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Synergistic antimicrobial effects of nisin and *p*-Anisaldehyde on *Staphylococcus aureus* in pasteurized milk

Ce Shi [a], Xingchen Zhao [a], Rizeng Meng [b], Zuojia Liu [c], Guannan Zhang [a], Na Guo [a, *]

[a] Department of Food Quality and Safety, College of Food Science and Engineering, Jilin University, 130062, Changchun, PR China
[b] Jilin Entry-exit Inspection and Quarantine Bureau, 130062, Changchun, PR China
[c] State Key Laboratory of Electroanalytical Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, 130022, Changchun, PR China

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ABSTRACT

Staphylococcus aureus (*S. aureus*) infections resistant to a variety of drugs have been increasingly documented in recent years. Drug resistance has appeared largely due to extensive use of antibiotics. Nisin has been widely used in food industry to prevent bacterial growth. However, nisin resistance has been found in several foodborne pathogenic bacteria. *p*-Anisaldehyde (AS), an extract from *Pimpinella anisum* seeds, is a very common digestive herb of north India. In this study, synergistic interactions of nisin combined with AS against *S. aureus* were observed. The synergy was confirmed by checkerboard microdilution method, with the fractional inhibitory concentration index values ranging from 0.25 to 0.375. The positive interactions were verified by the challenge tests in pasteurized milk and agar diffusion assays. In addition, when treatment with nisin or AS alone or in combination, an obvious loss of 260 nm absorbing materials was observed. And flow cytometry assay showed that nisin or AS alone or in combination caused bacterial membrane permeabilization. The LIVE/DEAD *Bac*Light experiment, scanning electron microscope and transmission electron microscopy observation further confirmed that the compounds could kill bacteria via disrupting membranes. All results above verified that the combination of nisin and AS which showed antibacterial activity by a membrane damage mechanism could not only reduce drug resistance but also be used as a new promising naturally sourced food preservative.

1. Introduction

In recent years, antibiotics have played an important role in fighting infectious diseases, however, uncontrolled long-term or inappropriate use of antibiotics leads to tolerant strains and drug resistance. *Staphylococcus aureus* (*S. aureus*) is undoubtedly one of the most prevalent and notorious examples of an antibiotic-resistant pathogen (DeLeo & Chambers, 2009). *S. aureus*, an opportunistic pathogen, is also associated with food poisoning and food spoilage (Argudin, Mendoza, & Rodicio, 2010). Microbial contamination can occur at any stage from food production to consumption. Moreover, milk and dairy products were implicated in staphylococcal outbreaks (Guzman-Hernandez et al., 2016). It is a growing concern around the world and is of concern in terms of foodborne infections, disease and food spoilage. Facing the risk of severe *S. aureus* epidemic and food spoilage, an increasing interest is aroused in uncovering the mechanisms of drug resistance and discovering new antibiotics and regimens to treat resistant strains. Nowadays, the combination of antibiotics has become one important approach to improve the efficacy of antibacterial therapy and overcome the resistance to an antibacterial agent (Tong et al., 2014). Synergism is a positive interaction created when two compounds combined and exert an inhibitory effect that is greater than the sum of their individual effects (Kumar, Lankalapalli, & Kumar, 2014). 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 heat-stable bacteriocin peptide produced by certain strains of *Lactococcus lactis*, exhibiting highly antibacterial activity towards a wide range of Gram-positive bacteria including spore-forming bacteria and other spoilage and pathogenic bacteria, but showing little or no activity against Gram-negative bacteria (Hui, Zhenzhen, Feng, Yongtao, & Xiaojun, 2016). Furthermore, nisin

* Corresponding author.
E-mail address: jlnaguo@126.com (N. Guo).

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has been permitted as a safe food additive around the world, being used for preservation of several food products such as dairy desserts, canned foods, processed cheese, plant protein foods, and cured meat (Shin et al., 2016).

p-Anisaldehyde (AS) (4-methoxybenzaldehyde), an extract from *Pimpinella anisum* seeds, is a very common digestive herb of north India, and it has been reported that AS exhibits antifungal activity against a number of yeast and mold strains in laboratory media, fruit purees and fruit juices (Shreaz et al., 2011). However, few studies have been reported on the use of nisin combined with AS against *S. aureus* in a food matrix. And to our knowledge, few research has been involved in the valid objective microorganisms of its effects of cellular morphology or structures. The purpose of this work was to study the synergetic effects of nisin and AS alone or in combination on Gram-positive bacteria *S. aureus* and to analyze their synergetic effects on cell membrane permeability, cell membrane integrity and cell morphology using the measurement of release of 260 nm absorbing cellular materials, flow cytometry (FCM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) assays. In summarize, this study aimed to reduce bacterial resistance, and verify the application feasibility of the combination of nisin and AS as a new promising antibacterial agent in food preservation.

2. Materials & methods

2.1. Chemical reagents and bacterial strains

Nisin and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Saint Louis, USA). *p*-Anisaldehyde (AS) was purchased from the Chinese Institute of Food and Drug Test (Beijing, China). Mueller-Hinton (MH) broth, Tryptic Soy Broth (TSB) and Baird-Parker selective agar were purchased from Qingdao Hope Biol-Technology Co., Ltd (Qingdao, China). The pasteurized milk (fat content: 3.6 g/100 ml) used in time-killing assays was purchased from the local supermarket (Changchun, China). Propidium Iodide (PI) was purchased from Invitrogen (USA). Thirteen food-borne *S. aureus* isolates were obtained from Jilin Entry-Exit Inspection and Quarantine Bureau. The quality control strain, *S. aureus* ATCC 29213, was acquired from the China Medical Culture Collection Center (Table 1).

2.2. Measurement of MIC values

The minimal inhibitory concentrations (MICs) of nisin and AS against the bacteria were determined on the basis of the Clinical and Laboratory Standards Institute guidelines (CLSI, 2009) using standard broth microdilution susceptibility testing method as previous research (Zhao et al., 2014). Briefly, the cells were diluted to a final concentration of 1×10^5 Colony Forming Unit (CFU)/ml in MH broth, 50 μl of prepared compounds dilutions and 50 μl of prepared bacterial inocula were added to individual wells of a 96-well microtiter plate in triplicate. Then the plate was incubated at 37 °C for 24 h. The MICs were defined as the lowest concentration of antibiotic that produced the complete inhibition of visible growth (Mariano, Sandra, Mariana, María, & Carmen, 2010).

Table 1
Synergistic effects of Nisin combined with AS against 14 *S. aureus* strains.

Strains	MIC of compound (mg/ml)				FICI	Outcome
	Alone		Combination			
	Nisin	AS	Nisin	AS		
JL-10011	0.016	2	0.002	0.25	0.25	Synergism
JL-10012	0.016	4	0.002	0.5	0.25	Synergism
JL-10013	0.016	2	0.002	0.25	0.25	Synergism
JL-10014	0.032	2	0.004	0.5	0.375	Synergism
JL-10015	0.016	2	0.002	0.25	0.25	Synergism
JL-10016	0.032	4	0.004	0.5	0.25	Synergism
JL-10017	0.032	2	0.004	0.5	0.375	Synergism
JL-10018	0.016	2	0.002	0.5	0.375	Synergism
JL-10019	0.016	4	0.002	0.5	0.25	Synergism
JL-10020	0.016	2	0.004	0.25	0.375	Synergism
JL-10021	0.016	2	0.002	0.5	0.375	Synergism
JL-10022	0.016	2	0.002	0.25	0.25	Synergism
JL-10023	0.032	4	0.004	0.5	0.25	Synergism
ATCC 29213	0.016	2	0.002	0.25	0.25	Synergism

2.3. Checkerboard microdilution test

The synergistic antibacterial effect of nisin and AS was studied by the checkerboard method. This assay was performed in a checkerboard configuration in a 96-well microtiter plate by broth microdilution, which was in conformance with established procedures (Moody, 2010). Serial 2-fold dilutions of nisin and AS were mixed in MH with final concentrations of the compounds ranged from 1/32 to 4 times the MIC for nisin and from 1/128 to 4 times the MIC for AS. 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. In order to evaluate the antibacterial effects of each combination, the data produced by the checkerboard assay were analyzed in terms of the fractional inhibitory concentration index (FICI) using the following equation:

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

where MIC_A and MIC_B are the MICs of compounds A and B when acting alone, C_A^{COMB} and C_B^{COMB} are the MICs of compounds A and B when in combination (Meletiadis, Pournaras, Roilides, & Walsh, 2010). Synergy was defined as an FICI of $\leq$0.5, and an additivity was defined as an FICI of >0.5 but <1. Indifferent was defined as an FICI of $\geq$1 but <4, whereas antagonism was defined as an FICI of $\geq$4 (Oliveira, Stamford, Gomes, & Souza, 2010).

2.4. Time-kill tests in TSB and pasteurized milk

Commercial pasteurized milk and TSB was inoculated with 1×10^6 CFU/ml of *S. aureus* 29 213 respectively and the time-kill synergy tests were carried out in six tubes containing an initial inoculum of 1×10^6 CFU/ml with a single or a combination of the compounds according to previous methods with slight modifications (García, Martínez, Rodríguez, & Rodríguez, 2010). The tube containing only bacteria without any compounds served as a control group. The pasteurized milk or TSB containing bacteria with or without compounds was incubated at 37 °C and samples were taken at predetermined time points (0, 3, 6, 9, 12 and 24 h). The bacterial counts was determined by plating appropriate dilutions on plates of Baird-Parker selective agar plates to allow for growth. The Baird-Parker selective agar plates were incubated at 37 °C for 16–24 h, the number of viable cells in each tube was calculated after counting bacterial colonies on plates and by multiplying by the appropriate dilution factor (dos Santos et al., 2008). In this assay, all the experiments conducted in triplicate were performed at 1/2 × MIC, and the results were showed as the mean values. Synergism was regarded as a decrease in the colony count of $\geq$2 $\log_{10}$ CFU/ml relative to the count that was obtained with the most active single compound. Antagonism was defined as a decrease of <2 $\log_{10}$ CFU/ml with respect to the least active compound (Knezevic, Curcin, Aleksic, Petrusic, & Vlaski, 2013).