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Antimicrobial activities and mechanisms of truncated and amino-acid-substituted peptides derived from bacteriocin PZJ5

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Abstract

Truncation and amino acid residue substitution are common methods to optimise the design of antimicrobial peptides (AMPs). In the present work, seven truncated and residue-substituted derivatives of Plantaricin ZJ5 (PZJ5) were designed and synthesised. PZJ5-5 was a truncation that simultaneously contained three substituted amino-acid residues, with enhanced antimicrobial activity and low haemolytic activity. The effects of PZJ5-5 on *Escherichia coli* microstructure were investigated using scanning and transmission electron microscopy, which indicated that its antibacterial mechanism was similar to PZJ5. C-terminal amidation of PZJ5-5 (PZJ5-7) was deleterious, and resulted in a dramatic reduction in potency against *E. coli* and *Listeria monocytogenes*, with no potency against the other three indicator bacteria. Truncation and residue substitution of bacteriocin PZJ5 changed its antimicrobial activities and specificities, which provided a rationale for bacteriocin design.

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Introduction

Preservatives are effective and widely used approach to prevent food spoilage. Artificial chemical additives are currently the most widely used food preservatives worldwide, but may cause safety risks (Foulerton, 1899; Rai *et al*., 2016). High concentrations of food preservatives reduce growth of human epithelial type 2 cells, whereas potassium and sodium nitrite are the two most toxic food preservatives among those tested (Maguire *et al*., 2016). Therefore, using natural preservatives such as bacteriocins to replace chemical preservatives is urgently needed in the food industry.

Bacteriocins are peptides with antibacterial activity that are synthesised by ribosomes during bacterial metabolism (Jack *et al*., 1995). Only a few commercial bacteriocins are used as natural food additives (De Vuyst and Leroy, 2007; Yang *et al*., 2014). Nisin is "generally recognised as safe" (GRAS) bacteriocin, and the only one approved for use as a food preservative by the US Food and Drug Administration (FDA). It is licensed as a food additive in over 45 countries (Settanni and Corsetti, 2008; Yang *et al*., 2014). Nisin has a broad range of activities against Gram-positive, but not Gram-negative bacteria (Harris *et al*., 1992).

Another commercially available bacteriocin is pediocin PA-1, marketed as Alta® 2341, which inhibits the growth of *Listeria monocytogenes* in meat products (De Vuyst and Leroy, 2007; Yang *et al*., 2014). Song *et al.* (2014a) constructed eight PA-1 variants, in which the N-termini were altered with cationic residues, and some of those peptides exhibited a two-fold increase in bacteriostatic activity.

Plantaricin ZJ5 (PZJ5), produced by *Lactobacillus plantarum*, is a linear helical bacteriocin composed of 22 amino-acid residues, resistant to high temperatures (Song *et al*., 2014b), biodegradable by enzymes, and considered safe. PZJ5 has significant inhibitory effects on Gram-positive and Gram-negative bacteria (Song *et al*., 2014b). Many studies have suggested that positive charge,

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hydrophobicity, amphipathicity, and terminal group structure are important features for the antibacterial activities of bacteriocins (Kazazic *et al*., 2002; Chen *et al*., 2007; Won *et al*., 2011a; Almaaytah *et al*., 2014; Mura *et al*., 2016). The amphipathic-helical antimicrobial peptides (AMPs) with positive charge are combined with the negatively charged bacterial phospholipid membrane by electrostatic action (Wieprecht *et al*., 2000). The hydrophobic part of the AMP is inserted into the bacterial membrane, following which the helices cluster to form channels and cytosolic leakage, which leads to bacterial death (Wieprecht *et al*., 2000). Therefore, hydrophobicity and amphipathicity are considered crucial for peptides that solely target the cytoplasmic membrane (Epand and Vogel, 1999; Chen *et al*., 2005; 2007; Won *et al*., 2011b).

