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Antibacterial activity and mechanism of bifidocin A against *Listeria monocytogenes*

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ABSTRACT

Bifidocin A, produced by *Bifidobacterium animalis* BB04, is a novel bacteriocin with antimicrobial activity against a wide range of gram-positive and gram-negative foodborne bacteria. The objective of this study was to investigate the antibacterial activity and mechanism of action of bifidocin A against *Listeria monocytogenes*, one of the most susceptible bacteria to this bacteriocin. The minimum inhibitory concentration (MIC) of bifidocin A for *L. monocytogenes* 35152 was 0.029 mg/mL. Time-kill assays showed that bifidocin A effectively inhibited the growth of *L. monocytogenes* in a time-and concentration-dependent manner. The mechanism of action of bifidocin A was studied by analyzing its effects at a MIC on the cell morphology, intracellular organization, membrane permeability, membrane integrity, and membrane proton motive force (PMF) of *L. monocytogenes*. Scanning and transmission electron microscopy analyses showed that bifidocin A induced alterations in the morphology and intracellular organization of *L. monocytogenes* cells. Confocal laser scanning microscopy images showed that *L. monocytogenes* cells treated with bifidocin A took up propidium iodide. Bifidocin A treatment also induced the leakage of K^+ and inorganic phosphate, the hydrolysis and release of ATP, and a collapse of the transmembrane electrical potential and pH gradient in *L. monocytogenes* cells. These results suggested that bifidocin A exerted its anti-*Listeria monocytogenes* effect through the dissipation of the cytoplasmic membrane PMF, increased membrane permeability, cell membrane pore formation, destruction of membrane integrity, and ultimately complete disintegration of the cells.

1. Introduction

Listeria monocytogenes is a gram-positive food-borne pathogen responsible for many food outbreaks worldwide. One of its remarkable features is the ability to survive and grow in adverse conditions, such as high salt concentration, and low pH and temperature. These flexible growth conditions enhance its potential as a contaminant of food products, even after long periods of refrigeration (McLauchlin, Mitchell, Smerdon, & Jewell, 2004). In spite of the various modern technologies and safety concepts such as HACCP, the control of this pathogen remains a major problem in food industry (Kramarenko et al., 2016).

Bacteriocins are antimicrobial peptides with variable antimicrobial spectrum, which are ribosomally synthesized by bacteria. Numerous reviews have suggested that some bacteriocin produced by lactic acid bacteria (LAB) were able to control the growth of food-borne pathogens and improve the safety of food products (Cleveland, Montville, Nes, & Chikindas, 2001; Galvez, Abriouel, Lopez, & Omar, 2007). During the last few years, a large number of new anti-*Listeria* LAB-bacteriocins have been identified, purified and characterized (Bendali, Gaillard-Martinie, Hebraud, & Sadoun, 2008; Gao, Jia, Gao, & Tan, 2010; Lu, Yi, Dang, Dang, & Liu, 2014).

An understanding about the mode of action of these bacteriocins against pathogens is important to ascertain their effective application as biopreservatives in the food industry (Moll, Konings, & Driessen, 1999). In general, most bacteriocins produced from

Abbreviations: BCECF, 2′,7′-bis-(2-carboxyethyl)5(and-6)-carboxyfluorescein; CLSM, confocal laser scanning microscopy; $DISC_3(5)$, 3,3-dipropylthia-dicarbocyanine iodide; LAB, lactic acid bacteria; MRS, de Man Rogosa and Sharpe; MIC, minimum inhibitory concentration; PI, propidium iodide; PMF, proton motive force; SEM, scanning electron microscopy; Δ;ψ, transmembrane electrical potential; TEM, transmission electron microscopy; Δ;pH, transmembrane pH gradient; TS, Tryptic Soy.

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Lactococcus, *Pediococcus* and *Lactobacillus* species appear to share a common mechanism of action against gram-positive bacteria. Specifically, they act on the cytoplasmic membrane and dissipate the proton motive force (PMF) through the formation of pores in the phospholipid bilayer, releasing intracellular proteins, nucleic acids, and ions (Hechard & Sahl, 2002). However, some differences have been observed between various antimicrobial systems (Bauer, Chikindas, & Dicks, 2005; Bendali et al., 2008; Castellano, Raya, & Vignolo, 2003; Zhou et al., 2008). To date, only a few bacteriocins from bifidobacteria have been reported, such as bifidocin B (Yildirim & Johnson, 1998), thermophilic in B67 (Uelivon, 2006), bifidin I (Cheikhyoussef et al., 2010) and bifidocin A (Liu, Ren, Song, Wang, & Sun, 2015). These bacteriocins have been purified to homogeneity and characterized (Martinez, Balciunas, Converti, Cotter, & de Souza Oliveira, 2013), however, their antibacterial mechanism has been seldom studied.

Bifidocin A, a novel 1198.68 kDa bacteriocin, is produced by *Bifidobacterium animalis* BB04, and exhibits inhibitory activity against a broad range of gram-positive and gram-negative food-borne spoilage and pathogenic bacteria such as *L. monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, as well as some yeast (Liu et al., 2015). In previous work, the production, purification, characterization and antibacterial potential of bifidocin A and its mode of action against *E. coli* have already been established in our lab (Liu et al., 2015, 2016).

