In silico prediction of bacteriocin gene in *Weissella cibaria* NM1 and *in vitro* enhancement of antipseudomonal activity through a combined action of antibiotics and bacteriocins from lactic acid bacteria

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Thesis submitted to the University of Nottingham for the degree of Master of Philosophy

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2020

Abstract

Multi-drug resistant *Pseudomonas aeruginosa* is the third-leading cause of nosocomial infections and is the predominant pathogen associated with the mortality of patients with cystic fibrosis. Infections caused by this bacterium is hard to treat due to its intrinsic, acquired and biofilm-associated antibiotic resistance. Hence, there is a pressing need for discovering new antipseudomonal agent and inhibitory strategy that could effectively inhibit this pathogen. The emerging antimicrobial peptides - bacteriocins produced by lactic acid bacteria are gaining much research attention with the shift from food industry into healthcare application. Hence, this study has two key goals: first, to screen for bacteriocin genes and ascertain its present in the genome of *Weissella cibaria* NM1 via *in silico* approach; Second, to investigate the antipseudomonal potency of crude bacteriocins and when in combinations with antibiotics against planktonic cells and biofilm of *P. aeruginosa* ATCC 10145.

This study started with a machine learning-based prediction approach combined with a homology-based search of highly conserved bacteriocin-associated genes in the genome of *W. cibaria* NM1. This prediction approach discovered a bacteriocin operon with a complete set of immunity gene, transporter gene, regulator gene and modifier gene with one bacteriocin genes (WC_2064) predicted. The genome of *W. cibaria* NM1 was screened on probiotic related genes and the genome was found to harboured specific genes that would contribute to acid and bile tolerance, adhesion on the host cell and exhibited cholesterol-reducing ability. On top of that, the genome also shows the absence of virulence and antibiotic resistance genes that have a high chance of being transferred which signifies the safety aspect as a potential probiotic candidate.

In vitro study was then conducted to investigate the antipseudomonal effect of crude bacteriocins WC, LG and PA respectively produced by *W. cibaria* NM1, *Lactococcus garvieae* NM2 and *Pediococcus acidilactici* NM3 against *P.*

aeruginosa ATCC 10145 in its planktonic cell and biofilm form. This study includes an investigation of the effect of the crude bacteriocin used alone and in combination with antibiotics Chloramphenicol (CHL), Tetracycline (TET) and Ciprofloxacin (CIP)

The Checkerboard assay confirmed that the combined treatments of the three crude bacteriocins with antibiotics CIP, CHL and TET exhibited a synergistic effect against planktonic cells of *P. aeruginosa* ATCC 10145 resulted in FIC indexes of 0.258 and 0.375. In a further attempt to confirm the crude bacteriocins can enhance antibiotic efficacy, time-kill assays were performed over a 24h treatment period. The antipseudomonal effect of combined treatment with WC-CHL which show synergistic effect is comparable with CIP treatment alone, and successfully inhibited *P. aeruginosa* ATCC 10145 after 6h of treatment. Followed by other treatment such as LG-CHL, PA-CHL, WC-TET, PA-TET which inhibited *P. aeruginosa* ATCC 10145 after 8h of treatment. Current findings suggested that WC is a potent antipseudomonal agent and when combined with CHL could enhance the antipseudomonal potency further against planktonic cells of *P. aeruginosa* ATCC 10145.

In the biofilm inhibition and eradication assays, crude bacteriocins alone successfully prevent the formation of biofilm from planktonic cells and eradicated 40%-50% of preformed biofilm. When combined with antibiotics, WC exhibited the most potent eradication effect towards preformed biofilm of *P. aeruginosa* ATCC 10145 and with WC-TET being the most potent combination with eradication effect of 93%. In hindsight, crude bacteriocin WC in combination with either TET and CH conferred strong effect towards the biofilm and planktonic cells of *P. aeruginosa*. These effects were apparent throughout the study through the reduction of MIC when in combination, the increased killing rate towards planktonic cells of *P. aeruginosa* and biofilm eradication capability. This output warrant for a more depth study of the crude bacteriocin in the future. Future studies can be extended into further validation of *in silico* prediction through an experimental approach using heterologous expression and the conventional way of bacteriocin purification. The obtainment of pure

bacteriocin will allow for its mode of action to be further understood especially against the planktonic and biofilm cell of *P. aeruginosa.*

Acknowledgement

All praise and thanks to the lord of the heavens and the earth, the most merciful and the most loving for allowing and assisting me to start, carry out and complete my MPhil journey. I am certainly will not be able to finish this journey except through His will, His grace and His aid. I am deeply grateful for the experience as I learned many valuable life lessons. Verily, this is all part of all the blessings I receive in this world which will later be questioned back in the day of judgment.

I would like to offer my deep gratitude the main supervisor, Dr Lim Yin Sze for her guidance, support and motivation of my study. Same goes to my co-supervisor, Dr Tan Boon Chin and Prof. Festo Massawe for their suggestions and advice for the research project. For financial support, I would like to thank the Ministry of High Education (MOHE) for funding this project and the studentship through Fundamental Research Grant Scheme (FRGS).

For technical support, I like to faculty of science's laboratory technician for their assistance regarding the experimental works. I also thank my fellow lab mates and friends for the companionship and support throughout the years.

Finally, I would like to thank my parents, wife, siblings and family members for moral support during the thesis writing period and my life in general.

List of Supervisory committee

The thesis was submitted to the University of Nottingham and has been accepted as fulfilment of the requirement for the degree of Master of Philosophy. The members of the Supervisory Committee are as follows:

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Abbreviations

ANOVA	One-way analysis of variance
CFS	Cell-free supernatant
CLSI	Clinical and Laboratory Standards Institute
CIP	Ciprofloxacin
CHL	Chloramphenicol
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine Tetra-acetic acid
FIC	Fractional Inhibitory Concentration
FICI	Fractional inhibitory concentration index
L.	*Lactococcus*
P.	*Pseudomonas*
Pd.	*Pediococcus*
W.	*Weissella*
LAB	Lactic acid bacteria
MDR	Multidrug resistance
MH	Mueller-Hinton
MIC	Minimum Inhibition Concentration
MRS	De Man, Rogosa and Sharpe
MRSA	methicillin-resistant *Staphylococcus aureus*
NCBI	National Centre for Biotechnological Information
PCR	Polymerase chain reaction
rDNA	Ribosomal DNA

℃	Degree Celsius
AU	Arbitrary Unit
AU/mL	Arbitrary Unit per millilitre
CFU/mL	Colony-forming unit per millilitre
Cm	Centimetre
g	Gravity
h	Hour
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre
TET	TETracycline
µl	Microliter
µm	Micrometer
nm	Nanometer
v/v	Volume/volume
WC	Crude bacteriocin of *Weissella cibaria*
LG	Crude bacteriocin of *Lactococcus garviae*
PA	Crude bacteriocin of *Pediococcus acidilactici*

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In this era, most classes of antibiotics are no longer effective in treating bacterial infection. Constant exposure to antibiotics due to misuse of antibiotics to consumer and lack of awareness in the community cause selective pressure upon the bacteria, giving birth to generations that are resistant to the antibiotics (Brown & Wright, 2016). As highlighted by the World Health Organization (WHO), antibiotic resistance is one of the greatest threats faced by human health (Hassan *et al.*, 2012). This global issue has become more critical of an issue as time passed through the increment in mortality and morbidity rates. The treatment options are becoming more limited as cases of antibiotic resistance increasingly being reported. Naturally, bacteria are encoded with a defence mechanism such as low outer membrane permeability, efflux pump system and enzyme that inactivate certain classes of antibiotics (Lambert, 2002). This natural form of defence mechanism differs from bacteria species to another and target a specific type of antibiotics classes.

Yet, resistance against new antibiotics can emerge by overexpression of its natural defence mechanism to adapt and survive the antibiotic attack. An example of this is the high gene expression or constant overexpression of efflux pump machinery due to a certain mutation of a related gene (Cabot *et al.*, 2011). For example, Extensively Drug-resistant (XDR) *P. aeruginosa* isolates from Spain was found to harbour multiple mutations in the genomes such as a mutation in *gyrA, gyrB, parA* genes which leads to Ciprofloxacin, highly effective antipseudomonal antibiotics and also overexpression of MexXY efflux pump that contributes to aminoglycoside resistance (Del Barrio-Tofiño *et al.*, 2017). A different example on this issue is Fosfomycin, a potentially useful antibiotic was initially considered as an option for the treatment of *P. aeruginosa* infection. The usage of Fosfomycin through combined therapy was found to be ineffective with high prevalence clinical strain of *P. aeruginosa* with a high mutation rate of the gene*, glpT* as it functions as the gene for Fosfomycin uptake in *P. aeruginosa* (Rodríguez-Rojas *et al.*, 2010). Due to this, a lot of antibiotic classes

that are discovered were no longer a viable option for bacterial infection treatment.

The pursuit to combat the antibiotic resistance has been an ongoing process with the usage of different antimicrobial compound and strategy such as bacteriocin, a natural compound from plants and bacteriophage therapy (Khameneh *et al.*, 2019; Romero-Calle *et al.*, 2019). Bacteriocin is an antimicrobial peptide produced by bacteria to gain an advantage over competing strain in their respective ecological niche (Dobson *et al.*, 2012). Bacteriocins from gram-positive lactic acid bacteria have been extensively used in the food industry for food preservation. Bacteriocins are with a diverse range of spectrum against competing strains and with a varying mode of action makes them good alternatives for the antibiotics. Hence, the researches done in past years have been heavily shifted toward the discovery of bacteriocin with the intent of therapeutic purpose.

The varying range of spectrum of bacteriocin activity is crucial for the usage against bacterial infection for which the bacteriocin can be deployed as "Sniper Bacteriocin" which kills specific pathogen only as remarked by Hols *et al.*, (2019). This is different from conventional antibiotics which are used are often broad spectrum which the effect will disrupt the microflora of human gut at the same killing the intended pathogen (Yoon & Yoon, 2018). Disruption of microflora in the gut will lead to an easier entrance for the pathogen as the microflora cannot longer provide the protection which often occurred for the case infection of *Clostridium difficile* (Bien *et al.*, 2013). Hence, the discovery of bacteriocin of a unique spectrum of activity is very essential for a specific targeted bacteriocin therapy which only intended for the pathogen can be used in the future.

Since an ideal bacteriocin that could target on MDR *P. aeruginosa* has yet to be found, new bacteriocin is continued to be discovered from the different environments through researches. This study is carried out with the same motivation of discovering putative bacteriocin of Lactic acid bacteria which can inhibit *P. aeruginosa*, known with its broad range of intrinsic resistance toward

many antimicrobials. This study also attempted to emulate a potent bacteriocin-antibiotic combination approach to inhibit the MDR *P. aeruginosa*. Many advantages conferred through this approach such as slowing down resistance emergence as the sub-inhibitory concentration of antibiotic still exhibit killing effect in combination of bacteriocin (Field *et al.*, 2016*b*). The bacteriocin has been used together with antibiotics for a stronger killing effect especially against the bacteria that already developed resistance toward the antibiotics (Ellis *et al.*, 2020).

The study adopted a different approach in discovering new bacteriocin which is through *in silico* prediction using the genome of the strain of interest which is very convenient compared to the conventional method of discovering bacteriocin which is through manual screening. The strain of interest used here is *W. cibaria* NM1 isolated from the fish gut. The published literature of bacteriocins produced by *Weissella* species is scarce when compared to *Lactococcus* species and *Lactobacillus* species. Only a few reports were found on weissellicin produced by *W. cibaria* 110 (Srionnual *et al.*, 2007) and weissellicin M, Y and L produced by *W. hellenica* (Masuda *et al.*, 2012; Leong *et al.*, 2013). Hence more effort is needed to understand the potential of bacteriocin produced by *Weissella* species and continue the search of new bacteriocin is essential.

1.1 Main aim and objectives of the study

The current study aimed to exploit the potential of using bacteriocins produced by selected lactic acid bacteria to strengthen the killing effect of those ineffective antibiotics against *P. aeruginosa* ATCC 10145. This study hypothesised that the bacteriocins produced can confer synergistic interaction with selected antibiotics to reverse antibiotic resistance ability of *P. aeruginosa* ATCC 10145.

There are three specific objectives in this study:

1) To predict the bacteriocin gene from the genome of *W. cibaria* NM1 using a bacteriocin prediction tool and assess the strain potential as a probiotic candidate by screening genes related to probiotic traits.

2) To assess the combined inhibition of crude bacteriocins from selected lactic acid bacteria and antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145.

3) To assess the combined inhibition of crude bacteriocins and antibiotics in eradicating the biofilm of *P. aeruginosa* ATCC 10145.

Chapter 2: Literature review

2.1 *P. aeruginosa* and its intrinsic resistance features towards antibiotic

2.1.1 Origin and history

P. aeruginosa is a Gram-negative, rod-shaped bacterium which can be found widely in the diverse biotic and abiotic environment such as plants, soil and water (Al-Wrafy *et al.*, 2017). This opportunistic pathogen is very adaptable as it can live in various environments due to its ability to form a biofilm. *P. aeruginosa* was classified in the *Bacillus* genus as *Bacillus pyocyaneus* and were not to be isolated in a pure culture until 1882 where Gessard published a paper entitled "On the blue and green colouration of bandages" (Gessard, 1984). It has been described as the causal agent for blue-green purulence between 1889 and 1894 (Villavicencio, 1998). The earliest work that attempts to elucidate *P. aeruginosa* which was called *B. pyocyaneus* at the moment was done by Leonard Freeman, a medical doctor in 1916 (Freeman, 1916).

2.1.2 Virulence and pathogenicity

P. aeruginosa is the leading causal agent of nosocomial infections worldwide as hospitalized patients are very susceptible to infection from this organism. *P. aeruginosa* can cause acute and chronic infection in the host which are the main cause of high morbidity and mortality. These infections can cause lung infection, meningitis, urinary tract infection, burn wound and much more. Like the pathogenicity of other pathogens, immunocompromised people, elderly people and babies are prone to the infection. On the contrary, healthy people rarely acquire the infection and the infection is usually mild as they are protected from the immune system and other human defence (Wagner *et al.*, 2016).

The advent of genome sequencing provided the genetic makeup of *P. aeruginosa* which is the largest bacteria genome. Its large genetic constituent

signifies the wide array of virulence factors in the infection process of this bacteria (Stover *et al.*, 2000). In general, the *P. aeruginosa* infection process consists of bacterial adhesion and colonization followed by local invasion and dissemination of systemic disease. Virulence factors are classified into two types which are a cell-associated factor and extracellular factor. A low amount of virulence factor will lead to chronic infection and vice versa (Strateva & Mitov, 2011). The entry of this pathogen is through breaching the first-line defence such as tissue injury from wound or surgery. As a result, from the breaching of first-line defence, cell-associated virulence factors such as fimbria will act as an adhesin to bind to a specific receptor of the host cells. Further invasion of the bacteria will allow its infection to spread through tissue degradation, host cell defence system and the physical barriers that are mediated by extracellular-virulence factors such as extracellular enzymes and toxins (Toder *et al.*, 1994).

These factors are controlled through quorum sensing as the secretion of these factors to the host is in a coordinated manner. Through this signalling language, it allows a coordinated adaptation of a bacterial population to environmental changes such as the formation of biofilm (Gellatly & Hancock, 2013). Moreover, quorum sensing plays an important role in pathogenicity as it controls the behaviour of the bacteria in releasing the virulence factor through the interplay of signalling pathways. When a certain threshold of autoinducer exceeds such as HSL(3-oxo-C12-homoserine lactone), BHL (N-butyrylhomoserine lactone or C4-HSL), PQS (2-heptyl-3-hydroxy-4-quinolone) and IQS 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (aeruginaldehyde), an autoinducer synthase that acts as transcriptional activators such as LasR, RhlR, or PqsR will activate genes to encode another group of proteins called cognate autoinducers such as LasI, RhlI, and PqsABCDH that will be exported out from the cells and imported again. The accumulation of the autoinducer will trigger the expression of alkaline protease, pyoverdine, phospholipase C, toxin A, LasA elastase, LasB elastase, hydrogen cyanide, pyocyanin, rhamnolipids and lectin. All of these

molecules are examples of virulence factors produced in response to address specific stimuli or stress (Moradali *et al.*, 2017).

2.2 Type of resistance exhibited by *P. aeruginosa*

P. aeruginosa possesses a wide range of antibiotic resistance mechanism that able to resist to antibiotics attack. Bacterial resistance mechanism consists of 3 different types of resistance which are intrinsic resistance, adaptive resistance and acquired resistance with the addition of biofilm structure as a form of phenotypic resistance. Intrinsic resistance is a form of resistance where it is naturally encoded in its chromosome. The bacteria are intrinsically resistant to β-lactam, Macrolides, Tetracyclines, Co-trimoxazole, and most Fluoroquinolones. One of the intrinsic resistances displayed by the bacteria is its outer membrane layer permeability. The permeability of the outer membrane of *P. aeruginosa* is 12-100 time less than *E. coli* (Hancock, 1998). Similar to any gram-negative bacteria, *P. aeruginosa* has a protein channel, termed as porin which is an outer membrane protein act as a water-filled channel (Hancock & Brinkman, 2002). Some porins are not substrate-specific and substrate-specific porin. Nonspecific porin is usually a large porin that does not bind to its substrate which is effective for a substrate that has high external concentration. Meanwhile, substrate-specific porin possessed a binding site for the substrate and this type of porin is prevalent in bacteria that thrive in a nutrient-poor environment. Example of substrate-specific porin for *P. aeruginosa* are OprP for phosphate and OprB for sugar (Benz & Hancock, 1987; Trias *et al.*, 1988)

2.2.1 Intrinsic resistance

Another form of intrinsic resistance exhibited by *P. aeruginosa* is the constitutive expression of resistance nodulation cell division (RND) multidrug efflux system. There are 4 fully characterized efflux pump (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM) and there are up to 13 uncharacterized efflux pumps. These pumps are a combination of three components which are the efflux transporter, fusion protein and outer membrane protein channel (Nikaido, 1994). The efflux pump can eject a wide range of substrate of antibiotics. For example, MexAB–OprM and MexXY–OprM can extrude out antibiotics from the class of β-lactam, Fluoroquinolones, Chloramphenicol, Macrolides Sulfonamides, Novobiocin, Trimethoprim and Aminoglycosides (Livermore, 2002; Schweizer, 2003).

2.2.2 Adaptive resistance

Next form of resistance is adaptive resistance which is a temporary increment of a bacterium ability to survive antibiotic attack through alteration of the regulatory gene or protein expression due to exposure to environmental stress such as the sub-inhibitory concentration of antibiotic and will be reverted to default condition once the environmental stress was no longer present (Fernández *et al.*, 2011; Breidenstein *et al.*, 2011; Fernández & Hancock, 2012). One example of adaptive resistance is clearly shown based on a report of increasing expression of MexXY-OprM efflux pump gene in the presence of aminoglycosides (Hocquet *et al.*, 2003). Another report of adaptive resistance is the production of β-lactamases. β-lactamases are inducible when the bacteria detect the presence of β-lactams antibiotic thus making *P. aeruginosa* intrinsically resistant to it due to the interplay between β-lactamases *AmpC* and the efflux pump MeXAB-OprM (Morita *et al.*, 2013).

2.2.3 Acquired resistance

The third form of resistance is acquired resistance which can be acquired through horizontal gene transfer and self-mutational resistance. Induction to β-lactamases can be acquired through horizontal gene transfer for Penicillin and Cephalosporins as mentioned by Sacha *et al.*, (2008) where novel β-lactamases was identified which *P. aeruginosa* acquired from horizontal gene transfer Mutation that occurs in the DNA can produce a breakthrough mutation that leads to a new resistance toward a class of antibiotics or enhance one of the existing intrinsic resistance. The frequency of mutation increased when the bacteria are in a biofilm state. Thus, increasing the rate of occurrence of the mutational resistance (Driffield *et al.*, 2008). Mutational resistance can appear in many forms such as constant overexpression of efflux pump, overproduction of β-lactamases and altered antibiotic target (Breidenstein *et al.*, 2011).

2.2.4 Biofilm structure as phenotypic resistance

Moreover, another mechanism of resistance is a phenotypic resistance where the microorganism employed a change in their structure to resist the attack of the antimicrobial compound. Hence, the features of *P. aeruginosa* in repelling the presence of antimicrobials is further strengthened with its ability to form a biofilm. Biofilm is comprised of enclosed communities of self-produced extracellular polymeric substance (EPS) adhered to a biological tissue or surfaces of medical devices (Arciola *et al.*, 2015). Most of the currently available antibiotics unsuccessful in treating biofilm infection due to various factors such as restriction of antibiotic penetration, limited growth at low oxygen tension, expression of biofilm-specific genes and the presence of persistent cells (Ciofu *et al.*, 2017). The failure of antibiotic treatment does not depend on only one form of resistance as the recalcitrant properties of the biofilm is due to the combination of different mechanism of tolerance interplay with one another. The bacteria within these microbial communities employed distinct

mechanisms to resist the action of antimicrobial agents (Mah *et al.*, 2003). The biofilm structure itself is a barrier for antibiotic penetration as antibiotics' penetration into the biofilm were affected to reach to its specific target. The performance of antibiotics was affected by the presence of cyclic glucan which is expressed gene *ndvB* present in the *P. aeruginosa* biofilm matrix. The antibiotics were sequestered away from their cellular target rendering them ineffective (Mah *et al.*, 2003). The research findings were supported with the discovery of a novel efflux pump PA1874-1877 that are constantly and actively expressed in *P. aeruginosa* in biofilm-associated cells (Zhang & Mah, 2008). This indicated the biofilm cells are equipped with a system that readily pumping out the antimicrobial compound. A study demonstrated the connection between efflux pump and with the ability to form biofilm as cells that have inactive efflux pump may have reduced the ability to form a biofilm (Kvist *et al.*, 2008). Besides that, the recalcitrance of the biofilm cells was further supported by the inner environment of the biofilm cells of depleting oxygen gradient and pH. The deeper layer of the biofilm have less oxygen gradient indicating the cells are less metabolically active hence increasing the tolerance as antimicrobial in general are most effective against fast-growing cells (Zheng & Stewart, 2004). It is also important to point out that certain antibiotics are less active in a low oxygen concentration state (Gupta *et al.*, 2016).

2.3 Current antibiotics treatment for *P. aeruginosa*

Similar to any other bacterial infection, *P. aeruginosa* related infection is treated with several selected antibiotics have antipseudomonal properties such as Aminoglycosides, Ticarcillin, Carbapenems, Ciprofloxacin, Ceftazidime, Levofloxacin. However, constant exposure to exposure to antimicrobials can lead to the emergence of a resistant strain of *P. aeruginosa* (Driscoll *et al.*, 2007). It is reported in Gujarat, India where 23% of *P. aeruginosa* clinical isolates were resistant to all 12 routine antipseudomonal drugs (Parmar *et al.*, 2013). With the availability of different class of antibiotics, it's are still not

sufficient to improve the healing for infection wound (O'Meara *et al.*, 2000). A further complication of antibiotic selection for treatment can also be seen by the usage of tobramycin aerosolized antibiotic treatment which shows effectiveness in vitro testing fails to eradicate infection Cystic Fibrosis patient (Hurley *et al.*, 2012). The patient infected with *P. aeruginosa* infection will be administered to an empirical therapy which used a combination of two different antibiotic class with the rationale of "double-coverage effect". The therapy will be reduced to a single in vitro antibiotic treatment once the most susceptible antibiotic for the particular *P. aeruginosa* isolate has been determined (Bassetti *et al.*, 2018). In summary, the healthcare system still relies on antipseudomonal antibiotics and at the same time, resistance profiling of the *P. aeruginosa* is required to determine efficient antibiotic therapy. However, numerous clinical reports have shown antipseudomonal such as Ciprofloxacin and Tobramycin has been losing the effectiveness for antibiotic therapy of lung infections for CF patient (Su *et al.*, 2013).

2.4 Antibiotic alternatives: bacteriocin

2.4.1 Introduction

The continuous effort of discovering antimicrobial compound has been carried out from all over the world from any possible sources. An antimicrobial peptide is a peptide sequence that has antimicrobial activity which is synthesized in any organism as a form of defence feature of the host (Zasloff, 2002). An antimicrobial peptide that is synthesized specifically by bacteria is termed bacteriocin. Bacteriocin is a ribosomally synthesized peptide that functions as a defence against competitive strain (Ahn & Jun, 2007). Early reports of bacteriocin from gram-positive bacteria and LAB specifically show inhibition of closely related species. As more of the group of bacteriocin is being discovered, a large repertoire of them has activity against the foodborne pathogens the

food industry. A few of these bacteriocins are now patented and being used in the food industry such as Nisin and Pediocin (Arthur *et al.*, 2014).

As antibiotic resistance issue started to be more alarming, a lot of researches have been conducted to shift the usage of bacteriocin from the only food industry and widen it for the therapeutic purpose and suggested the bacteriocin can become an alternative to antibiotic (Riley *et al.*, 2012). Bacteriocins have a lot of merits for it to be coined as the alternatives for antibiotic. Conventional antibiotics that were discovered early on such as Penicillin which is the earliest one discovered is a form of secondary metabolites possessed similar function such as bacteriocin. Current conventional antibiotics are chemically synthesized and multiple generations of antibiotics were branched off from the earlier generation with structure modification with an increment of antibacterial activity. However, one of the major differences in bacteriocin is that the bacteriocin is a ribosomally synthesized peptide. The proteinaceous aspect of bacteriocin allows it easily to disintegrate quickly compared to chemically synthesized antibiotics (Hols *et al.*, 2019). The remnant of antibiotics has been shown to able triggered the emergence of antibiotic resistance (Kohanski *et al.*, 2010). The rapid degradation of bacteriocin is an excellent trait to ensure the prevention of any resistance being emerged. However, one can say that it also can be a form of a drawback to the application of the healthcare industry. Alternately, the encapsulation technique can be used to address this issue (Langer & Folkman, 1976).

