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Enhanced killing of multidrug-resistant *Pseudomonas aeruginosa* ATCC10145 through a combined action of antibiotics and bacteriocin from *Pediococcus pentosaceus* TU2

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ABSTRACT

Aims: Due to its rapid development of resistance against most conventional antibiotics, there is an urgent need to develop new antimicrobial agents and strategies to overcome the challenges in combating multidrug-resistant *Pseudomonas aeruginosa* infections. This study aimed to determine the antipseudomonal potency of bacteriocin produced by *Pediococcus pentosaceus* TU2 when combined with conventional antibiotics.
Methodology and results: The checkerboard method and time-kill assay were conducted to investigate the antagonism interaction and kinetics of the bacteriocin TU2 and selected antibiotics against *Pseudomonas aeruginosa* ATCC10145. The scanning electron microscope (SEM) was used to observe the cell surface morphological changes of the treated *P. aeruginosa* ATCC10145. The combination of bacteriocin TU2 with ciprofloxacin and tetracycline resulted in a 4-fold reduction in minimum inhibitory concentration (MIC) and a fractional inhibitory concentration index (ΣFICI) of 0.5, indicating a synergistic interaction against *P. aeruginosa* ATCC10145. Similarly, the time-kill assay showed that the combination of bacteriocins TU2 respectively with chloramphenicol and tetracycline exerted enhanced bactericidal effect at 8 h and 10 h of treatments compared to treatment with antimicrobial agents alone. Results from SEM suggested that bacteriocin TU2 might cause pore formation on cells and thus enhanced the membrane permeability of antibiotics and intensified the membrane leakage that led to cell death of *P. aeruginosa* ATCC10145.
Conclusion, significance and impact of study: The combined antagonistic effect of bacteriocin TU2 and antibiotics could be a promising strategy in combating *P. aeruginosa* infections and may be applied in therapeutic industries.

Keywords: Bacteriocin, antibiotics, *Pediococcus pentosaceus*, *Pseudomonas aeruginosa*, antagonistic

INTRODUCTION

Antibiotic resistance is the new challenge of the 21st century. In 2017, World Health Organization (WHO) published a list of antibiotic-resistant "priority pathogens" that pose the greatest threat to human health. This priority pathogens list will be updated in the year 2022 (WHO, 2017a; WHO, 2020). WHO reported that *Pseudomonas aeruginosa*, Enterobacteriaceae and *Acinetobacter baumannii* are the key antibiotic-resistant strains that are linked with high mortality rates (WHO, 2017b). *Pseudomonas aeruginosa* is a well-known opportunistic pathogen and one of the most common causes of nosocomial infections and ventilator-associated pneumonia (Barbier *et al*., 2013). It presents a significant challenge in the clinical environment because of its multidrug resistance that leads to the increase in mortality, ranging from 28% (ward) to 48% (ICU) in patients with nosocomial bloodstream infection in the hospitals in United States (Wisplinghoff and Seifert, 2018), increased length of hospital stay and higher hospital costs (El Zowalaty *et al*., 2015). In addition, nosocomial infections associated with *P. aeruginosa* are often difficult to treat due to the intrinsic resistance to multiple conventional antibiotics such as most ß-lactams, chloramphenicol, tetracycline (Koo, 2015), aminoglycosides and fluoroquinolones (Pachori *et al.*, 2019).

This antimicrobial resistance problem is so critical that WHO is working closely with the Food and Agriculture Organization (FAO) of the United Nations and the World Organization for Animal Health (OIE) in a global effort to minimise the spread of resistance and to find new antimicrobial medicines and vaccines in combating the

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multidrug-resistant *P. aeruginosa* (WHO, 2018). WHO also initiated a Global Antibiotic Research and Development Partnership (GARDP) with the Drugs for Neglected Diseases Initiative (DNDi) and aims to develop and deliver new treatments against the drug-resistant bacteria identified by WHO by 2025 (WHO, 2020). With the number of effective antibiotics against the infection is reducing, therefore, the search for novel antimicrobial substances and the discovery of innovative therapy strategies are crucial.

In the past decade, bacteriocin has drawn the researchers' attention for use as a credible alternative to antibiotics (Hols *et al*., 2019). Bacteriocins are ribosomally synthesised, often post-translationally modified antimicrobial peptides and having either a broad or narrow spectrum of antimicrobial activity (Bierbaum and Sahl, 2009). The majority of bacteriocins produced by lactic acid bacteria (LAB) have the Generally Recognised as Safe (GRAS) status and their proteinaceous nature will ensure complete digestion in the gut and hence could be a safer alternative when compared to antibiotics which have many adverse effects to human and animal (Silva *et al*., 2018). In the past, studies were reported using bacteriocin solely as the antimicrobial agent (Al-Mathkhury *et al*., 2011; Smith *et al.*, 2012). However, the inhibitory effect shown by bacteriocins is not as potent when compared to the use of antibiotics. Hence the new approach is proposed to combine bacteriocins with antibiotics to increase the killing potency against the multidrug-resistant pathogen.

Several researchers have reported that the combination of bacteriocins with antibiotics have shown a promising killing effect on pathogens (Draper *et al*., 2013; Amensag *et al*., 2019; Ellis *et al*., 2019). Nisin with methicillin proved more effective against methicillin-resistant *Staphylococcus aureus* than either antimicrobial alone (Ellis *et al*., 2019). Lantibiotic lacticin 3147 was found to exhibit synergy when combined with polymyxin against *Chronobacter sakazakii* DPC6440 (Draper *et al*., 2013). Combination of pediocin PA-1 and Nisin A with antibiotics resulted in synergistic effects against *S. aureus* 400037 and *Acinetobacter baumannii* 280291 with MIC values ranging from 0.19-3.75 µg/mL (Amensag *et al*., 2019).

To the author's knowledge, the reported works on the combined treatment of bacteriocin and antibiotics against multidrug-resistant pathogens such as *S. aureus*, *Enterococcus* species, *Gardnerella vaginalis* and *Acinetobacter baumannii* are available (Algburi *et al.*, 2015; Cavera *et al*., 2015; Mathur *et al.*, 2017; Amensag *et al.*, 2019; Montalbán-López *et al.*, 2020; Zgheib *et al.*, 2020; Ovchinnikov *et al.*, 2021), but the reported works on *P. aeruginosa* is still limited (Kaur and Sharma, 2015; Field *et al*., 2016). Hence, this study aimed to evaluate the impact of using bacteriocin produced by *P. pentosaceus* TU2 in combination with selected conventional antibiotics in combating multidrug-resistant *P. aeruginosa* ATCC10145. The inhibitory synergism of the antimicrobial agents was determined using the checkerboard method and time-kill assay, and the cell surface morphological changes of the treated *P. aeruginosa* ATCC10145 were observed using a scanning electron microscope.

