V. anguillarum* infection. Meanwhile, according to Damodharan *et al.* (2015), the *in vitro* and *in vivo* studies of characterization of probiotic properties of *Pd. pentosaceus* strain, KID_7 revealed that the strain could be a potential probiotic strain which can be used to develop

cholesterol-lowering functional food. It was also found that LDL cholesterol levels in HCD-KID7 (atherogenic diet with KID_7 strain) group were decreased by 35.5% compared with HCD (atherogenic diet only) group.

Furthermore, *Pd. pentosaceus* produce various antimicrobial substances against Gram-positive and Gram-negative pathogens (Jamuna & Jeevaratnam, 2004). In a study by Jamuna & Jeevaratnam (2004), it was demonstrated that the bacteriocin from *Pd. pentosaceus* showed antimicrobial activities on *Salmonella typhimurium* ATCC 19430, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococus aureus* ATCC 25923 and *Micrococcus luteus* ATCC 10240. Other past study also revealed that *Pd. pentosaceus T1* isolated from kimchi could be a potential antilisterial agent in fish products as well as a starter to control over maturation of kimchi. Hence, *Pd. pentosaceus T1* was found to be effectively inhibited proliferation of *Listeria monocytogenes* in salmon fillet and was a stronger antilisterial agent than that of nisin (Jang *et al.,* 2015). Therefore, these past findings suggested that bacteriocin from *Pd. pentosaceus* might be useful in the food, clinical and agriculture applications (Nghe & Nguyen, 2014).

2.5 Bacteriocins

Bacteriocins are protein or antimicrobial peptide (AMP) synthesized by ribosome in the bacteria which can kill or inhibit other closely-related (narrow spectrum) or non-related (broad spectrum) bacterial strains using a natural bacterial immune weapon system (Yang *et al.,* 2014).

2.5.1 Applications of bacteriocins

Bacteriocins are considered as safe and natural product because they can be easily degraded by proteolytic enzymes of the mammalian gastrointestinal tract. Apart from that, most bacteriocin producers belong to lactic acid bacteria (LAB), a group that occurs naturally in foods and have a long history of safe use in food industry (Silva *et al.,* 2018).

a) Bacteriocins as natural preservatives in food products

In order to extend the shelf-life of foods, food preservatives are incorporated to delay microbial growth. However, most chemical preservatives such as nitrates, benzoate and sulfites are developed via chemical synthesis and long-term consumption may have adverse effects in human (Mowafy *et al.,* 2001). In contrast, bacteriocins produced by bacteria are suitable as natural preservatives in food products due to the sensitivity of bacteriocins to some proteases causing harmless bacteriocins digested in the guts (Bernbom *et al.*, 2006). Currently, bacteriocins produced by LAB are extensively studied due to their generally GRAS status. Hence, bacteriocins are considered as safe food additives after intake by the gastrointestinal system.

Nisin produced by *Lactococcus lactis* and was the first bacteriocin found in LAB. Originally reported in 1928, nisin has been used as a natural preservative in a variety of foods since the 1950s (FAO, 2016). It was approved by the Food and Agriculture Organization of the United Nations (FAO) and World Health Organization (WHO) in 1969 (FAO, 2016) and licensed as a food additive and marketed as Nisaplin® in over 45 countries (Settanni & Corsetti, 2008). According to Wessels *et al.* (1998), nisin has been added directly to cheese spread to control Clostridium spores. In another study reported by Rodrigez *et al.* (2001), a reduction of *Listeria monocytogenes* was observed in raw milk cheese using nisin-producing *Lactobacillus lactis* as starter culture.

Another commercially available bacteriocin is pediocin PA-1 produced by *Pediococcus* spp. marketed as Alta® 2341 or Microgard™ which inhibits the growth of *Listeria monocytogenes* in meat products (Garsa *et al.*, 2014). This bacteriocin has been shown to be more effective than nisin against some food-borne pathogens such as *L. monocytogenes, S. aureus* (Cintas *et al.,* 1998) and Gram-negative organisms such as *Pseudomonas* and *Escherichia coli (*Jamuna & Jeevaratnam, 2004). Recently, Verma *et al.* (2017) reported

that semi-purified pediocin containing fermented cheese whey was shown to be effective in reducing *S. aureus* counts and enhancing the shelf-life of raw buffalo milk.

Mills *et al.* (2011) studied bacteriocin plantaricin-producing *Lactobacillus plantarum* strain isolated from soft French artisanal cheese as an anti-listerial in the presence of nisin-producing starters for the manufacture of cheese. The study revealed that the combination of *Lb. plantarum* strain with a nisin producer reduced the growth of *Listeria innocua*. Moreover, it was also demonstrated that *Lb. plantarum* was much more effective in inhibiting *Listeria innocua* than the nisin producer alone.

b) Bacteriocins as antimicrobial agents in the treatment of pathogen-associated diseases

The rising problem with multiple drug-resistance pathogens is a global concern due to the misuse of antibiotics among the population. Bacteriocins are reported to inhibit many animal and plant pathogens and could be considered as one of the possible alternative treatment over the use of conventional antibiotics.

According to De Kwaadsteniet *et al*. (2009), nisin F produced by *L. lactis* subsp. *lactis* F10 was demonstrated to inhibit the growth of *Staphylococcus aureus* K in the respiratory tract of immunocompromised rats. This study revealed that nisin F was proved to be non-toxic and may be used to control the respiratory tract infections caused by *S. aureus* in animal model.

A study conducted by Corr and his co-workers (2007) demonstrated the ability of bacteriocin producer *L. salivarius* UCC 118 to inhibit pathogens in the gastrointestinal tract. The study found that the production of the bacteriocin Abp 118 from *L. salivarius* UCC 118 inhibited the growth of *L. monocytogenes* in the gastrointestinal tract of mice.

According to Martin-Visscher *et al*. (2011), three bacteriocins carnocyclin A (CclA), carnobacteriocin BM1 (CbnBM1) and piscicolin 126 (PisA) produced by *Carnobacterium maltaromaticum* UAL307 were evaluated against three Gram-negative bacteria (*Escherichia coli* DH5α, *Pseudomonas aeruginosa* ATCC 14207 and *Salmonella Typhimurium* ATCC 23564). The study revealed that the three bacteriocins inhibited Gram-negative bacteria when the outer membrane is weakened and the different classes of bacteriocins exerted unique mode of actions against pathogenic bacteria.

2.5.2 Mode of actions of bacteriocins

Bacteriocins can be classified into several types of classes based on their structures as well as the basis of mode of actions. Due to their great variety of chemical structures, bacteriocins affect different essential functions of the living cells such as transcription, translation, replication and cell wall biosynthesis. However, most of them act by forming membrane channels or pores that destroy the energy potential of target cells (Oscariz & Pisabarro, 2001). Furthermore, most of the bacteriocins produced from LAB exert their antibacterial effect by targeting the cell envelope-associated mechanisms (Cotter *et al.,* 2012).

Several lantibiotics and some class I bacteriocins target Lipid II, the main transporter in the peptidoglycan biosynthesis machinery within the bacterial cell envelope. This resulted in inhibition of peptidoglycan synthesis and therefore preventing correct cell wall synthesis leading to cell death (Breukink & de Kruijff, 2006).

Other bacteriocins use Lipid II as a docking molecule to initiate a process of membrane insertion and facilitate pore formation resulting in variation of the cytoplasm membrane potential and ultimately, cell death (Machaidze & Seelig, 2003). Class I bacteriocins such as nisin, the most studied lantibiotic, is capable of both mechanisms (Cotter *et al*., 2005).

Some Class II bacteriocins such as lactococcin A, damage or kill target cells by binding to the cell envelope-associated mannose phosphotransferase system (Man-PTS) (Cotter *et al*., 2012). In general, Class II peptides have amphiphilic helical structures, which allow them to insert into the membrane of the target cells, forming pores which lead to depolarization and death (Cotter *et al.,* 2012).

Other bacteriocins can kill their target cells by inhibition of gene expression for example, microcin B17 (MccB17) inhibits DNA gyrase, MccJ25 inhibits RNA polymerase and MccC7-C51 inhibits aspartyl-tRNA synthetase (Chen & Hoover, 2003; Cotter *et al.,* 2012).

Chapter 3: Isolation, screening and identification of bacteriocin-producing lactic acid bacteria from traditional fermented foods

3.1 Introduction

Due to the rising concerns on AMR, the need to seek for novel antimicrobial which derived from natural and safe sources are on rise. Bacteriocin from LAB are known to be safe, non-toxic and may inhibit multidrug-resistant pathogens such as *P. aeruginosa* (Mokoena, 2017). Bacteriocins are usually ribosomally synthesized antimicrobial peptides (AMP) that can inhibit closely-related or non-related bacteria. In fact, bacteriocins become powerful antimicrobial agents mainly due to large diversity of structures and functions, generally harmless to human body and environment, and being stable to heat (Yang *et al*., 2014).

Therefore, current chapter aimed to isolate and screen for bacteriocin-producing lactic acid bacteria from traditional fermented foods. The putative LAB strains were identified using API 50 CHL kit and 16S rDNA sequencing and bacteriocin activity were determined using critical dilution method against *P. aeruginosa* ATCC 10145.

3.2 Materials and methods

3.2.1 Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from three samples of local fermented foods, fermented cassava “tapai ubi”, fermented glutinous rice “tapai pulut” and fermented soybean cake “tempeh” obtained from different wet markets in Semenyih, Selangor, Malaysia. Initially, 10 g of each sample was weighed and added into 90 ml of peptone-buffered water (Oxoid, UK). After 30 min of homogenization using stomacher bags at room temperature, a ten-fold serial dilution (10^1 to 10^7) the sample was carried out. Diluted samples were spread evenly on de Man, Rogosa and Sharpe (MRS; Merck, Germany) agar plates. The plates were incubated for 48 h at 37℃ in the incubator (Memmert, Germany). After incubation, colonies with different colony

morphology were selected and streaked on new MRS agar plate for single colony. The isolate with single colony was sub-cultured in MRS broth and incubated for 24 h at 37℃ and stored as 20% (v/v) glycerol stock at -20℃ freezer till use.

3.2.2 Screening of bacteriocin producing lactic acid bacteria

a. Preparation of bacterial culture

The LAB isolates obtained from Section 3.2.1 were screened for production of bacteriocins. Then, 2%(v/v) of 24 h-old LAB isolate was inoculated in 10 ml of MRS broth and incubated at 37℃ for 24 h. The cell-free supernatant (CFS) was collected by centrifugation of the bacterial culture at 6000 *g* for 15 min. After that, the CFS were collected carefully and filtered through 0.22 µm milipore (Sartorius, Germany) into new sterile Eppendorf tubes, prior to agar-well diffusion assay.

b. Preparation of indicator pathogen

Pseudomonas aeruginosa ATCC 10145 was obtained from Microbiology Laboratory, University of Nottingham Malaysia Campus. Then, 2%(v/v) of 24 h-old *P. aeruginosa* ATCC 10145 was inoculated in 10 ml of Brain Heart Infusion (BHI) (Merck, Germany) broth and incubated at 37℃ for 24 h. After the incubation, the culture was ready to use for agar-well diffusion assay. *P. aeruginosa* ATCC 10145 was maintained in BHI broth and stored as 20%(v/v) glycerol stock till use.

3.2.3 Agar-well diffusion assay

The antagonistic activity of the forty-two LAB isolates were determined by using Agar-well diffusion method according to method of Clinical and Laboratory Standards Institute (CLSI, 2012). Six agar wells were punched on the surface of Brain Heart Infusion (BHI) agar using sterile Durham tube and 20 µL of CFS for the LAB isolates were pipetted respectively into each well and allowed it to dissolve into the well. After that, 50 µL of 24 h-old *P. aeruginosa* ATCC 10145 (1.73×10^9 CFU/ml) was pipetted on the agar

surface and swabbed evenly with sterile cotton swab. Plates were incubated at 37℃ for 24 h and zones of inhibition were observed and measured. The antagonistic activity of the CFS was determined by measuring the diameter of the clear zone formed around the agar well with calliper in mm.

3.2.4 Quantitative measurement of bacteriocin

3.2.4.1 Bacteriocin assay via critical dilution method

Critical dilution method (Mayr-Harting *et al*., 1972; Todorov & Dicks, 2005) was performed together with Agar-well diffusion method to determine the bacteriocin activity of the LAB isolates. The method used was similar to the method described in Section 3.2.3 with the additional step of the neutralization of the CFS to pH 6.5 with 1M NaOH. This step ensures the antagonistic activity is driven by bacteriocin and not organic acid produced by LAB. A two-fold serial dilution (2^0 to 2^{-5}) was carried out by pipetting 500 µl of neutralised CFS into Eppendorf tubes containing 500 µl of 0.85% (w/v) NaCl. The diluted samples were pipetted respectively into each agar well and allowed to dissolve at room temperature. After that, 50 µL of 24 h-old *P. aeruginosa* ATCC 10145 was pipetted on BHI agar surface and swabbed evenly with sterile cotton swab. After 24 h of incubation, the diameter of the clear inhibition zone formed around the wells with the highest dilution factor was measured.

Bacteriocin activity was expressed as Arbitrary Unit (AU) or Bacteriocin Unit (BU). AU was calculated from the reciprocals of the highest dilution yielding a clear zone of growth inhibition on the indicator lawn. Arbitrary Unit of bacteriocin was defined as amount of bacteriocin in 1ml of the bacteriocin preparation, as shown below:

$$\text{Bacteriocin Unit (AU/ml)} = \frac{D}{M}$$

Where:

D = Highest dilution yield a clear zone (AU)

M = Amount of sample apply into each well (ml)

The formula to calculate BU was modified to incorporate the diameter of inhibition zone and this calculation was shown below:

$$\text{Modified Bacteriocin Unit (AU/ml) (cm)} = \frac{D}{M \times Di}$$

Where:

D = Highest dilution yield a clear zone (AU)

M = Amount of mixture apply into each well (ml)

Di = Diameter of clear zone (cm)

3.2.5 Total viable plate count method via Miles misra method

The viable cell count of LAB isolates was determined using Miles misra method (Miles & Misra, 1938). A ten-fold serial dilution from 10^1 to 10^9 was carried out by pipetting 100 µl of bacterial culture into 900 µl of sterile peptone buffered water (Oxoid, UK). Five drops (20 µl/drop) of the ten-fold diluted samples were pipetted onto MRS agar plates and allowed to dissolve totally into the agar. All assays were carried out in triplicate. After the incubation, the agar plates with '3-30' colonies were counted and the total viable cell count was calculated based on following formula:

$$\text{Viable cell count (CFU/mL)} = \frac{C \times D}{V}$$

Where:

C = number of colonies counted

D = dilution factor

V = volume of sample plated (ml)

3.2.6 Identification of selected lactic acid bacteria

The selected bacteriocin-producing LAB isolates were identified using API 50 CHL kit and 16S rDNA sequencing.

3.2.6.1 Preliminary identification of selected lactic acid bacteria via API 50 CHL kit

API 50 CHL kit is used for the identification of lactic acid bacteria based on carbohydrates fermentation pattern (bioMérieux, France). The API test was conducted based on the manufacturer's protocol. An ampule of API suspension Medium (2 ml) was opened as indicated by the manufacturer's instruction. A heavy suspension (S) in the ampule was prepared by picking 24 h-old LAB isolates (1.65×10^8 CFU/ml) into the ampule using a swab. An ampule of API Suspension Medium (5 ml) was opened and a suspension with a turbidity equivalent to 2 McFarland (6.0×10^8 CFU/ml) was prepared by transferring three drops of suspension S into the ampule. An ampule of API 50 CHL Medium was opened and 6 drops of suspension S were inoculated into the ampule. The inoculated API 50 CHL Medium was homogenized and filled into the tubes and overlaid with mineral oil. The test tubes were incubated aerobically at 37℃ for 48 h. A positive test corresponds to acidification revealed by the bromocresol purple indicator contained in the medium changing to yellow. The obtained biochemical profile for the strain was identified using the apiweb™ identification software with database (V5.1) (bioMérieux, France).

3.2.6.2 Molecular identification of selected lactic acid bacteria via 16S rDNA sequencing

The selected bacteriocin-producing LAB strains were identified by using molecular technique of polymerase chain reaction (PCR) and 16S ribosomal DNA (rDNA) sequencing analysis. Prior to PCR and 16S rDNA sequencing, DNA extraction was performed to obtain DNA in a purified form.

a) DNA extraction method

The genomic DNA was extracted from 24 h-old LAB culture (1.65 x 10^8 CFU/ml) according to the procedure of Wizard® Genomic DNA Purification kit (Promega, USA). A 1 ml of 24 h-old LAB culture was added to a 1.5 ml of microcentrifuge tube and was centrifuged at 13000 *g* for 2 min to obtain the cell pellet. The supernatant was removed and the cells were resuspended thoroughly in 480 µl of 50mM EDTA (pH 8.0). The lysozyme buffer (Bio Basic Inc, Canada) was added to the resuspended cell pellet in a total volume of 120 µl and were mixed gently by pipetting. The sample was incubated at 37 ℃ in a water bath (Chemopharm, Malaysia) for 60 min. Then, the sample was centrifuged at 13000 *g* for 2 min and the supernatant were removed. The pellets were collected and Nuclei Lysis Solution (Promega, USA) was added and pipetted gently until the cells were resuspended. The sample was then incubated at 80 ℃ water bath for 5 min for cell lysis. After cooling the mixture to room temperature, 3 µl of Rnase solution (Promega, USA) was added to the cell lysate and the tubes were mixed by inverting for 5 times. The sample was incubated at 37 ℃ water bath for 30 min and then the sample was allowed to cool at room temperature.

A total of 200 µl of Protein Precipitation Solution (Promega, USA) was added to the cell lysate and the sample was then vortexed vigorously at high speed for 20 sec. The sample was then incubated on ice for 5 min, followed by centrifugation at 13000 *g* for 3 min. The supernatant was added with 600 µl of isopropanol and was mixed gently by inversion until the thread-like strands of DNA formed a visible mass. Then, the sample was centrifuged at 13000 *g* for 2 min and the supernatant was poured off carefully and the tube was drained on clean absorbent filter paper (Whatman, UK). After that, 600 µl of 70% (v/v) ethanol was added and the tube was inverted gently to wash the DNA pellet, followed by centrifugation at 13000 *g* for 5 min. The ethanol was aspirated carefully and the tube was drained on clean absorbent paper.

The pellet was air-dried for 15 min, followed by the addition of 50 µl of DNA Rehydration Solution (Promega, USA) to the tube and was incubated at 65 ℃ water bath for 1 h. Finally, the purified DNA was stored at 4 ℃ for further use and DNA purity was then measured using the ratio of the absorbance at 260 nm divided by the reading at 280 nm using spectrophotometer (BioTek, USA). Good quality DNA will have an A_{260}/A_{280} ratio of 1.7 – 2.0.

b) Amplification of 16S rDNA gene by Polymerase Chain Reaction (PCR)

DNA extracted from LAB isolates were amplified using Polymerase Chain Reaction (PCR). The universal primers used for the amplification of 16S rDNA genes were 8F forward primer (Bioline, UK): 5′-AGA GTT TGA TCC TGG CTC AG-3′ and U1492 reverse primer: 5′-GGT TAC CTT GTT ACG ACT T-3′ (James, 2010).

The PCR master mix for 10 reactions was prepared based on Table 3.1 using the DreamTaq DNA Polymerase kit (Thermo Fisher Scientific, USA). The reagents listed in the Table 3.1 were added into the sterile distilled water in a sterile 1.5 ml microcentrifuge tube while kept on ice. A total of 19 µL aliquots of the master mix was distributed into 200 µL PCR tubes and 1 µL of sample with a concentration of 100 ng/µL was added into each tube except for one tube, where 1 µL of distilled water was used instead as the PCR no-template control.

Then, the 200 µL PCR tubes were transferred into a thermal cycler (Eppendorf, Germany) with following: a) 1 cycle of initial denaturation at 95 °C for 5 mins; b) 35 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s; c) 1 cycle of final extension at 72 °C for 5 mins and d) storage at 10 °C till used. Finally, 5 µl of the PCR products were analyzed by 1.5% agarose gel electrophoresis stained with SYBR® safe DNA gel stain (Thermo Fisher Scientific, USA) in 1X TBE buffer (MP Biomedicals, USA) at 90 V for 45 mins.

Table 3.1 : Preparation of master mix for 10 reactions for PCR for LAB strains identification

Reagent	Stock concentration	Volume (µl)	Final concentration
DreamTaq Polymerase Buffer	10x	20	1x
DNTP Mix	2 mM	16	0.16 mM
Forward primer	10 mM	6	0.3 mM
Reverse primer	10 mM	6	0.3 mM
DreamTaq Polymerase	5 U/µl	2	1 U/µl
Sterile distilled water	0	140	0

c) 16S rDNA sequencing

The PCR products of the LAB isolates were outsourced to 1st BASE DNA Sequencing Services (Apical Scientific Sdn Bhd, Malaysia) for DNA sequencing. Then, the sequences generated for the three bacteriocin-producing LAB isolates were subjected to alignment in the genetic sequences database at GenBank® (BLAST, National Center for Biotechnology Information) to determine the identities of the isolates at species level.

3.2.7 Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 25 and each experiment was performed for at least three biologically independent replicates. All values reported were expressed as means ± standard deviation. Statistical comparisons of normal data were performed using one-way analysis of variance (ANOVA) between samples and statistical significances were indicated via labels of different letters. Duncan post hoc test was used at the chosen level of probability ($p < 0.05$) to determine significant differences between means.

3.3 Results and Discussion

3.3.1 Isolation of lactic acid bacteria from fermented foods

Table 3.2 : Number of LAB isolates obtained from three local fermented foods

Food samples	Number of LAB isolates
Tapai ubi	17
Tapai pulut	15
Tempeh	10
Total number of LAB isolates	**42**

A total number of forty-two lactic acid bacteria (LAB) were isolated from traditional fermented foods by using de Man, Rogosa and Sharpe (MRS) selective medium. MRS agar is a medium used for the isolation, enumeration and cultivation of lactic acid bacteria which includes species of the following genera*: Lactobacillus, Streptococcus*, *Pediococcus* and *Leuconostoc* (Marshall, 1992). MRS selective medium are enriched with nutrients such as peptone and beef extract which supply carbon and nitrogen sources to the lactic acid bacteria culture (Abbasiliasi *et al*., 2017). Apart from that, MRS medium also supplemented with yeast extract which provides vitamin B complex and dextrose as energy source (Abbasiliasi *et al*., 2017). These nutrients combine with all the necessary growth factors such as sodium acetate, ammonium citrate, potassium phosphate, magnesium sulphate and tween® 80 make MRS medium one of the best media for the cultivation of LAB (Abbasiliasi *et al*., 2017).

In this study, "tapai ubi" has the highest number of LAB isolates than the other traditional fermented foods (Table 3.2). Contrary to the current finding, report by Zareian *et al.* (2012) showed that "tempeh" has the highest number of LAB isolates followed by "tapai pulut" and "tapai ubi". Another

recent study conducted by Lim *et al.* (2019) also demonstrated that "tempeh" has the highest number of LAB isolates than the other local fermented foods. However, Suhartatik *et al.* (2014) revealed that "tapai pulut" has the highest number of LAB isolates, followed by "tapai ubi" and "tempeh". The different number of LAB isolated from different fermented foods in this study probably due to the variation in fermentation conditions such as temperature, pH, salinity or relative ingredients concentration exposed to these three food samples (Li *et al*., 2016). Hence, the differences in environmental variables during the food production may affect the microbial communities of fermented foods (Giraffa, 2004). Apart from that, the amount of LAB in fermented foods were different probably due to the different carbon sources found in three different fermented foods (Zareian *et al*., 2012).

Current findings were in accordance to a study reported by Ali (2011), that lactic acid bacteria were found abundantly in fermented foods as starter culture organisms used in fermentation process. According to Khalil *et al.* (2018), seventy-six LAB isolates were isolated from local fermented foods such as "tapai", "tempeh", pickles and "budu". Indeed, another study also revealed that traditional fermented foods such as "tapai ubi", "tapai pulut" and "tempeh" are considered as being vastly available sources of LAB (Chiang *et al.,* 2006).