Increasing the hydrophobicity of the non-polar face of the amphipathic-helical peptides also increases their antimicrobial activity (Dathe *et al*., 1997; Wieprecht *et al*., 1997; Avrahami and Yechiel, 2002; Chen *et al*., 2007). High amphipathic level generally leads to higher antimicrobial activity (Won *et al*., 2011a). The positive charge of AMPs is mainly derived from the lysine and arginine residues (Dennison *et al*., 2005; 2009). Many studies have shown that C-terminal amidation has a beneficial effect on the antibacterial activities of AMPs (Stromstedt *et al*., 2009; Tomita *et al*., 2015). The trend toward formation of alpha-helical structure of C-terminal amidated peptide is greater than that of non-amidated peptide, and C-terminal amidation can stabilise the secondary structure, and enhance the affinity of the peptide to the membrane (Cao *et al*., 2005; Dennison *et al*., 2009; Mura *et al*., 2016). Amidated peptides have a higher positive charge than the corresponding non-amidated peptides. The former is more active due to the aforementioned reasons (Mura *et al*., 2016). Many studies have shown that truncated AMPs are almost as active as full-length peptides or even more potent (Wang, 2008; Sikorska *et al*., 2009; Lv *et al*., 2014; Tomita *et al*., 2015). For instance, it has been reported that truncation analogs of LfcinB containing the RRWQWR sequence, which is the antimicrobial core of LfcinB, exhibit similar or more activity than LfcinB (Wakabayashi *et al*., 1999; Svenson *et al*., 2010; Tomita *et al*., 2015; de Jesus Huertas *et al*., 2017). Shortened peptides are easier to synthesise, and more suitable for large-scale manufacturing. Truncation and amino acid residue substitution are common methods to optimise the design of AMPs (Tomita *et al*., 2015; Wu *et al*., 2016). We have designed a series of truncated and residue-substituted derivatives of PZJ5 with different properties. However, the modification of one parameter (*e.g*., charge) often leads to a change in another (*e.g*., hydrophobic moment), thus making direct correlation between certain physicochemical properties and peptide activity challenging (Won *et al*., 2011a).

We obtained a derived peptide PZJ5-5 with perfect amphipathic structure and high activity against a wide range of bacteria. In the present work, we aimed to further optimise PZJ5.

Materials and methods

Bacterial strains were preserved in our laboratory, and listed in Table 1. All peptides were synthesised and purified by GL Biochem Ltd. (Shanghai, China). The peptides were purified to above 95% homogeneity by reverse-phase high-performance liquid chromatography (RP-HPLC), and the mass of the peptides was characterised by electrospray ionisation (ESI) mass spectrometry (Agilent Technologies, Palo Alto, USA). Peptides were then dissolved in distilled water at a concentration of 5 μg/μL, and the peptide solutions were stored at -20°C until subsequent analyses. Sheep red blood cells (SRBCs) were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

Table 1. Media and culture conditions used for indicator bacteria.

Type	Species	Strain	Culture	Incubation
Gram-negative	*E. coli*	O104	Luria-Bertani culture	37°C shake culture
	P. aeruginosa	MA1		
	Salmonella sp.	New Port	Brian Heart Infusion culture	
Gram-positive	*E. faecalis*	BMB	Brian Heart Infusion culture	37°C shake culture
	L. monocytogenes	Clip 11262		

Bacteriocin activity assay

Bacteriocin activity was measured using a microtiter plate assay system, as previously described (Lin *et al*., 2013). *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Salmonella* sp., and *L. monocytogenes* were selected as indicator bacterial strains (Table 1) to observe bacteriostasis of bacteriocin PZJ5 and its variants. Bacterial cells grown overnight were diluted in liquid medium to a density of 10^7 colony-forming units (CFU)/mL, and peptides were serially diluted for use. Each well of a sterile 96-well plate was seeded with 50 μL of test bacteria, and an equal volume of the peptide was added and mixed. The microtiter plate cultures were incubated at 37°C for 16 h, and the growth of the indicator strain was measured spectrophotometrically at 600 nm with the SpectraMax 190 microtiter plate reader (Molecular Devices, San Jose, USA). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of bacteriocin that inhibited the growth of the indicator strain. Medium containing bacterial cells was used as a positive control, and pure medium was used as a negative control. At least three independent measurements were performed.