The first discovery of bacteriocin, Colicin by Gratia in 1925 and Nisin was discovered shortly afterwards in 1928 by Rogers and Whittier. (Rogers & Whittier, 1928; Gratia, 2000). Decades had passed since the initial discovery of bacteriocin and the bacteriocin application has been intensively focused for food industry mainly focusing on food preservation. With the emergence of antibiotic resistance and the reduction of antibiotic effectiveness, the application of bacteriocin into biomedical and healthcare industries are

underway as many bacteriocins are in clinical studies stages (Ongey *et al.*, 2017).

With regards to the resistance issue, it has been reported that the effectiveness of bacteriocin is reduced as the emergence of resistance against it. (de Freire Bastos *et al.*, 2015). However, the report of emerged resistances is reported through a laboratory setting and not clinical setting (Cotter *et al.*, 2012). interestingly, no reported of Nisin resistance despite being used for a long period as a food preservative (Blake *et al.*, 2011).

Bacteriocin associated resistance can be classified into two forms which are innate or acquired (Collins *et al.*, 2012). Innate resistance is described whereby the presence of the immunity gene or absence of bacteriocin receptor. This immunity gene is found intrinsically in the species. A mutation in this gene resulting in increased sensitivity of bacteriocin toward the target is known as innate resistance, while the mutation of a gene found in the target bacteria resulting in increased resistance is considered as acquired resistance (de Freire Bastos *et al.*, 2015). Example of acquired resistance is the natural selection to reduced bacteriocin binding to the cell wall by alteration of the cell wall's composition. A study was done on Nisin by Crandall & Montville, (1998) demonstrated the alteration of *Listeria monocytogenes* ATCC 700302 in the ratio of fatty acid resulted in a more rigid cell wall which reduced Nisin insertion and binding. Nevertheless, resistance against bacteriocin does not hamper for bacteriocin research as it is natural for bacteria to gain resistance as it is part of the natural phenomenon of any living system to adapt to stress in the environment. There are many strategies to lessen the potential resistance emergence such as a combination of bacteriocin with other bacteriocin or conventional antibiotic which is described in section 2.4.5

2.4.2 Bacteriocins from LAB

The bacteriocins of LAB always gain the favour for further characterization due to its probiotic traits. hence, extensively studied bacteriocin such as Nisin which is used widely in the food industry. With the rising tide of antibiotic resistance, more attention is shifted medical application-related research using bacteriocin (Yang *et al.*, 2014). One of the merits of bacteriocin from lactic acid bacteria is the health benefits conferred by the strain itself. Many of the LAB are used as a form of supplements or probiotics after it went through a strict selection guideline. The requirement is it must be a non-pathogenic, have the capabilities to survive in the gastrointestinal environment with the presence of acid and bile salt, can be tolerated by the immune system and will not cause the formation of antibodies (Belicová *et al.*, 2013).

Due to the capability of LAB to produce bacteriocins and other metabolites such as lactic acid, hydrogen peroxide, several bacteriocins such as Nisin and Pediocin which have been approved by FDA and recognised as GRAS to be used as food preservatives to steer away from food associated pathogens. Moreover, more bacteriocin has been discovered to have an antimicrobial effect against a wide range of gram-negative which includes a lot of pathogenic bacteria and not just gram-positive (Dobson *et al.*, 2012). One example of bacteriocin with broad-spectrum is Enterocin E-760 which is produced by *Enterococcus* sp. The bacteriocin has broad antimicrobial activity against notable gram-negative pathogens such as *Shigella dysenteriae, Klebsiella pneumonia, Yersinia enterocolitica 03, Campylobacter sp.* and *Citrobacter freundii* (Line *et al.*, 2008).

2.4.3 Bacteriocin classification

The earliest classification of bacteriocin was proposed by Klaenhammer, (1993) with four class of bacteriocins which consist of four class and the fourth one was consisting carbohydrate and lipid structure such as Lactocin 27 and Leuconocin S. The fourth group was added after the discovery of bacteriocin that is inactivated by the addition of glycolytic and lipolytic enzymes. The fourth was renamed as bacteriolysins and was removed from the bacteriocin classes. Thus, bacteriocins now are classified into three classed (Liu *et al.*, 2014).

Bacteriocin of class 1 is known as lantibiotics that possessed modified residue of lanthionine. The presence of lanthionine residue leads to the formation of covalent bonds between amino acids resulting in internal rings (Perez *et al.*, 2014). Class 1 is separated into 3 subclasses. Each subclass contains specific modified amino acids such as Lantibiotic for class 1a, Labyrintipeptins for class 1b and Sanctibiotics for class 1c (Spencer *et al.*, 2003). Lantibiotics such as Nisin contains serine, threonine and thioether amino acids which lead to the formation of five lanthionine (Sen *et al.*, 1999)

Meanwhile as for class 2, in general, contains heat stable, non-modified peptides. Subclasses of class 2 are divided into pediocin like bacteriocin (class 2a), two peptides, unmodified (class 2b), circular (class 2c) and unmodified, linear and non-pediocin like bacteriocin (class 2d) (Belguesmia *et al.*, 2011). Lastly, class 3 is for bacteriocin which is large and heat-labile (Sun *et al.*, 2018). Example of class 3 bacteriocin is Colicin. Colicin is produced by many *E. coli* strains which inhibit *E. coli* other than its host by forming pores in the inner membrane. All Colicins consist of three domains which are central receptor binding domain, N-terminal translocation domain and C-terminal cytotoxicity domain. All of these domains help in binding to target cells, movement of colicins into the periplasm from the outer membrane and pore formation respectively (Jin *et al.*, 2018).

2.4.4 Bacteriocin's mode of action

Beside bacteriocin having the role as an antimicrobial agent for its producing strain, bacteriocin is hypothesized to have a secondary function which is a colonizing agent and signalling peptide (Dobson *et al.*, 2012). The interaction of the bacteriocin with the target cell is through interaction with the receptor. For example, Nisin, the most extensively studied bacteriocin form interaction with lipid 2 to form pores. It is said that in a pore, there will be 8 Nisin with 4 lipids II to cause destabilization and pore opening (Van Heusden *et al.*, 2002). Interestingly, whenever the lipid II is not available for interaction, a high concentration of Nisin can still cause cell membrane deformation (Prince *et al.*, 2016). Bacteriocin from different class such as class 2 is reported to have nonspecific interaction with the cell membrane through electrostatic interactions which cause pore formation (Gillor *et al.*, 2008). For example, lacticin Q produced by *L. lactis* QU5 form toroidal pores which cause protein leakage and cell death without any specific receptor (Yoneyama *et al.*, 2009).

Besides cell membrane disruption, bacteriocin also targeted other bacterial systems which conventional antibiotic targets such as protein synthesis inhibition and DNA inhibition (Chan *et al.*, 2011; Collin *et al.*, 2013). Classic drug targets are still significant for therapeutic application. However novel drug target will have a higher advantage. New cell target which is inhibition of septum formation was recently discovered. bacteriocin that possessed this mode of action is Garvicin A and Lactococcin 972 (Martínez *et al.*, 2000; Maldonado-Barragán *et al.*, 2013).

The field of research is constantly being explored and expanding. An understanding of the genetic organization of the bacteriocin, functionality of each gene in the genetic operon will open doors of opportunities to further exploit the antimicrobial activity of bacteriocin. This is due to the nature of bacteriocin being amenable to genetic engineering. Many strategies with different approach have been carried out such as improving one antibacterial activity through mutation study. An interesting example for this approach is the

work of Field *et al.*, (2019), where the work displayed a Nisin A derivative produce through the process of mutation at the specific amino acid residue that managed to increase the activity 20 times compared to the Nisin A itself and at the same time able to exhibit activity towards a strain that expressed Nisin resistance protein. This is work display one of the merits that bacteriocin have over conventional antibiotics as ribosomally peptide are amenable through genetic engineering.

2.4.5 Synergism application of bacteriocin and conventional antibiotic

With many of the conventional antibiotics developed are no longer viable options for therapeutic application. One of the strategies that could improve the efficacy of these antibiotics is through the combination of antibiotics with an adjuvant which acts as an augmenter. A successful combination between the two compound means the combination is synergistic to one another, in which the killing rate speeds up (Chi & Holo, 2018). As for the combination of conventional antibiotics with bacteriocins, there have been numerous studies being done in this. There are many advantages to employing combination therapy instead of monotherapy. One of them is to control resistance from being emerging. Secondly is to utilize an antimicrobial compound that has a high MIC breakpoint on a pathogen at a lower concentration when combined with a second compound (Cavera *et al.*, 2015). Nisin which is one of the heavily studied with high interest on application related to human health has been able to enhance its range of activity especially after being combined with various antimicrobials (Naghmouchi *et al.*, 2013; Shin *et al.*, 2016).

Examples of synergistic interaction can be seen from bacteriocin Subtilisin A when being combined with antimicrobial such as Aclindamycin and Metronidazole against the bacterial vaginosis associated pathogen *Gardnerella vaginalis* (Algburi *et al.*, 2015). A study using Nisin with the combination of ramoplanin against MDR pathogen such as MRSA also showed exceptional

potential. As there are many and limitless potentials to this strategy, the field is still at its infancy as there are always possibilities of the combination being antagonistic towards one another such as the case of Nisin and Chloramphenicol antagonizing one another when tested against MRSA (Brumfitt, 2002).

In vitro results are always straight forward and will not be similarly translated *in vivo* testing. *In vivo* testing of Nisin with a β-lactam antibiotic showed promising results as the membrane permeabilization of the β-lactam allow for Nisin to be uptake by the cells. Certainly, the field is exciting and at the same time requires more findings to validate the potential as there are always debate on the authenticity of the claims of advantages of combination therapy with mix results obtained (Tamma *et al.*, 2012).

2.4.6 Advancement in bacteriocin research

2.4.6.1 Extraction and purification strategies: conventional and alternatives

Discovery of bacteriocin has been targeted on a certain environment that will require for the community to have a certain form of antimicrobial capability. The most common location of the isolate is fermented food itself where LAB strive to grow. The mainstream approach of extracting bacteriocin has always been through initial concentration method known as ammonium sulphate pre which is common for any protein extraction work such as ammonium sulphate precipitation, solvent-based precipitation and followed up by a polishing step through chromatography steps utilizing columns such as ion exchange, gel filtration and reverse phase. Many of the extractions that have been done combining many types to increase the purity at the expense of quantity of the extracts (De Vuyst & Leroy, 2007). This common way of extracting bacteriocin is on a research scale is expensive but is reliable to produce a high purity extract which allows further study on the functionality of the compound. But often,

this conventional method of purification is tricky, complex and time-consuming.

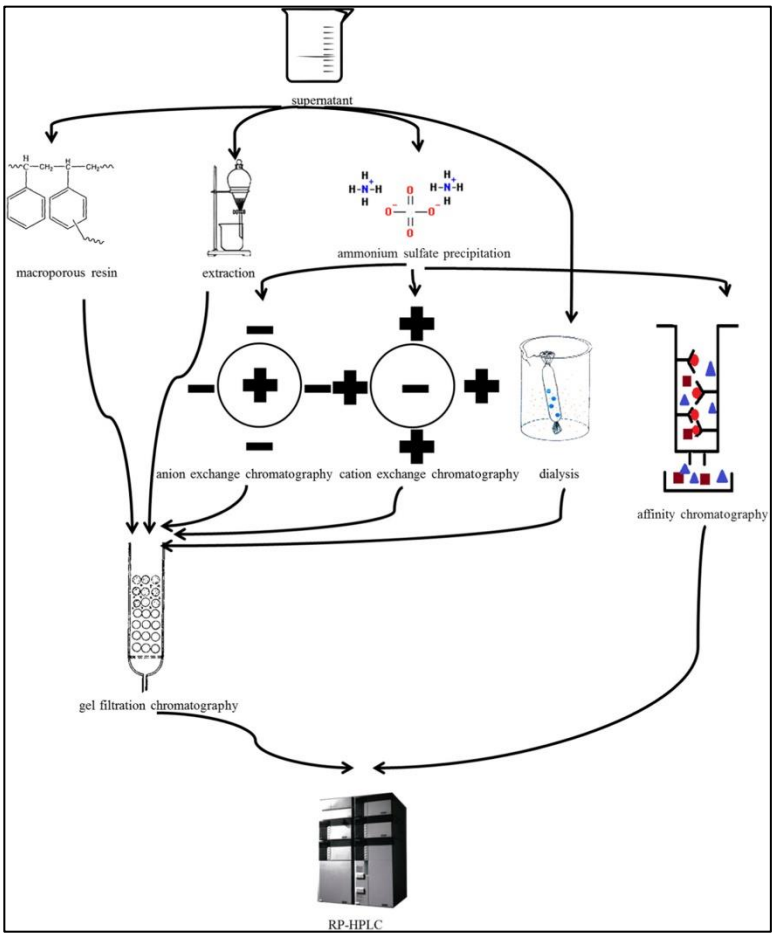

Figure 2.1: summarized approach of bacteriocin purification. Adapted from (Zou *et al.*, 2018).

Hence, other alternatives have been explored to try overcoming the bottleneck issues in bacteriocin purification fields. Of the issues are, inability to translate the bacteriocin purification scheme in a larger scale as the cost of bacteria medium and nutrient supplements and also the chromatography components are high or in another word up-scaling (Bali *et al.*, 2016).

The alternatives are such as expanded bed adsorption chromatography (EBA) that create an integrative unit operation through the continuous flow of sample that will pass through the bed and the pass-through will be the unwanted components and target protein will be captured simultaneously. This method combined clarification, product concentration and purification in one process

which has been applied into few studies along the years. one of the highest purification result based on this technique which is 47.6% activity and 140 times of the purification fold (Moreno *et al.*, 2001).

Another alternative is Aqueous two-phase system (ATPS) that provide purification through the simple process yet productive, cost-effective. The principle of this technique is by combining two different types of system (solute) that will create two different phases and the ideal system will cause the target protein to reside in one of the phases. This technique has been improved into different forms such as aqueous micellar two phase-system (AMPTPS). A detailed review of these approaches has been done by Jamaluddin *et al.*, (2018). The application of this technique demonstrated 81% activity yield with the purification of 0.96 on the extraction of Cerein A (Lappe *et al.*, 2012).

it is an important part of the research to integrate bacteriocin application in healthcare or any related field is through a more cost-effective production. At the same time, the ideal objective of bacteriocin purification is to obtain a high purity bacteriocin. The completion of this part of the process will allow for more in-depth information of the bacteriocin to be obtained.

2.4.6.2 bioinformatics approach in novel bacteriocin discovery

The extraction and purification are an important process in obtaining pure bacteriocin, removing unrelated compound present together with the bacteriocin. This is an ideal situation if the end goal is obtaining a known bacteriocin present in the CFS. However, a common approach for screening bacteriocin is often anchored together with extraction and purification process after inhibitory activity was observed. One of the limitations of this approach applies to the scenario where multiple bacteriocins are produced by a single strain as highlighted in the works of Campanero *et al.*, (2020).

The common approach of extraction and purifications such as the usage of chromatography will focus on the sample with inhibitory activity. Ideally,

bacteriocin is expected to be expressed in the bacterial media. In certain instances, the bacteriocin gene might not be active due to absence of accessory genes due to mutation or due to tight transcriptional regulation resulting no *in vitro* production of the bacteriocin (Collins *et al.*, 2018) Thus, extraction and purification may have yielded in a negative result. This finding was highlighted in the works of Yi *et al.*, (2016) where their bacteriocin extraction on *Lactobacillus crustorum* MN047 resulted in a discovery of a new bacteriocin. A subsequent study was used on the same strain through *in silico* approach resulted in another 7 possible bacteriocin genes which possessed antibacterial activity through heterologous production study (Yi *et al.*, 2018). This discovery highlights the importance and potential of bioinformatics approach which can help improve the limitation of the conventional approach of extraction and purification.

Bacteriocin research is transitioning parallel with technological advancement. The search for bacteriocin early on was focused on the screening, purification and characterization of a bacteriocin which has contributed to many great discoveries. However, this method is known for its labour-intensive process as alluded in the previous section which led researchers to developed for a more improved version using molecular biology. The approach improved by discovering the gene sequences by a conventional technique such as primer walking and PCR amplification (Suwanjinda *et al.*, 2007; Macwana & Muriana, 2012; Bibalan *et al.*, 2017). Through this approach, the additional wealth of information of these bacteriocins such as sequences of bacteriocin and genetic characterization of bacteriocin gene operon allows for further extended study of these developments such as function improvement study. This branch of the bacteriocin field is mainly focused on enhancing the capability of fully characterized bacteriocin such as Nisin (Field *et al.*, 2012).

With the advancement of next-generation sequencing, screening bacteriocin genes are done at another different level due to the availability of genome data and multiple bioinformatics tools. This recent approach is to tackle limitation that has persisted and ignored due to limitation of the commonly used

approach such inability to screen bacteriocin that is expressed in low quantity or not being expressed in a laboratory setting due to specific growth requirement are not satisfied.

Hence, to overcome this problem of limited coverage of bacteriocin detection from the conventional way of bacteriocin discovery, numerous works have been done on this. One of the strategies is to employ homology-based tools that detect the bacteriocin gene and its context gene based on available bacteriocin database (Collins *et al.*, 2017; Egan *et al.*, 2018; Van Heel *et al.*, 2018). The genome mining of bacteriocin allows for the discovery of many new bacteriocins.

Another bioinformatics approach is through screening a conserved gene that is related to bacteriocin biosynthesis homology-based search using the BLAST. This approach has allowed the discovery of lantibiotics such as Haloduracin and Lichenicidin (McClerren *et al.*, 2006; Begley *et al.*, 2009). Both bacteriocins are discovered through homology searches of the conserved processing enzymes. This works mentioned here is the earliest work on this prediction strategy which leads to numerous works of bacteriocin such as a new tool developed for bacteriocin prediction called Bacteriocin Operon and gene block Associator (BOA), bacteriocin gene are not conserved, hence most utilized bacteriocin prediction tool such as bagel will not detect bacteriocin gene at all (Morton *et al.*, 2015). BOA utilize HMMER search of context genes that will lead to bacteriocin discovery.

Development of tool such as BOA also gives rises to a newer tool which predict bacteriocin using machine learning, NeuBi (Hamid & Friedberg, 2019*a*). This tool utilizes the bacteriocin gene in a publicly available database to train the machine to predict a protein as a bacteriocin gene. This tool is crucial for the identification of the bacteriocin gene that is not similarities with other bacteriocin and no conserved motifs. Through bioinformatics approaches, the discovery of new bacteriocins can be speeded up with the availability of genome data.

2.4.6.3 Bioinformatics tool as a bridge for conventional bacteriocin research and heterologous expression study.

Bioinformatics or *in silico* work will just remain stagnant or hypothetical without further validation from laboratory experimental approach. With the abundance of tools at bay, bacteriocin genes or the biosynthetic operon can be emulated through a form of heterologous expression study. Heterologous expression of bacteriocin has been an active research area for 30 years. The production of the protein of interest using a heterologous expression system are based on three main purposes (Makrides, 1996). Firstly, for the functional elucidation of the proteins expressed which are in the case of heterologous expression of bacteriocin are the bacteriocin genes and the genes are associated with the bacteriocin such as transporter and immunity. Secondly, controlling the expression of the recombinant gene and thirdly, improvement of protein production higher than the native source. The importance of this approach reflected with the wealth of knowledge regarding the biology of bacteriocin being explored (Uzelac *et al.*, 2015; Noda *et al.*, 2018). Nisin is a perfect example of the usage of heterologous expression study being utilized in function improvement-based study. Nisin derivatives such as Nisin K12A which have been improved its function showed profound activity against clinical significance gram-positive microorganism (Molloy *et al.*, 2013). The bacteriocin production to be improved significantly through this approach vividly demonstrated in the works Telke *et al.*, (2019) where the production of Garvicin KS increased by 2000 times. This is very beneficial for industrial large scaling operation and it will not be achievable from the bacteriocin original producer. There are also heterologous expressions studies demonstrated where the production of bacteriocin using heterologous expression system was not effective compared to the native host such as the production of Hiracin JM79 in the work of Sánchez *et al.*, (2008) where all the transformants produced lower activity of Hiracin JM79 against *E. faecium* T136. Thus, many expression vectors are created and optimized for more efficient production of bacteriocin compare to the native host such as the work for of Mesa-Pereira *et al.*, (2017)

which demonstrate suitable expression of class II bacteriocins. Recently, a work by Collins *et al.*, (2018) demonstrated a unique approach in bridging between *in silico* prediction of *lactobacillus's* pangenome and heterologous expression study which were made possible even for silence bacteriocin gene that is not expressed *in vitro* due to incomplete bacteriocin gene. The works demonstrated the example of how the vast potential of vast genomic data containing the bacteriocin gene can be further exploited.

Chapter 3: *In silico* study of bacteriocin in *W. cibaria* NM1 genome

3.1 Introduction

The availability of genomic data opens the possibility of exploring different approaches in bacteriocin research. The approach of predicting bacteriocin has been utilized to analyse the large scale of publicly available genome data. Many different approaches have been used throughout the years such as finding highly conserved bacteriocin regulatory gene (driver gene) that often linked to a bacteriocin structural gene (Begley *et al.*, 2009) and extensive search on one bacterial genome of bacteriocin structural gene and genes that are associated to bacteriocin production. The latter approach is the working principle of Bagel4 tool. The development of Bagel4, dedicated webserver to predict genome data which allows a different form of strategy to identify bacteriocin such as bacteriocin gene prediction from a gut microbiome data (Walsh *et al.*, 2015). Another example is the usage of Bagel4 to assess the diversity of bacteriocin genes in the genus of *Geobacillus* (Egan *et al.*, 2018).

The work of Vezina *et al.*, (2020) provides a unique workflow on discovering putative circular bacteriocin through mining NCBI database against experimentally verified circular bacteriocin sequence. This study again highlights the swiftness of bacteriocin prediction approach in discovering new bacteriocin compared to the conventional method which requires many isolates to be tested against indicator strains. Another recent approach using machine learning prediction, Neural Bacteriocin Identifier (NeuBi) was developed attempting to overcome the limitation of homology-based prediction used by Bagel4 (Hamid & Friedberg, 2019*a*). Through this implementation, the hunt of bacteriocin from novel isolate can be more improved which new bacteriocin can be discovered.

One of the advantages is the connecting bridge of laboratory works and *in silico* analysis through data reconciliation or verification. Screening numerous bacteria isolate with bacteriocin capability with indicator strains is time-

consuming. Bioinformatics approach provides an alternate initial screening process that expedites the process of bacteriocin producing strain. However, this is not often the case as the presence of bacteriocin genes does not necessitate the strain exhibit antimicrobial strain in some cases where the bacteriocin producing trait of the strain is inactive due to missing essential gene linked to bacteriocin production (Collins *et al.*, 2018). Thus, it shows the importance of validation of experimental data as employed in many works (Oliveira *et al.*, 2017)

W. cibaria NM1 is a gram-positive bacterium from the LAB group that found in many environments such as human, fermented vegetables and animal gut. The species is highly studied of its probiotic characteristic recently but unexplored of the bacteriocin produced. Only the bacteriocins produced by *Weissella* species have been characterised such as Weissellicin Y, M, L from *W. hellenica* (Masuda *et al.*, 2012; Leong *et al.*, 2013) and Weissellicin 110 *from W. cibaria* 110 is (Srionnual *et al.*, 2007). Li *et al.*,(2017) demonstrated through a comparative genomic analysis where the bacteriocin operon of Weissellicin 110 are not conserved in other *W. cibaria.*

Thus, this study aims to execute a bioinformatics analysis of the genome of *W. cibaria* NM1 on the availability of the bacteriocin gene using publicly available bacteriocin prediction tool.

3.2 Objective

This chapter utilized an *in silico* approach to predict the availability of bacteriocin associated genes in the genome of *W. cibaria* NM1's using of machine learning-based prediction approach with homology search of highly conserved bacteriocin associated genes. This chapter also attempts to assess the probiotic potential of *W. cibaria* as a putative probiotic candidate through the identification of probiotic associated genes in its genome.

3.3 Materials and method

3.3.1 Bacterial culture

W. cibaria strain NM1 used in this study was isolated from the gut of Asian sea bass. The bacteria strain was provided by Dr Ivan Chiew. The strain was stored at −80 °C in MRS broth (Oxoid, UK) containing glycerol (25% v/v). Cultures were routinely grown at 37 °C for 24 h.

3.3.2 DNA extraction

The lytic enzyme solution was prepared earlier before beginning the DNA extraction by adding 50 mg of lysozyme (Sigma L6876) into 5 mL of 50 mM EDTA (pH 8.0). The solution was then mixed thoroughly.

LAB culture that has been grown for 24 h was used for genomic DNA extraction. The DNA extraction was done according to the protocol of Wizard® Genomic DNA Purification kit (Promega, USA). One mL of 24h old LAB culture was centrifuged at 13000 g for 2 min to obtain the cell pellet. The cell pellet was retained and was suspended in 480 µl of 50mM EDTA (pH 8.0) and followed by adding 120 µl of lytic enzyme solution. The sample was incubated in a water bath at 37℃ for 60 min. Then, the sample was subjected to centrifugation at 13000 g for 2 min. The supernatant was removed and the cell pellets were collected. Six hundred µL of Nuclei Lysis Solution (Promega, USA) was added to the tube. The cell pellet was resuspended by gentle pipetting and followed by incubation at 80℃ water bath for 5 min for cell lysis. After the sample was cooled to room temperature, 3 µl of Rnase solution (Promega, USA) was added to the sample and the tubes were mixed by inverting repeatedly. The sample was then incubated at 37℃ water bath for 30 min and then the sample was cooled to room temperature before 200µl of Protein Precipitation Solution (Promega, USA) is added to the cell lysate. The sample was then mixed 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 600µl of isopropanol and was mixed through repeated inversion of the sample 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). Then, 600 µl of 70% (v/v) ethanol was added into followed by gentle inversion of the tube for thorough washing of the DNA pellet which followed by centrifugation at 13000 g for 5 min. The aspiration of the ethanol was done carefully and the tube was further drained using clean absorbent paper. The pellet was then subjected to an air-drying process for 15 min. The pellet was rehydrated with the addition of 50µl of DNA Rehydration Solution (Promega, USA) and followed by incubation for 1 h in a water bath at 65℃. Finally, the purified DNA was stored at 4℃ until further use. The DNA quantity and purity were measured using a NanoDrop Spectrophotometer (Thermofisher, USA). The quality of DNA was further validated using 1.5% agarose gel electrophoresis stained by SYBR® safe DNA gel stain (Thermo Fisher Scientific, USA). The DNA band were viewed using the Chemidoc XRS+ system (Biorad, USA).

