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Synergistic antimicrobial effect of combined bacteriocins against food pathogens and spoilage bacteria

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ABSTRACT

This study was conducted to determine the possible synergistic antimicrobial activity of four bacteriocins (nisin, pediocin, enterocinMT104b and enterocinMT162b) against foodborne and food spoilage bacteria. Microbroth dilution assay was used to determine Minimum Inhibitory Concentration (MIC) of each bacteriocin and checkerboard technique was applied to determine the possible synergistic antibacterial effects among four bacteriocins. The combination of nisin with MT104b caused a synergistic effect on the elimination of *Staphylococcus aureus* whereas the combination of nisin plus pediocin, nisin plus MT162b, pediocin plus MT104b, and pediocin plus MT162b caused a synergistic effect against *Lactobacillus sakei*. Nisin caused an additive effect against *Listeria monocytogenes* when it was combined with pediocin. Thus, the combinations of different bacteriocins could act synergistically or additively to eliminate serious foodborne pathogens and food spoilage bacteria.

Keyword: Bacteriocin(s), nisin, pediocin, enterocin, pathogens.

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INTRODUCTION

Foodborne illness caused by consumption of food contaminated with pathogens or spoilage bacteria is of great concern in public health. Many known pathogens such as *Bacillus cereus, Campylobacter* spp*., Listeria monocytogenes, Salmonella* sp., *Staphylococcus aureus, Escherichia coli,* etc*.* are responsible for numerous illnesses and death (Scallan et al., 2011). *Lactobacillus sakei, L. curvatus and Leuconostoc mesenteroides* are common food spoilage organisms that can cause off-flavour and discoloration of refrigerated meats, especially, vacuum-packed products (Dias et al., 2013; Kalschne et al., 2015). Thus, controlling pathogens and spoilage bacteria in food products are important. Among different natural antibacterial agents, bacteriocins are of interest to be used in food products due to their safety and potential antibacterial effects (Galvez et al., 2008).

Bacteriocins are antimicrobial peptides or proteins ribosomally synthesized by some lactic acid bacteria (LAB) (Bruno and Montville, 1993; Abee et al., 1995; Cleveland et al., 2001). Bacteriocins are non-toxic to eukaryotic cells and are generally recognized as safe substances. Several classes of bacteriocins have been described, including lantibiotic (class I), small heat-stable non-lanthionine peptides (class II), large heat-labile bacteriocins (class III) and complex proteins that require the participation of carbohydrate or lipid moieties to express activity (class IV) (Abee et al., 1995). Each class can be further divided into subclasses as Class Ia, Ib, IIa, IIb, etc (Cleveland et al., 2001).

Nisin is a well-known bacteriocin belonging to class Ia that is active against Gram-positive pathogens associated with food (Cleveland et al, 2001; Thomas and Delves-Broughton, 2005). The use of nisin as a food biopreservative is limited because of its lesser effect

against Gram negative bacteria (Arques et al., 2004). Nisin is produced by some strains of *Lactococcus lactis* (Thomas and Delves-Broughton, 2005). Pediocin belongs to class IIa (Cleveland et al., 2001) and is produced by some strains of *Pediococcus acidilactici*, a commonly found and used bacterium in production of fermented sausage (Luchansky et al., 1992). Most pediocins are thermostable peptides and function under a wide range of pHs (Bhunia et al., 1988; Papagianni and Anastasiadou, 2009). Pediocin AcH has been proven to be effective against three pathogens such as *L. monocytogenes, S. aureus* and *C. perfringens* (Bhunia et al., 1988; Papagianni and Anastasiadou, 2009).

Enterocins are very diverse and belong to four classes of bacteriocins (Franz et al., 2007). Enterocins can prevent the growth of many foodborne and spoilage bacteria such as *S. aureus, L. monocytogenes*, *Escherichia coli, Pseudomonas* spp., *Bacillus* spp. and *Clostridium* spp. (Franz et al., 2007). Because enterocins are heat stable and active over a wide pH range, they can be used to enhance the shelf life of different food products. Among different species of *Enterococcus* that are able to produce bacteriocins for food preservation, *E. faecium* and *E. faecalis* are predominant (Javed et al., 2011).