Measurement of haemolytic activity

The haemolytic activity of the peptides was measured as the amount of haemoglobin released by the SRBCs, as previously described (Barksdale *et al*., 2017). Briefly, 200 μL of the 2% cell solution was incubated with 4, 16, and 64 μM of PZJ5 or PZJ5-5, for 1 h at 37°C in a 96-well plate. The solution was then centrifuged at 1,000 rpm for 2 min, and the supernatant was transferred to a new 96-well plate. The absorbance was measured at 540 nm with microtiter plate reader. SRBCs in PBS (haemolysis 0%) and sterile water (haemolysis 100%) without PZJ5 and PZJ5-5 were employed as negative and positive controls, respectively. The percentage of haemolysis was calculated using Eq. 1:

$$\text{Haemolysis (\%)} = \frac{A_{sample} - A_{negative}}{A_{positive} - A_{negative}} \times 100 \qquad \text{(Eq. 1)}$$

Scanning electron microscopy (SEM)

E. coli was grown to the exponential phase in LB liquid medium, as previously described (Jin *et al*., 2016). One millilitre of *E. coli* culture was then centrifuged at 2,000 *g* for 10 min, and the pellet was re-suspended in LB medium after washing twice with phosphate-buffered saline (PBS) (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China). *E. coli* solution was incubated with PZJ5 or PZJ5-5 (1× MIC) at 37°C for 30 min, and then centrifuged at 2,000 *g* for 10 min. The *E. coli* culture without peptides was used as control. The pellet was then washed with PBS twice, and re-suspended in 2.5% glutaraldehyde solution (Sinopharm Chemical Reagent Co. Ltd.) at 4°C overnight. *E. coli* suspension was centrifuged at 2,000 *g* for 10 min, and the pellet was washed with PBS. The pellet was fixed in 1% osmium tetroxide (SPI-CHEM, USA) in PBS for 1 h. The fixed cells were rinsed with PBS, dehydrated in a graded series of ethanol (Sinopharm Chemical Reagent Co. Ltd.) concentrations, dehydrated in the HCP-2 critical point dryer (Hitachi, Tokyo, Japan), coated with gold-palladium in the E-1010 ion sputter coater (Hitachi) for 4 - 5 min, and observed under the SU-8010 scanning electron microscope (Hitachi).

Transmission electron microscopy (TEM)

Bacterial samples were prepared by the same method as described for SEM. After prefixation with 2.5% glutaraldehyde (Sinopharm Chemical Reagent Co. Ltd.) at 4°C overnight, *E. coli* was embedded in agar, and then washed with PBS (Sinopharm Chemical Reagent Co. Ltd.) for three times. The pellet was fixed in 1% osmium tetroxide (SPI-CHEM) in PBS for 1 h. The fixed cells were rinsed with PBS, dehydrated in a graded series of ethanol (Sinopharm Chemical Reagent Co. Ltd.) concentrations, and transferred to absolute acetone for 20 min. The sample was placed in a 1:1 mixture of absolute acetone (Sinopharm Chemical Reagent Co. Ltd.) and the final Spurr resin (SPI-CHEM, West Chester, USA) mixture for 1 h at room temperature, and transferred to a 1:3 mixture of absolute acetone and the final Spurr resin (SPI-CHEM) mixture for 3 h, and thereafter in final Spurr resin mixture overnight. Samples were placed in Eppendorf tubes containing Spurr resin, and heated at 70°C for > 9 h. Blocks were trimmed to 70 - 90 nm thickness using the UC7 glass knives (Leica Microsystems Inc., Buffalo Grove, USA), and mounted on mesh grids. After staining with uranyl acetate (SPI-CHEM) followed by lead citrate, cells in each group were examined using the Hitachi H-7650 transmission electron microscope (Hitachi).