3.3.3 Bacterial genome extraction and sequencing

The DNA of *W. cibaria* NM1 was sent for genome sequencing service to BioEasy Sdn. Bhd. (Selangor, Malaysia) where the library preparation was done. Sequencing of *W. cibaria* NM1 was performed on the Illumina MiSeq® platform using 2 × 251 paired-end method (Malaysia Genome Institute, Malaysia). The resulted DNA sequence reads were adapter and quality trimmed and contaminant and read length filtered with a threshold of 30 using BBDuk (Bushnell *et al.*, 2017). The genome of *W. cibaria* NM1 was deposited in NCBI with the accession number of PRJNA640481.

3.3.4 Genome assembly and annotation

Raw sequences were assembled using St. Petersburg genome assembler (SPAdes) (Bankevich *et al.*, 2012) and annotated using Prokaryotic dynamic programming gene-finding algorithm (Prodigal) for protein-coding gene prediction (Hyatt *et al.*, 2010), ARAGORN (Laslett & Canback, 2004) for transfer RNA gene prediction, RNAmmer for ribosomal RNA gene prediction (Lagesen *et al.*, 2007), and HMMER for protein domain search (https://www.ebi.ac.uk/Tools/hmmer/; Potter *et al.*, 2018). The 16s rDNA sequence of *W. cibaria* NM1 which were predicted using RNAmmer tool was then searched against NCBI's 16s rRNA database using BLASTn standalone tool with the cutoff value of e value greater than -5. The hits from the BLAST search was used to form a phylogenetic tree through MEGA-X tool. (Kumar *et al.*, 2008). The statistical method used to form the tree is the Neighbour-joining method (Saitou & Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site (Tamura *et al.*, 2004).

3.3.5 Bacteriocin prediction

W. cibaria 110 sequences obtained from NCBI repository. *W. cibaria* NM1 and *W. cibaria* 110 nucleotide sequences were used for Bagel4 prediction while its protein sequences were used for NeuBi prediction. The inclusion of *W. cibaria* 110 in this study as a positive control for NeuBi tool as the strain has a bacteriocin sequence (Weissellicin 110) that has been validated experimentally. Both of the strains were predicted of the bacteriocin though Bagel4 dedicated web server (http://bagel4.molgenrug.nl/index.php; van Heel *et al.*, 2018). NeuBi tool was used following through instruction provided in the Github repository. (https://github.com/nafizh/NeuBi; Hamid & Friedberg, 2018) The protein sequences with a score higher than 0.95 were shortlisted. Accession ID of the context genes (genes associated to bacteriocin production; transport, regulator, immunity, modifier) was collected from the NeuBi's Github and then

were fetched from NCBI through Entrez tool (Maglott *et al.*, 2011). A custom BLAST database was created using makeblastdb.pl from the BLAST standalone tool suite (Camacho *et al.*, 2009). Protein sequences of *W. cibaria* NM1 and *W. cibaria* 110 were searched against the customised database with the cutoff value of e value greater than -5. Positive hits from the BLAST search were combined with NeuBi's bacteriocin prediction hits and were arranged in a coordinate manner according to its location in the genome. Bacteriocin gene that is surrounded with all type of context gene (modifier, immunity, regulator and transport) in the vicinity of 50 kb were selected as potential bacteriocin gene. The gene operon is visualized using dna_feature_viewer python script (https://github.com/Edinburgh-Genome-Foundry/DnaFeaturesViewer; Zulkower & Rosser, 2020)

3.3.6 Screening of probiotic related genes, virulence gene and antibiotic genes in the genome of *W. cibaria* NM1

Screening of probiotic associated genes of *W. cibaria* NM1 was done through BLASTp search (Camacho *et al.*, 2009) from the probiotic gene obtained from the works of Kumari *et al.*, (2020). The protein sequences of *W.* cibaria NM1 were searched of antimicrobial resistance genes using ResFinder 3.0 server (https://cge.cbs.dtu.dk/services/ResFinder/;Zankari *et al.*, 2012) and also through resistome data (RGI) from the Comprehensive Antibiotic Resistance Database (CARD; https://card.mcmaster.ca;Alcock *et al.*, 2020). Virulence genes were detected using VirulenceFinder (Joensen *et al.*, 2014).

3.4 Results

3.4.1 Assembly and analysis of the *W. cibaria* NM1 strain

Whole-genome sequencing generated a draft genome of 2,421,541 bp for *W. cibaria* NM1 with a GC content of 44.86%. The genome was assembled into 36 contigs with N50 of 151,860. The estimated assembly coverage was 42.32. Prodigal annotation predicted 2,248 coding sequences out of 2,320 genes (96% coding intensity). 72 RNA genes are consisting of 67 tRNA and 5 rRNA. The genome size of *W. cibaria* NM1 is similar to the median size of genome size deposited in NCBI 2.44 Mb. Figure 3.1 showed the sequences of the rDNA gene of *W. cibaria* NM1 showed 98.16% identical to *W. cibaria* II-59*,* resulting it to be clustered together in the phylogenetic tree. The closest related species to *W. cibaria* is *W. confusa* JSM 1093 with percentage identity of 97.68%

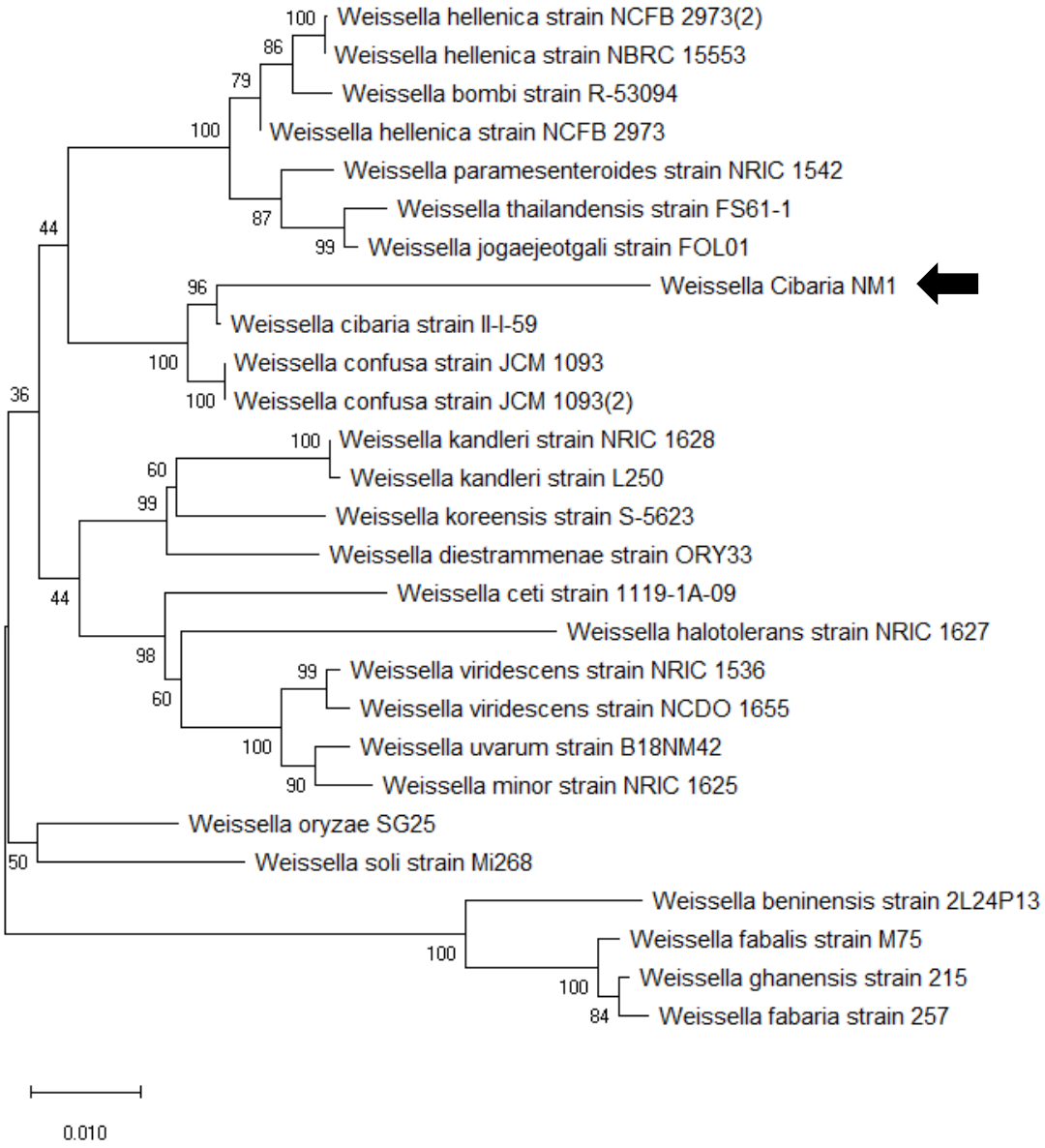

Figure 3.1: Phylogenetic tree of *W. Cibaria* NM1 of its 16s rDNA sequences against the 16s rDNA database. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed (Felsenstein, 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches

3.4.2 Bacteriocin prediction

3.4.2.1 Bacteriocin prediction of Weissella species

Bacteriocin prediction for *W. cibaria* NM1 and the reference strain *W. cibaria* 110 was performed using Bagel4. However, negative prediction result was obtained with no bacteriocin gene was found harboured in the genome of both *Weissella* strains. A similar finding was found for all the deposited *W. cibaria* genome in the NCBI database. NeuBi was also used for *W. cibaria* 110 and it predicts the protein sequence of Weissellicin 110, as a bacteriocin with the probability of 0.96. Figure 3.2 shows the genetic organization of bacteriocin operon of Weissellicin 110. The genetic organization generated in this study matched with the published works of Weissellicin 110 (Li *et al.*, 2017) which shows the NeuBi prediction approach can be in accordance with the experimental work. There are about 21 protein sequences of *W. cibaria* NM1 that have scored higher than 0.9. however, only one bacteriocin gene candidate that have all four type of context genes in the 25kb vicinity around the bacteriocin gene candidate which is WC_2064 (Figure 3.3).

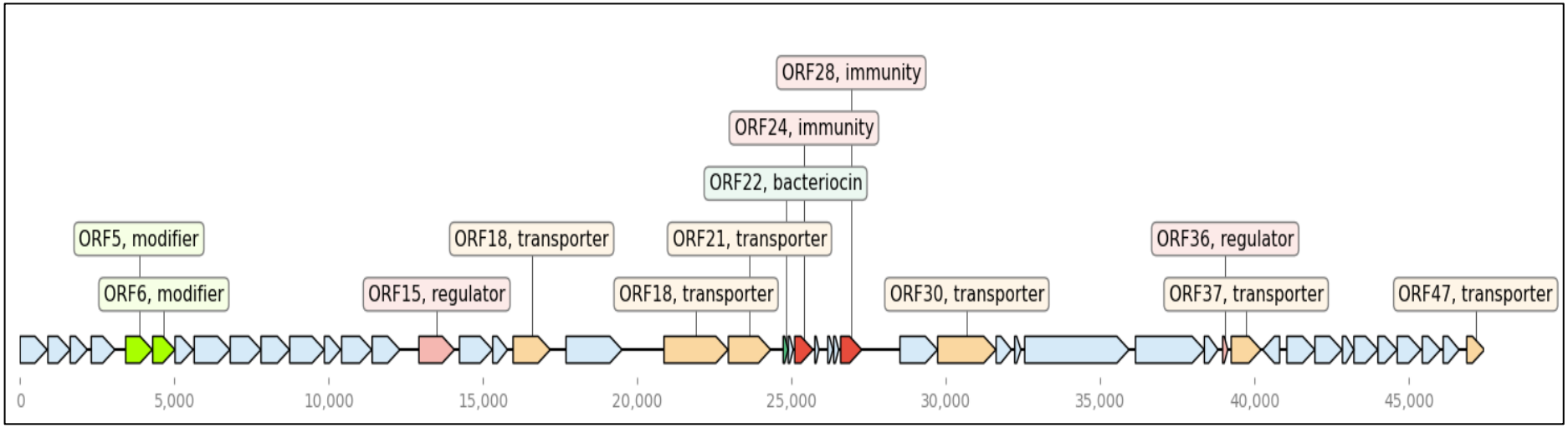

Figure 3.2: Illustration of predicted bacteriocin gene operon of *W. cibaria* 110. The unlabelled gene boxes are non-related genes.

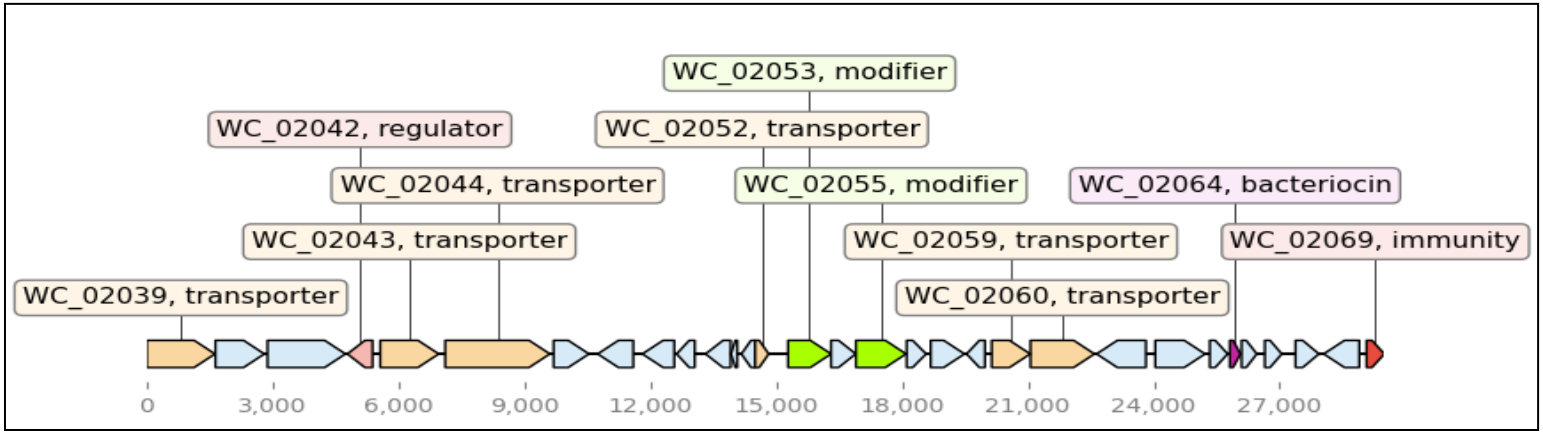

Figure 3.3: Illustration of predicted bacteriocin gene operon of *W. cibaria* NM1. The unlabelled gene boxes are non-related genes.

Table 3.1 listed out the complete set of context genes with the matching genes from the context gene database. Immunity gene was found upstream of the bacteriocin gene. Several transporter gene and modifier genes were found downstream of the bacteriocin gene and one regulator gene was found downstream of the bacteriocin gene.

Table 3.1: Gene predicted involved in bacteriocin biosynthesis with its predicted function

Gene ID	function	Gene name	E-value	Percentage similarity (%)	GenBank ID
WC_2039	Transporter	ABC Transporter	1.00e-75	30.43	EFM66193.1
WC_2042	Regulator	Tetr Family Transcriptional Regulator	2.00e-08	25.82	KKD14008.1
WC_2043	Transporter	MFS Family Major Facilitator Transporter	1.00e-39	28.6	AEA57849.1
WC_2044	Transporter	ABC Transporter ATP-Binding Protein	1.00e-09	31.25	COL50160.1
WC_2052	Transporter	Multidrug Transporter	8.00e-08	21.57	KQB72399.1
WC_2053	Modifier	Riboflavin Biosynthesis	9.00e-56	36.86	AIG26540.1
WC_2055	Modifier	Riboflavin Biosynthesis	5.00e-124	49.23	AIG26542.1
WC_2059	Transporter	Glycine Betaine Carnitine Choline ABC Transporter ATP-Binding Protein	3.00e-94	52.20	KRL50892.1
WC_2060	Transporter	Glycine Betaine Carnitine Choline ABC Transporter ATP-Binding Protein	5.00e-164	52.82	AGE38097.1
WC_2064	Bacteriocin	No Hits (NeuBi Prediction With Score Of 0.97)	-	-	-
WC_2069	Immunity	Putative Leucocin B Immunity Protein	3.00e-06	28.16	YP 009090140.1

No hits with any bacteriocin were found for WC_2064. Interestingly, the sequence of WC_2064 contains a double glycine leader at N-terminal which

indicates the sequence has precursor peptide that will be cleaved off by N-terminal domain of the transporter (figure 3.4) (Dirix *et al.*, 2004).

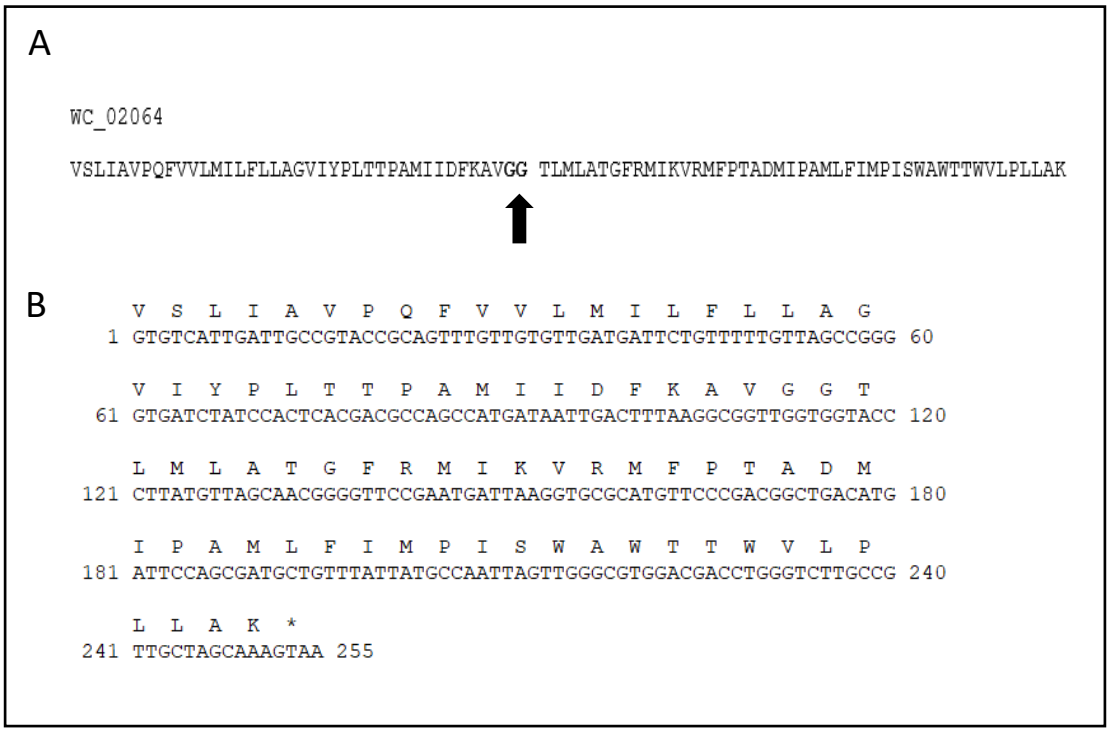

Figure 3.4: (A)Protein sequence of WC_2064. The double glycine motif in the sequence is shown by the arrow. (B) Alignment of the protein sequence and nucleotide sequence of WC_2064.

3.4.3 Probiotic potential of *W. cibaria* NM1 through genome screening.

Screening of probiotic attributes of *W. cibaria* was done at the genomic level. Table 3.2 shows the probiotic related genes extracted out from Kumari *et al.*, (2020) on closely related *W. cibaria* CH2. Probiotic related genes screened included gene function that is responsible for acid tolerance, bile tolerance, adhesion to the epithelial lining and functional trait such as cholesterol reduction. Genes that related to Acid tolerance were found in this strain such as of ATP synthase, and esterase. The screening of Bile tolerance related genes were also found in this strain such as ABC transporter, ATP dependent proteases subunit, and multidrug-resistant protein. *W. cibaria* NM1 also was found to consist a gene that is involved in adhesion ability in host cells and interestingly the strain encoded Catabolite control protein A, which is

recognised to involved in reducing cholesterol level. All screened genes resulted in hits with high percentage similarity and e-value. No virulence gene and antibiotic resistance gene were found encoded in *W. cibaria* NM1.

Table 3.2: List of probiotic related genes present in *W. cibaria* NM1 genome

Function	Protein	Gene hits	Percentage identity (%)	E-value
Acid Tolerance	Amino acid permease	WC_0193	100	9.31E-157
	ATP synthase F0F1 subunit C	WC_0515	100	1.97E-44
	F0F1 ATP synthase subunit A	WC_0516	99.583	4.10E-173
	ATP synthase F0F1 subunit B	WC_0514	100	9.72E-121
	ATP synthase F0F1 subunit delta	WC_0513	99.444	1.22E-129
	ATP synthase F0F1 subunit gamma	WC_0511	100	0
	Glucose-6-phosphate isomerase	WC_0687	99.776	0
	GTP pyrophosphokinase	WC_1382	99.731	0
	L-Lactate dehydrogenase	WC_0329	100	0
	Putative esterase	WC_0481	99.377	0
	Pyruvate kinase	WC_0797	99.577	0
Adhesion	sortase	WC_1443	75.934	9.07E-136
	Fibronectin-binding protein	WC_1897	79.9	0
	Mucus binding protein	WC_1688	95.038	0
	Competence protein ComGC	WC_0823	100	9E-75
	Elongation factor Tu	WC_1897	79.9	0
	Enolase	WC_0733	100	0
	Triosephosphate isomerase	WC_0047	100	1E-171
Acid And Bile Tolerance	Arginine-ornithine antiporter	WC_0584	100	0
	ATP-dependent Clp protease proteolytic subunit	WC_0358	100	8.94E-148
	Cyclopropane-fatty-acyl-phospholipid synthase	WC_1335	98.992	0
	ATP-dependent Clp protease ATP-binding subunit ClpC	WC_0500	99.88	0
	ATP-dependent Clp protease ATP-binding subunit ClpE	WC_1650	99.711	0
	ATP-dependent Clp protease ATP-binding subunit ClpX	WC_1894	64.216	0
	D-Alanyl-lipoteichoic acid biosynthesis protein DltB	WC_1553	100	0
	Multidrug resistance protein	WC_1775	47.951	9.40E-146
Cholesterol reduction	Catabolite control protein A	WC_0896	99.7	0

3.5 Discussion

This study depicts an *in silico* analysis of *W. cibaria* NM1 for bacteriocin gene. This part of the study act as a prelude study to discover the bacteriocin gene before carrying out experimental validation in the laboratory. The latter part of the study is an assessment of the genome for potential probiotic gene, antibiotic and virulence gene which is a key aspect in determining the suitability of a bacterial strain to become a probiotic candidate.

Wet-lab screening of bacteriocin is one of the commonly used approaches to identify bacteriocin in a bacterium. However, this method is time consuming filled with a lot trial and error process with regards to the process of screening bacteriocin producer strain, optimization process of bacteriocin production and purification process of the bacteriocin (Egan *et al.*, 2018). However, this time-consuming and conventional workflow can opt with the new advent of *in silico* screening process. To speed up the identification process, an *in silico* approach was used to predict the bacteriocin candidate genes in *W. cibaria* NM1.

3.5.1 Bacteriocin prediction

3.5.1.1 Bagel4 prediction

Bacteriocin prediction for both *W. cibaria* NM1 and reference strain *W. cibaria* 110 using Bagel4 was not successful as no bacteriocin gene was found harboured in the genome of these strains. Further investigation leads to a finding that no bacteriocin is identified from the genomes of *W. cibaria*. The work done by Abdulkarim *et al.*, (2020) on predicting bacteriocin gene on newly isolated *W. cibaria* D1 using bagel4 resulted in no bacteriocin gene found from the genome. A possible explanation of this occurrence is due to no similarity of the bacteriocin gene harboured in the genomes. A quick go-through over the Bagel4 database shows no related bacteriocin except for *W. paramesenteroides* and *W. hellenica* QU13 (http://bagel4.molgenrug.nl/databases.php). Hence,

this shows Bagel4 rely heavily on sequence similarity on the available bacteriocin database (Venegas-Ortega *et al.*, 2019).

Another finding that can be extracted out is the bacteriocin associated genes potentially harbouring in the genome have low similarity with the database of Bagel4. This finding agrees with the findings of Walsh *et al.*, (2015) on identifying bacteriocin gene cluster in the gastrointestinal tract using human microbiome genome database where the author stated that *in silico* prediction of bagel is dependent on the similarity to previously described bacteriocin associated genes. Another reason that yielded negative result in bagel prediction of the arrangement of the context genes which are not in close vicinity with one another. This form of arrangement also dampens the possibility of bacteriocin genetic organisation being detected (Hols *et al.*, 2019).