Recent years, antimicrobial resistance of many foodborne pathogens to current antibiotics or antimicrobial agents is a great concern of public health (Walsh and Fanning, 2008). The antimicrobial resistance has been linked mostly to the use of antimicrobial drugs in food-producing animals (Threlfall et al., 2000). Since genes encoding antimicrobial resistance are often linked to mobile genetic elements such as plasmids, transposons, and integrons; spreading of antibiotic resistance genes among bacteria, including bacteria causing infection in animal or humans, can be occurred (Sunde and Nordstrom, 2006). The resistance of bacteria to bacteriocins can also be occurred spontaneously (Bouttefroy and Milliere, 2000).

To overcome the antimicrobial resistance, a hurdle technology based on combined treatments for food preservation against foodborne pathogens or spoilage bacteria is necessary (Shalini and Singh, 2014; Severino et al., 2014; Severino et al., 2015). Further, hurdle technology may also reduce the dose or the concentrations of antibacterial agents required to eliminate bacteria due to possible synergistic or additive effects (Severino et al., 2014, 2015; Ndoti-Nembe et al., 2015).

It is expected that when microorganisms are treated by multiple antimicrobial agents, the capacity of their survival could be decreased due to synergic effects of combined antimicrobial agents. Previous study demonstrated that nisin, pediocin, and two enterocins had antibacterial effects against several foodborne and spoilage bacteria (Turgis et al., 2012). The obtained minimum inhibitory concentration values showed that pediocin and MT 162b have stronger antibacterial effects against target bacteria rather than nisin and MT 104b. However, their combined antibacterial effects have not been evaluated in our pervious study. Therefore, the aim of the present work was to evaluate the ability of combined bacteriocins (nisin, pediocin, and two enterocins) to eliminate some pathogens and food spoilage bacteria, including *Bacillus cereus, Lactobacillus sakei, Listeria monocytogenes,* and *Staphylococcus aureus.*

MATERIALS AND METHODS

Preparation of bacterial cultures

Stock cultures of *B. cereus* LSPQ 2872, *L. monocytogenes* HPB 2812, *S. aureus* ATCC 29213 were stored at -80°C in Mueller-Hinton broth medium (Oxoid LTD., Basingstoke, England) containing glycerol (10% V/V). Stock culture of *L. sakei* ATCC15521 was stored under the same conditions in Man Rogosa and Sharpe (MRS) broth medium (Difco Laboratories, Detroit, MI, USA). Prior to each experiment, stock cultures were grown through two consecutive 24 h growth cycles in Mueller-Hinton or MRS broth medium at 37°C. The cultivated cultures were centrifuged at 5000 ×g for 10 min and the obtained pellets were washed twice in saline solution (0.85% w/v) to obtain working cultures. The bacterial concentration was then adjusted to 10^6 CFU/ml with peptone water (0.1% W/V) for the antibacterial test.

Preparation of bacteriocins

Commercial nisin was purchased from Sigma-Aldrich (St. Louis, MO, USA). This is a powder product containing 2.5% nisin in NaCl and denatured milk solids. The nisin stock solution was prepared in distilled water (1% W/V) and was filter-sterilized through a 0.2 µm pore-size filter (Sarstedt, Montreal, QC, Canada). Pediocin was produced by *Pediococcus acidilactici* in our lab. This bacterium was obtained from Chr-Hansen (Hørsholm, Denmark). Two other bacteriocins MT 104b and MT 162b were produced by *Enterococcus faecium* MT 104 and MT 162, respectively, in our lab. These two strains of *E. faecium* were isolated from human intestine by our laboratories (Turgis et al., 2013). The bacteriocins MT 104b, MT 162b and pediocin were obtained from the culture of *E. faecium* MT 104, *E. faecium* MT 162 and *P. acidilactici*, respectively, in MRS broth after incubation for 24 h at 37°C. The supernatant containing bacteriocin was collected after centrifugation for 10 min at 2000 ×*g*. The obtained supernatant was filter-sterilized through a 0.2 µm pore-size filter. The antibacterial activity of these bacteriocins extracts against *L. sakei* was determined using a well-diffusion assay (Schillinger and Lucke, 1989). The antibacterial activity of pediocin-containing supernatant was 21,133 Arbitrary Units per ml (AU/ml). The antibacterial activity of bacteriocin MT104b-containing supernatant and bacteriocin MT162b-containing supernatant were both 800 AU/ml (Turgis et al., 2012).