Propidium iodide (PI) assay

As previously described (Wang *et al*., 2017), *E. coli* cells (1×10^7 CFU/mL) were harvested, washed three times, and re-suspended in PBS. PZJ5 and its variants (final concentration of 4.2 μM) were separately added to the suspension, and incubated for 4 h. Then, the suspensions were separately incubated with 2.5 μg/mL PI (BBI Life Sciences Corporation, Shanghai, China) for 30 min in the dark. Thereafter, 10 μL suspension was added to slides, and observed under the DM4000B fluorescence microscope (Leica).

Results and discussion

Peptide design

The amphipathic α-helical bacteriocin PZJ5 was used as a framework to design a series of analogs. PZJ5-1, the 16-residue internal segment of PZJ5, was designed by retaining the whole α-helical region of PZJ5. PZJ5-2, with five positive charges, was designed by replacing Thr at position 2 with Lys of the N-terminal truncation, which resulted in an 18-amino-acid helical region. To improve the amphipathicity of the peptide, analogs PZJ5-3, PZJ5-4, PZJ5-5, and PZJ5-6 with 1-4-residue substitutions were designed by replacing several residues with opposite polarity ones based on PZJ5-1. In the sequence of PZJ5-5, two Lys were employed for substitution at positions 6 and 13 for improving the cationicity. PZJ5-5 had the highest activity of all the variants, and was chosen to be the C-terminal amide; the new peptide was called PZJ5-7. The sequences and biophysical properties of the peptides are listed in Table 2, and the distribution of hydrophobic and hydrophilic residues in the helical region of the peptides is shown in Figure 1A. Amphipathicity of a peptide was determined by the mean hydrophobic moment (Eisenberg *et al*., 1984; Won *et al*., 2011a). The helical wheel facilitates straightforward examination of the amphipathicity of the α-helical peptide (Figure 1A).

Activity of PZJ5 and variant peptides

The antimicrobial activities, presented as MICs of the peptides against the indicator bacteria, are summarised in Table 3. The relative potency of variants as compared to PZJ5 is shown in Figure 1B. PZJ5-1 and PZJ5-2 showed no activity against the indicator strains (Table 3), which demonstrated that the non-helical region was essential for the antimicrobial activity of PZJ5. The lack of studies on the relationship between structure and function of PZJ5, and the lack of information regarding the core peptide of PZJ5, accounted for the design failure of these two truncated peptides. PZJ5-3, PZJ5-4, and PZJ5-6, which were designed with PZJ5-1 as the framework, also displayed no potency either. However, PZJ5-5, a truncation that simultaneously contained three substitute amino-acid residues, exhibited antimicrobial activity. Recovery of the potency of truncated AMP by substitution was also observed in another study (Zhu *et al*., 2014). Although PZJ5-4 and PZJ5-6 had good amphiphilicity, low positive charge may have limited their potency.

PZJ5-5 and the parent peptide PZJ5, both of which had four positive charges, showed potent antimicrobial activities against all the indicator bacteria, with varying activities against different bacteria (Table 3, Figure 1B). The antimicrobial activity of some AMPs may depend on the indicator bacteria (Fimland *et al*., 2000; Song *et al*., 2014a). Recovery of the antimicrobial activity of PZJ5-5 might have been due to its high amphiphilicity and appropriate positive charge. PZJ5-5 exhibited two-fold increase toward *E. coli* and *L. monocytogenes*, four-fold increase toward *P. aeruginosa* and *Enterococcus faecalis*, and four-fold decrease in potency toward *Salmonella* sp. as compared to PZJ5 (Table 3, Figure 1B). The positions of specific amino acids may influence the antimicrobial activities and specificities of AMPs, and amino-acid residues in the non-helical region may interact in a structurally-specified and restricted manner with the target cell.

