



Plantaricin NC8 from *Lactobacillus plantarum* causes cell membrane disruption to *Micrococcus luteus* without targeting lipid II

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Abstract

Plantaricin NC8, a two-peptide non-lantibiotic class IIb bacteriocin composed of PLNC8 α and PLNC8 β and derived from *Lactobacillus plantarum* ZJ316, has been shown to be highly potent against a range of bacteria and fungi. In this study, we assessed the antimicrobial mechanism of plantaricin NC8 against the most sensitive bacterial strain, *Micrococcus luteus* CGMCC 1.193. The results showed that plantaricin NC8 induced membrane permeabilization and caused cell membrane disruption to *M. luteus* CGMCC 1.193 cells, as evidenced by electrolyte efflux, loss of proton motive force, and ATP depletion within a few minutes of plantaricin NC8 treatment. Furthermore, scanning and transmission electron microscopy showed that plantaricin NC8 had a drastic impact on the structure and integrity of *M. luteus* CGMCC 1.193 cells. In addition, we found that either PLNC8 α or PLNC8 β alone exhibited membrane permeabilization activity, but that PLNC8 β had higher permeabilization activity, and their individual effects were not as strong as that of the combined compounds as plantaricin NC8. Finally, we showed that lipid II is not the specific target of plantaricin NC8 against *M. luteus* CGMCC 1.193. Our study reveals the antimicrobial mechanism of plantaricin NC8 against *M. luteus* CGMCC 1.193.

Keywords Cell membrane disruption · *Lactobacillus plantarum* ZJ316 · Plantaricin NC8 · Lipid II

Introduction

Bacteriocins, which are prokaryotic peptides or proteins that exhibit inhibitory activity against other prokaryotes, are promising candidates for bio-preservatives for food preservation and antimicrobial drugs for the prevention and control of bacterial infectious diseases (Jiang et al. 2016; Zhu et al.

2014). In particular, bacteriocins produced by lactic acid bacteria (LAB) have been the focus of recent research because LAB and their metabolic products are generally regarded as safe (Valenzuela et al. 2015).

Currently, there is no international standard classification for bacteriocins. The newest classification scheme divides LAB bacteriocins into five groups: class I includes small (< 5 kDa) linear peptides containing post-translationally modified amino acids, class II includes small (< 10 kDa) linear peptides without post-translationally modified amino acids, class III includes large (> 10 kDa) proteins, class IV includes small (< 10 kDa) circular peptides without post-translationally modified amino acids and an amide bond between the N- and C-termini, and class V are small (< 5 kDa) linear or circular peptides containing extensively post-translationally modified amino acids (Coelho et al. 2017).

Plantaricin NC8, which consists of PLNC8 α (29 amino acids) and PLNC8 β (34 amino acids), is a member of class IIb bacteriocins, a subclass of class II bacteriocins that normally consists of two different peptides. Typically, the combined activity of two peptides is significantly stronger than the sum of their individual activities (Nes and Holo 2000).

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Plantaricin NC8 derived from *Lactobacillus plantarum* ZJ316 was previously isolated from fecal samples of healthy newborn infants (Li et al. 2013) and has also been found in other *L. plantarum* isolates (Maldonado et al. 2003). Plantaricin NC8 mainly acts against Gram-positive bacteria (Jiang et al. 2016; Maldonado et al. 2003) as well as some Gram-negative bacteria (Jiang et al. 2016; Khalaf et al. 2016) and fungi (Jiang et al. 2016). It has been shown that the maximum inhibitory activity against the indicator strain *L. plantarum* 128/2 was achieved at a molar ratio of PLNC8 α to PLNC8 β of 1:16 (Maldonado et al. 2003). In contrast, a molar ratio of PLNC8 α to PLNC8 β of 1:2 was found to be effective against the periodontal pathogen *Porphyromonas gingivalis* (Khalaf et al. 2016). In our previous study, molar ratios of PLNC8 α to PLNC8 β of 1:1, 1:2, 1:4, 1:8, and 1:16 exhibited similar inhibitory activities ($p > 0.05$) (Jiang et al. 2016). Thus, a molar ratio of PLNC8 α to PLNC8 β of 1:1 was used for the current study.

The antimicrobial mechanism of plantaricin NC8 is believed to involve membrane binding, followed by permeabilization of the cell membrane, causing cellular distortion through detachment of the outer membrane and bacterial lysis (Khalaf et al. 2016). Other two-peptide bacteriocins, such as PlnEF (Zhang et al. 2016), plantaricin JK (Moll et al. 1999), lactacin F (Abee et al. 1994), lactocin 705 (Cuozzo et al. 2003), lactococcin G (Moll et al. 1998), and thermophilin 13 (Marciset et al. 1997) have been reported to permeabilize the cell membrane, leading to cell death.