The current study was designed to investigate the antibacterial activity and mechanism of action of bifidocin A against *L. monocytogenes* ATCC 35152, one of the most susceptible bacteria to this bacteriocin. The anti-*Listeria monocytogenes* activity of bifidocin A was evaluated by measuring the minimum inhibitory concentration (MIC) value and kill-time curves. The mechanism of action of bifidocin A was studied by analyzing its influence on the cell morphology, intracellular organization, membrane permeability, membrane integrity, and membrane PMF of *L. monocytogenes* 35152.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The bifidocin A producer *B. animalis* BB04 was isolated from a healthy centenarian's feces. This individual inhabited the Layisu longevity villages in the fourth macrobian district in Xinjiang, Uygur, an autonomous region of China (Liu et al., 2015). *B. animalis* BB04 was cultured in de Man Rogosa and Sharpe (MRS) medium (LuQiao, Beijing, China) supplemented with 0.05% (w/v) L-cysteine hydrochloride (Sigma-Aldrich, Shanghai, China) (MRS-C), and incubated anaerobically at 37 °C. *L. monocytogenes* 35152, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), was grown in Tryptic Soy (TS) broth (LuQiao, Beijing, China) supplemented with 0.6% (w/v) yeast extract (LuQiao, Beijing, China) (TSYE) at 37 °C, and used as the indicator strain to demonstrate and measure bacteriocin activity. Both bacteria were stored at −80 °C in MRS-C and TSYE broth, respectively, containing 15% (v/v) glycerol.

2.2. Purified bifidocin A preparation

Bifidocin A was purified from the supernatant of *B. animalis* BB04 using a four-step procedure consisting of adsorption and desorption onto/from producer cells, SP-Sepharose fast flow cation-exchange, Sephadex G10 gel filter chromatography, and reverse phase high-performance liquid chromatography, as described previously (Liu et al., 2015). Approximate 0.185 g of purified bifidocin A can be obtained from each 1000 mL cultures of *B. animalis* BB04. The specific activity of purified bacteriocin was 5535 arbitrary units (AU) /mg and one AU was defined as the reciprocal of the highest serial two-fold dilution showing a clear zone of growth inhibition of *L. monocytogenes* 35152.

2.3. Antibacterial activity assay

2.3.1. Determination of the MIC of bifidocin A against *L. monocytogenes* 35152

The MIC of bifidocin A against *L. monocytogenes* 35152 was determined as described by Lu et al. (2014), with minor modifications. Briefly, purified bifidocin A was first dissolved in 5 mM HEPES buffer (pH 7.0) and incorporated into TSYE medium to obtain a concentration of 1.85 mg/mL, and then serially diluted two-fold to achieve 1.85, 0.925, 0.463, 0.232, 0.116, 0.058, 0.029 and 0.015 mg/mL, respectively. Finally, 50 μL of bacterial suspensions (approximately 10^6 cells/mL) of *L. monocytogenes* 35152 were added in each test tube. All tubes were incubated at 37 °C for 24 h. Bacterial growth was monitored by counting viable cells on TSYE agar plates and all experiments were performed in triplicate. The MIC was defined as the lowest concentration of bifidocin A at which the growth of the indicator strain was inhibited completely.

2.3.2. Effect of bifidocin A on the viability of *L. monocytogenes* 35152

The time-kill curves test was performed based on the method of Bendali et al. (2008) to determine the influence of bifidocin A on the viability of *L. monocytogenes* 35152. Cultures of tested bacteria with a density of approximately 10^6 cells per ml were exposed to purified bacteriocin sample dilutions with the final concentrations ranging from 1 × MIC to 4 × MIC. Bacterial growth was monitored at appropriate times by counting viable cells on TSYE agar (LuQiao, Beijing, China) plates. *L. monocytogenes* 35152 grown in the absence of bifidocin A was used as a control.

2.4. Antibacterial mechanism

2.4.1. Preparation of *L. monocytogenes* 35152 cell suspensions for bifidocin A treatments

L. monocytogenes 35152 was grown in TSYE broth at 37 °C for 16 h. Cells were then harvested by centrifugation (8000 *g*, 10 min, 4 °C) and resuspended to approximately 10^6 cells/mL in 5 mM HEPES buffer (pH 7.0) supplemented with 10 mM glucose. Bifidocin A was added to the cell suspensions to obtain final concentrations of 1 × MIC, and then incubated at 37 °C for 3 h. Cells were monitored for changes in morphology and intracellular organization, as well as cell membrane permeability, integrity, and PMF. Cells suspensions without bifidocin A were used as controls.

2.4.2. Effect of bifidocin A on cell morphology and intracellular organization

Scanning electron microscopy (SEM) was used to observe the morphological changes in *L. monocytogenes* 35152 cells treated with bifidocin A (Yi et al., 2016). Control and bacteriocin-treated cell pellets were resuspended and fixed for 4 h in 2.5% (v/v) glutaraldehyde in 5 mM HEPES buffer (pH 7.0) at 4 °C. After the primary fixation, the suspended cells were post-fixed for 1 h with 1% osmium tetroxide in sodium cacodylate buffer (pH 7.4). Then the samples were dehydrated in a sequential graded ethanol (30%, 50%, 80%, 90%, 100%) and the ethanol was then replaced by 100% tertiary butyl alcohol. Finally, all samples were sputter-coated with gold in an ion coater for 2 min, followed by microscopic examinations by using a scanning electron microscope (S-4800, Hitachi Instruments Inc., Tokyo, Japan).

Transmission electron microscopy (TEM) was used to observe changes in the intracellular organization of *L. monocytogenes*