MATERIALS AND METHODS

Maintenance of bacterial cultures

Pediococcus pentosaceus TU2 was isolated from local fermented cassava "Tapai Ubi". *Pediococcus pentosaceus* TU2 (2% v/v) was maintained in 10 mL de Man, Rogosa and Sharpe (MRS; Merck, Germany) broth and incubated at 37 °C for 24 h prior to use. *Pseudomonas aeruginosa* ATCC10145 was purchased from American Type Culture Collection Centre and contributed by the Laboratory of Microbiology, University of Nottingham Malaysia. *P. aeruginosa* ATCC10145 (2% v/v) was incubated in 10 mL of Mueller Hinton (MH) broth (Oxoid, UK) and incubated at 37 °C for 24 h prior to use. Both cultures were supplemented with 20% (v/v) sterile glycerol and stored as frozen stock cultures at –20 °C freezer till use.

Identification of *Pediococcus pentosaceus* TU2

The bacterial identification was determined using API 50 CHL carbohydrate fermentation test kit and analysed with the database on APIWEB™ according to the manufacturer's protocol (BioMérieux, USA). The identity of the bacterium TU2 was further confirmed using 16S rRNA sequences. Genomic DNA of LAB isolate was first extracted with Wizard® Genomic DNA Purification kit (Promega, USA) and the amplification of the 16S rRNA gene was carried out using the universal primers: U8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and U1492R (5'-GGT TAC CTT GTT ACG ACT T-3') (James, 2010) followed by 16s rRNA gene sequencing. The sequence was then compared with those in the GenBank database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm the taxonomic identification at species level.

Production of bacteriocin by *Pediococcus pentosaceus* TU2

Pediococcus pentosaceus TU2 (2% v/v; 1.65×10^8 CFU/mL) was inoculated into 100 mL of MRS broth and incubated at 37 °C for 24 h. The cell-free supernatant (CFS) was obtained after centrifugation at 10,000× *g* for 15 min. The CFS was neutralised to pH 6.5 with 1 M NaOH to eliminate the inhibitory effect due to acid production and filter sterilised (Minisart 0.22 µm, Sartorius, Germany). The neutralised and filtrated CFS was freeze-dried for 72 h using the freeze dryer (Martin Christ, Germany) and stored at a –20 °C freezer till use. Prior to subsequent assays, the freeze-dried CFS with 1000 mg/mL concentration was prepared and designated as "bacteriocin TU2".

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Preparation of antibiotic solutions

Chloramphenicol (Sigma Aldrich, Germany), ciprofloxacin (Sigma Aldrich, Germany) and tetracycline (Sigma Aldrich, Germany) were used in this assay. These antibiotic stock solutions of 1.28 mg/mL were prepared according to manufacturer's protocol and were kept at –20 °C freezer till used.

Minimum inhibitory concentration (MIC)

Determination of MIC of bacteriocin TU2 and antibiotics against *P. aeruginosa* ATCC10145 were performed. A two-fold serial dilution of the bacteriocin TU2 and antibiotic chloramphenicol, ciprofloxacin and tetracycline were carried out using MH broth in a 96-well microtiter plate (SPL, Korea). After dilution, each well contained 50 µL of diluted bacteriocin with final concentration ranging from 0.98-500 mg/mL and antibiotics with final concentration ranging from 0.00025-0.128 mg/mL, respectively. After that, 50 µL of 24h-old *P. aeruginosa* ATCC 10145 was then added to each well. Positive control in this assay was the well containing *P. aeruginosa* ATCC10145 solely, while negative control was the well containing MH broth solely. The samples seeded in 96-well microtiter plate was incubated at 37 °C for 24 h. The optical density (OD) of bacterial growth is determined usually within the range of 600 nm (Duedu and French, 2017). Hence, the cell growth of *P. aeruginosa* ATCC10145 was measure at OD600 nm wavelength before and after incubation by using a microtiter plate reader (BioTek, United States). MIC is expressed as the lowest concentration of the crude bacteriocins and antibiotics that inhibited the visible growth of *P. aeruginosa* ATCC10145.

Minimum bactericidal concentration (MBC)

MBC is referred to as the minimum concentration of drug needed to kill most (≥99.9%) of the viable organisms after incubation for a fixed length of time (generally 24 h) (CLSI, 1999). In this study, an MBC assay was carried out to determine the lowest concentration of bacteriocin TU2 and antibiotics required to kill 99.9% *P. aeruginosa* ATCC10145. The MIC assay was first conducted and the content of each well used in the MIC assay with no growth of *P. aeruginosa* ATCC 10145 after incubation was streaked on a new MH agar plate. After the MH agar plate was incubated at 37 °C for 24 h, the colonies formed were counted. The lowest concentration of bacteriocin TU2 and antibiotics without growth of *P. aeruginosa* ATCC10145 was identified and recorded as MBC. When the ratio of MBC/MIC is ≤2.0, the antimicrobial agent is considered bactericidal or otherwise bacteriostatic (Shanmughapriya *et al*., 2008).

Checkerboard dilution assay

The antagonism interaction assay was performed using the checkerboard method with minor modification of the OD wavelength to 600 nm (Le *et al*., 2015). In order to prepare a range of concentrations that allows simultaneous detection of antagonism, indifference, additive and synergism, the assay was performed in such a way that each column of a 96-well microtiter plate contained a fixed 50 µL of 0.25× MIC of bacteriocin TU2 and eight serial dilutions of antibiotics at each row beginning with 8× MIC for ciprofloxacin and 4× MIC for chloramphenicol and tetracycline. Fifty microliters of *P. aeruginosa* ATCC10145 (1×10^6 CFU/mL) suspension was aliquoted into each well. The plates were incubated at 37 °C for 24 h and the absorbance at OD600 nm wavelength was measured before and after incubation. Positive control in this assay was the well containing the bacterial suspension of *P. aeruginosa* ATCC10145, while negative control was the wells containing only sterile MH broth. MIC of each combination is expressed as the lowest concentration of the bacteriocin TU2 and antibiotics that resulted in visual inhibition (no turbidity) of the *P. aeruginosa* ATCC10145.