Interactions between bacteriocins and the target cell membrane are mainly dependent on lipids (Nicolas 2009; Zhang et al. 2016). Among them, the membrane-bound cell wall precursor lipid II mediates the transport of disaccharide-pentapeptide units from the cytoplasm to the outside of the cell, where they are incorporated into the growing peptidoglycan network (Wiedemann et al. 2006a). Additionally, lipid II of susceptible bacteria is used as a docking molecule for pore formation by antibiotics such as vancomycin, ramoplanin (Breukink and de Kruijff 2006), teixobactin (Ling et al. 2015), and copsisin (Essig et al. 2014); bacteriocins such as nisin (Breukink et al. 1999), mersacidin (Brötz et al. 1998), lactacin 3147 (Wiedemann et al. 2006a), and plantaricin C (Wiedemann et al. 2006b); and the defensins human neutrophil peptide-1 (Varney et al. 2013) and plectasin (Schneider et al. 2010). In addition, some bacteriocins act on the specific membrane proteins. For example, a class IIb bacteriocins lactococcin G and enterocin 1071 used an undecaprenyl pyrophosphate phosphatase (Kjos et al. 2014), and a class IIc bacteriocin garvicin ML used the maltose ABC transporter as the docking molecule for pore formation (Gabrielsen et al. 2012). Also, a class IIa pediocin-like bacteriocins and class IID bacteriocin lactococcin A used the mannose phosphotransferase system (Diep et al. 2007), and the other class IID bacteriocin LsbB used the Zn-dependent metallopeptidase YvjB as the docking molecule for pore formation (Uzelac et al. 2013).

Few specific membrane targets of two-peptide bacteriocins have been identified. In addition, with the exception of PlnEF, antimicrobial mechanisms of two-peptide bacteriocins have mostly been studied using membrane mimicking entities rather than bacterial cells (Zhang et al. 2016). In the current study, we aimed to explore the antimicrobial mechanism of plantaricin NC8 against the most sensitive Gram-positive bacterial strain, *Micrococcus luteus* CGMCC 1.193. The results showed that plantaricin NC8 disrupts the cell membrane of *M. luteus* CGMCC 1.193 through electrolyte efflux, loss of proton motive force (PMF), and ATP depletion within a few minutes of treatment. Plantaricin NC8 also had a drastic impact on the structure and integrity of *M. luteus* CGMCC 1.193 cells. However, we found that lipid II is not the target of plantaricin NC8 pore formation.

Materials and methods

Production and purification of plantaricin NC8

The plantaricin NC8 peptides PLNC8 α and PLNC8 β (> 98% purity) were purchased from GL Biochem Ltd. (Shanghai, China) and synthesized using the 9-fluorenylmethoxycarbonyl (F-moc) solid-phase synthesis method. The peptides were purified using C₁₈ reversed-phase high-performance liquid chromatography (Waters, USA), and the molecular masses were confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (Shimadzu, Japan). The amino acid sequences of the PLNC8 α and PLNC8 β bacteriocin loci of *L. plantarum* ZJ316 (NCBI accession no. CP004082.1) obtained from the GenBank database were DLTTKLWSSWGYYLGKKARWNLKHPYVQF and SVPTSVYTLGIKILWSAYKHKRTIEKSFNKGFYH, respectively. A 10 mM stock solution of each peptide was freshly prepared in 0.05% (*w/v*) acetic acid (HAc) and stored at $-20\text{ }^{\circ}\text{C}$ (Wiedemann et al. 2001).

Determination of minimal inhibitory concentration against the indicator strain

M. luteus CGMCC 1.193 was used as the indicator strain and was grown in tryptone soy broth medium at $37\text{ }^{\circ}\text{C}$. The minimal inhibitory concentrations (MICs) of PLNC8 α or PLNC8 β alone or in combination at a molar ratio of 1:1 as plantaricin NC8 were assessed in *M. luteus* CGMCC 1.193 using the previously described method of Jiang et al. (2017) with some modifications. Overnight cultures of *M. luteus* CGMCC 1.193 at concentrations of approximately $2\text{--}4 \times 10^6$ colony forming units (CFU)/mL were collected, and 50 μL of each culture was grown in 96-well-microtiter plates (Bio-Rad, USA). PLNC8 α , PLNC8 β , and plantaricin NC8

were diluted twofold serially, and 50 μL was added to the bacterial culture. Plates were then incubated at 37 $^{\circ}\text{C}$ for 24 h. The MIC was defined as the minimum bacteriocin concentration at which 100% of microbial growth was inhibited and was measured by the absorbance at 600 nm.

Analysis of proton motive force

PMF was assessed using the method of Jiang et al. (2017) with some modifications. Briefly, the fluorescent probe 3, 3'-diethylthiadiazolium iodide [DisC₂(5)] (Sigma-Aldrich, USA) was used to measure transmembrane electrical potential ($\Delta\Psi$). Log phase cells of *M. luteus* CGMCC 1.193 were harvested, washed twice, resuspended in buffer A (250 mM glucose, 100 mM KCl, 10 mM K₃PO₄, and 5 mM MgSO₄, pH 7.0) at 4 $^{\circ}\text{C}$, and stored on ice for fluorescence measurements. The cells were then added to a fluorescence cuvette containing 0.1 μM DisC₂(5) and the fluorescence intensity was immediately detected. Fluorescence emissions were monitored for 10 min using a Cary Eclipse spectrofluorometer (Agilent, USA) with an emission wavelength (Em) of 670 nm and an excitation wavelength (Ex) of 650 nm. When the decrease in the signal was stabilized, PLNC8 α , PLNC8 β , or plantaricin NC8 were quickly added to the cuvette to a final concentration of 1.6 μM . Full dissipation of the membrane potential was induced by the addition of 1% (w/v) Triton X-100, which has the ability to disrupt the cell membrane. 0.05% (w/v) HAc, which is used to dissolve bacteriocins, was used as a negative control.