The C-terminal amidation of PZJ5-5 (PZJ5-7) was deleterious, which resulted in a dramatic reduction in potency against *E. coli* and *L. monocytogenes*, and no potency against *P. aeruginosa*, *E. faecalis*, and *Salmonella* sp. (Table 3, Figure 1B). Hydropathy plot analysis of PZJ5-5/ PZJ5-7 showed large hydrophobicity gradients in the C-terminus (Figure 1C). The gradient provided an asymmetric distribution of hydrophobicity along the α-helical long axis, which contributed to penetration of the bilayer core (Harris *et al*., 2009). Dennison *et al*. (2009) reported that in the case of large gradients, amidation could disrupt the gradient to decrease antimicrobial activity.

Table 2. Sequence and biophysical properties of PZJ5 and its variants.

Peptide	Sequence	Calculated MW[a]	Observed MW[b]	Net charge	H[c]	μH[d]
PZJ5	K T K Q Q F L I K A Q T Q L F K V F G Y T L	2631.10	2631.47	+4	0.49	0.31
PZJ5-1	. . . . .	1967.38	1967.61	+3	0.53	0.39
PZJ5-2	. K	2223.72	2223.99	+5	0.36	0.41
PZJ5-3	A	1910.29	1910.51	+2	0.61	0.44
PZJ5-4	A L . . . . . L	1880.34	1880.55	+2	0.85	0.63
PZJ5-5	A . . . . K . . . . . K . . .	1906.29	1906.53	+4	0.27	0.54
PZJ5-6	A I . . . Q . . F . . . Q . . .	1910.29	1910.51	+2	0.61	0.60
PZJ5-7	A . . . . K . . . . . K . . . -NH_2[e]	1905.30	1905.33	+5	0.27	0.54

[a]Calculated molecular weight, determined using the website http://aps.unmc.edu/AP/prediction/prediction_main.php. [b]Observed molecular weight, measured by mass spectroscopy (MS). [c]Hydrophobicity, determined using the website http://heliquest.ipmc.cnrs.fr/. [d]Hydrophobic moment, determined using the website http://heliquest.ipmc.cnrs.fr/. [e]The -NH_2 at the C-terminus of sequence indicates the amidation of a carboxylate group.