The inability of Bagel4 to predict bacteriocin from the species of *W. cibaria* might boils down to the species that harboured unique bacteriocin gene motifs does not have any similarity with the existing bacteria from other genera. Weissellicin 110 is the only bacteriocin representing *W. cibaria* with no similarity with any bacteriocin reported (Li *et al.*, 2017). The work of Li *et al.*, (2017) also showed genetic operon of Weissellicin 110 consist of context gene that has high similarity with other bacteriocin producers. This is a common observation for any bacteriocin producing strain which is the reason the prediction approach employed by this study and other bacteriocin prediction study such as McClerren *et al.*, (2006) and Begley *et al.*, (2009) utilize the similarity of context gene as a way to predict bacteriocin gene.

3.5.1.2 NeuBi prediction

The absence of bacteriocin predicted by Bagel4 resorted to the utilization of different and newly developed prediction tool. Bacteriocin Operon Associator (BOA), a bacteriocin prediction tool which was developed to target bacteriocin gene that is being unidentified due to a gene having unconserved gene motif and less similarity with other bacteriocin groups. These are the reason for the development of BOA as highlighted by Morton *et al.*, (2015). NeuBi has been developed to address the limitation of certain bacteriocin that does not have similarity with other bacteriocin genes which leads to many potential putative bacteriocin genes goes undetected which may be the case for Weissellicin 110 for Bagel4 prediction. NeuBi was developed with the notion to differentiate between bacteriocin and non-bacteriocin sequences using a deep recurrent Neural Network (RNN). Thus, NeuBi allows for a potential bacteriocin with low similarity with other bacteriocins to be detected and the prediction will be further verified with the presence of highly conserved context genes (Hamid & Friedberg, 2019*a*).

In this study, a bacteriocin candidate gene, WC_2064, did not have any hits with the context-gene database set up in this study. The presence of a double glycine-motif in WC_2064 indicated that it is a bacteriocin and its precursor peptide may be cleaved by ABC transporter upon maturation (Chatterjee & Raichaudhuri, 2017). WC_2064 to have immunity genes located at the upstream of the genes. These immunity genes were predicted to be teichoic acid glycosylation protein and were homologous to Leucoccin B immunity protein with an e-value of 3.00E-6 and with the percentage of similarity of 28.16%. Nonetheless, the presence of context genes around WC_2064 such as ABC transporter and immunity genes still carry a positive weight on indicating the possibility of these sequences are bacteriocin gene (table 3.1). However, a close inspection to the nearby ABC transporter adjacent to WC_2064, the transporter does not contain any C39 peptidase domain that functions specifically to process protein sequence with double glycine motif. This finding

does not justify the claim that bacteriocin candidate gene WC_2064 is bacteriocin with double glycine motif as the C39 peptidase domain is a well-characterized domain that specifically processed bacteriocin that possessed double glycine secretion signal (Dirix *et al.*, 2004). Nevertheless, work is done by Kjos *et al.*, (2010) described an *in silico* approach to discover putative bacteriocin through screening a bacteriocin immunity gene called Abi protein. The author listed several criteria for a sequence to be classified of potential bacteriocins such as length, containing N terminal secretion signal (double glycine motif), cationic protein sequence and containing related transport and immunity gene. No specific requirement for the ABC transporter was listed. Uniquely, the work also combined a successful heterologous expression study by assessing antimicrobial activity of the predicted genes. Perhaps the predicted bacteriocin gene may be part of different kind of bacteriocin system which little knowledge is known of especially with the case of *W. cibaria*. The way forward to further examine this is through heterologous expression study to further understand the function and interaction between these genes as demonstrated by Kjos *et al*., (2010).

Overall, this finding requires further validation from the experimental approach. Heterologous expression of the predicted operon in this study will strengthen the findings in this chapter such as providing further elucidation on the relationship of the context genes and the bacteriocin gene. This is crucial as the prediction of NeuBi presented almost 12 genes with a score higher than 0.95 but failed to be selected as the genes in the vicinity does not add up to the complete set of context genes. An interesting observation to be mentioned here that WC_1819 gene with NeuBi score of 0.98, does not have an immunity gene nearby to it despite having other types of context gene.

3.5.2 Probiotic assessment of *W. cibaria* NM1 through genome screening

Probiotic defined by Food and Agriculture Organization as live microorganism which when administered in sufficient amounts convene a health value to the host (Bielecka, 2006). Probiotics have several health-promoting effects by influencing gut microbiota and also improves intestinal immune response (Hemarajata & Versalovic, 2013). However, probiotic effects are varying from one strain to another and an in-depth investigation is needed to ensure the probiotic candidate does not harbour any ill effect toward the host. This part of the study utilizes a genome-centric strategy to assess *W. cibaria* NM1 for probiotic potential. The genome of *W. cibaria* NM1 was mined for genetic determinant that prelude to the capability on three important criteria which are survivability in the gut, not harbouring any virulence and antibiotic resistance and produce a beneficial property. *W. cibaria* has been studied of its probiotic capabilities in recent years (Lee *et al.*, 2012; Kang *et al.*, 2019; Kumari *et al.*, 2020).

This study presents the *W. cibaria* NM1 harbours gene that contributes for tolerance toward acid and bile such as the various subunit of FoF1 ATP synthase as remarked by Cotter & Hill, (2003) which are one of the contributing factors of acid tolerance capability. Clp proteases found the genome was reported by Whitehead *et al.*, (2008) contributes to bile tolerance through mutation study where mutant without the Clp protease unable to tolerate bile stress. Acid and bile tolerance are the criteria required for any probiotic to be able to survive and adapt during the transit through the gastrointestinal tract. The presence of the genes that contributed to these tolerances are in accordance to a finding done in a different study where *W. cibaria* NM1 strain have considerable tolerance toward bile salt at percentage 0.3% and acid tolerance at pH 3. The growth of this strain at pH 2 shown reduction after 1 hour of incubation but remained constant until 24 hours which suggest the acidic tolerance that related to the presence of acid tolerance presented in table 3.2 (Amalina, 2017). The result from this unpublished work tally with the result of Lee *et al.*,

(2012) that studied the capability of multiple Weissella strain such as *W. confusa* and *W. cibaria* exhibiting probiotic traits (Lee et al., 2012).

All the genes related to adhesion found in *W. cibaria* CH2 were also found harboured in the genome such as Mucus binding domain protein and fibronectin-binding protein. The work of Kumari *et al.*, (2020) also included *in-vitro* assay to determine the adhesion ability of CH2 strain which validate its adhesion ability. Adhesion characteristic of the probiotic is crucial for the increased gut residence time which helps in the colonization of the intestine and competitive exclusion of the pathogen (Collins *et al.*, 1998). In term of exhibiting beneficial effect, *W. cibaria* NM1 was found to harbour a Catabolite control protein A gene which was demonstrated by Lee *et al.*, (2010) to contribute to cholesterol reduction. The study was done through a random mutagenesis approach and further verified through *in vivo* approach where the rats were reduced of its total serum cholesterol by 20%. Recent work of Lakra *et al.*, (2020) demonstrated both *W. cibaria* MD1 and MD2 extracted from fermented batter exhibited a cholesterol-reducing ability of 67.11% and 78%, respectively. No virulence gene and antibiotic resistance gene indicates *W. cibaria* NM1 through RGI analysis suggest this strain to be safe for probiotic application. This finding is also in accordance with the safety assessment of *W. cibaria* CH2 through genome screening of antibiotic resistance (Kumari *et al.*, 2020).

3.6 Conclusion

In silico bacteriocin prediction of *W. cibaria* NM1 using NeuBi suggests the genome harboured a bacteriocin operon consists of potential bacteriocin gene with other genes that are related to the bacteriocin such as transportation, immunity, modification and regulatory. The *W. cibaria* NM1 also was shown to possessed adaptive capability in surviving the GIT and does not harbour any virulence and antibiotic resistance gene that will harm the host. Moreover, *W. cibaria* NM1 also exhibited beneficial effects such as the ability to reduce cholesterol through the presence of Catabolite control protein A gene and the potential production of bacteriocins. Hence, this chapter presented *W. cibaria* NM1 to be a bacteriocin producer as well as a suitable probiotic candidate.

Chapter 4: Assessment of crude bacteriocin in combination with antibiotics against the planktonic cells of *P. aeruginosa* ATCC 10145

4.1 Introduction

Combination of different classes of antibiotics dates back to 40 years ago as one of the strategies to prevent and delay the emergence of antibiotic resistance. (Eliopoulos & Eliopoulos, 1988; Cassir *et al.*, 2014). In this strategy, a combination of two different classes of antibiotics will be prescribed for the patient and will be continued with a specific type of antibiotic once the strain's antibiotic-resistant profile has been determined (Leekha *et al.*, 2011).

Ciprofloxacin, an effective antibiotic in the treatment of *P. aeruginosa's* infection as included in the WHO's list of essential medicine. It is the most widely used antibiotic for this bacterium, but data has shown across the years that the antibiotic efficacy is slowly reducing with the emergence of Ciprofloxacin resistant *P. aeruginosa* and other bacterial species as well from the clinical isolates. A study was done in 2018 on cancer patients showed that more than 89.3% of isolates conferred resistance to Ciprofloxacin with MIC higher than 32 mg/L (Hamed *et al.*, 2018). Ciprofloxacin resistance of *P. aeruginosa* that were isolated from wounds, catheters, blood, faeces, urine and sputum of hospitalized patients in burn wards between 2007-2014 was ranging from 14.3%-71.2% (Dou *et al.*, 2017). Hence this leads to the motivation to investigate the possibility of using bacteriocin and in combination with antibiotics to enhance the killing effect and reverse the antibiotic resistance.

Bacteriocins are antimicrobial peptides produced by bacteria such as *Lactococcus, Pediococcus, Lactobacillus* and other members of LAB. Bacteriocins have many properties that suggest that they are viable alternatives to antibiotics. These include their potency against clinically important pathogens, low toxicity for the treated host, and a broad and narrow spectrum of antimicrobial activity (Cotter *et al*., 2013). Recently, several studies have assessed the combination of bacteriocin such as Nisin with antibiotics to

enhance the antimicrobial potency of the antibiotics that are known to be ineffective and irrelevant to be used (Tong *et al.*, 2014; Chi & Holo, 2018). Hence, the motivation behind the study is to unveil the antipseudomonal potency of crude bacteriocins produced by three selected LAB and their killing efficiency when combined with antibiotics.

This chapter started with the preparation of crude bacteriocins produced by (*W. cibaria* NM1, *L. garviae* NM2 and *Pd. acidiclactici* NM3), followed by the selection of antibiotics based on sensitivity towards *P. aeruginosa* ATCC 10145. Then the Minimum Inhibition Concentration (MIC) of the selected antibiotics and bacteriocins against planktonic cells of *P. aeruginosa* was conducted. The checkerboard method was used to determine the synergistic interaction between bacteriocins and antibiotics. Time-kill assay was used to demonstrate the bactericidal activity of the combinations versus each antimicrobial alone.

4.2 Objectives

This chapter aimed to investigate the combined inhibition of crude bacteriocins produced by three LAB and antibiotics against the planktonic cells of *P. aeruginosa* ATCC 10145.

The specific objectives were:

- To determine the MIC of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145
- To evaluate the antagonism interaction between the combined treatment of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145 via the Checkerboard method
- To determine the killing kinetics of crude bacteriocins and antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145

4.3 Materials and method

4.3.1 Bacteria culture maintenance

Three LAB namely *W. cibaria* NM1, *L. garviae* NM2 and *Pd. acidilatici* NM3 were used in this study. *W. cibaria* NM1 and *L. garviae* NM2 were isolated from Siakap fish gut by Dr Ivan Chiew Kar Mun. *Pd. acidilactici* NM3 was isolated from fermented soybean curd by Mr Jason Chen Kok Ho. These isolates were definitively confirmed using 16S rDNA sequences and deposited at the Microbial Culture Collection Unit (UNICC), Institute of Bioscience, University Putra Malaysia. *P. aeruginosa* ATCC 10145 was purchased from American Type Culture Collection Centre and contributed by the Laboratory of Microbiology, University of Nottingham Malaysia. *P. aeruginosa* ATCC 10145 was used as the reference strain in this study. All LAB strains were maintained in De Man, Rogosa and Sharpe medium (MRS; Merck, Germany), while the indicator strain, *P. aeruginosa* ATCC 10145 was maintained in Brain Heart Infusion medium (BHI; Merck, Germany). Cultures were propagated twice in respective broth medium for 24 h at 37 ^{o}C or cultured on respective agar medium and incubated for 2 days at 37 ^{o}C. Cultures were maintained as frozen stock in respective broth medium at -20 ^{o}C supplemented with 20 % (v/v) of sterile glycerol till used.

4.3.2 16S rDNA sequencing and Phylogenetic tree analysis of the LAB

The DNA extraction was done following the previously described in section 3.3.2. PCR amplification of 16s rDNA was done and details on the procedures are listed in Appendix A. The PCR products were sent for sequencing via services from 1st BASE DNA Sequencing services (Selangor, Malaysia). The obtained 16s rDNA sequences for the LAB were searched against the 16s rDNA database using BLAST standalone tool (Camacho *et al.*, 2009). The 16s rDNA sequences were listed in Appendix B. The phylogenetic tree was constructed as described in section 3.3.3 and the constructed trees are placed in Appendix C.

4.3.3 Production of crude bacteriocins

In this assay, 1% (v/v) of 24h old LAB strains (8.78-9.05 Log_{10} CFU/mL) were respectively grown in 10mL MRS broth for 24 h at 37 °C. After incubation, the culture was centrifuged at 9600 rpm for 20 min. The cell-free supernatant (CFS) was withdrawn, neutralized to pH 6.5 using 1M NaOH to eliminate the inhibitory effect due to acid production, and then filter sterilized with a 0.45µm syringe filter (Minisart, Sigma Aldrich, United States). The neutralized and filtrated CFS was then stored in a -80 ^{o}C freezer for 24 h and then subjected to freeze-drying using a Freeze-Dryer (Christ Alpha 1-2 Ldplus, Denmark) for 72 h. The freeze-dried CFS powder was designated as "crude bacteriocin". A stock solution of 1g of crude bacteriocin dissolved in 1 mL of sterile water was prepared freshly before broth microdilution assay, Checkerboard assay, and Time-kill assay. Crude bacteriocins produced by *W. cibaria* NM1, *L. garvieae* NM2 and *Pd. acidilactici* NM3 were designated as WA, LG and PA, respectively.

4.3.4 Quantification of bacteriocin inhibitory activity

The bacteriocin inhibitory activity of the neutralized filtrated CFS and crude bacteriocin were quantified using the modified method of Todorov & Dicks, (2005). In this assay, a two-fold serial dilution (2^1 - 2^5) of bacteriocin was carried out with 0.85% (w/v) NaCl. The diluted bacteriocins were pipetted respectively into pre-punched agar wells on BHI agar. The BHI agar plate was then swabbed with 100ul (8.9 Log_{10} CFU/mL) of *P. aeruginosa* ATCC 10145 and incubated at 37^{o}C for 24h. Bacteriocin activity was calculated from the reciprocal of the highest dilution yielding a clear zone of growth inhibition on the indicator lawn according to the formula below:

$$\text{Bacteriocin activity} = \frac{\text{Highest dilution yield a clear zone (AU)}}{\text{Amount of mixture apply to each well (ml)}} \times \text{Diameter of clear zone (cm)}$$

$$= \text{AU.cm.ml}^{-1}$$

4.3.5 Antibiotic susceptibility test

Antibiotic susceptibility of the *P. aeruginosa* ATCC 10145 was determined by the disk diffusion method or Kirby Bauer test (Ahmed *et al.*, 2013). 1% (v/v) of 24h-old *P. aeruginosa* ATCC 10145 was inoculated into 10 mL BHI broth for 24 h at 37 °C. After incubation, 100 µL (8.9 Log_{10} CFU/mL) of *P. aeruginosa* was swabbed on BHI agar plate. Then seven antibiotic discs (Sigma Aldrich, Germany), namely Amoxicillin (10 µg), Chloramphenicol (30 µg), Gentamicin (10 µg), Tetracycline (30 µg), Sulfamethazine (25 µg), Ciprofloxacin (5 µg) and Cephalexin (30 µg) were placed on the BHI agar plate. The plate was then incubated at 37 °C for 24 h. After incubation, the formation of a clear inhibition zone was observed and the diameter (mm) of the zone was measured. The susceptibility of the *P. aeruginosa* to the antibiotic tested was based on the diameter of the inhibition zone, whereas the absence of zone represents the resistance of *P. aeruginosa* towards the antibiotic tested.

4.3.6 Preparation of antibiotic stock solution

Based on the results from the antibiotic susceptibility test (Section 4.4.2), three antibiotics were selected for subsequent assays. The antibiotic Chloramphenicol (CHL), Tetracycline (TET) and Ciprofloxacin (CIP) (Sigma Aldrich, Germany) were prepared for a stock concentration of 1.28 mg/mL according to the manufacturer's protocol. The stock solutions were kept at -20 ℃ until used. CIP was selected as it is one of the potent antibiotics against *P. aeruginosa* while CHL and TET are the antibiotics that are ineffective due to intrinsic resistance of the pathogen.

4.3.7 Broth microdilution assay

Determination of Minimum Inhibition Concentration (MIC) of crude bacteriocins and antibiotics were performed using broth microdilution assay according to CLSI guidelines (Subramaniam & Nandan, 2011). In this assay, 1% (v/v) of 24h-old *P. aeruginosa* ATCC 10145 was grown in Mueller Hinton (MH)

broth for 24 h at 37°C. After incubation, the absorbance of the bacterial cell suspension was measured at OD600nm wavelength. The bacterial cell suspension was diluted with MH broth to achieve absorbance of 0.10 which corresponds to a viable cell count of 1 x10^8 CFU/ml. Further dilution was made using the ratio of 1:100 to obtain the final viable cell count of 1 x10^6 CFU/ml.

A two-fold serial dilution of the crude bacteriocins and antibiotics was carried out using MH broth in 96 well microtiter plates (SPL, Korea). After dilution, each well contained 50 µl of diluted crude bacteriocins with final concentrations ranging from 0.98-500 mg/mL, and 50 µl of diluted antibiotics ranging from 0.00025-0.128 mg/mL, respectively. The range for the MIC range was chosen based on the known MIC for *P. aeruginosa* in CLSI guideline and taking into consideration the number of well present per each column in the 96 well plates used. Then, 50 µl of the diluted cell bacterial suspension was added into each well. In this assay, the well with solely bacterial cell suspension served as a positive control, while the well with solely MH broth served as a negative control.

The microtiter plates were incubated at 37°C for 24 h and the absorbance at OD600 nm wavelength was measure using a microtiter plate reader (BioTek, United States). The absorbance values were recorded before and after incubation. MIC was read as the concentration of antimicrobials (crude bacteriocins/antibiotics) producing complete inhibition on the visible growth of the indicator *P. aeruginosa* ATCC 10145. This assay was carried out in three independent replicates.

4.3.8 Checkerboard assay

A checkerboard assay was performed to detect the simultaneous, indifference and synergism interaction between crude bacteriocins and antibiotics (Basri *et al.*, 2014). The bacterial cell suspension of *P. aeruginosa* ATCC 10145 is prepared as described in Section 4.3.7 This assay comprises 9 combinations of bacteriocin and antibiotics (WC-CIP, WC-CHL, WC-TET, LG-CIP, LG-CHL, LG-TET, PA-CIP, PA-CHL and PA-TET). The combinations will be tested twice where one of the pairs for each combination will have a fixed volume while the other will antimicrobial will have varying concentrations which were achieved through two-fold dilutions 9 times. The assay was performed in such a way that each column of a 96-well microtiter plate contained a 25 µl of fixed and final concentration (0.25x MIC) of bacteriocins/antibiotics and 25 µL of two-fold serially diluted bacteriocin/antibiotics.

The combination with a fixed final concentration of all crude bacteriocin and varying concentration of antibiotics will consist of 31.25 mg/mL for all of the crude bacteriocin and the concentration of antibiotics ranging from 0.004 -2.00 µg/mL for CIP, 0.25-128 µg/mL for CHL, and 0.125-64 µg/mL for TET. Meanwhile, the combination with a fixed final concentration of antibiotics and varying concentrations of crude bacteriocin will consist of a fixed concentration of 0.25 µg/mL for CIP, 16 µg/mL for CHL and 8 µg/mL for TET with varying crude bacteriocin ranging from 0.25-125 mg/mL. Positive control of this assay is the well with only bacterial suspension and negative control is the well contained only broth. Then, 50 µl of the bacterial cell suspension was added into each well. In this assay, the well with solely bacterial cell suspension served as the positive control, while the well with solely MH broth served as the negative control. The microtiter plates were incubated at 37°C for 24 h and the absorbance at OD600nm wavelength was measure using a microtiter plate reader (BioTek, United States). The absorbance values were recorded before and after incubation. MIC of each combination was recorded as described in Section 4.3.7. Fractional inhibitory concentration index (FICI) of each

combination of crude bacteriocins and conventional antibiotics are calculated based on the formula below:

$$\text{FIC index} = \frac{\text{MIC combination A}}{\text{MIC of A alone}} + \frac{\text{MIC combination B}}{\text{MIC of B alone}}$$

The combination of antimicrobials is considered:
"Synergistic" if the FICI is ≤0.5; "Partial synergistic" if FICI is > 0.5 < 1.0; "Addition" if the FICI is 1.0; "Indifference" if FICI is > 1 < 4; and "Antagonistic" if the FICI is > 4.

All assays were carried out in three independent replicates.

4.3.9 Time-kill assay

Time-kill assay was performed to evaluate the killing rate of the single and combined treatment of crude bacteriocins (WC, LG and PA) and antibiotics (CIP, CHL and TET) against planktonic cells of *P. aeruginosa* ATCC 10145. A total of 9 combinations of bacteriocins and antibiotics were tested in this study. The bacterial cell suspension of *P. aeruginosa* ATCC 10145 is prepared as described in Section 4.3.7. A microtitre 96-well plate is prepared where the wells are filled with a single treatment of crude bacteriocin or antibiotics or filled with the combination of both crude bacteriocin and antibiotics with a final concentration of 1X MIC for each antimicrobial agent. Then, 50 µl of the diluted cell bacterial suspension was added into each well that was filled with combined treatment of crude bacteriocin and antibiotic. The plates were then incubated at 37℃ for 24 h. For every time intervals (0, 1, 2, 4, 6, 8, 10, 24 h), aliquots of 30 µl of samples were withdrawn and ten-fold serially diluted with peptone buffered water ($10^0 – 10^4$). Then, 30 µl of each dilution were plated on MH agar and incubated at 37℃ for 24 h. The colonies formed were counted and expressed as Colony Forming Unit per millilitre (CFU/mL) of the sample by using the viable plate count method (Pankuch *et al.*, 1994). This assay is the

sample that only consists of *P. aeruginosa* ATCC 10145 and negative control is the well containing MH broth only. The time-kill curve was plotted using the viable plate count (Log_{10} CFU/mL) versus incubation duration (h). Positive control for this assay is untreated treatment with only *P. aeruginosa* ATCC 10145 while negative control for this assay is well containing MH broth.

Synergistic interaction was defined as more than 2 log_{10} decreases in CFU/mL reduction in colony count within a specific period over 24 h between the combination and the most active agent. Additive or indifference was defined as less than 2 log_{10} decreases in CFU/mL reduction in colony count within a specific period over 24 h between the combination and the most active agent. Antagonism interaction was defined as more than 2 log_{10} increase in CFU/mL reduction in colony count within a specific period over 24 h between the combination and the most active agent (Lee *et al.*, 2017).

The activity of the antimicrobial agent is considered bactericidal if the reduction of total plate count is more than 3 log_{10} CFU/mL and considered bacteriostatic if the reduction of total plate count is less than 3 Log_{10} CFU/mL when compared with the starting inoculum (Basri *et al.*, 2014). All assays were carried out in three independent replicates.

4.3.10 Statistical analysis

Data were analyzed using IBM SPSS Statistic version 24.0 (SPSS Inc, Chicago, IL, USA). All the test was done in triplicate. A paired T-test was used to compare the bacteriocin activity of crude bacteriocins and CFS. All assays were conducted in three independent replicates.

4.4 Results

4.4.1 Antipseudomonal potency of CFSs and crude bacteriocins of LAB

In this chapter, the antipseudomonal potency of CFSs and crude bacteriocins produced by *W. cibaria* NM1, *L. garviae* NM2 and *Pd. acidiclactici* NM3 were determined. The CFSs of these LAB were neutralized to eliminate the inhibitory effect due to acid and filter sterilized with a 0.45µm syringe filter. The CFSs were then concentrated via the freeze-drying technique. The freeze-dried CFSs were termed as crude bacteriocins and were used throughout this study. A comparison of the antipseudomonal potency of CFSs and crude bacteriocins (freeze-dried CFSs) was shown in Table 4.1. The freeze-drying technique had significantly enhanced the antipseudomonal potency of crude bacteriocin with more than 14.93 times of increment in bacteriocin activity. All the crude bacteriocins and CFS are not significant with one another.

Table 4.1: Antipseudomonal potency of CFSs and crude bacteriocins of LAB

LAB	Bacteriocin activity (AU.cm.ml^{-1}) against *P. aeruginosa* ATCC 10145	
	CFS	Crude bacteriocin
W. cibaria NM1	73.33 ± 11.57$^{A;b}$	1373.33 ± 244.00$^{A;a}$
L. garviae NM2	100.00 ± 40.00$^{A;b}$	1493.00 ± 184.00$^{A;a}$
Pd. acidiclactic NM3	40.00 ± 20.00$^{A;b}$	1120.00 ± 160.00$^{A;a}$

Notes:

The values are mean with standard deviation (n = 3).

^{ab} Different superscripts within a row denote values that are significantly different at $P < 0.05$.

^{AB} Different superscripts within a column denote values that are significantly different at $P < 0.05$.

4.4.2 Antibiotics susceptibility of *P. aeruginosa* ATCC 10145

Table 4.2 shows the inhibitory effect of seven antibiotics on *P. aeruginosa* ATCC 10145 via the disc diffusion method. *P. aeruginosa* ATCC 10145 was resistant to five out of the seven antibiotics tested and only susceptible to CIP (5 µg) and partially susceptible to gentamicin (10 µg). The inhibitory activity of CIP was at least 2-fold higher than gentamicin and with 2-fold lesser dosage used than gentamicin. The inhibition zone's diameter breakpoint for CIP falls under the range of susceptible (> 21 mm), while gentamicin falls under the range of intermediate (13-14 mm). All the other antibiotics are remarked to be intrinsically resistant by *P. aeruginosa* hence, no value of susceptibility testing is required.