3.3.2 Determination of antagonistic activity of lactic acid bacteria via Agar-well diffusion assay

Agar-well diffusion assay was used to determine the antagonistic activity of the cell free supernatant (CFS) of forty-two LAB isolates against *P. aeruginosa* ATCC 10145. Only the CFS of seven LAB isolates successfully inhibited *P. aeruginosa* ATCC 10145 (Table 3.3). Among the seven isolates, isolate TU3 from "tapai ubi", isolate TP2 from "tapai pulut" and isolate TE1 from "tempeh" have significantly higher inhibitory activity ($p<0.05$) than the

remaining LAB isolates. Hence, current finding supports the hypothesis that some of the lactic acid bacteria from traditional fermented foods are able to produce antimicrobial compounds that inhibit the growth of *P. aeruginosa* ATCC 10145.

Table 3.3 : Antagonistic activity of seven LAB isolates against *P. aeruginosa* ATCC 10145 using Agar-well diffusion assay

LAB isolates (source)	Diameter of inhibition zone (mm)
TU1 (Tapai ubi)	66.67 ± 0.06^{b}
TU2 (Tapai ubi)	63.33 ± 0.06^{b}
TU3 (Tapai ubi)	86.67 ± 0.06^{a}
TU4 (Tapai ubi)	63.33 ± 0.06^{b}
TP1 (Tapai pulut)	73.33 ± 0.06^{b}
TP2 (Tapai pulut)	86.67 ± 0.06^{a}
TE1 (Tempeh)	90.00 ± 0.00^{a}

Notes:

* Positive control (well contained MH broth only) has no inhibition zone.

Values reported are means ± standard deviation; n=3

$^{a, b}$ Within a column, values with different superscripts are significantly different at $p < 0.05$.

Similar observations were also reported by Affhan *et al.* (2015) and Yuksekdag *et al.* (2004), in which the CFS derived from LAB were able to inhibit *P. aeruginosa* as determined by agar-well diffusion assay. Putra *et al.* (2018) also reported that LAB isolated from traditional fermented fish in their study also exhibited inhibition against *P. aeruginosa*. According to Castellano *et al.* (2017) and (De Vuyst & Leroy, 2007), LAB able to produce antimicrobial substances such as organic acid and bacteriocins which showed antagonistic activity against pathogens.

3.3.3 Determination of bacteriocin activity of lactic acid bacteria

LAB able to produce antimicrobial substances such as organic acid and bacteriocins which inhibited pathogens. Hence in order to eliminate the effect of organic acid, the CFS of seven LAB were neutralised to pH 6.5 using 1 M NaOH. The bacteriocin activity of these CFS were determined using combination of Critical dilution method and Agar-well diffusion assay. Table 3.4 showed that the neutralised CFS of the seven LAB isolates successfully inhibited *P. aeruginosa* ATCC 10145. Hence, further confirmed that the inhibitory activity is most likely due to production of bacteriocin by LAB. The TU2 isolated from "tapai ubi" has significantly higher bacteriocin activity ($p < 0.05$) than the remaining isolates, followed by TP1 from "tapai pulut".

Table 3.4: Bacteriocin activity of selected LAB isolated from traditional fermented foods using Critical dilution method.

LAB isolates (source)	Average Bacteriocin Activity (AU/ml)
TU1 (tapai ubi)	50.00 ± 0.00^{c}
TU2 (tapai ubi)	200.00 ± 0.00^{a}
TU3 (tapai ubi)	50.00 ± 0.00^{c}
TU4 (tapai ubi)	50 ± 0.00^{c}
TP1 (tapai pulut)	100 ± 0.00^{b}
TP2 (tapai pulut)	50 ± 0.00^{c}
TE1 (tempeh)	50 ± 0.00^{c}

Notes:

Values reported are means ± standard deviation; n=3

$^{a, b, c}$ Within a column, values with different superscripts are significantly different at $p < 0.05$.

Bacteriocin activity (AU/ml) was calculated from the reciprocals of the highest dilution yielding a clear zone of growth inhibition on the indicator lawn (Batdorj *et al*., 2006). However, the diameter of inhibition zone was not taken into consideration in the calculation. Based on the results in Table 3.4, isolates of TU1, TU3, TU4, TP2 and TE1 have the same bacteriocin activity even though each of these isolates have different diameter of inhibition zone. Thus, bacteriocins with positive inhibition at the same dilution endpoint, but with different diameter of inhibition zone will have the same bacteriocin activity.

Although critical dilution method is straightforward and have been widely used in many applications, it gives only little value when statistical analysis on quantitative measurement of bacteriocin activity are to be performed (Parente *et al*., 1995). Hence, to improve the accuracy of the measurement of bacteriocin activity, the calculation of bacteriocin activity was modified by inclusion of the diameter of inhibition zone. The modified bacteriocin activity of the seven LAB isolates is shown in Table 3.5. The result shows that TU2 isolated from "tapai ubi" has the highest modified bacteriocin activity, followed by TP1 isolated from "tapai pulut". Whereas, TU4 from "tapai ubi" has the lowest modified bacteriocin activity, which is 75% lower than the modified bacteriocin activity of TU2.

Table 3.5: The modified bacteriocin activity of selected LAB isolated from traditional fermented foods using Critical dilution method.

LAB isolates (source)	Average Modified Bacteriocin Activity (AU.cm/ml)
TU1 (tapai ubi)	33.33 ± 2.89 [d]
TU2 (tapai ubi)	126.67 ± 11.55 [a]
TU3 (tapai ubi)	56.67 ± 20.21 [c]
TU4 (tapai ubi)	31.67 ± 2.89 [d]
TP1 (tapai pulut)	73.33 ± 5.78 [b]
TP2 (tapai pulut)	43.33 ± 2.89 [cd]
TE1 (tempeh)	45.00 ± 0.00 [cd]

Notes:

Values reported are means ± standard deviation; n=3

[a, b, c, d] Within a column, values with different superscripts are significantly different at $p < 0.05$.

This modified bacteriocin activity generated more accurate results since it incorporated the size of inhibition zones into the quantification. The result in Table 3.5 clearly indicated that the LAB isolates showed different degree of bacteriocin activity against *P. aeruginosa* ATCC 10145. This finding was in accordance to the selective inhibition characteristic of LAB against the indicator microorganism reported by Park *et al*. (2016).

Current study also confirmed that the seven LAB isolates able to produce bacteriocin that inhibited *P. aeruginosa* ATCC 10145. This was in line with the finding published by Jagadeeswari *et al.* (2010) which reported bacteriocin from LAB isolated from various traditional fermented foods showed strong inhibitory activity against *P. aeruginosa*. Jamuna & Jeevaratnam (2004) also revealed that bacteriocin from LAB isolated from traditional fermented foods

showed a broad inhibitory spectrum against Gram-negative bacteria including *Pseudomonas* DFR 219.

3.3.4 Identification of bacteriocin-producing lactic acid bacteria

Three out of the seven bacteriocin-producing LAB namely, TU2, TP1 and TE1 with the highest modified bacteriocin activity representing each fermented foods were selected for identification using biochemical and molecular identification methods.

3.3.4.1 Biochemical identification of selected lactic acid bacteria isolates

Biochemical identification of LAB was conducted using API 50CHL test kit. The carbohydrate fermentation patterns of three bacteriocin-producing LAB isolates TU2, TP1 and TE1 are shown in Table 3.6 and Appendix A. The isolates TU2, TP1 and TE1 showed 99.7%, 99.7% and 97.9% similarity to *Pediococcus pentosaceus*.

A)

Strip	API 50 CHL V5.2				
Significant taxa	% ID	T	Tests against		
Pediococcus pentosaceus 1	99.7	0.79	ESC 100%		
Next taxon	% ID	T	Tests against		
Lactococcus lactis ssp *lactics* 1	0.1	0.66	ESC 90%	LAC 90 %	TAG 9%

B)

Strip	API 50 CHL V5.2					
Significant taxa	% ID	T	Tests against			
Pediococcus pentosaceus 1	99.7	0.19	ESC 100%	XLT 0%	DFUC 0%	
Next taxon	% ID	T	Tests against			
Lactococcus lactis ssp *lactics* 1	0.1	0.06	ESC 90%	LAC 90 %	XLT 0%	TAG 9%
			DFUC 0%			

C)

Strip	API 50 CHL V5.2					
Significant taxa	% ID	T	Tests against			
Pediococcus pentosaceus 1	97.9	0.59	GAL 100%	ESC 100%		
Next taxon	% ID	T	Tests against			
Lactococcus lactis ssp *lactics* 1	1.8	0.57	GAL 90%	ESC 90%	LAC 90%	TAG 9%

Table 3.6: Identification of selected LAB isolated from traditional fermented foods using API 50 CHL test kit: A) TU2; B) TP1; C) TE1

All three LAB isolates, TU2, TP1 and TE1, were able to ferment 15 carbohydrates as listed in Appendix A. Meanwhile, only isolates TU2 and TP1 able to ferment D-galactose while only isolate TP1 able to ferment L-fucose. Baradaran *et al.* (2012) also reported similar observation with K1 strain isolated from Malaysian herb, *Polygonum minus,* that showed 97% similarity to *Pediococcus pentosaceus.* K1 strain showed positive results with 14 carbohydrates similar to TU2, TP1 and TE1 isolates, except that K1 strain was able to ferment β-methyl-D-xyloside and esculin.

Meanwhile, a study conducted by Jung *et al.* (2012) revealed a different carbohydrate fermentation pattern of IJ-K1 strain isolated from fermented sauce-type *kimchi* where the IJ-K1 strain showed 99.2% similarity to *Pediococcus pentosaceus*. The IJ-K1 strain was able to ferment esculin, D-lactose, D-melibiose and D-raffinose. In contrast to TU2, TP1 and TE1 isolates, IJ-K1 strain did not utilize D-xylose.

Taxonomic identification by API 50 CHL which are based on biochemical characteristic of isolates in fermenting carbohydrate is still widely used. Carbohydrates were fermented to acids during incubation which produced a decrease in pH. This was detected by the colour change of the indicator. Isolates with high fermentation activity will change the colour from bromcresol purple to yellow and isolates without fermentation activity would

not change the colour of basal medium of bromcresol purple at all (Yelnetty *et al*., 2014). These biochemical profiles of bacteria were then cross-referenced to the API® databases using APIweb.

3.3.4.2 Molecular identification of selected lactic acid bacteria isolates

The molecular identification of LAB isolates TU2, TP1 and TE1 were determined via 16S rDNA gene sequencing technique (Table 3.7 – 3.9; Appendix B). The gene sequences obtained were aligned with the genetic sequences database at GenBank (BLAST) and results obtained showed that all three LAB isolates (TU2, TP1 and TE1) had respectively 96.77%, 96.91% and 96.28% similarity to *Pediococcus pentosaceus* ATCC 25745.

Phenotypic characterization based on sugar fermentation reveals biochemical properties of the microorganisms (Dimitonova *et al*., 2008) but may not always provide a strong basis for LAB identification (Conter *et al*., 2005). Hence, genotype-based methods such as 16S rDNA sequencing which is robust in identifying bacteria was used as a complement or alternative to phenotypic methods (Weisburg *et al*., 1991). 16S rDNA sequence analysis is also a powerful technique for determining the phylogenetic relationships of microorganisms (Mora *et al*., 1997). In this study, both API and 16S rDNA sequencing method had confirmed the identity of TU2, TP1 and TE1 as *Pediococcus pentosaceus*.

Current study successfully isolated bacteriocinogenic *Pd. pentosacues* from local fermented foods. Similar findings were reported by Sujaya *et al*. (2010) in which *Pd. pentosaceus* was predominant bacterial flora in all types of "tapai" from different sources. Tapai is a traditional fermented food in South East Asian countries which is made from starch and "ragi" (Kuriyama *et al.,* 1997). "Ragi' naturally contains filamentous fungi, bacteria and yeasts which is used as one of the fermentation starters made from the mixtures of rice flour, spices and water or sugarcane juice (Kofli & Dayaon, 2010). According

to Uchimura *et al*. (1998), *Pd. pentosaceus* strains were most abundantly isolated from newly collected "ragi" samples in Indonesia. Hence, it is not unusual for current study to discover *Pd. pentosaceus* strains in "tapai ubi", "tapai pulut" and "tempeh" that commonly used "ragi" as starter culture. Nevertheless, fermented foods are also good reservoir for LAB and hence researchers also managed to isolate a range of LAB from these fermented foods.

Moreno *et al.* (2002) has successfully isolated bacteriocinogenic LAB from "tempeh" and the LAB isolate was further identified as *Enterococcus faecium* and the bacteriocin was characterized as enterocin, which was found to exhibit antagonistic activity against various indicator organisms.

According to Thanh *et al.* (2010), bacteriocin produced from *L. plantarum* I-UL4 isolated from "tapai ubi" has been shown to have broad inhibitory spectrum against various Gram-positive such as *Bacillus cereus*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Enterococcus faecalis*, and Gram-negative bacteria such as *Escherichia coli* and *Salmonella typhimurium*.

In another study, Rahayu and her co-workers (2015) revealed that bacteriocinogenic *Lactobacillus plantarum* isolated from "tapai ubi" was demonstrated to inhibit three pathogenic bacteria including *S. dysenteriae* dky-4, *E. coli* OK111, and *E. coli* ST.

A recent study by Adeyemo *et al.* (2018) demonstrated that four species of LAB namely *L. plantarum*, *L. acidophilus*, *L. brevis* and *L. casei*, have been successfully isolated from fermenting cassava, or also known as "pupuru". The bacteriocin produced from these four species of LAB have been shown to inhibit *P. aeruginosa* and *S. aureus*.

Another recent study by Ukwuru & Ohaegbu (2018) also revealed that the predominant LAB isolates namely *Lactobacillus fermentum, L. plantarum* and *L. pentosus* have been successfully isolated from fermenting

cassava. The bacteriocin produced from these LAB isolates was inhibitory to many food spoilage microorganisms including *P. aeruginosa*.

Table 3.7: Top five identities of LAB isolate TU2 via 16S rDNA gene sequence

Sequences Alignments	Query cover	E-value	Identity	Accession
1) *Pediococcus pentosaceus* ATCC 25745, complete genome	94%	0.0	96.77%	NC_008525.1
2) *Pediococcus acidilactici* strain ZPA017, complete genome	94%	0.0	95.40%	NZ_CP015206.1
3) *Pediococcus stilesii* strain DSM 18001 Scaffold41, whole genome shotgun sequence	94%	0.0	95.39%	NZ_JQBX01000041.1
4) *Pediococcus argentinicus* strain DSM 23026 Scaffold64, whole genome shotgun sequence	94%	0.0	94.10%	NZ_JQCQ01000064.1
5) *Pediococcus claussenii* ATCC BAA-344, complete genome	94%	0.0	94.03%	NC_016605.1

Table 3.8: Top five identities of LAB isolate TP1 via 16S rDNA gene sequence

Sequences Alignments	Query cover	E-value	Identity	Accession
1) *Pediococcus pentosaceus* ATCC 25745, complete genome	94%	0.0	96.91%	NC_008525.1
2) *Pediococcus stilesii* strain DSM 18001, Scaffold41, whole genome shotgun sequence	94%	0.0	95.40%	NZ_JQBX01000041.1
3) *Pediococcus acidilactici* strain ZPA017, complete genome	94%	0.0	95.40%	NZ_CP015206.1
4) *Pediococcus argentinicus* strain DSM 23026 Scaffold64, whole genome shotgun sequence	94%	0.0	94.23%	NZ_JQCQ01000064.1
5) *Pediococcus claussenii* ATCC BAA-344, complete genome	94%	0.0	94.03%	NC_016605.1

Table 3.9: Top five identities of LAB isolate TE1 via 16S rDNA gene sequence

Sequences Alignments	Query cover	E-value	Identity	Accession
1) *Pediococcus pentosaceus* ATCC 25745, complete genome	97%	0.0	96.28%	NC_008525.1
2) *Pediococcus stilesii* strain DSM 18001, Scaffold41, whole genome shotgun sequence	97%	0.0	94.97%	NZ_JQBX01000041.1
3) *Pediococcus acidilactici* strain ZPA017, complete genome	97%	0.0	94.90%	NZ_CP015206.1
4) *Pediococcus argentinicus* strain DSM 23026 Scaffold64, whole genome shotgun sequence	97%	0.0	93.67%	NZ_JQCQ01000064.1
5) *Pediococcus claussenii* ATCC BAA-344, complete genome	97%	0.0	93.54%	NC_016605.1

3.4 Summary

Forty-two LAB were isolated from traditional fermented foods, “tapai ubi”, “tapai pulut” and “tempeh’. A total of seven LAB isolates produced bacteriocin and able to inhibit *P. aeruginosa* ATCC 10145 resulted in modified bacteriocin activity that ranged from 31.67 – 126.67 (AU.cm)/ml. Three LAB isolates, namely TU2, TP1 and TE1 that have higher bacteriocin activity representing each type of fermented foods were identified as *Pediococcus pentosaceus* using biochemical and molecular identification methods.

Chapter 4: Determination of the synergistic interactions between crude bacteriocins and selected antibiotics against *Pseudomonas aeruginosa* ATCC 10145

4.1 Introduction

The growing emergence of multidrug-resistant microorganisms leads to urgent need for the discovery of novel antimicrobial agent and strategy to address the antibiotic resistant crisis. Bacteriocin is an antimicrobial peptide which has been proposed as a potential solution to inhibit multidrug-resistant microorganism (da Costa *et al.,* 2019). Brumfitt *et al.* (2002) reported that the combination of bacteriocin nisin produced by *Lactococcus lactis* with antibiotic ramoplanin resulted in synergistic interactions that inhibited 14 out of 20 Methicillin-resistant *Staphylococcus aureus* (MRSA) strains assessed. This is an important finding that suggest a potential strategy to reverse the antibiotic resistance of multidrug-resistant pathogen by using bacteriocin and conventional antibiotics. However similar study was limited to the author's knowledge.

Hence, current chapter aimed to determine the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of crude bacteriocins and selected antibiotics. The obtained MIC values of crude bacteriocins and selected antibiotics will be used to assess the combinatory antimicrobial effect of crude bacteriocins produced by *Pd. pentosaceus* with selected antibiotics against multidrug-resistant *P. aeruginosa* ATCC 10145 using checkerboard method and time-kill assay.

4.2 Materials and methods

4.2.1 Preparation of *P. aeruginosa* ATCC 10145

A 2% (v/v) of 24-h old *P. aeruginosa* ATCC 10145 was grown in 10 ml of Mueller Hinton (MH) Broth (Oxoid, UK) for 24 h at 37 ℃ which gave the final cells densities of 1.8 x 10^8 CFU/ml. The culture was diluted to 1 x 10^8 CFU/ml

(Absorbance of 0.08-0.1 at OD_{600nm}) by using MH broth. A 100-fold dilution was made to obtain the final concentration of 1 x 10^6 CFU/ml.

4.2.2 Antibiotic susceptibility test

Disc Diffusion or the Kirby-Bauer test (Bauer *et al*., 1966) was carried out to determine susceptibility of antibiotics against *P. aeruginosa* ATCC 10145. *P. aeruginosa* ATCC 10145 was swabbed evenly on the surface of MH agar. All the antibiotic-impregnated discs were placed on the swabbed plate according to the manufacturer's instructions (Oxoid) and incubated at 37 ℃ for 24 h. After incubation, the diameter of inhibition zone formed was measured.

The antibiotic discs used in this assay were:

1. Amoxicillin (Oxoid, UK), 10 μg per disc
2. Cephalexin (Oxoid, UK), 30 μg per disc
3. Chloramphenicol (Oxoid, UK), 50 μg per disc
4. Ciprofloxacin (Oxoid, UK), 5 μg per disc
5. Gentamicin (Oxoid, UK), 10 μg per disc
6. Sulfamethoxazole (Oxoid, UK), 25 μg per disc
7. Tetracycline (Oxoid, UK), 30 μg per disc

4.2.3 Determination of minimum inhibition concentration (MIC) of crude bacteriocins

4.2.3.1 Preparation of crude bacteriocins

Isolates TU2, TP1 and TE1 were prepared according to the procedure described in Section 3.2.2.(a). The CFS obtained after centrifugation at 10,000 *g* for 15 min was adjusted to pH 6.5 using 1 M NaOH. The neutralised CFS were then filtered through sterile 0.22 μm pore size syringe filter (Sartorius, Germany).

The neutralised CFS was then subjected to overnight freezing at -80 ℃ freezer (GE Healthcare, USA). After that, the frozen neutralised CFS was freeze-dried using the freeze dryer (Martin Christ, Germany) for 72 h. The freeze-dried CFS were then adjusted to a concentration of 1000 mg/ml using

sterile water and designated as "crude bacteriocins". The crude bacteriocin was kept at -20 ℃ freezer till use.

Critical dilution method (as described in Section 3.2.4.1) was carried out to determine the bacteriocin activity of neutralised CFS and crude bacteriocin.

4.2.3.2 Preparation of antibiotic solutions

Chloramphenicol (Sigma Aldrich, Germany), ciprofloxacin (Sigma Aldrich, Germany) and tetracycline (Sigma Aldrich, Germany) were used in this assay and antibiotic stock solutions of 1.28 mg/ml were prepared according to manufacturer's protocol. The antibiotic stock solutions were kept at -20℃ freezer till used.

4.2.3.3 Determination of minimum inhibition concentration (MIC) of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145

Determination of minimum inhibitory concentration (MIC) of crude bacteriocins of TU2, TP1 and TE1 against *P. aeruginosa* ATCC 10145 were performed and *P. aeruginosa* was prepared according to Section 3.2.2(b).

A two-fold serial dilution of the crude bacteriocins (TU2, TP1 and TE1) and antibiotics (chloramphenicol, ciprofloxacin and tetracycline) were carried out using MH broth in a 96 well microtiter plate. After dilution, each well contained 50 µl of diluted crude bacteriocins with final concentration ranging from 0.98 – 500 mg/ml, respectively and antibiotics with final concentration ranging from 0.00025 – 0.128 mg/ml, respectively after 50 µl of *P. aeruginosa* ATCC 10145 was then added into each well.

Positive control in this assay was the well containing *P. aeruginosa* ATCC 10145 only, while negative control was the well containing MH broth only.

The arrangement of crude bacteriocins (TU2, TP1 and TE1) and antibiotics (chloramphenicol, ciprofloxacin and tetracycline) on 96 well plates are shown in the Appendix C. The plate was incubated at 37℃ for 24 h and the absorbance at OD_{600nm} wavelength was measured before and after incubation.

MIC is expressed as the lowest concentration of the crude bacteriocins and antibiotics that resulted in inhibition of the visible growth of *P. aeruginosa*. All assays were carried out in triplicate.

4.2.3.4 Determination of minimum bactericidal concentration (MBC) of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145

MBC is referred as the minimum concentration of drug needed to kill most (≥99.9%) of the viable organisms after incubation for a fixed length of time (generally 24 hours) (CLSI, 1999). In this study, MBC assay was carried out to determine the lowest concentration of crude bacteriocins and antibiotics required to kill 99.9% *P. aeruginosa* ATCC 10145. The MIC assay was first conducted and the content of each well used in the MIC assay with no growth of *P. aeruginosa* ATCC 10145 after incubation was streaked on a new MH agar plate. After the MH agar plate was incubated at 37℃ for 24 h, the colonies formed were counted. The lowest concentration of crude bacteriocins and antibiotics without bacterial growth of *P. aeruginosa* was identified and taken as minimum bactericidal concentration (MBC). When the ratio of MBC/MIC is ≤2.0, the antimicrobial agent is considered as bactericidal or otherwise bacteriostatic (Shanmughapriya *et al*., 2008).

4.2.3.5 Determination of Fractional Inhibitory Concentration (FIC) of crude bacteriocins and selected antibiotics

A checkerboard plate assay is used to test the synergism, addition, indifference or antagonism activities of the combination of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145 by determining the ∑FICs of the combination tested. The checkerboard method was carried out using National Committee for Clinical Laboratory Standards (NCCLS) checkerboard method with slight modifications (Le *et al.,* 2015).

