Food Control 84 (2018) 442–448

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Antibacterial and antibiofilm activity of phenyllactic acid against *Enterobacter cloacae*

Fang Liu [a], Fengting Wang [a], Lihui Du [b, *], Tong Zhao [c], Michael P. Doyle [c], Daoying Wang [a], Xinxiao Zhang [a], Zhilan Sun [a, **], Weimin Xu [a]

[a] Institute of Agricultural Products Processing, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, China
[b] College of Food Science and Engineering, Collaborative Innovation Center for Modern Grain Circulation and Safety, Key Laboratory of Grains and Oils Quality Control and Processing, Nanjing University of Finance and Economics, Nanjing, 210023, China
[c] Center for Food Safety, College of Agriculture and Environmental Sciences, University of Georgia, 1109 Experiment Street, Griffin, GA, 30223, USA

ARTICLE INFO

Article history:
Received 20 June 2017
Received in revised form
1 September 2017
Accepted 2 September 2017
Available online 4 September 2017

Keywords:
Phenyllactic acid
Biofilm
Enterobacter cloacae
Cell damage

ABSTRACT

Previous studies revealed that 3-Phenyllactic acid (PLA) was an antimicrobial compound with broad-spectrum activity. In this study, the efficacy of PLA to inactivate *Enterobacter cloacae* planktonic cells, biofilms, and its antibacterial mechanism were evaluated. Results indicated that 1% PLA reduced *E. cloacae* populations by about 6 log CFU/ml for planktonic cells and by 2.83 log CFU/ml for biofilm cells after 10-min treatments. TEM images revealed the damaged cell structure and leakage of the intracellular components. The permeability of the cytoplasmic membrane of *E. cloacae* was increased when the treated PLA concentrations was raised. Flow cytometry analysis further revealed that almost all the bacterial cells were damaged after treatment with 1% PLA for 30 min. The damages on bacterial cell membrane in the biofilms caused by 1% PLA treatment were also observed by confocal laser scanning microscopy analysis. These findings revealed that PLA was effective in inactivating planktonic and biofilm (or biofilm-associated) *E. cloacae* cells mainly by the mechanisms of cell membrane damage and leakage of the intracellular components.

1. Introduction

Enterobacter cloacae is part of the normal flora of the gastrointestinal tract of 40%–80% of humans and domestic animals, and is widely distributed in the environment (Nyenje, Green, & Ndip, 2013). *E. cloacae* is also an important nosocomial opportunistic pathogen in neonatalunits, responsible for a variety of infections, including catheter associated urinary tract infections (CAUTIs) and lower respiratory tract infections (Dalben et al., 2008; Oteo et al., 2013; Pérez et al., 2012). This organism has been frequently isolated from food processing plants, fresh vegetables, rice, meat and meat products (Canganella, Nespica, Giontella, & Trovatelli, 1999; Geser, Stephan, & Hächler, 2012; Gran, Wetlesen, Mutukumira, Rukure, & Narvhus, 2003; Sala, Morar, Colibar, & Morvay, 2012; Schwaiger, Helmke, Hölzel, & Bauer, 2011; Shaker, Osaili, Al-Omary, Jaradat, & Al-Zuby, 2007), and can produce cadaverine and putrescine which lead to food spoilage (Greif, Greifová, & Karovičová, 2006; Lavizzari, Breccia, Bover-Cid, Vidal-Carou, & Veciana-Nogués, 2010; Liu et al., 2015; Lorenzo et al., 2010; Pons-Sánchez-Cascado, Bover-Cid, Teresa Veciana-Nogués, & Carmen Vidal-Carou, 2005). In the past year, two food poisoning incidents in China were caused by *E. cloacae* (Gao, Zhang, & Fang, 2016; Sun, Cui, & Wu, 2016). The clinical symptoms of these patients were nausea, vomiting, diarrhea, and abdominal cramps which were similar with those of salmonellosis. *E. cloacae* can form biofilms on many different types of surfaces (Nyenje et al., 2013; Zhou et al., 2014; Majtan, Bohova, Horniackova, Klaudiny, & Majtan, 2014; dos Reis Ponce, Martins, de Araujo, Mantovani, & Vanetti, 2012). Biofilms are defined as matrix-enclosed bacterial populations which adhere to each other and to surfaces (Costerton, Lewandowski, Caldwell, Korber, & Lappin-Scott, 1995). The presence of pathogenic and food spoilage bacteria biofilms in food processing plants is an important hazard because the biofilms can contaminate food products that contact or pass over contaminated surfaces, which can lead to issues of food safety or spoilage.