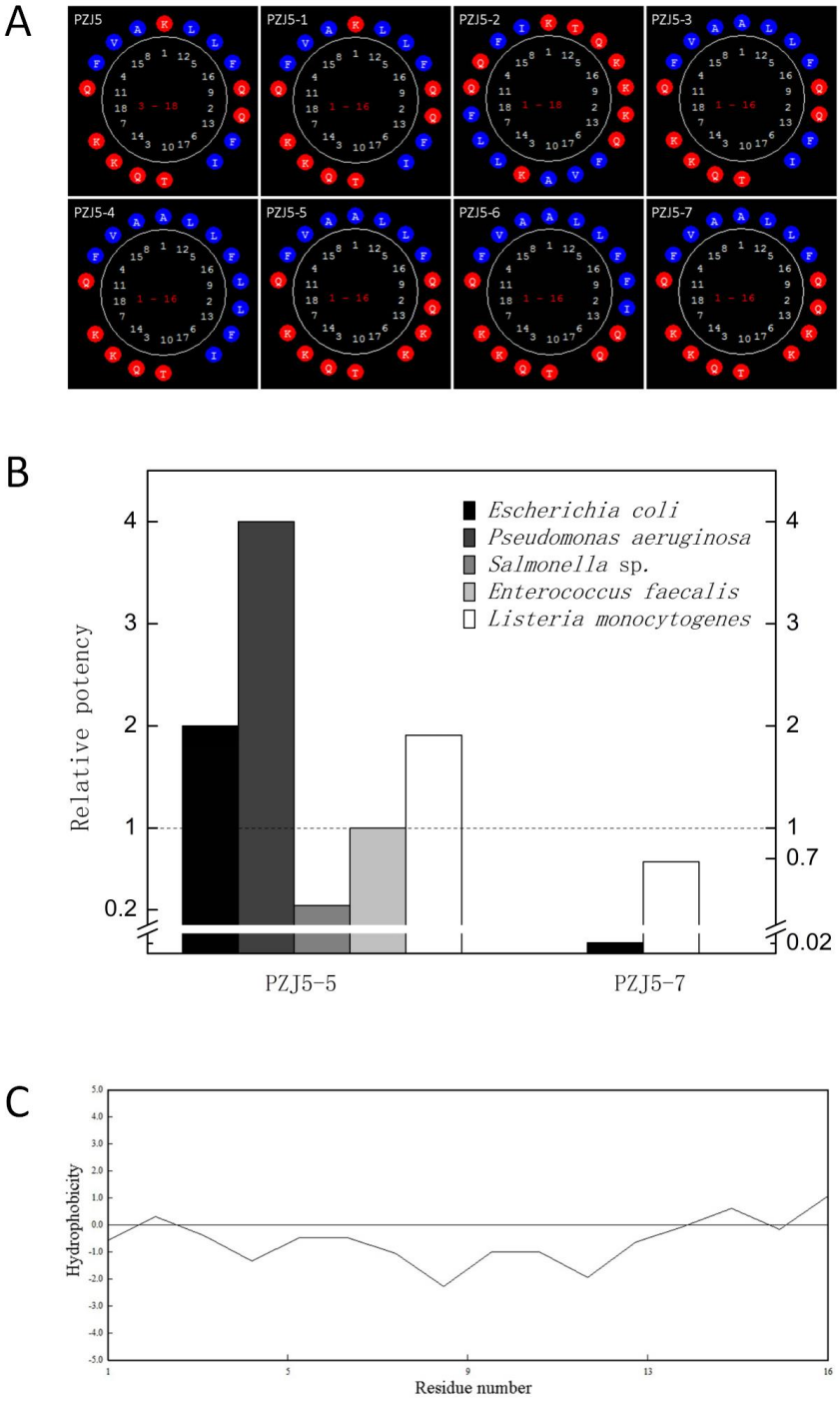

Figure 1. Properties of the derived peptides. **(A)** Helical wheel projections by ANTHEPROT6.0. Hydrophilic residues are in red, and hydrophobic residues in blue. **(B)** Relative potency of different variants (as calculated from the data in Table 3), where relative potency is defined as the MIC for PZJ5 divided by the MIC for the variant. The peptide had similar potency as PZJ5 when the relative potency was equal to 1 (indicated by the dashed line) and was more active than PZJ5 if it was > 1 (above the dashed line). Values are means. **(C)** Hydropathy plot analysis of PZJ5-5/PZJ5-7 by DNAMAN 8.

Table 3. Potency of bacteriocin PZJ5 and its variants toward various indicator bacteria.

Peptide	MIC (μM)				
	E. coli	***P. aeruginosa***	***Salmonella* sp.**	***E. faecalis***	***L. monocytogenes***
PZJ5	7.9 ± 0.7	31.7 ± 2.8	7.9 ± 1.2	7.9 ± 2.5	63.5 ± 8.6
PZJ5-5	4.2 ± 2.6	8.3 ± 1.1	33.3 ± 4.7	8.3 ± 2.5	33.3 ± 3.1
PZJ5-7	376.1 ± 63.2	NA	NA	NA	94.0 ± 0.8
PZJ5-1, PZJ5-2, PZJ5-3, PZJ5-4, PZJ5-6	NA	NA	NA	NA	NA

MIC, minimum inhibitory concentration; NA, not available. Values are mean ± standard deviation from triplicate determinations.