A two-fold serial dilution of the crude bacteriocins (TU2, TP1 and TE1) and antibiotics (chloramphenicol, ciprofloxacin and tetracycline) were carried out

using MH broth in a 96 well microtiter plate. After dilution, each well contained 50 µl of diluted crude bacteriocins with final concentration ranging from 3.906–250 mg/ml, respectively and ciprofloxacin with final concentration ranging from 0.016-2.00 µg/ml, respectively and chloramphenicol and tetracycline with final concentration ranging from 0.001-0.128 mg/ml, respectively after 50 µl of *P. aeruginosa* ATCC 10145 was then added into each well.

Positive control in this assay was the well containing *P. aeruginosa* ATCC 10145 only, while negative control was the well containing MH broth only. The plate was incubated at 37℃ for 24 h and the absorbance at OD_{600nm} wavelength was measured before and after incubation. MIC is expressed as the lowest concentration of the crude bacteriocins and antibiotics that resulted in visual inhibition (no turbidity) of the *P. aeruginosa* ATCC 10145. All assays were carried out in triplicate.

A fractional inhibitory concentration index (ΣFICI) was calculated as following (Bassolé & Juliani, 2012):

ΣFICI = FIC A + FIC B,

where FIC A is the MIC of bacteriocin in the combination/MIC of bacteriocin alone,

and FIC B is the MIC of antibiotic in the combination/MIC of antibiotic alone.

The combination is considered:

"Synergistic" if the ΣFIC Index is ≤0.5;

"Addition" if the ΣFIC Index is 0.5 – 1;

"Indifference" if ∑FIC Index is > 1 and

"Antagonistic" if the ∑FIC Index is > 4

4.2.3.6 Time-Kill Assay

Time-kill assay was carried out to determine the killing rate of *P. aeruginosa* ATCC 10145 by crude bacteriocins (TU2, TP1 and TE1) and selected antibiotics (chloramphenicol, ciprofloxacin and tetracycline).

A two-fold serial dilution of the crude bacteriocins (TU2, TP1 and TE1) and antibiotics (chloramphenicol, ciprofloxacin and tetracycline) were carried out using MH broth in a 96-well microtiter plate. After dilution, each well contained 50 µl of diluted crude bacteriocins with final concentration ranging from 3.906–250 mg/ml, respectively and ciprofloxacin with final concentration ranging from 0.016-2.00 µg/ml, respectively and chloramphenicol and tetracycline with final concentration ranging from 0.001-0.128 mg/ml, respectively after 50 µl of *P. aeruginosa* ATCC 10145 was then added into each well. Positive control in this assay was the well containing *P. aeruginosa* ATCC 10145 only, while negative control was the well containing MH broth only.

After that, 10-fold serial dilutions of each well in the plate were made using MH broths. Aliquots of 30 µl of dilution (10^0 – 10^4) was pipetted and spread evenly on the MH agar plate at time intervals of 0, 1, 2, 4, 6, 8, 10 and 24 h. The MH plate was incubated at 37℃ for 24 h. The colony formation (CFU/ml) was observed and the agar plates with "30-300" colonies were counted using viable plate count method (Pankuch *et al*., 1994). The time-kill curves were obtained by plotting $\log_{10}$ CFUs against time.

4.2.4 Statistical Analysis

All assays were carried out in triplicates. The statistical analysis was performed using IBM SPSS Statistics Version 25. Mean values and the standard deviation (SD) were calculated for each experiment. One-way analysis of variance (ANOVA) was used to determine the differences between various treatments. The paired t-test was used for comparison of modified bacteriocin activity before and after freeze-drying process. Duncan post hoc

test was used at the chosen level of probability ($p < 0.05$) to determine significant differences between means.

4.3 Results and Discussion

4.3.1 Antibiotic susceptibility of *P. aeruginosa* ATCC 10145

Figure 4.1 and Table 4.1 show the inhibition of seven antibiotics against *P. aeruginosa* ATCC 10145 after incubated for 24 h at 37ºC. Ciprofloxacin showed the largest inhibition zone with diameter of 30 mm. According to CLSI (2017), if the diameter of inhibition zone is more than or equal to the acceptable breakpoint range of 21 mm, in this case with 30 mm in diameter, *P. aeruginosa* was interpreted to be highly susceptible to ciprofloxacin.

Both chloramphenicol and gentamicin resulted in inhibition zone of 15 mm and 13 mm in diameter, respectively. These results were within the CLSI (2017) acceptable breakpoint range of 13-17 mm in diameter for chloramphenicol and 13-14 mm in diameter for gentamicin. Hence, *P. aeruginosa* was interpreted to be intermediately susceptible or resistant to chloramphenicol and gentamicin.

On the other hand, tetracycline revealed the smallest inhibition zone with 12 mm in diameter. Current finding is within the CLSI (2017) breakpoint range of less than or equal to 14 mm in diameter. Hence, *P. aeruginosa* was interpreted to be resistant to tetracycline. Whereas, the remaining antibiotics of amoxicillin, cephalexin and sulfamethoxazole had failed to inhibit *P. aeruginosa*. Thus, *P. aeruginosa* was interpreted to be highly resistant to amoxicillin, cephalexin and sulfamethoxazole.

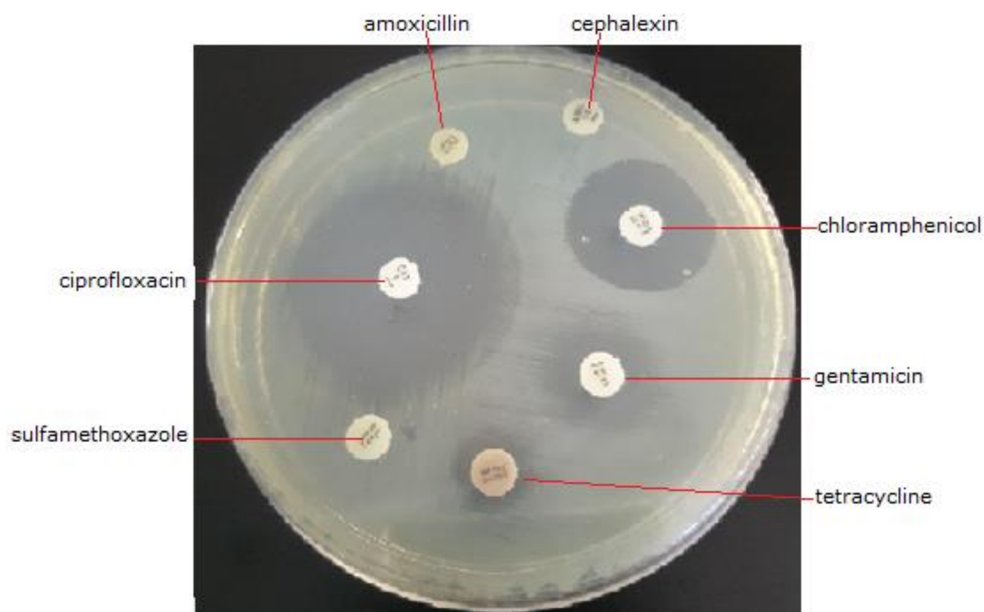

Figure 4.1: Antibiotic susceptibility test of *Pseudomonas aeruginosa* ATCC 10145 using Disc diffusion method

Antibiotic disc	Antibiotic concentration on test disc (µg)	Diameter of inhibition zone (mm)	Interpretation of inhibition according to CLSI (2017)
Amoxicillin	10	0 ± 0*	Resistant
Cephalexin	30	0 ± 0*	Resistant
Chloramphenicol	50	20 ± 0**	Intermediate
Ciprofloxacin	5	30 ± 0***	Susceptible
Gentamicin	10	15 ± 0**	Intermediate
Sulfamethoxazole	25	0 ± 0*	Resistant
Tetracycline	30	10 ± 0*	Resistant

Table 4.1: Antibiotic susceptibility of *P. aeruginosa* ATCC 10145 to seven antibiotics

Notes: Values reported are means ± standard deviation; n=3 where data obtained were based on the average of three replication experiments.

* weak inhibition zone, ** moderate inhibition zone and *** high inhibition zone (see Appendix D).

Current finding suggested that out of the seven antibiotics tested, only ciprofloxacin able to inhibit *P. aeruginosa* ATCC 10145, whilst *P. aeruginosa* was intermediate susceptible or resistant to chloramphenicol and gentamicin. Whereas *P. aeruginosa* ATCC 10145 was resistant to the remaining four antibiotics (amoxicillin, cephalexin, sulfamethoxazole and tetracycline) tested. Current finding is alarming due to *P. aeruginosa* ATCC 10145 was resistant to almost all the antibiotics tested and only one antibiotic tested is highly susceptible to *P. aeruginosa* ATCC 10145. Hence, further efforts in overcoming this antibiotic resistance issue of MDR *P. aeruginosa* is crucial.

In this study, ciprofloxacin was demonstrated to be the most effective antibiotic against *P. aeruginosa* ATCC 10145. Ciprofloxacin belongs to the fluoroquinolone class of antibiotic that commonly used to treat several bacterial infections such as urinary tract infections, pneumonia, skin, bone and joint infections, gastrointestinal infections, lower respiratory tract infections and sexually transmitted diseases (Davis *et al.,* 1996). Ciprofloxacin inhibits DNA replication by inhibiting bacterial DNA topoisomerase and DNA-gyrase (Campoli-Richards *et al.,* 1988).

Of the fluoroquinolone class, ciprofloxacin is the most potent against Gram-negative bacteria especially *Pseudomonas aeruginosa* (LeBel, 1988). This was in line with the current study that demonstrated *P. aeruginosa* ATCC 10145 was highly susceptible to ciprofloxacin. Current finding was also in accordance with published works from Lomholt & Kilian (2003) and Fazlul *et al.* (2011). Lomholt & Kilian (2003) reported that ciprofloxacin resistance was still rare among *P. aeruginosa* isolated from eye infections in Europe countries. Whereas Fazlul *et al.* (2011) reported a high susceptibility pattern (83.3% of sensitivity) of ciprofloxacin on clinical isolates of *P. aeruginosa* obtained from Hospital Selayang, Malaysia.

On the other hand, chloramphenicol was found to be intermediate susceptible or resistant against *P. aeruginosa* ATCC 10145 in this study. Chloramphenicol

is an antibiotic derived from *Streptomyces venezuelae,* but currently it is produced entirely by chemical synthesis (National Center for Biotechnology Information, n.d). It was the first antibiotic to be manufactured synthetically on a large scale and is a potent broad-spectrum antibiotic, but the use of chloramphenicol is now limited (Madhavan & Bagyalakshmi, 2014). Chloramphenicol was first used for the treatment of typhoid but with the global presence of multiple drug-resistant *Salmonella* Typhi, it loses its clinical value. It is widely used for the treatment of bacterial conjunctivitis, staphylococcal brain abscesses, meningitis and pneumonia (WHO, 2001). Chloramphenicol binds to the 50S ribosomal subunit and inhibits fundamental ribosomal function such as peptidyl transferase (PTase) activity, thereby hindering the protein chain elongation during protein synthesis (Madhavan & Bagyalakshmi, 2014).

However, chloramphenicol is not active against *Pseudomonas aeruginosa* (Mir & Abbas, 2010). This report was in line with the current study that demonstrated *P. aeruginosa* ATCC 10145 was intermediate susceptible or resistant to chloramphenicol. Current finding was also in accordance with published works from Morita *et al.* (2001) and Strateva & Yordanov (2009). Morita *et al.* (2001) reported that *P. aeruginosa* was said to be intrinsically resistant to chloramphenicol due to the MexAB-OprM system. Meanwhile, Strateva & Yordanov (2009) reported that most strains of *P. aeruginosa* were significantly resistant to chloramphenicol (Strateva & Yordanov, 2009). Gentamicin is a broad-spectrum antibiotic belongs to the aminoglycoside class produced by *Micromonospora purpurea* (WHO, 2001). It is used for the treatment of pneumonia, endocarditis, neonatal meningitis and acute cholecystitis (WHO, 2001). Gentamicin irreversibly binds to the bacterial 30S ribosomal subunit. Specifically, gentamicin is embedded between 16S rRNA and S12 protein within 30S subunit. This leads to interference with translational initiation complex, misreading of mRNA, thereby interfering

protein synthesis and resulted in bactericidal effect (National Center for Biotechnology Information, n.d).

In this study, *P. aeruginosa* ATCC 10145 was found to be intermediate susceptible or resistant to gentamicin. A similar finding was reported by Yayan *et al.* (2015) in which the clinical isolates of *P. aeruginosa* were found to have high resistance to gentamicin in patients with pneumonia in Hospital in Germany. Pitt *et al.* (2003) also demonstrated that 47% of clinical isolates of *P. aeruginosa* were resistant to gentamicin in patients with cystic fibrosis in UK. Hence, these reports on *P. aeruginosa* resistances towards gentamicin supported the current finding in this study.

Meanwhile, tetracycline is a broad-spectrum antibiotic which exhibited activity against a wide range of microorganisms including Gram-positive and Gram-negative bacteria, chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites (Chopra & Roberts, 2001). Tetracycline was also used for the prevention of malaria and in some countries including United States, tetracycline is added to animal feeds to act as growth promoters (Chopra & Roberts, 2001). Furthermore, tetracycline inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the bacterial ribosome acceptor (A) site (Schnappinger & Hillen, 1996).

However, resistance to tetracycline has now emerged in pathogenic bacteria and has limited their effectiveness (Roberts, 2003). In this study, *P. aeruginosa* ATCC 10145 was demonstrated to be resistant to tetracycline. This current finding was in accordance with published works from Li *et al.* (1994) and Sivanmaliappan & Sevanan (2011). Li *et al.* (1994) reported that *P. aeruginosa* strains were revealed to be intrinsically resistant to tetracycline due to efflux mechanism and low permeability of the outer membrane. Moreover, Sivanmaliappan & Sevanan (2011) revealed that *P. aeruginosa* isolated from foot ulcers of diabetes patients showed 83.3% resistance to

tetracycline in hospital in India. This study also revealed that ciprofloxacin retained high levels of antipseudomonal activity against *P. aeruginosa.*
Amoxicillin is a broad-spectrum antibiotic belongs to penicillin-class antibiotic (National Center for Biotechnology Information, n.d). It is used in the treatment of acute pharyngitis, pneumonia, acute gastritis and osteomyelitis (WHO, 2001). Amoxicillin binds to and inactivates penicillin-binding proteins (PBPs) located on the inner membrane of the bacterial cell wall. Inactivation of PBPs interferes with the cross-linkage of peptidoglycan chains which results in the weakening of the bacterial cell wall and causes cell lysis (National Center for Biotechnology Information, n.d).
Whereas, cephalexin is a beta-lactam and first-generation cephalosporin antibiotic (National Center for Biotechnology, n.d). Cephalexin is used in the treatment of susceptible bacterial infections through inhibition of cell wall synthesis (Food and Drug Administration, 2005). In this study, *P. aeruginosa* ATCC 10145 was demonstrated to be resistant to amoxicillin and cephalexin. This current finding was in line with the study conducted by Gad and his co-workers (2008). Gad and his co-workers (2008) revealed that *P. aeruginosa* skin infection isolates were 100% resistant to amoxicillin and 87% resistant to cephalexin in three Egyptian hospitals.
Sulfamethoxazole is a sulfonamide antibiotic that is commonly used in combination with trimethoprim (National Center for Biotechnology Informarion, n.d). It is mostly prescribed for urinary tract infection and other infections caused by *Pneumocystis jiroveci, Toxoplasma gondii, Stenotrophomonas maltophilia* and community-associated methicillin-resistant *Staphylococcus aureus* (Ho *et al.,* 2011). Sulfamethoxazole interferes with folic acid synthesis in susceptible bacteria. It acts as a competitor of p-aminobenzoic acid (PABA) during the synthesis of dihydrofolate (Kemnic & Coleman, 2019).

In this study, *P. aeruginosa* ATCC 10145 was demonstrated to be resistant to sulfamethoxazole. This current finding was in line with the study conducted by Kohler and his co-workers (1996). Kohler and his co-workers (1996) demonstrated that *P. aeruginosa* strains were intrinsically resistance to sulfamethoxazole due to the mexAB-OprM multidrug efflux system. A similar finding by Shin *et al.* (2015) also revealed that 30% of *P. aeruginosa* isolates were found to be resistant to trimethoprim-sulfamethoxazole.

Current findings indicated that *P. aeruginosa* ATCC 10145 was resistant to multiple antibiotics and hence gives rise to concern on the limited therapeutic choices available in treating severe *P. aeruginosa* infections in future. Therefore, this study seeks to find a new strategy by combining antibiotics with novel bacteriocin from *Pd. pentosaceus* isolated from traditional fermented foods to combat the multidrug-resistant *P. aeruginosa*.

In this study, chloramphenicol, ciprofloxacin and tetracycline have been selected to proceed with MIC assay and further tests. Chloramphenicol, ciprofloxacin and tetracycline have been chosen because each one of the antibiotics representing intermediate susceptible or resistant, susceptible and resistant respectively, towards *P. aeruginosa* ATCC 10145 based on the antibiotic susceptibility test performed. In order to determine the synergism antagonism interactions of crude bacteriocin with selected antibiotics, each group of intermediate, susceptible and resistant antibiotics must be included in this study.

4.3.2 Determination on the effect of freeze-drying on the activity of crude bacteriocins

In order to allow determination of bacteriocin concentration for use in MIC, MBC and FIC assay, the neutralised CFS was freeze-dried to obtain crude bacteriocin powder. Current work was conducted to confirm the effect of freeze-drying step on the inhibitory activity of bacteriocins.

Table 4.2 compares the modified bacteriocin activity of isolates TU2, TP1 and TE1 before and after the freeze-drying process. The modified bacteriocin activity of the freeze-dried crude bacteriocins of TU2, TP1 and TE1 were significantly higher ($p<0.05$) than the neutralised CFS. Bacteriocin activity for all crude bacteriocins TU2, TP1 and TE1 were increased by three-fold after freeze-drying process.

Table 4.2: Comparison of the modified bacteriocin activity of isolates TU2, TP1 and TE1 before and after the freeze-drying process.

Isolates	**Before freeze-drying process (neutralised CFS)**	**After freeze-drying process (crude bacteriocins)**
	Modified Bacteriocin Activity (AU.cm/ml)	Modified Bacteriocin Activity (AU.cm/ml)
TU2	126.67 ± 11.55^{a}	320 ± 0.00^{a}
TP1	73.33 ± 5.78^{b}	226.67 ± 23.10^{b}
TE1	45.00 ± 0.00^{c}	140 ± 0.00^{c}

Notes:

Values reported are means ± standard deviation; n=3 where data obtained were based on the average of three replication experiments.

$^{a, b, c}$ Within a column, values with different superscripts are significantly different at $p < 0.05$.

The freeze-drying process has been commonly used in sample pretreatment, particularly for sample preconcentration and preservation. It is a process that starts with the freezing of bacteriocins followed by sublimation and desorption to remove water content (Bergenholtz *et al*., 2012). This method helps to concentrate biogenic amines and metabolites from biological fluids containing low concentrations of protein and other interfering substances (de Castro & Izquierdo, 1990). Dimitrieva-Moats & Unlu (2012) demonstrated

that freeze-drying to be a feasible approach to prepare concentrated and stable bacteriocin-containing powders for prospective food applications. Current finding was also in accordance with study reported by Sharma *et al*. (2006) that an increase in bacteriocin activity was observed after lyophilization of lenticin isolated from traditional fermented foods with an increase of 33.3 % in inhibitory zone size against various indicators. After confirmed the bacteriocin activity of freeze-dried crude bacteriocins, further investigations on MIC, MBC and FIC were conducted.

4.3.3 Determination of minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145

MIC and MBC are used to determine the susceptibility of microorganism to antimicrobial agent (Andrews, 2001). In this study, the obtained MIC and MBC values were used to evaluate the synergism antagonism interactions of crude bacteriocins and selected antibiotics which have been determined via FIC and time-kill assays.

Table 4.3 summarizes the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145 after overnight incubation. Crude bacteriocin TU2, TP1 and TE1 had the same MIC of 15.63 mg/ml against *P. aeruginosa* ATCC 10145. Only ciprofloxacin showed MIC of 0.25 µg/ml against *P. aeruginosa*. This MIC of 0.25 µg/ml is within the CLSI (2017) breakpoint of 0.25-1.00 µg/ml for susceptible inhibition.

Table 4.3: Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145.

Antimicrobial	**MIC**	**MBC**	**Interpretation according to MBC test**
Bacteriocin			
TU2	15.63 ± 0 mg/ml	31.25 ± 0 mg/ml	Bactericidal
TP1	15.63 ± 0 mg/ml	31.25 ± 0 mg/ml	Bactericidal
TE1	15.63 ± 0 mg/ml	31.25 ± 0 mg/ml	Bactericidal
Antibiotics			
Chloramphenicol	32.00 ± 0 μg/ml	no MBC	Bacteriostatic
Ciprofloxacin	0.25 ± 0 μg/ml	0.25 ± 0 μg/ml	Bactericidal
Tetracycline	32.00 ± 0 μg/ml	no MBC	Bacteriostatic

Notes:

Values reported are median ± standard deviation; n=3 where data obtained were based on the average of three replication experiments.

MIC = Minimum inhibition concentration; MBC = Minimum bactericidal concentration

In this study, all three crude bacteriocins TU2, TP1 and TE1 showed MIC values of 15.63 mg/ml against *P. aeruginosa* ATCC 10145 which were much higher than other published works.

Lin & Pan (2019) reported that the purified cell-free supernatant (CFS) of *Lactobacillus plantarum* NTU 102 isolated from homemade Korean cabbage pickles showed MIC value of >3.35 mg/ml against *P. aeruginosa*.

In Benin, the antibacterial effect of the starter used in traditional beer "kpètè-kpètè" were investigated in one study. The starter's neutralized CFS

showed that MIC values varies from 0.62 - 5 mg/ml against *P. aeruginosa* (Christine *et al*., 2017).

Shelburne *et al*. (2007) demonstrated that MIC value of purified bacteriocin subtilosin A isolated from *Bacillus subtilis* against *P. aeruginosa* showed to be 0.05 mg/ml.

The MIC values are not standard for all types of bacteriocins because the MIC values of bacteriocins varied, depending on the strain of producer bacteria, sources of the strain, indicator pathogens and methods for the determination (N'tcha *et al*., 2017).

According to Oscariz & Pisabarro (2001), different classes of bacteriocins may have different mode of actions on the bacterial target membrane. Hence, different doses of bacteriocins are required to inactivate the different mechanisms of resistance of *P. aeruginosa*.

Meanwhile, *P. aeruginosa* ATCC 10145 was highly susceptible to ciprofloxacin with MIC value equals to 0.25 µg/ml. Current finding was in accordance to study conducted by Jayaraman *et al*. (2010), where the MIC of ciprofloxacin against *P. aeruginosa* DR3062 was demonstrated to be 0.25 µg/ml. In contrast, Grillon *et al*. (2016) also reported that 35% of *P. aeruginosa* isolates were shown to be resistant to ciprofloxacin in an *in vitro* study.

On the other hand, *P. aeruginosa* ATCC 10145 was resistant to tetracycline and chloramphenicol with MIC value of 32 µg/ml in this study. The MIC value for tetracycline was in the CLSI (2017) acceptable breakpoint range of 8-32 µg/ml for inhibition against *P. aeruginosa* ATCC 10145. However, since *P. aeruginosa* appeared to be intrinsically resistant to chloramphenicol that susceptibility testing is unnecessary, hence there was no breakpoint range for chloramphenicol (CLSI, 2017).

Similar to finding by Jayaraman *et al*. (2010), all five strains of *P. aeruginosa* tested showed MIC values of 32 µg/ml for tetracycline. However, Mawabo *et al.* (2015) demonstrated that the MIC of chloramphenicol and tetracycline on

P. aeruginosa were 16 µg/ml and 32-128 µg/ml, respectively. These results indicating that the antagonistic activity of the indicator pathogens can vary not only from one species to another, but also from one strain to another strain of the same species (Takeo *et al*., 2004).