The fractional inhibitory concentration index (ΣFICI) was calculated as following: ΣFICI = FIC A + FIC B, where FIC A is the MIC of bacteriocin in the combination divided by MIC of bacteriocin alone and FIC B is the MIC of antibiotic in the combination divided by MIC of antibiotic alone (Le *et al*., 2015). The combination is considered "Synergistic" if the ΣFIC Index is ≤0.5; "Addition" if the ΣFIC Index is ranging from 0.5–1; "Indifference" if ∑FIC Index is >1; and "Antagonistic" if the ∑FIC Index is >4.

Time-kill assay

Time-kill assay was performed to evaluate the killing kinetics of single and combined treatment of bacteriocin TU2 and antibiotics against *P. aeruginosa* ATCC10145. For single antimicrobial treatment, each well was added with single antimicrobial agent of 50 µL of 1× MIC of bacteriocin TU2 and 1× MIC of antibiotics. For combined antimicrobial treatment, each well was added with 50 µL of 0.25× MIC of bacteriocin TU2 and 0.25× MIC of antibiotics. After that, 50 µL of *P. aeruginosa* ATCC10145 (1×10^6 CFU/mL) suspension was aliquoted into each 96-well microtiter plate.

The plates were incubated at 37 °C for 24 h. For every time interval (0, 1, 2, 4, 6, 8, 10 and 24 h), 30 µL of aliquot was withdrawn from each well and ten-fold serially diluted with peptone buffered water. The aliquot was plated on MH agar plate and incubated at 37 °C for 24 h and the colonies formed were counted and expressed as colony forming unit per millilitre (CFU/mL) of a sample by using the viable plate count method (Roberts *et al*., 1995). 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).

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Scanning electron microscopy

Pseudomonas aeruginosa ATCC10145 (1×10^6 CFU/mL; 50 µL) was treated with 50 µL of a single antimicrobial agent (1× MIC bacteriocin TU2 or 1× MIC antibiotics) and with a combination of 0.25× MIC bacteriocin TU2 and 0.25× MIC antibiotics in a 96-well microtiter plate. The plate was incubated at 37 °C for 24 h. Positive control in this assay containing *P. aeruginosa* ATCC 10145 only, while negative control containing MH broth only. Four time-intervals (1, 2, 4 and 8 h) were chosen based on the time-kill assay to detect any morphological changes on bacterial cells after the treatments. For every time interval, 30 µL of aliquot was withdrawn from each well and plated on an MH agar plate and then incubated at 37 °C for 24 h for colony formation.

Karnovsky's fixation method with minor modifications of the glutaraldehyde concentration to 2.5% and the molarity of phosphate buffer to 0.1 M was used to prepare the bacterial sample for scanning electron microscopy (SEM) (Karnovsky, 1965). The sterile glass coverslip was placed and dabbed onto the colony formed on each agar plate so that the bacterial cells were attached to the glass coverslip. Then, the coverslip was immersed in 2% (v/v) formaldehyde (Sigma Aldrich, Germany) and 2.5% (v/v) glutaraldehyde (Sigma Aldrich, Germany) buffered with 0.1 M phosphate buffer solution (PBS) (Sigma Aldrich, Germany) and it was kept at 4 °C chiller for 24 h. Then, the sample was washed three times in 0.1 M PBS for 3 min. The sample was then dehydrated in each concentration of ethanol (Systerm Chemicals, Malaysia) from 50, 70, 80, 90, 95 to 100% (v/v) for 15 min. The sample was then subjected to air-drying in the fume hood. The dried coverslip was placed on aluminium stubs and coated with platinum using a sputtering device (Quorum, UK) and observed with the scanning electron microscope (FEI, USA) at 20 KV under standard operating conditions.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 25 (IBM Corp, 2017) and each experiment was performed in three independent replicates. All values reported were expressed as mean ± standard deviation. Statistical comparisons of normal data were performed using one-way analysis of variance (ANOVA) between samples and statistical significances were indicated via labels of different letters. Duncan post hoc test was used to determine the statistical differences between pairs of means at the chosen level of probability (p<0.05).

RESULTS

Antipseudomonal potency of bacteriocin TU2 and antibiotics

BioMérieux's API 50 CHL identification kit is a well-established method for the identification of Gram-positive bacteria to the species level based on their carbohydrate fermentation patterns (BioMérieux, 2021). Table 1 shows the API 50 CHL carbohydrate fermentation pattern of *Pediococcus pentosaceus* TU2 and the result was analysed using the database of APIWEB™ and confirmed that the bacteriocin TU2 producer showed 99.7% similarity to *P. pentosaceus*. Further verification with 16S rDNA gene sequencing technique has resulted in 96.77% similarity to *P. pentosaceus* ATCC 25745 (Table 2). Bacteriocin TU2 was challenged against *P. aeruginosa* ATCC10145 via MIC and MBC assays and resulted in MIC value of 15.63 mg/mL and MBC value of 31.25 mg/mL (Table 3). *Pseudomonas aeruginosa* ATCC 10145 was resistant to tetracycline and chloramphenicol with the MIC value of 32 µg/mL. The MBC and MIC values of ciprofloxacin against *P. aeruginosa* ATCC 10145 was

Table 1: Carbohydrate fermentation pattern of *P. pentosaceus* TU2 using API 50 CHL test.

Carbohydrate	48 h	Carbohydrate	48 h	Carbohydrate	48 h	Carbohydrate	48 h
Control	-	D-mannose	+	Salicin	+	Gentibiose	+
Glycerol	-	L-sorbose	-	D-cellobiose	+	D-turanose	-
Erythritol	-	L-rhamnose	-	D-maltose	+	D-lyxose	-
D-arabinose	-	Dulcitol	-	D-lactose	-	D-tagatose	+
L-arabinose	+	Inositol	-	D-melibiose	-	D-fucose	-
D-ribose	+	D-mannitol	-	D-saccharose	-	L-fucose	-
D-xylose	+	D-sorbitol	-	D-trehalose	+	D-arabitol	-
L-xylose	-	Methyl-α-D-mannoside	-	Inulin	-	l-arabitol	-
D-adonitol	-	Methyl-α-D-glucopyranoside	-	D-melezitose	-	Potassium gluconate	-
Methyl-β-D-xyloside	-	N-acetylglucosamine	+	D-raffinose	-	2-ketogluconate	-
D-galactose	+	Amygdalin	+	Starch	-	5-ketogluconate	-
D-glucose	+	Arbutin	+	Glycogen	-		
D-fructose	+	Esculin	-	Xylitol	-		

Notes: "+" indicates utilisation of carbohydrates; "-" indicates no utilisation of carbohydrates.