3-Phenyllactic acid (2-hydroxy-3-phenylpropanoic acid or β-

* Corresponding author.
** Corresponding author.
E-mail addresses: ddabc_2000@163.com (L. Du), zhilan408@163.com (Z. Sun).

http://dx.doi.org/10.1016/j.foodcont.2017.09.004
0956-7135/

phenyllactic acid, PLA), an organic acid, is an antimicrobial compound with broad-spectrum activity against bacteria (Dieuleveux, Van Der Pyl, Chataud, & Gueguen, 1998). Dieuleveux et al. (1998) initially purified and characterized PLA, being a novel anti-*Listeria* compound produced by *Geotrichum candidum*. Subsequently, it was reported that PLA can be produced by a wide range of lactic acid bacteria species (Mu, Yu, Zhu, Zhang, & Jiang, 2012) and can inhibit many Gram-positive bacteria, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Bacillus cereus*, and Gram-negative bacteria, including *Salmonella enterica* and *Escherichia coli* (Mu et al., 2012). PLA can also inhibit yeasts and molds (Lavermicocca, Valerio, & Visconti, 2003; Schwenninger et al., 2008). With this broad spectrum antimicrobial activity, PLA could possibly control bacterial contamination in food products and processing plants.

Although PLA reportedly has broad-spectrum antibacterial activity, its antimicrobial activity against *E. cloacae* planktonic cells and biofilms has not been reported. Because *E. cloacae* is one of the main food spoilage bacteria, its inactivation in food products and processing plants is important for consumers' health. In this study, the efficacy of PLA to inactivate *E. cloacae* planktonic cells was first evaluated, and the changes in the organism's cell membrane integrity including the release of ATP and UV-absorbing substances, and changes in cell membrane potentials were determined to help elucidate the antibacterial mechanism of PLA. In addition, the cell membrane damage to *E. cloacae* cells in biofilms was determined to evaluate the activity of PLA on *E. cloacae* cells in biofilms.

2. Materials and methods

2.1. Bacterial strain and preparation of a single-species culture

E. cloacae was isolated from frozen chicken meat and stored at −20 °C. This strain was identified using 16S rDNA sequencing in our previous study (Liu et al., 2015). Before each experiment, the culture was thawed and first grown at 37 °C in Tryptic Soy Broth (TSB, Becton Dickinson, Sparks, MD) for 24 h, and then streaked on to Tryptic Soy Agar (TSA) and grown at 37 °C for 24 h. A single bacterial colony was selected and inoculated into a test tube containing 10 ml of TSB and transferred at least three times at 24-h intervals before use. The bacteria were grown to the exponential phase to obtain a bacterial count of approximately 10^9 CFU/ml which was confirmed by plating serial dilutions (1:10) on TSA.

2.2. Antimicrobial activity under planktonic condition

PLA (≥98%, Sigma, St. Louis, MO) solutions at the concentrations of 0.1%, 0.3%, 0.5% and 1%, and lactic acid (LA, 90%, Sigma, St. Louis, MO) at 1% were freshly made in deionized water (w/v).