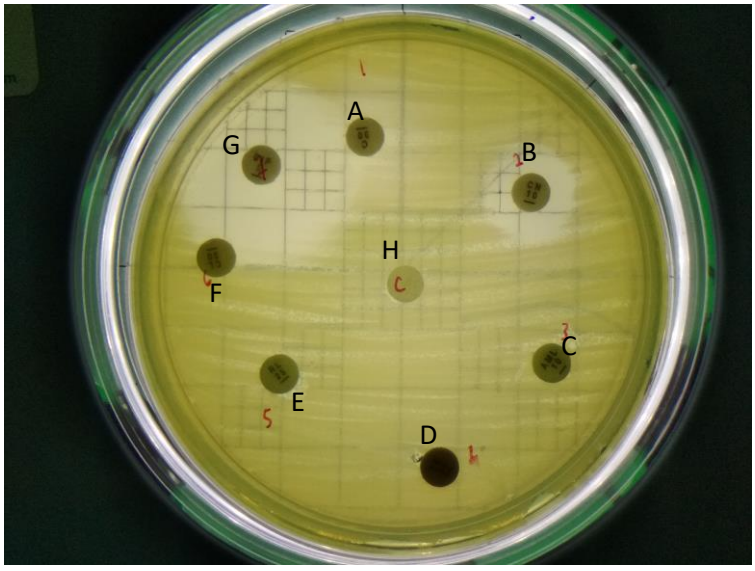

Figure 4.1: Antibiotic susceptibility test of Pseudomonas aeruginosa ATCC 10145 using Disc diffusion method. Disks labelled A until H are Amoxicillin, Gentamicin, Tetracycline, Cephalexin, Sulfamethoxazole, Chloramphenicol, Ciprofloxacin and Control respectively

Table 4.2*:* Antibiotic susceptibility of *P. aeruginosa* ATCC 10145 via disc diffusion method

Antibiotics	Concentration (µg)	Diameter of inhibition zone (mm)	Antibiotic susceptibility of P. aeruginosa ATCC 10145 (Resistant/Intermediate/Susceptible)
Chloramphenicol	30	0.00	Resistant
Gentamicin	10	13.30 ± 1.15	Intermediate
Amoxicillin	10	0.00	Resistant
Tetracycline	30	0.00	Resistant
Sulfamethoxazole	25	0.00	Resistant
Cephalexin	30	0.00	Resistant
Ciprofloxacin	5	32.00 ± 0.00	Susceptible

Notes:

Antibiotic susceptibility is based on CLSI guidelines (Subramaniam & Nandan, 2011).

The current study aimed to assess the antipseudomonal potency of crude bacteriocins when combined with antibiotics to improve the efficacy of conventional antibiotics that are known to be ineffective to be used. Hence based on Table 2 and figure 4.1, two antibiotics CHL and TET that were ineffective to *P. aeruginosa* ATCC 10145 were selected for subsequent assays. Concurrently, CIP that is with antipseudomonal potency was also selected to investigate if there is any enhancement with regards to the antipseudomonal potency through a combination of both CIP and the crude bacteriocins.

4.4.3 Minimum inhibition concentration of the crude bacteriocins and antibiotics

Table 4.3 shows the MIC of crude bacteriocins and selected antibiotics needed to inhibit *P. aeruginosa* ATCC 10145 by using the broth microdilution method. CIP is most effective in inhibiting *P. aeruginosa* ATCC 10145. Both CHL and TET are ineffective to *P. aeruginosa* as it is intrinsically resistant toward the antibiotics. The minimum concentration of TET and CHL are 32 and 64 µg/ml in broth microdilution assay, respectively. The MIC of broth microdilution based on the CLSI standard for CIP and TET are 0.25-1 µg/ml and 8-32 µg/ml while CHL is not provided. The MIC obtained through this study is in accordance with the breakpoint range provided by CLSI (Subramaniam & Nandan, 2011) where CIP reported MIC of 1 µg/ml and TET reported MIC of 32 µg/ml.

Table 4.3: Minimum inhibition concentration of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145

Antimicrobial agent	MIC
Crude bacteriocins	
WC	62.5 ± 0.0 mg/ml
LG	62.5 ± 0.0 mg/ml
PA	62.5 ± 0.0 mg/ml
Antibiotics	
Chloramphenicol	64.0 ± 0.0 µg/ml
Ciprofloxacin	1.0 ± 0.0 µg/ml
Tetracycline	32.0 ± 0.0 µg/ml

4.4.4 interaction of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145 via Checkerboard method

A checkerboard method was performed to determine whether a bacteriocin can work synergistically with an antibiotic against *P. aeruginosa* ATCC 10145. A total of 9 combinations of bacteriocins and antibiotics were tested in this study and the FIC indexes were shown in Table 4.4. The FIC index is obtained through the ratio of a minimum inhibitory concentration of combined treatment with a single treatment. The FIC indexes of 0.258 and 0.375 confirmed that a combination of respective crude bacteriocins WC, LG and PA with antibiotics CIP, CHL and TET exhibited a synergistic effect against *P. aeruginosa* ATCC 10145.

As shown in Table 4.4, the MIC of crude bacteriocins was reduced by 4 times in the combined treatment with antibiotics. While the MIC of CIP, CHL and TET were reduced by 128, 0.8 and 12.8 times respectively in the combined treatment against *P. aeruginosa* ATCC 10145. Current findings indicated that crude bacteriocins can enhance antibiotic efficacy against *P. aeruginosa* ATCC 10145.

Table 4.4: Interaction between crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145 via Checkerboard method

Combination	Antimicrobial agents	MIC (mg/mL) for crude bacteriocin / (µg/mL) for antibiotic		FIC		Interaction
		Single	Combination	Individual	Index	
1	WC	62.5	15.625	0.25	0.258	Synergistic
	CIP	1.00	0.0078	0.008		
2	WC	62.50	15.625	0.25	0.375	Synergistic
	CHL	64.00	8.00	0.125		
3	WC	62.50	15.625	0.25	0.328	Synergistic
	TET	32.00	0.250	0.078		
4	PA	62.50	15.625	0.25	0.258	Synergistic
	CIP	1.00	0.0078	0.008		
5	PA	62.50	15.625	0.25	0.375	Synergistic
	CHL	64.00	8.00	0.125		
6	PA	62.50	15.625	0.25	0.328	Synergistic
	TET	32.00	0.250	0.078		
7	LG	62.50	15.625	0.25	0.258	Synergistic
	CIP	1.00	0.0078	0.008		
8	LG	62.50	15.625	0.25	0.375	Synergistic
	CHL	64.00	8.00	0.125		
9	LG	62.50	15.625	0.25	0.328	Synergistic
	TET	32.00	0.250	0.078		

Notes: The combination of antimicrobials is considered “Synergistic” if the FIC index (FICI) is ≤0.5; “Partial synergistic” if FICI is > 0.5 < 1.0; “Addition” if the FICI is 1.0; “Indifference” if FICI is > 1 < 4; and “Antagonistic” if the FICI is > 4.

4.4.5 Killing rate of crude bacteriocins and antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145

In a further attempt to confirm that crude bacteriocins can enhance antibiotic efficacy, time-kill assays were performed to establish the effect of crude bacteriocin and antibiotic alone or in combination on *P. aeruginosa* ATCC 10145 planktonic cell numbers following 0, 1, 2, 4, 6, 8, 10 and 24 h treatment periods (Figure 4.2– 4.4). The antipseudomonal effect of WC-CHL is comparable with CIP alone, and successfully reduced the *P. aeruginosa* ATCC 10145 planktonic cell number to an undetectable level after 6h of treatment. This combination has the most rapid killing effect compared to other treatment. Followed by WC alone that inhibited *P. aeruginosa* ATCC 10145 after 8h of treatment together with PA, PA-CHL, PA-TET and WC-TET. LG and all the combinations of crude bacteriocin with CIP able to reduce the cell numbers to an undetectable level after 24h. Current findings suggested that WC and PA are potent antipseudomonal agents and when combined with CHL and TET can enhance the antipseudomonal potency

Figure 4.2A-4.2C showed that CHL alone could reduce the growth of *P. aeruginosa* ATCC 10145 by 48% up until 10 h of the treatment and the cell count continue to increase after 24 h of the treatment. Compared to CHL, all three crude bacteriocins alone were able to inhibit the *P. aeruginosa* to an undetectable level within 6-8h of treatment, with WC-CHL being the most potent antimicrobial agent. All combinations of crude bacteriocin with CHL have stronger antipseudomonal potency than its single counterpart except for PA-CHL which both PA and PA-CHL similarly managed to inhibit the cells to an undetectable level after 8h (Figure 4.2C). The combination successfully inhibits the *P. aeruginosa* to an undetectable level within 6h (WC-CHL) and 8h (PA-CHL and LG-CHL), respectively. WC-CHL and LG-CHL showed synergistic interaction through more than 2 Log_{10} decreases in CFU/mL between the combination and the most active single agent at 6h and 8h respectively. Meanwhile, PA-CHL showed indifference or additive which is justified through less than 2 Log_{10}

decreases in CFU/mL between the combination and the most active single agent at any specific time point throughout 24 h incubation.

TET on the other hand shows the least potent antipseudomonal activity than CHL (Figure 4.3A-4.3C). TET alone could reduce the growth of *P. aeruginosa* ATCC 10145 by 26% up until 8 h of the treatment and the cell count continue to increase after 24 h of the treatment. All crude bacteriocin and its combination with TET managed to inhibit the *P. aeruginosa* to an undetectable level after 8h of treatment except for LG-TET and LG which took 10h and 24h respectively. The killing pattern for the crude bacteriocin and TET are not distinguishably different except for the LG and LG-TET on the latter stage of the incubation period where LG-TET activity was stagnant (8h-10h). LG-TET showed synergistic interaction justified through more than 2 Log_{10} decreases in CFU/mL between the combination and the most active single agent at 10 h. Meanwhile, WC-TET and PA-TET showed indifference or additive which is justified through less than 2 Log_{10} decreases in CFU/mL between the combination and the most active single agent at any specific time point throughout 24 h incubation.

Unfortunately, as seen in figure 4.4A-4.4C, all crude bacteriocins in combination with CIP required 24h to inhibit *P. aeruginosa* to an undetectable level which contrasted with CIP alone that took 6h to achieve its complete inhibition effect. The killing pattern of crude combination with CIP and single crude bacteriocin was observed to be almost similar especially for LG-CIP and LG. Both WC-CIP and PA-CIP also show a slow killing effect at the latter stage of the incubation period. Interestingly, all combinations of CIP and crude bacteriocins showed antagonism interaction justified through more than 2 Log_{10} CFU/mL increase between the combination and the most active single agent at any specific time point throughout 24 h incubation.

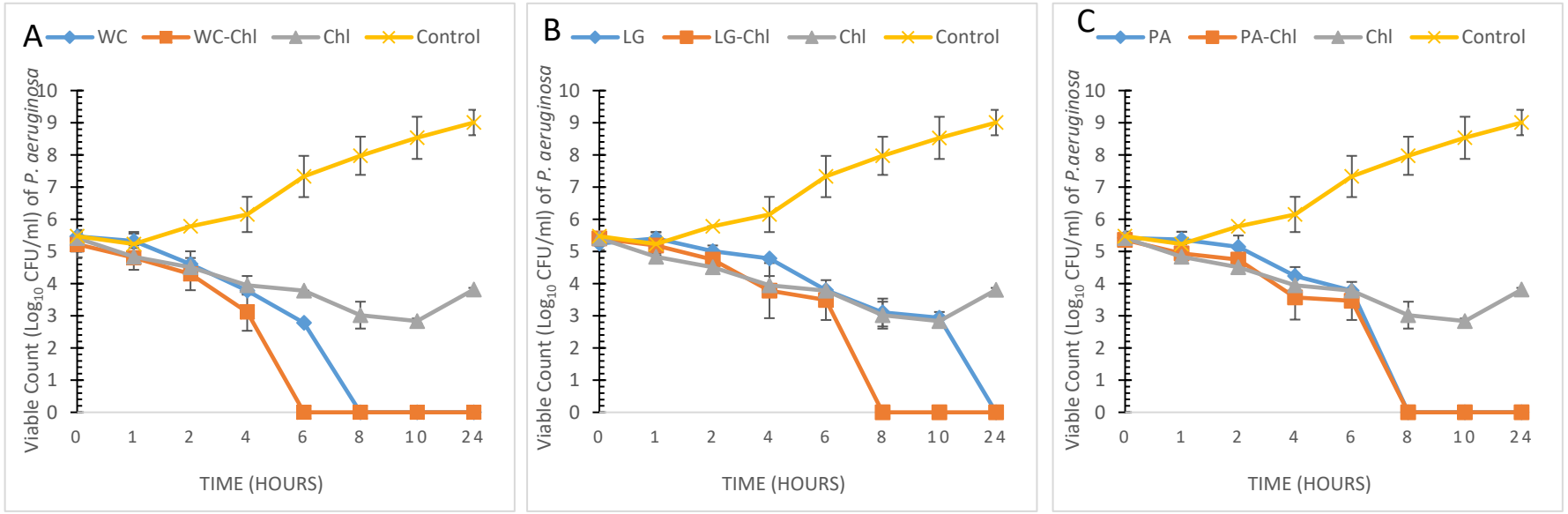

Figure 4.2: Time-kill growth curves of (a) combination of WC-CHL, WC alone, and CHL alone;(b) combination of LG-CHL, LG alone, and CHL alone; (c) combination of PA-CHL, PA alone, and CHL alone.

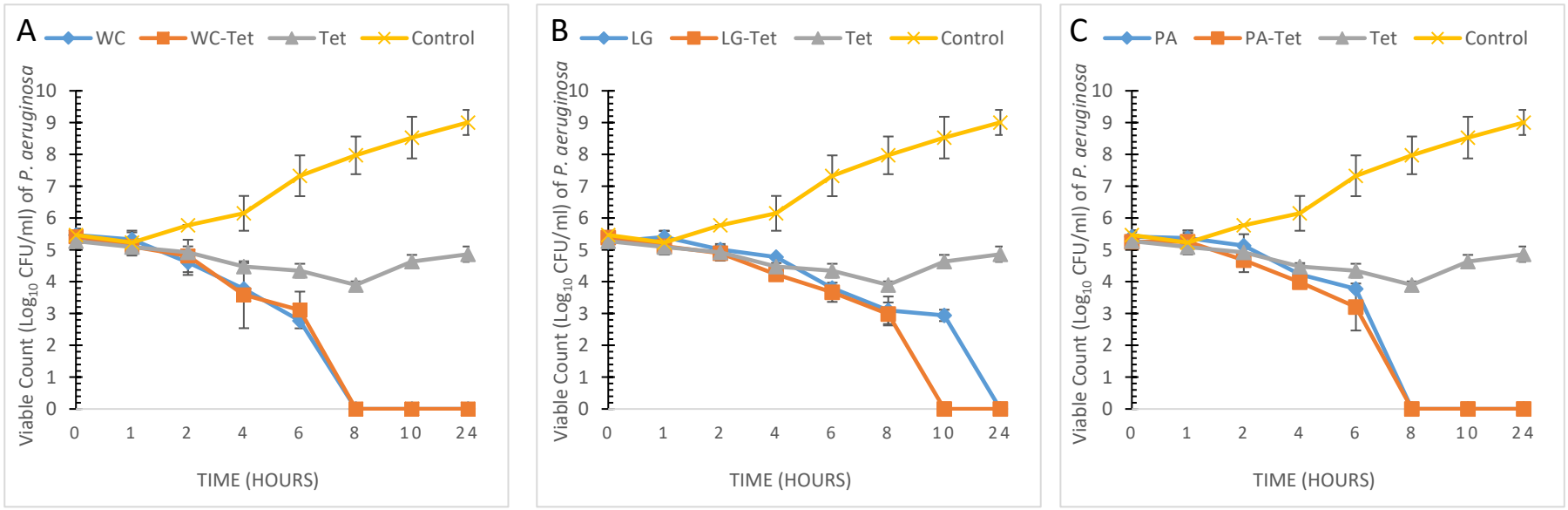

Figure 4.3: Time-kill plots of (a) combination of WC-TET, WC alone, and TET alone;(b) combination of LG-TET, LG alone, and TET alone; (c) combination of PA-TET, PA alone, and TET alone.

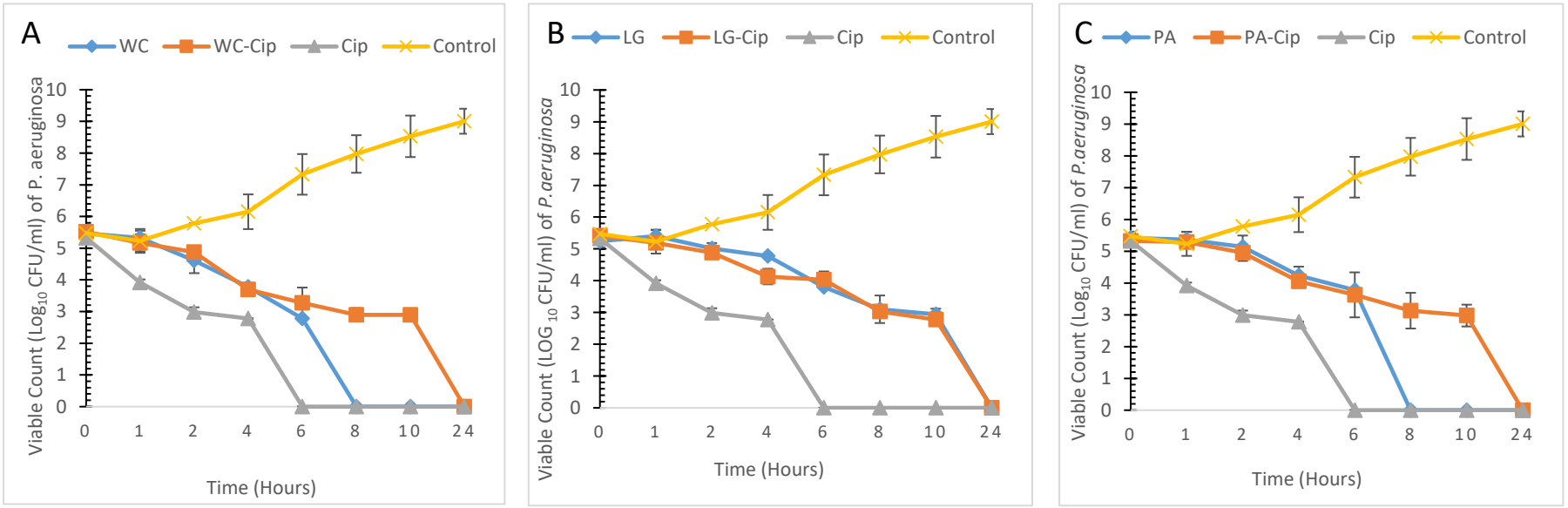

Figure 4.4: Time-kill growth curves of (a) combination of WC-CIP, WC alone, and CIP alone; (b) combination of LG-CIP, LG alone, and CIP alone; (c) combination of PA-CIP, PA alone, and CIP alone.

4.5 Discussion

This chapter depicts the combined inhibition of crude bacteriocins (WC, LG and PA) produced by three LAB and antibiotics against the planktonic cells of *P. aeruginosa* ATCC 10145. Any synergistic and antagonistic interaction of combined crude bacteriocins and antibiotics treatments were elucidated via Checkerboard assay and Time-kill assay.

4.5.1 Antipseudomonal activity of crude bacteriocins

The three crude bacteriocins (WC, LG and PA) were produced by LAB *W. cibaria* NM1, *L. garviae* NM2, and *Pd. acidilactici* NM3 have shown promising inhibitory activity against planktonic cells of *P. aeruginosa* ATCC 10145 (Table 4.1) and resulted in the MIC of 62.5 mg/ml (Table 4.2). To the author's knowledge, the published work related to the antipseudomonal effect of bacteriocins is scarce. Only the report from Lin and Pan (2019) is slightly relevant to current work. Their findings showed that the CFS sample from *Lactobacillus plantarum* NTU 102 exhibited a MIC of > 3.35 mg/ml against *P. aeruginosa and* showed a stronger antipseudomonal potency than the crude bacteriocins tested in this study.

Based on Bactibase (http://bactibase.hammamilab.org/main.php), an online bacteriocin database repositories for characterized bacteriocins, there are about seven bacteriocins that have inhibitory activity toward *P. aeruginosa* and all of these bacteriocins are produced by Gram-positive bacteria that have broad-spectrum activities (Hammami *et al.*, 2010). These bacteriocins are Thuricin S from *Bacillus thuringiensis,* Subtilosin from *Bacillus subtilis,* Enterocin AS-48 from *Enterococcus faecalis,* Bacteriocin L-1077 from *Lactobacillus salivarius,* Laterosporulin from *Brevibacillus sp.,* Plantaricin 163 from *Lactobacillus plantarum*, and Elgicins from *Paenibacillus elgii* (Galvez *et al.*,

1986; Chehimi *et al.*, 2007; Shelburne *et al.*, 2007; Svetoch *et al.*, 2011; Singh *et al.*, 2012; Teng *et al.*, 2012; Hu *et al.*, 2013).

Neutralized CFS of *W. cibaria* isolates from different food sources such as goat milk, Idli batter and Thai fermented fish were found to exhibit activity against *P. aeruginosa* through Agar well diffusion assay (Elavarasi, 2014; Deatraksa *et al.*, 2018). *W. confusa* A3, a member of the same genus as *W. cibaria,* produced a bacteriocin that possessed broad-spectrum activity and able to inhibit *P. aeruginosa* PA7 with MIC of 18.5 µg/mL. The bacteriocin which is a heat-stable bacteriocin also shown inhibition on Bacillus *cereus, E. coli*, *M. luteus*, *L. lactis* and *E. faecium* (Goh & Philip, 2015). *L. garviae* isolated from cow's milk was found to exhibit antimicrobial activity against *P. aeruginosa* (Suneel & Basappa, 2013). Lee *et al.*, (2020) demonstrated *Pd. acidilactici* HW01 produced HW01 bacteriocin that has antibiofilm activity against *P. aeruginosa* yet not against its planktonic cells. Related species of *Pd. acidilactici* such as *Pd. Pentosaceus* and *Pediococcus sp.* were reported to exhibit antipseudomonal activity (Todorov & Dicks, 2009; Sukumar & Ghosh, 2010).

Till to date, there are several published reports on the antimicrobial activity of the bacteriocins produced by *W. cibaria*, *L. garviae*, and *Pd. acidilactici.* Weissellicin 110 produced by *W. cibaria* that have a narrow inhibitory activity such as against strains from *Lactococcus* genus and related species such as *W. kandleri, W. halotolerans, W paramesenteroides* and *Leuconostoc mesenteroides* (Srionnual *et al.*, 2007). While Garvicin KS produced by *L. garvieae* exhibit broad inhibitory activity such as against *Staphylococcus aureus* and *Bacillus cereus and Escherichia coli* (Chi & Holo, 2018) Similarly, Pediocin SA-1 produced by *Pd. acidilactici* could inhibit a wide spectrum of bacteria such as *listeria monocytogenes* and other *Pd. acidilactici* strains (Anastasiadou *et al.*, 2008). Nevertheless, the study on the antipseudomonal effect of these bacteriocins is still lacking. Thus, current findings achieve an important milestone to address this research gap through an investigation of the antipseudomonal potential of bacteriocins WC, LG and PA.

4.5.2 Antibiotic susceptibility of *P. aeruginosa* ATCC 10145

Prior to Checkerboard and Time-kill assay, the antibiotic susceptibility of *P. aeruginosa* ATCC 10145 was determined via Antibiotic disc diffusion assay and MIC assay. In the antibiotic disc diffusion assay (Table 4.2), *P. aeruginosa* ATCC 10145 is intrinsically resistant to CHL, TET, Amoxicillin, Sulfamethoxazole and Cephalexin. The antibiotic resistance profile of *P. aeruginosa* ATCC 10145 is conformed with the CLSI performance standard (Subramaniam & Nandan, 2011).

In this study, only CIP and Gentamicin are the antibiotic that *P. aeruginosa* is not intrinsically resistant against. Both CIP and aminoglycosides such as gentamicin are antipseudomonal antibiotic alongside with, Ticarcillin, Ureidopenicillins, Ceftazidime, Cefepime, Aztreonam and Carbapenems (Giamarellou & Antoniadou, 2001). The susceptibility testing through disk diffusion and broth microdilution assays further verified the intrinsic resistance of *P. aeruginosa* is at play. This is achieved through the correlation of the data from this study and the report's standard. The intrinsic resistance system of *P. aeruginosa* is based on features the bacterium possessed early on and not added through gene mutation such as impermeable cell wall, efflux pump, production of an enzyme that inactivates antibiotic (Breidenstein *et al.*, 2011). Amoxicillin and Cephalexin are β-lactam groups of antibiotics are known to be inactive against *P. aeruginosa* due to the interplay of the degradation activity of β-lactamase AmpC and efflux pump action of MexAB-OprM (Masuda *et al.*, 1999). *P. aeruginosa* is also known to be resistant toward sulphonamides which is the antibiotic class that sulfamethoxazole originates from (Huovinen, 2001). MexAB-OprM has broad action in pumping out unwanted substances which plays a major role in the intrinsic resistance system of *P. aeruginosa* (Köhler *et al.*, 1996), which also responsible for the CHL and TET pumping out from *P. aeruginosa* system (Llanes *et al.*, 2004; Morita *et al.*, 2013). The finding of two different forms of susceptibility testing is in excellent agreement with CLSI breakpoints. Disc diffusion assay was carried out to perform a selection of

antibiotics to be chosen for this study while broth microdilution assay was carried out to determine the MIC for each antibiotic and crude bacteriocin used for the *P. aeruginosa* ATCC 10145.