After the MIC for crude bacteriocins and antibiotics were confirmed, study was proceeded to confirm the MBC of crude bacteriocins and antibiotics. MBC is defined as the lowest concentration of antimicrobial that will prevent the growth of microorganism after sub-culture on to antibiotic-free media (Andrews, 2001). MBC test can be used to determine whether the antimicrobial agent is bactericidal or bacteriostatic. Antibacterial agent is usually regarded as bactericidal if the MBC is not more than four times the MIC (CLSI, 1999).

An antimicrobial agent is said to be bactericidal if it can kill 99.9% of indicator pathogen within 24 hours of incubation (Pankey & Sabath, 2004). In this study, bacteriocin TU2, TP1 and TE1 were shown to be effective bactericidal agents against *P. aeruginosa* ATCC 10145 at MBC of 31.25 mg/ml which are much higher than other published works.

Bholay *et al.* (2017) reported that the MBC of bacteriocin isolated from *Bacillus subtilis* against *S. aureus* and *E. coli* was 80 mg/l respectively, whereas for *A. niger* and *C. albicans* was 96 mg/l respectively. Meanwhile, a study conducted by Huang *et al.* (2016) demonstrated that the MBC of bacteriocin (thuricins) produced by *Bacillus thuringiensis* (Bt) against *B. cereus* strains 0938 and ATCC 10987 were 12.5 µg/ml and 6.25 µg/ml, respectively.

According to N'tcha *et al.* (2017), the MBC of bacteriocins vary, depending on the type of strain of producer bacteria and sources of the strain. Hence, the MBC of crude bacteriocins in the current study were different from the MBC of bacteriocins in other published reports.

In this study, MIC of crude bacteriocins was found to be 15.63 mg/ml whereas, MBC of crude bacteriocins was 31.25 mg/ml against *P. aeruginosa* ATCC 10145. This was probably due to crude bacteriocins can only have an inhibiting effect at concentration of 15.63 mg/ml on *P. aeruginosa* ATCC 10145 and the concentration of crude bacteriocins was not enough to completely kill the bacteria. Whereas higher concentration 31.25 mg/ml is needed to completely kill *P. aeruginosa* ATCC 10145.

The MBC and MIC values of ciprofloxacin against *P. aeruginosa* ATCC 10145 was shown to be 0.25 µg/ml, indicating that ciprofloxacin is a bactericidal agent. This finding was consistent with the study reported by Chalkley & Koornhof (1985) that ciprofloxacin was found to be an effective bactericidal agent against *P. aeruginosa*.

On the other hand, chloramphenicol and tetracycline were shown to be bacteriostatic agents against *P. aeruginosa* ATCC 10145 in this study. An antimicrobial agent is said to be a bacteriostatic agent if it could not kill but rather inhibit the growth of indicator microorganism (Pankey & Sabath, 2004).

Current finding was in line with the study reported by Morita *et al.* (2014) which demonstrated that chloramphenicol and tetracycline were bacteriostatic agents against *P. aeruginosa* which due to *P. aeruginosa* intrinsic resistance mechanism of MexAB/MexXY efflux systems.

Overall, MIC and MBC tests can be powerful and inexpensive methods for screening of antimicrobial agents by potency. Prior information of susceptibility will be useful to reduce mortality and morbidity caused by *P. aeruginosa* among patients in hospital.

4.3.4 Determination of fractional inhibitory concentration (FIC) of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145

In this study, FIC assay was used to evaluate the synergism antagonism interaction between crude bacteriocins and selected antibiotics in combination against *P. aeruginosa* ATCC 10145 (Fratini *et al.* 2017).

Table 4.4 summarizes the fractional inhibitory concentration (FIC) of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145 after overnight incubation. In this study, crude bacteriocins TU2, TP1 and TE1 were tested in combination with chloramphenicol, ciprofloxacin and tetracycline using checkerboard assay. All three bacteriocins were demonstrated to be synergy with ciprofloxacin and tetracycline, and indifference with chloramphenicol against *P. aeruginosa* ATCC 10145. Interestingly, none of the combination of bacteriocins and antibiotics show antagonism.

Table 4.4: Fractional inhibitory concentration (FIC) of crude bacteriocins and selected antibiotics against *P. aeruginosa* ATCC 10145

	FIC Index (according to CLSI, 2006)		
Crude bacteriocin	**Chloramphenicol**	**Ciprofloxacin**	**Tetracycline**
TU2	2.25 ± 0, indifference	0.50 ± 0, synergistic	0.50 ± 0, synergistic
TP1	2.25 ± 0, indifference	0.50 ± 0, synergistic	0.50 ± 0, synergistic
TE1	2.25 ± 0, indifference	0.50 ± 0, synergistic	0.50 ± 0, synergistic

Notes:

Values reported are means ± standard deviation; n=3

$\sum FIC = FIC\ A + FIC\ B$, where

FIC A = MIC of A in the combination/ MIC of A alone;
FIC B = MIC of B in the combination/ MIC of B alone
ΣFIC Index is ≤ 0.5, means synergistic;
ΣFIC Index is 0.5 to 1 means addition;
ΣFIC Index is > 1 means indifference;
ΣFIC is > 4 means antagonistic

Based on the Table 4.4, it was shown that the FIC index resulting from the combination of respective crude bacteriocins with ciprofloxacin and tetracycline was 0.5, indicating a synergistic interaction in inhibiting *P. aeruginosa* ATCC 10145. The MIC of bacteriocins, ciprofloxacin and tetracycline combinations resulted in a 4-fold reduction when compared to the MIC of bacteriocins and antibiotics alone.

According to Saiman (2007), the antimicrobial combinations that result in a 4-fold reduction in the MIC compared with the MICs of agents alone are synergistic. Synergy is a positive interaction in which the combined effect of the two antimicrobial agents is significantly greater than the effect of either antimicrobial agent alone or greater than the sum of the effects of the individual antimicrobial agent (Cappelletty & Rybak, 1996). Hence, both combinations were producing synergistic interactions against *P. aeruginosa* ATCC 10145 as indicated by checkerboard assay.

To the author's knowledge, no relevant published work was found on combination of bacteriocins produced by *Pd. pentosaceus* and antibiotic against *P. aeruginosa* ATCC 10145. A few studies were available on nisin against *P. fluorescens* and *E. faecalis*. The synergistic inhibitory effect was observed when *P. fluorescens* were treated with combination of nisin and antibiotics such as penicillin, streptomycin, chloramphenicol and rifampicin (Naghmouchi *et al*., 2012). While Tong *et al.* (2014) reported that the synergistic interactions between nisin in combination with either penicillin or

chloramphenicol against three *E. faecalis* strains were also demonstrated with checkerboard assays.

On the other hand, it was apparent that the combination of crude bacteriocins TU2, TP1 and TE1 and chloramphenicol had indifferent effects, with FIC index of 2.25 against *P. aeruginosa* (Table 4.4). Indifference indicates that the effect of the combination of the two antimicrobial agents is the same as that of the most potent of these antimicrobial agents used alone (Rennerberg, 1993). The MIC of bacteriocins in combination with chloramphenicol resulted in a 4-fold reduction, whereas, the MIC of chloramphenicol in combination with bacteriocins resulted in a 2-fold addition when compared to the MIC of bacteriocins and antibiotics alone.

The indifferent interactions of crude bacteriocins and chloramphenicol were due to the higher MIC values obtained from the resistance of *P. aeruginosa* ATCC 10145 to chloramphenicol (Olajuyigbe & Afolayan, 2012). This was also probably due to only one metabolic pathway can be growth-limiting at any time of interaction (Rennerberg, 1993), and in this interaction, the concentration of chloramphenicol is low and not enough to inhibit the growth of *P. aeruginosa* ATCC 10145. Hence, the higher concentration of chloramphenicol was needed to inhibit *P. aeruginosa* ATCC 10145.

The indifferent effect of *P. aeruginosa* ATCC 10145 between crude bacteriocins and chloramphenicol was also probably due to different mode of actions utilized by crude bacteriocins alone as well as in combination with antibiotics. The mode of action of bacteriocin from *Pd. pentosaceus* isolated from traditional fermented foods is unknown, but study reported by Jiang *et al.* (2017) demonstrated that the cell membrane was the target of pentocin against the pathogenic bacteria. Chloramphenicol on the other hand act intracellularly by binding to the 50S ribosomal subunit and inhibits fundamental ribosomal function such as peptidyl transferase (PTase) activity,

thereby hindering the protein chain elongation during protein synthesis (Madhavan & Bagyalakshmi, 2014).

Bacteriocins and antibiotics are known to have different targets on bacterial cell membrane and study reported by Delcour (2009) revealed that the synergistic interaction of nisin and polymyxin B against Gram-negative bacteria can be attributed to disruption of the outer membrane by polymyxin B allowing access of the bacteriocins to the target cell membrane. However, further investigations such as scanning electron microscope will aid in confirming the mechanism of actions of crude bacteriocin and antibiotics against *P. aeruginosa* ATCC 10145 in this study.

4.3.5 Determination of the inhibitory effects of crude bacteriocins against *P. aeruginosa* ATCC 10145 via time-kill assay

Crude bacteriocins TU2, TP1 and TE1 produced by *Pd. pentosaceus* could inhibit the growth of *P. aeruginosa* ATCC 10145. Hence, time-kill assay was performed to determine the inhibitory effect of crude bacteriocins against *P. aeruginosa* ATCC 10145.

Figure 4.2 shows the time-kill curves for *P. aeruginosa* ATCC 10145 treated with bacteriocin TU2, TP1, TE1 for 24 h. *P. aeruginosa* ATCC 10145 without any treatment was used as a control. The control *P. aeruginosa* ATCC 10145 entered log phase after 2 h of incubation and with a steady increment till 9.24 $\log_{10}$ CFU/ml at 24 h of incubation. Meanwhile, when treated with crude bacteriocins TU2, TP1 and TE1, *P. aeruginosa* ATCC 10145 maintained a long lag phase from 0 h – 10 h and started to decrease after 10 h of incubation. Time-kill assay also revealed that crude bacteriocins TU2, TP1 and TE1 caused 1-$\log_{10}$ CFU/ml reduction of *P. aeruginosa* ATCC 10145 after 24 h of incubation. Although only 1-$\log_{10}$ CFU/ml reduction were observed after 24 h, these findings suggested that treatment of crude bacteriocins alone were able to exert inhibitory effect against *P. aeruginosa* ATCC 10145.

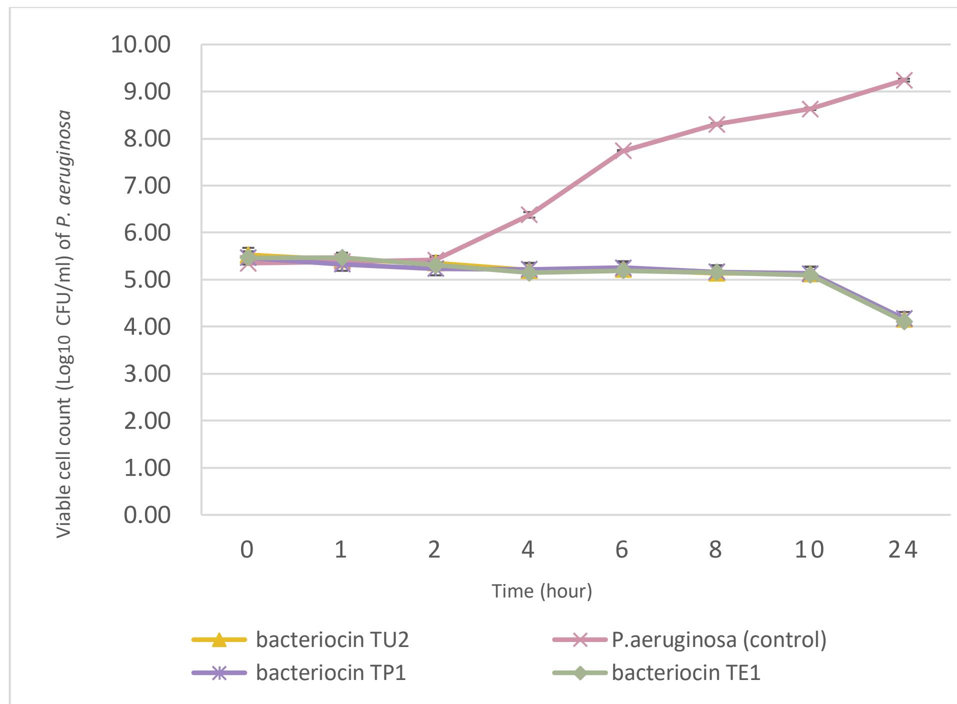

Figure 4.2: Effect of treatment of bacteriocin TU2, TP1 and TE1 on the growth of *P. aeruginosa* ATCC 10145

Similar finding was reported by Chi & Holo (2018) which revealed that the result of time-kill assay showed that 1x MIC of nisin and garvicin KS caused 3-$\log_{10}$ CFU/ml reduction of *S. aureus* within 4 h when tested alone. This time-kill assay also showed that the addition of bacteriocin nisin and garvicin KS alone could not completely kill *S. aureus* even after 48 h exposure. Another report by Beric *et al.* (2013) also revealed that the result of time-kill assay showed that 1x MIC of crude bacteriocin isolated from *B. licheniformis* VPS50.2 caused 3-$\log_{10}$ CFU/ml reduction of *L. monocytogenes* within the first 2 h of incubation in the presence of bacteriocin alone.

4.3.6 Determination of the inhibitory effect of selected antibiotics against *P. aeruginosa* ATCC 10145 via time-kill assay

Figure 4.3 shows the time-kill curves for *P. aeruginosa* ATCC 10145 treated with chloramphenicol, ciprofloxacin, tetracycline for 24 h. Time-kill curve revealed that 1x MIC of ciprofloxacin (0.25 µg/ml) successfully inhibited *P. aeruginosa* ATCC 10145 totally by 5-$\log_{10}$ CFU/ml reduction after 2 h of

incubation. Hence, this finding further confirmed the bactericidal action of ciprofloxacin against *P. aeruginosa* ATCC 10145.

In contrast, 1x MIC of chloramphenicol (32 μg/ml) and 1x MIC of tetracycline (32 μg/ml) had suppressed the growth of *P. aeruginosa* ATCC 10145 by 1-$\log_{10}$ CFU/ml but unable to inhibit it totally after 24 h. Thus, these findings confirmed that both chloramphenicol and tetracycline exerted bacteriostatic effect against *P. aeruginosa* ATCC 10145.

In this study, ciprofloxacin exerted bactericidal effect against *P. aeruginosa* ATCC 10145 by 5-$\log_{10}$ CFU/ml reduction within 2 h of incubation. Meanwhile, both chloramphenicol and tetracycline exerted bacteriostatic effect against *P. aeruginosa* ATCC 10145 by 1-$\log_{10}$ CFU/ml after 24 h of incubation.

Current finding was also in accordance to a study reported by Oyedemi *et al.* (2016) which revealed that sub-inhibitory concentration of ciprofloxacin exerted a bactericidal effect against *P. aeruginosa* ATCC 19582 by 5-$\log_{10}$ CFU/ml reduction within 24 h of incubation. According to Drago *et al.* (2001), 4x MIC of ciprofloxacin caused a bactericidal effect against *E. coli* with a 3-$\log_{10}$ CFU/ml decrease in bacterial count after 24 h of incubation in time-kill assay. Hence, these reports further confirmed the current finding that ciprofloxacin exerted bactericidal effect against *P. aeruginosa* ATCC 10145.

Current finding was also in line with a study reported by Zheng *et al.* (2017) which revealed that 2x MIC of tetracycline exerted bacteriostatic effect against *P. aeruginosa* strain PA14 by 1-$\log_{10}$ CFU/ml reduction within 4 h in a time-kill assay.

Interestingly, Foerster *et al.* (2016) demonstrated a similar observation on *Neisseria gonorrhoeae.* The time-kill analysis revealed that ciprofloxacin induced a bactericidal effect in all six different strains of *Neisseria gonorrhoeae*. The most susceptible DG666 strain experienced the most rapid killing at 1 h of incubation. Meanwhile, the time-kill curves for tetracycline

and chloramphenicol looked similar and both exerted bacteriostatic effects with almost no killing of *Neisseria gonorrhoeae* within 4 h of incubation.

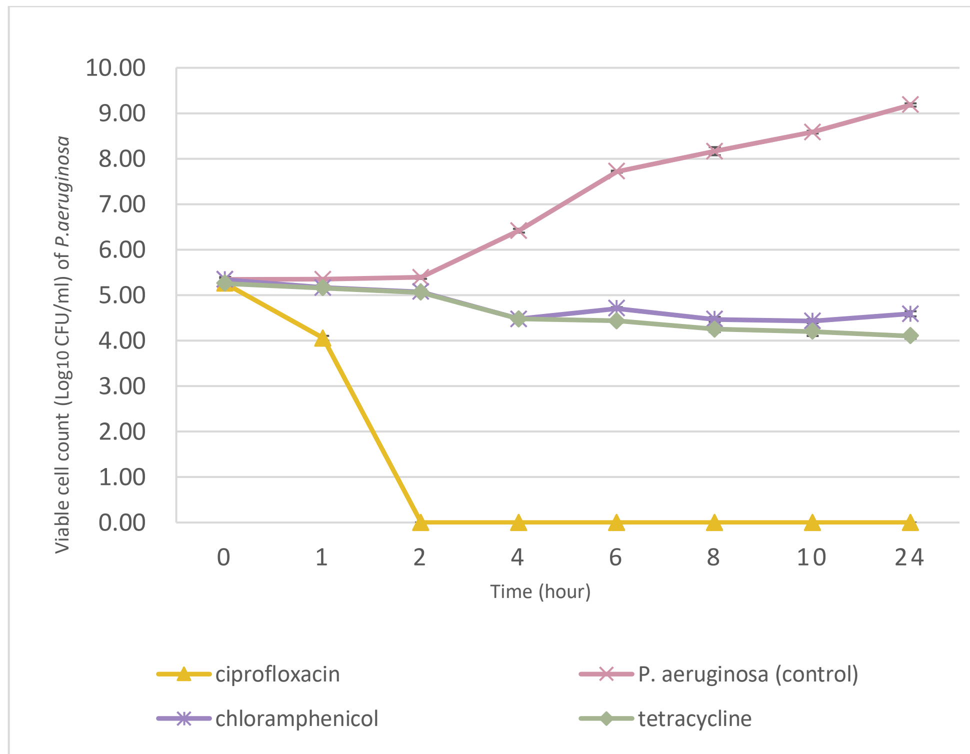

Figure 4.3: Effect of treatment of chloramphenicol, ciprofloxacin and tetracycline on the growth of *P. aeruginosa* ATCC 10145

4.3.7 Determination of the combined inhibitory effect of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145 via time-kill assay

Time-kill assay was conducted to determine the combined inhibitory effect of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145.

Figure 4.4, 4.5 and 4.6 show the time-kill curves for *P. aeruginosa* ATCC 10145 treated with combination of crude bacteriocins TU2, TP1 and TE1 and chloramphenicol. Time-kill curves showed that combination of crude bacteriocins TU2, TP1 and TE1 with chloramphenicol successfully inhibited *P. aeruginosa* ATCC 10145 totally after 8 h of incubation. Hence, combination of crude bacteriocins TU2, TP1 and TE1 with chloramphenicol had exerted bactericidal effect against *P. aeruginosa.* When compared to treatment with crude bacteriocin alone and chloramphenicol alone, the combination of both antimicrobial agents has successfully speed up the killing of *P. aeruginosa* ATCC 10145.

Hence, the killing rate against *P. aeruginosa*:

Chloramphenicol + Bacteriocin > Bacteriocin alone > Chloramphenicol alone

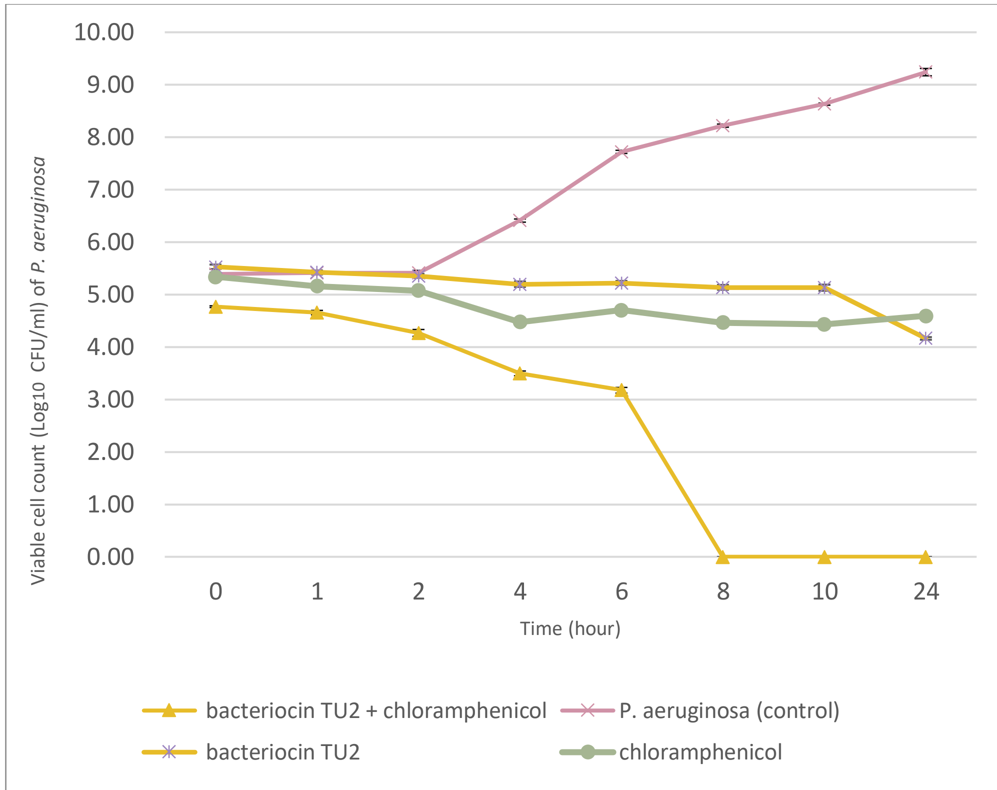

Figure 4.4: Effect of combination of crude bacteriocin TU2 and chloramphenicol on the growth of *P. aeruginosa* ATCC 10145

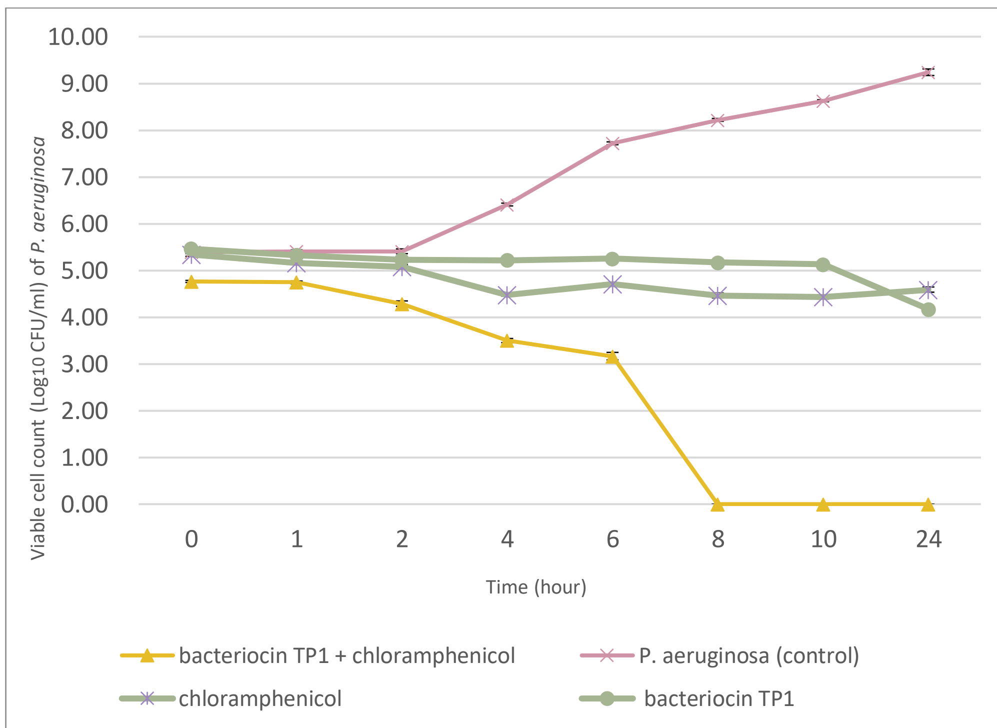

Figure 4.5: Effect of combination of crude bacteriocin TP1 and chloramphenicol on the growth of *P. aeruginosa* ATCC 10145