The fluorescent pH probe 2', 7'-bis-(2-carboxyethyl)-5 (and-6) carboxyfluorescein, acetoxymethyl ester (BCECF AM; Beyotime, China) was used for transmembrane pH gradient (ΔpH) detection. Log phase cells of *M. luteus* CGMCC 1.193 were harvested, washed twice, resuspended in 5 mM HEPES buffer (Sigma-Aldrich, USA), and incubated at room temperature for 30 min. Next, 1 μM BCECF AM was added and the solution was incubated at 37 $^{\circ}\text{C}$ for 1 h in the dark. Subsequently, 1 mL of the incubated solution and PLNC8 α , PLNC8 β , or plantaricin NC8 at a final concentration of 1.6 μM were added to a fluorescence cuvette. Immediately after mixing, the fluorescence intensity was monitored for 10 min at 1 min intervals at an Ex of 488 nm and an Em of 535 nm using a Cary Eclipse spectrofluorometer.

Analysis of intracellular ATP

Intracellular ATP levels were measured using a firefly luciferase-based ATP assay kit (Beyotime, China) according to the manufacturer's instructions. Log phase *M. luteus* CGMCC 1.193 cells were treated as described for ΔpH detection. The 10 mM glucose-energized *M. luteus* CGMCC 1.193 cell suspension was then added to a 1.5 mL Eppendorf tube containing PLNC8 α , PLNC8 β , or plantaricin NC8 at a final concentration of 1.6 μM . Samples were collected by centrifugation (12,000 $\times g$,

3 min, 4 $^{\circ}\text{C}$) every 4 min for 40 min. Subsequently, 25 μL ATP-detecting lysate and 100 μL ATP-detecting working solution was added to the cell suspension. Luminance was immediately measured using a JC15-KPS-I Chemiluminescent Analyzer (Beixin Co., China).

Analysis of electric conductivity

Log phase *M. luteus* CGMCC 1.193 cells were treated as described for ΔpH detection. PLNC8 α , PLNC8 β , or plantaricin NC8 were added to the *M. luteus* CGMCC 1.193 suspension at a final concentration of 1.6 μM . Cell suspensions were then incubated at room temperature and their electric conductivity was measured every 4 min for 60 min using a DDS-307A Digital-EC-Meter (Leici Co., China).

Examination of cell morphology by microscopy

Scanning electron microscopy (SEM) was performed as previously described by Jiang et al. (2017) with some modifications. Briefly, PLNC8 α , PLNC8 β , or plantaricin NC8 were added to a *M. luteus* CGMCC 1.193 suspension (1×10^6 CFU/mL) to a final concentration of 1.6 μM and incubated at 37 $^{\circ}\text{C}$ for 10 min. Cells with no bacteriocins added were used as the control. Next, cells were harvested by centrifugation (6000 $\times g$, 5 min, 4 $^{\circ}\text{C}$) and gently washed twice with 500 μL PBS (0.1 M, pH 7.4). Cells for SEM scanning were fixed in 2.5% glutaraldehyde at 4 $^{\circ}\text{C}$ for 16 h, gently washed twice with 500 μL PBS, dehydrated with an ethanol gradient (30, 50, 70, 80, 90, and 100%), and centrifuged (6000 $\times g$, 15 min, 4 $^{\circ}\text{C}$). The cells were then freeze-dried, coated with gold, and imaged using an SU8010 Scanning Electron Microscope (Hitachi, Japan).

For transmission electron microscopy (TEM), control and bacteriocin-treated cells were harvested and processed for fixation as described for SEM. After washing, the cells were post-fixed in 1% OsO₄ at 4 $^{\circ}\text{C}$ for 3 h and gently washed twice with 500 μL PBS. The cells were dehydrated with an ethanol gradient followed by two washes with acetone for dehydration. The fixed cells were embedded in epoxy resin and polymerized at 65 $^{\circ}\text{C}$ for 48 h. Ultrathin sections (70 nm thick) were prepared using an ultramicrotome (LKB Co., Sweden). The sections were then stained with uranyl acetate and lead citrate for 10 min each and gently washed with distilled water. Cell morphology was examined using a 7650 Transmission Electron Microscope (Hitachi).

Analysis of interactions between plantaricin NC8 and lipid II

The lipid II synthesis and purification assay was provided by Breukink (2003) and purified lipid II was stored in chloroform:methanol (1:1 v:v) at -20 $^{\circ}\text{C}$.

First, the agar-well diffusion test was used to analyze the interactions between plantaricin NC8 and lipid II according to the previously described protocol of Jiang et al. (2016) with some modifications. Briefly, 1 mL of log phase *M. luteus* CGMCC 1.193 cells were mixed with 100 mL semi-solid culture and poured onto Petri dishes. Then, under sterile conditions, four wells (8 mm diameter) were punched into the plates and filled with either (1) 50 μ L of 10 μ M plantaricin NC8, (2) 50 μ L of 10 μ M plantaricin NC8 mixed with 20 μ M lipid II, (3) 50 μ L of 10 μ M nisin, or (4) 50 μ L of 10 μ M nisin mixed with 20 μ M lipid II. Plates were incubated overnight at 37 °C, and the clear zone of inhibition, if observed, was considered to indicate the presence of antimicrobial activity.

