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Synergetic effects of high-pressure carbon dioxide and nisin on the inactivation of *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Synergetic effects of high-pressure carbon dioxide (HPCD) and nisin on *Escherichia coli* and *Staphylococcus aureus* were evaluated. Changes in morphology, interior structure, and membrane permeability were analyzed by scanning and transmission electron microscopy, and flow cytometry. Synergetic effects were found, especially in *S. aureus*. HPCD alone or with nisin led to morphological and intracellular alterations in both bacteria, but nisin alone led to these damages only in *S. aureus*. A positive correlation between membrane damage and inactivation was found, but ratios of inactivation were higher, probably because of viable but non-culturable state. Mechanisms were proposed for synergism: for *E. coli*, outer membrane was damaged first by HPCD, and then HPCD and nisin jointly acted on and destroyed the cytoplasmic membrane, leading to further intracellular damage by HPCD; for *S. aureus*, HPCD and nisin acted on the cytoplasmic membrane together leading to cell death.
Industrial Relevance: Escherichia coli and *Staphylococcus aureus* are two common microorganisms, which exist widely in the environment and easily contaminate food such as vegetables and dairy products, respectively. Considering heat treatment may destroy some heat-sensitive quality of the products, this study evaluated synergetic effects of high-pressure carbon dioxide (HPCD) combined with the bacteriocin nisin. The investigations provided evidence for potentially combined application of HPCD and nisin to help keep food safe in the industry.
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1. Introduction

As a novel non-thermal technology, high-pressure carbon dioxide (HPCD) has been widely investigated for inactivating the microorganisms in foods (Ballestra & Cuq, 1998; Corwin & Shellhammer, 2002; Liao, Hu, Liao, et al., 2007; Liao, Zhong, Liao, & Hu, 2014), as well as producing a few side effects and no marked change in sensory quality (Gasperi, Aprea, Biasioli, et al., 2009; Gunes, Blum, & Hotchkiss, 2005; Pozo-Insfran, Balaban, & Talcott, 2006). In the past decades, many researchers have investigated a noticeable inactivation effect of HPCD on microorganisms in real food systems or buffers (Damar & Balaban, 2006; Liao et al., 2007; Liao et al., 2010a, b; Soares, Lerin, Cansian, Oliveira, & Mazutti, 2013). The HPCD resistance of different microorganisms is related to treatment time, pressure, and temperature (Damar & Balaban, 2006; Garcia-Gonzalez, Geeraerd, Spilimbergo, et al., 2007; Garcia-Gonzalez et al., 2009). It was generally noticed that Gram-positives were more resistant to HPCD than Gram-negatives (Debs-Louka, Louka, Abraham, et al., 1999; Garcia-Gonzalez et al., 2009). Dillow, Dehghani, Hrkach, et al. (1999) found that Gram-positive *S. aureus* and *Listeria innocua* showed a susceptibility equal or lower to that of Gram-negative *Salmonella* Salford, *Proteus vulgaris*, *Legionella dunnifii*, *Pseudomonas aeruginosa*, and *E. coli* when treated by HPCD at 20.5 MPa, 34 °C for 36 min and 3 pressure cycles. More HPCD resistance of Gram-positive bacteria occurs due to their thicker cell wall, which has a negative effect on diffusion of CO_2 into the cellular membrane (Garcia-Gonzalez et al., 2009). In recent years, several studies have discussed the inactivation mechanism of microorganisms by HPCD, mainly regarding cellular damage including the modification of morphology, changes in membrane permeability and fluidity, loss of cellular components, and changes in the intracellular organization of microorganisms induced by HPCD as possible inactivation mechanisms (Damar & Balaban, 2006; Garcia-Gonzalez et al., 2007; Li, Deng, Chen, & Liao, 2012a; Liao et al., 2010a).