Haemolytic activity of PZJ5-5

The haemolytic activity of AMPs against the SRBCs was determined as a measure of their toxicity to mammalian cells (Table 4). The results showed less than 0.2% haemolysis even at the maximum concentration of 64 Mm PZJ5 or PZJ5-5, thus suggesting that the peptide was not significantly toxic to mammalian cells.

Table 4. Haemolytic activities of PZJ5-5 against sheep red blood cells.

Peptide concentration (μM)	Haemolytic activity (%)	
	PZJ5	**PZJ5-5**
4	0.14	0.14
16	0.16	0.15
64	0.19	0.17

Values are mean ± standard deviation from triplicate determinations.

Effects of bacteriocin PZJ5-5 on microstructure of E. coli

SEM and TEM revealed morphological differences between *E. coli* treated with PZJ5 or PZJ5-5, and those without PZJ5 and PZJ5-5 treatment. The untreated *E. coli* had well-distributed cytoplasm (Figure 2b) with an intact cell wall and smooth outer membrane (Figure 2a). After PZJ5 or PZJ5-5 treatment, the cells had uneven edges, wrinkled, and roughened surfaces and perforations in the cell membrane, from which cytosolic content might have leaked. In addition, some cells were severely deformed (Figure 2a). The bacteria were vacuolated (Figure 2b). These changes indicated that the peptides targeted the plasma membrane of *E. coli* and caused cytosolic leakage as a result of perforations, due to which cell metabolism could not be carried out normally, which eventually led to death of the bacteria.

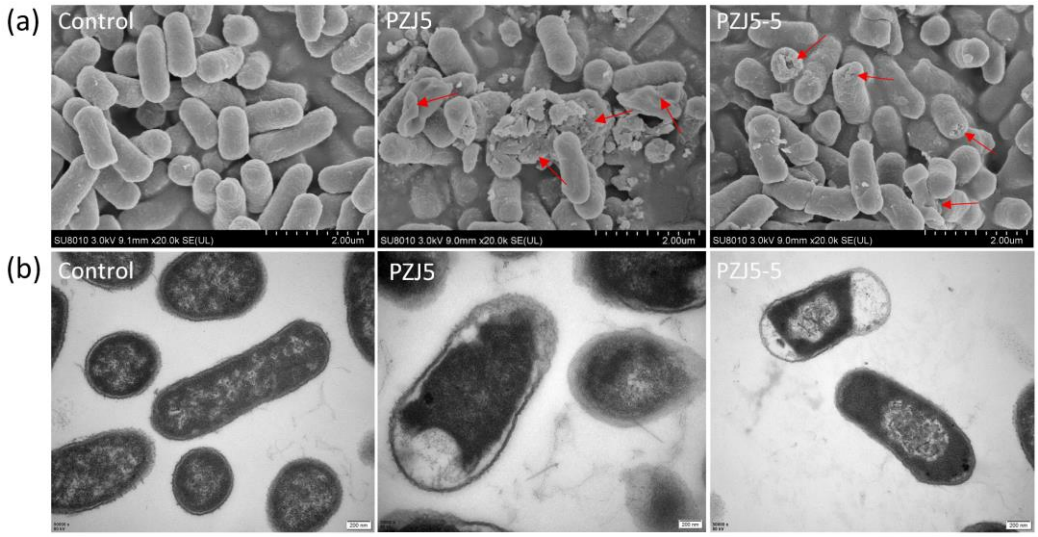

Figure 2. Effect of PZJ5-5 on the morphology of *E. coli*. **(a)** SEM, and **(b)** TEM. When compared with the control cells, 1× MIC PZJ5 or PZJ5-5 caused cell wall/membrane breakage and vacuolisation. The arrows indicate perforations on the membranes of *Escherichia coli* and the deformed cells.