4.5.3 Interaction of crude bacteriocins and antibiotics against *P. aeruginosa* ATCC 10145 via Checkerboard method

The combination of antimicrobial agents that have different modes of action is one of the suggested approaches to address antibiotic resistance (Mathur *et al.*, 2017). In this study, the interaction of combined treatments of crude bacteriocins and antibiotics against MDR *P. aeruginosa* was assessed via the Checkerboard method.

The fractional inhibitory concentration (FIC) index obtained from the Checkerboard method is a scale that indicates the inhibition effect of the combination of two different antimicrobial agents. A lower FIC is an indication of synergism while a high FIC will indicate an incompatible combination (Qin *et al.*, 2013). FIC aims to evaluate the MIC of an antimicrobial agent when in combination with another antimicrobial agent. This aim is based on the goal of combination strategy which is to combine the use of antibiotics at a lower concentration in combination with other antimicrobial agents without affecting the efficacy of the antibiotic and at the same time, reduce the emergence of resistance (Doern, 2014).

Nine combinations of crude bacteriocins and antibiotics were tested in this study and all combinations have FIC index below 0.5 indicating synergistic antipseudomonal potency (Table 4.4). Furthermore, the results obtained have met the goal of a combination strategy reported by Doern, (2014) n which lower MICs of crude bacteriocins and antibiotics are needed when compared to treatment with antimicrobial agents alone. In this study, treatment with CHL alone required MIC of 0.064 mg/ml to inhibit *P. aeruginosa* ATCC 10145, but when combined with crude bacteriocins, the MIC was reduced to 0.008 mg/ml. Similarly, a reduction in the MIC of crude bacteriocins was also observed (Table 4.4).

No study was found to use a similar combination of bacteriocins and antibiotics specifically against *P. aeruginosa* which suggests the work presented here is promising based on the author's knowledge. Several *E. faecalis* strains were not inhibited completely by CHL alone yet the inhibitory effect become more potent when combining with Nisin, with the individual MIC of CHL against *E. faecalis* ATCC 29212 reduces from 32 mg/L to 1 mg/L when combined with Nisin (Tong *et al.*, 2014). However, an earlier study done by Brumfitt (2002) showed that Nisin in combination CHL exhibited addition effect against methicillin-resistant *Staphylococcus aureus* (MRSA) strain with FIC index greater than 2. The same study also showed that the combination of Nisin with CHL resulted in synergetic effect for nine tested strains of Vancomycin-resistant *enterococci* (VRE) with FIC index ranging from 0.21-0.58 and 10 other strains showed addition (FIC index not mentioned). The FIC index that the author had set in the study is lower or equal than 0.7. This study showed that the effects of the combination of antibiotic-bacteriocin are a case to case basis even for different strains in the same species.

Fredalina Basri *et al.*, (2014) reported that it is essential to include an additional assay to validate the synergistic interaction of antimicrobial agents through Time-kill assay. The FIC index is to evaluate the MIC of an antimicrobial agent when in combination with another antimicrobial agent. Whereas the Time-kill assay allowed the monitoring of changes in viable cell count of pathogen throughout the treatment period and further validate the bactericidal activity of the combination of antimicrobial agents. Hence, in the subsequent section, a Time-kill assay was conducted to validate the synergistic interaction of crude bacteriocins with antibiotics.

4.5.4 Killing rate of crude bacteriocins and antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145

In a further attempt to validate the synergistic interaction of crude bacteriocins and antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145 and observe the antipseudomonal activity of the crude bacteriocin and its

combination with the antibiotic in a more dynamic fashion, Time-kill assays were performed over 24h of the treatment period (Figure 4.2 – 4.4). Treatment with CHL and TET alone only resulted in a bacteriostatic effect on *P. aeruginosa* ATCC 10145 through $\log_{10}$ CFU/ml reduction less than 2 (Figure 4.2 and 4.3). CHL and TET are two of the antibiotics that *P. aeruginosa* intrinsically resistant towards due to the presence of efflux pumps such as MexAB-OprM and MexCD-OprJ that extrude out the antibiotics (Masuda *et al.*, 2000). A similar finding was found for TET activity against a planktonic cell of *P. aeruginosa* where (Zheng *et al.*, 2017). All crude bacteriocin was found to exhibit antipseudomonal with bactericidal effect with WC being the most one followed by PA and LG. Pham *et al.*, (2004) reported that bacteriocin of LAB eliciting lethal actions against cell membrane of the sensitive strains, such as disruption of protein motive force eludes to the dissipation of electric potential, pore formation leading to efflux of intracellular content such as potassium ion and important macromolecules.

When both CHL and TET were combined with crude bacteriocins WC, LG and PA, the bactericidal effects also were detected for all combinations with varying levels and WC-CHL is the most potent of the combination treatment. WC-CHL (Figure 4.2A) displayed a synergistic effect equivalent to the capability of CIP alone (Figure 4.4) and successfully induced bactericidal effect to planktonic cells of *P. aeruginosa* ATCC 10145 after 6h of treatment. Rapid killing effects of WC-CHL through the time-kill assay signify the effectiveness of the combination in eliminating the bacteria. The shorter time to clear the bacteria is one of the key advantages of an antimicrobial peptide. However, it is known seen in any of the crude bacteriocins possibly due to its crude and impure state. Development of antimicrobials or combination of antimicrobials with rapid killing effect to perform eradication will contribute to reducing resistance development of the pathogens (Chi & Holo, 2018).

Other combinations able to inhibit the cells to an undetectable level at 8h, and only LG-TET inhibited the *P. aeruginosa* after 10h of treatment (Fig 4.2-4.4). The combination of antimicrobial agents that exhibit two different targets often produces synergistic interaction with one another (Tong *et al.*, 2014). The

synergistic interaction of Nisin with other antibiotics was highlighted by the author to be involving the intracellular penetration forming pores on the membrane, allowing the other antimicrobials to enter the bacteria and exert its effect. Danesh *et al.*, (2017) reported that the bacteriocin Haloduracin was tested with CHL and synergistic interactions were observed against all tested strains. The author suggested that the fast CHL influx into the planktonic cells of *P. aeruginosa* is assisted through the pore formed by Haloduracin and preventing protein synthesis alongside other forms of leakage of the pore causes a higher killing rate (Danesh *et al.*, 2017). This proposed mode of action in both studies could potentially be applied to the current findings on the antipseudomonal efficacy of combined treatments of crude bacteriocins with CHL and TET, nevertheless further study to affirm the inhibitory mechanisms needed.

Unfortunately, the combination of crude bacteriocins with CIP has not resulted in a similar enhancement in antipseudomonal potency (Figure 4.4). Breukink *et al.*, (1999) suggested the antagonistic activity of the combination of Nisin with vancomycin, in which vancomycin has inhibited the cell wall biosynthesis, hence reducing the accessibility of lipid II to the Nisin as a docking molecule to exert its pore formation ability. In this study, there are possibilities that the presence of CIP may have blocked the binding of crude bacteriocins to its target receptor on the cell membrane and hence suppress its pore formation ability. However further works needed to elucidate the mechanism involved such as investigation on the mode of action of the purified bacteriocin via qualitative assay such as membrane leakage assay will be essential (Pham *et al.*, 2004).

Out of all combinations of crude bacteriocin and antibiotic, the combination such as PA-CHL, WC-TET, PA-TET displayed indifference interaction and all crude bacteriocin combined with CIP showed antagonistic interaction based on the interpretation on the time-kill assay which contrast Checkerboard assay outcome. Fredalina Basri *et al.*, (2014) reported the similar contradicting outcomes of Time-kill assay and Checkerboard assay which the outcome of Time-kill assay that reported antagonistic effect is more favoured than the

synergistic effect displayed by checkerboard assay. There is no gold standard for synergy determination (Doern, 2014). However, a study was done by Cappelletty & Rybak, (1996) shown no correlation between checkerboard assay and time-kill assay and shown time-kill showed more consistent synergism at various concentration of the antimicrobial while checkerboard only can exhibit indifference. Steenbergen *et al.*, (2009) also agreed that the outcome from the Time-kill assay has more weight to it than the FIC index as it is more established.

4.6 Conclusion

The combined treatment with crude bacteriocins has enhanced the antipseudomonal efficacy of CHL and TET. The inhibitory effect of combined WC-CHL is comparable with CIP alone, and successfully induced bactericidal effect on planktonic cells of *P. aeruginosa* ATCC 10145 after 6h of treatment. The finding of the time-kill assay for this combination of WC-CHL is in accordance with the synergistic outcome of Checkerboard assay. Treatment using WC and PA alone also exhibited antipseudomonal potency after 8h of treatment suggested that WC and PA could be a potent antipseudomonal agent. Future studies to further elucidate the mode of action of these combined treatments of crude bacteriocins and antibiotics will be essential.

Chapter 5: Combined inhibition of crude bacteriocins and antibiotics in eradicating the biofilm of *P. aeruginosa* ATCC 10145

5.1 Introduction

Biofilms are communities of microorganisms attached to a surface. These microorganisms undergo profound changes during their transition from planktonic organisms to cells that are part of a complex, surface-attached community (O'Toole et al., 2000). Multiple factors appear to contribute to the overall resistance of biofilm bacteria. These include reduced metabolic and growth rates, protection by extracellular polymeric substances and specific resistance mechanisms conferred by the altered physiology of biofilm bacteria compared with planktonic bacteria (Davies, 2003).

With the persistence and unique characteristic of the biofilm, MDR bacteria that formed biofilms have been reported to be resistant to almost all classes of antibiotics such as Aminoglycosides, Cephalosporins, Fluoroquinolones and Carbapenems (Breidenstein *et al.*, 2011). *P. aeruginosa* is known to form biofilms that aggregate in the viscous sputum in the lungs of patients with cystic fibrosis. The recalcitrance of the biofilm cells allowed them to persist despite antibiotic treatment was subjected toward the patients (Staudinger *et al.*, 2014). Bacteriocins have been studied extensively with the constant discovery of new bacteriocin from novel isolates which exhibit potent effects on MDR pathogens (Ghodhbane *et al.*, 2015). Bacteriocins also have been investigated for its application against biofilm with a notable mention of Nisin, from the lantibiotic group of bacteriocin (Mathur *et al.*, 2018). Due to the high tolerance of biofilm in general toward antibiotics, among the strategy employed with regards to bacteriocin application is by combining both antimicrobials which have shown promising results (Mathur *et al.*, 2017).

In the previous chapter, the crude bacteriocins WC, LG and PA successfully enhanced the antipseudomonal efficacy of CHL and TET. Therefore, the current study was aimed to investigate further the *in vitro* ability of these crude

bacteriocins in the application of inhibiting or eradicating the biofilm of *P. aeruginosa* ATCC 10145 alone in combination with the selected antibiotics.

5.2 Objectives

The current chapter was aimed to evaluate the combined inhibition of crude bacteriocins and antibiotics in eradicating the biofilm formed by *P. aeruginosa* ATCC 10145. The specific objectives were:

1. To assess the ability of the crude bacteriocins in preventing the formation of biofilm by *P. aeruginosa* biofilm ATCC 10145 via Biofilm inhibition assay.
2. To assess the ability of crude bacteriocins and antibiotics in removing preformed biofilm of *P. aeruginosa* ATCC 10145 via Biofilm eradication assay.

5.3 Material and Methods

5.3.1 Bacteria culture maintenance

All three LAB strains (*W. cibaria* NM1, *L. garvieae* NM2, and *Pd. acidilactici* NM3) were maintained in De Man, Rogosa and Sharpe medium (MRS; Merck, Germany), while the indicator strain, *P. aeruginosa* ATCC 10145 was maintained in Brain Heart Infusion medium (BHI; Merck, Germany). Cultures were propagated twice in respective broth medium for 24 h at 37 ^{o}C or cultured on respective agar medium and incubated for 2 days at 37 ^{o}C. Cultures were maintained as frozen stock in respective broth medium at -20 ^{o}C supplemented with 20 % (v/v) of sterile glycerol till used.

5.3.2 Preparation of crude bacteriocins and antibiotics

The crude bacteriocins WA, LG and LA were prepared according to the method described in Section 4.3.3. A stock solution of 1g of crude bacteriocin dissolved in 1 mL of sterile water was prepared freshly before Biofilm inhibition assay and Biofilm eradication assay. The antibiotic CHL, TET and CIP (Sigma Aldrich, Germany) were prepared to a stock concentration of 1.28 mg/ml according to the manufacturer's protocol. The stock solutions were kept at -20 ℃ until used.

5.3.3 Biofilm inhibition assay

Biofilm inhibition assay was performed according to Santiago *et al.*, (2015) with a minor modification. In this assay, the 24h-old *P. aeruginosa* ATCC 10145 was diluted with BHI broth (that added with 1% glucose) to reach a final viable cell count of 1 x10^6 CFU/ml. Then, 100 μl of the bacterial suspension was then added to the wells of a 96-well microtiter plate that were pre-loaded with 100 μl of 1X MIC and 0.5X MIC of crude bacteriocins, respectively. The positive control is the well with bacterial suspension and the negative control is the well with only BHI broth. The 96-well microtiter plate was incubated at 37 ℃ for 24 h. After the incubation, the biofilm produced was quantified according to the Crystal violet staining method in Section 5.3.5. The experiment was done in three independent replicates.

5.3.4 Biofilm eradication assay

The biofilm eradication assay was performed according to the modified method of Santiago *et al.*, (2015). In this assay, the biofilm cells of *P. aeruginosa* ATCC 10145 were grown for 24 h. The content of each well was aspirated slowly, and the wells were washed through gentle repetition of aspiration and dispensation three times with sterile water to remove unattached bacteria and left to dry.

After that different treatments were added into each well as following:

1. Single treatment of crude bacteriocins (WC, LG, and PA) with a final concentration of 0.25X MIC, 0.50X MIC and 1.0X MIC.
2. Single treatment of antibiotics (CIP, CHL and TET) with a final concentration of 0.25X MIC, 0.50X MIC and 1.0X MIC.
3. Combined treatment crude bacteriocins (WC, LG, and PA) and antibiotics (CIP, CHL and TET), both with a final concentration of 1.0X MIC.

100 µl of the bacterial suspension was added into the wells loaded with treatment. The positive control is the well with only bacterial suspension and the negative control is the well with only BHI broth. The 96-well microtiter plate was incubated at 37 ^{o}C for 24 h. After the incubation, the biofilm production was quantified according to the Crystal violet staining method in Section 5.3.5. The experiment was done in three independent replicates.

5.3.5 Quantification of biofilm via crystal violet staining method

Quantification of the biofilm formed was determined using the crystal violet staining method (Durham-Colleran *et al.*, 2010). The microtiter plates were washed three times with 250 µl of peptone buffered water to remove the free flow or non-attached bacteria and the plate was left to dry for 15 min. After that. 200 µL of 99% methanol (Fisher Scientific Chemicals, USA) was added into each well for the fixation process. The plates were left for 15 min to fix the biofilm to the bottom of the well and the solvent was removed afterwards and were left to dry for another 15 minutes. Then, the wells were stained with 0.1% (v/v) crystal violet (R&M Chemicals, UK) for 5 minutes. The excess stain was discarded gently with distilled water, and the plate was left to be air-dried. Then 200ul of 95% (v/v) ethanol (Fisher Scientific Chemicals, USA) was added to treated wells to dissolve the crystal violet's stain. The plates were shaken for 10 min on an orbital shaker (Fisher Scientific 260300 Ocelot Orbital, USA). Finally, the absorbance was read at 595nm wavelength using a microplate spectrophotometer (BioTek, USA). The percentage recovery of the biofilm was determined by dividing the absorbance recorded for the treated well with the

absorbance recorded for the positive control well. Negative control well was also included for no biofilm growth reading

5.3.6 Statistical analysis

Data were analysed using IBM SPSS Statistic version 24.0 (SPSS Inc, Chicago, IL, USA). All Experiment was done in three independent replicates. All data obtained are the average of three replicates and are represented as the mean ± standard deviation. One-way ANOVA was used to compare data between different treatments. P-value <0.05 was considered as being statistically significant.\

5.4 Results

5.4.1 Anti-biofilm formation ability of crude bacteriocins

This assay was performed to assess the ability of the crude bacteriocins in preventing the formation of *P. aeruginosa* biofilm. Figure 5.1 showed the recovery of *P. aeruginosa* ATCC 10145 biofilm with and without crude bacteriocins treatment. The application of 0.5X and 1.0X crude bacteriocins successfully inhibit the formation of biofilm, with less than 10 % of biofilm was recovered after 24h of incubation. All three crude bacteriocins showed strong capability in preventing the formation of biofilm.

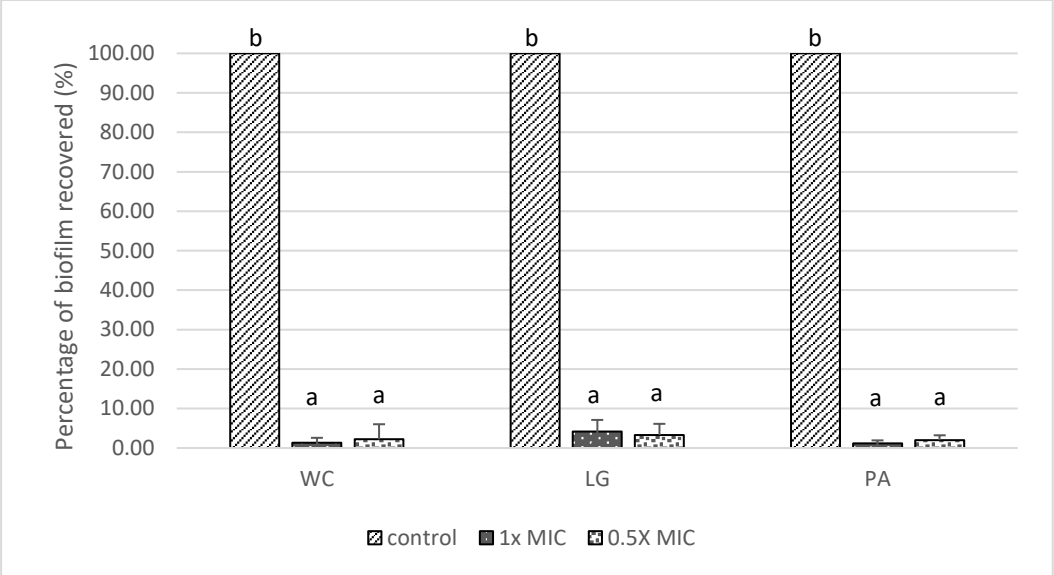

Figure 5.1: The recovery of *P. aeruginosa* biofilm formed after 24-hour incubation with crude bacteriocins.

Notes: Control – Only with *P. aeruginosa* ATCC 10145 and without antimicrobial agents; The treatments are significantly different from control ($p < 0.05$).

5.4.2 Biofilm eradication ability of crude bacteriocins and antibiotics

This assay was performed to assess the ability of the crude bacteriocins and antibiotics in eradicating the preformed biofilm of *P. aeruginosa* ATCC 10145. This assay consists of two sections; the first section is to evaluate the ability of a single antimicrobial agent in eradicating the preformed biofilm. The second section is to evaluate the ability of combined crude bacteriocins and antibiotics in eradicating the preformed biofilm.

5.4.2.1 Biofilm eradication ability of treatment with a single antimicrobial agent

Figure 5.2 showed that treatment with 1X MIC of crude bacteriocins can significantly eradicate the preformed biofilm, with only 50%-60% of biofilm recovered after the treatment when compared to the control treatment. However, although the treatments with 0.25X MIC and 0.5X MIC of crude bacteriocins eradicated 1%-7% and 14%-31% of the biofilm, respectively. This

reduction was not significantly different from the control treatment. The ability of crude bacteriocins to eradicate biofilm is strongly dependent on dosage. The higher the MIC of crude bacteriocin, the stronger the biofilm eradication ability.

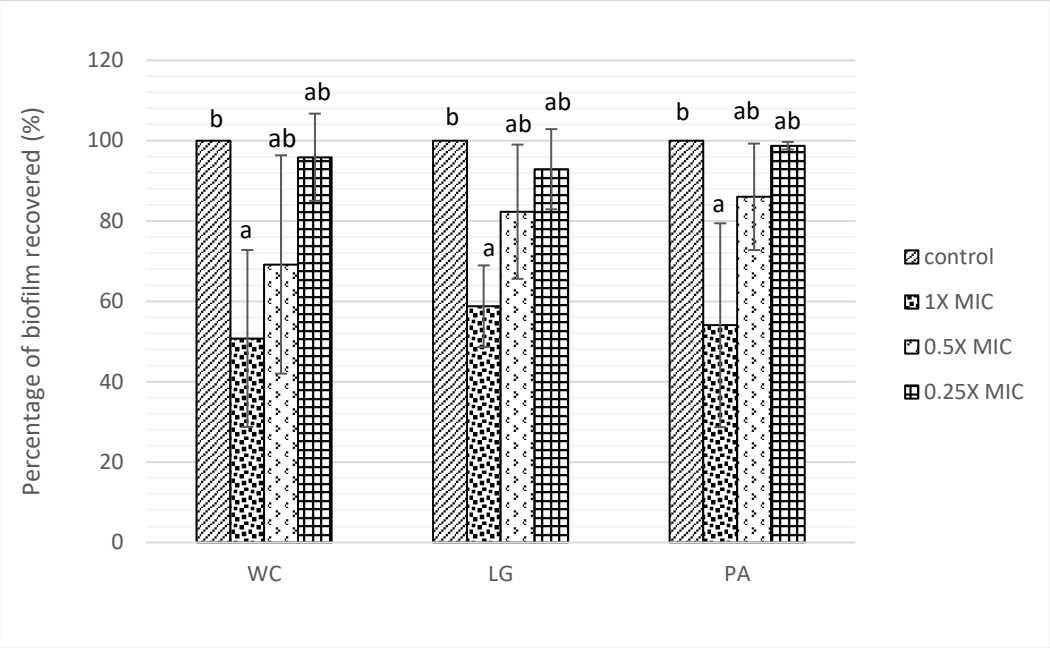

Figure 5.2: Percentage recovery of *P. aeruginosa* biofilm after treated with crude bacteriocins.

Notes: Different superscripts within the bar denote values that are significantly different at P <0.05.

Figure 5.3 showed that treatment using antibiotics for all the tested concentrations could only eradicate 0.03% - 18.19% of the preformed biofilm. However, none of the biofilm recoveries is significantly different from Control. Current findings also displayed the persistency and unique characteristic of the biofilm that enhanced the resistant of *P. aeruginosa* ATCC 10145 to antibiotics CHL, CIP and TET.

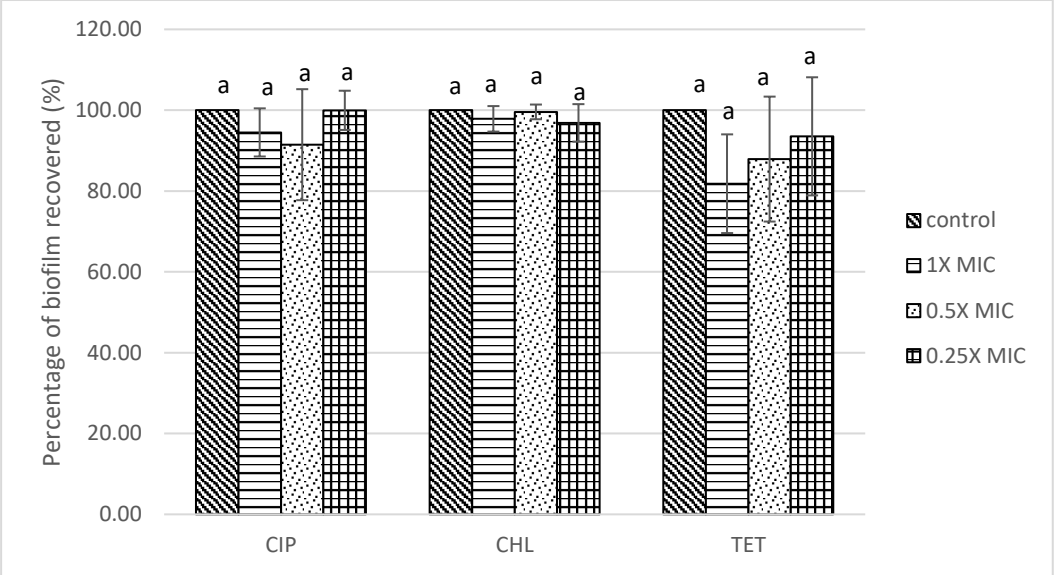

Figure 5.3: Percentage recovery of *P. aeruginosa* biofilm after treated with antibiotics.

Notes: Different superscripts within the bar denote values that are significantly different at P <0.05.

5.4.2.2 Biofilm eradication ability of combined treatment with crude bacteriocins and antibiotics

When compared the treatments between antibiotic alone and crude bacteriocin alone (Figure 5.2 and 5.3), the crude bacteriocin treatment exhibited a stronger ability to eradicate biofilm of *P. aeruginosa* ATCC 10145. Hence in this section, the biofilm eradication ability of combined treatments with crude bacteriocins and antibiotics was performed. Among the combinations of crude bacteriocins and antibiotics, All the WC with antibiotics, certain concentrations from the combination of LG-CIP, LG-TET, PA-CIP and PA-CHL had significantly eradicated the preformed biofilm when compared to the untreated control.

Figure 5.4 showed that the combined crude bacteriocin WC and antibiotics treatments for all tested concentrations have significantly eradicated more than 49% of the preformed biofilm compared to the untreated control. Only the combination of 1X MIC of WC and 1X MIC of TET showed a significant increment of eradication effect when compared between the single treatment of 1X MIC of WC. The combination of crude bacteriocin WC with CIP and CHL, did not show any significant effect when compared with WC alone. However, the combined treatments still provide a significant increment of eradication effect compared to the untreated control with WC-TET being the most potent one with an eradication effect of 93% of preformed biofilm cells.