Nisin is a heat-stable bacteriocin peptide produced by certain strains of *Lactococcus lactis*, exhibiting highly antimicrobial activity toward a wide range of Gram-positive bacteria (Brewer et al., 2002; Samelis et al., 2003; Kim, Choi, & Kim, 2009; Cabo, Herrera, Crespo, et al., 2009; Pinto, de Carvalho, Pires, et al., 2011), while showing little or no activity against Gram-negative bacteria (Boziaris & Adams, 1999; Helander & Mattila-Sandholm, 2000). Nisin has been permitted as a safe food additive all over the world, being used for preservation of dairy products, canned foods, plant protein foods, and cured meat (De Arauz, Jozala, & Mazzola, 2009; Tong, Ni, & Ling, 2014). The target

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of nisin on bacteria, mainly in Gram-positive bacteria, is proposed the cytoplasmic membrane, and the mechanism of its effect has been stated as the widely accepted pore formation on cell membrane (De Arauz et al., 2009). Its interaction with anionic lipids on the cytoplasmic membrane results in pore formation, causing an efflux of adenosine triphosphate (ATP), amino acids, preaccumulated rubidium, or the collapse of vital ion gradients, and ultimately leads to cell death (Tong et al., 2014).

Our previous work found a synergistic inactivation of aerobic bacteria by HPCD at 10 MPa and 32 ~ 52 °C for 5 ~ 30 min combined with nisin (Li, Zhao, Wu, Zhang, & Liao, 2012b). Bi et al. (2014) also reported that HPCD enhanced the sensitization of *E. coli* O157:H7 to nisin in phosphate buffered saline and carrot juice. To our knowledge, no research has been involved in the valid objective microorganisms of this synergistic inactivation or its effects of cellular morphology or structures. The purpose of this work was to compare the synergetic effects of HPCD and nisin on Gram-negative bacteria *E. coli* and Gram-positive bacteria *S. aureus* and to analyze their synergetic effects on cell morphology, interior structure, and the cellular permeability using scanning electron microscopy (SEM), transmission electron microscopy (TEM), and flow cytometry (FCM).

2. Materials and methods

2.1. Preparation of bacterial strains

E. coli (CGMCC1.90) and *S. aureus* (CGMCC1.1861) were obtained from China General Microbiological Culture Collection Center (CGMCC, Beijing, China) and were maintained on slants of Nutrient Agar (NA, Beijing Aoboxing Biological Technology Co. Ltd., Beijing, China). Each strain was inoculated in nutrient broth (NB, Beijing Aoboxing Biological Technology Co. Ltd., Beijing, China), and then incubated at 37 °C for 15 h (*E. coli*) and 16 h (*S. aureus*), respectively, to obtain the initial stationary phase. The stationary phase was determined by timing measurement of optical density (OD) values during incubation in nutrient broth using a spectrophotometer at 600 nm. A working solution was prepared by diluting the above subculture into sterile 0.85% NaCl solution at a volume ratio of 1:10, with the final bacterial concentration 10^7 ~ 10^8 cfu/mL.

2.2. Preparation of nisin solution

Two grams of a commercial nisin preparation containing 1×10^6 IU/g (Zhejiang Silver-Elephant Bio-engineering Co., Zhejiang, China) was dissolved into 100 mL of 0.02 M sterile citric acid solution at approximately pH 2.50, and then filtered through a 0.2 μm-pore-size Millipore filter (Beijing Lanyi Chemicals Co., Beijing, China). The above solution was then added into the prepared working solution at a final concentration of 200 ppm before immediate HPCD treatment.