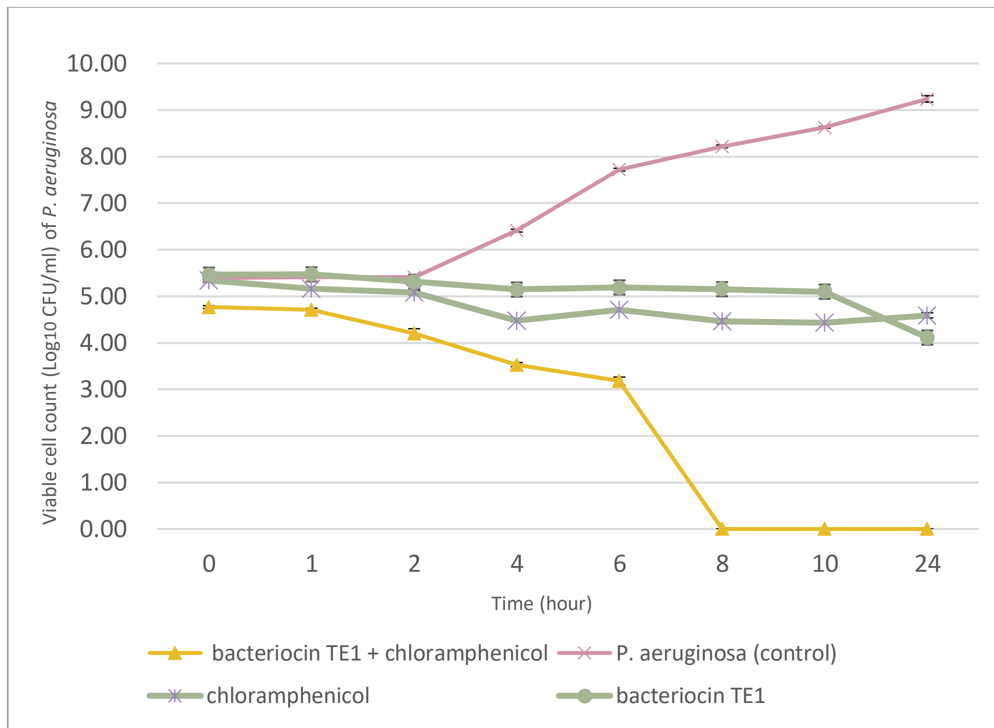

Figure 4.6: Effect of combination of crude bacteriocin TE1 and chloramphenicol on the growth of *P. aeruginosa* ATCC 10145

Figure 4.7, 4.8 and 4.9 show the effect of combination of crude bacteriocins TU2, TP1 and TE1 and ciprofloxacin on the growth of *P. aeruginosa* ATCC 10145. The time-kill curves showed that treatment with ciprofloxacin alone was more effective in killing *P. aeruginosa* ATCC 10145 within 2 h of incubation. Meanwhile, the combination of crude bacteriocin and ciprofloxacin only able to inhibit *P. aeruginosa* ATCC 10145 totally after 8 h of incubation. While treatment with crude bacteriocin alone only resulted in 1-$\log_{10}$ CFU/ml reduction in growth of *P. aeruginosa* ATCC 10145 after 24 h of incubation. Hence, the killing rate against *P. aeruginosa*:

Ciprofloxacin alone > Bacteriocin + ciprofloxacin > Bacteriocin alone

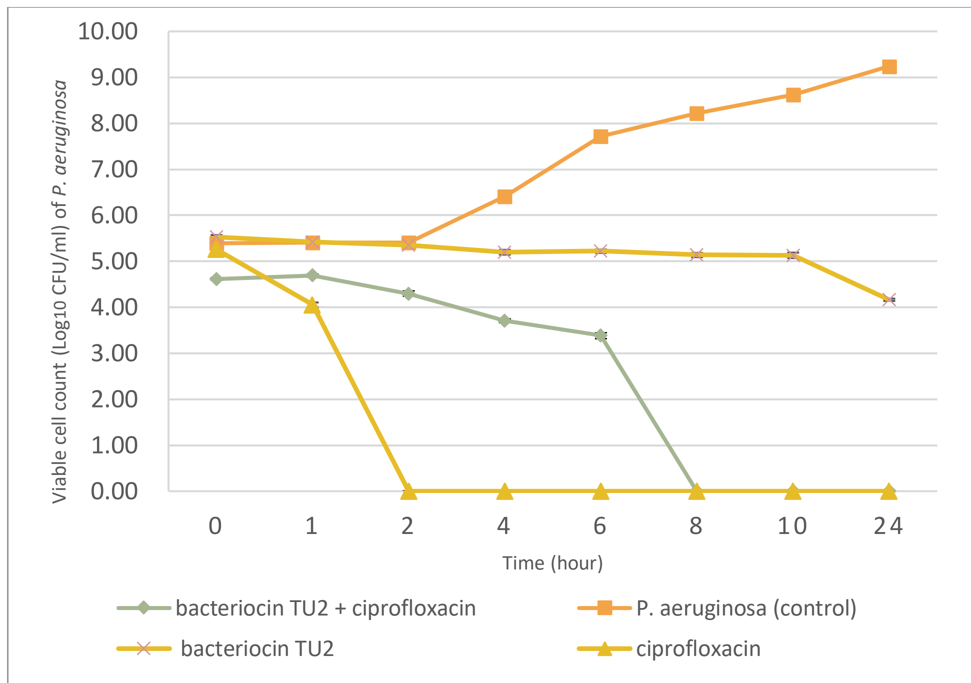

Figure 4.7: Effect of combination of crude bacteriocin TU2 and ciprofloxacin on the growth of *P. aeruginosa* ATCC 10145

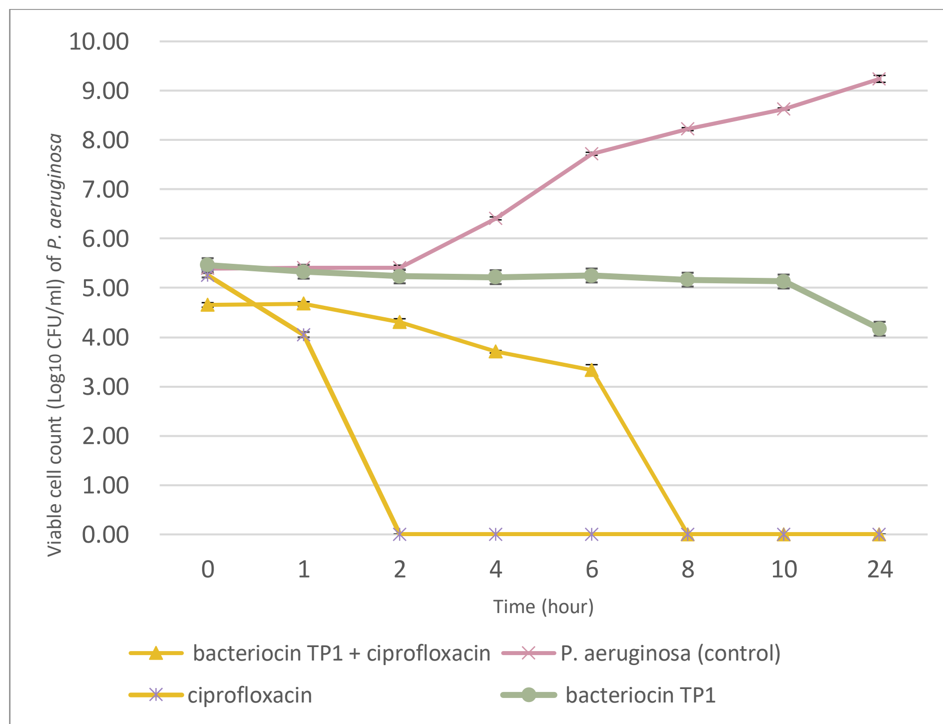

Figure 4.8: Effect of combination of crude bacteriocin TP1 and ciprofloxacin on the growth of *P. aeruginosa* ATCC 10145

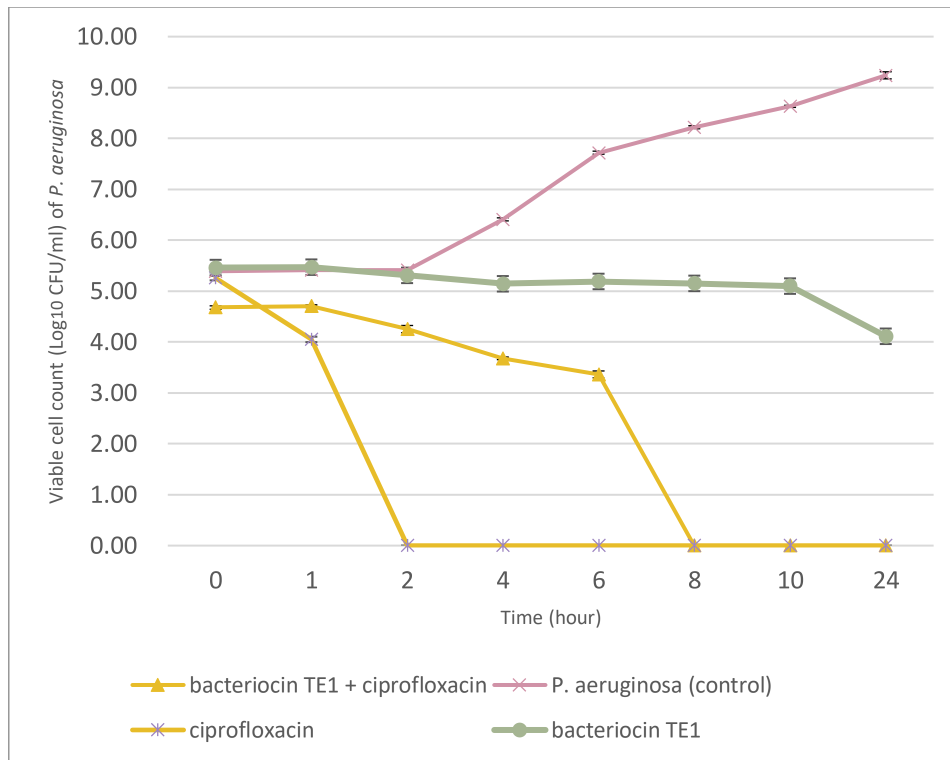

Figure 4.9: Effect of combination of crude bacteriocin TE1 and ciprofloxacin on the growth of *P. aeruginosa* ATCC 10145

Figure 4.10, 4.11 and 4.12 show the time-kill curves for combination of crude bacteriocins TU2, TP1 and TE1 and tetracycline against *P. aeruginosa* ATCC 10145 were similar with combination of crude bacteriocins with chloramphenicol. Combination of crude bacteriocins and tetracycline able to enhance the inhibition of *P. aeruginosa* at 10 h of incubation. Whereas, treatment with crude bacteriocin alone only resulted in 1-$\log_{10}$ CFU/ml reduction after 24 h of incubation. While tetracycline alone only able to suppress the growth of *P. aeruginosa* ATCC 10145 and unable to inhibit it. Hence, the killing rate against *P. aeruginosa*:

Tetracycline + Bacteriocin > Bacteriocin alone > Tetracycline alone

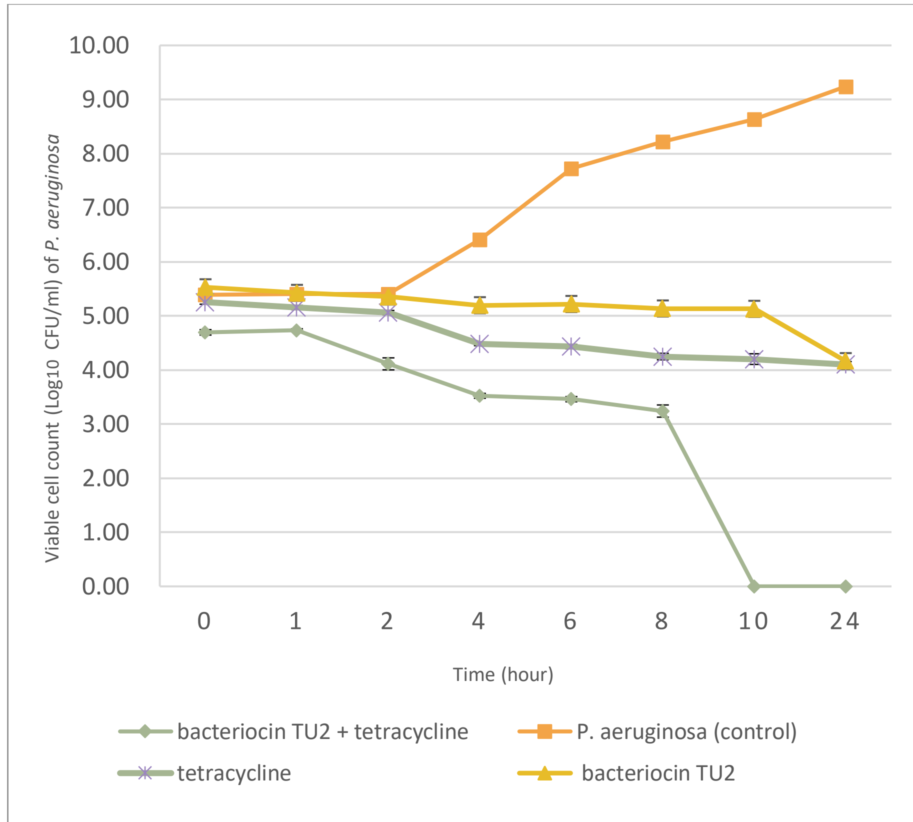

Figure 4.10: Effect of combination of bacteriocin TU2 and tetracycline on the growth of *P. aeruginosa* ATCC 10145

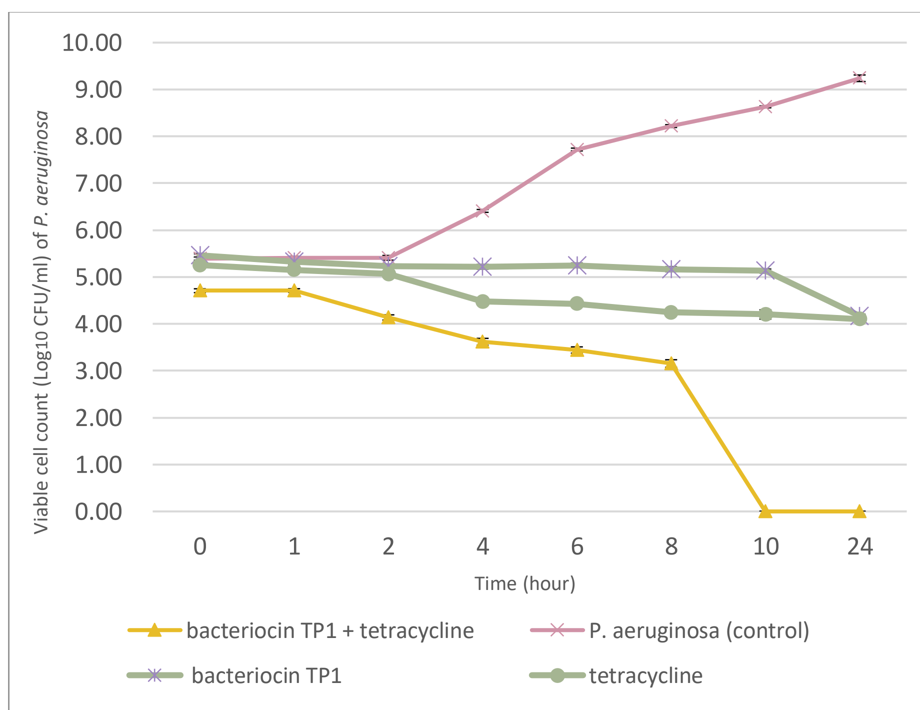

Figure 4.11: Effect of combination of bacteriocin TP1 and tetracycline on the growth of *P. aeruginosa* ATCC 10145

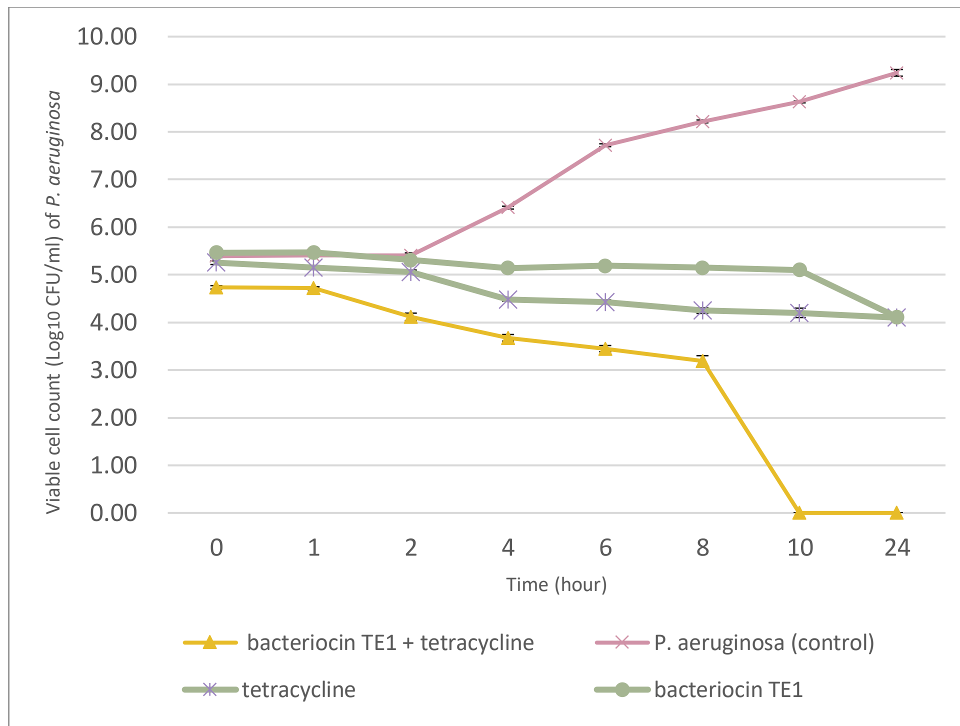

Figure 4.12: Effect of combination of bacteriocin TE1 and tetracycline on the growth of *P. aeruginosa* ATCC 10145

Interestingly, all three crude bacteriocins TU2, TP1 and TE1 tested exhibited similar killing profiles with all the antibiotics against *P. aeruginosa* ATCC 10145. These findings suggested that the three bacteriocins produced by *Pd. pentosaceus* could be of similar type of bacteriocin, hence produced similar inhibiting patterns.

Synergy between crude bacteriocins and chloramphenicol and tetracycline caused a complete killing of *P. aeruginosa* ATCC 10145 in the time-kill assay. Whereas treatment of these antimicrobial agents alone only able to suppress the growth of *P. aeruginosa* ATCC 10145 and unable to inhibit it. Hence, the synergy indicates that crude bacteriocins from *Pd. pentosaceus* and chloramphenicol and tetracycline have different modes of action, which is different from the crude bacteriocin and antibiotic when used alone.

In contrast, the combination of crude bacteriocins with ciprofloxacin was found to delay the killing rate at 8 h when compared to the treatment of ciprofloxacin alone which caused the complete killing of *P. aeruginosa* ATCC

10145 at 2 h. A study conducted by Grillon *et al.* (2016) also revealed that treatment of ciprofloxacin alone was found to be the most effective bactericidal agent against *P. aeruginosa* when compared to other treatment of antibiotics in time-kill study.

To the author's knowledge, no relevant findings on time-kill assay of combination of antibiotics and crude bacteriocin from *Pd. pentosaceus* were found. A study reported by Naghmouchi *et al.* (2013) demonstrated that the combination of nisin A or pediocin A with antibiotic colistin produced synergistic effect against Gram-negative bacteria such as *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 35150. Similar finding was reported by Field *et al.* (2016) which revealed that the combination of nisin with polymyxin produced synergistic effect against *P. aeruginosa* PA-01 biofilm. Hence, current study of using combination of crude bacteriocins and selected antibiotics might enhance the efficacy of treatment against MDR pathogens.

4.4 Summary

The modified bacteriocin activity of freeze-dried crude bacteriocins was higher than the neutralized CFS suggested the freeze-drying successfully concentrated the bacteriocin. All three crude bacteriocins demonstrated MIC of 15.63 mg/ml against *P. aeruginosa* ATCC 10145. While the MIC value for ciprofloxacin is 0.25 µg/ml, more effective than chloramphenicol and tetracycline. Ciprofloxacin was shown to be an effective bactericidal agent against *P. aeruginosa* ATCC 10145*.* In contrast, all three crude bacteriocins, chloramphenicol and tetracycline were demonstrated to be bacteriostatic agents against *P. aeruginosa* ATCC 10145*.* The time-kill assays revealed that the combination of crude bacteriocins with chloramphenicol and tetracycline showed synergistic interaction of *P. aeruginosa* ATCC 10145. Whilst the combination of crude bacteriocins and ciprofloxacin produced an indifferent effect against *P. aeruginosa* ATCC 10145. These findings suggested that the combination of bacteriocins produced by *Pd. pentosaceus* with selected antibiotics could be a promising strategy to revoke the antibiotic resistance of *P. aeruginosa* ATCC 10145.

Chapter 5: Effect of crude bacteriocins and selected antibiotics on the cell surface morphological changes of *P. aeruginosa* ATCC 10145

5.1 Introduction

Combination of crude bacteriocins from *Pd. pentosaceus* with antibiotics have effectively inhibited *P. aeruginosa* ATCC 10145 via time-kill assay. Hence, this chapter will investigate the cell surface morphological changes on *P. aeruginosa* ATCC 10145 using scanning electron microscope (SEM).

In the previous chapter, it was revealed that crude bacteriocins from *Pd. pentosaceus* isolated from traditional fermented foods have synergistic interaction with chloramphenicol and tetracycline, and able to inhibit *P. aeruginosa* ATCC 10145. However, the action mechanisms of these combinations were still unknown.

In this chapter, the scanning electron microscope was used to observe the changes on cell surface morphology of *P. aeruginosa* ATCC 10145 after treated with crude bacteriocins and antibiotics. This may feasibly provide some fundamental understanding on the action mechanism of these antimicrobial agents.

5.2 Materials and methods

5.2.1 Preparation of Karnovsky's fixative solution

The formaldehyde-glutaraldehyde fixative solution was prepared according to the procedure described by Karnovsky's fixation method with minor modifications (Karnovsky, 1965). Fixation 2% formaldehyde and 2.5% glutaraldehyde was prepared by adding 5 ml of 40% formaldehyde (Sigma Aldrich, Germany) to 10 ml 25% glutaraldehyde (Sigma Aldrich, Germany) and buffered with 85 ml of 0.1 M Phosphate-buffered saline (Sigma Aldrich, Germany) and kept in Schott bottle at 4℃ chiller.

5.2.2 Experimental design

P. aeruginosa ATCC 10145 used in this study was prepared according to Section 3.2.2(b). This experiment was carried out using MH broth in a 96 well microtiter plate. Only bacteriocin TU2 was chosen to represent the three crude bacteriocins in this study. TU2 bacteriocin was selected due to it has the highest modified bacteriocin activity among the three crude bacteriocins, hence it will exhibit strong inhibitory effect against *P. aeruginosa* ATCC 10145.

For the treatment of crude bacteriocin only, each well contained 50 µl of crude bacteriocin with concentration of 1x MIC (15.63 mg/ml) after 50 µl of *P. aeruginosa* ATCC 10145 was then added into each well. For the treatment of antibiotics only, each well contained 50 µl of antibiotics with concentration of 1x MIC (32 µg/ml for chloramphenicol and tetracycline, and 0.25 µg/ml for ciprofloxacin) after 50 µl of *P. aeruginosa* ATCC 10145 was then added into each well.