The bacterial cells of 50 ml at exponential phase of growth (37 °C for 16 h) were collected by centrifugation at 6000 × g for 10 min at 4 °C. The cell pellet was washed by sterile distilled water for 3 times, and resuspended in flasks containing 50 ml PLA at different treatment concentrations and stirred with a magnetic stir bar at 100 rpm in room temperature. At predetermined sampling time (0, 10, 30, 60, 120, and 180 min), 1.0 ml of the bacterial suspension was removed and mixed with 9.0 ml of 0.1 M phosphate buffer (PBS) to neutralize the pH. Then, the solution was serially (1:10) diluted in 0.01 M PBS, and 0.1 ml from each dilution tubes was surface-plated on TSA agar plates in duplicate. The plates were incubated at 37 °C for 48 h and *E. cloacae* colonies were enumerated.

2.3. Examination of cell morphology by TEM

Transmission electron microscopy (TEM) was used to determine the morphological changes occurring in *E. cloacae* cells treated with PLA. *E. cloacae* was cultured to the late exponential phase in 20 ml of M17 broth at 37 °C. The cells were sedimented by centrifugation at 6000 × g for 10 min and resuspended in 0.01 M PBS to adjust the OD value to 1.0. The cell suspension was divided into two portions, with one used as the control, and the other treated with 1% PLA for 30 min at 25 °C. After treatment, the treated and control cells were separately sedimented by centrifugation at 6000 × g for 10 min and washed with 0.1 M phosphate buffer. These cells were then fixed with 2.5% (v/v) glutaraldehyde overnight at 4 °C. After serial dehydration, digital images of the treated and untreated fixed *E. cloacae* cells were subjected to TEM (H-7650, Hitachi, Japan) at an operating voltage of 80 kV.

2.4. Membrane permeability assay

Cell membrane permeability was determined by confocal laser scanning microscopy (CLSM). Exponential phase *E. cloacae* cells were prepared according to the protocol described above. After treatment with 0.1%, 0.3%, and 0.5%, and 1% LA for 30 min, the bacterial cells were sedimented by centrifugation at 6000 × g for 10 min and resuspended in PBS buffer, then combined with two probes, i.e., 5(6)-Carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) at final concentrations of 100 μM and 30 μM, respectively. cFDA, a non-fluorescent precursor can only enter into the intact cell membrane and yield green fluorescent compound by the effect of nonspecific cellular esterase. When the cell membrane was damaged, PI entered into cells and bind to DNA to form a red fluorescent DNA-complex. The probe-treated cells were incubated in the dark for 15 min at 25 °C and then washed twice with 0.01 MPBS buffer (pH 7.0). The cells were examined under an Ultra ViewVoX spinning disk confocal microscope (Perkin Elmer, Waltham Mass, USA) with laser light at 488 nm.

2.5. Measurement of extracellular ATP and UV-absorbing substances

Exponential phase *E. cloacae* cells were prepared according to the protocol described above and then treated with 0.1%, 0.3%, 0.5%, and 1% PLA, respectively. At predetermined sampling times (0, 10, 30, 60, 120, and 180 min), 1.0 ml of the bacterial suspension was removed and mixed with 9.0 ml of 0.1 M PBS to neutralize the pH. The supernatant fluids of the cell suspensions obtained by centrifugation were used to detect the release of ATP and UV-absorbing substances. The extracellular ATP levels were determined using the luciferin-luciferase method following the protocols of ATP detection kit (Beyotime, Shanghai, China). The luminescence of 100 μl supernatant sample was assayed using an Infinite 200 PRO microplate reader (Tecan, Switzerland) together with 100 μl ATP detection buffer. The standard curve of ATP concentration was prepared from a known serial concentrations (10 nM-10 μM). The UV-absorbing substances were determined at anOD260 by a UV-VIS Spectrophotometer (Mapada, Shanghai, China).

2.6. Membrane potential depolarization

The membrane potential of bacterial cells was measured using the fluorescence probe 3,3′-dipropylthiadicarbocyanineiodide [DiSC3(5)], as described by Huang and Yousef (2014). Exponential phase *E. cloacae* cells were prepared according to the protocol described above. The bacterial cells were suspended in 5 mM HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid)