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Synergistic interactions of nisin in combination with cinnamaldehyde against *Staphylococcus aureus* in pasteurized milk

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is the leading cause of a broad range of human infections and associated with food poisoning worldwide. Nisin can be considered as a natural preservative and is widely used by the food industry. However, resistance of nisin has been reported in several species of bacteria. Identification of antibacterial agents that are synergistic with nisin is a method of minimising resistance to nisin. Cinnamaldehyde (CA), a well-studied essential oil isolated from cinnamon bark, has been reported to exhibit a broad spectrum of antimicrobial activity across a wide range of microorganisms. Previous studies have demonstrated that CA could be used in the food industry due to its active antibacterial activity and its safety. In this study, the synergistic antibacterial activity in vitro of nisin and CA against 13 food-borne isolates of *S. aureus* and *S. aureus* ATCC 29213 were evaluated by a checkerboard assay. The FICI values of nisin combined with CA ranged from 0.1875 to 0.375, which demonstrated a synergistic effect. The positive interactions were verified using the challenge tests in pasteurized milk and agar diffusion assays. The LIVE/DEAD BacLight experiment and scanning electron microscope (SEM) assay were also performed to distinguish the intact and damaged both of cell walls and membranes with the compounds, the combination group displayed stronger damage both of cell wall and cell membrane than when nisin or CA was used alone. These findings indicated that the combination of nisin and CA could not only be used as a promising naturally sourced food preservative but also to reduce the problem of bacterial resistance.

1. Introduction

Staphylococcus aureus (*S. aureus*), an opportunistic pathogen, is the leading cause of a broad range of human infections and associated with food poisoning (Argudin, Mendoza, & Rodicio, 2010). It is a growing concern worldwide and is of concern in terms of both foodborne disease and food spoilage (Loir, Baron, & Gautier, 2003). In recent years, despite the application of the antibacterial therapy, the morbidity and mortality of foodborne diseases infected by *S. aureus* maintain a high level due to the emergence and development of antibiotic resistant strains (Kuroda, Kuroda, Cui, & Hiramatsu, 2007; Zetola, Francis, Nuermberger, & Bishai, 2005). Based on the inevitable trend towards bacterial resistance, new approaches for killing and eliminating bacterial pathogens effectively as well as limiting the evolution of bacterial resistance are necessary. Nowadays, combinations of antibiotics is one important approach to improve the efficacy of antibacterial therapy and overcome resistance to an antibacterial agent (Afeltra, Vitale, Mouton, & Verweij, 2004; Oo, Cole, Garthwaite, Willcox, & Zhu, 2010; Tong et al., 2014). Antimicrobial combination therapy may be used to extend spectrum coverage, prevent the emergence of resistant mutants and gain synergy between antimicrobials (Eliopoulos, 1989). Synergy is believed to be brought about by the combination of compounds due to their function on one or more different targets in a metabolic pathway. Furthermore, understanding the mechanisms of synergistic effects will enable the development of a new generation of safe and standardized drug combinations with higher efficacies than currently available options (Wagner & Ulrich-Merzenich, 2009).

Nisin, a bacteriocin or natural antibacterial peptide with 34 amino acid residues, is secreted from *Lactococcus lactis* subsp. *Lactis*

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(Kindrachuk et al., 2012). Nisin was permitted and widely used in the food industry as a safe and natural food preservative in over 50 countries around the world (Pimentel-Filho, Martins, Nogueira, Mantovani, & Vanetti, 2014). In addition, nisin is highly active against a wide range of Gram-positive bacteria such as *S. aureus*, *Listeria monocytogenes*, *Lactobacillus* spp., *Micrococcus* spp., *Bacillus* spp., and *Clostrdium* spp. (Periago & Moezelaar, 2001; Severina, Severin, & Tomasz, 1998). Nevertheless, the antibacterial efficacy of nisin in food preservation has been compromised by the occurrence of nisin resistance in various bacteria because of extensive use (Zhou, Fang, Tian, & Lu, 2014). Until now, the resistance to nisin has been reported in several species of bacteria, including *S. aureus* (Blake, Randall, & Neill, 2011). For years, a serious health problem might be posed due to the continued widespread use of chemical preservatives, reflecting high daily intake and the likely development of resistance through both food spoilage and food pathogenic bacteria (Kito, Onji, Yoshida, & Nagasawa, 2002). Natural plant extracts have established a strong position as leads for drug discovery (Dias, Urban, & Roessner, 2012). Cinnamaldehyde (CA) is well established essential oil isolated from cinnamon bark. CA has been reported to exhibit a broad spectrum of antimicrobial activity across a wide range of microorganisms. Previous studies have demonstrated that CA could be used in the food industry due to its active antibacterial activity and its safety (Sanla-Ead, Jangchud, Chonhenchob, & Suppakul, 2012). However, the synergistic antibacterial activity of nisin and CA against *S. aureus* is currently unknown. To consider the synergistic antibacterial effect of nisin in combination with CA for practical application, time-killing assays were carried out in pasteurized milk, and agar diffusion tests were also performed. An attempt was also made to understand the mechanism underlying the antibacterial action of these compounds using the LIVE/DEAD BacLight experiment and the scanning electron microscope (SEM) assay.

2. Materials and methods

2.1. Chemical reagents

Nisin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Cinnamaldehyde (CA) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). Mueller-Hinton (MH) broth, Tryptic Soy Broth (TSB), Nutrient Agar (NA) medium and Baird-Parker selective agar were purchased from Qingdao Hope Biol-Technology Co., Ltd. The pasteurized milk (fat content: 3.6 g/100 mL) used in time-killing assays was purchased from the local supermarket.

2.2. Bacterial strains and culture conditions

Thirteen food-borne *S. aureus* isolates (Table 1) were obtained from Jilin Entry-Exit Inspection and Quarantine Bureau. *S. aureus* ATCC 29213, the quality control strain, was acquired from the China Medical Culture Collection Center. Bacterial cells were cultured in TSB overnight at 37 °C with constant shaking and used to test the antibacterial activity of the compounds.

2.3. Determination of MIC values of the antibacterial agents

The minimal inhibitory concentration (MIC) values of nisin and CA to the bacteria were determined by standard broth microdilution susceptibility testing method as a described in previous studies (Wiegand, Hilpert, & Hancock, 2008; Zhao et al., 2014) and described by the Clinical and Laboratory Standards Institute (CLSI, 2009). In brief, the cells were diluted to a final concentration of 1×10^5 CFU/mL in MH broth. A volume of 50 μL of prepared compounds dilutions earlier and 50 μL of prepared bacterial inocula were added to individual wells of a 96-well microtiter plate in triplicate. Finally, the plate was incubated for 24 h at 37 °C. The MIC values were defined as the lowest concentration of antimicrobial that inhibited the growth of microorganism by visual reading (Mariano, Sandra, Mariana, María, & Carmen, 2010).

Table 1
Synergistic effects of nisin with CA against 14 *S. aureus* strains.

Strains	MIC (range) of compound (mg/mL)				FICI	Outcome
	Alone		Combination			
	Nisin	CA	Nisin	CA		
JL-10001	0.016	0.25	0.002	0.05	0.325	Synergism
JL-10002	0.016	0.25	0.001	0.0625	0.3125	Synergism
JL-10003	0.032	0.5	0.002	0.125	0.3125	Synergism
JL-10004	0.016	0.5	0.002	0.125	0.375	Synergism
JL-10005	0.032	0.5	0.002	0.125	0.3125	Synergism
JL-10006	0.016	0.25	0.001	0.0625	0.3125	Synergism
JL-10007	0.016	0.25	0.002	0.0625	0.375	Synergism
JL-10008	0.016	0.25	0.001	0.0625	0.3125	Synergism
JL-10009	0.032	0.5	0.002	0.125	0.3125	Synergism
JL-10010	0.032	0.25	0.004	0.0625	0.375	Synergism
JL-10011	0.016	0.25	0.001	0.0625	0.3125	Synergism
JL-10012	0.016	0.25	0.002	0.0625	0.375	Synergism
JL-10013	0.032	0.5	0.002	0.125	0.3125	Synergism
ATCC 29213	0.032	0.25	0.002	0.05	0.2625	Synergism

2.4. Checkerboard synergy testing

Mechanistic interactions between compounds are usually measured with the broth dilution checkerboard assay (He, Starr, & Wimley, 2015). A checkerboard assay, performed in a checkerboard configuration in a 96-well microtiter plate by broth microdilution, was determined for the interactive inhibition between nisin and CA of different concentrations and the assay was in conformance with established procedures (Moody, 2010). The serial 2-fold dilutions of compounds against the organism were tested. The inocula were adjusted to final concentrations of 1×10^5 CFU/mL for each well, and the plate was incubated at 37 °C for 24 h. The CLSI guideline was used to ensure the accurate microbiological assay. In order to evaluate the antibacterial effects of each combination, the fractional inhibitory concentration indices (FICIs) were calculated as the ratio of the MIC of agents A and B in combination to the MIC of agent A (or B) alone. The FICI was calculated as follows:

$$FICI = FICI_A + FICI_B = (C_A^{COMB}/MIC_A) + (C_B^{COMB}/MIC_B)$$

where MIC_A and MIC_B are defined as the MIC of agents A and B acting alone and C_A^{COMB} and C_B^{COMB} are the MICs of agents A and B when in combination (Meletiadis, Pournaras, Roilides, & Walsh, 2010). The interpretation of the FICI was as follows: a FICI value of $\leq$0.5 represented synergy, a FICI value of 0.5–4 represented indifference, and a FICI value of >4 represented antagonism (Odds, 2003).

2.5. Time-kill synergy testing

According to the results of checkerboard synergy testing, the synergy between nisin and CA against *S. aureus* ATCC 29213 strain was chosen for time-kill curve synergy tests. The time-kill synergy assays were performed in six test tubes containing an initial inoculum of 1×10^6 CFU/mL in pasteurized milk and in TSB respectively with a single or a combination of the compounds according to modification of a previous method (García, Martínez, Rodríguez, & Rodríguez, 2010; Kent, Bakhtiar, & Shanson, 1992; Singh, Bacon,