For the combination of crude bacteriocin and antibiotic treatment, each well contained 50 µl of crude bacteriocin with concentration of 0.25x MIC (3.906 mg/ml), respectively and ciprofloxacin with concentration of 0.25x MIC (0.063 µg/ml), respectively and chloramphenicol and tetracycline with concentration of 0.25x MIC (8 µg/ml), respectively after 50 µl of *P. aeruginosa* ATCC 10145 was then added into each well. Positive control in this assay was the well containing *P. aeruginosa* ATCC 10145 only, while negative control was the well containing MH broth only.

The plate was incubated at 37 ℃ for 24 h prior to fix on glass coverslip using Karnovsky method. An aliquot of 30 µl was pipetted from each well and spread onto MH agar plates at some interval times. The agar plates were then incubated at 37 ℃ for 24 hours.

5.2.3 Preparation of samples for SEM viewing

Samples from each treatment in Section 5.2.2 were fixed using Karnovsky's fixation method with minor modifications (Karnovsky, 1965). The sterile glass cover slip was placed and dabbed onto the colony formed on the agar plates obtained from Section 5.2.2 so that the bacterial cells were attached to the glass cover slip. Then, the cover slip was immersed in 2%(v/v) formaldehyde (Sigma Aldrich, Germany) and 2.5%(v/v) glutaraldehyde (Sigma Aldrich, Germany) buffered with 0.1 M phosphate buffer solution (Sigma Aldrich, Germany) and was kept at 4 ℃ chiller for 24 h. Then, the sample was washed three times in 0.1 M phosphate buffer solution for 3 min. The sample was then dehydrated in each concentration of ethanol (Systerm Chemicals, Malaysia) by 50%(v/v), 70%(v/v), 80%(v/v), 90%(v/v), 95%(v/v) and 100%(v/v) for 15 min. Then, the sample was subjected to air-drying in the fume hood. The dried cover slip was placed on aluminium stubs using double sided foam tape and coated with platinum using sputtering device (Quorum, UK) and observed with scanning electron microscope (FEI, USA) at 20 KV. The SEM images of untreated and treated *P. aeruginosa* ATCC 10145 with crude bacteriocin and antibiotics were adjusted at magnifications of x20000, x50000 and x80000. Any significant cell surface morphological changes such as size, shapes and structures on the cell surface of *P. aeruginosa* ATCC 10145 at these magnifications were captured and further examined.

5.3 Results and Discussion

5.3.1 Cell surface morphology of *P. aeruginosa* ATCC 10145

Scanning electron microscope (SEM) is a useful tool that uses electron beam radiated to a biological sample and scans the surface of the sample to form an image at a greater resolution than a light microscope (Micheva *et al.*, 2007). Hence, SEM allows examination of the cell surface morphology of large numbers of whole intact microorganism at high magnification (Kashi *et al*., 2014).

Figure 5.1 show the scanning electron microscope (SEM) images of cell surface morphology of *Pseudomonas aeruginosa* ATCC 10145 at 4 and 8 h of incubation. The time at 4 and 8 h of incubation were chosen because *P. aeruginosa* ATCC 10145 was at the initial growth of a log-phase at 4 h and growing rapidly between 4 to 8 h of incubation based on the time-kill assay carried out previously. Based on the Figure 5.1(A), the *P. aeruginosa* ATCC 10145 cells were rod-shaped, smooth and intact cell walls and membrane without any morphological changes at 4 h of incubation.

This finding was in line with studies reported by Iglewski (1996) and Waisbren *et al.* (1980). Iglewski (1996) described *P. aeruginosa* as a rod-shaped *bacillus* belonged to bacterial family Pseudomonadaceae while Waisbren *et al.* (1980) reported that *P. aeruginosa* were observed to have smooth and intact cell walls and membranes without any morphological changes. Apart from that, *P. aeruginosa* also formed a monolayer and was evenly distributed throughout the surface of glass coverslip at 4 h as seen in the Figure 5.1(B).

After 8 h of incubation, *P. aeruginosa* formed clumps of cells as showed in Figure 5.1(C) and (D). This observation suggested that *P. aeruginosa* cells have started to aggregate and formed biofilms as strong cohesive adherence to solid surface. The ability of *P. aeruginosa* to grow in a biofilm may help the bacteria to resist and protect them from the host defenses mechanisms

(Mulcahy *et al*., 2014). A similar finding was also reported by Deligianni *et al.* (2010) and O' Driscoll *et al.* (2018) which demonstrated that *P. aeruginosa* formed a monolayer and clump of cells on glass coverslip when observed under scanning electron microscope.

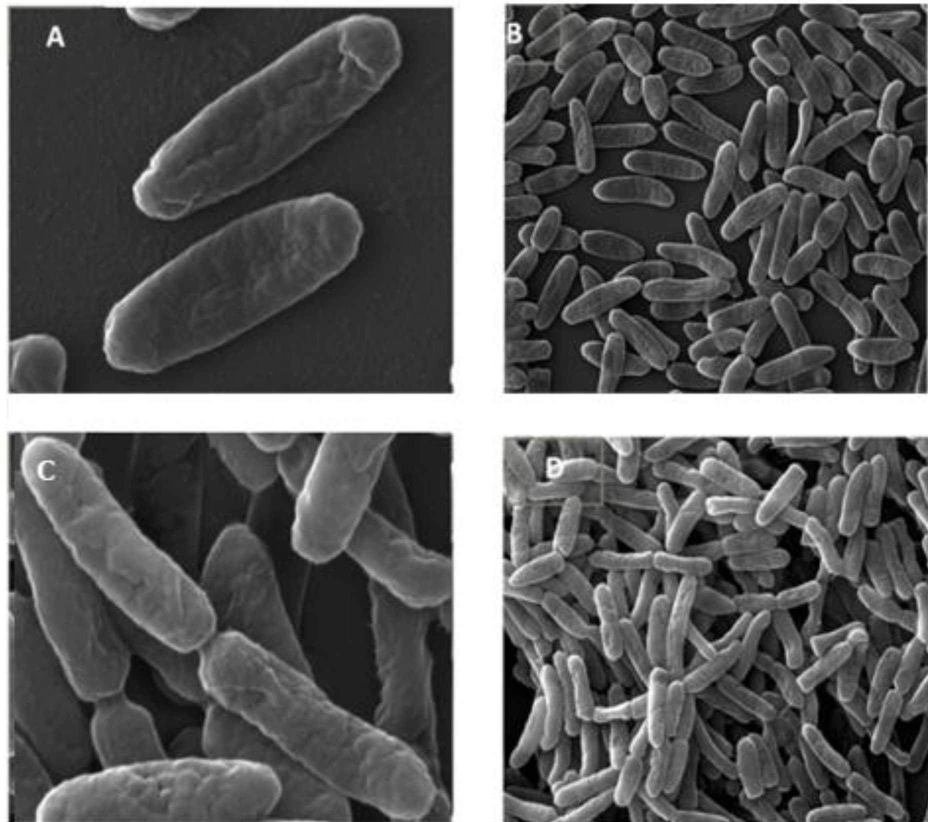

Figure 5.1: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 after incubation with Mueller Hinton broth for 4 h (A)(B) and 8 h (C)(D).

A) Control culture of the untreated *P. aeruginosa* at 4 h (x80,000).

B) Control culture of the untreated *P. aeruginosa* at 4 h (x20,000).

C) Control culture of the untreated *P. aeruginosa* at 8 h (x80,000).

D) Control culture of the untreated *P. aeruginosa* at 8 h (x20,000).

5.3.2 Cell surface morphology of *P. aeruginosa* ATCC 10145 after treated with crude bacteriocin

Figure 5.2 show the scanning electron microscope images of *Pseudomonas aeruginosa* ATCC 10145 treated with crude bacteriocin TU2 at 4 and 8 h. Interesting change in cell surface morphology was observed such as cell was elongated by 8% at 4 h as observed in Figure 5.2(A) when compared to control *P. aeruginosa* ATCC 10145 cells (not treated with crude bacteriocin) at 4 h of incubation (Figure 5.1A).

After 8 hours of incubation with crude bacteriocin TU2, circular holes or pores formed on the cell surface (yellow arrows) in Figure 5.2(C). In addition to that, cell wall was ruptured where leakage of the cellular content was observed (blue arrows) in Figure 5.2(D).

These findings were in accordance with the studies reported by Yusra & Efendi (2007), Wen *et al.* (2016) and Indiraa *et al.* (2018), which demonstrated pore formation and leakage of the cellular contents after *P. aeruginosa* cells were exposed to crude bacteriocin. Okuda *et al*. (2013) also suggested that the pore formation in the membrane of *P. aeruginosa* resulted in membrane leakage of ions leading to disruption of membrane potential and ATP depletion. Hence, this will lead to diffusion of low molecular cystosolic compounds out of the cell and resulting in cell death. In general, the antimicrobial compound of bacteriocin disrupted the cell membrane target, such that the cells lost their functions (Oscariz & Pisabarro, 2000). However, scanning electron microscope revealed that crude bacteriocin TU2 was not able to inhibit 100% of *P. aeruginosa* cells at 8 h of incubation as observed in Figure 5.2(D). Cell lysis were seen in a few *P. aeruginosa* cells and only approximately 20% of the cells were affected by the presence of crude bacteriocin. This finding was consistent with the result from the time-kill assay that shown the bacteriostatic effect of crude bacteriocins against *P. aeruginosa* ATCC 10145 (Figure 4.2).

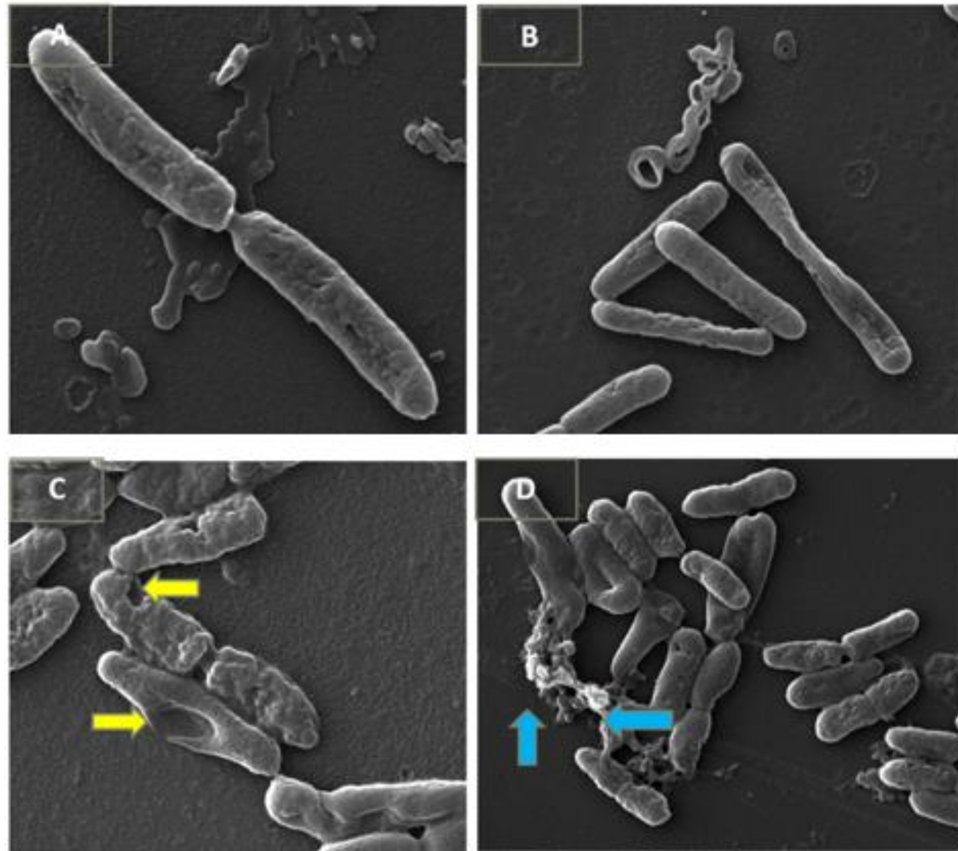

Figure 5.2: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 treated with crude bacteriocin for 4 h (A)(B) and 8 h (C)(D).

A) and B) Cell elongation to form chains in the presence of bacteriocins (x80,000) (x50,000)

C) Holes formation (yellow arrows) in the presence of bacteriocin (x80,000).

D) Cell walls were broken where efflux of the cellular contents was observed (blue arrows) in the presence of bacteriocin (x50,000).

5.3.3 Cell surface morphology of *P. aeruginosa* ATCC 10145 after treated with selected antibiotics

5.3.3.1 Chloramphenicol

Figure 5.3 show the SEM images of *P. aeruginosa* ATCC 10145 treated with chloramphenicol for 2, 4 and 8 h. Among the cell surface morphological changes observed were large pores or circular holes on the cell surfaces at 2 h (Figure 5.3 A) and cell walls ruptured at 4 h (Figure 5.3 B) and 8 h (Figure 5.3 C).

After treated with chloramphenicol alone, the formation of holes and the cell membranes ruptured in *P. aeruginosa* ATCC 10145 cells indicated partial disruption of the cell walls, bacterial outer membranes and inner membranes. These observations suggested that the formation of pores helped chloramphenicol to gain access into the bacterial inner membrane.

According to Morita *et al.* (2014), chloramphenicol bound reversibly to the peptidyl transferase component of the 50S ribosomal subunit which may inhibit protein synthesis and preventing the transpeptidation process of peptide chain elongation. The interference of protein synthesis was accompanied by the cell membrane disruption, leading to the cell-wall rupture as seen in Figure 5.3(D).

However, only approximately 20% of the *P. aeruginosa* ATCC 10145 cells were examined to have cell lysis and the presence of chloramphenicol alone could not kill 100% *P. aeruginosa* cells at 8 h of incubation as observed in Figure 5.3 (D). Hence, this finding was consistent with the time-kill assay previously conducted where chloramphenicol was found to be bacteriostatic against *P. aeruginosa*. In the time-kill assay, chloramphenicol only inhibited *P. aeruginosa* by 1-log_{10} CFU/ml reduction after 8 h of incubation (Figure 4.3).

According to Delcour (2009), the greater resistance of *P. aeruginosa* towards antibiotic such as chloramphenicol may be associated with the presence of

the outer membrane (OM), low abundance of porins combined with numerous and highly efficient efflux mechanism.

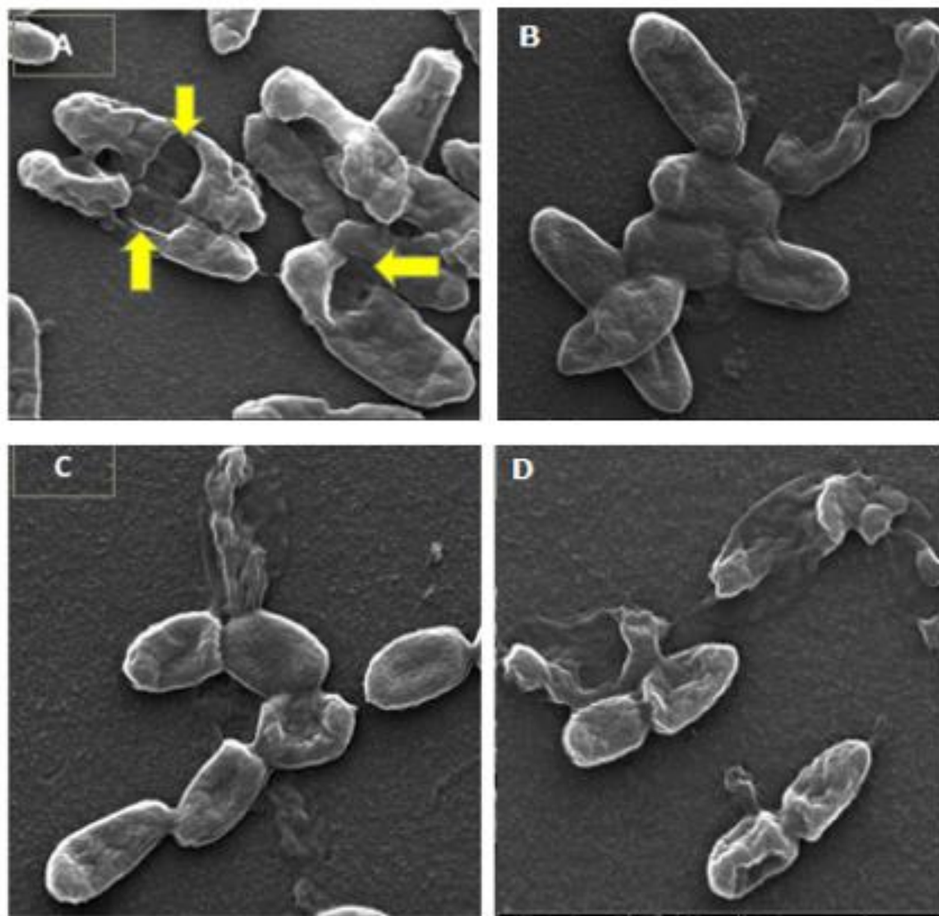

Figure 5.3: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 treated with chloramphenicol for 2 h (A) 4 h (B) and 8 h (C)(D).

A) Holes formation (yellow arrows) in the presence of chloramphenicol (x50,000).

B) Cell walls started to rupture in the presence of chloramphenicol (x50,000).

C) Cell walls and cell membranes were ruptured in the presence of chloramphenicol (x50,000).

D) Cell lysis were observed in a few cells in the presence of chloramphenicol (x50,000).

5.3.3.2 Ciprofloxacin

Figure 5.4 show the SEM images of *P. aeruginosa* ATCC 10145 treated with ciprofloxacin at 1 and 2 h after incubation. The chosen time at 1 and 2 h of incubation were shorter than the other treatments because ciprofloxacin was the most effective antimicrobial agent against *P. aeruginosa* ATCC 10145 and able to inhibit the cells totally within 2 h of incubation based on the previous time-kill assay conducted (Figure 4.3). Cell surface morphological changes were seen in cells exposed to ciprofloxacin such as large pore or hole (yellow arrow) on the cell surface at 1 h in Figure 5.4(A). Apart from that, some of the cells have broken cell walls where efflux of the cellular contents (blue arrow) were observed at 1 h in Figure 5.4(B).

In Figure 5.4(C), nub formation (red arrow) which consisting of small and roughly spherical surfaces were also examined at 2 h under the scanning electron microscope. This finding was consistent with the past study by Siqueira *et al.* (2014) that demonstrated spherical surfaces were observed in the significant number in 1071-meropenem resistant *P. aeruginosa* (MRPA) cells exposed to 0.5 x MIC (32 μg/ml) of ciprofloxacin at 3 h.

Meanwhile, a study conducted by Reid (1994) found that *P. aeruginosa* AK1 strains were malformed and elongated after exposure to 0.5 μg/ml of ciprofloxacin at 1 h of incubation. According to LeBel (1988), ciprofloxacin was known to be the most active quinolone against *Pseudomonas aeruginosa* and bind non-covalently to two target proteins, DNA gyrase and topoisomerase IV, leading to double-strand DNA breaks and hence, leading to cell lysis.

The SEM images in this study revealed that 100% of *P. aeruginosa* ATCC 10145 cells treated with ciprofloxacin were examined to have morphological changes leading to cell lysis within 2 hours as observed in Figure 5.4(D). Hence, this finding was in line with the result demonstrated in the previous time-kill assay conducted in this study that ciprofloxacin was an effective

bactericidal agent against *P. aeruginosa* ATCC 10145 by 5-log_{10} CFU/ml reduction within 2 h of incubation (Figure 4.3).

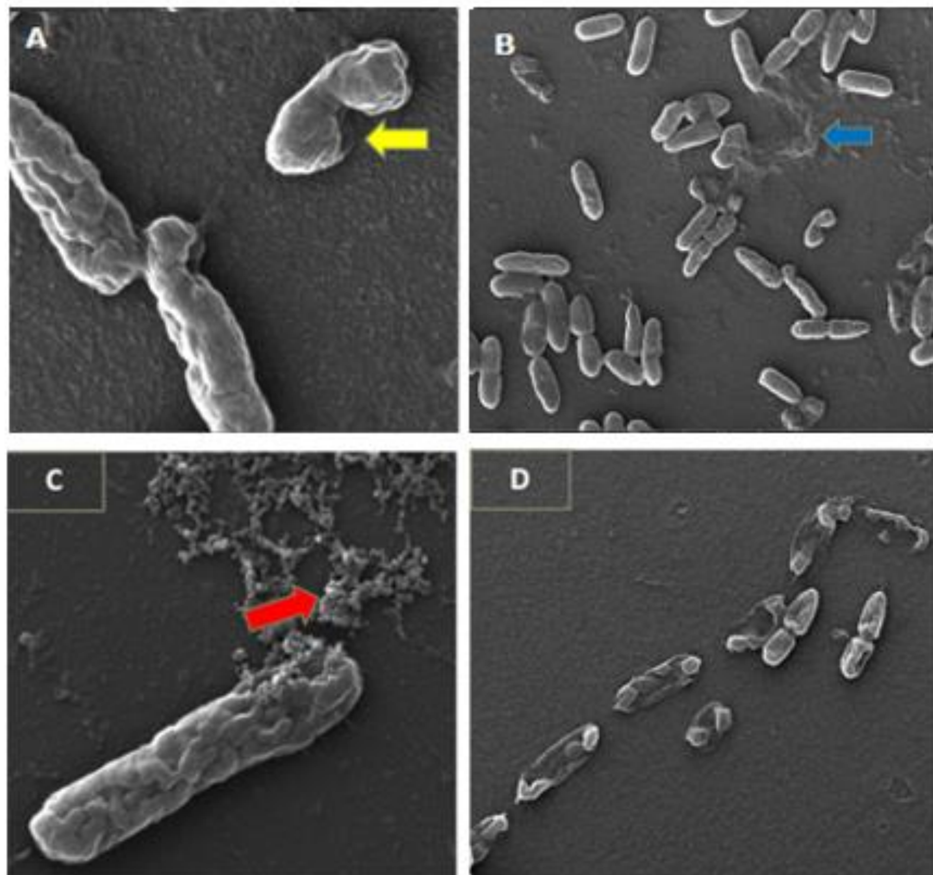

Figure 5.4: Scanning electron microscopy images of *Pseudomonas aeruginosa* treated with ciprofloxacin for 1 h (A)(B) and 2 h (C)(D).

A) Hole formation (yellow arrow) in the presence of ciprofloxacin (x80,000).

B) Some of the cells have broken cell walls where efflux of the cellular contents was observed (blue arrow) in the presence of ciprofloxacin (x20,000).

C) Nub formation (red arrow) which consist of small and roughly spherical surfaces were examined in the presence of ciprofloxacin (x80,000).

D) Cell death occurred in the presence of ciprofloxacin (x20,000)

5.3.3.3 Tetracycline

Figure 5.5 show the SEM images of *P. aeruginosa* ATCC 10145 treated with tetracycline at 2, 4 and 8 h after incubation. Cell surface morphological changes were seen in cells exposed to tetracycline such as pores formation on the cell surfaces (yellow arrows) at 2 h in Figure 5.5(A). In Figure 5.5 (B), a roundish cell (red arrow), cell elongation (green arrow) and stalked nubs formation (blue arrow) were observed at 4 h of incubation. Apart from that, cell death occurred in a few cells were observed at 4 h in Figure 5.5(C) and at 8 h in Figure 5.5(D).

These cell surface morphological changes observed were consistent with the finding reported by Waisbren *et al*. (1980) which found elongation of cells and stalked nubs in *P. aeruginosa* cells when treated with tetracycline via scanning electron microscope. In another study conducted by Moghoofei *et al*. (2015), microscopic image of *P. aeruginosa* revealed that the cells were elongated after exposure to antibiotic. According to Ofek *et al*. (1979), the elongated forms of cells were less adherent than the normal forms, hence decreased the adhesion ability of *P. aeruginosa* to form biofilm. Since the adhesion of planktonic cells is the first step to form biofilms (Ebrey *et al*., 2004), hence antibiotic helps to prevent the further biofilm formation by adjusting their morphology via cell elongation.