Second, the $\Delta\Psi$ in the membrane interior of *M. luteus* CGMCC 1.193 was used to analyze the interactions between plantaricin NC8 and lipid II. When the decrease in the signal was stabilized, a final concentration of 0.8 μ M plantaricin NC8, 1.6 μ M lipid II in combination with 0.8 μ M plantaricin NC8, 0.8 μ M nisin, or 1.6 μ M lipid II in combination with 0.8 μ M nisin was added to the cuvette respectively. The negative control was 0.05% (*w/v*) HAC.

Statistical analysis

All experiments were performed in triplicate. Results are expressed as mean \pm standard deviation. Data analysis was performed using SPSS 19.0 (IBM Corp., USA) and Origin 8.0 (OriginLab, USA). Independent sample *t* test *p* values < 0.05 were considered statistically significant.

Results

Analysis of proton motive force, intracellular ATP, and electric conductivity

The MICs against *M. luteus* CGMCC 1.193 of PLNC8 α or PLNC8 β alone or in combination at a molar ratio of 1:1 as plantaricin NC8 were determined to be 3.2, 1.6, and 0.8 μ M, respectively, suggesting that PLNC8 α and PLNC8 β alone possessed independent activity but were most active in combination. In further experiments evaluating $\Delta\Psi$, Δ pH, intracellular ATP, and extracellular electrolytes, a final concentration of twice the MIC of plantaricin NC8 (1.6 μ M) was used.

PMF consists of $\Delta\Psi$ and Δ pH (Zhang et al. 2016). The membrane potential-sensitive fluorescent probe DisC₂(5) was used for $\Delta\Psi$ detection (Fig. 1a). Stabilization of the decrease in fluorescence intensity of DisC₂(5) indicated that the dye that accumulated in the membrane interior of energized cells was strongly quenched. After a stable signal was observed, the addition of 1.6 μ M PLNC8 α , PLNC8 β , or plantaricin NC8 (indicated by the first arrow in Fig. 1a) caused a rapid increase in fluorescence due to the collapse

of the ion gradients that generate the membrane potential (van Kan et al. 2002). Once the fluorescence signal stabilized, the addition of 1% (*w/v*) Triton X-100 (indicated by the second arrow in Fig. 1a), which can fully collapse the membrane potential, caused a small increase in the fluorescence of cells treated with PLNC8 α or PLNC8 β but almost no change in the fluorescence of cells treated with plantaricin NC8. These results suggest that plantaricin NC8 caused nearly 100% cell membrane permeabilization and that the cell membrane permeabilization ability of PLNC8 β was higher than that of PLNC8 α . The fluorescence of the negative control sample, to which 0.05% (*w/v*) HAC was added, did not change during the 10-min experiment.

The pH probe BCECF AM was used for Δ pH detection (Fig. 1b). When BCECF AM is absorbed into the cell membrane, it is cleaved by an esterase into BCECF, a fluorescent Δ pH probe (Jiang et al. 2017). As shown in Fig. 1b, when 1.6 μ M plantaricin NC8 was added to the sample, the fluorescence intensity of BCECF increased immediately within 2 min and became slower increase or stable at later time points, indicating that the Δ pH of *M. luteus* CGMCC 1.193 can be rapidly dissipated by plantaricin NC8. The final fluorescence intensity of the cells treated with 1.6 μ M plantaricin NC8 was as high as that of cells exposed to 1% (*w/v*) Triton X-100, indicating that Δ pH was almost completely dissipated by 1.6 μ M plantaricin NC8. However, the final fluorescence of the cells treated with 1.6 μ M PLNC8 α or PLNC8 β was lower than that of the cells exposed to 1.6 μ M plantaricin NC8. Also, 0.05% (*w/v*) HAC added did not cause any change in fluorescence.

As shown in Fig. 1c, the level of intracellular ATP remained almost stable when cells were exposed to 0.05% (*w/v*) HAC. In comparison, fluorescence intensity values decreased significantly when cells were treated with 1.6 μ M PLNC8 α , PLNC8 β , or plantaricin NC8 or with 1% (*w/v*) Triton X-100 (*p* < 0.05). Fluorescence intensity values reached their minimum at around 12 min. The final intracellular ATP level of cells treated with 1.6 μ M plantaricin NC8 was lower than that of cells treated with 1.6 μ M PLNC8 α or PLNC8 β alone, suggesting that the two-peptide bacteriocin inhibited intracellular ATP synthesis to a greater degree than either bacteriocin alone.

As shown in Fig. 1d, in contrast to cells exposed to 0.05% (*w/v*) HAC, the electric conductivity of the cell suspension treated with bacteriocins or 1% (*w/v*) Triton X-100 increased dramatically within 4 min of treatment and then almost kept stable with 1.6 μ M PLNC8 α , plantaricin NC8, and 1% (*w/v*) Triton X-100 respectively or increased slightly with 1.6 μ M PLNC8 β up to 60 min. The increase in electric conductivity was greater in cells treated with 1.6 μ M plantaricin NC8 than in those treated with 1.6 μ M PLNC8 α or PLNC8 β alone, suggesting that the two-peptide bacteriocin induced more leakage of electrolytes than either bacteriocin alone.