Evaluation of combined effects of bacteriocins against different bacteria

Previous study showed that nisin, pediocin, enterocin MT 104b and enterocin MT 162b have antibacterial effects against different foodborne and spoilage bacteria in a microbroth dilution assay (Turgis et al., 2012); however, the possible synergistic effects against these bacteria is not known. Therefore, in this study, the checkerboard method was chosen to assess the efficacy of possible interaction between bacteriocins which could be synergistic, additive, or exhibiting no interaction or antagonist against the pathogens (Davidson and Parish, 1989). In this method,

Fractional Inhibitory Concentration (FIC) index of bacteriocin in combinations were used.

In this study, nisin was assayed at serial concentrations of 172, 86, 43, 21.5, 10.75, 5.38, 2.68 and 1.34 ppm, pediocin at serial concentrations of 21133, 10566, 5283, 2641, 1320, 660, 330, and 165 AU/ml, MT 104b and MT 162b at serial concentrations of 800, 400, 200, 100, 50, 25, 12.5 and 6.25 AU/ml. Each of the two selected bacteriocins was two-fold diluted with Mueller-Hinton in two separate microplates of 96 wells. Then the bacteriocins were transferred into the main microplate which contained a serial concentration of 25 µl of bacteriocin 'a' along the X axis and the serial concentration of same volume of bacteriocin 'b' along the Y axis. In total, there were a 8 × 8 matrix in which there was a combination of bacteriocin 'a' and bacteriocin 'b' at different concentrations in each well. Subsequently, 90 µl of Mueller-Hinton (MH) medium containing approximately 2×10^6 CFU/ml of one target bacterium were added to the wells. Plates were incubated at 37°C for 24 h and optical density (OD) was measured at 600 nm. The MIC of each bacteriocin alone or in combination with other bacteriocin was taken as the lowest concentration that inhibited bacterial growth completely after 24 h. All assays were performed in triplicate.The fractional inhibitory concentration (FIC) index was calculated by the following formula:

FICa = (MICa combined / MICa alone)
FICb = (MICb combined / MICb alone)

FIC = FICa + FICb

Where
'MICa alone' is the MIC value of bacteriocin 'a' tested alone; 'MICb alone' is the MIC value of bacteriocin 'b' tested alone; 'MICa combined' is the MIC value of bacteriocin 'a' tested in combination with bacteriocin 'b'; 'MICb combined' is the MIC value of bacteriocin 'b' tested in combination with bacteriocin 'a'.
The results are considered as synergistic when FIC ≤ 0.5, additive when 0.5 < FIC ≤ 1, not interactive for 1 < FIC ≤ 4 and antagonist for the FIC > 4 (Gutierrez et al., 2009; Turgis et al., 2012).

RESULTS AND DISCUSSION

The MIC values of nisin and pediocin were respectively 172 ppm and 21133 AU/ml (Aribtary Unit in each ml of supernatant) for all bacteria except for *S. aures* which were not detected. MIC value of MT 104b was 800 AU/ml for *B. cereus*, *L. sakei* and *L. monocytogenes* and 600 AU/ml for *S. aureus*. For MT 162b the MIC values were found much lesser including 100, 200, 300 and 400 AU/ml for *L. sakei, L. monocytogenes, S. aureus and B. cereus,* respectively (Turgis et al., 2012).

The antibacterial effects of combined bacteriocins against tested bacteria are described in terms of the FIC indices and presented in Table 1. Results show that the combined nisin and pediocin displayed a synergic activity against *L. sakei* and caused an additive effect against *B. cereus* and *L. monocytogenes.* The combination of nisin and MT 104b caused a synergic activity against *S. aureus* but caused no interactive effect against other bacteria. The combination of nisin with MT 162b caused a synergic effect against *L. sakei*. Combined pediocin and enterocin MT 104b led to a synergic effect against *L. sakei*. Similarly, pediocin in combination with MT 162b caused a synergistic activity against *L. sakei*. Thus, it can be found that there were different synergistic or additive actions depending on bacteriocin types and target bacteria.