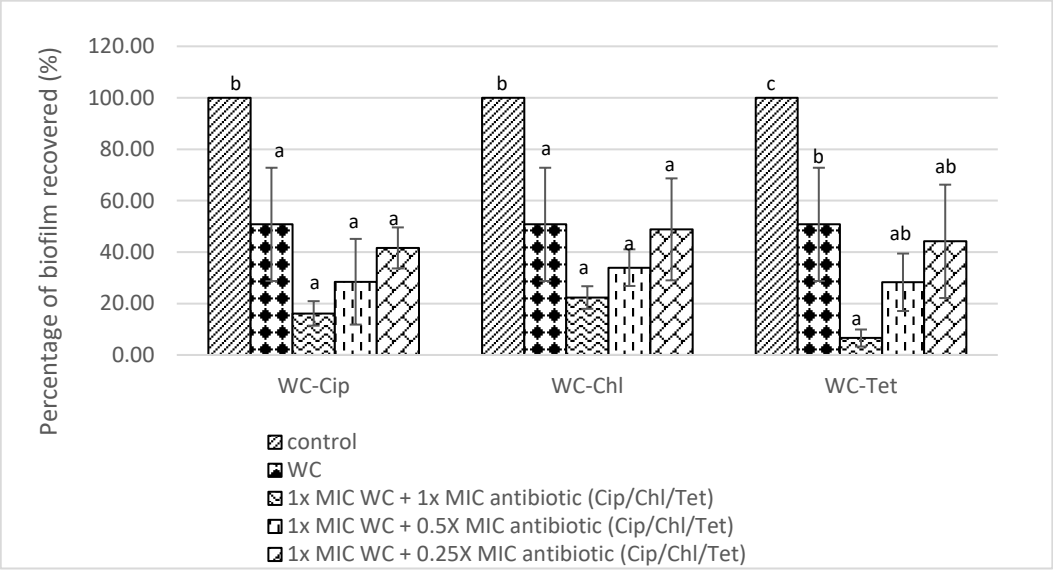

Figure 5.4: Percentage recovery of *P. aeruginosa* biofilm after treated with crude bacteriocin WC in combination with antibiotics.

Notes: Different superscripts within the bar denote values that are significantly different at P <0.05.

Figure 5.5 showed that the preformed biofilm eradication ability of combined treatment with crude bacteriocin LG and antibiotics. Both combined treatments of 1X MIC LG with 1X and 0.5X MIC of CIP significantly eradicated 61% and 69% of the preformed biofilm when compared to control, respectively. The combination of 1X MIC LG with 1X MIC TET has the highest eradication

effect on preformed biofilm when compared with control. Meanwhile, the combination of 1x MIC LG with 1X MIC CHL produced an unexpected outcome where biofilm recovered was not significantly different from control. However, both combined treatments of 1X MIC of LG with 0.5X CHL and 0.25X CHL significantly eradicated 65% and 62% of preformed biofilm when compared to the untreated, respectively.

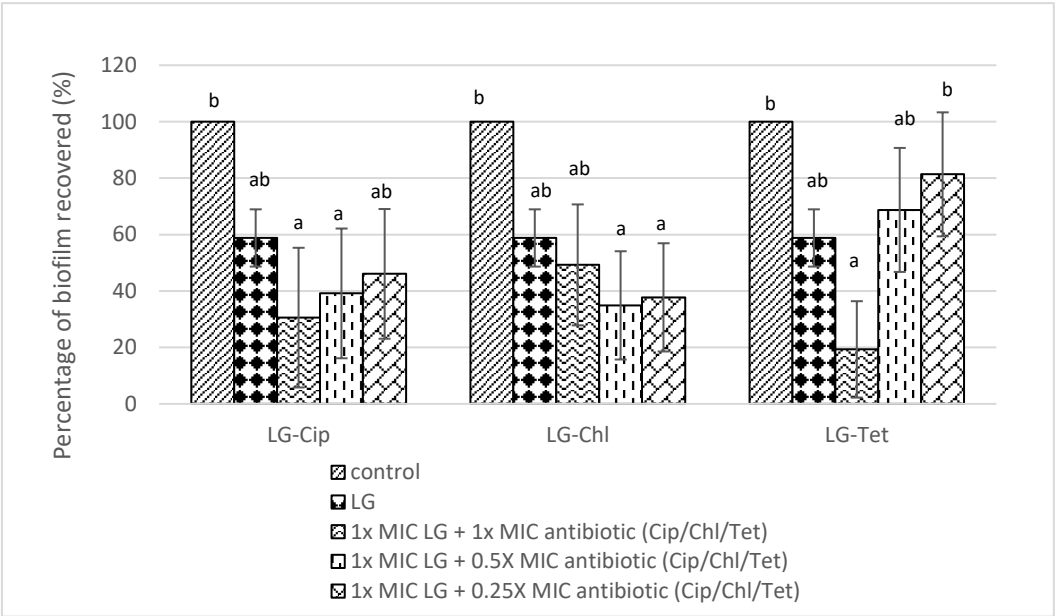

Figure 5.5: Percentage recovery of *P. aeruginosa* biofilm after treated with crude bacteriocin LG in combination with antibiotics.

Notes: Different superscripts within the bar denote values that are significantly different at P <0.05.

Figure 5.6 showed that the preformed biofilm eradication ability of combined treatment with crude bacteriocin PA and antibiotics. Both combined treatments of 1X MIC PA with 1X and 0.5X MIC of CIP significantly eradicated 55% and 78% of the preformed biofilm when compared with Control,

respectively. The most prominent eradication effect can be seen from the combination of 1x MIC PA with 1X MIC of CIP. The combination treatments of PA with CHL for all tested concentrations have significantly eradicated 42%-59% of the preformed biofilm when compared with Control. Although the eradication was not significantly different when compared to treatment with PA alone. As for the combinations of PA-TET, although biofilm eradication effect was observed nevertheless the recovery of biofilm was not significantly different from Control.

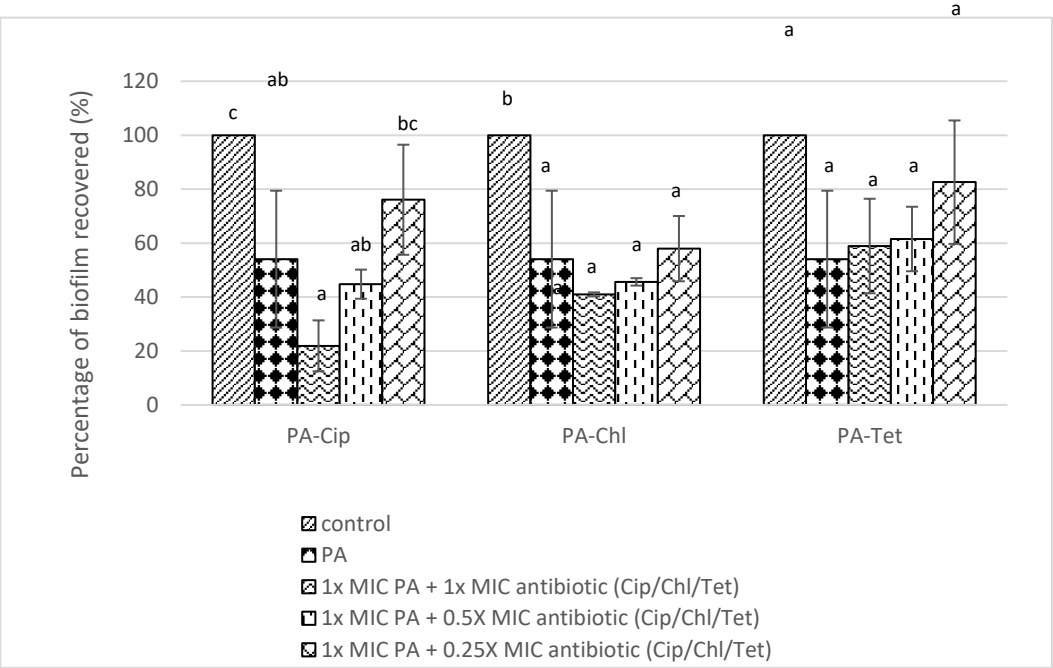

Figure 5.6: Percentage recovery of *P. aeruginosa* biofilm after treated with crude bacteriocin PA in combination with antibiotics.

Notes: Different superscripts within the bar denotes values that are significantly different at P <0.05

5.5 Discussion

The chapter aimed to assess the ability of crude bacteriocins and antibiotics in preventing the formation of biofilm of *P. aeruginosa* ATCC 10145 and also the ability of the crude bacteriocin and antibiotics in eradicating the preformed biofilm.

5.5.1 Anti-biofilm formation ability of crude bacteriocins and antibiotics

Biofilm can be suppressed in three different ways which are through inhibition of the initial planktonic population or prevention of the initial adhesion of cells to the surface or removal of established biofilm. Biofilm is known to be less susceptible to antibiotics which is one of the reasons anti-biofilm therapy is more focused on the inhibition of biofilm formation (Dosler & Karaaslan, 2014). The anti-biofilm formation ability of crude bacteriocins was proven in this study. The incubation of planktonic cells of *P. aeruginosa* ATCC 10145 with 0.5X and 1.0X MIC of WC, LG and PA had successfully inhibited the formation of biofilm (Figure 5.1). Over the years, numerous bacteriocins have been discovered exhibiting antibiofilm properties against a wide variety of crucial biofilm-producing pathogens. *Pd. acidilactici* HW01 through its bacteriocin exhibited antibiofilm properties against biofilm cells of *P. aeruginosa* demonstrated by the bacteriocin significantly inhibited biofilm production of the pathogen on the surface of stainless steel with 57% and 83% reduction at 24 h and 72 h, respectively. The study further demonstrated the bacteriocin suppressed the twitching motility and significantly reduced the production of virulence factors by *P. aeruginosa* KCTC 2004 such as pyocyanin, protease and rhamnolipid which are the factors that involve in biofilm formation (Lee *et al.*, 2020).

A study was done Mahdi *et al.*, (2019) by using Salivaricn LHM produced by *L. salivarius* against multiple isolates of *P. aeruginosa.* The bacteriocin was found to exhibit antibiofilm activity against biofilm of *P. aeruginosa* strains by preventing biofilm formation signifies by reduction of OD value. Sonorensin is produced by *Bacillus sonorensis* MT93, was found to have an antibiofilm effect against biofilm of *S. aureus* which is achieved through biofilm inhibition assay and furthered visualize of its effect through scanning electron microscopy (SEM) analysis. Sonorensin was found to have the ability to target non-multiplying cells of *S. aureus* and targets its bacterial membrane. (Chopra *et al.*, 2015). A study conducted by Al-Seraih *et al.*, (2017), showed bacteriocin B3A-B3B extracted from *E. faecalis* B3A-B3B can inhibit the biofilm formation of *Listeria monocytogenes* with the concentration of 0.0064 mg/mL and the combination of B3A-B3B and Nisin managed to lower the concentration of Nisin from 16 mg/mL to 4 mg/mL to prevent biofilm formation. The report by Cirkovic *et al.*, (2016) showed that utilization of crude bacteriocin in the form of partially purified bacteriocin able to inhibit biofilm formation at a sub-inhibitory concentration (0.5X MIC and 0.25X MIC) against *L. monocytogenes.*

5.5.2 Biofilm eradication ability of treatment with a single antimicrobial agent.

Figure 5.2 showed that treatment with 1X MIC of crude bacteriocins can significantly eradicate the preformed biofilm, with only 50%-60% of biofilm recovered after the treatment. The ability of crude bacteriocins to eradicate biofilm is strongly dependent on dosage and reduced with lower MIC. Nisin with dose-dependent effect display high antibiofilm activity against *S. aureus* MR23 biofilm through a high reading of ATP efflux, increased fluorescence intensity of dead cells and high membrane potential of biofilm cells (Okuda *et al.*, 2013). This study also compared another two bacteriocins from different classes which are Nukasin-ISK-1 and Lacticin Q. Nisin demonstrated high bactericidal activity against biofilm cells which are supported with high ATP-efflux suggesting bacteriocin that can consistently form pores on biofilm cells are a good candidate for treating biofilm infections. When compared the ability

of crude bacteriocins in preventing the formation of biofilm and eradicating the preformed biofilm, it is expected that the crude bacteriocins will be more efficient in preventing biofilm formation. This observation is also supported by Camargo *et al.*, (2016) where the bacteriocin-containing cell-free supernatants (CFS) from *Lactobacillus curvatus* ET31 effectively inhibited the formation of biofilms but not effective toward preformed biofilm. However, in contrast with the current study, the crude bacteriocins tested can significantly eradicate the preformed biofilm for 1X MIC.

None of the antibiotics CHL, CIP and TET tested showed significant eradication activity on preformed biofilm of *P. aeruginosa* ATCC 10145 (Figure 5.3). Current findings have proven the fact that antibiotics are ineffective when used against biofilm with the increment of resistance by around 1000-fold (Bjarnsholt *et al.*, 2009). When coming into contact with antibiotics, the biofilm of *P. aeruginosa* would trigger the increase in expression of efflux pumps such as MexAB-OprM and also novel efflux pumps such as PA1874-1877 (Zhang & Mah, 2008; Soto, 2013). Furthermore, Linares *et al.* (2006) also reported that the formation of biofilm will increase when being exposed to antibiotics CIP and TET. When compared both findings from a single treatment with crude bacteriocins and antibiotics (Figure 5.2 and 5.3), the crude bacteriocins exhibited a stronger ability to eradicate biofilm of *P. aeruginosa* ATCC 10145.

5.5.3 Biofilm eradication ability of combined treatment with crude bacteriocins and antibiotics treatment

Among the combinations of crude bacteriocins and antibiotics, all combinations of WC with antibiotics diminished their recovered biofilm with a significant eradication effect compared to the untreated control (Figure 5.4). As for other crude bacteriocins, notable effects can be seen from LG-CIP, LG-TET, PA-CIP and PA-CHL concerning the comparison between the combined treatment and untreated control. The mode of action for these combinations is still unclear with not many reports of bacteriocin-antibiotic combination against biofilm focused on the underlying effects toward the biofilm.

Nisin displayed an effective eradication effect when compared with CIP or daptomycin against 24h old MRSA biofilm through the time-kill curve method. the study also demonstrated the synergy of the Nisin with CIP or daptomycin with more than 2-log 10 decreases of the colony count after 24 hours (Dosler & Mataraci, 2013). In a different study using different variant Nisin, Nisin V-CHL and Nisin 14V-CHL caused the metabolic activity of 48 h old biofilm of *S. aureus* SA113 to be significantly reduced when compared to Nisin A-CHL (Field *et al.*, 2016*a*). Combination of Nisin with Penicillin, CIP and CHL increased the killing effect of the 24 hour grown biofilm cells of *E. faecalis* visualized through Confocal Laser Scanning Microscopy analysis (CLSM). Different effects were visualized when single antibiotic treatment and combined nisi-antibiotic subjected against the biofilm cells (Tong *et al.*, 2014).

Among the combination of antibiotics with the crude bacteriocin that showed the most significant increment in eradication effect compared to the Control is WC-TET, both with 1X MIC (figure 5.4). It is well known that antibiotics used in this study; TET, CHL and especially CIP are reduced in its killing effect when used against biofilm cells due to low oxygen availability as displayed by the works of Borriello *et al.*, (2004).

P. aeruginosa's biofilm ability to tolerate antibiotics is also contributed from the low oxygen level environment in the biofilm cell. The tolerance of biofilm cells is multivariate that involves factors such as specific genetic determinant such as ndvB that involves in the production of cyclic glucans that interacts with antibiotics such as CIP by sequestering them (Mah *et al.*, 2003), and extracellular matrix component such as Psl that protect the antibiotics (Billings *et al.*, 2013). Billings work showed that Psl contributes to the defence of biofilm from any antimicrobial activities through the finding of Colistin treatment caused more damage to Psl mutant of *P. aeruginosa's* biofilm cells compared to the wild type. It is deducible from the findings that the successful combined treatment such as WC-TET managed to overcome the aforementioned key players in making biofilm becoming tolerant toward antibiotics, yet the mechanism behind the mode of action of the combined crude bacteriocin-

antibiotic treatment are still unclear and required further study such as the usage of scanning electron microscope to observe the effect of the treatments on cell surface changes will allow further understanding to depict any possible explanation.

5.6 Conclusion

Crude bacteriocins used in this study exhibited the capability to prevent biofilm formation of *P. aeruginosa* ATCC 10145. The study finding also showed the crude bacteriocin's significant eradication ability against the biofilm cells at 1X MIC. The crude bacteriocin combination with antibiotics displayed a significant eradication effect for certain combinations especially WC-TET being the most potent combination. This combination also displayed a significant eradication effect compared to WC alone and the untreated control.

Chapter 6: General Discussion

Antibiotic resistance is a global issue that emerged into an alarming state which efficiency of available antibiotics is dropping with the rise of multidrug-resistant pathogen been reported frequently in these few decades. One of the alternatives is the other type of antimicrobial compound produced by bacteria known as bacteriocin, a small peptide which is less than 10 kDa. An interesting fact of bacteriocin is the diversity of this antimicrobial peptide. Bacteriocin has been one of the main components for bacteria defence systems and recently discovered function as a signalling system (Chikindas *et al.*, 2018). Throughout the years of bacteriocin research, bacteriocin has been found in all major lineage in bacteria with a varied spectrum of activity as some have a broad-spectrum activity such as antibiotics while some have narrow and specific activity. The discovery of bacteriocin with the detailed elucidation of the target strain of each bacteriocin can be formulated into a strategy of "sniper bacteriocin" in the healthcare industry targeting specific pathogen with the benefit of not disrupting the microflora of the gut system contrasting to the impact of antibiotic have the gut microflora (Hols *et al.*, 2019). The discovery of bacteriocin is further boosted up with the integration of the bioinformatics approach using genomic data with the discovery of new bacteriocins without the need for screening thousands of strains in the laboratory.

The current study entails the research on the prediction of the bacteriocin gene in the genome of *W. cibaria* NM1 with machine learning-based prediction tool, and the notion of the presence of bacteriocin genes and probiotic associated genes alluded to the bacteriocin producing capability of *W. cibaria* NM1. The study continues with *in vitro* testing of the antipseudomonal potency of crude bacteriocin WC from *W. cibaria* NM1 alongside the crude bacteriocins from two other putative LAB, *L. garvieae* NM2 and *Pd. acidilactici* NM3. The initial intention to start this *in vitro* study with crude bacteriocins was mainly since this is a cost-effective and least time-consuming method that allowed confirmation on the antipseudomonal potency of the crude bacteriocins and

their efficacy in antibiotic enhancement. These data can then justify further studies with purified bacteriocins to investigate the diversity of the antimicrobials characteristics that allowed their use in health care industries. Furthermore, the possibility of using crude bacteriocins as alternative antimicrobials toward MDR bacteria could be an economical approach that reduces the industrial production cost that might have incurred on the extraction and purification steps.

6.1 *In silico* study of bacteriocin in *W. cibaria* NM1 genome

In this study, an *in silico* approach was used to identify bacteriocin, probiotics-related and antibiotic resistance genes in *W. cibaria* NM1. *In silico* prediction using NeuBi resulted in a prediction of WC_2064 as a bacteriocin gene with the probability score of 0.97. The prediction was supported with multiple genes that are found homologous with necessary genes for bacteriocin production such as immunity gene, transporter gene, regulatory gene and modifier gene. WC_2064 does not have any similarity with any bacteriocin genes and this is expected as NeuBi was developed to predict bacteriocin with less or no similarity from the known ones. *W. cibaria* 110 which produced the only characterized bacteriocin from *W. cibaria*, weissellicin 110 is also was found not to have any similarity from other bacteriocins. Usage of Bagel4, a common tool used for bacteriocin prediction resulted in no bacteriocin was found for both *W. cibaria* NM1 and *W. cibaria* 110, despite Weissellicin 110 is an experimentally verified bacteriocin. Meanwhile, NeuBi can detect the protein sequence of Weissellicin 110 as a bacteriocin gene. The inability of Bagel4 to predict bacteriocin that has low similarity is known which is the reason different tool such as BOA and NeuBi were developed to counteract this issue (Morton *et al.*, 2015; Hamid & Friedberg, 2019*b*). Morton *et al.*, (2015) emphasized the nature of the bacteriocin gene is highly diverse and motifs of certain bacteriocin are not conserved which will leave the bacteriocin gene being unpredicted. This finding is supported by Fields *et al.*, (2017) and Venegas-Ortega *et al.*, (2019) where the prediction of bacteriocin using homology-based prediction heavily

relies on dedicated bacteriocin database such as Bagel4 will be limited due to low significant similarity of the bacteriocin.

The finding of *in silico* analysis possibly be extended through heterologous expression especially in understanding how the context genes linked with the predicted bacteriocin gene. As remarked by Collins *et al.*, (2018), it was mentioned that the *in silico* works reported in the published literature has been a disconnected form of work as there is no continuation toward the findings by presenting the availability of the bacteriocin genes. Collins *et al.*, (2018) presented a form of continuation by carrying out a heterologous expression study of these bacteriocin genes to assess the antimicrobial activity. This should be expected for any form of *in silico* work to produce a form of validation to the results as there is always a possibility of bacteriocin genes are not being expressed and inactive. It is important to point out the *in silico* prediction of the bacteriocin gene cannot affirm the functionality of the gene is being expressed out to exert antimicrobial activity. Hence future studies that could combine both *in silico* prediction and experimental validation will be essential.

This chapter also discovered the presence of probiotic associated genes as shown in section 3.4.3. The results correlate with an initial study that was done early on by Amalina, (2017) demonstrating acid and bile tolerance of *W. cibaria* NM1 through *in vitro* experiments correlates with a recent study by Kumari *et al.*, (2020) which utilize the genome of *W. cibaria* CH2 for screening probiotic associated gene at a larger scale by covering another aspect of a probiotic associated gene such as the beneficial properties conferred by the strain. This covers amino acid production and anticancer enzymes. The absence of both virulence and antibiotic genes that are transferable are also in accordance with the work done by Kumari *et al.*, (2020). The presence of Catabolite control protein A, which eluded to contributed to cholesterol reduction ability by Lee *et al.*, (2010) was supported by the work of Lakra *et al.*, (2020) which demonstrated *in vitro* findings of *W. cibaria* MD1 and MD2 in reducing cholesterol level. The finding for this part of the study highlights the suitability of *W. cibaria* NM1 as a suitable candidate for the probiotic strain which overall

in accordance with other *Weissella* strains that were tested through *in vitro* for probiotic properties (Lee *et al.*, 2012).

6.2 Combined inhibition of crude bacteriocins and antibiotics against the planktonic cells of *P. aeruginosa* ATCC 10145

After predicted the presence of bacteriocin gene in the genome for *W. cibaria* NM1 and to prepare for future studies to construct or purify the antimicrobial peptide, the *in vitro* inhibitory assays were performed to validate the antipseudomonal potency of crude bacteriocins and when combined with antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145. In this chapter, the crude bacteriocins WC, LG and PA respectively produced by *W. cibaria* NM1, *L. garvieae* NM2 and *Pd. acidilactici* NM3 and antibiotics were subjected to broth microdilution assay to determine the minimum inhibitory concentration (MIC) against *P. aeruginosa* ATCC 10145. The MIC of the crude bacteriocin and antibiotics is important as they will be used for the subsequent assay for the checkerboard assay to determine the synergism between the combination of crude bacteriocin and conventional antibiotics.

All three crude bacteriocins exhibited MIC of 62.5 mg/mL that was 970 times higher than the antibiotics. Nevertheless, when crude bacteriocins were used in combination with antibiotics CHL, TET and CIP in Checkerboard assay, the FIC indexes confirmed that the synergistic interaction of crude bacteriocins with antibiotics towards planktonic cells of *P. aeruginosa* ATCC 10145 (Table 4.4). No specific finding specifically tailored of bacteriocin in combination antibiotic used in this study except for the study conducted by Aminnezhad *et al.*, (2012) showed CIP exhibited an indifference effect when combined with CFS of *L. plantarum* ATCC 8014 against *P. aeruginosa* with FIC index of 1.6. Similar findings on the synergistic interaction were also reported through the combination of Enterocin CRL35 with CHL (FIC index of 0.4), TET (FIC index of 0.25) but the additive effect with CIP (FIC index of 0.9) (Minahk *et al.*, 2004). The study tested the combination against *Listeria innocua* 7. In contrast,

Brumfitt, (2002) reported that the interaction of Nisin with CHL against nine strains of MRSA resulted in antagonism with FIC index higher than 2, while the other nine strains were found to result in additive when combined.

In a further attempt to confirm the crude bacteriocins can enhance antibiotic efficacy, time-kill assays were performed over a 24h treatment period (Figure 4.2 – 4.4). Combined treatment with WC-CHL is comparable with CIP treatment alone, and successfully killed planktonic cells of *P. aeruginosa* ATCC 10145 after 6h of treatment. WC-CHL interaction between one another works in accordance with both Checkerboard and Time-kill assay suggesting the synergistic nature of the combination. WC improved the activity of CHL toward *P. aeruginosa* despite the antibiotic is being intrinsically resistant towards it. This outcome suggested that the right combination of antimicrobials can reverse the antibiotic-resistant of *P. aeruginosa* ATCC 0145. A recent study was done on the similar concept of reversing antibiotic resistance carried out by Ellis *et al.*, (2020) using Nisin Z in combination with Methicillin against MRSA. The combination yielded a partial synergy that translates into effective cell killings up to 9 hours, followed by increased cell number until the end of the experiment. Despite the increment of cell number, the stress effect of treatment was exerted toward the bacteria through a much smaller colony. Interestingly, the application with WC alone also induced a bactericidal effect on *P. aeruginosa* ATCC 10145 after 8h of treatment. The current finding suggested that WC is a potent antipseudomonal agent.