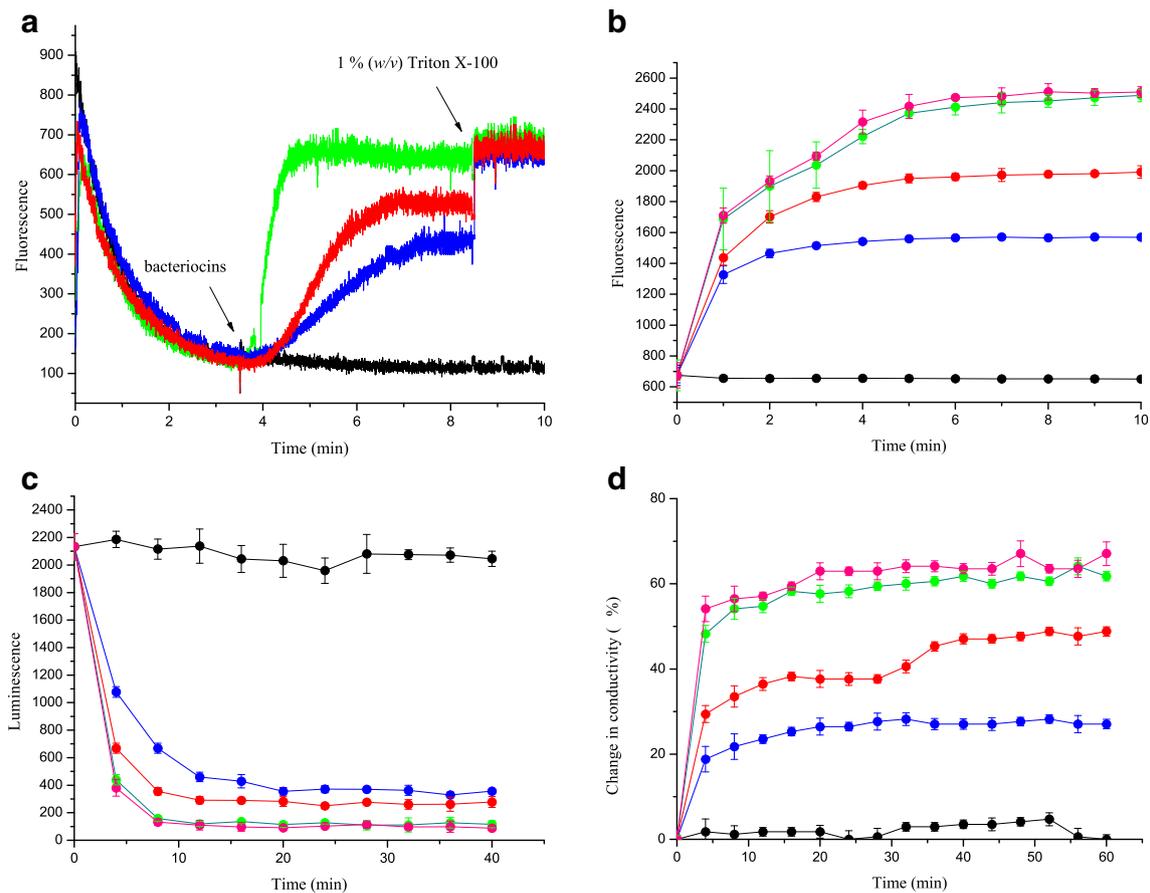


Fig. 1 Analysis of **a** $\Delta\Psi$, **b** ΔpH , **c** intracellular ATP, and **d** electric conductivity of *M. luteus* CGMCC 1.193 cells treated with 1.6 μM PLNC8 α (blue line), PLNC8 β (red line), or plantaricin NC8 (green line). The negative control was 0.05% (*w/v*) HAc (black line). The

positive control was 1% (*w/v*) Triton X-100 (Fig. 1b–d, pink line). In Fig. 1a, the addition of 1% (*w/v*) Triton X-100 is indicated by the second arrow

Examination of cell morphology by microscopy

SEM and TEM were used to further demonstrate membrane damage to *M. luteus* CGMCC 1.193 cells caused by 1.6 μM PLNC8 α , PLNC8 β , or plantaricin NC8.

Morphological changes in *M. luteus* CGMCC 1.193 cells after 10 min of exposure to the bacteriocins are presented in Fig. 2.

Under SEM, in contrast to the smooth surface of the control cells (Fig. 2a, b), bacteriocin-treated cells were