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Table 2: Top five identities of *P. pentosaceus* TU2 via 16S rRNA sequencing.

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

Table 3: Minimum inhibitory concentration and minimum bactericidal concentration of bacteriocin TU2 and antibiotics against *P. aeruginosa* ATCC10145.

Antimicrobial agents	Inhibition against *P. aeruginosa* ATCC10145		
	Minimum inhibitory concentration MIC (µg/mL)	Minimum bactericidal concentration MBC (µg/mL)	MBC/MIC ratio
Bacteriocin TU2	15630	31250 (Bactericidal)	1.99 (Bactericidal)
Chloramphenicol	32.00 (Resistant)	No inhibition	NIL
Ciprofloxacin	0.25 (Sensitive)	0.25 (Bactericidal)	1.00 (Bactericidal)
Tetracycline	32.00 (Resistant)	No inhibition	NIL
Combination of antimicrobial agents	MIC of bacteriocin + MIC of antibiotic (µg/mL)		
Bacteriocin TU2 + Chloramphenicol	3906 + 16.000		
Bacteriocin TU2 + Ciprofloxacin	3906 + 0.125		
Bacteriocin TU2 + Tetracycline	3906 + 16.000		

Table 4: Fractional inhibitory concentration of combined bacteriocin TU2 and antibiotics against *P. aeruginosa* ATCC10145.

Combination of antimicrobial agents	Inhibition against *P. aeruginosa* ATCC10145	
	Fractional inhibitory concentration (ΣFICI)	Interpretation
Bacteriocin TU2 + Chloramphenicol	2.25	Indifference interaction
Bacteriocin TU2 + Ciprofloxacin	0.50	Synergistic interaction
Bacteriocin TU2 + Tetracycline	0.50	Synergistic interaction

shown to be 0.25 µg/mL, indicating that ciprofloxacin is a bactericidal agent.

Table 4 shows that the FIC index resulting from the combination of respective bacteriocin TU2 with ciprofloxacin and tetracycline was 0.5, indicating a synergistic interaction in inhibiting *P. aeruginosa* ATCC10145. The MIC of bacteriocin TU2 used in these combined treatments was 4-fold less than the MIC of bacteriocin TU2 alone (Table 3). While the MIC of the antibiotics used in these combined treatments was 2-fold less than the MICs of ciprofloxacin and tetracycline alone (Table 3). Hence, both combinations were producing synergistic inhibition and enhanced anti-pseudomonal effect. On the other hand, it was apparent that the combination of bacteriocin TU2 and chloramphenicol had resulted in an indifferent inhibitory effect, with the FIC index of 2.25 against *P. aeruginosa* ATCC10145 (Table 4).

Time-kill assay of bacteriocin TU2 and antibiotics

Time-kill assay was performed to determine the antipseudomonal effect of bacteriocin TU2 and antibiotics alone and in combination, following 24 h of the treatment period (Figures 1-3). Figure 1 shows the time-kill curves for *P. aeruginosa* ATCC10145 treated with a combination of bacteriocin TU2 and chloramphenicol. Time-kill curves showed that the combination of bacteriocins TU2 with chloramphenicol successfully inhibited *P. aeruginosa* ATCC10145 totally after 8 h of incubation. Hence, this combination of bacteriocin TU2 with chloramphenicol had exerted a bactericidal effect against *P. aeruginosa*

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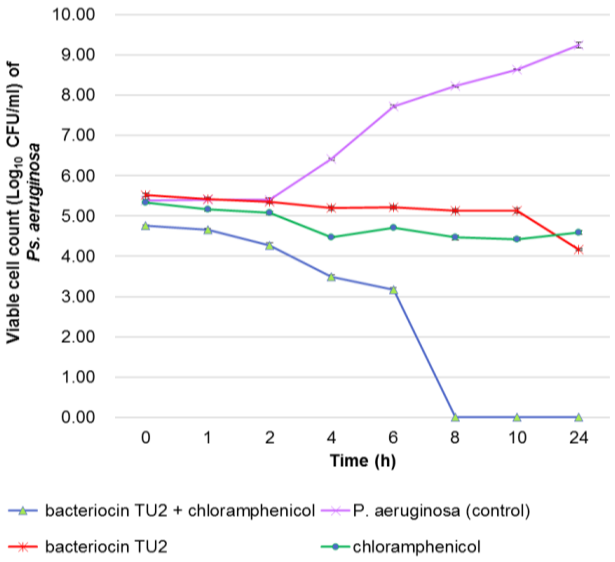

Figure 1: Effect of combined bacteriocin TU2 and chloramphenicol on the growth of *P. aeruginosa* ATCC10145.

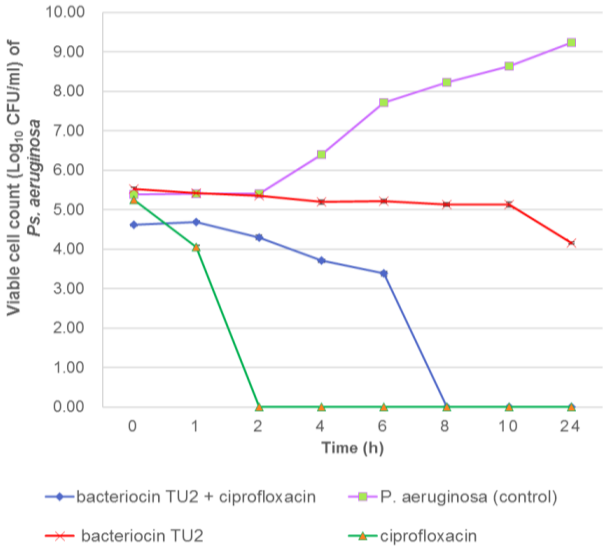

Figure 2: Effect of combined bacteriocin TU2 and ciprofloxacin on the growth of *P. aeruginosa* ATCC10145.