In contrast, a combination of ineffective antibiotic against a strain of bacteria with bacteriocin does not always ensure the improvement of the antibiotic performance reaching synergistic interaction with another. A study by Draper *et al.*, (2013) reported the usage of Polymyxin B (MIC: >375 µg/ml) with combination Lacticin 3147 against *E. faecium* DO resulted in indifference effects with FIC index of 1. A similar study also reported the usage Lacticin 3147 combined with polymyxin E against *Salmonella Typhimurium* LT2 resulted in antagonism with FIC index of 4. Interestingly, the MIC of *Salmonella Typhimurium* LT2 using polymyxin E is 0.3125 µg/mL yet no MIC was achieved

when the antibiotic was used in combination with Lacticin 3147. In another word, the presence of bacteriocin did not improve the performance of the antibiotic as seen in other combinations in that particular study.

In general, the efficacy of the combination of bacteriocin and antibiotic is case to case basis with different mechanisms that are at play for specific combinations for specific strains of bacteria. This study showed the usage of CIP in combination with the crude bacteriocin although did not reach the state of antagonistic interaction showed the presence of crude bacteriocin simply did not aid the antibiotic in improving further the performance of the antibiotic.

6.3 Combined inhibition of crude bacteriocins and antibiotics in eradicating the biofilm of *P. aeruginosa* ATCC 10145

After assessing the antipseudomonal potency of crude bacteriocins and when in combination with antibiotics against planktonic cells of *P. aeruginosa* ATCC 10145, the biofilm cells of *P. aeruginosa* ATCC 10145 were investigate using crude bacteriocins and when combined with antibiotics through *in vitro* inhibitory assays to validate the anti-biofilm potency. In the biofilm inhibition and eradication assays, crude bacteriocins alone successfully prevent the formation of biofilm from planktonic cells and eradication of preformed biofilm. Current findings suggested that 0.5X MIC of crude bacteriocins are substantial in preventing the formation of biofilm. While 1X MIC of crude bacteriocin displayed significant eradication activity with 50%-60% of preformed biofilms recovered compared to the untreated control. Nisin A exhibited excellent antibiofilm properties when used against MRSA displaying high efflux of ATP suggesting pore formation of the biofilm cells. Vancomycin was used in the study was effective against the planktonic cell of MRSA but did not perform well against its biofilm cells (Okuda *et al.*, 2013). in a different study, EntV, a bacteriocin produced by *E. faecalis* is found to prevent the biofilm formation of *Candida albicans* and also active against the mature biofilm (Graham *et al.*, 2017). Sonorensin also exhibited antibiofilm properties against biofilm *S.*

aureus with the capability to kill active and non-multiplying bacteria. The features of Sonorensin able to target non-multiplying bacteria outshines most antibiotics that fail to do so.

The current finding also indicates that the antibiotics CHL, CIP and TET were unable to eradicate the preformed biofilm (Figure 5.3). The findings were in accordance with the reports from the work of Field *et al.*, (2016), in which 2X MIC and 1X CHL were treated against biofilm cells of *S. aureus* SA113 and *S. pseudintermedius* DSM 21284 resulted in biofilm recovery reading similar to the untreated control. CIP and TET were described in the work of Linares *et al.*, (2006), to increases biofilm formation especially at a sub-inhibitory concentration which is demonstrated in this study using 1X MIC. The early work of Suci *et al.*, (1994) demonstrated CIP transport into the biofilm cells was limited, preventing CIP from penetration due to diffusion limitation set by the matrix of biofilm cells. The latter works refined the observation of Suci *et al.*, (1994) into a more complex picture. The biofilm tolerance toward any antibiotic in general is dependent on multiple factors such as expression of efflux pump (Zhang & Mah, 2008), production of glucosyltransferase for sequestration of antibiotic (Mah *et al.*, 2003), oxygen limitation and low metabolic activity reduce of the biofilm cells (Walters III *et al.*, 2003) and involvement of exopolysaccharide matrix produced by biofilm cells such Psl, Pel and alginate which improve antibiotic tolerance once overexpressed (Goltermann & Tolker-Nielsen, 2017). However, when combined antibiotics with crude bacteriocin WC, the eradication effect towards preformed biofilm of *P. aeruginosa* ATCC 10145 were enhanced and with WC-TET being the most potent combination (Figure 5.4). Similar findings were reported by Field *et al.*, (2016) where the study reported the usage of Nisin 14V in combination with CHL against both biofilm cells of *S. aureus* SA113 and *S. pseudintermedius* DSM 21284 resulted in significantly low recovery of the biofilm cells compared to the untreated control and other combination. the finding was verified using CLSM which biofilm cell death occurred and the reduction of biofilm density.

Okuda *et al.*, (2013) also demonstrated the Nisin with high anti-biofilm activity to be able to form pores on the biofilm cells with the evidence of high ATP efflux reading and high fluorescence reading from membrane leakage. The finding of Okuda *et al.*, (2013) is in accordance with the work of Davison *et al.*, (2010), where Nisin alone treated against biofilm cells of *Staphylococcus epidermidis* was found to able to access the interior biofilm cells at a higher speed compared to other treatment such as chlorine and Nisin were not suppressed or neutralized by the matrix of biofilm which is indicated through time taken for Nisin to penetrate the biofilm cells which is similar to the time taken to permeabilize planktonic cells. Nisin permeabilization into the biofilm cells able to disrupt the membrane cells yet not able to remove biofilm cells. The inability of removal of biofilm cells by Nisin is also in accordance with the CLSM analysis in the work of Field *et al.*, (2016) where Nisin 14V unable to reduce the biofilm cells when used alone but the combination of CHL with Nisin 14V improved the treatment causing the removal of biofilm cells.

Due to the diversity of antimicrobial activity of the bacteriocins, it is considered a suitable candidate for the next generation antibiotic. Bacteriocin such as Nisin is suitable required more *in vivo* and clinical studies to obtain more efficacy and safety data. Numerous in vitro studies were conducted over the years have demonstrated its effectiveness in killing the infectious pathogen. Another potential application of bacteriocin is to be used as a form of disinfectant especially for implanted medical equipment or surgical equipment. Presence of biofilm cells on the surface of implanted medical devices can be the cause of 60%-70% of nosocomial infections (Bryers, 2008). The common causal agent of the medical device's associated infection such as biofilm cells of *S. epidermis* can be killed effectively by Nisin (Davison *et al.*, 2010).

6.4 Summary and future studies

In summary, this study was carried out to investigated antipseudomonal activity of crude bacteriocins produced by a novel isolate of LAB. Bacteriocin is suggested to be an alternative for antibiotics due to its antimicrobial potential and advantages over antibiotics. Antibiotics have been used for decades as a means of treating bacterial infection. However, the disadvantages of using antibiotic are the high chance of continuous emergence of antibiotic resistance strain pathogenic bacteria as extreme as MDR. A total of 700 000 fatal cases were reported yearly due to AMR infections. By 2050, it is predicted the number will rise to 10 million deaths (Nicolaou & Rigol, 2018). Bacteriocin such as Nisin is known for the food preservative application, and there is no bacteriocin yet to be used for medical application. The bacteriocins produced from LAB were found to exhibit low toxicity level towards human and animal tissues which suggested its suitability for human therapeutic purposes (Maher & McClean, 2006). LAB have been used extensively in fermented foods signifying the GRAS recognition by the FDA. Thus, the bacteriocins produced from them are safe for human consumption and do not cause any negative impact on the microbiota (Egan *et al.*, 2016).

Bacteriocin that is active against gram-negative pathogen specifically *P. aeruginosa* will be promising due to the pathogen's clinical significance and broad intrinsic resistance. Thus, the study was designed to identify the antipseudomonal activity of the crude bacteriocin extracted from newly isolated LAB. In conjunction, the study also explored the possibility of finding bacteriocin genes through a machine prediction tool. The study was conducted in a manner where a crude form of the bacteriocins was utilized with selected conventional antibiotics against planktonic and biofilm cells of *P. aeruginosa* ATCC 10145. The rationale on antibiotic selection is based on the efficacy towards the pathogen. CIP representing effective antibiotics while CHL and TET represent ineffective antibiotics.

Crude bacteriocin displayed antipseudomonal effects which its killing effect was further shown in time-kill assays. All three crude bacteriocin displayed almost similar killing effects. Combination of WC with CHL is the most effective compared to other combinations and slightly low in terms of its killing rate compared to CIP alone. Crude bacteriocins against biofilm displayed interesting results where biofilms. In terms of eradicating the biofilms, crude bacteriocins were also managed to eradicate 40%-50% of the biofilm cells whereas conventional antibiotics unable to do the same. Combination of crude bacteriocin with antibiotics displayed apparent eradication effects, and WC-TET displayed the highest eradication effects with 93% of the preformed biofilm cells. Combination of the crude bacteriocins with the antibiotics was found to be able to elicit more harmful effects to the both planktonic and biofilm cells of *P. aeruginosa*. In the case of treatment against planktonic cells, the boost of ineffective CHL and TET through the addition of crude bacteriocins is somewhat interesting finding and similarly, CIP being less effective when combined with crude bacteriocins. The biology behind biofilm cells is different from planktonic cells. This is evident with the major increment in resistance towards antibiotics. However, crude bacteriocins were able to elicit eradication effects towards it and a certain combination of antibiotics further increased the effects. This finding required more in-depth in order to further understand the interaction of the crude bacteriocin against the biofilm cells.

However, one of the limitations in conducting this study is the method selected for producing crude bacteriocin. The crude bacteriocins were formed through concentrating the CFS by freeze-drying method followed by rehydration. The limitation lies where the quantity of bacteriocin in the CFS might differ from the different batch at a different production time. Evaluation of the bacteriocin activity will not be consistent because the bacteriocin production is not optimised. Thus, the method might not be consistent in presenting the actual activity of the bacteriocin. Through the search from the literature, Crude bacteriocin can be defined as the precipitate from ammonium salt precipitation (Kim *et al.*, 2019; Lei *et al.*, 2020). Through using this method, crude bacteriocin

can be standardized out its protein content. Using this form of crude bacteriocin can aid in extracting active component from the supernatant of LAB and at the same time be used in this study to identify antipseudomonal activity. The advantage of this method is that it acts as a foundation study that can be further progressed into purification study. The crude bacteriocin can be brought to a further purification study using chromatography techniques.

Future works can be extended through the possible inclusion of heterologous expression of the in silico predicted bacteriocin genes and other related genes in its vicinity to validate the prediction. Presence of antimicrobial activity elicited from the successful expression of bacteriocin will suggest the accuracy of in silico prediction. Heterologous expression allows for the bacteriocin to be amenable for modification. Besides that, purification of bacteriocin through conventional method can work as a complementary work for the in silico prediction work which can serve as another form of a validation study. Through this, both approaches of *in silico* prediction and conventional prediction can be compared which can be essential for this study as this work utilize new tool which has not been verified through an experimental approach based on the author knowledge.

The acquirement of pure bacteriocin compound will allow for many other extensive studies such as investigation on the mode of action of targeted bacteriocin. This can be achieved through a series of experimental approaches such as investigating the membrane potential and level of ATP leakage of indicator strains upon treatment. moreover, SEM analysis also can help elucidate the mode of action of the bacteriocin against both planktonic and biofilm cells. Increasing the knowledge on the mode of action of bacteriocin is crucial especially with regards to the bacteriocin-antibiotic combination study. All of these are crucial in evaluating their antipseudomonal potency and to decide the best approach forward for health care industries.

Chapter 7: Conclusion

This study is comprised of two parts, one via *in silico* prediction to screen for bacteriocin genes and ascertain its presence in the genome of *W. cibaria* NM1. The second approach was to investigate antipseudomonal potency of crude bacteriocins and when in combinations with antibiotics against planktonic cells and biofilm of *P. aeruginosa* ATCC 10145.

In the first part of the study, the *in silico* workflow predicted a bacteriocin gene associated operon. The bacteriocin gene was predicted through a method specifically predicting the bacteriocin gene with no homology with the existing list of bacteriocin genes. The genome of *W. cibaria* NM1 was also investigated for the genes that are related to probiotic capabilities such as adherence in the gut, survivability in the gut, harbouring no virulence and antibiotic resistance gene. The findings displayed the prospect of *W. cibaria* NM1 as a probiotic candidate containing potential bacteriocin gene and having the capability to survive in the gastrointestinal tract without harbouring any bad effects such as virulence gene. In the second part of the study, the crude bacteriocin produced by three selected LAB, namely WC, LG and PA shown to exhibit antipseudomonal activity and displayed synergistic interaction when in combination with selected antibiotics. The combination of WC-CHL displayed antipseudomonal activity corresponding to the inhibitory activity of CIP and capable to reduce the planktonic cells of *P. aeruginosa* to an undetectable level after 6h of treatment. The 1X and 0.25X MIC of crude bacteriocins also exhibited antibiofilm capability against *P. aeruginosa*. The WC-TET was the most potent combined treatment of all with significant biofilm eradication when compared to the untreated control and WC alone. These findings show the promising ability of crude bacteriocin WC which possess antimicrobial activity against *P. aeruginosa* planktonic cells and biofilm cells.

Overall, while several questions remain unanswered, this study demonstrates the capability of *in silico* prediction tool to discover potential bacteriocin and improves the workflow of the discovery of potential bacteriocin gene and also

highlights the promising aspect of the crude bacteriocin against *P. aeruginosa* with and without the combination of antibiotic, at the same time, the needs for further extensive studies such as obtaining pure bacteriocin to properly understand its mode of action.

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List of Appendices

Appendix

Appendix A: Protocol for PCR amplification of bacteria 16s rDNA gene.

The universal primers set applied in this study is according to James (2010).

8F forward primer: 5'-AGA GTT TGA TCC TGG CTC AG-3' and U1492 reverse primer: 5'-GGT TAC CTT GTT ACG ACT T-3' (James, 2010).

PCR master mix was prepared using DreamTaq DNA Polymerase kit (Thermo Scientific). The reagents for Master mix for 10 reactions was prepared based on Table 3.1. The reagents were added into a sterile 1.5 mL microcentrifuge tube while being kept on ice. The prepared master mix was distributed into 200 µL PCR tubes by aliquoting 19 µL into the tube. Then, 1 µL of the sample with a concentration of 100 ng/µL is added into each except for one tube, which designated as a negative control. The tube was added with 1 µL of distilled water.

The 200 µL PCR tubes were then transferred into a thermal cycler with settings as follows: a) 1 cycle of initial denaturation at 95 °C for 5 min; 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 min and, d) storage at 10 °C till used.

Table A1: Preparation of master mix for 10 reactions for PCR for bacteria identification

Reagent	Stock concentration	Final concentration	Volume for one reaction (µL)	Volume for a master mix (µL)
DreamTaq Polymerase Buffer	10x	1x	2	20
DNTP Mix	2 mM	0.16 mM	1.6	16
Forward primer	10 mM	0.3 mM	0.6	6
Reverse primer	10 mM	0.3 mM	0.6	6
DreamTaq Polymerase	5 U/µL	1 U	0.2	2
Sterile distilled water	n/a	n/a	14	140

Appendix B: 16s rDNA sequences

a. 16s rDNA sequences of *L. garvieae* NM2 using primer sets U8F & U1492R

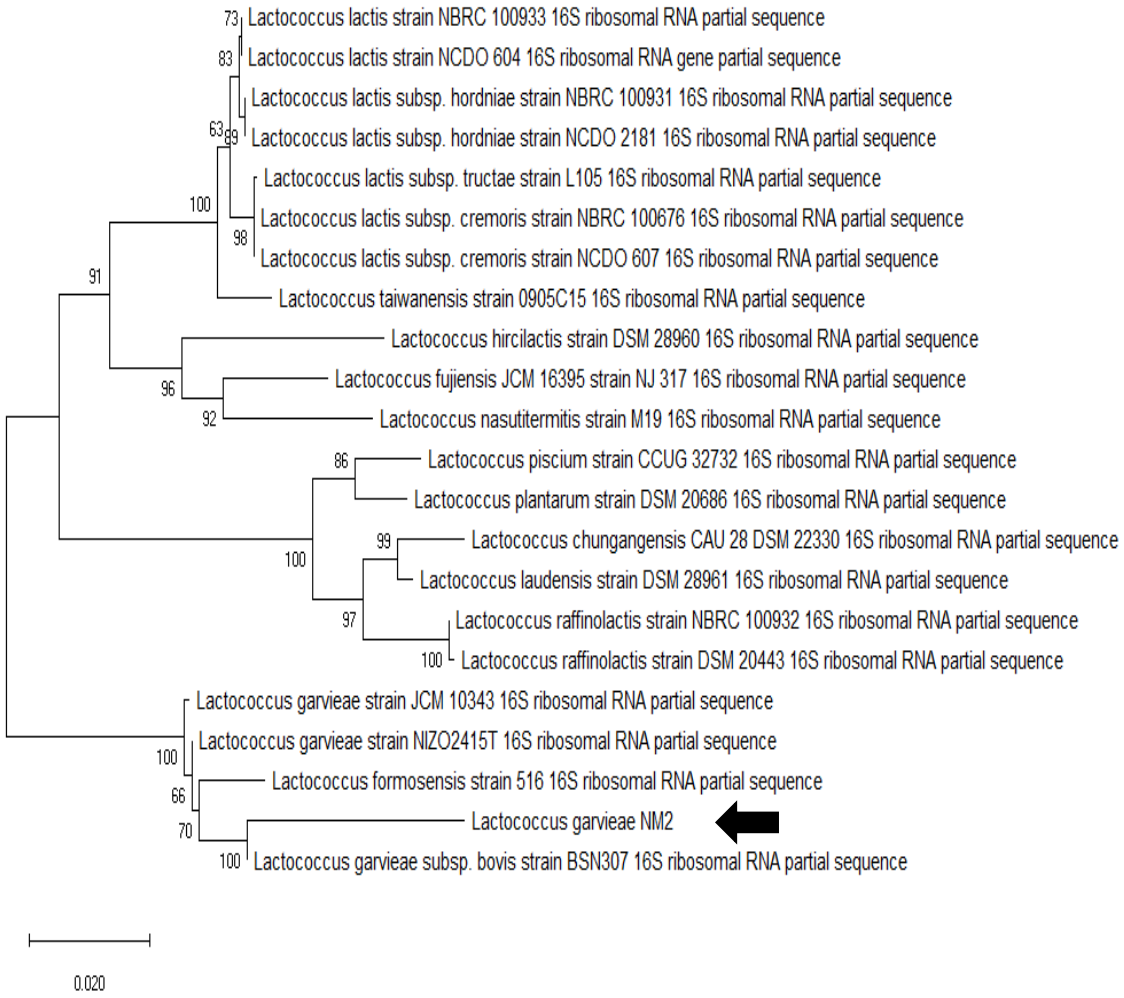
NNNNNNNNNNNNNNNNCNNTAGATGNAAGTCGAGCGATGTATTAGCNAGATAGCTTGCTATTTTC
ATGAAGAGCGGCGAACGGGTGAGTAACGCGTGGGAAATCTGCCGAGTAGCGGGGGACAACGTTTGG
AAACGAACGCTAATACCGCATAACAATGAGAATCGCATGATTCTTATTTGAAAGAAGCAATTGCTTCA
CTACTTGATGATCCCGCGTTGTATTAGCTAGTTGGTAGTGTAAAGGACTACCAAGGCGATGATACATA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTAGGGAATCTTCGGCAATGGGGGCAACCCTGACCGAGCAACGCCGCGTGAGTGAAGAAGGTTTT
CGGATCGTAAAACTCTGTTGTTAGAGAAGAACGTTAAGTAGAGTGGAAAATTACTTAAGTGACGGTAT
CTAACCAGAAAGGGACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTCCCAAGCGTTGTC
CGGATTTATTGGGCGTAAAGCGAGCGCAGGTGGTTTCTTAAGTCTGATGTAAAAGGCAGTGGCTCAA
CCATTGTGTGCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAGGAACACCGGAGGCGAAAGCGGCTCTCTGGCCTGTAACTGACACT
GAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAGCTGTAGGGAGCTATAAGTTCTCTGTAGCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGT
ACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT
AATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATACTCGTGCTATCCTTAGAGNTAAGGAGT
TCCTTCGGGACACGGGATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCATCATTAAGTTGGGCANTCTAGTGNGACTGC
CGGTGATAAACCGGAAGAAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACC
ACGTGCTACATGGATGGTACAACCAGTCGCCAACCCGNGAGGGTGCGCTAATTCTTAAAACCTTTNNC
AATTCGGATTGCAGGCTGCACNCCCCTGCTNNAANTCGGAATCCCTTNAATCCCGAAANNCCCCCCCG
GGGAAATCTTTCCCGGCCTTTTAACCCCCCCCNNNNCCCGAAAGTGGGGAACCCCAAAAGNNTCCTA
CCCNGAGGGGCCNTCAAGAAACCAAGGGGAGGGGGGGAAAAAAAATTGGTANNAAGGGAAACTTT

b. 16s rDNA sequences of *Pd. acidilactici* NM3 using primer sets U8F & U1492R

NNNNNNTNGNNGGNNNCTATACATGCAAGTCGAACGAACTTCCGTTAATTGATCAGGACGTGCTTG
CACTGAATGAGATTTTAACACGAAGTGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCAG
AAGCAGGGGATAACACCTGGAAACAGATGCTAATACCGTATAACAGAGAAAACCGCCTGGTTTTCTTT
TAAAAGATGGCTCTGCTATCACTTCTGGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGC
TCACCAAGGCGATGATGCGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGCAAGTCTGATGGAGCAACGCC
GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACGTGGGTGAGAGTAAC
TGTTCACCCAGTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTCTTTTAAGTCTAAT
GTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAC
AGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGC
TGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTA
GTCCATGCCGTAAACGATGATTACTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCA
TTAAGTAATCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAAGAATTGACGGGGGCCCGCAC
AAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATCTTCTGC
CAACCTAAGAGATTAGGCGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTACTAGTTGCCAGCATTCAGT
TGGGCACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATG
CCCCTTATGACCTGGGCTACCCCCGTGCTACANNGGAGGGNNAACCAGTCCCGAAACCCNGGGGTTT
NCTAATNNTTAAAACCTTTTCCATTCGNAATGNAGGGNGNAANNCCCCNNCNAAAATCGGAATCNTT
NAANTCCGGAAANNAANCCCCGGGGAAAANTTTCCCGGGCTTTTAACCCCCCNNTCNNCCTGGNAAT
TN

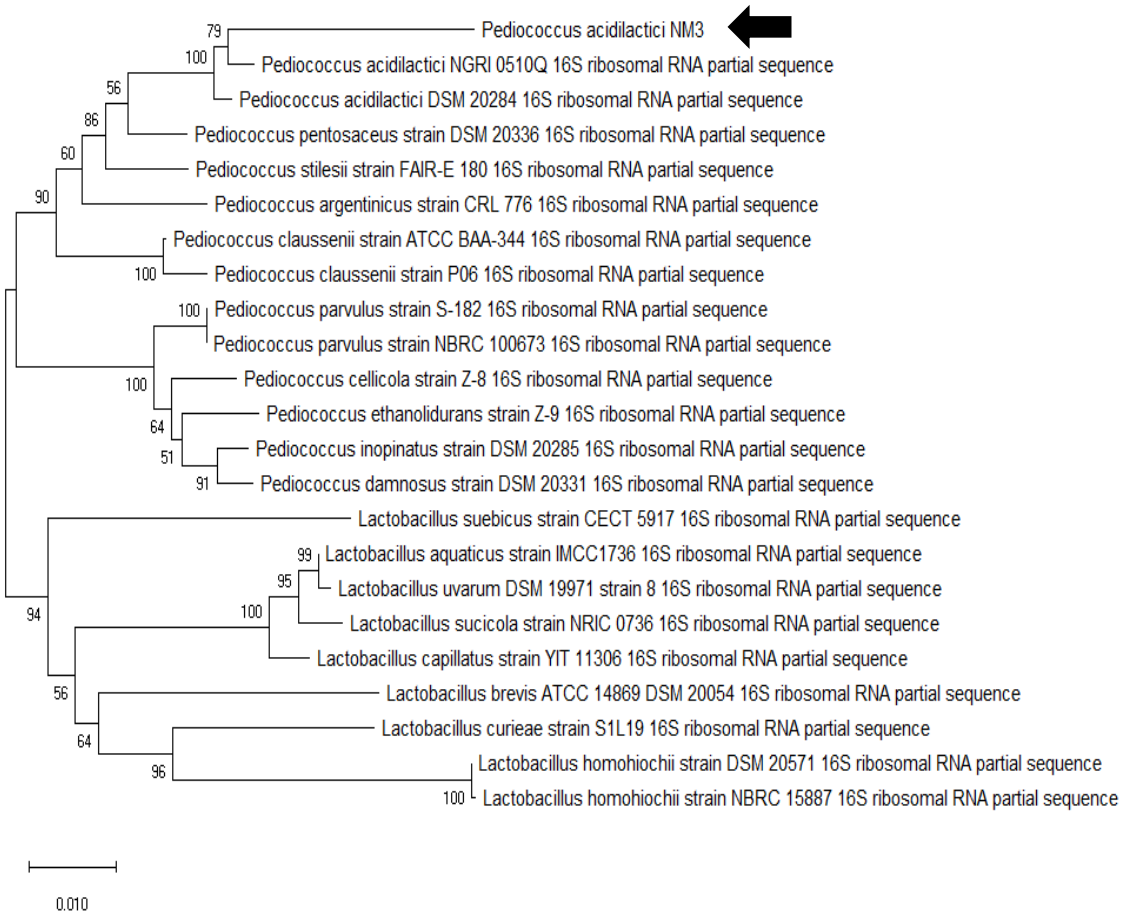
Appendix C: Phylogenetic tree

a. Phylogenetic tree of *L. garvieae* NM2



Phylogenetic tree of *L. garvieae* NM2 of its 16s rDNA sequences against the 16s rDNA database. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

b. Phylogenetic tree of *Pd. acidilactici* NM3



Phylogenetic tree of *Pd. acidilactici* NM3 of its 16s rDNA sequences against the 16s rDNA database. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.