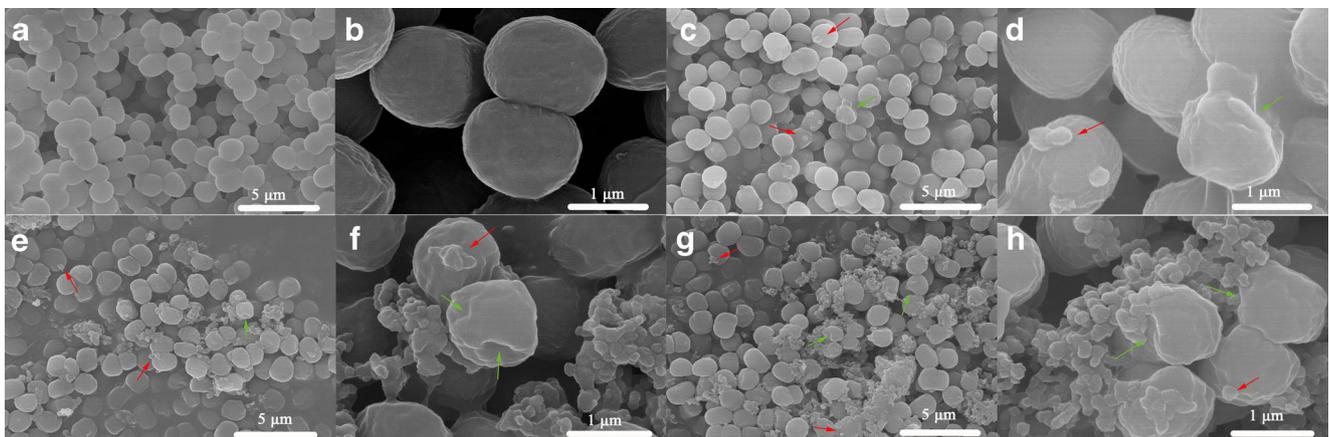


Fig. 2 Scanning electron micrographs of *M. luteus* CGMCC 1.193 cells either untreated (**a**, **b**) or treated with 1.6 μM PLNC8 α (**c**, **d**), PLNC8 β (**e**, **f**), or plantaricin NC8 (**g**, **h**) for 10 min

disrupted and appeared deformed and shrunken with cavities visible on their surfaces (Fig. 2c–h, green arrows). In addition, blebs (Fig. 2c–h, red arrows) protruded into the cell surface, demonstrating that bacteriocins act on the cell surface. Maximum damage, including disruption, deformation, and the appearance of blebs, was observed in 1.6 μM plantaricin NC8-treated cells, many of which were completely lysed (Fig. 2g, h). In contrast, only a few blebs and wrinkles were visible in 1.6 μM PLNC8 α -treated cells (Fig. 2c, d).

Under TEM, the untreated control cells exhibited a typical cell wall and a smooth and intact cell membrane. The entocyte was dense and full without any defects (Fig. 3a, b). After exposure to 1.6 μM PLNC8 α , PLNC8 β , or plantaricin NC8 for 10 min, abnormal septation (Fig. 3c, yellow arrow), an irregular cell wall (Fig. 3d, green arrow), discontinuity and disruption of the cell membrane (Fig. 3c, f, red arrows), a deformed nucleus, extensive shrinking of the cytoplasmic contents, and vacuolization (Fig. 3e, blue arrows) were observed.

Analysis of interactions between plantaricin NC8 and lipid II

As shown in Fig. 4, clear zones of inhibition with similar diameters were observed upon the addition of plantaricin NC8 either with or without excess lipid II. However, when

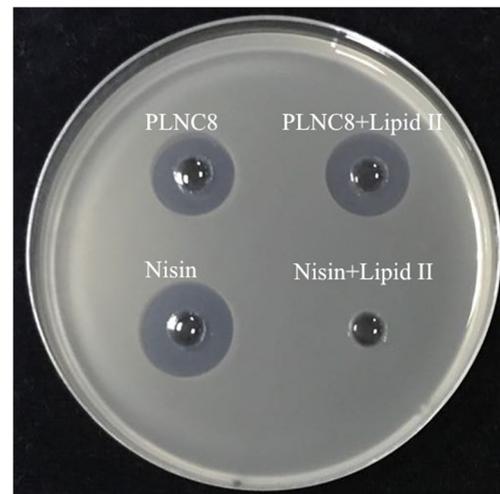


Fig. 4 Agar-well diffusion test of plantaricin NC8 with or without the addition of excess lipid II. Nisin was used as a positive control

nisin was mixed with excess lipid II, the clear zone almost disappeared.

As shown in Fig. 5a, the final fluorescence intensity was significantly lower after the addition of 1.6 μM lipid II in combination with 0.8 μM nisin (Fig. 5a, blue line) than after the addition of 0.8 μM nisin alone (Fig. 5a, red line; $p < 0.05$). In contrast, there was no obvious change in the final fluorescence intensity after the addition of 0.8 μM plantaricin NC8 (Fig. 5b, red line) or 1.6 μM lipid II in combination with