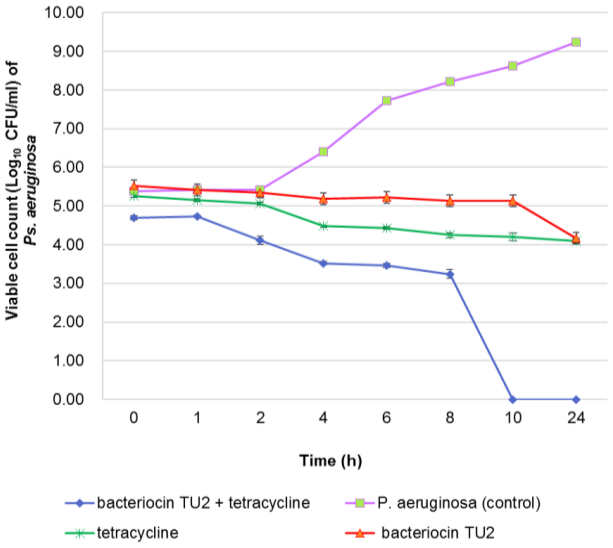

Figure 3: Effect of combined bacteriocin TU2 and tetracycline on the growth of *P. aeruginosa* ATCC10145.

ATCC10145. When compared to treatment with bacteriocin TU2 alone and chloramphenicol alone, the combination of both antimicrobial agents has successfully sped up the killing of *P. aeruginosa* ATCC10145. Hence, the killing rate against *P. aeruginosa* ATCC10145 is as following: chloramphenicol + bacteriocin TU2 > bacteriocin TU2 alone > chloramphenicol alone.

Figure 2 shows the effect of the combination of bacteriocins TU2 and ciprofloxacin on the growth of *P. aeruginosa* ATCC10145. The time-kill curves showed that treatment with ciprofloxacin alone was more effective in killing *P. aeruginosa* ATCC10145 within 2 h of incubation. Meanwhile, the combination of bacteriocin TU2 and ciprofloxacin was only able to inhibit *P. aeruginosa* ATCC10145 totally after 8 h of incubation. While after 24 h of incubation, the treatment with bacteriocin TU2 alone only resulted in 1 $\log_{10}$ CFU/mL reduction in the growth of *P. aeruginosa* ATCC10145. Hence, the killing rate against *P. aeruginosa* ATCC10145 is as following: ciprofloxacin alone > bacteriocin TU2 + ciprofloxacin > bacteriocin TU2 alone.

Figure 3 shows the time-kill curves for the combination of bacteriocin TU2 and tetracycline against *P. aeruginosa* ATCC10145 were similar to the combination of bacteriocin TU2 with chloramphenicol. The combination of bacteriocin TU2 and tetracycline can enhance the inhibition of *P. aeruginosa* ATCC10145 at 10 h of incubation. Whereas treatment with bacteriocin TU2 alone only resulted in 1 $\log_{10}$ CFU/mL reduction after 24 h of incubation. At the same time, tetracycline alone can only suppress the growth of *P. aeruginosa* ATCC10145 and failed to inhibit it. Hence, the killing rate against *P. aeruginosa* ATCC10145 is as following: tetracycline + bacteriocin TU2 > bacteriocin TU2 alone > tetracycline alone.

Cell surface morphological changes of treated *P. aeruginosa* ATCC10145

In order to further elucidate the possible interaction of bacteriocin TU2 and antibiotics when inhibiting *P. aeruginosa* ATCC10145, the visualisation of the cell surface morphological changes on treated *P. aeruginosa*

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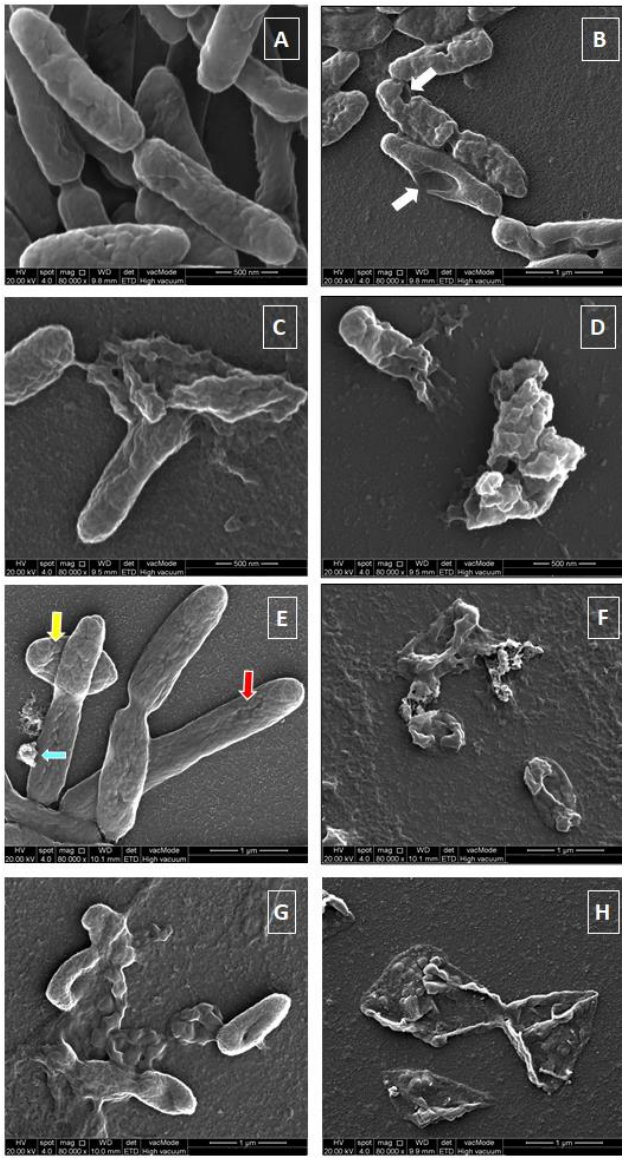

Figure 4: Scanning electron microscopy images of *P. aeruginosa* ATCC10145 after incubation with Mueller Hinton broth for 2 h and 8 h (A-H) (image magnification at 80,000×).
A) Control culture of the untreated *P. aeruginosa* at 8 h.
B) Holes formation (white arrows) in the presence of bacteriocin TU2 only at 8 h.
C) Some part of cell membrane was ruptured in the presence of chloramphenicol only at 8 h.
D) Cell wall was broken where efflux of the cellular contents was observed in the presence of ciprofloxacin only at 2 h.
E) Roundish cell formed (yellow arrow), cell elongation (red arrow) and stalked nubs formation (blue arrow) in the presence of tetracycline only at 8 h.
F) Destruction of all cells (cell lysis) in the presence of bacteriocin TU2 and chloramphenicol at 8 h.
G) Cell membranes were ruptured and release of the cellular contents in the presence of bacteriocin TU2 and ciprofloxacin at 8 h.
H) Cells death in the presence of bacteriocin TU2 and tetracycline at 8 h.