PI staining

PI is a DNA stain that can pass through damaged cell membranes, and stain the nucleus (Wang *et al*., 2017). Therefore, PI was used as a fluorescent probe to detect the ability of peptides to permeate the bacterial membrane (Wang *et al*., 2017). In the present work, logarithmic phase bacterial cells were stained after incubation with PZJ5 or PZJ5-5, and normal logarithmic phase bacterial cells were used as the negative control group. Light microscopy (Figure 3a) and fluorescence microscopy (Figure 3b) showed that all bacteria were viable. The red fluorescence of *E. coli* treated with PZJ5 and PZJ5-5 (Figures 3c and 3d) suggested that the two peptides destroyed the structure and function of the bacterial cell membrane. The fluorescence of *E. coli* treated with PZJ5-5 was stronger than that with PZJ5 (Figure 3B), which correlated with the higher antimicrobial activity of PZJ5-5 against *E. coli* as compared to PZJ5.

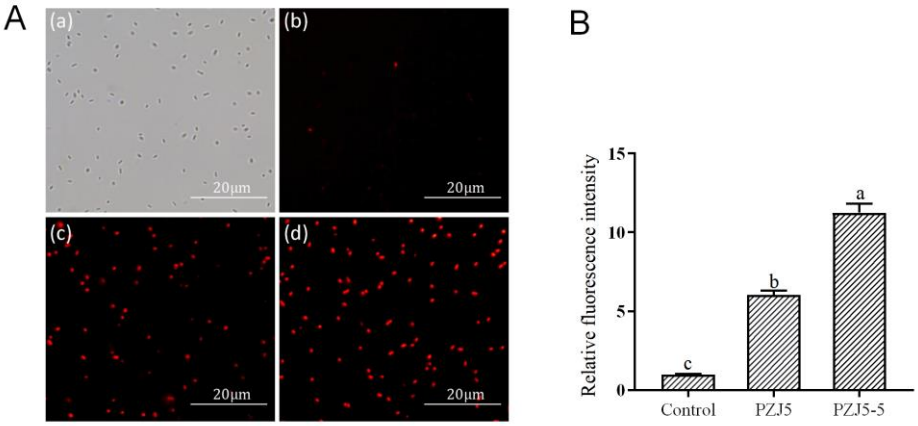

Figure 3. Effect of different peptides on membrane integrity of *E. coli*. **(A)** Fluorescence microscopy of *E. coli* treated with different peptides. **(a)** Bacteria untreated with bacteriocin (control) observed under light microscope; **(b)** bacteria untreated with bacteriocin (control) observed under fluorescence microscope; **(c)** bacteria treated with 4 μM PZJ5 observed under fluorescence microscope; **(d)** bacteria treated with 4 μM PZJ5-5 observed under fluorescence microscope. **(B)** Relative fluorescence intensity of PI after 4 μM PZJ5 or PZJ5-5 on *E. coli*. Different lowercase letters indicate statistically significant differences between groups ($p < 0.05$, respectively).

Conclusion

In the present work, we used three approaches to design the bacteriocins. A series of truncated amino-acid-substituted derivatives of PZJ5 were synthesised, and their bacteriostatic effects on three Gram-negative bacteria and two Gram-positive bacteria were evaluated. PZJ5-1, a fragment of the α-helical region of PZJ5 did not show potency, although the α-helical region is known to be vital to antimicrobial activity. Focusing on amphiphilicity, PZJ5-3, PZJ5-4, PZJ5-5, and PZJ5-6 were designed based on helical wheel projections by substituting several amino-acid residues of PZJ5-1. Only PZJ5-5 retained potency although all four peptides had greater amphiphilicity as compared to PZJ5-1. Amphipathicity was not the only element that determined the antimicrobial activities of AMPs. At its MIC, PZJ5-5 perforated the membrane of bacterial cells, thus causing cytosolic content leakage, which led to cell death. PZJ5-5 was not obviously haemolytic. PZJ5-7, the C-terminal amidated counterpart of PZJ5-5, showed weak antimicrobial activities against *E. coli* and *L. monocytogenes*. These findings provided a rationale for peptide design and clarify the relationship between the structure and function of bacteriocin PZJ5, which could help with future development of natural food preservatives.

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