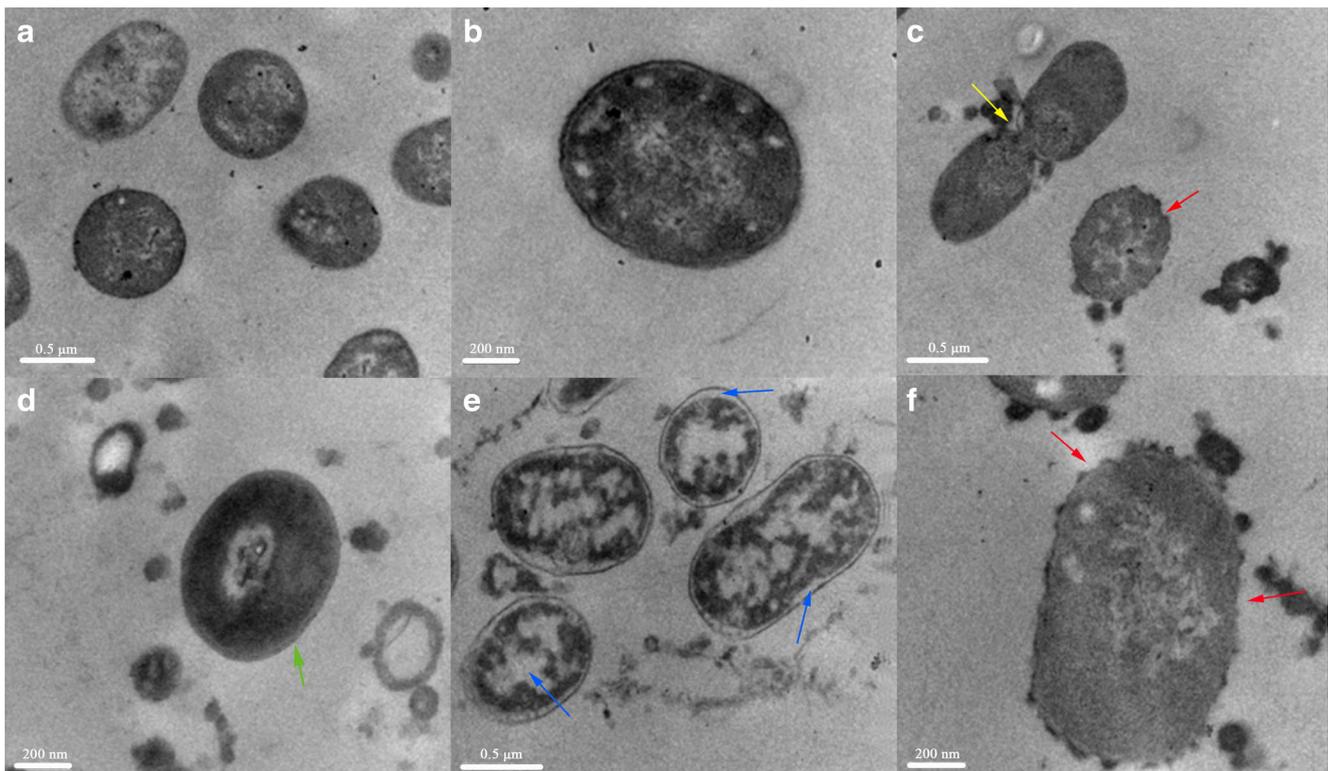


Fig. 3 Transmission electron micrographs of *M. luteus* CGMCC 1.193 cells either untreated (a, b) or treated with 1.6 μM PLNC8 α (c), PLNC8 β (d), or plantaricin NC8 (e, f) for 10 min

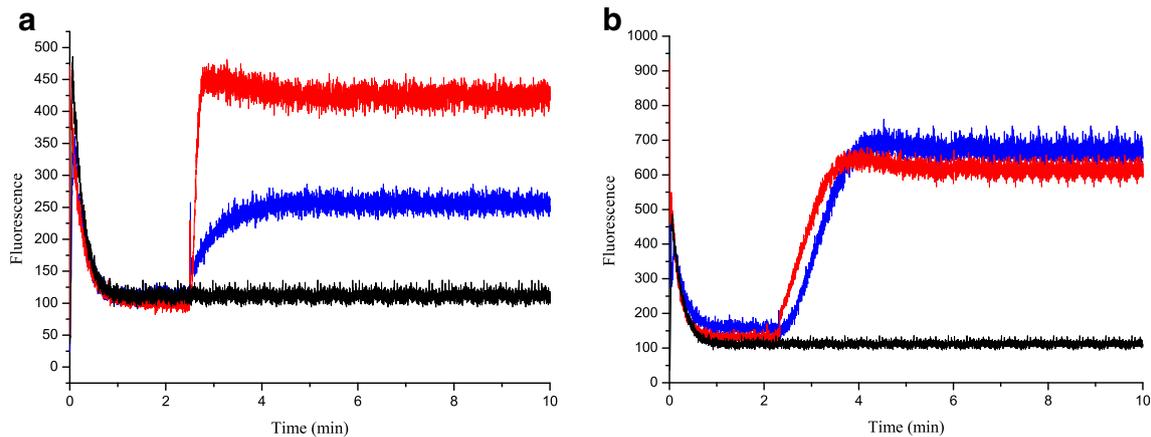


Fig. 5 Analysis of the $\Delta\Psi$ of *M. luteus* CGMCC 1.193 cells treated with **a** 0.8 μM nisin (red line) or 1.6 μM lipid II in combination with 0.8 μM nisin (blue line) and **b** 0.8 μM plantaricin NC8 (red line) or 1.6 μM lipid

II in combination with 0.8 μM plantaricin NC8 (blue line). The negative control was 0.05% (*w/v*) HAc (black line)

0.8 μM plantaricin NC8 (Fig. 5b, blue line). The addition of 0.05% (*w/v*) HAc as a negative control did not result in any changes to the fluorescence intensity.

Discussion

Anionic cell membranes of microorganisms are the first barrier to cationic bacteriocins. Therefore, many known bacteriocins kill their target microorganisms via permeabilization of the cell membrane (Abee et al. 1994; Cuozzo et al. 2003; Marciset et al. 1997; Moll et al. 1998, 1999; Roces et al. 2012; Zhang et al. 2016). In this study, the membrane permeabilization action of plantaricin NC8 against *M. luteus* CGMCC 1.193 was revealed. Plantaricin NC8 dissipated the $\Delta\Psi$ and ΔpH , which constitute PMF, of *M. luteus* CGMCC 1.193 via membrane permeabilization and subsequent electrolyte efflux. Dissipation of the $\Delta\Psi$, ΔpH , and PMF of *M. luteus* CGMCC 1.193 resulted in inhibition of intracellular ATP, leading to cell death. Our results suggest that the components of plantaricin NC8, PLNC8 α , and PLNC8 β , have some activity against *M. luteus* CGMCC 1.193 when applied individually but are only fully active when applied in combination. Such synergistic effects have been observed for many other two-peptide bacteriocins, typically at a molar ratio of components of 1:1 (Nissen-Meyer et al. 2010).