Meanwhile, approximately 10% of *P. aeruginosa* cells were examined to have roundish cells at 8 h of incubation when treated with tetracycline in the current study. A similar finding was reported by Fonseca & Sousa (2007) which found that 62% of all *P. aeruginosa* strains have roundish cells formed under the influence of 0·5x MIC of Imipenem. In another study reported by Yokochi *et al*. (2000), the rod-shaped *P. aeruginosa* cells became rounded within 2 hours after the treatment of Imipenem. These morphological changes were believed to be related to the susceptibility of round *P.*

aeruginosa cells to phagocytosis of peritoneal macrophages (Yokochi *et al*., 2000).

However, scanning electron microscope in this study revealed that approximately 20% of *P. aeruginosa* ATCC 10145 cells treated with tetracycline were examined to have cell lysis (Figure 5.5D). The treatment of tetracycline alone could not inhibit *P. aeruginosa* completely, hence, this finding suggested that tetracycline was a bacteriostatic agent against *P. aeruginosa* as demonstrated via time-kill assay in this study. In the previous time-kill study, tetracycline was only able to inhibit *P. aeruginosa* by 1-$\log_{10}$ CFU/ml reduction after 8 h of incubation (Figure 4.3).

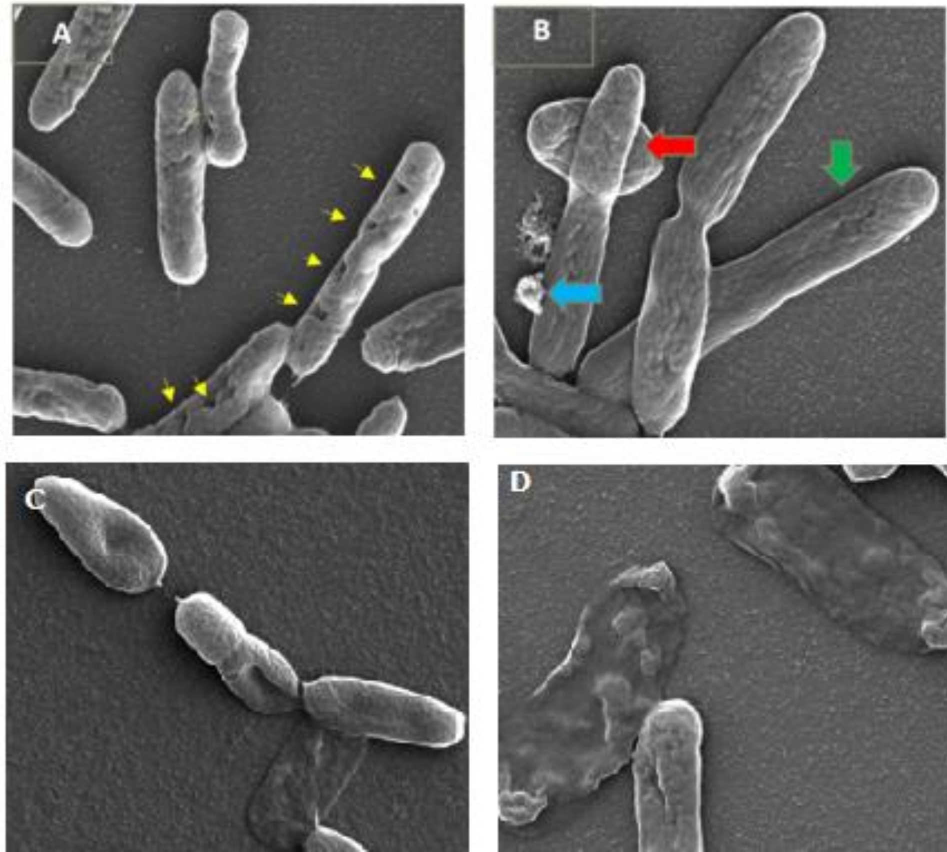

Figure 5.5: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 treated with tetracycline for 2 h (A), 4 h (B)(C) and 8 h (D).

A) Pores formation (yellow arrows) in the presence of tetracycline (x80,000).

B) Roundish cell formed (red arrow), cell elongation (green arrow) and stalked nubs formation (blue arrow) in the presence of tetracycline in the presence of tetracycline (x80,000).

C) Cell death occurred in one cell in the presence of tetracycline (x80,000).

D) Cell deaths occurred in a few cells in the presence of tetracycline (x80,000).

5.3.4 Cell surface morphology of *P. aeruginosa* ATCC 10145 after treated with antibiotics and crude bacteriocin

5.3.4.1 Chloramphenicol and crude bacteriocin

Figure 5.6 show the SEM images of *P. aeruginosa* ATCC 10145 with combination of crude bacteriocin and chloramphenicol for 2, 4 and 8 h. Among the cell surface morphological changes observed were formation of pores (yellow arrows) on the cell membranes at 2 h in Figure 5.6 (A). Other than that, cells elongation was also observed in Figure 5.6 (B) at 2 h. At 4 h, the cell membranes started to rupture and the cellular contents were leaked as seen in Figure 5.6(C). After 8 h, the *P. aeruginosa* cells were ruptured and leading to cell death as observed in Figure 5.6(D).

A study reported by Tong *et al.* (2014) demonstrated that the combination of nisin and chloramphenicol also resulted in many *E. faecalis* cells lost their original morphology and showed distinct cellular disruption.

When *P. aeruginosa* treated respectively with crude bacteriocin and chloramphenicol, some cell surface morphological changes such as pore formation, cell wall ruptured and cell lysis were observed but unable to cause severe damage to all cells tested after 8 h of treatment. However, when *P. aeruginosa* cells were treated with the combination of crude bacteriocin and chloramphenicol, majority of the cells were severely lysed and resulted in cell death within 8 h of treatment (Figure 5.6 D). Hence, this finding suggested that the combination of crude bacteriocin with chloramphenicol was bactericidal and the most effective in killing *P. aeruginosa* ATCC 10145 as demonstrated via time-kill assay in this study (Figure 4.4).

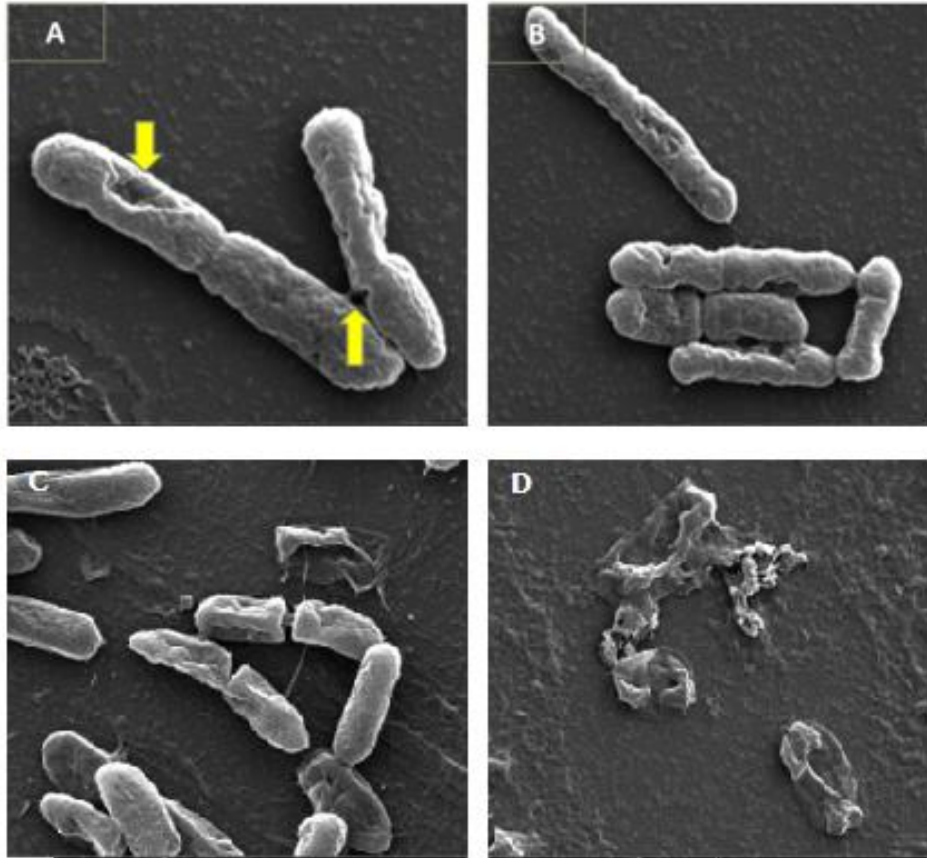

Figure 5.6: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 treated with crude bacteriocin and chloramphenicol for 2 h (A) and (B), 4 h (C) and 8 h (D).

A) Formation of pores (yellow arrows) in the presence of bacteriocin and chloramphenicol (x80,000).

B) Cell elongation in the presence of bacteriocin and chloramphenicol (x80,000).

C) Cell membranes were ruptured and release of the cellular contents in the presence of bacteriocin and chloramphenicol (x80,000)

D) Destruction of all cells in the presence of bacteriocin and chloramphenicol (x80,000).

5.3.4.2 Ciprofloxacin and crude bacteriocin

Figure 5.7 show the SEM of *P. aeruginosa* ATCC 10145 treated with crude bacteriocin and ciprofloxacin for 2, 4 and 8 h. Among the cell surface morphological changes observed were formation of pore (yellow arrow) on the cell membrane at 2 h in Figure 5.7(A). In Figure 5.7(B), cells became shorter and formed chains at 2 h, and the cell membranes started to rupture and the cellular contents were leaked at 4 h in Figure 5.7(C). After 8 h of treatment, cell lysis was observed in about 100% of *P. aeruginosa* ATCC 10145 cells and leading to cell death as observed in Figure 5.7(D).
Current finding suggested that the combination of crude bacteriocin and ciprofloxacin treatment was effective in inhibiting *P. aeruginosa* ATCC 10145 after 8 hours of incubation. This finding was in line with the result of time-kill assay of combination of crude bacteriocin and ciprofloxacin carried out in this study (Figure 4.7). The SEM images of *P. aeruginosa* treated with bacteriocin alone resulted in some cell surface morphological changes but unable to lyse all the cells (Figure 5.2D). However, treatment with ciprofloxacin alone was the most effective in killing *P. aeruginosa* ATCC 10145 cells after 2 h of incubation (Figure 5.4D).

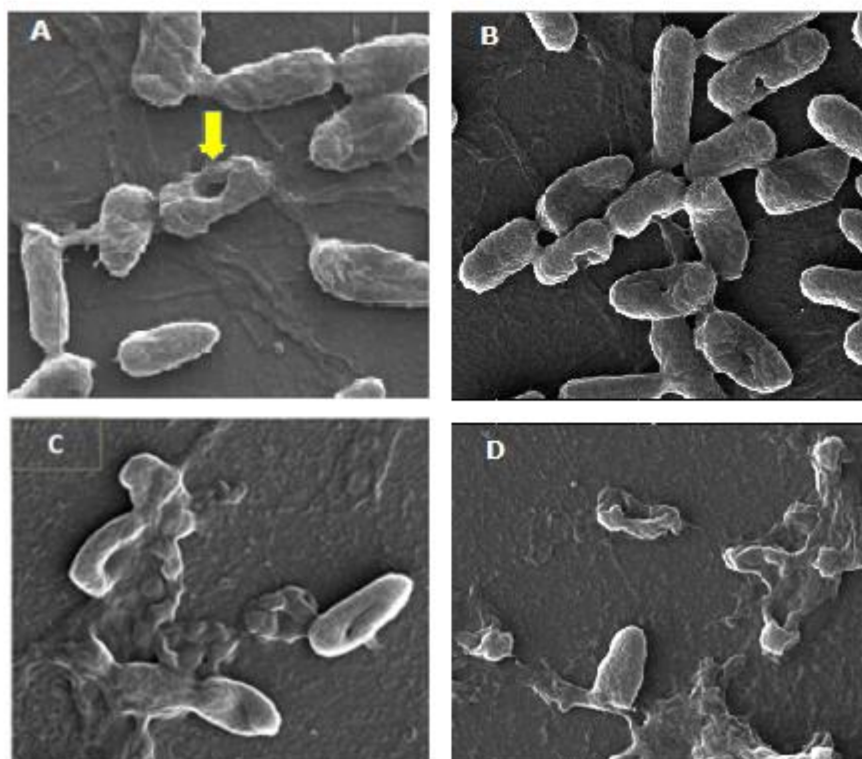

Figure 5.7: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 treated with crude bacteriocin and ciprofloxacin for 2 h (A) and (B), 4 h (C) and 8 h (D).

A) Formation of pore (yellow arrow) in the presence of bacteriocin and ciprofloxacin (x80,000).

B) Cells became shorter and formed chains in the presence of bacteriocin and ciprofloxacin (x80,000).

C) Cell membranes were ruptured and release of the cellular contents in the presence of bacteriocin and ciprofloxacin (x80,000)

D) Destruction of all cells in the presence of bacteriocin and ciprofloxacin (x80,000).

5.3.4.3 Tetracycline and crude bacteriocin

Figure 5.8 show the SEM images of *P. aeruginosa* ATCC 10145 treated with crude bacteriocin and tetracycline for 2, 4 and 8 h. Among the cell surface morphological changes observed were formation of pores (yellow arrows) on the cell membranes after 2 h treatment (Figure 5.8A). In Figure 5.8(B), cell membranes ruptured and released of the cellular contents were observed in some of the cells after 4 h incubation. After 8 h of incubation, cells lysis occurred and the cytoplasmic contents were leaked as observed in Figure 5.8(C), resulting in cell death as observed in Figure 5.8(D).

Scanning electron microscope revealed that the treatment of crude bacteriocin in combination with tetracycline was bactericidal and resulted in 100% killing of *P. aeruginosa* ATCC 10145 after 8 h of incubation. This finding was consistent with the observation shown by time-kill assay of combination of crude bacteriocin and tetracycline treatment (Figure 4.10) in this study. The SEM images of *P. aeruginosa* ATCC 10145 treated with crude bacteriocin and tetracycline alone resulted in some cell surface morphological changes but ineffective in killing all *P. aeruginosa* ATCC 10145 cells (Figure 5.2D and Figure 5.5D). Hence it can be concluded that the combination of crude bacteriocin with tetracycline was the most effective in killing *P. aeruginosa* ATCC 10145.

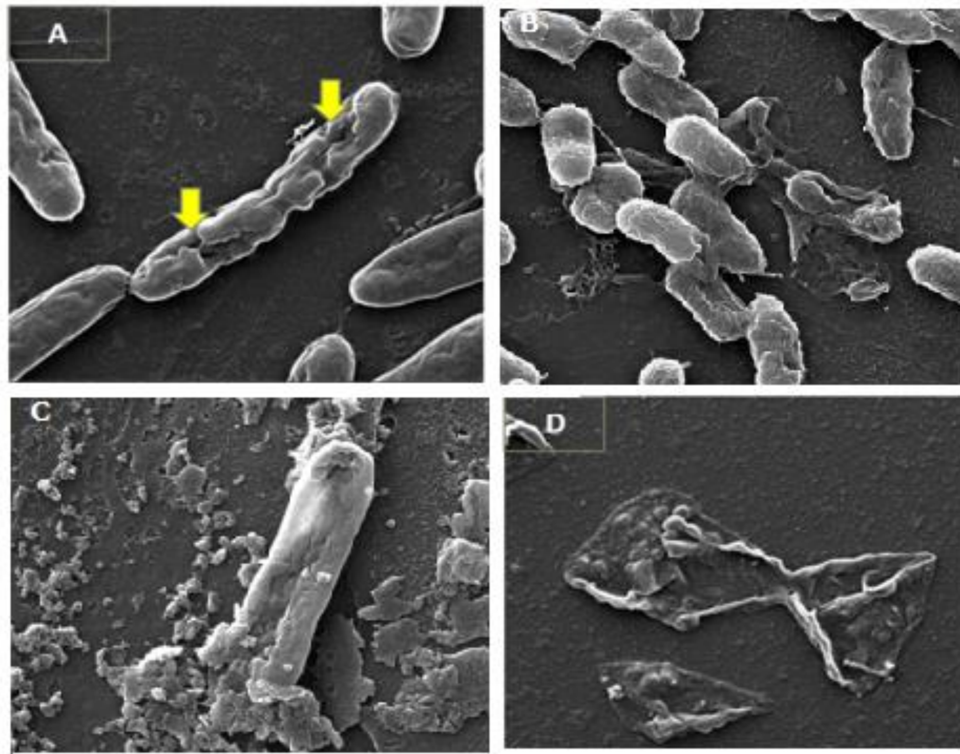

Figure 5.8: Scanning electron microscopy images of *Pseudomonas aeruginosa* ATCC 10145 treated with crude bacteriocin and tetracycline for 2 h (A), 4 h (B) and 8 h (C) and (D).

A) Formation of pores (yellow arrows) in the presence of bacteriocin and tetracycline (x80,000).

B) Some of the cells with the cell membranes were ruptured and release of the cellular contents in the presence of bacteriocin and tetracycline (x80,000).

C) Cell lysis in the presence of bacteriocin and tetracycline (x80,000)

D) Cell death in the presence of bacteriocin and tetracycline (x80,000).

SEM images of *P. aeruginosa* ATCC 10145 treated with combination of crude bacteriocin and all antibiotics (chloramphenicol, ciprofloxacin and tetracycline) have similar cell surface morphological changes such as pores formation, ruptured cell membranes and cell walls, and cell lysis leading to cell death. Similar cell surface morphological changes have also been

observed on the *P. aeruginosa* ATCC 10145 treated respectively with crude bacteriocin and antibiotics.

Pore formation on the bacterial cell membrane has been reported to be one of the known antibacterial action mechanisms of the bacteriocin from lactic acid bacteria (Perez *et al*., 2014). In a study reported by Sharma *et al.* (2018), the morphological changes on the surface of *E. coli* treated with bacteriocin showed pores formation by bacteriocin isolated from *B. subtilis* GAS101.

According to Breukink & Kruijff (2006) and Singh *et al.* (2013), nisin uses Lipid Π as "docking molecule" to form pores on the cell membrane surface in a targeted manner and at a nanomolar level. In another study, bacteriocin lacticin Q was found to form a huge toroidal pore (HTP) around 4.6-6.6 nm in size that was large enough to cause leakage of intracellular components such as ions, ATP, as well as large molecules such as proteins resulting in cells damaged (Yoneyama *et al.*, 2009). Hence, these findings suggested that one mode of action of bacteriocin might involve membrane disruption of the targeted cell by forming pores on the cell membrane.

Meanwhile, treatment of all three antibiotics respectively also demonstrated to form pores on the *P. aeruginosa* ATCC 10145 cells. According to Montero *et al*. (2007), chloramphenicol was known as a bacteriostatic agent that blocks the elongation of peptides during the biosynthesis of proteins by binding primarily to 50S ribosomal subunit of the target bacteria. On the other hand, ciprofloxacin was believed to act intracellularly and bind noncovalently to two target proteins, DNA gyrase and topoisomerase Ⅳ (Hooper, 1999). Whereas, tetracycline acted intracellularly by inhibiting the initiation of translation by binding to the 30S ribosomal subunit which is made up of 16S rRNA and 21 proteins (Pioletti *et al*., 2001).

However, mechanism of actions for crude bacteriocin and antibiotics respectively and the combination of crude bacteriocin and antibiotics could

not be determined precisely based on the scanning electron microscope examinations in this study. The use of SEM alone has limitation and could only reveal the morphological changes of the cell surfaces such as shapes, sizes and the overall physical appearances of the treated cells. Nonetheless, SEM images and the previous time-kill assay conducted revealed that the antibacterial activity of antibiotics was improved in the presence of crude bacteriocin. Current finding also suggested that the combined mode of action from crude bacteriocin of *Pd. pentosaceus* TU2 and antibiotics resulted in cell death of *P. aeruginosa*. Current finding was in line with a study reported by Selegard *et al.* (2019) which revealed that bacteriocin plantaricins (PlnEF) was found to enhance the effect of tetracycline by 500-fold against *Staphylococcus epidermidis*. Hence, the combination of crude bacteriocin with antibiotics has potential for use in the inhibition of multidrug-resistant *P. aeruginosa*.

5.4 Summary

Treatment of crude bacteriocin TU2 in combination with all antibiotics (chloramphenicol, ciprofloxacin and tetracycline) resulted in similar cell surface morphological changes of *P. aeruginosa* ATCC 10145 when examined under scanning electron microscope. Among the cell surface morphological changes observed were formation of pores on the cell membranes at 2 h of incubation. Apart from that, cell membranes ruptured and released of the cellular contents were observed after 4 h of treatment. After 8 h of incubation, cell lysis occurred, resulting in cell death. The synergistic interactions of crude bacteriocin TU2 and antibiotics were believed to involve two-step actions, first step by forming pores in the target membrane of *P. aeruginosa*, and second step to allow the diffusion of antibiotics into the inner cell membranes and causing the cellular leakages.

Chapter 6: General Discussion and Conclusion

Antibiotics have been found to cure various kind of infectious diseases that once killed people for decades. However, some serious infections have become difficult to treat and antibiotic-resistant strains are starting to emerge (Boucher *et al.*, 2009; Savjani *et al.,* 2009).

P. aeruginosa presents a great challenge in the clinical environment because of its multidrug-resistance (MDR) (El Zowalaty *et al*., 2015) and hence, this study focusses on the crude bacteriocins produced by *Pd. pentosaceus* strains which could be used as putative anti-pseudomonal agents that might induce synergistic antagonism with selected antibiotics to reverse the antibiotic resistance ability of *P. aeruginosa*. Therefore, the three main objectives in this study were (i) to isolate, screen and identify bacteriocin-producing LAB from traditional fermented foods; (ii) to determine the synergistic interactions between crude bacteriocins and selected antibiotics against multidrug-resistant *P. aeruginosa;* and (iii) to examine changes in cell surface morphology of *P. aeruginosa* after treated with crude bacteriocin and antibiotics.

6.1 Isolation, screening and identification of bacteriocin-producing lactic acid bacteria from traditional fermented foods

In this study, a total of forty-two LAB were isolated from traditional fermented foods and seven isolates were able to produce bacteriocin and successfully inhibited *P. aeruginosa* ATCC 10145. This finding was consistent with several studies that demonstrated the bacteriocin from LAB isolated from various traditional fermented foods showed strong inhibitory activity against *P. aeruginosa* and other pathogens (Castellano *et al*., 2017; Jagadeeswari *et al.,* 2010).

The bacteriocins produced by the seven LAB isolates showed different degree of inhibition against *P. aeruginosa* ATCC 10145, ranging from 31.67 to

126.67 AU.cm/ml. This finding suggested that each bacteriocin is unique and selective against the *P. aeruginosa*. Future study should be conducted to purify and identify the type of bacteriocin present.

Out of seven bacteriocin-producing LAB isolates, only three representatives LAB isolates from each fermented food, namely TU2, TP1 and TE1 with the highest modified bacteriocin activity were selected for further biochemical and molecular identification. These three isolates were with high similarity (>96%) to *Pediococcus pentosaceus* ATCC25745. *Pd. pentosaceus* is known to exhibit antimicrobial activities against many pathogenic bacteria including *Pseudomonas aeruginosa* as previously reported by Nghe & Nguyen (2014).

6.2 Determination of the synergistic interactions between crude bacteriocins and selected antibiotics against *Pseudomonas aeruginosa* ATCC 10145

Once the three bacteriocin-producing LAB were identified, the next step was to determine the inhibitory activity of these bacteriocins when combined with antibiotics against *P. aeruginosa* ATCC 10145. The minimum inhibition concentration (MIC) and the minimum bactericidal concentration (MBC) of these antimicrobial agents were first determined, followed by evaluation on the synergistic interaction of bacteriocin and selected antibiotics using FIC checkerboard method and time-kill assay.

The crude bacteriocins TU2, TP1 and TE1 exhibited MIC of 15.63 mg/ml against *P. aeruginosa* ATCC 10145. This finding was contradictory with other published studies by Lin & Pan (2019) and Shelburne *et al.* (2017). The MIC values of bacteriocins might varied according to the strain of bacteria, sources of the strains, indicator pathogens and methods for the determination (N'tcha *et al.,* 2017).