2.3. Treatments of HPCD or combined HPCD and nisin (HPCD + nisin)

The microbial inactivation by HPCD was carried out using an HPCD system, which was described by Liao et al. (2007). Prepared working solutions were transferred into a 50 mL sterile glass tube secured with a breathable, plastic film. Samples were then placed into the HPCD vessel, which was sanitized and preheated to the experimental temperature. The vessel was pressurized by a plunger pump, held the pressure set for the required time, and then was depressurized by opening the pressure relief valve. Sample tubes were then taken out from the vessel and immediately cooled in an ice bath for further analysis. The CO_2 purity of the HPCD system used was 99.9% (Beijing Analytical Apparatus Co., Beijing, China). Experiments were performed using HPCD at 10 MPa and 32 °C for 15 min. The untreated working solutions with and without nisin were performed as two controls. CO_2 was pumped in supercritical state to the treatment cell, like liquid, but its diffusivity is different from pure liquid CO_2.The bacterial cells were maintained in liquid media during processing. Each treatment was performed in triplicate.

2.4. Enumeration of viable cells

To count the viable cells of *E. coli* and *S. aureus*, the total plate count was used. Samples were serially diluted and plated onto duplicate plates of nutrient agar. After incubation at 37 °C for 48 ± 2 h, the colonies were counted. Log N/N_0 was calculated to determine the inactivation, where N_0 was the number of initial cell counts in the untreated sample, and N was the corresponding cell counts after treatments.

2.5. SEM and TEM analysis

The procedures of preparing sections were referenced of Liao et al. (2010a). Observations and photomicrographs were carried out with a Hitachi S-3400 N SEM (Hitachi Instruments Inc., Japan) and a JEM-1230 TEM (JEOL Japan Electronics Co., Ltd., Japan).

2.6. FCM analysis

Cell suspensions were adjusted to 10^8–10^9 CFU/mL after rinsing twice and re-suspended in sterile 0.85% NaCl solution. Then 50 μL of the cell suspensions was incubated with 0.15 μL of dye mixture containing equivalent SYTO9 (Invitrogen, USA) and propidium iodine (PI, Sigma–Aldrich, USA). After 15 min incubation at room temperature in darkness, the mixture of cells and dye was then immediately analyzed with a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif. USA) equipped with an air-cooled argon ion laser emitting 15 mW blue light at 488 nm and with the standard filter setup. About 30,000 cells were collected for each sample. Forward scatter, side scatter were recorded for each cell sample, and the fluorescence signal was collected in the FL1 (green fluorescence of SYTO 9 at 502 nm) and FL3 (red fluorescence of PI at 613 nm) channels (Amor et al., 2002). Data were collected using Cell Quest software (Becton Dickinson, San Jose, Calif. USA).

2.7. Statistical analysis

Significant differences between cell counts were determined by simple *t* test. Significance was accessed at an error probability of 5% ($P < 0.05$).

3. Results and discussion

3.1. Inactivation of E. coli and S. aureus by nisin, HPCD, and HPCD + nisin

The inactivation of *E. coli* and *S. aureus* by nisin (De Melo, Cook, Miles, & Poole, 1996) or HPCD (Huang et al., 2009; Liao et al., 2010a) was previously studied, but their inactivation by HPCD + nisin has not been reported up to date. Based on our preliminary experiments, the HPCD treatment condition of 10 MPa and 32 °C for 15 min was chosen to investigate the effects of nisin, HPCD, and HPCD + nisin on the inactivation of *E. coli* and *S. aureus*.

As shown in Fig. 1, single treatment of nisin caused 0.16 logs reduction in *E. coli*, which indicated that *E. coli* was not sensitive to nisin. This result was consistent with previous studies (Boziaris & Adams, 1999; Helander & Mattila-Sandholm, 2000), which showed that nisin had no effect on *E. coli* inactivation. Actually, Gram-negative bacteria are normally not sensitive to nisin, because cellular targets for nisin are shielded by their outer membrane (De Arauz et al., 2009; Gänzle, Hertel, & Hammes, 1999; Masschalck, Garcí, Van Haver, & Michiels, 2000; Millette, Smoragiewicz, & Lacroix, 2004). However, cell counts of *E. coli* were significantly reduced by 2.24 logs by HPCD ($P < 0.05$). The inactivation effect of HPCD to *E. coli* was also investigated by Garcia-Gonzalez et al. (2009), who found that approximately 4 logs

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