The electrolyte efflux rate dramatically increased within 4 min of bacteriocin addition, which may have been related to PMF dissipation. Plantaricin NC8 inserted into the target cell membrane to form ion-permeable pores. Prior to saturation, as plantaricin NC8 levels increased, more pores formed resulting in an increasing electrolyte efflux rate and an increasing PMF dissipation level. By around 2 min, PMF was nearly entirely dissipated. Subsequently, intracellular ATP synthesis was almost completely inhibited by 12 min. Similar results

have been found for PlnEF, another two-peptide bacteriocin (Zhang et al. 2016). The fact that the dissipation of $\Delta\Psi$ and ΔpH was nearly complete within 2 min shows that the membrane permeabilization caused by plantaricin NC8 is a rapid process. This is in accordance with other antibiotic peptides that target the membrane, such as the well-known antibiotic peptides clavamin (Van Kan et al. 2002) and pentocin JL-1 (Jiang et al. 2017). However, some membrane-targeted peptides cause gradual dissipation of membrane potential, including lactocin 705 (Castellano et al. 2003), pentocin 31-1 (Zhou et al. 2008), and PlnEF (Zhang et al. 2016). We also found that PLNC8 α or PLNC8 β alone had membrane permeabilization activity, with PLNC8 β having a more pronounced effect. However, at the same concentration of plantaricin NC8 (twice the MIC), neither PLNC8 α nor PLNC8 β caused complete cell membrane permeabilization of *M. luteus* CGMCC 1.193.

SEM and TEM were used to further demonstrate the membrane-damaging effects of plantaricin NC8 on *M. luteus* CGMCC 1.193 cells. Morphological changes in the ultrastructure of *M. luteus* CGMCC 1.193 cells were observed after a 10-min exposure to 1.6 μM plantaricin NC8, PLNC8 α , or PLNC8 β . Disruption, deformation, shrinkage, the appearance of cavities on the cell surface, and cell lysis were observed using SEM. These are typical characteristics of cell membrane damage caused by bacteriocins (Zhang et al. 2016). Similar membrane damage has been reported as a result of nisin, pediocin, and PlnEF treatment (Kalchayanand et al. 2004; Pattanayaying et al. 2014; Zhang et al. 2016). Moreover, the formation of blebs, which are a type of extracellular vesicle, induced by external stimuli might play a crucial role in cell-cell communication (Zhang et al. 2016). In addition, discontinuity and disruption of the cell membrane, abnormal septation, irregular cell walls, deformed nuclei, extensive shrinking of cytoplasmic contents, and vacuolization were observed using TEM, all of which might be attributed to membrane damage caused by plantaricin NC8 treatment.

Finally, we studied the interactions between plantaricin NC8 and lipid II. Nisin, a well-known lipid II target bacteriocin, forms complexes with lipid II at a ratio of 8:4 during the pore formation process (Breukink and de Kruijff 2006). As shown in Fig. 5a, when excess outer lipid II combines with nisin, the ability of nisin to target the inner lipid II of *M. luteus* CGMCC 1.193 cells is affected. Thus, the pore-forming ability of nisin is reduced, leading to a decrease in the $\Delta\Psi$ of *M. luteus* CGMCC 1.193 cells. However, Fig. 5b shows that the $\Delta\Psi$ of *M. luteus* CGMCC 1.193 cells was similar when treated with plantaricin NC8 with or without the addition of excess outer lipid II. Therefore, lipid II is not the target of plantaricin NC8 pore formation. There might be other potential mechanisms how plantaricin NC8 induces membrane permeability, i.e., via specific membrane proteins. Thus whole-genome sequencing of spontaneous plantaricin NC8-resistant mutants can be used to identify a possible specific membrane receptor of plantaricin NC8 (Kjos et al. 2014). Furthermore, it has been reported that the synergistic effect of these two-peptide bacteriocins might be related to helix-helix interactions involving GxxxG motifs, which are necessary for membrane permeabilization (Zhang et al. 2016). In our two-peptide plantaricin NC8, only PLNC8 α has a GxxxG motif but with the weakest antimicrobial activity. Thus whether this motif is relevant for the mutual interaction of PLNC8 α and PLNC8 β should be further discussed.

In conclusion, the present study demonstrated that the two-peptide bacteriocin plantaricin NC8 from *L. plantarum* ZJ316 caused cell membrane disruption of *M. luteus* CGMCC 1.193, as evidenced by electrolyte efflux, loss of PMF, and ATP depletion within a few minutes of treatment. Additionally, we showed that plantaricin NC8 has a drastic impact on the structure and integrity of *M. luteus* CGMCC 1.193 cells but does not target lipid II. In the future, the interplay and synergistic effect of PLNC8 α and PLNC8 β will be studied. The specific targets of plantaricin NC8 leading to permeation of the cell membrane will also be further researched.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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