ATCC10145 was carried out using scanning electron microscopy (SEM). SEM image showed that the cell surface morphology of untreated *P. aeruginosa* ATCC10145 was rod-shaped, smooth, with intact cell walls and membrane without any morphological changes at 8 h of incubation (Figure 4A). On the other hand, treatment of 1× MIC bacteriocin TU2 alone on *P. aeruginosa* ATCC10145 cells resulted in circular holes or pores on the cell surface at 8 h of incubation (Figure 4B).

In the meantime, SEM image revealed that treatment of ciprofloxacin alone (1× MIC) on *P. aeruginosa* ATCC10145 cells resulted in cell lysis within 2 h (Figure 4D), whereas treatment of chloramphenicol (1× MIC) and tetracycline (1× MIC) alone caused only a few of the cell lysis and could not inhibit *P. aeruginosa* ATCC10145 cells completely (Figure 4C and 4E). Hence, these findings were in line with the results demonstrated in the time-kill assays that showed ciprofloxacin was an effective bactericidal, and chloramphenicol and tetracycline were bacteriostatic agents against *P. aeruginosa* ATCC10145 (Figures 1, 2 and 3). Meanwhile, all three combinations of bacteriocin TU2 (0.25× MIC) with antibiotics chloramphenicol (0.25× MIC), ciprofloxacin (0.25× MIC) and tetracycline (0.25× MIC) treatments, respectively caused shrinkage of the volumes, cell membranes ruptured and released of the cellular contents, resulting in cell death at 8 h of incubation (Figure 4F, 4G and 4H). These findings were also in accordance with the time-kill assays carried out in this study (Figures 1, 2 and 3).

DISCUSSION

Antipseudomonal potency of bacteriocin TU2 and antibiotics

Pseudomonas pentosaceus TU2 was isolated from Malaysian fermented cassava “tapai ubi” and with 96.8% similarity to *P. pentosaceus* ATCC25745. *Pseudomonas pentosaceus* TU2 was able to ferment D-galactose and 15 other carbohydrates that are being tested in API 50 CHL test kit.

Similar to the other bacteriocinogenic lactic acid bacteria, *P. pentosaceus* TU2 can produce antimicrobial substances such as bacteriocin and organic acid that could inhibit other bacteria. The bacteriocin TU2 used in this study has been neutralised to eliminate the effect of organic acid. When bacteriocin TU2 was challenged against *P. aeruginosa* ATCC10145, it has successfully inhibited the pathogen with the MIC value of 15.63 mg/mL (Table 1), which is much higher than those bacteriocins reported by Lin and Pan (2019) and Shelburne *et al*. (2007). Lin and Pan (2019) reported that the purified cell-free supernatant (CFS) of *Lactobacillus plantarum* NTU 102 isolated from homemade Korean cabbage pickles

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showed the MIC value of >3.35 mg/mL against *P. aeruginosa*. Meanwhile, Shelburne *et al*. (2007) demonstrated that the MIC value of purified bacteriocin subtilosin A isolated from *Bacillus subtilis* against *P. aeruginosa* showed 0.05 mg/mL. These reports suggest that there is a variation in the inhibitory activity of bacteriocins produced by different producers. Bacteriocins produced by *L. plantarum* NTU 102 and *B. subtilis* tend to exhibit a stronger inhibitory activity against *P. aeruginosa*, when compared to *P. pentosaceus* TU2.

Pseudomonas aeruginosa is an antibiotic-resistant bacterium that caused an estimated 32,600 infections among hospitalised patients and 2,700 estimated deaths in the United States in 2017 (CDC, 2019). *Pseudomonas aeruginosa* ATCC10145 used in this study was resistant to antibiotic chloramphenicol and tetracycline resulted in the MIC value of 32 µg/mL (Table 3). Similar findings were also reported by Jayaraman *et al.* (2010) and Mawabo *et al.* (2015), the *P. aeruginosa* strains tested showed MIC values of 32 µg/mL for tetracycline. However, Mawabo *et al.* (2015) demonstrated a lower MIC value of 16 µg/mL for chloramphenicol.

Meanwhile, *P. aeruginosa* ATCC10145 was highly susceptible to ciprofloxacin with a MIC of 0.25 µg/mL. A similar finding was reported by Jayaraman *et al*. (2010), where the MIC of ciprofloxacin against *P. aeruginosa* DR3062 was demonstrated to be 0.25 µg/mL. In contrast, Grillon *et al.* (2016) reported that 35% of *P. aeruginosa* isolates were shown to be resistant to ciprofloxacin in an *in vitro* study. These results indicated that the antagonistic activity of the indicator pathogen could vary not only from one species to another but also from one strain to another strain of the same species (Ono *et al*., 2004).

When the ratio of MBC/MIC is ≤2.0, the antimicrobial agent is considered as bactericidal or otherwise bacteriostatic (Shanmughapriya *et al.*, 2008). In this study, the ratio of MBC/MIC for bacteriocin TU2 was 1.99 and hence considered as bactericidal agent. However, ciprofloxacin exhibited a strong bactericidal effect with the MBC/MIC ratio of 1.0 (Table 3). This finding was consistent with the study reported by Chalkley and Koornhof (1985) that ciprofloxacin was found to be an effective bactericidal agent against *P. aeruginosa*. It has been proposed that treatment of ciprofloxacin may help in the formation of cytotoxic hydroxyl radicals (OH-) and increased total amount of reactive oxygen species (ROS), which was accompanied by a decreased in biofilm viability. As a result, this may lead to the killing of *P. aeruginosa* (Jensen *et al*., 2014).