In this study, nisin plus pediocin caused an additive effect against *B. cereus* and *L. monocytogenes* while in other studies, an additive antibacterial effects were obtained by combination of nisin with leucocin F10 (Parente et al.,1998); or nisin with curvaticin 13 (Bouttefroy and Milliere, 2000) against *L. monocytogenes*. For example, Parente et al., (1998) found that combined nisin and leucocin F10 (produced by *Leuconostoc carnosum* F10) together at the concentration of 50 to 80 AU/ml in Tryptic Soya Broth plus Yeast Extract (TSBYE) were more bacteriocidal against a pool of three strains of *L. monocytogenes* than each bacteriocin applied alone. Bouttefroy and Milliere (2000) demonstrated that utilization of nisin (50 IU/ml) or curvaticin 13 (160 AU/ml) caused an immediate transitory bactericidal effect against *L. monocytogenes*, then the regrowth of bacteria occurred after 6 or 12 h. However, simultaneous utilization of nisin (50 IU/ml) and curvaticin 13 (160 AU/ml) at beginning of incubation (t = 0) caused a greater inhibitory effect against *L. monocytogenes* than each bacteriocin, causing a reduction of bacterial population to undetectable level (Bouttefroy and Milliere, 2000). Thus, combined nisin and pediocin may be applied to food products to control the growth of *L. monocytogenes* or *B. cereus* and/or also to reduce the occurrence of spontaneous resistance of these bacteria to individual bacteriocin.

Our study also found that combined nisin and enterocin MT 104b caused a synergic activity against *S. aureus* ATCC 29213. This is standard strain for antimicrobial susceptibility testing and is not a methicillin-resistant *S. aureus* (MRSA). However, based on obtained result, it may be useful to evaluate the combined effect of nisin and enterocin MT 104b against MRSA strains. It should be mentioned that MRSA has emerged not only in hospital but also in the community and even in food products (Kluytmans, 2010).

It has been known that *L. sakei* is popular among spoilage bacteria found in meat products (Dias et al., 2013; Kalschne et al., 2015). Therefore, controlling the growth of this bacterium in meat products is important. In current study, there were four combined bacteriocins including nisin plus pediocin, nisin plus enterocin MT 162b, pediocin plus enterocin MT 104b or pediocin plus enterocin MT 162b that caused a synergic activity against *L. sakei* (Table 1). The obtained results are of interest since there is less information on the antibacterial effects of combined enterocin with other bacteriocins against food pathogens or food spoilage bacteria. In fact, two enterocins (MT 104b and MT 162b) used in current study were found to be stable at different pH ranging from 2 to 11 and were active after different treatments such as heat, enzymes, detergents, and γ-irradiation (Turgis et

Table 1. The fractional inhibitory concentration (FIC) indices of bacteriocin combination against different target bacteria.

Bacteria	Nisin + Pediocin		Nisin + MT 104b		Nisin + MT 162b		Pediocin + MT 104b		Pediocin + MT 162b		MT 104b + MT 162b	
	FIC	Activity*	FIC	Activity*	FIC	Activity*	FIC	Activity*	FIC	Activity*	FIC	Activity*
Bacillus cereus	1.0	AD	1.5	I	1.8	I	1.2	I	2.3	I	3.0	I
Lactobacillus sakei	0.01	S	1.0	AD	0.1	S	0.1	S	0.1	S	4.6	A
Listeria monocytogenes	0.6	AD	1.1	I	2.1	I	1.1	I	4.1	A	2.0	I
Staphylococcus aureus	1.2	I	0.5	S	1.4	I	1.6	I	2.9	I	4.0	I

*FICa = (MICa combined/MICa alone) and FICb = (MICb combined/MICb alone). FIC = FICa + FICb. FIC ≤ 0.5: synergistic effect (S); 0.5 < FIC ≤ 1: additive effect (AD); 1 < FIC ≤ 4: no interactive effect (I); FIC > 4: antagonistic effect (A).

al., 2013). These properties are important for food preservation. Based on obtained results, future study on antibacterial effects of combined enterocin MT 162b with either nisin or pediocin or combined enterocin MT 104b with pediocin against *L. sakei* in vacuum-packed meat should be conducted.

CONCLUSIONS

In the current study, different bacteriocins in combination caused synergistic or additive effects against different target bacteria. The synergistic effects of bacteriocins in combination against *S. aureus* have been obtained using a mixture of nisin and MT104b, against *L. sakei* using different mixtures of Nisin plus enterocin MT104b, nisin plus pediocin, pediocin plus enterocin MT104b, and pediocin plus MT162b. Moreover, an additive antimicrobial effect against *L. monocytogenes* and *B. cereus* was obtained using the mixture of nisin and pediocin. These mixtures represented as potential candidates applied in food system for controlling food pathogenic or food spoilage bacteria. Future studies on utilization of the potential bacteriocin mixtures alone or in combination with other antibacterial agents such as organic acid salts, NaCl, essential oils for preservation food products will be conducted.

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