Meanwhile, *P. aeruginosa* ATCC 10145 displayed high sensitivity towards ciprofloxacin with MIC value equals to 0.25 μg/ml which was within the CLSI (2017) breakpoint of 0.25-1 μg/ml for susceptible inhibition. Current finding

was in accordance to a study conducted by Jayaraman *et al*. (2010), where the MIC of ciprofloxacin against *P. aeruginosa* DR3062 was demonstrated to be 0.25 µg/ml. In contrast, Grillon *et al*. (2016) also reported that 35% of *P. aeruginosa* isolates were shown to be resistant to ciprofloxacin in an *in vitro* study.

On the other hand, tetracycline and chloramphenicol were reported as resistant against *P. aeruginosa* with MIC value of 32 µg/ml in this study. The obtained MIC value for tetracycline was still in the CLSI (2017) acceptable breakpoint range of 8-32 µg/ml for inhibition against *P. aeruginosa* ATCC 10145. However, since *P. aeruginosa* appeared to be intrinsically resistant to chloramphenicol that susceptibility testing is unnecessary, hence there was no breakpoint range for chloramphenicol (CLSI, 2017).

In this study, bacteriocin TU2, TP1 and TE1 were shown to be effective bactericidal agents against *P. aeruginosa* ATCC 10145 at MBC of 31.25 mg/ml which were much higher than other published works reported by Bholay *et al.* (2017) and Huang *et al.* (2016). The differences in the MBC values of the bacteriocins was probably depending on the type of strain of producer bacteria and sources of the strain (N'tcha *et al.*, 2017).

The MBC and MIC values of ciprofloxacin against *P. aeruginosa* ATCC 10145 was shown to be 0.25 µg/ml, indicating that ciprofloxacin is a bactericidal agent. On the other hand, chloramphenicol and tetracycline were shown to be bacteriostatic agents against *P. aeruginosa* ATCC 10145 in this study. Current findings on the bactericidal and bacteriostatic actions of these three antibiotics were in accordance to reports published by Chalkley and Koornhof (1985) and Morita *et al.* (2014).

The FIC checkerboard assay revealed that combination of crude bacteriocins with ciprofloxacin and tetracycline indicated a synergistic interaction against *P. aeruginosa* ATCC 10145. On the other hand, the combination of crude

bacteriocins and chloramphenicol had indifferent effects against *P. aeruginosa*.

To the author's knowledge, no relevant published work was found on combination of bacteriocins produced by *Pd. pentosaceus* and antibiotic against *P. aeruginosa* ATCC 10145. Published studies are only available for combination of nisin produced by *Lactococcus lactis* with chloramphenicol that resulted in synergistic interaction against *P. fluorescens* and *E. faecalis* using checkerboard assay (Naghmouchi *et al*., 2012).

Time-kill assay further confirmed the synergistic interaction observed in FIC assay. The combination of crude bacteriocins with tetracycline produced synergistic interactions and successfully speed up the killing of *P. aeruginosa* ATCC 10145 within 10 h of incubation.

In contrast to the result of FIC assay, the time-kill curves of crude bacteriocins with combined chloramphenicol produced synergistic interaction and successfully inhibited *P. aeruginosa* ATCC 10145 totally after 8 h of incubation. When compared to treatment with crude bacteriocin and chloramphenicol respectively, the combination of both antimicrobial agents has successfully speed up the killing of *P. aeruginosa* ATCC 10145.

Contradict to the result of FIC assay, the time-kill assay revealed that treatment with ciprofloxacin alone was more effective in killing *P. aeruginosa* ATCC 10145 within 2 h of incubation. Meanwhile, the combination of bacteriocin and ciprofloxacin only able to inhibit *P. aeruginosa* ATCC 10145 totally after 8 h of incubation. While treatment with bacteriocin alone only resulted in 1-$\log_{10}$ CFU/ml reduction in growth of *P. aeruginosa* ATCC 10145 after 24 h of incubation.

The contradictory results observed between FIC and Time kill assays could be due to the limitation of Checkerboard assay. The checkerboard method has no standardized protocol and might have variations in the end point used for

FICI interpretation (Mathur *et al*., 2017). Thus, time-kill assay is a more accurate way to confirm the synergistic action of antimicrobial agents.

6.3 Effect of crude bacteriocins and selected antibiotics on the cell surface morphological changes on *P. aeruginosa* ATCC 10145

In order to further elucidate the mode of action of crude bacteriocin and antibiotic against *P. aeruginosa* ATCC 10145, the scanning electron microscope (SEM) was used to reveal the cell surface morphological changes in *P. aeruginosa* ATCC 10145 after treated with crude bacteriocin TU2 and antibiotics.

After treated with crude bacteriocin TU2, the cell of *P. aeruginosa* was elongated by approximately 8% at 4 h of incubation. After 8 h of incubation, circular holes or pores were formed on the cell surface of *P. aeruginosa* and the cell wall was ruptured where leakage of the cellular content was observed.

These findings were in accordance with the studies reported by researchers which demonstrated that the pore formation in the membrane of *P. aeruginosa* resulted in membrane leakage of ions leading to disruption of membrane potential and ATP depletion. Hence, this will lead to diffusion of low molecular cystosolic compounds out of the cell and resulting in cell death (Indiraa *et al*., 2018; Okuda *et al*., 2013; Wen *et al*., 2016; Yusra & Efendi, 2007).

After treated with chloramphenicol, large pores or circular holes were formed on the cell surfaces of *P. aeruginosa* at 2 h and cell walls ruptured at 4 h and 8 h. These observations indicated partial disruption of the cell walls, bacterial outer membranes and inner membranes. These observations suggested that the formation of pores helped chloramphenicol to gain access into the bacterial inner membrane.

After treated with ciprofloxacin, large pore or hole was formed on the cell surface and some of the cells have broken cell walls where efflux of the

cellular contents was observed at 1 h of incubation. Nub formation leading to cell lysis was observed after 2 h of incubation.

Hence, this finding was in line with the result demonstrated in the previous time-kill assay conducted in this study that ciprofloxacin was an effective bactericidal agent against *P. aeruginosa* ATCC 10145 by 5-log_{10} CFU/ml reduction within 2 h of incubation.

Meanwhile, the SEM images of *Pseudomonas aeruginosa* ATCC 10145 treated with tetracycline showed pores formation on the cell surfaces at 2 h. Apart from that, a roundish cell, cell elongation and stalked nubs formation were observed at 4 h of incubation. Furthermore, cell death occurred in a few cells were observed at 4 and 8 h of incubation. These cell surface morphological changes observed were consistent with the finding reported by Waisbren *et al*. (1980) which found elongation of cells and stalked nubs in *P. aeruginosa* cells when treated with tetracycline via scanning electron microscope.

Treatment of crude bacteriocin TU2 in combination with all antibiotics (chloramphenicol, ciprofloxacin and tetracycline) resulted in similar cell surface morphological changes of *P. aeruginosa* ATCC 10145 when examined under scanning electron microscope. Among the cell surface morphological changes observed were formation of pores on the cell membranes at 2 h, cell membranes ruptured and released of the cellular contents at 4 h and cell lysis occurred, resulting in cell death at 8 h of incubation. However, when compared the three combinations of crude bacteriocin TU2 with all antibiotics, the combination of crude bacteriocin TU2 with chloramphenicol showed distinct cell surface morphological changes such as cell elongation at 2 h of incubation. Whilst *P. aeruginosa* ATCC 10145 cells revealed to be shorter and formed chains when treated with crude bacteriocin TU2 in combination with ciprofloxacin.

When compared the three combinations of bacteriocin TU2 with all antibiotics, only the combination of crude bacteriocin TU2 with

chloramphenicol caused 100% killing of *P. aeruginosa* cells in the shortest time at 8 h based on the results of scanning electron microscope. Furthermore, this combination of crude bacteriocin TU2 with chloramphenicol was effective against *P. aeruginosa* ATCC 10145 by 5-log_{10} CFU/ml reduction in the shortest time at 8 h of incubation. Hence, the synergistic interaction of crude bacteriocin TU2 with chloramphenicol produced the best combination against *P. aeruginosa* ATCC 10145 in this study.

Pore formation on the bacterial cell membrane has been reported to be one of the known antibacterial action mechanisms of the bacteriocin from lactic acid bacteria (Perez *et al*., 2014). In another study, bacteriocin lacticin Q was found to form a huge toroidal pore (HTP) around 4.6-6.6 nm in size that was large enough to cause leakage of intracellular components such as ions, ATP, as well as large molecules such as proteins resulting in cells damaged (Yoneyama *et al.*, 2009). Hence, these findings suggested that one mode of action of bacteriocin might involve membrane disruption of the targeted cell by forming pores on the cell membrane.

Meanwhile, treatment of all three antibiotics respectively also demonstrated to form pores on the *P. aeruginosa* ATCC 10145 cells. According to Montero *et al*. (2007), chloramphenicol was known as a bacteriostatic agent that blocks the elongation of peptides during the biosynthesis of proteins by binding primarily to 50S ribosomal subunit of the target bacteria. On the other hand, ciprofloxacin was believed to act intracellularly and bind noncovalently to two target proteins, DNA gyrase and topoisomerase IV (Hooper, 1999). Whereas, tetracycline acted intracellularly by inhibiting the initiation of translation by binding to the 30S ribosomal subunit which is made up of 16S rRNA and 21 proteins (Pioletti *et al*., 2001).

However, mechanism of actions for crude bacteriocin and antibiotics respectively and the combination of crude bacteriocin and antibiotics could not be determined precisely based on the scanning electron microscope

examinations in this study. The use of SEM alone has limitation and could only reveal the morphological changes of the cell surfaces such as shapes, sizes and the overall physical appearances of the treated cells. Hence, further studies are required to investigate the action mechanisms of crude bacteriocin and antibiotics against *P. aeruginosa* at molecular level. These key findings and the limitation gap found in this study would help in contributing for the future direction of the study in this area.

Nonetheless, SEM images and the previous time-kill assay conducted revealed that the antibacterial activity of antibiotics was improved in the presence of crude bacteriocin. Current finding also suggested that the combined mode of actions from crude bacteriocin from *Pd. pentosaceus* and antibiotics resulted in cell death of *P. aeruginosa*. Hence, the combination of crude bacteriocin with antibiotics has potential for use in the inhibition of multidrug-resistant *P. aeruginosa*.

6.4 Conclusion & Future work

In conclusion, three bacteriocinogenic LAB (TU2, TP1 and TE1) were isolated from "tapai ubi", "tapai pulut" and "tempeh" and identified as *Pediococcus pentosaceus.* The crude bacteriocins showed promising inhibitory activity against *P. aeruginosa* ATCC 10145. The combination of crude bacteriocin TU2 with antibiotics chloramphenicol and tetracycline exhibited synergistic inhibition against multidrug-resistant *Pseudomonas aeruginosa* ATCC 10145. Mode of actions for crude bacteriocin and antibiotics could not be determined precisely in this study, hence, further studies are required to investigate the action mechanisms of crude bacteriocin and antibiotics against *P. aeruginosa* at molecular level. Future work should include transmission electron microscope (TEM) and epifluorescence image-based screening as complimentary to SEM to examine the inhibitory effect of the combination of crude bacteriocin and antibiotics in the target cell membranes. The use of SEM in the current study could only revealed the cell surface morphology of

P. aeruginosa ATCC 10145 whereas, TEM and epifluorescence image-based screening could provide the details on membrane permeability changes such as broken cell envelope and the outflow of cytoplasm substances.

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Appendix A

Carbohydrate fermentation patterns of TU2, TP1 and TE1 via API 50 CHL

Carbohydrate fermentation test	LAB isolate		
	TU2	TP1	TE1
Control	-	-	-
Glycerol	-	-	-
Erythritol	-	-	-
D-arabinose	-	-	-
L-arabinose	+	+	+
D-ribose	+	+	+
D-xylose	+	+	+
L-xylose	-	-	-
D-adonitol	-	-	-
Methyl-β-D-xyloside	-	-	-
D-galactose	+	+	-
D-glucose	+	+	+
D-fructose	+	+	+
D-mannose	+	+	+
L-sorbose	-	-	-
L-rhamnose	-	-	-
Dulcitol	-	-	-
Inositol	-	-	-
D-mannitol	-	-	-
D-sorbitol	-	-	-
Metyhl-α-D-mannoside	-	-	-
Metyhl-α-D-glucopyranoside	-	-	-
N-acetylglucosamine	+	+	+
Amygdalin	+	+	+
Arbutin	+	+	+
Esculin	-	-	-
Salicin	+	+	+
D-cellobiose	+	+	+
D-maltose	+	+	+
D-lactose	-	-	-
D-melibiose	-	-	-
D-saccharose	-	-	-
D-trehalose	+	+	+
Inulin	-	-	-
D-melezitose	-	-	-
D-raffinose	-	-	-
Starch	-	-	-
Glycogen	-	-	-
Xylitol	-	-	-
Gentibiose	+	+	+
D-turanose	-	-	-
D-lyxose	-	-	-
D-tagatose	+	+	+
D-fucose	-	-	-
L-fucose	-	+	-
D-arabitol	-	-	-
l-arabitol	-	-	-
Potassium gluconate	-	-	-
2-ketogluconate	-	-	-
5-ketogluconate	-	-	-

Notes:

Analysis of bacteria biochemical profile; n=3

+ refers to a positive result; - refers to a negative result

Appendix B

Identification of TU2, TP1 and TE1 via 16S rDNA gene sequencing

a) 16S rDNA gene sequence of TU2 using primer sets U8F & U1492R

TGGGAGTGCGGGTGCTATACATGCAAGTCGAACGAACTTCCGCTATAATTG
ATTATGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAAC
GGGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGA
AACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAA
GATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGT
GAGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAAT
CGGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAG
TGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGT
AAGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAAC
TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTT
ATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCT
TCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAG
AGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGA
ACACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGA
AAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAAC
GATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGC
ATTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTAC
GCGAAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTAGAG
GTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGT
TGCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAG
GAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACCA
CGTGCTACAATGGATGGTCAACGAGTCGCGGAACCCCGAGGTTAAGCTAAT
CCCTTAAAACCTTTTCAGTTCGGAATGGAGGCTGGCAACTCCCCCAACAAAA
TCGGAATCCCTTGAAACCCGGATAACCATGCCCCGGGGAAAACTTTCCCGG
GCCTTGGTAAACCGCCGTTCACTTGAAGTTTGTAACCCCAACCCGGGGGGA
CCTTTTAGAACCCTAAGGGCAATGGGTTGGGGGGGGGTTTTCCCCCGCACA
AAAAAAAAACAACCGTTAGTTTTTGGGGGTTTTG

b) 16S rDNA gene sequence of TP1 using primer sets U8F & U1492R

GGCTGGCGGCGTGCTATACATGCAGTCGAACGAACTTCCGTATAATTGATTA
TGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACGGGT
GAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGAAACA
GATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAGATG
GCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTGAG
GTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATCGG
CCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGTGA
AGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTAAG
AGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTAC
GTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATT
GGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTTCG
GCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGG

ACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACA
CCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAAA
GCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGA
TGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCAT
TAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATT
GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACG
CGAAGAACCTTACCAGGTCTTGACATCTTCTGACAGTCTAAGAGATTAGAGG
TTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTG
TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTT
GCCAGCATTAAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGG
AAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACCAC
GTGCTACAATGGAAGGGTACAACGAGTCCCGAGACCGCGAGGTTAACCTAA
TCTCTTAAAACCTTTCCCATTTCGGAATGTAGGCTGCAATCCGCCCACCAAA
ATCGGAATCCCTTATAATCCGGAAACACATGCCCCGGGGAAAACTTTCCCGG
GCCTTTTAACACCCCCCCTCCACTAGAAATTTGAAACCCAAACCGGGGGGG
GACCTTTAGAACCCTAAGGGCAATAGAGAAAGAAAAAAGGGGGATCGGTTG
AAAACAAAACAAAACAAGTGGGGTTGGGGGTCGGGGGG

c) 16S rDNA gene sequence of TE1 using primer sets U8F & U1492R

GTGATGCGGCGTGCTATACATGCAAGTCGTACGAGCTTCACGCTCTAATTGA
TTATGACGTACTTGTACTGATTGAGATTTTAACACGAAGTGAGTGGCGAACG
GGTGAGTAACACGTGGGTAACCTGCCCAGAAGTAGGGGATAACACCTGGAA
ACAGATGCTAATACCGTATAACAGAGAAAACCGCATGGTTTTCTTTTAAAAG
ATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGTATTAGCTAGTTGGTG
AGGTAAAGGCTCACCAAGGCAGTGATACGTAGCCGACCTGAGAGGGTAATC
GGCCACATTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
AGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCCGCGTGAGT
GAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTA
AGAGTAACTGTTTACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACT
ACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTA
TTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAATGTGAAAGCCTT
CGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGA
GGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAA
CACCAGTGGCGAAGGCGGCTGTCTGGTCTGCAACTGACGCTGAGGCTCGAA
AGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG
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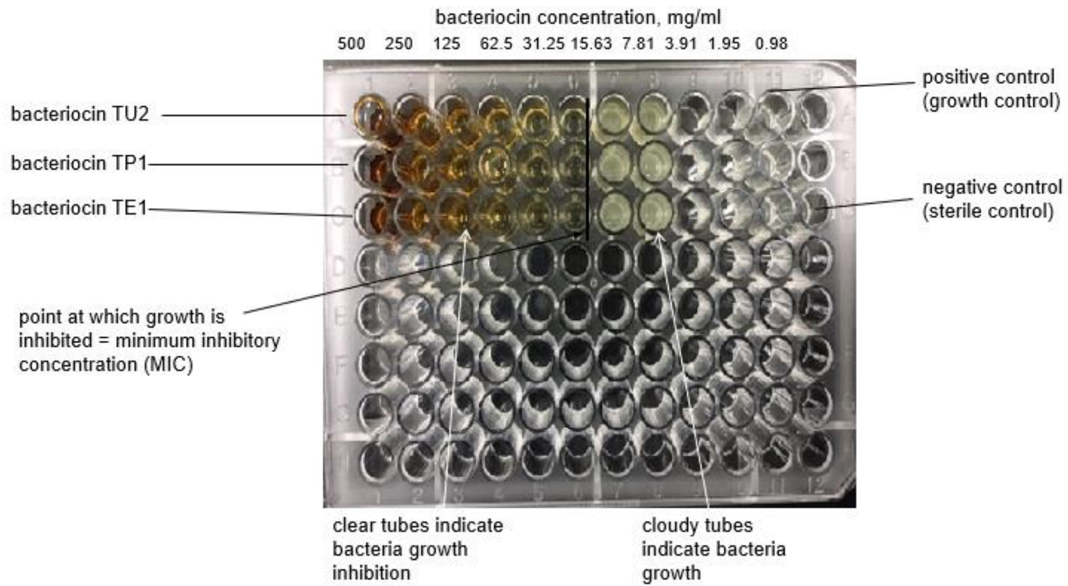
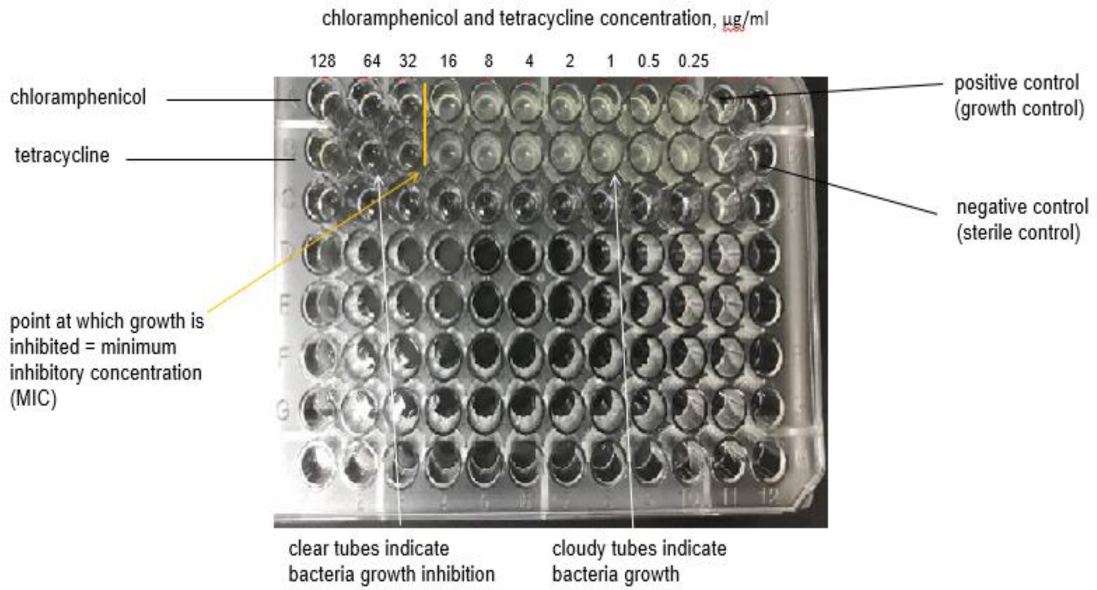
Appendix C

The arrangement of crude bacteriocins (TU2, TP1 and TE1) and antibiotics (chloramphenicol, ciprofloxacin and tetracycline) on 96 well plates for MIC determination

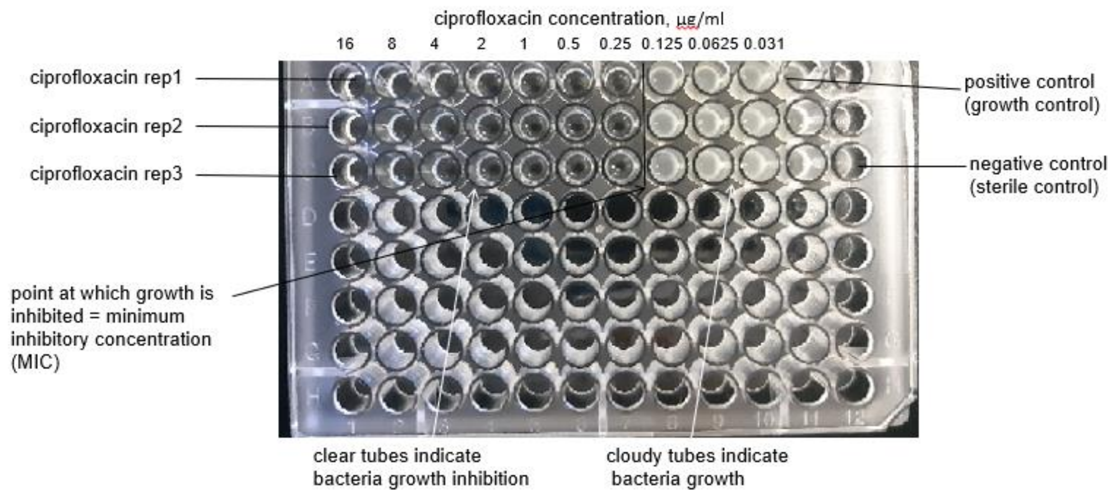
a) Figure of the arrangement of bacteriocins (TU2, TP1 and TE1) on 96 well plate



b) Figure of the arrangement of antibiotics (chloramphenicol and tetracycline) on 96 well plate



c) Figure of the arrangement of antibiotic (ciprofloxacin) on 96 well plate



Appendix D

Standard scale of antibiotic susceptibility (inhibition zone) of *P. aeruginosa* according to CLSI (2017)

Antibiotics	**Antibiotic concentration on test disc (µg)**	**Susceptibility/High inhibition zone (mm)**	**Intermediate/Moderate inhibition zone (mm)**	**Resistant/Weak inhibition zone (mm)**
Amoxicillin	10	>18	14-17	<13
Cephalexin	30	>18	15-17	<14
Chloramphenicol	50	≥18	13-17	≤12
Ciprofloxacin	5	≥21	16-20	≤15
Gentamicin	10	≥15	13-14	≤12
Sulfamethoxazole	25	≥16	11-15	≤10
Tetracycline	30	≥19	15-18	≤14