Both chloramphenicol and tetracycline exhibited bacteriostatic effects, which were unable to inhibit *P. aeruginosa*. The current finding was in line with the study reported by Morita *et al*. (2014), which demonstrated that chloramphenicol and tetracycline were bacteriostatic agents against *P. aeruginosa* due to its good intrinsic resistance mechanism of MexAB/MexXY efflux systems. Intrinsic resistance is a major reason for *P. aeruginosa* refractory to many antibiotics. Different from adaptive and acquired resistance mechanisms, intrinsic resistance has hereditarily occurred, and among the intrinsic resistance in *P. aeruginosa* is chromosomally encoded low outer membrane permeability and constitutively over-expressed efflux systems (Idowu *et al*., 2019). For example, an important mechanism of resistance to chloramphenicol and tetracycline by *P. aeruginosa* is by actively expelling these antibiotics out of the cell using its abundantly expressed efflux pumps (Chopra and Roberts, 2001). The MexAB/MexXY efflux systems play a prominent role in the multi-drug resistance of *P. aeruginosa* and are produced at low levels by the gene operon in all strains of *P. aeruginosa*. This gene operon becomes overexpressed, and the resulting efflux pump overproduction in turn increases the MICs for several antibiotics, including chloramphenicol and tetracycline (Aeschlimann, 2003).

Combined antagonism interaction of bacteriocin TU2 and antibiotics

Checkerboard dilution assay was used to evaluate the combined antagonism interaction of bacteriocin TU2 and antibiotics against *P. aeruginosa* ATCC10145. Table 4 shows that bacteriocin TU2 with ciprofloxacin and tetracycline indicated a synergistic interaction in inhibiting *P. aeruginosa* ATCC10145. The MIC of these combinations resulted in a 4-fold reduction compared to the MICs of bacteriocin TU2 and antibiotics alone. According to Saiman (2007), the antimicrobial combinations that result in a 4-fold reduction in the MIC compared with the MICs of agents alone are synergistic. Synergy is a positive interaction in which the combined effect of the two antimicrobial agents is significantly greater than the effect of either antimicrobial agent alone or greater than the sum of the impact of the individual antimicrobial agent (Cappelletty and Rybak, 1996). Hence, both combinations were producing synergistic inhibition and an enhanced anti-pseudomonal effect. A similar synergistic inhibitory effect was observed when *P. fluorescens* were treated with the combination of nisin and antibiotics such as penicillin, streptomycin, chloramphenicol and rifampicin (Naghmouchi *et al*., 2013). While Selegard *et al.* (2019) reported that bacteriocin plantaricins (PlnEF) was found to enhance the effect of tetracycline by 500-fold against *Staphylococcus epidermidis* with checkerboard assay.

The combination of bacteriocin TU2 and chloramphenicol had resulted in an indifferent antagonistic effect indicates that the effect of the combination of these two antimicrobial agents is the same as that of the most potent of these antimicrobial agents used alone (Rennerberg, 1993). Similar indifferent/additive inhibitory effects were observed when *P. aeruginosa* strains were treated with the combination of bacteriocins (such as cecropin P1, indolicidin, magainin II, nisin and ranalexin) and antibiotics (doxycycline, ofloxacin, meropenem, netilmicin, ceftazidime and piperacillin), respectively (Giacometti *et al*., 1999). At the same time, Aminnezhad *et al.* (2012) reported that the combination of cell-free supernatant of *Lactobacillus plantarum* ATCC8014 with either amikacin or ciprofloxacin against *P. aeruginosa* ATCC27853 were

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also demonstrated indifference actions. Bacteriocins and antibiotics are known to have different targets on the bacterial cell membrane, and a study reported by Delcour (2009) revealed that the synergistic interaction of nisin and polymyxin B against Gram-negative bacteria could be attributed to disruption of the outer membrane by polymyxin B, allowing access of the bacteriocins to the target cell membrane.

Time-kill kinetics of bacteriocin TU2 and antibiotics

The current study has proven that combined treatments of bacteriocin TU2 with chloramphenicol and tetracycline have enhanced the antipseudomonal effect (Figures 1 and 3). In contrast, treatment with these antimicrobial agents alone can only suppress the growth of *P. aeruginosa* ATCC10145 and has failed to inhibit it. The exact mechanism by which bacteriocin TU2 with chloramphenicol and tetracycline produce a synergistic effect against *P. aeruginosa* ATCC10145 is still unclear. Whereas the combination of crude bacteriocin TU2 with ciprofloxacin was found to delay the killing rate at 8 h compared to the treatment of ciprofloxacin alone which caused the complete killing of *P. aeruginosa* ATCC10145 at 2 h (Figure 2). Current finding suggested that the treatment of ciprofloxacin alone is more potent than the treatment of combination of the bacteriocin TU2 with ciprofloxacin against *P. aeruginosa* ATCC10145.

A study conducted by Grillon *et al.* (2016) also found the treatment of ciprofloxacin alone to be the most effective bactericidal agent against *P. aeruginosa* when compared to other treatments of antibiotics in time-kill study. Ciprofloxacin is a broad-spectrum antibiotic with a higher efficacy against Gram-negative bacteria such as *P. aeruginosa* (Cheng *et al*., 2015). Ciprofloxacin is one of the most used quinolones that interfere with bacterial metabolism by interacting with bacterial DNA gyrase, therefore lead to separate of bacterial DNA, inhibit the cell division and eventually causing cell death (Salem *et al.*, 2020). Hence, the high concentration of ciprofloxacin on the surface of *P. aeruginosa* may improve its bactericidal effect (Lu *et al*., 2013).

The specificity and safety features of bacteriocins have been recognized as with advantages over conventional antibiotics. Nonetheless, many aspects concerning their efficacy remained unknown. Although bacteriocins are found to be active at low concentrations, they have low *in vivo* stability and are susceptible to degradation by proteolytic enzymes, which will greatly impact the feasibility of large-scale production (Meade *et al*., 2020). Hence, only a few studies have investigated the combination of antibiotics and bacteriocins against other pathogenic bacteria using time-kill assays (Le *et al.*, 2015; Ellis *et al*., 2019). A study reported by Naghmouchi *et al*. (2013) demonstrated that the combination of nisin A or pediocin A with antibiotic colistin produced a synergistic effect against Gram-negative bacteria such as *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 3515029. A similar finding was reported by Field *et al.* (2016), which revealed that the combination of nisin with polymyxin produced a synergistic effect against *P. aeruginosa* PA-01 biofilm.

Bacteriocin and antibiotics are known to have different targets on bacterial cells and a potential mechanism of this combination could be that antibiotic acts first on the bacterial cell, and then bacteriocin reaches other targets (Naghmouchi *et al*., 2013). Nisin, when combined with glycolipodepsipeptide ramoplanin resulted in synergistic interaction against methicillin-resistant *Staphylococcus aureus* (MRSA) strains and both target lipid II within the bacterial cell envelope. Hence, it was postulated that the mechanism of action of both antimicrobials is by targeting lipid II, which resulted in the inhibition of peptidoglycan synthesis (Brumfitt *et al*., 2002).

Therefore, the current study of using a combination of bacteriocin TU2 with chloramphenicol and tetracycline have improved the killing kinetics and eradication of multidrug-resistant *P. aeruginosa.* The growth of *P. aeruginosa* was inhibited totally by the combination of bacteriocin TU2 with chloramphenicol and tetracycline after 8 h and 10 h of treatments. While the *P. aeruginosa* treated solely with each of the antimicrobial agents remains viable at 4 to 5 $\log_{10}$ CFU/mL after 8 h and 10 h of treatments (Figures 1 and 3).

Chloramphenicol is a broad-spectrum antibiotic that functions by binding to the 50S ribosomal subunit and hindering the protein chain elongation during protein synthesis (Madhavan and Bagyalakshmi, 2014). Tetracycline, on the other hand, is a broad-spectrum antibiotic that inhibits protein synthesis by preventing the attachment of aminoacyl-tRNA to the bacterial ribosome acceptor (A) site (Schnappinger and Hillen, 1996). Meanwhile, the study by Cotter *et al.* (2012) revealed that bacteriocin class IIa from LAB cross the outer membrane of Gram-negative bacteria by binding to the cell envelope-associated mannose phosphotransferase system (Man-PTS), resulting in pore formation of the outer membrane. It has been claimed that the synergism between antibiotic and bacteriocin is based on the ability of bacteriocin to permeabilise the outer membrane and allowing antibiotics to gain access to their internal target sites (Kaur and Sharma, 2015).

Cell surface morphological changes of treated *P. aeruginosa* ATCC10145

The visualisation of the cell surface morphological changes on *P. aeruginosa* ATCC10145 after being treated with bacteriocin TU2 and antibiotics was carried out using scanning electron microscopy (SEM). Figure 4B shows that treatment of 1× MIC bacteriocin TU2 alone on *P. aeruginosa* ATCC10145 cells resulted in circular holes or pores on the cell surface at 8 h of incubation. Pore formation on the bacterial cell membrane has been reported to be one of the known antibacterial action mechanisms of the bacteriocin from lactic acid bacteria (Perez *et al*., 2014). In a study reported by Sharma *et al.* (2018), the morphological changes on the surface of *E. coli* treated with bacteriocin showed pores formation by bacteriocin isolated from *B. subtilis* GAS10144. Okuda *et*

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al. (2013) also suggested that the pore formation in the membrane of *P. aeruginosa* resulted in membrane leakage of ions leading to disruption of membrane potential and ATP depletion. Hence, this will lead to the diffusion of low molecular cytosolic compounds out of the cell and resulting in cell death.

Figure 4D revealed that treatment of ciprofloxacin alone (1× MIC) resulted in cell lysis within 2 h. In contrast, Figure 4C and 4E revealed respectively that the treatment of chloramphenicol (1× MIC) and tetracycline (1× MIC) alone could not completely lyse the cells of *P. aeruginosa* ATCC10145. Ciprofloxacin is usually active against *P. aeruginosa* (Gillespie and Masterton, 2002), whereas the intrinsic resistance of *P. aeruginosa* towards chloramphenicol and tetracycline have been associated with the low permeability of the outer membrane, which prevents the access of the antibiotics to the cytoplasmic membrane, combined with numerous and highly efficient efflux mechanism (Delcour, 2009).

Meanwhile, all three combinations of bacteriocin TU2 (0.25× MIC) with antibiotics (0.25× MIC) resulted in distinct cellular disruption and cell death after 8 h of incubation (Figure 4F, 4G and 4H). A study reported by Tong *et al.* (2014) demonstrated that the combination of nisin and chloramphenicol also resulted in *Enterococcus faecalis* cells lost their original morphology and showed distinct cellular disruption. While Regmi *et al.* (2016) demonstrated that bacteriocin CSpK14 and ß-lactams had a synergistic effect against MRSA strains. In this study, the combined effect demonstrated that the MRSA strain carrying plasmid-borne copies of Tn1546 could produce the altered cell wall precursor and may inhibit the production of PBP2a, a key component of the ß-lactams resistance mechanism in MRSA. These works suggested that the membrane disruption of the targeted cell triggered by bacteriocin might have enhanced the permeability of antimicrobial agents such as antibiotics that were initially repelled by pathogens to their target sites.

To date, the precise mechanism of action involved remains unclear, and the use of SEM alone in this study has limitations. It could only reveal the morphological changes of the cell surfaces, such as shapes, sizes and the overall physical appearances of the treated cells. Nonetheless, SEM images and the previous time-kill assay conducted revealed that the antipseudomonal activity of antibiotics was improved in the presence of bacteriocin TU2. Future work could include transmission electron microscope (TEM) and epifluorescence image-based screening as complementary to SEM to examine the combined inhibitory effect of bacteriocin TU2 and antibiotics on the target cells.

CONCLUSION

With the alarming trend of antibiotics that are rapidly losing their efficacies, the urge to find alternative treatments is becoming more prevalent. In this study, the FIC and time-kill assays show the synergy and indifference interaction between the bacteriocin TU2 and chloramphenicol, ciprofloxacin and tetracycline. Furthermore, the SEM images also revealed that the combined effects of bacteriocin TU2 and antibiotics resulted in cell surface disruption and lead to cell death of *P. aeruginosa*. Hence, the combination of bacteriocin TU2 with antibiotics represent a promising strategy to combat the multidrug-resistant *P. aeruginosa* infection.

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